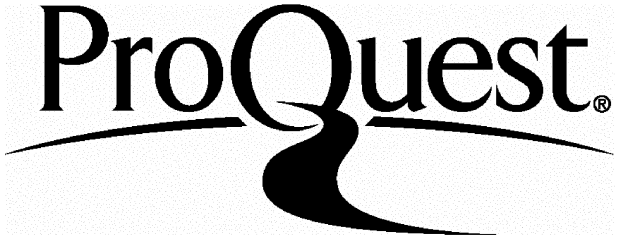
Regulation of the Urokinase-Type Plasminogen Activation System in Oral Squamous Carcinoma Cell Lines by the β6 Integrin Subunit

A thesis submitted to University College London for the degree of Doctor of Philosophy in the Faculty of Science, 2003

By

Nafisa Dalvi

Eastman Dental Institute
University College London



DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

This thesis is dedicated to my loving father

my inspiration and motivation

ACKNOWLEDGEMENTS

First and foremost I wish to thank my supervisor, Dr Simon Whawell. His excellent guidance and continual support and encouragement have been invaluable and I owe a huge debt of gratitude to him. I must also thank Professor Paul Speight for giving me the opportunity to carry to my scientific research in his laboratories and for his helpful advice.

I would like to acknowledge the following persons for aiding me in my research:
Dr Matthew Morgan for practical and theoretical advice. I am truly grateful for his infallible enthusiasm, encouragement and generosity with his time.
Dr Vince Ellis for enabling me to carry out some research in his laboratory and Dr Rosemary Bass for being extremely helpful with this.
Dr John Marshall for providing me with some cells important to this research.

Most importantly without the unconditional love, patience and support of my family and my dear friends Sarah, Angie and Mariana, this thesis would not have been possible, so I thank them.

Finally, I would like to thank all of my friends at the Eastman, past and present for their kindness and humour and for making my time there a most enjoyable one.

ABSTRACT

Over-expression of components of the urokinase system is well documented in cancer and is thought to enable tumour cells to migrate and invade. Changes in integrin expression are also a common feature of tumours and have been linked to changes in protease activity. The αv integrin family has been correlated with cancer progression; particularly the αvβ6 integrin is neo-expressed in a number of epithelial carcinomas and has been associated with metalloproteinase expression.

Oral SCC keratinocytes, which had been transfected to express different integrin subunits, were used in this study. Cells expressing αvβ6 showed enhanced urokinase-type plasminogen activator (uPA) secretion compared to control cells. This was unaffected by plating on a variety of matrix ligands including fibronectin. However, cells over-expressing αvβ6 showed decreased urokinase receptor (uPAR) mRNA and protein expression, associated with decreased cell surface plasminogen activation. Therefore, uPAR levels and cell surface plasminogen activation were inversely related to αvβ6 expression. The up-regulation of uPA secretion and down-regulation of uPAR expression and function by the αvβ6 integrin was mediated through the unique 11 amino acid extension of the β6 cytoplasmic tail.

Transfection of αvβ6 into melanoma cell lines did not affect the urokinase system, suggesting this is cell-specific. Morpholino antisense oligonucleotides targeted against the αv and β6 integrins and an inducible β6 gene expression system were also used in order to confirm effects of αvβ6 on the urokinase system. The roles of αvβ6 and uPA in cell migration and invasion were also investigated. Expression of αvβ6 into the keratinocytes promoted only fibronectin-dependent cell migration that was not uPA dependent but required plasmin activity. The dual regulation of the urokinase system by αvβ6 integrin suggests a complex system where levels of proteolysis in cancer invasion are precisely regulated.

CONTENTS

CHAPTER 3 – αvβ6 MODULATION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR (uPA) IN ORAL SCC CELL LINES

CHAPTER 4 – αvβ6 MODULATION OF THE UROKINASE RECEPTOR (uPAR) IN ORAL SCC CELL LINES

CHAPTER 6 – THE ROLE OF αvβ6 AND uPA ON CELLULAR FUNCTION

CHAPTER 7 – THE ROLE OF αvβ6 IN MELANOMA CELLS

ABBREVIATIONS

αvβ6	ALPHA V BETA 6 INTEGRIN
αvβ5	ALPHA V BETA 5 INTEGRIN
αvβ3	ALPHA V BETA 3 INTEGRIN
β1	BETA 1 INTEGRIN
BFGF	BASIC FIBROBLAST GROWTH FACTOR
ECM	EXTRACELLULAR MATRIX
EGF	EPIDERMAL GROWTH FACTOR
ERK	EXTRACELLULAR REGULATED KINASE
FAK	FOCAL ADHESION KINASE
FN	FIBRONECTIN
GFR	GROWTH FACTOR RECEPTOR
IAP	INTEGRIN ASSOCIATED PROTEIN
JNK	JANUS KINASE
MAPK	MITOGEN ACTIVATED PROTEIN KINASE
PAI	PLASMINOGEN ACTIVATOR INHIBITOR
PDGF	PLATELET DERIVED GROWTH FACTOR
TGFβ	TRANSFORMING GROWTH FACTOR-β
TM4SF	TRANSMEMBRANE-4 SUPERFAMILY
tPA	TISSUE-TYPE PLASMINOGEN ACTIVATOR
uPA	UROKINASE-TYPE PLASMINOGEN ACTIVATOR
uPAR	UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR
VN	VITRONECTIN

LIST OF FIGURES

LIST OF TABLES

Introduction

1.1 CANCER METASTASIS

A cancer cell can be defined as a cell with an abnormal phenotype that exhibits uncontrolled growth and loss of normal cellular function. This is generally the result of a spontaneous mutation that causes activation of oncogenes or the loss of tumour suppressor genes, enabling the cell to escape regulation from the cell cycle (Yarnold, 1992). The uncontrolled proliferation of the cancer cell results in the formation of a tumour mass. The characteristics of a malignant tumour include uncontrolled growth, local invasion of adjacent tissue and the propensity to establish distant metastases (Hanahan and Weinberg, 2000).

Tumour progression involves the coordination of several signal-transduction pathways to drive or regulate the different events of tumour development, such as cancer cell proliferation leading to tumour growth, migration and invasion into surrounding tissues, remodelling of the surrounding extracellular matrix (ECM), angiogenesis and metastasis formation. These signalling events are activated upon binding of the cells to ECM components. In addition there is bi-directional signalling between the epithelial cancer cells and stromal cells, including activated fibroblasts, endothelial cells, smooth muscle cells and inflammatory cells (Alessandro and Kohn, 2002).

Tumour cell invasion is an extremely complex multi-step process that involves cell detachment from the primary tumour site, proteolysis of matrix components and

invasion and migration of cells through the disrupted matrix. Cells then penetrate vessels and pass to distant sites in the circulation or lymphatics. The malignant cells may then attach to vessels of the target tissues and adhere via integrins, allowing the cells to migrate into the tissue, where they are then able to adhere to cell and ECM components of the target tissue. Further cell division and differentiation may then form secondary metastases (figure 1), (Stetler-Stevenson et al, 1993). There are no qualitative differences between cancerous cells and normal cells with respect to the basic processes of cell adhesion, migration and invasion. However, in cancer, these processes occur at times and places incompatible with normal cellular behaviour and it is the ability of malignant cells to cross tissue boundaries, which distinguishes true malignancy from proliferative disorders (Liotta and Stetler-Stevenson, 1991, Johnsen et al, 1998). The metastatic spread of tumours to different organs is the primary cause of death in cancer patients (Chambers et al, 2002), so the ability to block the migratory and invasive capacity of tumour cells offers an important approach to treating patients with malignant disease.

Degradation of the basement membrane, as well as breakdown of components of the extracellular matrix via proteolytic enzymes is a critical event in the metastatic cascade by allowing tumours to disseminate (Reuning et al, 1998). Enzymatic degradation of basement membrane and ECM involves proteases from several families and it was first suggested over 50 years ago that the phenotype of multiple human cancers might be dependent on the presence of proteases, (Fisher, 1925). There are two major classes of ECM-degrading enzymes: the serine proteases such as

the plasminogen activator system and the matrix metalloproteases. Increased production of these enzymes has been associated with the invasive and/or metastatic phenotype in many tumours (reviewed by Dano et al, 1985, Liotta and Stetler-Stevenson, 1991, Mignatti and Rifkin, 1993).

A critical aspect of invasive and metastatic behaviour involves adhesive interactions of tumour cells with other cells or with the extracellular matrix. Such interactions occur as tumour cells migrate locally from the primary tumour mass, as circulating tumour cells adhere to vascular endothelial cells, and during the seeding of metastases. An adequate blood supply is also required for the tumour mass to grow, which involves endothelial cells becoming angiogenic (Folkman, 1992). During this process both the cancer cells and endothelial cells develop altered affinity and avidity for their extracellular matrix and other cell membrane proteins. Many of the adhesive interactions involved in tumour progression are mediated by a family of structurally versatile cell-surface adhesion molecules known as the integrins (Hood and Cheresh, 2002, Hynes, 1999, Miranti and Brugge, 2002).

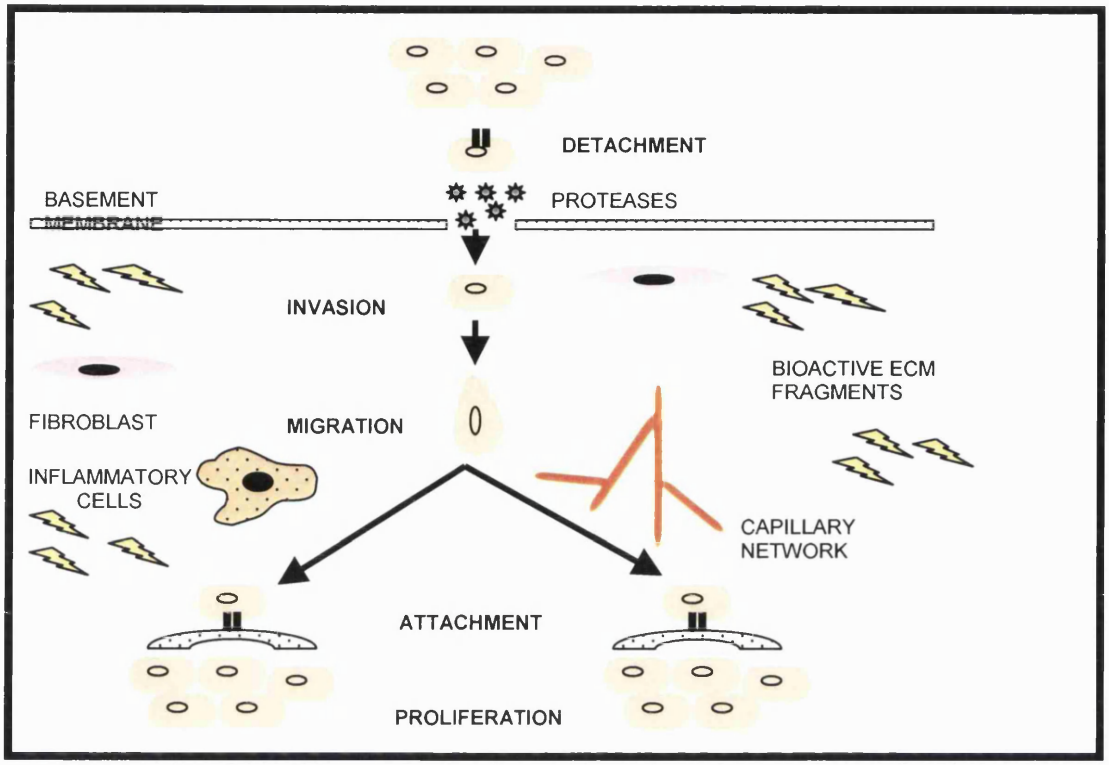


Figure 1.1 Cancer Metastasis

Following detachment of the cancer cell from the primary tumour site, proteolytic enzymes are released which degrade the basement membrane enabling cell invasion and migration through the extracellular matrix. Interaction with the microenvironment plays a critical role in the development of a malignant phenotype. The heterogenous stromal cell population including fibroblasts and endothelial cells modifies the extracellular matrix by the production of cytokines and proteases. Growth factors, ECM molecules and bioactive fragments derived by degradation of ECM components, stimulate the expression of an invasive and motile phenotype of the malignant cell. The cell then attaches to the target site. Local blood flow through a capillary network enables growth and proliferation of cancer cells to form secondary metastases.

1.2 INTEGRINS

Integrins are cell adhesion molecules that are important in maintaining tissue integrity and homeostasis. The expression of integrins is normally tightly regulated, thereby regulating physiological processes such as cell adhesion, migration, cell shape, proliferation, differentiation and survival. Many of these processes are misregulated in malignant tumours and it has been shown that many of the characteristics of tumour cells are attributable to the aberrant expression or function of integrins (Thomas and Speight, 2001). Thus, integrins play a crucial role in tumour metastasis as well as contributing to many diseases in humans (Ruoslahti and Pierschbacher, 1987, Hynes, 1992, Felding-Habermann et al, 2003). Numerous studies have shown dramatic differences in the surface expression and distribution of integrins in malignant cells compared with pre-neoplastic tumours of the same type (Mizejewski, 1999). The ability to interfere with integrin function using antibodies or peptides may provide many opportunities for therapeutic intervention in a number of diseases.

1.2.1 INTEGRIN STRUCTURE

Integrins are transmembrane glycoproteins composed of an alpha and a beta subunit, which are non-covalently linked to form a heterodimer. Eighteen α and 8 β subunits have been discovered to date which associate to form at least 24 different integrin receptors. Integrins are expressed on a wide variety of cells, and most cells express several different integrin heterodimers. The α subunits vary in size between 120 and 180 kd and are each non-covalently associated with a β subunit (90–110 kd). Many α

subunits can associate with a single β subunit, for example β1 has several α-subunit partners (including α2β1, α3β1, α5β1). However, a number of α subunits (α4, α6 and αv) can associate with more than one β subunit with αv being particularly promiscuous to form several different heterodimers, including αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 (figure 1.2), (Jones et al, 1997). Each subunit has a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain (50 amino acids or less). Integrin heterodimers consist of a globular 'head' that is extracellular and contains the amino terminal ends of the subunits. The remainder of the heterodimer comprises two tails, which span the remaining extracellular region, transmembrane and cytoplasmic domains to form the carboxyl terminal ends of the subunits (figure 1.3). The first crystal structure of the extracellular portion of an integrin was recently reported (Xiong et al, 2001). A quarternary structure prediction, suggests that integrin structure resembles that of heterotrimeric GTP-binding proteins (G proteins).

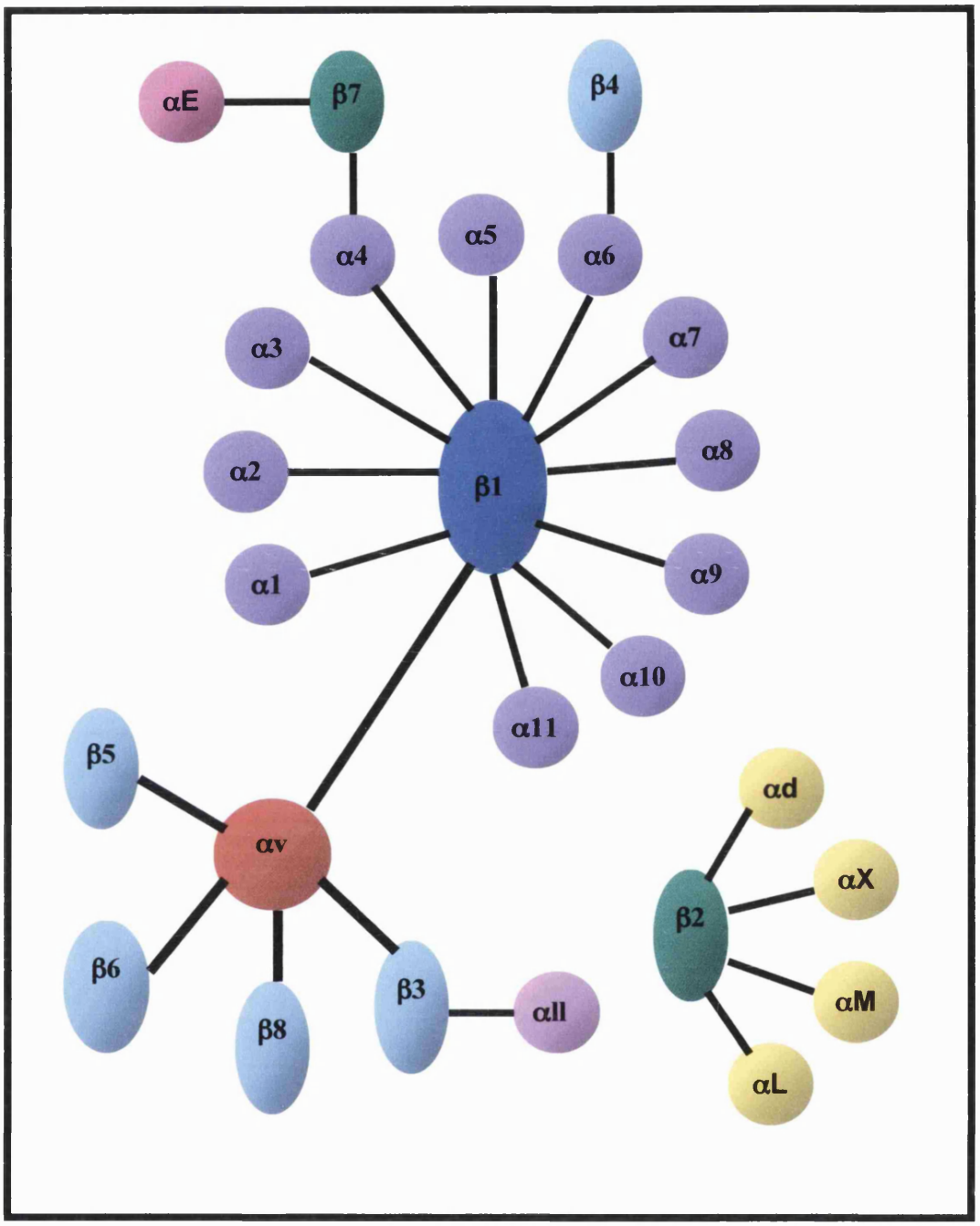


Figure 1.2 Integrin Subunit Association

Eighteen α and 8 β integrin subunits associate to form 23 different heterodimeric combinations, with the αv and β1 subunits being particularly promiscuous.

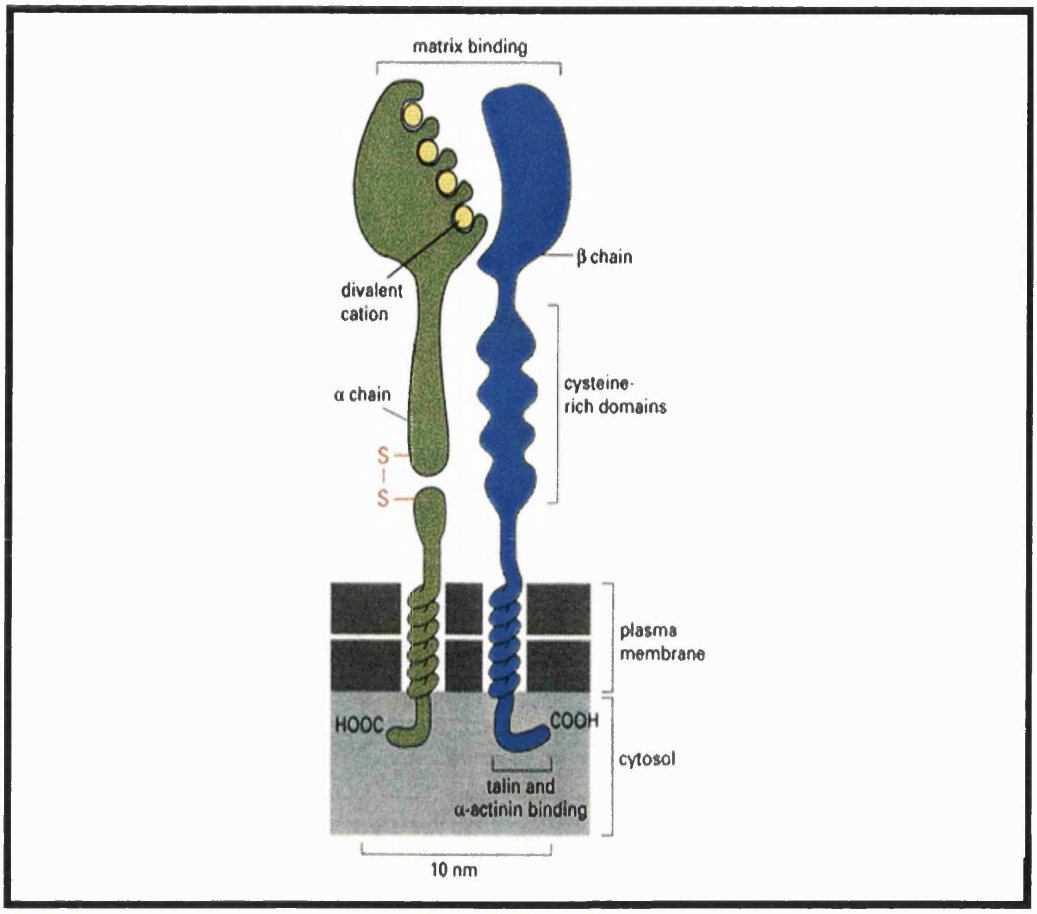


Figure 1.3 Integrin Structure

Integrins consist of a large globular head, which is extracellular and two tails which span through the plasma membrane to the cell interior. Integrin heterodimers are composed of an α and a β subunit, which both have a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain (diagram taken from Alberts et al, 1994).

1.2.2 INTEGRIN-LIGAND INTERACTIONS

The extracellular domains of integrins bind to various proteins within the ECM and the intracellular domains connect directly to the actin cytoskeleton, therefore integrins serve as integrators of the ECM and cytoskeleton (Hynes, 1992). Extracellular matrices are made up of an insoluble meshwork of protein and carbohydrate that is laid down by cells and fills most of the intercellular spaces. Matrices in different locations in the body consist of different combinations of collagens, proteoglycans, elastin, hyaluronic acid and various glycoproteins such as fibronectin and laminin (Ruoslahti and Pierschbacher, 1986).

The variations in subunit associations of the integrins confer ligand binding specificity so that different integrins can recognise the same ligand (for example, at least eight integrins bind to fibronectin (integrins $\alpha5\beta1$, $\alpha4\beta1$, $\alpha4\beta7$, $\alpha v\beta1$, $\alpha v\beta6$, $\alpha v\beta8$, $\alpha v\beta3$ and $\alpha IIb\beta3$) and five bind to laminin ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$ and $\alpha6\beta4$). Conversely integrins composed of a common subunit can bind to several ligands. This is most notably demonstrated by the $\beta1$ subunit, which can bind to fibronectin, laminin and collagen (Hynes, 1992, Ruoslahti and Pierschbacher, 1987). In addition, each integrin heterodimer is specific for a unique set of ligands, for example $\alpha v\beta3$ binds a wide range of ECM molecules, including fibronectin, fibrinogen, von Willebrand factor, vitronectin and proteolysed forms of collagen and laminin, whereas integrin $\alpha5\beta1$ selectively binds fibronectin (van der Flier, 2001). This suggests a degree of compensation amongst integrins, so that they may have overlapping functions. Despite this apparent redundancy, many integrins have unique

and irreplaceable functions within cells (Hynes, 1999 and 2002). Genetically altered mice have provided great insight into defining integrin functions. β1 is a promiscuous integrin, unsurprisingly genetic ablation of β1 causes embryonic lethality (Fassler and Meyer, 1995, Stephens et al, 1995) and it is also involved in angiogenesis (Bloch et al, 1997). Mice lacking β3 are viable and fertile (Hodivala-Dilke et al, 1999) as are mice lacking β5, β6, or any pairwise combination of β3, β5 and β6 (Huang et al, 2000, Huang et al, 1996, Reynolds et al, 2002). Genetic ablation of the αv integrin subunit in mice, thus eliminating all five αv integrins, allowed considerable development and organogenesis, particularly vasculogenesis and angiogenesis. This suggests that the blood supply required to sustain tumour growth is not inhibited by the absence of αv integrins (Bader et al, 1998). Results from genetic studies could underestimate the importance of αv integrins because of overlapping functions or some sort of compensation among the integrins. Future experiments, employing cell-type specific and/or regulated gene ablation may provide additional information on integrin functions.

The binding of integrins to extracellular ligands is a dynamic and highly regulated event involving the induction of conformational changes within the integrin structure, which can alter ligand-binding affinity (Humphries, 2002). A common feature of integrin ligands is a short Arg-Gly-Asp (RGD) peptide sequence as an essential part of the receptor recognition site. This motif is present in a number of ECM proteins including fibronectin and vitronectin and has been identified as the major ligand-binding site (Ruoslahti and Pierschbacher, 1987). More recently, it has been shown

that RGD collaborates with additional 'synergy' peptidic sites to generate full adhesive activity. Therefore, it seems likely that a ligand interacts with an integrin at two or more distinct sites within the heterodimer (Aota et al, 1994, Newham and Humphries, 1996). Several regions in both the α and β integrin subunits are thought to act together to form the ligand binding surface. The α A-domain, the β A-domain and N-terminal repeats all contain regions critical for the binding of ligands, although evidence is still lacking as to how these structures are organised and how they interact to form a ligand binding pocket (Fernandez et al, 1998).

1.2.3 INTEGRIN FUNCTION

Integrins were primarily thought to play structural roles as cell adhesion molecules, linking the internal cytoskeleton to extracellular matrix proteins. It is now clear that integrins also play important roles in developmental processes by transmitting molecular signals regarding the cellular environment to influence cell behaviour, cell shape, cell migration, tissue architecture, cell survival, cell proliferation and gene transcription (Aplin et al, 1999). Integrins also cooperate with growth factors to promote cell proliferation and they regulate adhesion, which is necessary for cells to exit from the cell cycle and differentiate. Integrins are able to influence cell survival by mediating apoptosis, since release of adherent cells from the surrounding ECM causes them to forfeit survival signals. They are also able to regulate gene transcription by activation of intracellular signalling pathways.

1.2.4 INTEGRINS AND CELL MIGRATION

Cell migration is central to many biological and pathological processes including embryogenesis, the inflammatory response, tissue repair and regeneration and cancer. It can be viewed as a multi-step cycle, which involves membrane extension and formation of lamellipodia at the leading edge of a cell. These penetrate and form stable attachments to the target tissue's matrix proteins via integrins, followed by cell contraction and translocation of the cell body forward so that existing ECM contacts are broken at the trailing edge and there is retraction at the cell rear. This allows the cell to pull itself forward into the tissue (Lauffenburger and Horwitz, 1996). Extension of lamellipodia is induced by actin polymerisation and facilitated by a localised decrease in membrane tension (Raucher and Sheetz, 2000). Retraction of the cell edge is dependent on the adhesive environment and occurs by breaking the cell-ECM linkage in highly adhesive environments, during slow migration or by simple dissociation of integrins, in less adhesive environments, during fast migration (Palecek et al, 1998, Palecek et al, 1999).

1.2.5 INTEGRIN SIGNALLING

In addition to their structural role, integrins also regulate intracellular signalling pathways that modulate cellular functions. These signalling pathways are induced following binding of integrins to their ligands. Integrin ligands are generally immobilised, consequently the signalling is usually localised to discrete regions of the plasma membrane. Integrins are able to signal through the cell membrane in either direction, and therefore are known as bi-directional signalling receptors (Giancotti

and Ruoslahti, 1999). The extracellular ligand binding activity of integrins, i.e. adhesive affinity is modulated in response to cellular physiology from the inside of the cell (inside-out signal transduction). In this process, there is interaction of specific intracellular proteins with the integrin cytoplasmic tail to promote a structural change in the integrin conformation that is transmitted across the plasma membrane to the extracellular domain, causing altered ligand-binding affinity. Alternatively, the binding of the ECM ligand may elicit signals that are transmitted into the cell to induce changes in protein activities or gene expression (outside-in signal transduction).

As integrins cooperatively bind to the multivalent ECM ligands, they become clustered in the plane of the cell membrane at distinct sites known as focal contacts or focal adhesions. These contain ECM proteins, integrins and cytoskeletal proteins, which assemble into aggregates on each side of the membrane and provide a link between the ECM proteins and the cytoskeletal-signalling complex (Burridge and Chrzanowska-Wodnicka, 1996). The different actin-associated proteins present within this complex include, α-actinin, vinculin, tensin and paxillin, which promote assembly of actin filaments figure 1.4). The reorganization of actin filaments into larger stress fibres, in turn causes more integrin clustering thus enhancing matrix binding in a positive feedback system (Sastry et al, 2000).

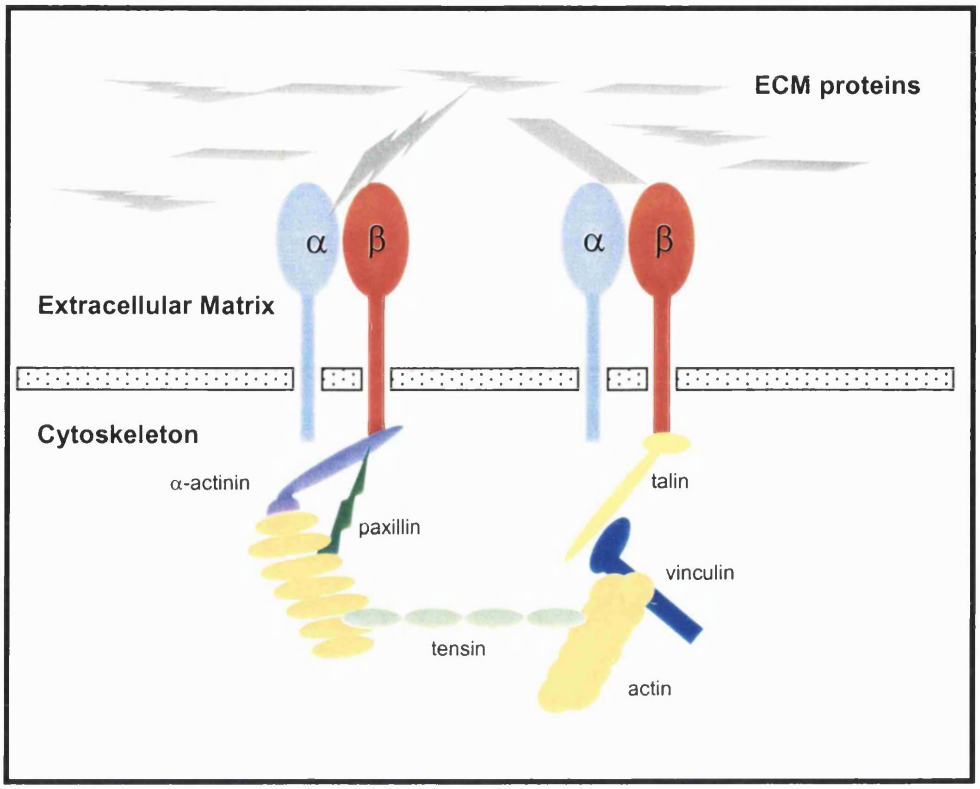


Figure 1.4 Transmembrane connections between the extracellular matrix (ECM) and the cytoskeleton. Ligand activation of integrins by ECM proteins induce the recruitment of cytoskeletal proteins which promote the assembly of actin filaments, in turn inducing integrin aggregation at the cell membrane.

The cytoplasmic tails of integrins provide anchors for the actin cytoskeleton and are generally short and always devoid of enzymatic features, therefore integrins require an association with adaptor proteins that connect the integrin to the cytoskeleton, cytoplasmic kinases, and trans-membrane growth factor receptors in order to transduce signals (Giancotti and Ruoslahti, 1999). The detailed sequence of events following ECM binding to the integrin is unclear but it is thought that integrins undergo a conformational change, which allows the intracellular β-subunit cytoplasmic domain to interact directly with focal-adhesion proteins such as talin and α-actinin, which then interact with both vinculin and paxillin (figure 1.4, Liu et al, 2000). However, it is now becoming clear that, like binding to the ECM, integrin signalling is determined by both α and β subunits (Giancotti, 2000).

Many, perhaps all integrins except α6β4, use a core signalling machinery that can regulate the actin cytoskeleton and activate the mitogen-activated protein kinase (MAPK) pathway (Howe et al, 1998). Subsets of integrins and even individual integrins recruit specific signalling components. The protein tyrosine kinase, focal adhesion kinase (FAK) is central to many integrin-mediated intracellular signalling events, regulating cell adhesion and/or migration (Schlaepfer and Hunter, 1998). Following attachment to the ECM, FAK localises to focal adhesions where it is tyrosine phosphorylated. It then combines with Src-family kinases (Src or Fyn), which phosphorylate paxillin and p130 cas. Both of these molecules are then able to initiate a signalling cascade by recruiting various adaptors and signalling intermediates. There are a vast number of signalling proteins linked to integrin

activation. These include small GTPases, protein tyrosine kinases, Src family kinases and adaptor proteins (Table 1.1). The mechanism by which these proteins are activated, how they couple with each other, and how their activation by integrins affects different cell functions are still under investigation (Miranti and Brugge, 2002).

Small GTPases	Rho, Rac, cdc42
Protein tyrosine kinases	FAK
Src family kinases	Abl, Syk/ZAP, Csk, Ras, Raf, Mek, Erk, PKC, Cbl, Pyk2, protein kinase A, Etk, Ack2, LAR, PEST
Adaptor proteins	Crk, Nck, Grb-2

Table 1.1 Signalling Proteins linked to Integrin Activation.

Integrins often synergise with growth factor receptors to enhance their activity since they have many common elements in their signalling pathways. In addition, they are able to regulate sodium-proton antiporters and protein kinase C has been shown to associate with integrin-containing focal adhesions. Integrins are also able to inhibit or activate other integrins, resulting in local modulation of cell adhesiveness. Therefore, there is the potential for a broad range of integrin signals however, if uncontrolled they may result in tumourigenesis. The MAPK pathway is particularly important in promoting cancer growth *in vivo*, since MAP kinases have been shown to be important in experimental tumour metastases (Mansour et al, 1994) and are

highly activated during the late progression of colorectal cancer (Licato et al, 1997). High frequencies of MAP kinase activation have also been observed in a large study of primary tumours of diverse origins (Hoshino et al, 1999) and very significantly, a highly potent inhibitor of MAP kinase activation has been identified which is capable of inhibiting human cancer growth in immune-deficient mice (Sebolt-Leopold et al, 1999).

The MAP kinase pathway is initiated by activation of Ras leading to the sequential stimulation of the protein kinase Raf. This is then able to activate one of the three separate MAPK pathways: activation of MEK followed by the extracellular signal regulated kinases, ERK1 and ERK2, the p38 MAPK pathway and finally the Janus kinase (JNK) pathway. A number of integrin and growth-factor signals converge at multiple points within these pathways, suggesting that there is a great deal of complexity within integrin signalling. This complexity, where multiple and possibly parallel and intersecting pathways activate specific signalling proteins is best illustrated for ERK activation. Although FAK is capable of activating ERK through the recruitment of Grb2, Shc or Src, other mechanisms that result in ERK activation have also been described. These include integrin coupling with caveolin leading to the recruitment of Fyn resulting in Shc phosphorylation and transactivation of the epidermal growth factor (EGF) receptor by integrins (Schwartz and Ginsberg, 2002, Giancotti and Ruoslahti, 1999, Howe et al, 1998, Hynes, 2002). Figure 1.5 shows a summary of integrin-mediated signalling of the MAP Kinase pathways.

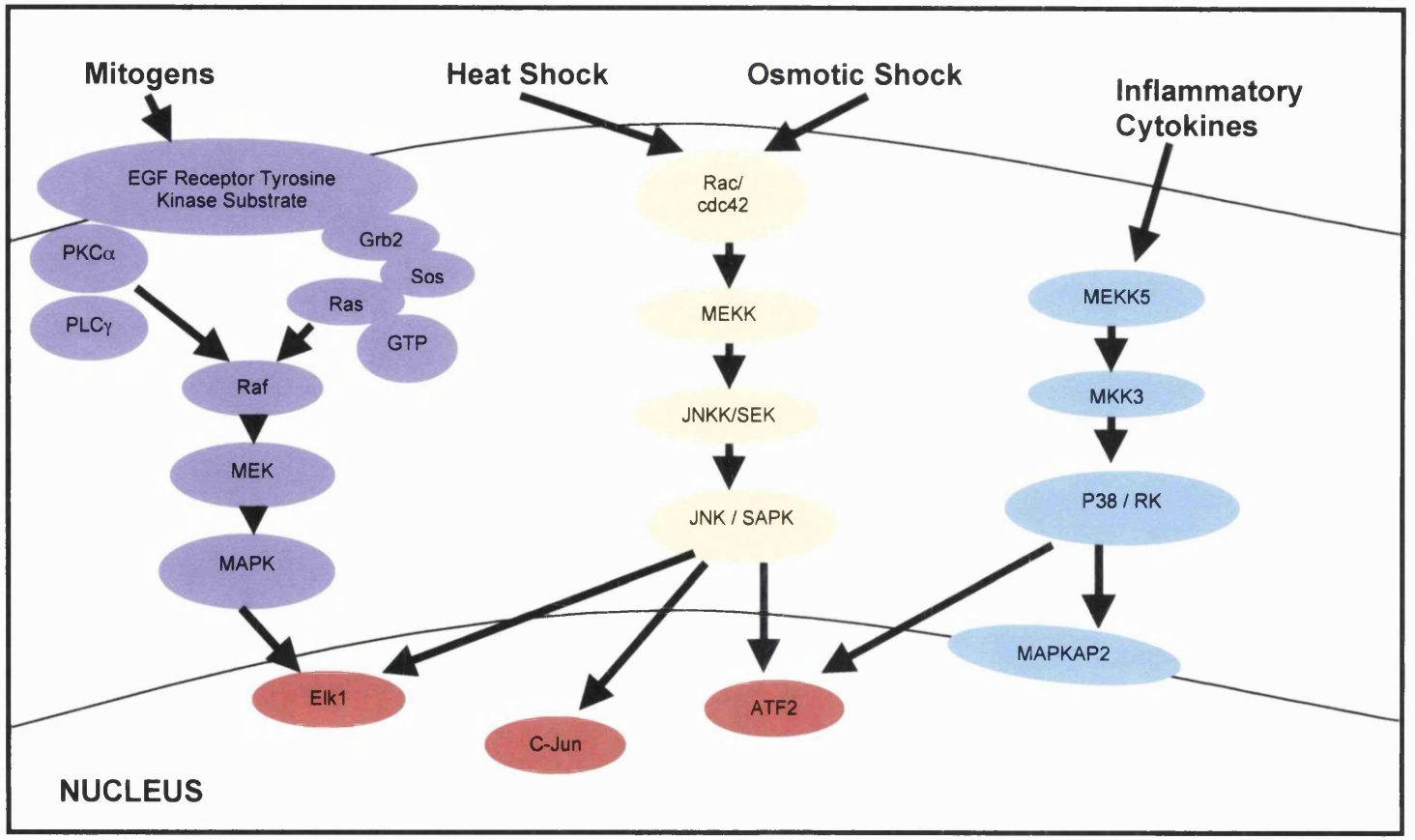


Figure 1.5 Integrin Mediated Signalling of the MAP Kinase Pathways

A range of stimuli via integrins can activate the three intracellular MAP Kinase signalling pathways. This leads to the activation of transcription factors within the nucleus resulting in regulation of gene expression and function.

1.2.6 INTEGRIN CYTOPLASMIC DOMAINS

The cytoplasmic domain of integrins is fundamental for gene expression, cell proliferation and cell cycle regulation (Clark and Brugge, 1995). Integrin cytoplasmic domains bind directly to many different cytoskeletal and regulatory proteins, which affect integrin functions, such as the regulation of binding affinity, signal transduction and integrin-mediated uptake of ligands (Calderwood et al, 2002, Han et al, 2001a, Liu et al, 2000). This is dependent on the integrity of the cytoplasmic domain of the β-subunit (Dedhar and Hannigan, 1996, Hynes et al, 1992, O'Toole et al, 1995), since several cytoskeletal proteins including talin, α-actinin, filamin and paxillin have been shown to interact with integrin β subunits (Horwitz et al, 1986, Loo et al, 1998, Otey et al, 1990, Sharma et al, 1995, Hughes and Pfaff, 1998). In addition, several intracellular signalling proteins have been reported to associate with integrin β cytoplasmic domains (Table 1.2). These include cytohesin-1, FAK, ILK, β3-endonexin, ICAP-1. Most recently 14-3-3 proteins, which are proto-oncogene and oncogene products have been shown to bind to β1 cytoplasmic domains by yeast two hybrid screen (Han et al, 2001b). The NXXY motif, which is located within 30 residues of the transmembrane domain is highly conserved in the cytoplasmic domains of β1, β2, β3, β5, β6 and β7 integrins (Fernandez et al, 1998). This sequence is thought to be important in controlling integrin affinity by its involvement in inside-out signalling and its interaction with cytoskeletal components. Mutations that disrupt this motif have been shown to interfere with both integrin activation (O'Toole et al, 1995) and talin binding (Calderwood et al, 1999). It is thought that the β-turn formed by the NPXY motif in integrins is required for the

activation of the phosphotyrosine-like domain of talin. The α-subunit cytoplasmic domains have also been shown to be important in regulating integrin-mediated signalling events (Giancotti, 2000) and have been shown to bind to the calcium binding proteins, calreticulin and CIB.

CYTOPLASMIC PROTEINS	INTEGRIN CYTOPLASMIC INTERACTION	REFERENCE
Cytohesin-1	β2	Kolanus et al, 1996
FAK	β-subunits	Schaller et al, 1995
Integrin-linked kinase (ILK)	β1, β2 and β3	Hannigan et al, 1996
β3-endonexin	β3	Shattil et al, 1995
Integrin cytoplasmic domain-associated protein-1 (ICAP-1)	β1	Chang et al, 1997, Zhang and Hemler, 1999
14-3-3 proteins	β1	Han et al, 2001b
Calreticulin	α-subunits	Coppolino et al, 1995
Calcium and integrin binding protein (CIB)	αIIb	Naik et al, 1997

Table 1.2 Proteins which interact directly with integrin cytoplasmic domains

1.2.7 INTEGRIN RECYCLING

Many surface receptors, including integrins participate in an endo/exocytic cycle (Bretscher, 1992). They are internalised, delivered to endosomes and then recycled to the plasma membrane for re-utilisation. It is suggested that this cycle may facilitate focal complex assembly by internalising integrins at the rear of the cell and transporting them forward within vesicles for exocytosis at the leading edge, indicating that integrin recycling is most prominent in situations where there is cell locomotion (Bretscher, 1996). The precise mechanism of integrin recycling is as yet unclear, but it may be that sorting signals on the early endosome may regulate recycling. A mechanism by which growth factors can regulate integrin recycling has recently been described. Following treatment with platelet derived growth factor (PDGF), $\alpha v\beta 3$ integrin, but not $\alpha 5\beta 1$, was rapidly recycled directly back to the plasma membrane from the early endosomes via a rab4-dependent mechanism (Roberts et al, 2001). The integrin endo/exocytic cycle has also been shown to be functionally important, influencing cell adhesion and motility (Roberts et al, 2001, Fabbri et al, 1999, Ng et al, 1999).

1.2.8 INTEGRIN-INTERACTING MEMBRANE PROTEINS

As well as interacting with molecules on neighbouring cells or the ECM, integrins can also form cis interactions with other receptors on the same cell to form multi-receptor complexes. These complexes recruit signalling molecules to sites of cell-cell or cell-matrix adhesion, such as focal complexes or focal adhesions. Several membrane proteins, referred to as integrin-associated proteins stably couple with

integrins and modulate integrin responses. These include urokinase-type plasminogen activator receptor (uPAR), integrin-associated protein (IAP; CD47), tetraspanin proteins (TM4SFs), growth factor receptors (PDGF), insulin and EGF receptors) and syndecan heparan sulphate proteoglycan receptors. These proteins have been shown to regulate intracellular signalling and control integrin adhesion, migration, invasiveness and matrix assembly (Porter and Hogg, 1998).

uPAR is attached to the cell membrane via a glycosyl phosphatidylinositol (GPI)-anchor and focuses proteolytic activity to this site. uPAR has been described as a signalling molecule, which regulates cellular functions such as adhesion and migration independent of its proteolytic role. The lack of a transmembrane domain in uPAR suggests an association with adaptor proteins in order to transmit molecular signals between the cells interior and exterior. A number of studies have shown physical and functional associations of uPAR with various integrins (Preissner et al, 2000, Chapman and Wei, 2001), which is discussed in detail in section 1.5.

Integrin-associated protein (IAP; CD47) is a single-chain protein that has five membrane-spanning domains and a short cytoplasmic tail. It has been shown to be associated with αvβ3 and αIIbβ3 as well as having a potential association with αvβ5 (Lindberg et al, 1996). More recently, IAP has been shown to associate with α2β1 in human smooth muscle cells (Wang and Frazier, 1998). It is suggested that IAP does not modulate the activity of the β1 and β3 integrins by direct physical interaction but

actually forms a functional membrane unit that signals through heterotrimeric G-proteins (Chung et al, 1997).

The transmembrane-4 superfamily (TM4SF), also called tetraspans include at least 21 different proteins, which are expressed on cell membranes. They have four membrane-spanning domains, resulting in two extracellular loops and intracellular N- and C-termini (Hemler et al, 1996). The tetraspans CD9, CD53, CD63, CD81, CD82, CD151/PETA-3 and NAG-2 have been shown to physically associate in separate complexes with certain integrins as demonstrated by reciprocal co-immunoprecipitation studies (Hemler et al, 1996, Yanez-Mo et al, 1998, Tachibana et al, 1997). The integrins that have been identified in complexes with tetraspans are $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 6\beta 1$, $\alpha 4\beta 7$ and $\alpha IIb\beta 3$ (Hemler et al, 1996). These tetraspan-integrin complexes vary depending on cell-type, and one integrin can associate with more than one tetraspan (Mannion et al, 1996). Tetraspan-integrin complexes have been observed in membrane lamellipodia at the leading edge of migrating cells where recycling of integrin occurs (Berditchevski et al, 1995) and it has been shown that in cells with reduced levels of tetraspans, the rate of integrin recycling is substantially decreased (Berditchevski et al, 1997). Therefore tetraspans have a potentially important role in integrin recycling and integrin localisation on the cell surface and may affect integrin-mediated events such as cell adhesion, motility and metastatic spread.

The localisation of growth factor receptors to focal adhesion complexes and the fact that adhesive interactions are essential to both cell cycle progression and proliferative responses initiated by growth factor receptors (GFRs), suggests a possible association of GFRs with integrins (Miyamoto et al, 1996). GFRs appear to be used by integrins for signal transduction modulated by cell adhesion suggesting a functional association, however no direct physical association has been shown.

Caveolae are plasma membrane invaginations present in most cells (Bohuslav et al, 1995). They are involved in transcytosis and recruit GPI-linked receptors and signalling proteins upon clustering or activation (Okamoto et al, 1998). The main structural component of caveolae is caveolin, a 22kDa membrane-spanning protein, which associates with signalling molecules, such as heterotrimeric G proteins, Ha-Ras and Src-family tyrosine kinases, via its N-terminal cytosolic domain. A functional and physical interaction between β1 integrin, caveolin and uPAR has been described in a model system of uPAR-transfected human kidney cells (Wei et al, 1999). Although only a small pool of the total β1 integrins is involved, the formation of these complexes switches the ligand specificity of cell binding from integrin-mediated fibronectin binding to uPAR-mediated vitronectin binding, emphasising the significance of even a small number of integrin-partner complexes.

1.2.9 INTEGRINS IN CARCINOMA

Carcinomas are characterised by invasion of malignant cells into the underlying connective tissue and the ability of cells to metastasise to distant sites. In order for this to occur, a tumour cell must change its ability to interact with other cells and the ECM. This may be associated with changes in integrin expression and function. In addition to invasion, integrins may also affect malignant transformation by modulating cell proliferation, apoptosis and gene expression. Altered integrin expression has been reported in epithelial tumours, including squamous and basal cell carcinomas of the skin and oral cavity and adenocarcinomas of the colon, breast and pancreas (Jones et al, 1997, Thomas and Speight, 2001. Changes in integrin expression are thought to be important in the progression of head and neck carcinoma. Oral squamous cell carcinoma (SCC) accounts for 96% of all tumours of the oral cavity and is characterised by local invasion of malignant cells into the underlying connective tissue. Integrin expression is altered in SCCs but the expression patterns are variable, both between tumours and in different regions of the same tumour. Furthermore, within a given tumour different integrins may have different expression patterns (Thomas et al, 1997). The tumour stroma is a specialised form of tissue that is associated primarily with neoplasms of epithelial origin with mesenchymal-epithelial interactions being important during carcinogenesis (van Roozendaal et al, 1992). Normal epithelial cells generally express the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins as major receptors for basement membrane components as well as the $\alpha 6\beta 4$ integrin which is associated with hemidesmosomes. These integrins are frequently lost or redistributed in poorly differentiated SCC,

concomitant with loss of basement membrane anchorage and normal epithelial organisation (Jones et al, 1993, Downer et al, 1993).

The αv integrin subfamily is important in the progression of epithelial carcinomas. αv integrins, which primarily recognise components of the interstitial extracellular matrix (e.g. vitronectin, fibronectin, tenascin, osteopontin, laminin and collagen) display a complex expression pattern in carcinomas. The αv integrin subunit is only weakly expressed in normal oral mucosa (Jones et al, 1993) but expression is markedly increased during wound healing (Haapasalmi et al, 1996, Larjava et al, 1993, Larjava et al, 1996). The αvβ3 integrin is not detected in most carcinomas, but was shown to play a role in prostate cancer (Cooper et al, 2002) and breast cancer metastasis [Felding-Habermann et al, 2001. It seems that αvβ3 is more important in the progression of melanoma since αvβ3 is strongly expressed at the invasive front of malignant melanoma cells and angiogenic blood vessels but weakly expressed on pre-neoplastic melanomas and quiescent blood vessels (Brooks et al, 1994). Furthermore, inducing the expression αvβ3 in a melanoma cell line increases metastatic potential (Felding-Habermann et al, 1992, Filardo et al, 1995). αvβ3 is also up-regulated significantly during the latter, more invasive stages of both melanoma and glioblastoma (Felding-Habermann et al, 2002).

1.2.10 αvβ6 IN CARCINOMA

The integrin αvβ6 is an epithelial cell-restricted integrin that binds to RGD sites in fibronectin, vitronectin and tenascin and the TGFβ latency-associated peptide (LAP) (Busk et al, 1992, Prieto et al, 1993, Huang et al, 1998, Munger et al, 1999). The αvβ6 integrin is unusual in that it is either absent or expressed at very low levels in fully differentiated normal epithelia with no expression on keratinocytes in adult oral epithelium or epidermis. However, it becomes highly expressed during times of morphogenesis, tissue repair, inflammation and tumourigenesis (Breuss et al, 1995). Over the last few years, evidence has accumulated to suggest that αvβ6 may play a role in tumour progression. αvβ6 is the most commonly up-regulated integrin in epithelial tumours with *de novo* expression of αvβ6 seen in malignant colonic and oral epithelium (Breuss et al, 1995, Jones et al, 1997). In oral SCC, this expression was correlated with a downregulation of αvβ5 (Jones et al, 1997). More recently, several studies have found high expression of αvβ6 in oral squamous cell and other carcinomas such as colon, lung, advanced stages of breast carcinoma and ovarian carcinoma (Arihiro et al, 2000, Hamidi et al, 2000, Ahmed et al, 2002a). αvβ6 appears to promote the transition to a malignant phenotype, since expression was seen in a high percentage of oral epithelial dysplasias where it was correlated with disease progression. Also, induction of αvβ6 expression in oral leukoplakia appears to be a necessary pre-requesite for progression to squamous cell carcinoma (Hamidi et al, 2000). A role for αvβ6 in tissue repair and/or remodelling was suggested since increased αvβ6 expression was seen on wound keratinocytes (Haapasalmi et al, 1996). Huang et al, (1998) also demonstrated this since a strong expression of αvβ6

was found in cultured cells exposed to an environment that mimicked a wound. Moreover, αvβ6 is concentrated at the invading margin of epithelial malignancies, which is where matrix-degrading enzymes appear to be concentrated (Breuss et al, 1995, Hewitt et al, 1991). Somewhat paradoxically cutaneous wounds heal uneventfully in β6-/- mice (Huang et al, 1996), although in this situation early deletion of β6 may lead to other integrins fulfilling the role of the αvβ6 heterodimer.

αvβ6 modulates several processes in keratinocytes, including migration on fibronectin and vitronectin (Huang et al, 1998) and also fibronectin-dependent upregulation of MMP-9 (Thomas et al, 2001a and 2001c). Increased invasion in oral keratinocytes was also demonstrated to be αvβ6-dependent (Thomas et al, 2001b). A recent study has shown that the progression of oral squamous carcinoma *in vivo* can be significantly impeded using inhibitory anti-αvβ6 antibodies (Xue et al, 2001).

1.2.10.1 Identification of the β6 integrin subunit.

The complete sequence of the β6 integrin subunit was identified over a decade ago (Sheppard et al, 1990). Amplification of guinea pig airway epithelial cell DNA with oligonucleotide primers designed to recognise consensus integrin β subunit sequences led to the identification of a novel partial cDNA sequence. Clones containing portions of this sequence were used to screen cDNA libraries constructed from the human pancreatic carcinoma cell line FG-2 and identified a series of overlapping clones encoding the full-length sequence of the human homologue of this protein. This sequence of 788 amino acids produces a novel protein of 106 kDa. It is 43, 38

and 47% identical to the sequences of β1, β2 and β3 respectively. As in each of the other integrin β subunits, the novel β6 protein shares some features including the positions of all the 56 cysteine residues in the extracellular domain, a single transmembrane domain and a short cytoplasmic domain. However, the β6 subunit contains a unique 11-amino acid extension at the carboxyl terminus, which is not present in any of the other β-subunits, suggesting that it distinctly interacts with cytoplasmic components.

1.2.10.2 β6 cytoplasmic tail

The short cytoplasmic tails of β1, β2 and β3 have been suggested as important sites of interaction with the cytoskeleton and are probably critical regions for the transduction of signals initiated by interactions of the large extracellular domains with ligands. The cytoplasmic tails may also be important targets for regulation of integrin function. The portion of the β6 cytoplasmic domain that is homologous to β1 and β3 is sufficient to support functions that are shared with β1 and β3 integrins, i.e. localisation to focal contacts and promotion of cell adhesion and spreading. The unique extension of the cytoplasmic tail of β6 (figure 1.6) implies that its regulation or its pathways for signal transduction may be different from those of β1, β2 and β3. The unique 11-amino acid extension of the β6-subunit was shown to be critical for αvβ6-mediated stimulation of cell proliferation (Agrez et al, 1994) as well as inducing MMP-9 secretion in colon cancer cells as shown by cytoplasmic tail deletion mutants (Niu et al, 1998).

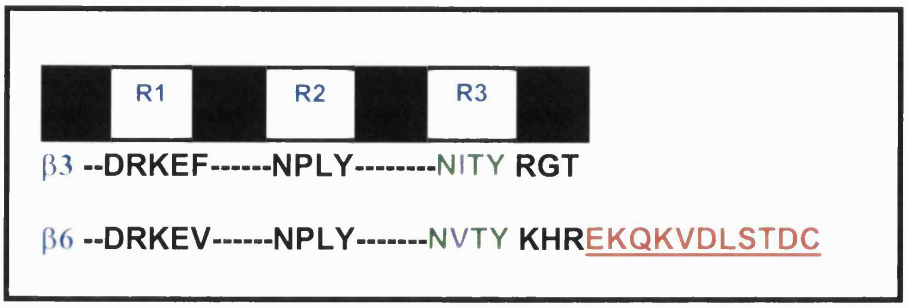


Figure 1.6 Sequence Homology between the β3 and β6 Cytoplasmic Domains

There is high sequence homology between the β3 and β6 integrin cytoplasmic domains. Within the three highly conserved regions of β6 and β3, there is only a difference of 2 amino acids. In addition, β6 has a unique 11 amino acid cytoplasmic extension.

1.3 PROTEASES

Plasminogen activation, catalysed by the serine proteases uPA (urokinase-type plasminogen activator) and tPA (tissue-type plasminogen activator), is thought to play an important role in a variety of physiological and pathological processes. Proteolytic enzymes mediate the dissolution of the extracellular matrix that is necessary for the passage of migrating cells through tissue barriers in a range of biological processes such as trophoblast implantation, embryo morphogenesis, angiogenesis, inflammation, wound healing and tissue remodelling (Johnsen et al, 1998). In these situations, proteolysis is of limited extent and duration and therefore must be subject to strict regulation to initiate, localise and terminate the proteolytic activity (Ellis et al, 1992).

The serine proteases of the plasminogen activation system have traditionally been considered as part of the haemostatic mechanism owing to the dissolution of fibrin clots by plasmin (fibrinolysis). In the majority of conditions known to entail a risk of thrombosis, changes in the levels of fibrinolytic proteins are seen (Dobrovolsky and Titaeva, 2002). In atherosclerosis, smooth muscle cells proliferate and migrate through the vessel wall, which is associated with increased production of extracellular proteinases resulting in local lysis of the surrounding extracellular matrix (van Leeuwen, 1996). Furthermore, plasminogen activators (tissue-type and urokinase-type) and streptokinase are used clinically in the treatment of myocardial infarction (Bell, 2002, Baker, 2002).

Extracellular proteolysis may also contribute to changes in cell-surface proteins like antigens and receptors and therefore may control cellular functions including responses to growth factors and hormones, immunologic and inflammatory stimuli, cell migration, tissue degradation and involution, expansion of individual cell clones, invasion of normal tissues by monocyte-macrophage cells or by cancer cells (Blasi et al, 1986). The diverse targets of this proteolytic system have led to it being implicated in a range of pathologies, including tumour invasion and metastasis, arthritis, cardiovascular disease and neurodegeneration; all linked by their dependence on cell migration and tissue degradation or remodelling.

1.3.1 PROTEASES AND CANCER

A hallmark of cancer is the ability of malignant cells to cross tissue boundaries through proteolytic degradation. The release of proteolytic enzymes from tumours facilitates cancer cell invasion into the surrounding normal tissue by degradation of the basement membrane and extracellular matrix (Dano et al, 1985, Liotta et al, 1991, Mignatti and Rifkin, 1993), which involves proteases from several families. The major class of ECM-degrading enzymes are the matrix metalloproteinases and the serine proteases, which include the plasminogen activators. Increased production of these enzymes has been associated with the invasive and/or metastatic phenotype in many tumours (Reuning et al, 1998).

1.3.2 THE PLASMINOGEN ACTIVATION SYSTEM

Plasminogen activators released from cancer cells catalyse the proteolytic conversion of the inactive zymogen plasminogen to the active proteinase plasmin, which in turn catalyses degradation of proteins in basement membranes and extracellular matrix (ECM), thus facilitating cancer cell invasion into the surrounding tissue (for reviews see, Dano et al, 1985, Mignatti and Rifkin, 1993, Werb, 1997). Since plasmin has wide substrate specificity, unrestrained plasmin generation is potentially hazardous to cells; thus, the process of plasminogen activation in a healthy organism is strictly controlled through the availability of PAs, localised activation and interaction with specific inhibitors (PAIs). The ability of tumour cells to express an invasive phenotype depends in part on the balance between tumour proteases and their inhibitors and in several experimental and human tumours; the balance between proteases and their inhibitors is usually shifted towards a constitutive pericellular proteolysis (high proteases/low inhibitors) (Mueller et al, 1996).

There are two types of plasminogen activator, urokinase-type (uPA) and tissue-type (tPA). Despite their common enzymatic activities, the two PAs appear to play distinct roles *in vivo*. uPA plays a role in localised cell-associated proteolysis and is the enzyme of most relevance to tumour biology. tPA has a high affinity for fibrin and its enzymatic activity is enhanced by fibrin binding, so its primary role is the generation of plasmin for fibrinolysis in blood vessels and is the enzyme that is physiologically important in maintaining homeostasis of the fibrinolytic state (Blasi et al, 1986).

1.3.3 COMPONENTS OF THE PLASMINOGEN ACTIVATION SYSTEM

1.3.3.1 Urokinase-type plasminogen activator

uPA is produced by a variety of cell types including epithelial cells, fibroblasts and connective tissue cells from most organs and tissues including the gastrointestinal tract, lung, pancreas, kidneys, urinary tract, placenta and involuting mammary glands in normal physiological situations, with uPA being more highly produced by malignant tumour cells (Dano et al, 1985). The human uPA gene codes for a 53kDa serine protease produced as a single-chain protein (scuPA or pro-uPA) (Gunzler et al, 1982). Secreted pro-uPA is converted to the active two-chain form, by cleavage of a single peptide bond (Lys 158 - Ile 159) by plasmin (Dano et al, 1985). Other proteins including plasma kallikrein, blood coagulation factor XIIa and cathepsins also catalyse this reaction (Andreasen et al, 1997). The single-chain zymogen form, pro-uPA has 250-fold less activity than the two-chain form uPA, and pro-uPA bound to its specific cell-surface receptor (uPAR) is activated by plasmin much faster than when free in the fluid-phase (Ellis et al, 1992). This is accomplished by increased efficiencies of both plasminogen activation and the reciprocal activation of pro-uPA by plasmin (Ellis et al, 1991). uPA consists of two disulfide bridge-linked polypeptide chains containing three functional domains (figure 1.7). In the carboxyl-terminal region is the B-chain, which contains the serine protease domain (SPD, residues 144-411), making up most of the low-molecular weight (LMW) uPA with full specific activity. The non-catalytic amino-terminal fragment (ATF), corresponding to the A-chain, contains the kringle domain (residues 47-135) and the epidermal growth factor (EGF)-like domain (GF, residues 4-43). The two chains are

linked by a connecting peptide (reviewed by Andreasen et al, 2000). Thus, the uPA molecule consists of two parts: the catalytic carboxyl-terminal chain and the non-catalytic amino-terminal chain, of which the initial GF domain of 32 residues is responsible for the specific interaction with uPAR.

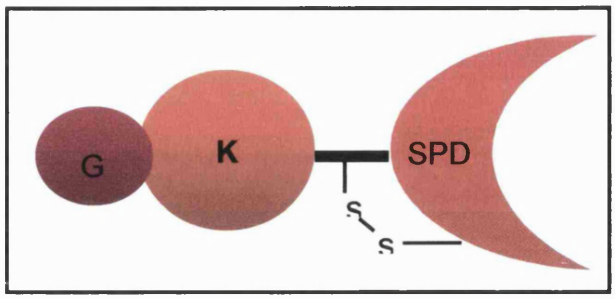


Figure 1.7 uPA Molecule

uPA consists of a B chain containing the serine protease domain (SPD), and an A chain containing a kringle domain (K) and a growth factor-like domain (G), linked by a connecting peptide.

The plasmin generated by uPA-catalysed plasminogen activation triggers a proteolytic cascade that involves the activation of MMPs, which are responsible for collagen degradation (Ossowski, 1992, Mazzieri et al, 1997). uPA is also able to degrade several extracellular matrix components, such as fibronectin (Alonso et al, 1996, Ellis et al, 1991, Keski-Oja and Vaheri, 1982). uPA is able to inactivate it's own specific receptor by cleaving a peptide bond between domains D1 and D2 of uPAR (Hoyer-Hansen et al, 1992) as well as cleaving it's own inhibitor PAI-1 in a plasminogen-independent manner (Andreasen et al, 1986, Nielsen et al, 1986). uPA

is also able to directly activate pro-hepatocyte growth factor/scatter factor (HGF/SF) (Naldini et al, 1992, Mars et al, 1993).

1.3.3.2 Tissue-type plasminogen activator

In contrast to the relatively widespread expression of uPA, tPA is expressed in very few cell types and constitutes only a small part of the total amount of protein in whole tissues, blood plasma and blood vessel perfusates (Dano et al, 1985). Vascular smooth muscle cells express significant levels of tPA subsequent to vascular injury (Clowes et al, 1990). tPA is a 70kDa protein which is secreted as a precursor in single-chain form which is enzymatically active (Pennica et al, 1993). Plasmin cleaves the peptide bond Arg275 – Ile276 to give a two-chain form held together by a single inter-chain disulfide bond. The tPA molecule is composed of four functionally distinct domains: (1) an amino-terminal region known as the fibronectin-like domain, (2) an EGF-like domain, (3) two kringle regions and (4) a serine protease region (figure 1.8). There is little evidence for a cellular receptor for tPA, although two broad classes of binding sites have been identified: those involved in tPA clearance such as the low-density lipoprotein receptor-related protein (LRP) and those involved in the regulation of tPA activity. These have been reported in a variety of cell types including vascular smooth muscle cells (Ellis and Whawell, 1997), hepatocytes (Bakhit et al, 1987), brain (Verrall and Seeds, 1989), endothelial (Fukao et al, 1997) and pre-implantation embryonic cells (Carroll et al, 1993), although these sites do not equal uPAR in terms of specificity or affinity. Due to its high affinity for fibrin and activation by fibrin binding, the main biological role of tPA seems to be associated

with fibrinolysis (Lijnen and Collen, 1982). Expression of tPA is observed in locations close to fibrin clots (Kooistra et al, 1994) and is induced in physiological situations of thrombosis, such as ischaemia (Schneiderman et al, 1991), wounding (Grondahl-Hansen et al, 1988) and ovulation (Deutinger et al, 1988). Unlike uPA, most malignant tumour cells do not show high tPA expression, although some tumour cell lines do secrete tPA, particularly those derived from melanomas (Dano et al, 1999). However, there may be interchangeability of the roles of uPA and tPA in some situations. This was demonstrated when either of the two genes were deleted from mice, skin wound healing proceeded normally, but when both genes were inactivated wound healing was severely impeded (Romer et al, 1996). Urokinase and tPA both play a role in the degradation of fibrin and thrombi *in vivo*, as shown with transgenic mice deficient in one or both of the plasminogen activators. Inactivation of the tPA gene impairs clot lysis, and inactivation of the uPA gene results in occasional fibrin deposition in the mouse, while plasminogen deficiency or combined tPA and uPA deficiency cause extensive fibrin deposition (Carmeliet et al, 1994, Carmeliet et al, 1995).

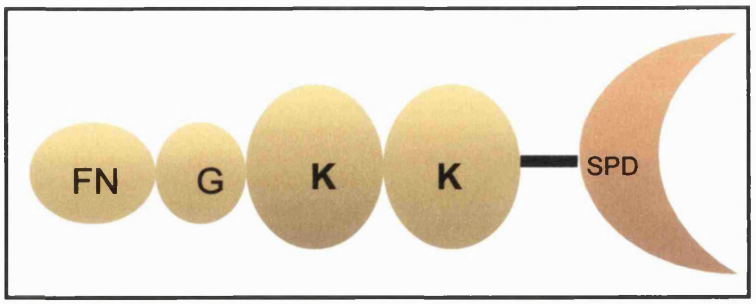


Figure 1.8 tPA Molecule

tPA is composed of four domains; a fibronectin-like domain (FN), an EGF-like domain (G) and two kringle regions which are linked to serine protease domain by a connecting peptide.

1.3.3.3 Plasminogen

Plasminogen is the one-chain zymogen counterpart of the serine protease plasmin (Mr approximately 90kDa). It is produced by the liver and has a circulating blood concentration of 2μM, but there is also a large pool of extravascular plasminogen (reviewed by Mignatti and Rifkin, 1993). The molecule comprises an N-terminal A chain, containing five kringle domains and a C-terminal B chain containing the serine protease domain that is responsible for catalytic activity (figure 1.9). Plasminogen is converted into plasmin by proteolytic cleavage of a single peptide bond (Arg561 – Val562), which can be catalysed by either uPA or tPA. Plasminogen has an activity 10^4- to 10^6-fold lower than that of plasmin. The lysine-binding sites on the kringle-domains of plasminogen enable binding to a wide variety of molecules via specific cellular binding sites (Plow et al, 1986, Miles et al, 1988). These lysine-binding sites of the individual kringles vary in structure, thus affinity and selectivity for lysine,

other aminocarboxylic acids and their derivatives (Rejante et al, 1991). The necessity for the cellular binding of plasminogen for efficient plasmin generation was demonstrated using aminocarboxylic acid analogues of lysine, that act as inhibitors of plasminogen binding (Ellis et al, 1989, Ellis et al, 1991).

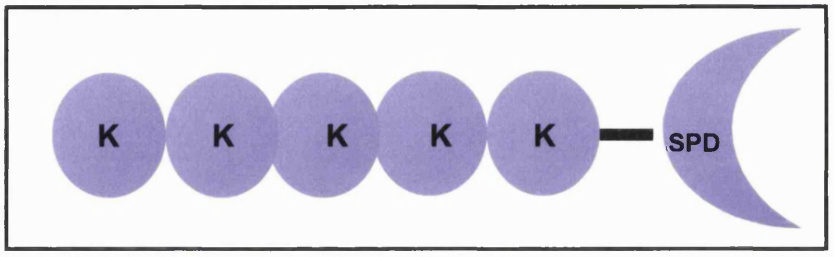


Figure 1.9 Plasminogen

The serine protease domain of plasminogen is responsible for catalytic activity and the five kringle domains harbour lysine-binding sites that enable binding to specific cellular targets.

1.3.3.4 The PA Inhibitors

Unrestrained proteolytic activity by even low levels of uPA is potentially hazardous for cells, therefore, as a form of protection cells secrete a surplus of inhibitor to prevent excessive pericellular proteolysis. The serine protease inhibitor superfamily (SERPIN) has amongst its members PAI-1, PAI-2, protease nexin 1, and protein C inactivator (PCI). Generally SERPINS are specific, fast-acting inactivators, present in most body fluids, tissues and cell lines (Potempa et al, 1994).

Plasminogen activator inhibitor type 1

PAI-1 is a single-chain 45-50kDa glycoprotein secreted by many cell types including epithelial cells and vascular smooth muscle cells. It reacts quickly with both uPA and tPA to inactivate them, however, it is rapidly transformed into a dormant conformation. High-affinity binding of PAI-1 to vitronectin stabilises the active conformation, enabling it to remain active in the matrix for prolonged periods of time, unlike the secreted form (Deng et al, 1995). PAI-1 reacts rapidly with free and uPAR-associated uPA (Ellis et al, 1990). By coupling with uPAR-bound uPA, PAI-1 is able to inhibit ECM degradation initiated by cell-associated uPA (Cubellis et al, 1989). PAI-1 activity is regulated by forming part of a quarternary complex of uPAR-uPA-PAI-1 with α2-MR/LRP or with gp330, which is internalised. uPA-PAI is then engulfed by lysosomes and digested, while uPAR is recycled to the cell surface (Planus et al, 1997, Nykjaer et al, 1997).

Plasminogen Activator Inhibitor Type 2 (PAI-2)

PAI-2 is a single-chain protein of 47kDa, which is able to inhibit both uPA and tPA, although it reacts more slowly than PAI-1 with its inhibitory potency towards receptor-bound uPA being 15 times less than that of PAI-1 (Ellis et al, 1990). PAI-2 has the unique feature of existing in both secreted and cytosolic forms (Kruithof et al 1995). In its secreted form, PAI-2 participates in the control of tissue remodelling and fibrinolysis. In its cytosolic form, it plays an important role in intracellular proteolysis involved in processes such as apoptosis and inflammation (Antalis et al, 1998).

1.3.3.5 Urokinase receptor (uPAR)

The specific high-affinity cellular receptor for uPA (uPAR), is a 55kDa molecule, which consists of three cysteine-rich, homologous, independently folded domains connected by short linker regions (figure 1.10), so stable receptor fragments with internal disulfide bridges can be produced by limited proteolysis (Ploug and Ellis, 1994, Kristensen et al, 1991). The amino-terminal domain (D1) has uPA-binding activity; the other two domains (D2 and D3) bind vitronectin (Hoyer-Hansen et al, 1992, Solberg et al. 1994). Both pro-uPA and uPA bind to uPAR present on the surface of many cell types (Blasi, 1986). These, along with DFP-inactivated uPA and ATF all bind with the same affinity to uPAR (Cubellis et al, 1986). uPAR can also bind integrins at sites distinguishable from its uPA and vitronectin-binding sites (Hoyer-Hansen et al, 1992b, Solberg et al, 1994). The presence of uPAR on the surface of cells leads to the formation of an efficient system for the activation of plasminogen. In kinetic terms, uPAR increases uPA activity by decreasing the Km for plasminogen activation by up to 200-fold (Ellis et al, 1991), which is dependent on the cellular binding of plasminogen since this effect can be abolished by aminocarboxylic acid lysine analogues and cannot be replicated with soluble forms of uPAR (Ellis, 1996). uPAR is anchored to the outer plasma-membrane by a glycosyl phosphatidyl inositol anchor (GPI) chain (Moller, 1993), although anchorless, soluble uPAR variants have also been identified in conditioned medium from various cell lines and in body fluids from cancer patients. This may arise by differential splicing, by proteolysis, or by phospholipase C cleavage of the GPI anchor (Kristensen et al, 1991). By targeting uPA to the plasma membrane, uPAR acts as a focal point for the

assembly of multi-molecular complexes, where uPA/uPAR interact with a variety of ligands, including plasminogen, vitronectin and integrins. This suggests a multi-functional role for uPAR, where it regulates efficient and spatially restricted plasmin generation. uPAR exhibits lateral mobility on the cell membrane so that during cell migration, it is redistributed to the leading edge to assist in the directional invasion of migrating cells (Kjoller, 2002). It is now becoming increasingly evident that uPAR, via signal transduction mediated events elicits a plethora of cellular responses that include cellular adhesion, differentiation, proliferation and migration in a non-proteolytic fashion (Yu et al, 1997, Ellis, 1997, Blasi and Carmeliet, 2002).

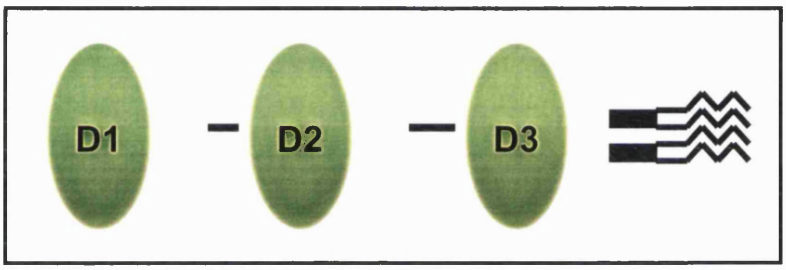


Figure 1.10 uPAR Molecule

uPAR consists of three cysteine homologous domains connected by short linker regions. The amino-terminal domain (D1) binds uPA, whereas domains (D2 and D3) bind vitronectin.

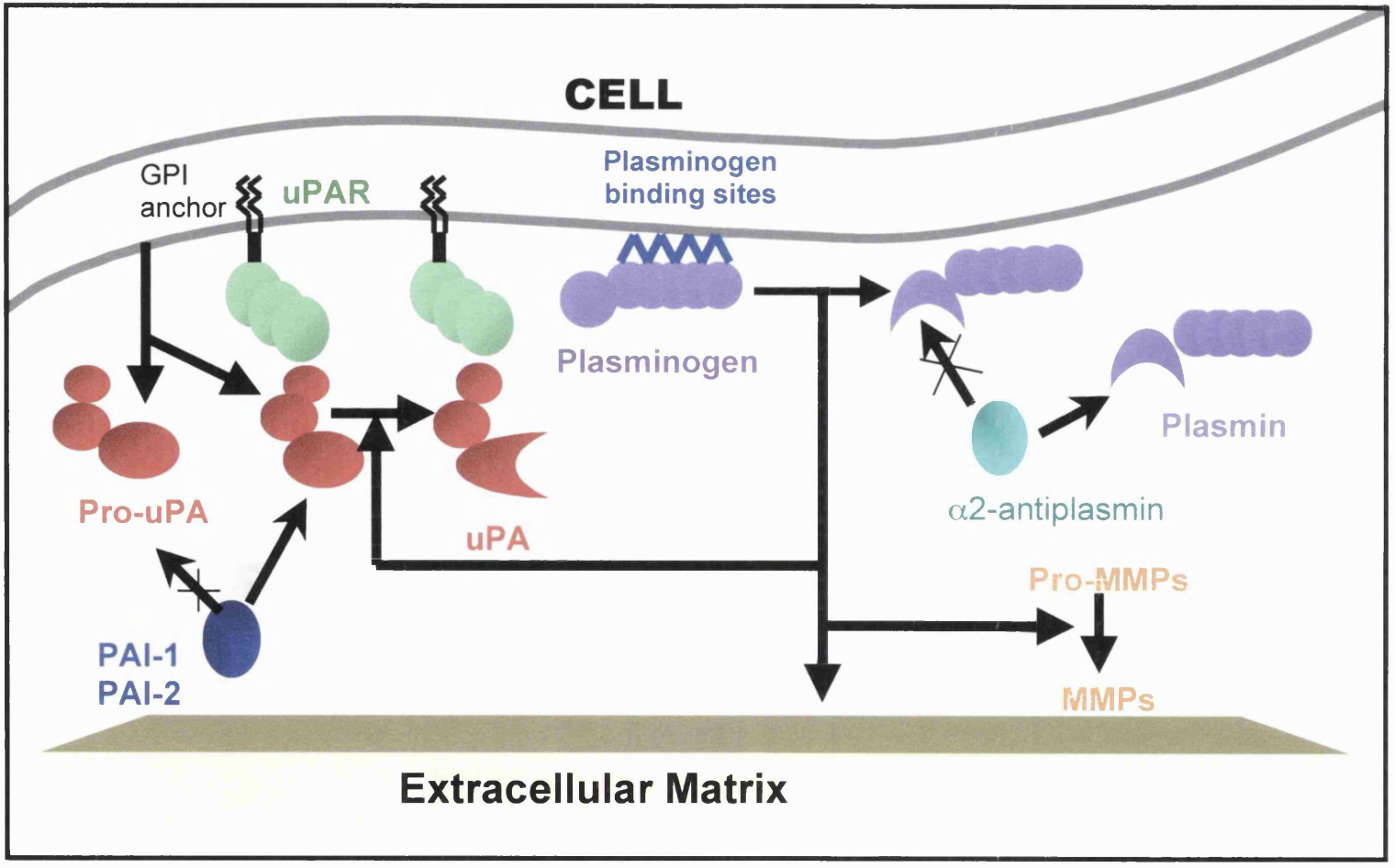
1.3.4 PLASMINOGEN ACTIVATION CASCADE

Plasminogen activation is a very efficient process due to the amplification of proteolytic activity resulting in a protease cascade, i.e. small amounts of plasminogen activator can generate large amounts of plasmin (Bass and Ellis, 2002). The activation of plasminogen by uPA occurs mostly at the cell surface. pro-uPA is secreted from the cell and binds to uPAR where it is converted to the active form. uPA then catalyses the cleavage of plasminogen bound to the cell surface into plasmin. The binding of secreted pro-uPA to uPAR leads to a reciprocal zymogen activation system in which uPA preferentially activates cell-bound plasminogen, and cell-bound plasmin can efficiently activate receptor-bound pro-uPA (Ellis et al, 1989, Ellis et al, 1991). The binding of pro-uPA to uPAR potentiates plasmin generation, which is dependent on the concurrent cellular binding of plasminogen. A non-active site interaction between uPA and plasminogen is necessary for the assembly and efficiency of cell-surface plasminogen activation complexes (Ellis et al, 1999). Therefore, interactions between the molecules are important in increasing the efficiency of the process. Once generated, plasmin can degrade most components of the extracellular matrix and basement membrane, such as fibronectin, laminin and collagen (Blasi et al, 1997), can activate certain matrix metalloproteases (Murphy, 1992) and activate or release matrix-bound growth factors such as TGFβ and basic fibroblast growth factor (BFGF). Physiological inhibitors also modulate the plasminogen activation pathway. Pro-uPA is not inhibited by PAIs but uPA bound to uPAR is available for inhibition by PAIs (Lukes et al, 1999). α2-antiplasmin, which is abundant in plasma acts to further localise plasmin activity at the cell surface, as

any plasmin that is generated is fully protected from inhibition while it remains bound, but is rapidly inhibited upon dissociation from the cell surface (Ellis et al, 1991). Therefore, plasmin activity is dynamically regulated at the cell surface with α2-antiplasmin suppressing the dissemination of plasmin activity and PAI-1 inhibiting its generation (figure 1.11).

1.3.5 PLASMINOGEN ACTIVATION SYSTEM AND CANCER

The plasminogen activation system plays a central role in tumour biology. It is not only important in cancer cell migration and invasion but is also involved in other processes in tumours, collectively called cancer cell-directed tissue remodelling. This enzyme system was first implicated in cancer when the oncogenic transformation of cells was shown to up-regulate the expression of plasminogen activators (PAs) and with the histological localisation of PAs to invasive foci of experimental tumours (Dano et al, 1985). Evidence to show a correlation between the uPA system and cancer has accumulated over the past several decades, from experimental invasion and metastasis models and from expression patterns of components of the uPA system in tumours and normal tissues.



Adapted from Ellis, 1999

Figure 1.11 The Plasminogen Activation Cascade

1.3.6 LEVELS OF PLASMINOGEN ACTIVATION SYSTEM COMPONENTS IN TUMOURS AND PATIENT PROGNOSIS

A direct correlation between plasminogen activation and cancer was shown when plasminogen activators were identified by biochemical and immunological methods in a large number of malignant neoplasms as well as in normal tissues (Dano et al, 1985). Human malignant tumours showed significantly higher levels of uPA, uPAR and PAI-1 expression than in the corresponding normal tissue. However, the levels of these components in a given tumour type vary considerably between individual patients (Duffy, 1993 and Duffy et al, 1996). uPA was the first protease shown to be a prognostic marker in human malignancy (Duffy et al, 1988) with over-expression in several human tumours including mammary, lung, bladder, kidney, colorectal, stomach, brain, ovary, endometrium, and melanoma (Markus, 1988). Also, in a variety of malignancies, high tumour uPA levels are correlated with a shorter disease-free interval and a shorter overall survival, than patients with a low level of uPA.

uPAR is also a prognostic marker in tumours since elevated levels of uPAR on cancer cells and high levels of soluble uPAR in blood plasma and other body fluids are correlated with a poor prognosis (reviewed by Andreasen et al, 1997 and Schmitt et al, 1997). Expression of uPAR and uPA in disseminated cancer cells in bone marrow of patients with gastric and breast cancers, respectively, predicted an early relapse (Heiss et al, 1995, Solomayer et al, 1997). In squamous cell carcinomas of the skin, uPAR mRNA was detected focally in well-differentiated tumours and was detected on cancer cells at the edge of invasively growing strands of tumour (Romer et al,

2001). In colon adenocarcinomas uPAR has been identified in both cancer cells and macrophages at invasive foci, whereas neighbouring fibroblast-like cells express uPA (Grondahl-Hansen et al, 1991, Pyke et al, 1991, 1994). In ductal breast carcinomas uPAR is located in tumour-associated macrophages (Pyke et al, 1993). Therefore, in several tumour types, while uPAR can be expressed in the neoplastic cells, the source of uPA is not the tumour cells but the stromal fibroblasts (Hewitt and Dano, 1996). In this situation the tumour cell may produce cytokines, angiogenic factors or other factors that modulate the stromal environment, which can promote the expression of uPA, other proteases or angiogenic factors by fibroblasts or macrophages in contact with the tumour.

tPA levels in contrast are correlated with a good prognosis (reviewed by Duffy et al, 1995). Surprisingly, PAI-1 is also a strong independent prognostic marker in many human cancers, with elevated levels of PAI-1 being correlated with a more aggressive disease and poor prognosis. However, there is some discrepancy in the role of PAI-2 in cancer, with high levels of PAI-2 expression being correlated with both a good and poor prognosis (Andreasen et al, 1997).

1.3.7 THE PLASMINOGEN ACTIVATOR SYSTEM IN EXPERIMENTAL TUMOURS IN ANIMALS

Studies with *in vitro* assays and the staining of tissue sections provided evidence for an important role of the uPA system in migration and invasion at the level of individual cells. However, the situation in tumours *in vivo* is more complex, where

non-malignant cells may also be migratory and invasive during processes of cancer cell-directed tissue remodelling. Therefore, components of the uPA system may contribute to tumour progression and metastasis, even if they do not participate directly in cancer-cell migration and invasion. Numerous studies in animal model systems show convincingly that uPA-catalysed plasminogen activation is rate limiting for tumour growth, local tumour invasion and formation of distant metastases.

The first animal model system for studying the role of uPA in cancer involved implantation of human tumour cells onto the chorioallantoic membrane of chicken embryos (Ossowski and Reich, 1983). Using this model it was shown that antibodies against human uPA resulted in inhibition of metastasis to the lungs of the embryo and that binding of uPA to uPAR was necessary for the effect of uPA on invasion and metastasis (Ossowski, 1988). Using the model of dissemination of human tumours in nude mice, it was reported that antibodies against human uPA prevented local invasion of cancer cells (Ossowski et al, 1991). Using the same method Quax et al, 1991 [reported a correlation between cancer cell uPA expression and lung metastasis with a series of human melanoma cell lines].

The generation of genetically modified mice in recent years has been instrumental in evaluating the role of the uPA system in tumour biology. The development of plasminogen -/- mice provided evidence for a crucial role of the plasminogen activation system in metastasis. Plg -/- mice displayed a slower growth of transplanted Lewis lung carcinoma with fewer regional lymph node metastases. They

also lived longer after the transplantation than control mice (Bugge et al, 1997). The incidence of lung metastases from mammary tumours induced by mouse mammary tumour virus and polyoma middle T antigen was up to 10-fold lower in Plg -/- mice than in control mice (Bugge et al, 1998). Studies in uPA knockout mice have shown reduced local invasion and a drastically reduced progression to malignant melanomas in uPA -/- mice compared to control mice (Shapiro et al, 1996).

1.3.7.1 uPAR in animal model systems

Most experiments with animal model systems showed that to support tumour metastasis, uPA must be bound to uPAR. Using the nude mouse model for studying a series of human melanoma cell lines, a correlation between cancer-cell uPAR expression and lung metastasis was demonstrated (Quax et al, 1991). Saturation of uPAR with an enzymatically inactive but uPAR-binding uPA variant inhibited metastasis of primary tumours produced by inoculating PC3 prostate carcinoma cells in nude mice (Crowley et al 1993). Reduction of uPAR expression in implanted human cancer cells by transfection with antisense sequences reduced both local invasion and lung metastasis in the nude mouse model (Kook et al, 1994). Studies with uPAR -/- mice should provide further insight into the role of cell-bound plasminogen activation in tumour metastasis.

1.3.8 MATRIX METALLOPROTEINASES (MMPS)

The MMP family is composed of at least 25 zinc-dependent extracellular endopeptidases, with well-characterised structural and catalytic properties. They can be divided into groups based on their structure and substrate specificity: collagenases, gelatinases, stromelysins and the membrane-type (MT)-MMPs. All of the enzymes are secreted in the pro-enzyme form and are converted to the active form by cleavage of the pro-peptide, by other proteolytic enzymes. The urokinase system has a recognised role in the initial stage of MMP activation, since plasmin is a potent activator of a number of latent MMPs including MMP-1, -3 and –9 (Carmeliet et al, 1997). Other serine proteases are also capable of *in vitro* MMP activation (Ramos-DeSimone et al, 1999). Therefore extracellular proteolysis of matrix components and the basement membrane is a complex process involving a number of enzymes acting directly or indirectly, through activation of other proteolytic enzymes.

1.3.9 OTHER SERINE PROTEASES

The proteolytic activities of membrane-anchored proteins such as ADAMs and MT-MMPs are thought to play central roles in cell-surface activating events. In contrast, most of the members of the serine protease family are either secreted enzymes or sequestered in cytoplasmic storage organelles awaiting signal-regulated release. Recently a family of transmembrane proteins containing C-terminal extracellular serine protease domains have been identified. Currently, there are 17 members of this Type II Transmembrane Serine Protease (TTSPs) family (reviewed by Hooper et al, 2001). These enzymes interact with other proteins on the cell surface as well as

soluble proteins, matrix components and proteins on adjacent cells. In addition, these membrane-spanning proteins have cytoplasmic N-terminal domains, suggesting possible functions in intracellular signal transduction. Since serine proteases are capable of activating MMPs, it is postulated that the TTSPs may also play a role in MMP activation. The multi-domain structure of the TTSPs suggests that they may be able to interact with multiple partners to form signalling complexes, as is the case for uPA/uPAR.

1.4 CO-OPERATION OF PROTEASES AND INTEGRINS

Changes in integrin and protease activities are a common feature of many metastatic tumour cells, suggesting that intricate interactions between proteolytic enzymes and the integrin family of adhesion receptors coordinate focal extracellular matrix attachments and detachments central to cell migration. Co-operation between integrins and proteases are thought to operate at several levels. Firstly, integrin clustering and the consequent intracellular signalling induces proteolytic enzyme expression. This was first reported for the MMP, collagenase by fragments of fibronectin acting through $\alpha5\beta1$ (Werb et al, 1989), but has more recently been observed with other integrins and proteases, for example, $\alpha3\beta1$ clustering induces uPA expression (Ghosh et al, 2000) and intracellular signalling via p38α MAP Kinase activity is required for uPA/uPAR expression (Chen et al, 2001). Secondly, proteases localise on the cell surface to sites of integrin clustering. An example is the localisation of MMP2 to the surface of invasive cells by interaction with $\alpha v\beta3$ (Brooks et al, 1996). This enables the activation of proteolytic cascades to sites of

adhesion, thereby focusing the pathways of migration. Protease activity also results in the creation of fragments of extracellular matrix proteins that are biologically active themselves, for example, proteolytic fragments of elastin and collagen are chemotactic (Senior et al, 1989 and fragments of plasminogen and collagen have anti-angiogenic activity (Griffioen and Molema, 2000, Hagedorn and Bikfalvi, 2000). Thirdly, as integrins cluster at sites of their matrix ligands the calcium stimulated calpains localise to the cytoplasmic tails of integrin clusters and regulate cytoskeletal reorganisation important to integrin migration (Kulkarni et al, 1999). Thus proteases surrounding integrins both outside and inside the plasma membrane are important to regulation of integrin function.

1.5 INTEGRIN AND uPAR ASSOCIATION

Most studies examining integrin/uPAR interaction have utilised co-immunoprecipitation or resonance energy transfer techniques. However, there are relatively few demonstrations of an actual physical association of uPAR with integrins. uPAR was co-immunoprecipitated with β1 integrins, although only a small proportion of either uPAR or integrin was committed to the uPAR-integrin complex (Wei et al 1996). A physical association was also shown between uPAR and the β2 integrins Mac-1 and LFA-1 in monocytes, which were in a complex with tyrosine kinases (Xue et al, 1994, Bohuslav et al, 1995). A degree of functional association between uPAR and the integrins αvβ5 and β3 has also been demonstrated (reviewed by Chapman, 1997). Although, it is apparent that uPAR complexes with integrins, the precise nature of this interaction is still unclear. Chapman et al (2001) have

proposed a model whereby uPAR binds to an integrin α chain at two distinct sites, likely changing the conformation of both the integrin and uPAR. They identified a phage display peptide that binds to uPAR and disrupts uPAR/integrin interactions and found sequence homology at two sites on the α-chain of the β2 integrin (Wei et al, 2001). The studies demonstrating a physical or functional association between uPAR and various integrin subunits have been summarised in Tables 1.3, 1.4 and 1.5 (reviewed by Chapman, 1997, Ossowski and Aguirre-Ghiso, 2000, Chapman and Wei, 2001).

Some studies have demonstrated that integrins may affect the expression of uPAR. In metastatic melanoma cells, expression of αv integrin subunit was correlated with increased uPAR expression (Nip et al, 1995). However, in CHO cells over-expression of β3 integrin was associated with decreased uPAR expression (Hapke et al, 2001a) and in ovarian carcinoma cells αvβ3 ligation to vitronectin decreased uPAR and uPA expression (Hapke et al, 2001b).

uPAR, besides its uPA-binding role, also functions as a vitronectin receptor and has been shown to participate in tumour cell migration and invasion (Stahl and Mueller, 1997). It has been suggested that uPAR may actually act as an integrin ligand, that is RGD-independent and that this interaction regulates integrin and uPAR functions, suggesting mechanisms of cross-talk between uPAR and integrins (Chapman and Wei, 2001). The urokinase system may influence integrin function through "outside-in" signalling, where a conformational change induced by uPA binding to uPAR

promotes integrin signalling, thereby leading to activation of other integrins, to alter cell shape and adhesiveness. This was seen in normal kidney cells overexpressing uPAR, but not uPA, which showed enhanced spreading and migratory behaviour on vitronectin, however the binding function of β1-integrin to fibronectin was blocked, increasing the migratory capability of the cells (Wei et al, 1996). This group subsequently showed that α3β1/uPAR complex formation promoted adhesion to fibronectin (Wei et al, 2001). Also, in human epidermoid carcinoma cells, an association between α5β1 and uPAR promoted cell migration to fibronectin via MEK/ERK signalling (Aguirre-Ghiso et al, 1999a). In human pancreatic carcinoma cells, uPA, uPAR and αvβ5 were shown to functionally co-operate to promote cell migration (Yebra et al, 1996). Conversely, uPAR activation by uPA binding promoted the association of uPAR with β1 integrin (Yebra et al, 1999). It is clear that uPA promotes uPAR/integrin interactions suggesting that known pathways of uPA clearance, i.e. through complexes of PAI-1 and the LRP scavenger receptor, may partially or completely reverse uPAR/integrin interactions, which may be an interesting avenue for further study (Cubellis et al, 1990, Conese et al, 1995). *In vivo*, there is circumstantial data to suggest that uPAR and integrins cooperate to facilitate migration, however, the extent to which migratory cells actually use uPAR/integrin complexes to optimise function still needs clarification.

Table 1.3 β1 INTEGRIN AND uPAR ASSOCIATION

INTEGRIN	CELLS	RESPONSE	REFERENCE
β1	Human embryonic kidney 293	Over-expression of uPAR ↑ migration towards VN but ↓ migration towards FN.	Wei et al, 1996
β1	Human embryonic kidney 293 chondrocytes	uPAR was shown to be in a complex with β1 and caveolin	Wei et al, 1999 Stahl and Mueller, 1995 Schwab et al, 2001
β1	Prostate carcinoma LNCaP	uPA binding to uPAR promotes association of uPAR with β1.	Yebra et al, 1999
α5β1	Human epidermoid carcinoma (Hep3)	α5β1/uPAR association promotes cell migration towards FN via MEK/ERK signalling. Also suPAR promotes tumour progression in vivo via α5β1 signalling.	Aguirre-Ghiso et al, 1999a
β1	Thyroid cells	Cleavage of domain 1 of uPAR abolishes most of β1/uPAR association	Montuori et al, 1999
α4β1	T lymphocytes	uPAR inhibits α4β1-mediated (but not a5β1-mediated) adhesion to FN, which is uPA-independent.	Olivier et al, 1999
α3β1	Kidney 293 cells and MDA-MB231 breast epithelial cells	α3β1/uPAR complex promotes adhesion to VN. Spreading and FAK activation on FN and COL1 was promoted by addition of uPA.	Wei et al, 2001
β1	MB231 breast epithelial cells	Disruption of uPAR/integrin complexes using p25 peptide ↑ β1-mediated adhesion to FN.	van der Pluijm et al, 2001
β1 and β3	Human fibrosarcoma, HT1080 cells	Engagement of matrix ligands, FN, VN and laminin suggested as association between uPAR and β1/β3.	Xue et al, 1997

Table 1.4 β2 INTEGRIN AND uPAR ASSOCIATION

INTEGRIN	CELLS	RESPONSE	REFERENCE
β2 (Mac-1)	Monocytes	uPAR and β2 co-cluster in presence of Abs. uPAR associates with the integrin heterodimers, αxβ2 or αmβ2 in a reversible manner depending on the activation state of the cell.	Xue et al, 1994 Kindzelskii et al, 1996
β2	Monocytes	Src tyrosine kinase was found in a complex with uPAR and β2. Src kinase became activated upon uPA binding to uPAR.	Bohuslav et al, 1995
β2	Kidney 293 cells	A direct interaction between purified Mac-1 and uPAR was promoted by active state of β2.	Wei et al, 1996
β2	Monocytes	uPAR binding to VN promoted β2 function of binding to and degradation of fibrinogen.	Simon et al, 1996a
β2	Monocytes	β2 and uPAR co-distributed on fibrinogen. β2-mediated adhesion was promoted by un-occupied uPAR. Also, uPAR knockdown resulted in defective β2-dependent adhesion.	Sitrin et al, 1996
β2	uPAR-deficient mice	β2-dependent recruitment of neutrophils to inflammatory sites was inhibited.	May et al, 1998

Table 1.5 αV INTEGRIN AND uPAR ASSOCIATION

INTEGRIN	CELLS	RESPONSE	REFERENCE
αvβ3	Metastatic melanoma cells	Antisense of αv caused ↓ uPAR mRNA synthesis. Also, Ab-mediated ligation of αvβ3 ↑ uPAR mRNA expression	Nip et al, 1995
αvβ3	CHO cells	Over-expression of β3 ↓ uPAR expression.	Hapke et al, 2001a
αvβ3	Human ovarian carcinoma OVMZ-6 cells	αvβ3 ligation to VN ↓ uPAR and uPA expression.	Hapke et al, 2001b
αvβ5	Human keratinocytes	uPA, uPAR and αvβ5 were localised in focal contacts and plasmin generated at the cell surface abrogated αvβ5-mediated adhesion to VN.	Reinartz et al, 1995
αvβ5	FG human pancreatic carcinoma cells	uPA, uPAR and αvβ5 were shown to functionally co-operate to promote cell migration.	Yebra et al, 1996
αvβ5	Breast carcinomas HT1080 human fibrosarcoma and MCF-7 human breast adenocarcinoma	αvβ5 and uPAR were co-purified. Exposure of cells to uPA and VN enhanced uPAR/αvβ5 association, leading to a functional interaction, suggesting that uPAR directs cytoskeletal rearrangement and cell migration by altering αvβ5 signalling specificity.	Carriero et al, 1999

1.6 UROKINASE AND SIGNALLING

Activation of components of the classical mitogen-activated protein kinase (MAPK) pathway are involved in the up-regulation of uPA/uPAR. The sources of these signals are derived from receptor tyrosine kinases (RTK) or oncogene derived signals. For example, uPA expression can be regulated by growth factors that bind to tyrosine kinase receptors and activate the Ras signalling pathway (Aguirre-Ghiso et al, 1999b). In fact, oncogenic Ras was shown to activate uPA transcriptional activity through a MAPK signalling pathway (Lengyel et al, 1995). This involves the activation of the mitogen-activated protein kinase (MAPK) kinase, MEK, by c-Raf, which in turn activates ERK. Targets of ERK include transcription factors such as c-Myc and the Ets-family member Elk-1 that trigger expression of c-Fos (Hill and Treisman, 1995). In transformed PDV keratinocytes, Ras-ERK signalling is necessary for TGF-β1 to stimulate uPA production and cell migration/invasiveness (Santibanez et al, 2000). These results are in agreement with other observations demonstrating that activation of ERK regulates epithelial cell motility and migration across the extracellular matrix (Klemke et al, 1998, Montesano et al, 1999) and that the MEK inhibitor PD098059 concomitantly reduces uPA expression and invasiveness in carcinoma cell lines characterised as avid secretors of uPA (Simon et al, 1996b).

Ras can activate other c-Raf-independent pathways; for example that involving the c-Jun N-terminal kinase (JNK) (Minden et al, 1994). Therefore, in addition to the classical ERK1/2 pathway, other mitogen activated protein kinases such as JNK-

SAPK induce up-regulation of uPA and uPAR (Gum et al, 1998, Miralles et al, 1998). The p38 MAP Kinase pathway has also been shown to regulate protease expression, since inhibition of the p38 led to reduced uPA and MMP expression and invasion by tumour cells (Simon et al, 1998). However, p38 was also found to regulate uPA mRNA stability in carcinoma cells (Montero and Nagamine, 1999).

The signalling pathways activated by uPA/uPAR appear to be identical to those that induce their own expression. uPA binding to uPAR activates several tyrosine kinases from the Src family (Fyn, Lck, Hck, Yes) (Bohuslav et al, 1995) and may also stimulate the Jak/Stat (Dumler et al, 1998) and MAPK pathways (Nguyen et al, 1998, Konakova et al, 1998). uPA, through its uPAR-binding domain, or soluble uPAR are able to directly initiate intracellular signalling events. Thus, it is possible that overexpression of the uPA/uPAR system in tumour cells leads to a signalling loop and/or activation of additional mechanisms dependent on these molecules that contribute to enhanced pericellular proteolysis, migration, survival and proliferation.

uPAR has been recently shown to mediate proteolysis-independent signalling events, including protein tyrosine phosphorylation (Dumler et al, 1993), diacylglycerol formation (Del Rosso et al, 1993), activation of a serine kinase (Busso et al, 1994) and activation of the JAK/STAT signalling pathway (Koshelnick et al, 1997, Dumler et al, 1998). uPA may influence cell migration and differentiation, by binding to uPAR, which then mediates endothelial cell signal transduction that involves the activation of FAK and MAPK (Tang et al, 1998). uPA bound to its receptor activates

intracellular signalling through a still unknown adaptor protein, β1 and β2 integrins being the best candidates (Chapman et al, 1997, Dumler et al, 1998, Nguyen et al, 1998). This involves signalling molecules, which are also activated by integrin signalling. For example, activation of MAP Kinase, myosin light chain kinase and other intracellular tyrosine kinases as a consequence of cellular binding of urokinase are consistent with signalling through integrins (reviewed by Ossowski and Aguirre-Ghiso, 2000). Down-regulation or function blocking of αv integrins inhibits endogenous p38 MAPK activity and uPA expression in invasive MDA-MB-231 breast cancer cells whereas engaging αv integrins with vitronectin activates p38 MAPK and up-regulates uPA expression (Chen et al, 2001). It was recently demonstrated that αv integrin ligation activated the small GTPase Rac1, followed by MKK3 and then p38-MAPK/MAPK-activated protein kinase 2, which regulated uPA mRNA stability (Han et al, 2002). The association of uPAR with an atypical isoform (PKCε) of protein kinase C (PKC) has been documented in human epithelial cells, suggesting that a PKC-based signalling system may be available to uPAR (Busso et al, 1994). The signalling pathways associated with uPA/uPAR expression and function have been summarised in Table 1.6 (reviewed by Aguirre-Ghiso et al, 1999b, Ossowski and Aguirre-Ghiso, 2000, Dear and Medcalf, 1998).

Table 1. 6 UROKINASE AND SIGNALLING

uPA/ uPAR	Signalling Pathway Activated	Cell type	Response	Reference
uPA	Oncogenic Ras activates MAPK (Ras→Raf→ERK →cMyc→Elk1→ cFos		Activation of uPA transcription	Lengyel et al, 1995
uPA	MAPK	Carcinoma cell line	MEK inhibitor PD98059 ↓ uPA expression and invasion.	Simon et al, 1996b
uPA	Growth factors binding to tyrosine kinase receptors activate Ras.		Regulation of uPA expression	Aguirre-Ghiso et al, 1999b
uPA	MAPK (Ras→ERK)	Transformed PDV keratinocytes	TGFβ1 stimulates uPA production and cell migration/invasion.	Santibanez et al, 2000
uPA	MAPK (FAK→Src→Ras →ERK)	LLC-PK1 kidney epithelial cells	Cytoskeletal reorganization leads to uPA induction	Irigoyen and Nagamine, 1999
uPA	p38 MAPK	Tumour cells	p38 inhibitor ↓ uPA expression and invasion.	Simon et al, 1998
uPA	p38 MAPK	Carcinoma cell line	Regulation of mRNA stability	Montero and Nagamine, 1999
uPA / uPAR	JNK/SAPK		Activation of this pathway ↑ uPA and uPAR expression.	Gum et al, 1998, Miralles et al, 1998
uPAR	JAK/STAT	Vascular smooth muscle cells	uPAR regulates cell migration through this pathway.	Koshelnick et al, 1997, Dumler et al, 1998
uPA / uPAR	FAK→MAPK	Endothelial cells	uPA binding to uPAR influences cell migration and differentiation	Tang et al, 1998

uPA / uPAR	MAPK MLCK Intracellular tyrosine kinase		uPA binding to uPAR activates intracellular signalling pathways through adaptor proteins (β1/2 integrins).	Chapman 1997 Dumler et al, 1998 Nguyen et al, 1998
uPAR	PKC→Ras→Raf →serine /threonine kinase →MAPK	Human epithelial cells	uPAR through this pathway possibly mediates modulation of gene expression	Busso et al, 1994
uPAR	ERK1/2	Hep 3 cells	uPA/uPAR/α5β1 complexes maintain ERK activity to sustain tumour growth	Aguirre-Ghiso et al, 1999a
uPA / uPAR	Ras→MEK,ERK, MLCK	MCF-7 breast cancer epithelial cells	uPA binding to uPAR initiates signalling cascade to promote cell migration to VN in a β1 and αvβ5-selective manner.	Nguyen et al, 1999
uPA / uPAR	Ras→ERK	MDA-MB231 breast cancer epithelial cells	uPA signals through uPAR to maintain activated ERK => inhibiting apoptosis.	Ma et al, 2001
uPA	p38 MAPK	MDA-MB231 breast cancer epithelial cells	↓ αv inhibits p38 activity and uPA expression. Also αv/VN ligation activates p38 and uPA expression.	Chen et al, 2001
uPA	GTPase→Rac1→ MKK3, p38 MAPK/ MAPKAP2	MDA-MB231 breast cancer epithelial cells	αv/VN ligation regulates mRNA stability through this pathway.	Han et al, 2002

1.7 SUMMARY

Cancer Metastasis is a complex multi-step process that involves adhesion to and migration and invasion through the extracellular matrix, which are primarily mediated by integrins and ECM-degrading enzymes. Integrins are a family of cell-surface adhesion molecules composed of an α and β-subunit, which are non-covalently linked to form a heterodimer (Hynes, 1992). Aberrant integrin expression is a common feature of many epithelial tumours, including squamous and basal cell carcinomas of the skin and oral cavity and adenocarcinomas of the colon, breast and pancreas. The αv integrin subfamily is important in the progression of oral squamous cell carcinoma (Jones et al, 1997). In particular, the αvβ6 integrin, which is epithelial-cell restricted is either absent or expressed at very low levels in fully differentiated normal epithelia (Breuss et al, 1995). However, it becomes highly expressed during times of morphogenesis, tissue repair, inflammation and tumourigenesis. αvβ6 is the most commonly up-regulated integrin in epithelial tumours with *de novo* expression seen in malignant colonic and oral epithelium. High expression is also seen in lung and advanced stages of breast carcinoma.

Variations in integrin subunit associations confer ligand-binding specificity. Integrins can bind to various ligands via the RGD site that is present in a number of ECM proteins. In addition synergy peptidic sites within the integrin generate full adhesive activity, so a ligand can interact with an integrin at two or more distinct sites within the heterodimer. Several regions in both α and β-subunits are thought to act together

to form the ligand-binding surface (Fernandez et al, 1998). Evidence is still lacking as to how these sites interact to form the ligand-binding pocket. Since many integrins can bind to a certain ligand and a specific integrin can bind to various ligands, there may be compensation amongst integrins with regard to their function. This apparent redundancy could account for overlapping functions of integrins, however they still have unique and irreplaceable functions within the cell. Further experiments with cell-type specific and/or regulated gene ablation may provide additional information on integrin functions, which include influencing cell shape, tissue architecture, cell migration, adhesion, proliferation, differentiation, apoptosis and gene transcription.

Integrin-mediated regulation of cell behaviour involves integrins coupling with the cytoskeleton, signals being transduced bi-directionally and reciprocal cross-talk between integrins and other receptors. Various intracellular signalling pathways are associated with integrins suggesting a great deal of complexity within integrin signalling (Giancotti and Ruoslahti, 1999).

Integrin cytoplasmic domains bind directly to numerous cytoskeletal and regulatory proteins that affect integrin functions. Certain motifs within the β cytoplasmic domains are highly conserved and the β-subunits are thought to be important in controlling integrin affinity states and function (Dedhar and Hannigan, 1996). The α-subunit cytoplasmic domains have also been shown to be important in regulating integrin-mediated signalling events. Questions remain as to the precise nature of the cytoplasmic domains and how they control integrin functions. Is there synergism

between certain sites in the α and β cytoplasmic domains? Do the α and β subunits have specific roles and/or interconnecting roles? What signalling pathways do they activate and through which motifs in the cytoplasmic domains?

Integrin expression on the cell surface is regulated at a number of stages, which are not yet completely defined. Further insight needs to be gained into how the heterodimers are assembled, for example how long it takes from transcription of the integrin subunits to assembly on the cell surface. Are there intracellular storage pools of integrins? How long are they expressed on the cell surface for? Are they degraded or recycled back to the cell surface? How long is integrin turnover time and what determines this? Extracellular, or intracellular signals? Therefore, there is large gap in the literature with regard to integrin expression and function.

In addition to interacting with molecules on neighbouring cells or with the extracellular matrix, integrins can also form cis-interactions with other receptors on the same cell to form multi-receptor complexes that are able to recruit signalling molecules. These include urokinase receptor (uPAR), integrin associated protein (IAP), tetraspans, growth factor receptors (GFRs) and caveolae; the functional significance of these integrin/partner complexes is not yet fully established (Porter and Hogg, 1998).

αvβ6 modulates several processes in keratinocytes including migration on fibronectin and vitronectin and also fibronectin-dependent up-regulation of MMP-9 secretion.

Increased invasion of oral SCC cells was also shown to be αvβ6-dependent (Thomas et al, 2001c). *In vivo*, progression of oral SCC was significantly impeded using inhibitory anti-αvβ6 antibodies. The β6 cytoplasmic tail has a unique 11 amino acid extension which is crucial for αvβ6-mediated specific functions, such as stimulation of proliferation and MMP-9 secretion (Agrez et al, 1994, Niu et al, 1998). Further investigation into the role of the 11 amino acid extension, should provide insight into whether this sequence mediates other integrin-specific functions and if the sequence has specificity for binding certain signalling molecules in order to initiate the response.

The release of proteolytic enzymes from tumours facilitates cancer cell invasion into the surrounding normal tissue by degradation of the extracellular matrix and basement membrane. A major class of these enzymes are the serine proteases of the plasminogen activation system. Increased production of these enzymes has been associated with the metastatic/invasive phenotype. A direct correlation between plasminogen activation and cancer was shown when plasminogen activators were identified by biochemical and immunological methods in a large number of malignant neoplasms as well as in normal tissues (Dano et al, 1985). Human malignant tumours showed significantly higher levels of uPA, uPAR and PAI-1 expression than in the corresponding normal tissue.

Plasminogen activation in a healthy organism is strictly controlled via various components, the plasminogen activators, their specific receptors and interaction with

plasminogen activator inhibitors. In invasive tumours, the balance between proteases and inhibitors is usually shifted towards constitutive pericellular proteolysis. The plasminogen activation cascade is a very efficient process since small amounts of plasminogen activator can generate large amounts of plasmin. uPA is synthesised and secreted as an inactive pro-enzyme. It is bound to a specific cell-surface receptor, uPAR and converted to the active form. uPA then catalyses the cleavage of plasminogen bound to the cell surface into plasmin, which can degrade most components of the ECM and basement membrane and can activate certain matrix metalloproteases. In addition, cell-associated plasmin acts in a reciprocal zymogen activation loop, preferntially activating pro-uPA bound to uPAR (Bass and Ellis, 2002).

Changes in integrin and protease activities are a common feature of many metastatic tumour cells, suggesting that intricate interactions between proteolytic enzymes and integrins coordinate focal extracellular matrix attachments and detachments central to cell migration. Co-operation between integrins and proteases are thought to operate at several levels. Integrin clustering and the consequent intracellular signalling induces proteolytic enzyme expression. Also, as proteases localise on the cell surface to sites of integrin clustering, proteolytic cascades are activated to sites of adhesion, thereby focusing the pathways of migration. Proteases surrounding integrins both outside and inside the plasma membrane are important to the regulation of integrin function (Ossowski and Aguirre-Ghiso, 2000). Much research of late has focussed on the association between uPAR and integrins. There are only a few demonstrations of

an actual physical association of uPAR with integrins. uPAR has been shown to co-immunoprecipitate with β1 integrins and colocalise with β2 integrins. A degree of functional association between uPAR and αvβ5 and αvβ3 integrins has also been demonstrated. It has been suggested that uPAR may actually act as an integrin ligand, that is RGD-independent and that this interaction regulates integrin and uPAR functions, suggesting mechanisms of cross-talk between uPAR and integrins. At present this data is conflicting with no consistently detected molecular interaction. Some studies have reported that engagement of matrix ligands promotes association between uPAR and integrins, others that engagement of uPA to uPAR may promote integrin function such as migration. Over-expression of either integrin or uPAR may result in changes in the other one's expression or function. It is difficult to assess the exact role of integrin/uPAR association because integrin expression and function seems to be cell-type specific (Chapman and Wei, 2001).

The signalling pathways activated by uPA/uPAR appear to be identical to those that induce their own expression. Activation of MAP Kinase, myosin light chain kinase and other intracellular tyrosine kinases as a consequence of cellular binding of urokinase are consistent with signalling through integrins, which suggests a further association between uPA/uPAR and integrins.

1.8 AIMS

It is clearly established that integrins and the urokinase system are intimately linked but exactly how they are linked and what role this plays in normal physiological systems as well as in cancer remains unclear. Therefore the aim of this study is to investigate further how integrins and the plasminogen activator system interact to promote cancer metastasis. A model system where oral squamous cell carcinoma keratinocytes have been genetically manipulated to express different integrin heterodimers will be used to investigate the effects on the urokinase system. Specifically αvβ6 integrin has been studied, which is neo-expressed in oral SCC. The specific aims of this study are:

- Initially to determine any modulation of uPA and uPAR expression and function by αvβ6 overexpression.
- In order to confirm any αvβ6 effects on the urokinase system, a number of different approaches will be employed:
    - Ligand activation
    - The use of morpholino antisense oligonucleotides to inhibit αvβ6
    - An attempt to develop an inducible β6 expression system
- The cell specificity of αvβ6 functions will be investigated by overexpression into melanoma cell lines.
- The role of αvβ6 and uPA in cancer metastasis will be studied by *in vitro* invasion and migration assays.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL LINES

Cell lines derived from oral squamous cell carcinomas (SCC) provided a useful experimental model to study the role of integrins and proteases in tumour progression. H357 is a stable cell line established from a well-differentiated, grade 1 carcinoma from the tongue of an elderly male (Prime et al., 1990). H357 has previously been shown to lack the αv subunit (Sugiyama et al., 1993). Full-length αv cDNA in the pRC/CMV vector was transfected into these cells. The V3 cell line was one generated population, expressing high levels of αv, mainly as the αvβ5 heterodimer. An empty expression plasmid, containing only the drug resistance gene (neomycin) was also transfected, to create the control cell line IC6 (Jones et al, 1996).

The V3 cell line was further manipulated to study the effects of the αvβ6 integrin heterodimer. Various integrin cDNA sequences were ligated into the retroviral plasmid pBabepuro (Stratagene, Amsterdam, The Netherlands). Maxiprep DNA of this was transfected into the AM12 amphotrophic retroviral packaging cell line and selected in puromycin. Target cells (V3) were exposed to retrovirus-containing conditioned medium and selected in puromycin. As controls, (C1 cells) were created by infection with the retroviral vector (pBabe puro) alone. Specific integrin expressing cell populations were selected by two rounds of magnetic bead sorting using mouse anti-integrin antibodies, see section 7.2.2 (Thomas et al, 2001b). All of the cell lines used in this study have been previously established and characterised.

The cell lines created from the V3 cells are:

VB6 – transfected with full-length human β6 cDNA (Thomas et al, 2001b).

$VB6_{\Delta 11aa}$ – transfected with mutated human β6 cDNA, lacking the 11 amino acid C-terminal cytoplasmic extension (Morgan et al., 2002 (unpublished))

V3B3- transfected with full-length human β3 cDNA (Thomas, thesis, 2000).

The oral squamous cell carcinoma cell lines used in this study are depicted in Figure 2.1. A description of the cell lines used in this study are summarised in Table 2.1

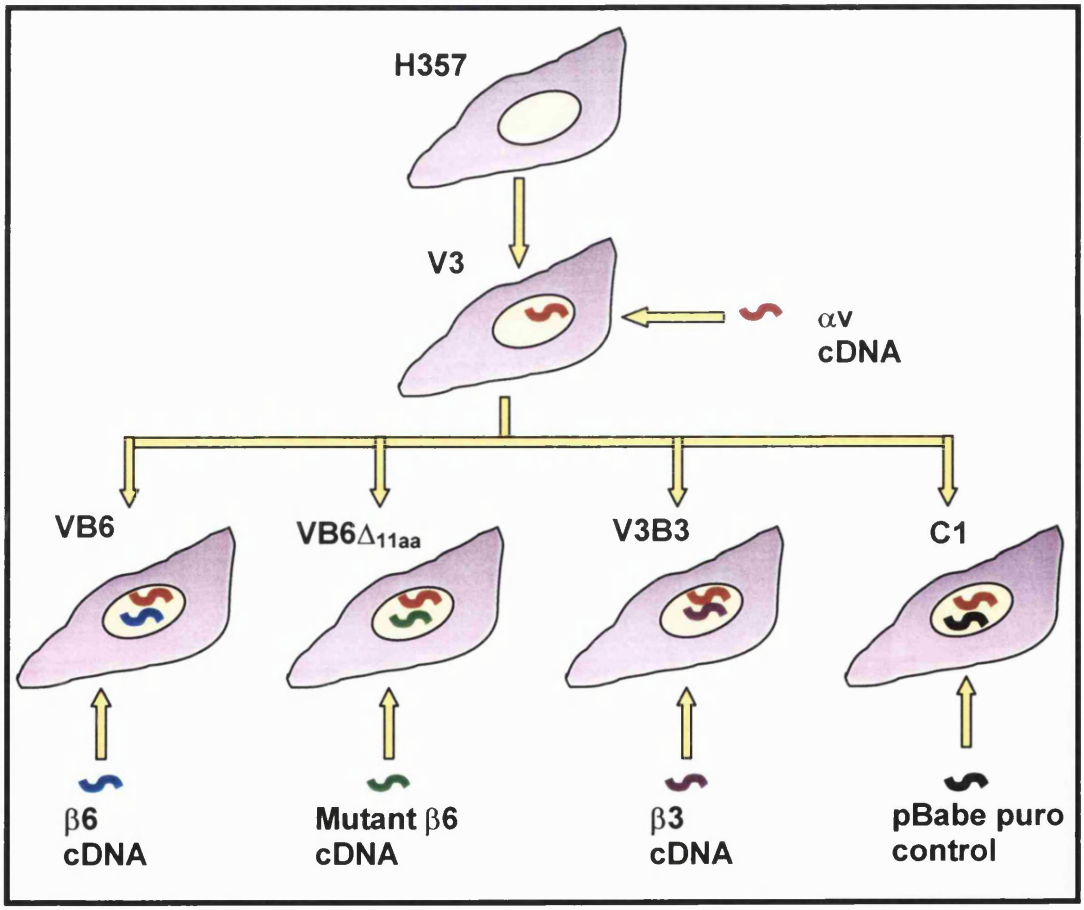


Figure 2.1 Oral Squamous Cell Carcimoma Cell Lines

CELL LINE	DESCRIPTION
H357	Oral SCC cell line lacking αv integrin expression
V3	Transfected with αv, so expresses predominantly the αvβ5 integrin heterodimer.
IC6	Control cell line for V3. Lacks αv expression.
VB6	V3 cells transfected with full-length β6 gene, so they overexpress the αvβ6 integrin heterodimer.
$VB6\Delta_{11aa}$	V3 cells transfected with truncated β6 gene, so they overexpress the mutated αvβ6 integrin heterodimer.
V3B3	V3 cells transfected with the β3 gene, so they overexpress the αvβ3 integrin heterodimer.
C1	Control cells for VB6, $VB6_{\Delta 11aa}$ and V3B3, so only express αv in the form of the αvβ5 integrin heterodimer.

Table 2.1 Description of Cell Lines Used in this Study

2.2 CELL CULTURE

All cells were grown in a humidified incubator in 5% CO_2 at 37°C.

2.2.1 Routine Subculture of Keratinocyte Cell Lines

Human oral keratinocytes were maintained in standard keratinocyte growth medium (Appendix 1). Sub-confluent or just confluent cells were washed with PBS

(Invitrogen, Paisley, UK), which was aspirated and discarded. Cells were harvested by incubation at 37°C with Trypsin/EDTA (0.25% w/v, 5mM, Invitrogen) at 1ml/75cm^2. Once cells had detached, trypsinisation was terminated by the addition of 10mls KGM, with the serum in the KGM inhibiting any further enzymatic action. Cells were transferred to a tube and centrifuged for 5 minutes at 1000rpm, the supernatant was discarded and the cells re-suspended in KGM before seeding at the desired density. Cells were routinely maintained in 75cm^2 flasks (Nunc, Invitrogen, Paisley, UK).

2.2.2 Cell Counting

Cell concentration was determined by counting the number of cells within a defined area of known depth, using a haemocytometer. This contains two chambers (volume of 0.1mm^3 or 1x10^4/ml each). A glass coverslip was adhered firmly to the top of the moistened haemocytometer until Newton's rings were visible at the sides of the chamber. 10µl of cell sample was loaded into each chamber by capillary action and was viewed under a light microscope at X100 magnification. The grid was located and the cells were counted in the four primary squares. Only the cells that are within or that touch the left or top boundaries were counted and the value was averaged for the four primary squares. The number of cells/ml = number of cells/square x10^4.

2.2.3 Cell Freezing

Following trypsinisation, cells were washed in complete medium and resuspended in medium containing 10% DMSO at 2x10^6 cells/ml. One ml of cell suspension was

placed in a cryotube (Nunc, Invitrogen, Paisley, UK) in an insulated container containing propan-2-ol and placed at -70°C for 8 hours, before transferring the vials to liquid nitrogen (-196°C) for storage.

2.2.4 Cell Thawing

Frozen vials of cells were recovered from liquid nitrogen and thawed rapidly in a 37°C water bath. As soon as the vial contents were thawed they were transferred to a centrifuge tube containing 10ml growth medium. Cells were centrifuged at 1000rpm for 5 minutes, the supernatant discarded and cells transferred to a $75cm^2$ flask containing fresh medium.

2.3 FLOW CYTOMETRY

Subconfluent cells were washed twice with PBS and harvested with trypsin/EDTA. The cells were washed once with PBS containing 10% FCS incubated with primary antibody for 1 hour on ice. Cells were washed three times with PBS/10% FCS and incubated with either FITC-conjugated or RPE-conjugated secondary antibody (Appendix 2) for 30 minutes at 4°C. The cells were then washed three times and resuspended in 0.5ml of PBS/10% FCS. Control samples were labelled with secondary antibodies alone. For dual labelling of cells, the cells were first incubated with a murine monoclonal primary antibody, followed by RPE-conjugated secondary antibody. The cells were then labelled with a polyclonal primary antibody recognising a different antigen, followed by a FITC-conjugated secondary antibody (a complete list of antibodies used is given in Appendix 2). In between each stage of

antibody labelling, cells were washed twice with PBS/10% FCS. A FACScaliber cytometer (Becton-Dickinson, Cowley UK), running Cell Quest™ software was used to analyse 1 x 10^4 labelled cells.

2.4 PLASMINOGEN ACTIVITY ANALYSIS

2.4.1 Preparation of cultured supernatants and cell extracts for PA determination

1 x 10^5 cells were seeded in KGM in uncoated 24-well plates (Nunc, Invitrogen, Paisley, UK) and allowed to adhere for 4 hours. Cells were then washed twice with PBS and incubated with 0.5ml serum and additive free medium, 0.1% BSA (SFM), (Sigma, Poole, UK). After 15 hours incubation at 37°C, supernatants were collected, cleared of cellular debris by centrifugation at 3000g for 10 minutes and stored at –20°C prior to use.

In order to optimise the assay conditions, a time course was carried out. After the addition of SFM, samples were taken every hour for 18 hours and assayed for PAA (see section 2.4.2) in order to determine PA secretion into the supernatant (figure 2.2). PA could be detected in the supernatants between 12-18 hours, and a time point of 15-hours deemed the most suitable. In order to determine if cell confluency affected PA secretion, cells were seeded so that they were 100% and 50% confluent, supernatants collected and assayed for PA activity (figure 2.3). Cells secreted two-fold more PA when 50% confluent, therefore, experiments were routinely carried out on subconfluent cells.

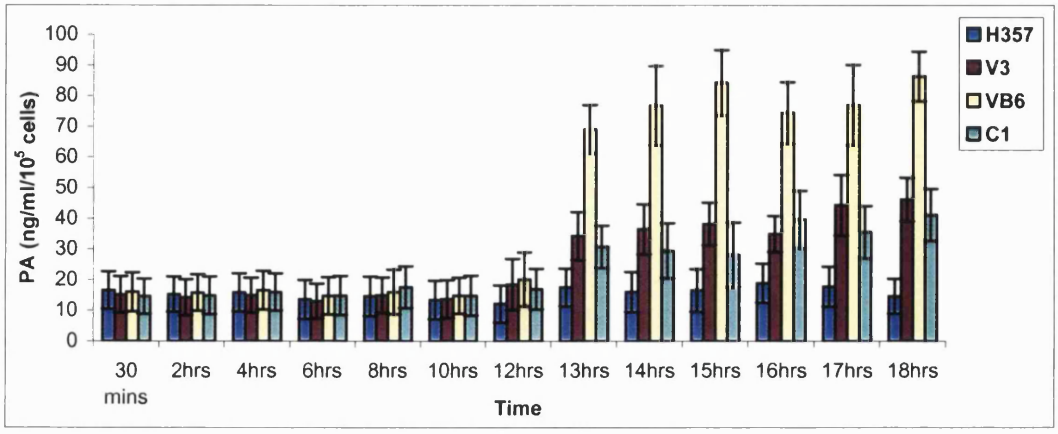


Figure 2.2 Time Course of Secreted Plasminogen Activator Activity.

Cells were seeded into 24-well plates at 10^5/ml in SFM and incubated at 37°C. Cultured supernatants were collected at different time points and assayed for PA activity using a chromogenic assay. Measurable levels of PA could be detected between 13 and 18 hours incubation. Figure represents mean ±SD of an experiment in triplicate.

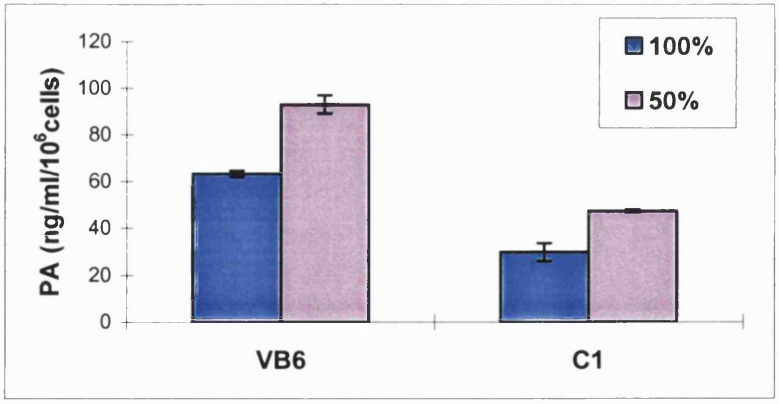


Figure 2.3 Effect of Cell Confluency on Secreted Plaminogen Activator Activity.

Cells were seeded into 6-well plates at 100% and 50% confluency. Cultured supernatants were collected after 15 hours incubation and assayed for PA activity using a chromogenic assay. PA levels were normalised to 10^6 cells and figure represents mean ±SD of 2 experiments in triplicate.

For ligand studies wells were pre-coated with matrix proteins before seeding of the cells in SFM. For antibody-blocking studies, cells were pre-incubated on ice with antibody prior to seeding on wells pre-coated with fibronectin (see section 3.2.3). Cell extracts were prepared by washing monolayers with PBS and lysing cells with 150µl of 100mM NaCl/50mM Tris/0.1% Triton-X-100 at pH 7.4, on ice for 30 minutes. Cells were scraped and the lysate was cleared by centrifugation at 5000g for 10 minutes and the supernatant stored at –20°C.

2.4.2 Plasminogen Activator Activity (PAA) Assay.

Cultured supernatants and cell extracts were assayed for PAA by a chromogenic assay (Ellis, 1996). In a 96-well plate, 50µl of sample was assayed with the plasmin-specific chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251) at 25µg/ml (Quadratech Epsom, UK) and 160nM human glu-plasminogen (Enzyme Research Labs Ltd. Swansea, UK) in a 50mM Tris/100mM NaCl/0.1% Tween 80 buffer (pH 7.4) to make a final volume of 100µl. Samples were incubated for 90 minutes at 37°C and the absorbance was measured at 405nm on a spectrophotometer (SLT Instruments, Vienna, Austria). Plasminogen activator activity was determined by interpolation from a standard curve using known concentrations of uPA (Urokinase, Medac UK, Unidrug Distribution Group, South Normanton, UK) and corrected for cell number.

2.4.3 ELISA

Levels of secreted uPA and tPA antigen in the conditioned media were determined using commercial ELISA kits according to the manufacturers instructions (Biopool International, Alpha labs, Ensleigh, UK). ELISA detects all forms of uPA; pro-uPA, activated uPA and that bound to PAI-1. The assay is based on a double antibody principle. 20µl of sample or standard was added to wells pre-coated with antibody and PA detected by a peroxidase labelled Fab' antibody. The reaction was stopped by the addition of an acid solution and the resultant colour change measured at 405nm on a spectrophotometer. The amount of yellow colour developed is directly proportional to the amount of PA present in the sample and levels were determined by interpolation from uPA and tPA standard curves, the limit of detection being 0.1ng/ml (uPA) and 1.5ng/ml (tPA).

2.5 CELL SURFACE PLASMIN GENERATION

2 x 10^5 cells were seeded in 0.5ml complete medium in 48-well plates (Nunc, UK). After 24 hours incubation at 37°C, the rate of cell surface plasminogen activation was determined using endogenously bound uPA. Confluent monolayers were washed in serum and additive free medium (SFM) and pre-incubated with 25µl PA Assay buffer (2%BSA/ 50mM Tris/ 100mM NaCl / 0.01% Tween 80) for 5 minutes at 37°C. The substrate comprising PA assay buffer and the plasmin-specific peptide substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin, (HDVLK-AMC, 250µM, Bachem, UK ltd, St Helens, UK) was incubated at 37°C for 5 minutes. The substrate was activated by the addition of 48nM human Lys-plasminogen (1.8µg/ml, Enzyme Research Labs

Ltd.) and 225µl of this was added to each well. The fluorogenic substrate was continuously hydrolysed by the generated plasmin to liberate the highly fluorescent 7-amido-4-methylcoumarin (AMC) group, which was quantified spectrophotometrically (Ellis et al, 1993). Plasminogen activation was followed kinetically for 30 minutes at 37°C, using a SPECTRAmaxTM GEMINI XS, Dual Scanning Microplate Spectrofluorimeter (Molecular Devices, Wokingham, UK) at excitation/emission wavelengths of 360/440nm respectively. Results were analysed using SOFTmax PRO software.

Cell surface plasmin generation was also determined using exogenous pro-uPA, so that all cells were exposed to the same levels of uPA ensuring all urokinase receptors were saturated. Following washing with warmed SFM, the cells were stripped of endogenously bound pro-uPA by the addition of 0.5ml of 50mM Glycine/100mM NaCl/pH 3.0 to each well and incubated for 3 minutes at room temperature, followed by neutralisation with 0.5ml of 500mM HEPES/100mM NaCl/pH 7.5 (Stoppelli et al, 1986). This was removed and 0.5ml of PA assay buffer (2% BSA/Tris/NaCl) with 10µl of 100mM pro-uPA (M.Ploug, Finsen Laboratory, Copenhagen, Denmark) was added to each well and incubated at 37°C for 20 minutes. Cells were washed in PA assay buffer and the assay carried out as described above.

2.6 RNA ANALYSIS

2.6.1 RNA isolation

Cells were grown to subconfluence in 75cm^2 flasks in KGM and in serum and additive free medium. Total cellular RNA was extracted according to the method of Chomczynski and Sacchi (1987). The medium was aspirated and cells were washed with PBS, which was completely removed. 750µl of denaturing solution (Appendix 3) was pipetted across the cells to ensure complete lysis before being scraped and transferred to an Eppendorf tube. 75µl of 2M sodium acetate (pH 5.2), 750µl water-saturated phenol and 150µl chloroform were then added to the lysate. This was vortexed and spun in a microfuge at 13000rpm for 5 minutes. 750µl of the resultant upper aqueous phase was transferred to another tube, followed by the addition of 750µl isopropanol. This was vortexed, incubated on ice for 5 minutes and centrifuged at 13000rpm for 5 minutes so that a translucent white/light brown pellet was visible. The supernatant was completely removed, the pellet washed with 900µl of 75% ethanol and centrifuged again. The ethanol was completely removed and the pellet resuspended in 105µl dH_2O. 5µl of this was diluted to 80µl in dH_2O, which was used to measure the RNA concentration and purity in a UV spectrophotometer. Absorbance was measured at 260nm and 280nm, corrected for the dilution factor and expressed as ng/µl.

RNA samples were then adjusted to a standard concentration. In order to precipitate the RNA, 2.5 volumes of 100% ethanol and 1/10th volume of 3M sodium acetate were added. This was vortexed and spun at 13000rpm for 5 minutes. The supernatant was

completely removed and the RNA pellet dissolved in Formamide at a concentration of 5μg/μl. Samples were stored at -80°C until use.

2.6.2. Northern Blotting

2.6.2.1. Gel Electrophoresis and Transfer

RNA samples were electrophoresed in agarose gels and transferred onto nylon membrane as described in Sambrook et al, 1989. The gel was prepared by dissolving 1% agarose (of final gel volume) in dH_2O in a microwave and cooled to 50°C. 10% (of final gel volume) of 10X MOPS buffer (Appendix 3) and 10% of 37% formaldehyde stock solution was added to this, mixed and poured into the gel mould. Once set and cooled, the gel was placed into the tank, filled with 1X MOPS buffer and 10% formaldehyde solution in dH_2O, followed by removal of the combs so the wells were formed.

The samples were prepared by the addition of 4μl RNA sample loading buffer (Appendix 3) to 1μl of RNA (5μg/μl concentration). The samples were heat denatured at 80°C for 5 minutes, then transferred immediately to ice, before loading. The gel was electrophoresed at 150V for 2½ hours, with a cooling system operating and every 30 minutes buffer within the tank was transferred from the cathode to the anode side.

Following electrophoresis, the gel was washed in at least 5 volumes of 10X SSC for 30 minutes. This was repeated once more in order to completely remove the

formaldehyde. The RNA from the gel was transferred onto Hybond N Nylon membrane (Amersham Biosciences, Cambridge, UK) by capillary action. The transfer apparatus was bathed in 10X SSC (Appendix 3) and the gel was placed onto pre-soaked (in 10X SSC) Whatman filter paper wicks. The gel was overlayed with pre-soaked nylon membrane, followed by a further two layers of Whatman filter paper, a 5cm thickness of paper towels and a weight. The gel was allowed to blot overnight, after which the nylon membrane was removed and allowed to dry. The RNA was fixed onto the membrane by baking for 2 hours at 80°C and stored at -70°C until use. At this stage an image was taken under UV light, of the Ethidium Bromide stain of the ribosomal RNA bands.

2.6.2.2 Generation of cDNA probes

Full-length uPA and uPAR genes in pCR2.1 vectors were a kind gift from Dr J. Marshall (Cancer Research UK, St Thomas' Hospital, London, UK). Plasmid DNA was amplified (see section 8.2.1), subjected to restriction enzyme digestion and the uPA and uPAR genes were extracted and purified from an agarose gel (see section 8.2.2).

2.6.2.3 Probe labelling and hybridisation

The α-[^{32}P]-dATP radiolabelled nucleotide stock, with an activity of 3000Ci/μmol or 10μCi/μl (Amersham Biosciences, Little Chalfont, UK) was allowed to warm before probe labelling. 10ng of DNA probe was added to dH_2O to make a final volume of 4.5μl in a 0.5ml Eppendorf tube. The DNA solution was denatured by incubation at

96°C for 2 minutes then cooled on ice before a pulse spin in a microfuge to collect the DNA solution. In a separate 0.5ml Eppendorf tube 2µl of reaction buffer, 1µl of primer mix, 1µl Klenow enzyme (Megaprime DNA labelling system, Amersham Biosciences) and 1.5µl α-[^{32}P]-dATP were added. This 5.5µl reaction mix was then added to the DNA solution and the contents mixed carefully using the pipette. The DNA labelling mixture was then incubated at 37°C for 30 minutes in an Eppendorf thermal cycler, to allow incorporation of the radioisotope into the newly synthesised strands of DNA using the random primer method.

During the incubation the nylon membrane was pre-soaked in 0.1% SDS. 10mls of QuickHyb hybridisation buffer (Stratagene) was added to a large hybridisation bottle (thick walled glass tube) and the internal surfaces coated with buffer. The nylon membrane was placed into the bottle, ensuring the membrane was covered with buffer with no air bubbles. The bottle was placed into the rotisserie within the hybridisation oven and incubated at 68°C, rotating for 30 minutes pre-hybridisation.

50µl of salmon sperm DNA (Sigma Chemical Co.) was then added to the DNA labelling mixture and the solution denatured at 96°C for 5 minutes. The salmon sperm DNA, in effect acted as a 'carrier' for the single stranded probe DNA. The probe mixture was added to a separate Eppendorf tube containing 1ml of QuickHyb and mixed carefully. The QuickHyb mix containing the labelled DNA was then added to the hybridisation bottle and rotated to ensure complete coating of the membrane with no air bubbles. The bottle was returned to the rotisserie within the

hybridisation oven and incubated at 68°C for 4 hours to allow hybridisation of the DNA probe to the nylon membrane.

Following hybridisation, the bottle was removed from the oven and allowed to cool to room temperature, before careful removal and disposal of the hybridisation buffer. Unbound probe was then removed from the membrane by post-hybridisation washes, using 2XSSC, 0.1%SDS on the membrane. The contents of the bottle were initially washed with 50ml of this. The membrane was then washed for 15 minutes rotating at room temperature, then a further 15-minute wash at 65°C. This was followed by a high stringency wash using 0.2XSSC, 0.1%SDS, for 30 minutes at 65°C. The nylon membrane was finally rinsed again in this wash mixture, and then wrapped in Saranwrap. This was placed into an X-ray cassette, overlayed with autoradiographic film and allowed to expose for 3-4 days at -70°C. The film was developed using an automated developer.

In order for further hybridisation of the nylon membrane with another radiolabelled cDNA probe, the membrane was stripped by shaking in a container with pre-boiled 0.1%SDS, for 15 minutes. This procedure was repeated and the membrane was dried before re-use.

2.7 PROTEIN ANALYSIS -WESTERN BLOTTING

2.7.1 Preparation of Cell Lysates

2.7.1.1 Total Cell Lysates

Western blotting for integrins was carried out using total cell lysates in a lysis buffer (Appendix 4) specifically designed for the solubilisation of membrane proteins (personal communication from Dr J.Marshall). Medium was aspirated from the cell monolayer and the cells washed twice with PBS and kept on ice. Protease inhibitor cocktail (Sigma Chemical Co.) was added to the ice-cold lysis buffer at a 1:20 dilution. The buffer was then added to the cells (500µl/75cm^2), ensuring complete coverage for 1 hour on ice with occasional agitation. The lysate was then scraped into an Eppendorf tube centrifuged at 5000rpm for 5 minutes to pellet any cellular debris. The supernatant was transferred to another tube and 20µl was aliquotted for use in a protein assay. Samples were stored at -20°C until use.

2.7.1.2 Cell Membrane Extracts

To prepare extracts for uPAR Western blotting, hydrophobic temperature dependent phase separation using Triton X-114 was used (Behrendt et al, 1993). This serves to concentrate membrane proteins such as uPAR by up to 50-fold. The medium of three confluent 75cm^2 flasks was aspirated and the cells washed twice with PBS. Cells were harvested by scraping in 5ml PBS on ice and transferred to a centrifuge tube. This was followed by centrifugation at 3000rpm for 10 minutes and washing of the pellet with 1ml PBS with further centrifugation at 5000rpm for 5 minutes. The PBS was completely removed and the cell pellet gently re-suspended in 400µl acid wash

buffer, pH 3.0 (Appendix 4) for exactly 3 minutes at room temperature in order to release endogenously bound pro-uPA. This treatment was terminated by the addition of 100µl neutralising buffer (Appendix 4) followed by gentle agitation. Cells were immediately isolated by centrifugation at 5000rpm for 5 minutes, the supernatant removed and the cell pellet transferred to ice. Protease inhibitor cocktail was added to the ice-cold lysis buffer (Appendix 4) at a 1:20 dilution. 400µl of this was added to the cells, gently mixed and incubated on ice for 10 minutes. The lysate was clarified by high-speed centrifugation at 13000rpm for 10 minutes at 4°C (critical temperature control was essential). The clarified lysate was incubated in a water bath at 37°C for 10 minutes, so the detergent (Triton X-114) became immiscible with water. The detergent phase was collected by centrifugation at 13000rpm for 10 minutes at room temperature. The upper aqueous phase was discarded and the lower detergent phase was transferred to a separate tube. The volume of detergent phase was noted and made up to the original volume of 400µl with ice-cold 0.1M Tris-HCl, pH 8.1. This was warmed to 37°C in the water bath and incubated for 10 minutes, followed by centrifugation at 13000rpm for 10 minutes. The upper aqueous phase was removed and the lower detergent phase was transferred to a separate tube and made up to a final volume of 120µl with 0.1M Tris-HCl. 20µl was aliquotted for a protein assay. 1µl of 25% CHAPS was added to the 100µl protein extracts to prevent any renewed phase separation and samples were stored at -20°C until use.

2.7.2 Determination of Protein Concentration

The Bio-Rad DC Protein Assay (Biorad, Hemel Hempstead, UK) was used, according to the manufacturer's instructions, in order to determine protein concentration of the cell extracts. This colorimetric assay is based on the reaction of the protein with an alkaline copper tartrate solution and the subsequent reduction of Folin reagent by the copper-treated protein, resulting in a blue colour that was measured spectrophotometrically.

A standard solution of 10mg/ml BSA in protein lysis solution was prepared and further diluted to form a standard curve. 5µl of each of the standards and cell lysates in triplicate were added to a 96-well plate. 20µl of reagent S (10% SDS) was added to 1ml of reagent A (an alkaline copper tartrate solution) and 25µl was added to each well containing the standards and samples. 200µl of reagent B (a dilute Folin reagent) was added to the wells and the plate left for 15 minutes at room temperature, after which the absorbance was measured at 620nm. A standard curve was then plotted and relative concentrations of the cell lysates calculated.

2.7.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was carried out according to the manufacturer's protocols (Biorad). Glass plates and spacers (0.75mm) were assembled in the clamp and clipped into the casting stand ensuring no leaks. The 10% acrylamide separating gel was prepared (Appendix 4) and poured into the plates to two-thirds full, propan-2-ol was overlaid and the gel left for 15 minutes to polymerise. The propan-2-ol was poured off and

completely removed using filter paper. The 4% acrylamide stacking gel was prepared (Appendix 4) and added to the top of the separating gel. The comb was carefully inserted into the stacking gel between the glass plates ensuring there were no air bubbles and the gel left to set for 10 minutes. The clamps were then transferred from the casting stand to the electrode assembly and clipped in. 1X running buffer (Appendix 4) was poured into the inner chamber and the combs slowly removed, running buffer was also poured into the tank to cover the bottom of the electrode assembly to complete the circuit.

Samples were prepared by the addition of sample buffer (Appendix 4) to the required volume of protein (as calculated from the protein assay). Under reducing conditions, β-mercaptoethanol was added to the sample buffer (Appendix 4) and protein samples were boiled at 90°C for 5 minutes in order to denature the proteins. The prepared samples and 10μl of molecular weight markers were then loaded into the wells. The gels were run at 100V for 10 minutes and then at 120V for a further 60-90 minutes until the bromophenol blue dye front of the protein sample had reached the bottom.

2.7.4 Membrane Transfer

The proteins were transferred from the gel to a nitrocellulose membrane by wet electroblotting, where a sandwich of gel and nitrocellulose was compressed in a cassette and immersed in buffer between two parallel electrodes. A current was passed through causing the separated proteins to electrophorese out of the gel and onto the nitrocellulose membrane.

Following gel electrophoresis, the electrode assembly was removed from the tank, the glass plates gently separated and the stacking gel discarded. The remaining gel containing protein was placed in 1X transfer buffer (Appendix 4) to equilibrate. The cassette for transfer was then assembled, which consisted of a sponge, followed by 4 sheets of Whatman filter paper soaked in transfer buffer, then the gel, the membrane, a further 4 sheets of soaked filter paper and finally another sponge, to complete the sandwich. Any air bubbles were rolled out and the cassette closed and placed in a transfer cell ensuring that the gel was on the side of the cathode, since proteins transfer from the cathode to the anode. The transfer cell was placed in a tank containing transfer buffer and allowed to run at 26V overnight. Alternatively, for the transfer of certain proteins such as uPAR, transfer was carried out at 60V for 4 hours, but was kept cool using ice packs. For this quick transfer method, 20% Methanol was added to the transfer buffer.

2.7.5 Immunodetection of Antigen

Post-transfer the nitrocellulose membrane was placed in blocking buffer (Appendix 4). After overnight transfer, the membrane was blocked in a 50ml tube on a roller for 1 hour at room temperature. After a 4-hour transfer, the membrane was blocked in an enclosed box at 4°C overnight. This stage blocks remaining hydrophobic binding sites on the membrane, to prevent any non-specific binding of the antibody. The blot was then washed three times for 15 minutes in PBST (Appendix 4). The primary antibody was diluted in 2ml of PBST and pipetted over the membrane in the tube. This was incubated on the roller for 1 hour at room temperature to enable the

antibody to bind to the specific antigenic sites on the membrane. The wash step was repeated and the membrane incubated with horseradish peroxidase labelled secondary antibody (diluted in 2ml PBST), for 30 minutes on a roller at room temperature (see Appendix 2 for antibodies and concentrations used). The membrane was then washed as before and protein detected by enhanced chemiluminescence (ECL, Amersham Biosciences).

2.7.6 Development of Blot

ECL is a method for the detection of immobilised specific antigens, conjugated directly or indirectly to horseradish peroxidase (HRP) labelled antibodies. An oxidation reaction occurs between the HRP molecules and the ECL reagent, to cause light to be emitted, which can then be detected on radiographic film. ECL was carried out in the dark room under safe light. Excess PBST was blotted off and the membrane placed on a sheet of Saranwrap. 2ml of ECL reagent 1 was mixed with 2ml of ECL reagent 2, pipetted over the blot and left for 1 minute. The excess was drained off; the blot wrapped in Saranwrap and placed protein side up into an X-ray cassette. A sheet of autoradiographic film was placed on top of the membrane and the cassette closed. Exposure times varied from 15 seconds to 20 minutes depending on the antibody used. The film was then immersed in developer for a few minutes until bands appeared, then placed in fixer for 5 minutes before being washed in water and dried.

2.8 WOUND ASSAYS

2 x 10^5 cells were seeded in 24-well plates in 1ml of KGM. After 24 hours incubation, cells were 'wounded' using a 200µl pipette tip, by making a cross through the centre of the well. An image, at time point 0, was taken under a light microscope under X200 magnification. The part of the wounded cross that was used was noted and the same sites were used for imaging at further time points of 4 hours and 6 hours. These images were then used to determine the rate of wound closure of the cells. Using Scion image software, at each time point, the width of the wound was measured at 5 points and averaged, so the rate of wound closure was determined. This was also measured in cells treated with 0.15µM Lys-plasminogen, post-wounding.

2.9 Migration Assays

Haptotactic cell migration assays were performed using matrix coated polycarbonate filters (8µm pore size, Transwell®, Becton Dickinson, Cowley, UK). The filters were placed in 24-well plates and the under-surface of the membrane was coated with 250µl of fibronectin (10µg/ml) diluted in PBS at 37°C for 1 hour. Both sides of the filters were then blocked with migration buffer (0.5% BSA in 3 parts DMEM, 1 part HAMS F12) for 30 minutes at 37°C. Before seeding cells were washed in migration buffer to remove the traces of trypsin. For blocking experiments, cells were incubated with specific antibodies and inhibitors (see chapter 6) for 20 minutes prior to seeding. The migration buffer from the inside of the Transwell insert was removed and 1X10^5 cells in 100µl migration buffer was added into the filter. The filter was

placed into a well containing 600µl migration, containing any appropriate antibodies or chemical inhibitors and keratinocytes were allowed to migrate towards fibronectin for 4 hours (figure 2.4). Cells migrated to the underside of the filter were fixed in 10% Formalin for 10 minutes, then stained with 0.5% Crystal Violet in 10% ethanol for 10 minutes. Inserts were washed twice in dH_2O and cells on the upper surface of the filter were gently wiped. When dry the membrane filter was carefully cut out and mounted on slides. Five random fields on each filter were counted under a light microscope at X200 magnification, to determine the average cell migration.

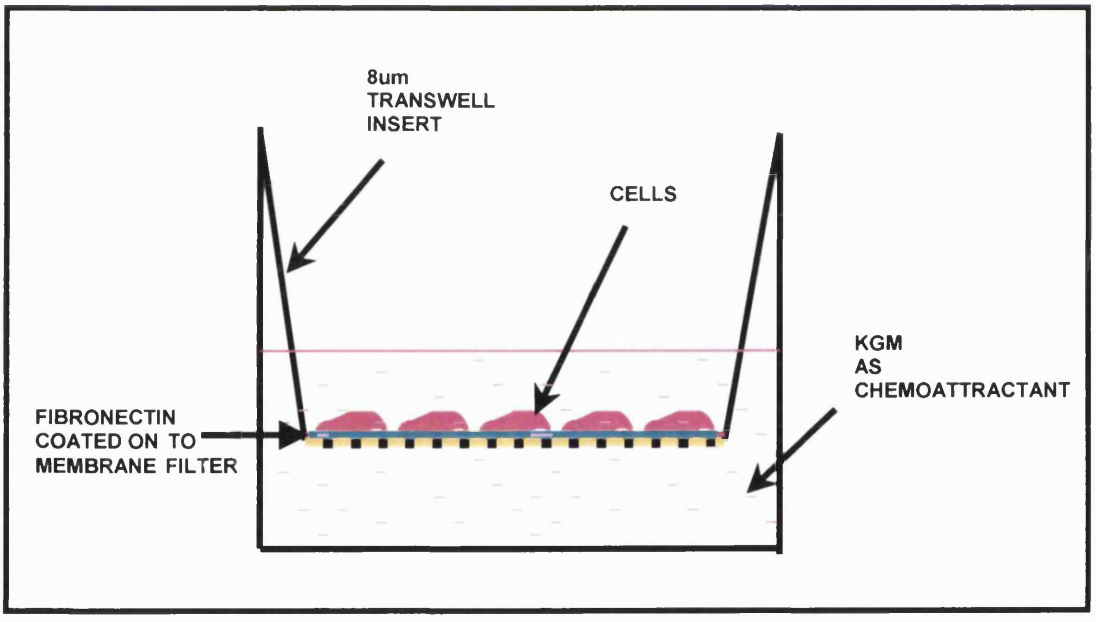


Figure 2.4 Haptotatic Cell Migration Assay.

2.10 INVASION ASSAYS

In vitro cell invasion assays were performed using polycarbonate filters (8μm pore size, Transwell®, Becton Dickinson, UK). In order to mimic the process of invasion, the filters were coated with a layer of matrigel (Becton Dickinson, UK) a basement membrane preparation composed of mainly collagen IV and laminin. The matrigel was allowed to thaw on ice overnight, then diluted 1:40 and 1:14 in dH_2O. 70μl of matrigel was added to the inside of the filters so the membranes were completely covered. This was allowed to set at 37°C for 1 hour so a solid layer of matrigel coated the membrane. Cells were washed in serum free medium (SFM) in order to completely remove any traces of serum. 2 X 10^5 cells in 100μl SFM were added onto the layer of matrigel. The filter was then placed into a well containing 750μl KGM which acts as a chemoattractant for the cells. Filters were incubated at 37°C for 48 hours, then fixed in 10% Formalin and stained with 0.5% Crystal Violet in 10% ethanol. The filters were washed in dH_2O and the cells on the inside were gently wiped away leaving those that had invaded through to the underside of the membrane. The membranes were cut out and mounted onto microscope slides. The cells in five random fields on each filter were counted under X200 magnification and the average cell invasion determined.

2.11 IMMUNOCYTOCHEMISTRY

Immunocytochemistry is a method that can be used to visualise proteins on or within a cell using labelled antibodies. An indirect method of detection was used where the primary antibody bound to the desired protein was detected by a FITC conjugated

secondary antibody and viewed using fluorescence, under the microscope. 13mm diameter glass coverslips (BDH) were sterilised by baking at 200°C for 2 hours. Cooled coverslips were placed into each well of a 24-well cell culture plate and coated with 10μg/ml plasma fibronectin, for 1 hour at 37°C. Matrix-coated coverslips as well as non-coated control coverslips were then blocked with 1% BSA for 30 minutes at 37°C. 10^5 cells were plated out onto each coverslip in serum free medium and incubated overnight before staining. Cells were washed 3 times with PBS then fixed in 1ml ice-cold 4% paraformaldehyde for 10 minutes on ice. Cells were blocked with 0.1M glycine/PBS for 5 minutes then washed 3 times with PBS. Cells were further blocked with 1% BSA/PBS for 30 minutes at room temperature, to eliminate any non-specific binding. Each well was then covered with 200μl of primary antibody diluted in 1% BSA/PBS and incubated at 37°C for 30 minutes. Cells were washed 4 X 5 minutes in PBS/0.1% Tween 20, incubated with a FITC-conjugated secondary antibody for 37°C for 30 minutes (see Appendix 2 for antibody concentrations and dilutions), washed 4 X 5 minutes in PBS/0.1% Tween 20, before a further wash for 1 minute in dH_2O to remove the PBS. Stained coverslips were the mounted and analysed under a fluorescent microscope (Leica, DMIRB, Milton Keynes, UK).

2.12 EXTRACTION AND PURIFICATION OF PLASMID DNA

2.12.1 Plasmid Vectors

Plasmid vectors are double-stranded closed circular DNA molecules that are found in a variety of bacterial species, where they behave as accessory genetic units that replicate. They contain a multiple cloning site (MCS) into which the gene of interest is inserted, enabling the production of large quantities of the desired gene (Sambrook et al, 1989). All of the plasmid vectors contained an ampicillin resistance gene; therefore bacterial cells were routinely grown in the presence of ampicillin.

2.12.2 Growth of Bacterial Culture

The XL1-Blue MRF' strain of *Eschericia Coli* (Stratagene) which has a very high transformation efficiency of > 1 x 10^9 transformants/μg was used for all bacterial work in this study. This strain carries the tetracycline (tet) gene to select for the F' episome, so bacterial growth was routinely carried out in the presence of 15μg/ml tetracyline. Under sterile conditions, a single bacterial colony of *E.Coli* was picked from an antibiotic-selective agar plate and used to inoculate 5ml of LB medium (Appendix 7) containing 15μg/ml tetracycline and 100μg/ml ampicillin in a 25ml sterile tube. Cultures were allowed to grow for 8-12 hours at 37°C with vigorous shaking until bacterial growth reached the late log phase. Bacteria were recovered by centrifugation at 4000 rpm for 5 minutes. The supernatant was completely removed and the bacteria stored at -20°C until lysis and extraction of plasmid DNA.

2.12.3 Minipreparations of Plasmid DNA

Plasmid DNA was extracted and purified using Qiaprep Spin Miniprep Kits (Qiagen, Crawley, UK) using a microcentrifuge and silica-gel-membrane technology. This procedure involves the alkaline lysis of bacterial cells followed by clearing of the bacterial lysate. Using QIAprep spin columns (Qiagen), the DNA was adsorbed onto silica-gel membrane in the presence of high salt. Following washes of the column with ethanol the plasmid DNA was eluted with 10mM Tris-Cl.

2.12.4 Large-Scale Preparations of Plasmid DNA and Purification (Maxiprep)

A single bacterial colony from a freshly streaked selective plate was used to inoculate a 5ml LB-medium containing tet/amp. This was incubated for 8 hours at 37°C with vigorous shaking (300 rpm). 1ml of this starter culture was then diluted into 100ml LB-medium, containing tet/amp and allowed to grow at 37°C for 12-16 hours with vigorous shaking. The bacterial cells were then harvested by centrifugation at 6000 rpm for 15 minutes at 4°C and then purified using the EndoFree™ Plasmid Kit according to the manufacturer's protocol (Qiagen). Endotoxin-free DNA improves the efficiency of transfection into sensitive or immunologically active cells. The method was based on a modified alkaline lysis procedure, where bacterial lysates were cleared by filtration with QIAfilter cartridges and plasmid DNA was purified by gravity flow through QIAGEN Anion-Exchange Resin tips under low salt and pH conditions. Any impurities were removed by a medium-salt wash. The plasmid DNA was then eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

2.13 CLONING IN PLASMID VECTORS

Cloning in plasmid vectors involved cleavage of the plasmid DNA with a restriction enzyme resulting in a linearised plasmid. This was then ligated to foreign DNA, which had been digested with the same restriction enzyme, so they had complementary sequences. Following ligation, the resulting recombinant plasmids were then used to transform bacteria to produce large quantities of the DNA.

2.13.1 Restriction Digests

2.5µl of 10X restriction enzyme buffer (AB gene Ltd., Surrey, UK) and 0.1U of enzyme (AB gene Ltd.) was added to 10µg DNA, mixed and incubated at 37°C for 4 hours. Following digestion, the restriction enzymes were inactivated by heat treatment at 70°C for 10 minutes.

2.13.2 Electroelution of Digested Fragments

The restriction digests were separated on a 1% agarose gel in TAE buffer (Appendix 7). The desired fragments were excised from the gel and placed into a 0.5ml Eppendorf tube, then purified using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Heating to 50°C in a high salt buffer dissolved the agarose and the DNA was bound to the silica-gel membrane of a spin column. All traces of agarose were removed to enhance purity and the DNA washed in ethanol before being eluted in 0.1M Tris/EDTA; pH8.0.

2.13.3 Ligation

The vector and insert DNA were mixed and diluted to a volume of 7µl in dH_2O, to which 2µl of T4 DNA ligation buffer and 0.7µl T4 DNA ligase (Promega, California, USA) was added. This was incubated at 16°C for 14 hours; 20µl dH_2O was then added to the ligation mix and the enzyme heat inactivated at 70°C for 10 minutes. The efficiency of ligation is dependent on the vector to insert ratio and needs to be determined experimentally.

2.13.4 Preparation of Antibiotic-Selective Agar Plates

1.5g of bacterial agar was added to 100ml of LB broth (Appendix 7) and autoclaved to dissolve. Under sterile conditions, whilst still hot, the antibiotics, 100µl of 10mg/ml ampicillin and 133µl of 12mg/ml tetracycline were added to the LB-agar. 10ml of this was poured into each petridish and left to set for 20 minutes and then stored at 4°C.

2.13.5 Preparation of Competent Bacterial Cells

In order for efficient bacterial transformation a transient state of 'competence' needs to be induced in the recipient bacteria, so that they are able to take up the DNA (Cohen et al, 1972). XL1-Blue *E.Coli* was made competent using the following method. 5µl of bacterial stock was streaked onto a tetracyline selective agar plate and incubated overnight at 37°C. A single bacterial colony was used to inoculate 5ml of LB-medium containing 15µg/ml tet and allowed to grow overnight at 37°C with vigorous shaking. This was diluted 1:100 into LB-medium plus tet and incubated at

37°C in a shaker for 3-4 hours until the bacteria reached the exponential growth phase. The bacteria were then pelleted at 4000 rpm for 10 minutes at 4°C and washed in ice-cold dH_2O. The pelleted bacteria were pooled and re-suspended into 40ml ice-cold 10% glycerol in dH_2O ensuring thorough mixing. This was centrifuged at 4000 rpm at 4°C for 15 minutes and the supernatant aspirated until there was about 100µl left. 800µl of 10% glycerol in dH_2O was added to this so that the bacterial pellet was re-suspended to a volume of 1ml. This was vortexed, kept on ice and 60µl aliquots of these competent bacteria were stored at -80°C.

2.13.6 Transformation of Competent E.Coli

Electroporation was used to introduce DNA into the *E.Coli*. For the transformation the electroporator was set at 1500V, 25mA, 25W, 50µF and 150Ohms. Sterile electroporation cuvettes were cooled and competent bacterial cells thawed on ice. 6µl DNA was added to 60µl of bacterial cells, transferred into the cuvette, placed in the electroporator and pulsed for 1 second. 250µl SOC buffer (Appendix 7) was immediately added to the bacteria, gently mixed and transferred to an eppendorff tube. This was incubated at 37°C for 15 minutes, to enable the bacteria to express their antibiotic resistance genes. The bacteria were then spread out onto selective agar plates, allowed to dry and incubated inverted at 37°C, overnight.

2.14 POLYMERASE CHAIN REACTION (PCR)

PCR results in the selective amplification of a DNA molecule by hybridisation of two short oligonucleotides, which act as primers to the border sequences of the defined region of the DNA molecule to be amplified. Thermostable Taq DNA polymerase is added to the primed template DNA and incubated so that the enzyme synthesises new complementary strands. This is heated to 94°C, so that the newly synthesised strands detach from the template and cooled enabling more primers to hybridise to their respective positions, including positions on the newly synthesised strands. The Taq polymerase then catalyses a second round of DNA synthesis. The cycle of denaturation-hybridisation-synthesis is repeated, 25-30 times, resulting in the eventual synthesis of several hundred million copies of the amplified DNA fragment.

For a 20µl PCR reaction mix, the following were added to a PCR tube on ice:

2µl 10X Taq polymerase buffer (Promega)

2µl $MgCl_2$ (Promega, UK)

0.4µl 5' primer and 0.4µl 3' primer (Sigma)

1.6µl dNTPs (this is a master mix of the nucleotide bases: Adenine, Cytosine, Guanine and Thymine (Promega)

1µl template DNA

12.2µl dH_2O

0.4µl Taq polymerase

The PCR reaction mix was overlayed with 10µl mineral oil (Sigma) and immediately placed in the PCR machine for amplification.

The PCR programme used in this study was an initial denaturation at 95°C for 5 mins. This was followed by denaturation at 94°C for 30 secs, annealing at 62°C for 1 min (the optimal annealing temperature is determined by the nature of the primers) and extension at 72°C for 30 secs. This step was repeated for 30 cycles, followed by a final extension of the newly synthesised DNA strands at 72°C for 2 mins.

CHAPTER 3

αvβ6 MODULATION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR (uPA) IN ORAL SCC CARCINOMA CELL LINES

3.1 INTRODUCTION

In a number of physiological and pathological situations, intricate interactions between proteolytic enzymes and integrins exist in order to regulate cell-matrix interactions that are central to the processes of cell migration and invasion. Increased uPA expression is a feature of many malignant cancers and it is an independent prognostic marker (Duffy et al, 1988). uPA is secreted as an inactive precursor (pro-uPA), that binds with high affinity to uPAR. It converts enzymatically inactive plasminogen abundant in the extracellular fluid into plasmin, which can degrade most components of the extracellular matrix (ECM) either directly or via activation of matrix metalloproteases (MMPs), thus promoting cellular migration (reviewed by Irigoyen, 1999).

Altered integrin expression is also a common feature of tumours and the αvβ6 integrin is neo-expressed in a number of epithelial carcinomas (Breuss et al, 1995, Jones et al, 1997). αvβ6 has previously been shown to promote MMP-9 secretion from epithelial carcinoma cells (Thomas et al, 2001a, Niu et al, 1998). Since activation of pro-MMP-9 may be catalysed by plasmin generated from the plasminogen activation cascade, it was thought that αvβ6 may also directly regulate the expression of uPA. Of late studies have suggested a direct link between the plasminogen activator system and integrins, with expression levels of uPA being influenced by β1 integrins (Ghosh et al, 2000) and αvβ3 integrin (Hapke et al, 2001b).

3.2 MATERIALS AND METHODS

3.2.1 Cell Lines

The human oral SCC line H357 and its derivatives, created to express different integrin heterodimers: V3, VB6, VB6Δ_{11aa}, V3B3 and C1 were used in this study (see section 2.1).

3.2.2 Flow Cytometry

Integrin expression on the cell surface was determined by flow cytometry (see section 2.3). Cells were labelled with monoclonal primary antibodies (see Appendix 2) for 1 hour at 4°C:

-αv integrin : clone L230 (50μg/ml)

-αvβ5 integrin : clone P1F6 (100μg/ml)

-αvβ6 integrin : clone E7P6 (100μg/ml)

-β1 integrin : clone P1D6 at 1:25 dilution

-αvβ3 integrin : clone 13C10 (100μg/ml)

Cells were then washed and incubated with secondary antibody: Rabbit anti-Mouse FITC-conjugated or Goat anti-Mouse RPE-conjugated both at a 1:50 dilution for 30 minutes at 4°C (Appendix 2).

3.2.3 Plasminogen Activator Activity Assay

Cells were seeded at 1 X 10^5 per ml in 24-well plates. PA activity in cultured supernatants was measured using a chromogenic assay (see section 2.4.2). The

supernatants were collected as described in section 2.4.1 and basal levels of secreted PA activity were measured from cells plated on plastic.

Cell-associated extracts were also collected where cells were washed in PBS, then harvested by scraping in 200μl of 50mM Tris / 100mM NaCl / 0.1% Triton X-100; pH7.4 on ice. Experiments were routinely carried out in triplicate.

In order to see any integrin-ligand effects on secreted PA activity, a number of methods were employed.

1) **Matrix-coated wells.** 24-well tissue-culture plates were coated for 1 hour at 37°C with 100μl of various matrix proteins diluted in PBS. These were plasma fibronectin (pFN, 10μg/ml), cellular fibronectin (cFN, 10μg/ml), collagen 1 (COL1, 10μg/ml) and vitronectin (VN, 250μg/ml) (all from Sigma Chemical Co., Poole, UK). Wells were then blocked with 0.1% BSA for 1 hour at 37°C and then washed twice with PBS. Cells were washed twice in SFM (0.1%BSA), to remove all traces of KGM before seeding the cells in SFM. Cells were seeded onto plastic coated with 0.1% BSA as a control. After incubation for 16 hours, supernatants were collected and assayed.
2) **Incubation with soluble ligand.** Cells were washed twice in SFM (0.1% BSA), before seeding in SFM (0.1% BSA). After incubation for 14 hours, cells were washed once with PBS. Plasma fibronectin was diluted in SFM to a final concentration of 10μg/ml. Cells were incubated with 0.5mls of this

soluble fibronectin and with SFM alone as a control, for 8 hours at 37°C, after which the supernatants were collected.

3) **Treatment with matrix protein and antibody-conjugated latex beads.** Latex beads were first conjugated to antibody and fibronectin as previously described (Ghosh et al, 2000). A 10% stock of latex beads (2.97μm, Sigma), were diluted in 100mM MES; pH 6.1 to give a 1% solution. Beads were allowed to conjugate to antibody (anti-β1/P4C10, anti-αvβ6/E7P6 and mouse IgG for the control) and plasma and cellular fibronectin at 75μg/ml final concentration, in a 0.5ml Eppendorf tube, overnight at 4°C with gentle agitation, then kept at 4°C until use. 2 x 10^4 cells were seeded in 100μl in 96-well plates in KGM and allowed to adhere for 4 hours. The medium was removed, cells washed twice with PBS, then incubated with 100μl SFM for 1 hour. During this incubation, the conjugated beads were blocked with 10mg/ml BSA for 90 minutes at room temperature with gentle agitation, and then centrifuged at 3000rpm for 3 minutes. The supernatant was removed and the volume of beads remaining noted. These were then washed in 2 volumes of SFM (0.1% BSA) before being re-suspended in SFM at a final concentration of 1% by volume. The supernatant was removed, cells treated with 100μl of conjugated beads in SFM and incubated at 37°C for 15 hours; supernatants were then collected, cleared of debris and assayed for PA activity (see section 2.4.2).

4) **Antibody blocking.** Wells were coated with plasma fibronectin and BSA for the controls as described above. Following trypsinisation, cells were washed

in SFM and incubated with the anti-αv antibody, L230 (10μg/ml) and anti-αvβ6 antibody, 10D5 (10μg/ml) for 30 minutes on ice. 1 x 10^5 cells were then seeded onto the coated wells in 500μl SFM. Further antibody at a 10μg/ml final concentration was added to the medium. Following a 15-hour incubation at 37°C, supernatants were collected and assayed for PA activity.

5) **Treatment with Signalling Pathway Inhibitors.** Cells were pre-treated with the chemical inhibitors (i) UO126 (100μM, Calbiochem, San Diego, California), which inhibits ERK1/2 of the MAP Kinase pathway, (ii) SB203580 (20μM, Calbiochem) which inhibits the p38 MAP Kinase pathway and (iii) Calphostin C (100nM, Calbiochem) which inhibits the protein kinase C pathway, by incubation of the cells for 20 minutes at room temperature. Cells were then seeded into 24-well plates in SFM containing chemical inhibitors and incubated for 15 hours at 37°C. Supernatants were collected and assayed for PA activity.

3.2.4 ELISA

The levels of uPA and tPA antigen in the cultured supernatants were determined using commercial ELISA kits (Biopool International, Alpha labs, Ensleigh, UK) (see section 2.4.3).

3.2.5 Immunocytochemistry

Cells were seeded onto sterile glass coverslips (BDH, Poole, UK) in 24-well plates at a density of 1 x 10^5 per well in 0.5ml KGM. After 4 hours, the medium was

removed; cells were washed twice with PBS and incubated with 0.5ml SFM. After a 15-hour incubation, cells were stained for fibronectin using the monoclonal antibody (see section 2.11).

3.2.6 Northern Blotting

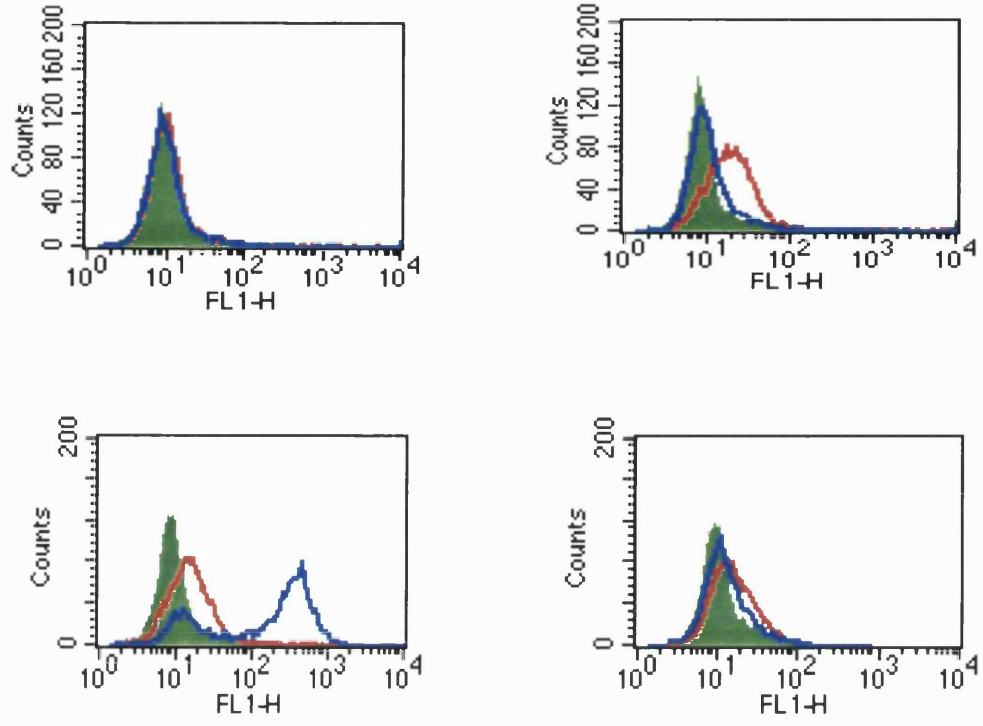
The cDNA probe for uPA was generated as described in section 2.6.2.2. RNA extracts of all the cell lines were prepared and subjected to Northern blotting (see section 2.6). Levels of stable uPA mRNA expression were determined by densitometric analysis using Scion image software and normalised to ribosomal RNA levels as shown by ethidium bromide staining.

3.3 RESULTS

3.3.1 Integrin Expresssion of Oral Carcinoma Cell Lines

Integrin expression of the cell lines was determined by FACS analysis. The results confirm the lack of αv-integrin expression in the H357 cell line, initially demonstrated by Prime et al, 1990 (figure 3.1A). The V3 cell line, which was created by transfection with αv cDNA, display a marked increase in αv-integrin subunit expression, but this is not in the form of the αvβ6 heterodimer, since αvβ6 expression was minimal (Jones et al, 1997). The V3 cells were further infected with β6 cDNA to create the VB6 cell line, which expressed αvβ6 at very high levels but αv expression was maintained at the same level as V3 cells (Thomas et al, 2001c). The integrin expression pattern of the control C1 cells was the same as that of the V3 cells, with positive αv-subunit expression but no αvβ6 expression. Integrin expression was quantified using the geometric mean values, which represents the population of cells positively stained with integrin antibody (figure 3.1B). All cell lines expressed similar levels of the β1-integrin subunit (data not shown).

A)



B)

	negative control	αv	αvβ6
H357	9.74	10.39	8.77
V3	11.54	25.32	13.15
VB6	9.97	22.41	155.83
C1	10.06	17.4	11.55

Figure 3.1

Integrin Expression of the Oral Carcinoma Cell Lines

Expression of αv integrin (red) and αvβ6 integrin (blue). Cells were stained with anti-αv (L230) and anti-αvβ6 (10D5) primary antibodies and then a FITC-conjugated secondary antibody. Integrin expression was quantified using the geometric mean values. A representative result is shown.

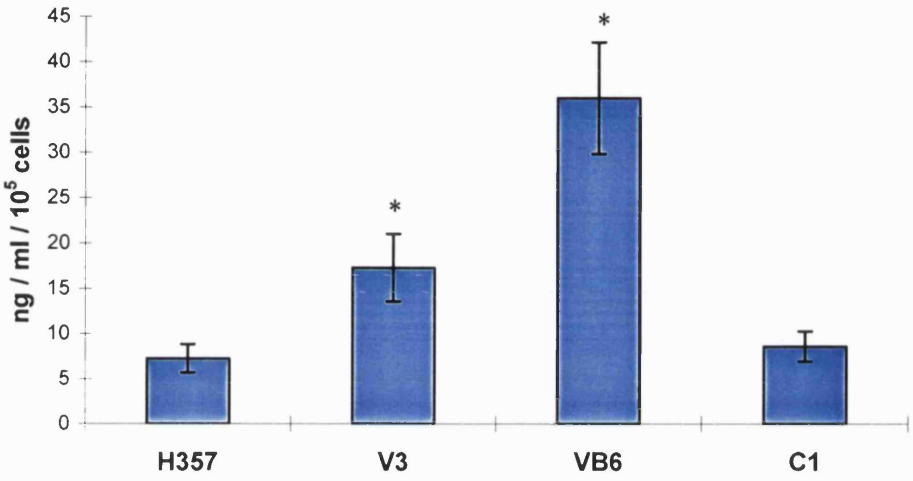


Figure 3.2 Secreted Plaminogen Activator Activity

Supernatants were assayed for uPA activity using CS-2251 and Glu-Plg in excess. Absorbance was measured at 405nm and plasmin generation expressed as ng/ml by comparison with uPA standards and corrected for cell number. Figure shows the mean ± SD of a representative experiment done in triplicate. VB6 cells produce a significantly greater level of PA than V3 and H357 parental cells ($p < 0.05$, Mann-Whitney-U-Test).

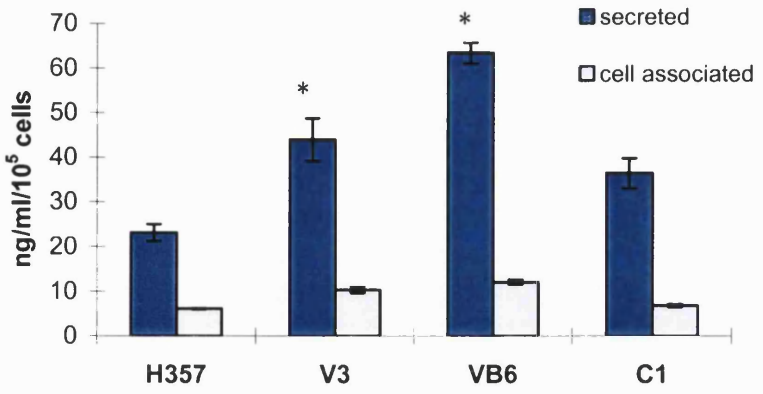


Figure 3.3 Secreted and Cell-Associated PAA

Supernatants and cell-extracts were assayed for uPA activity using CS-2251 and Glu-Plg in excess. Absorbance was measured at 405nm and plasmin generation expressed as ng/ml by comparison with uPA standards and corrected for cell number. Figure shows the mean ±SD of a representative. VB6 cells produce a significantly greater level of PA than V3 and H357 parental cells ($p < 0.05$, Mann-Whitney-U-Test).

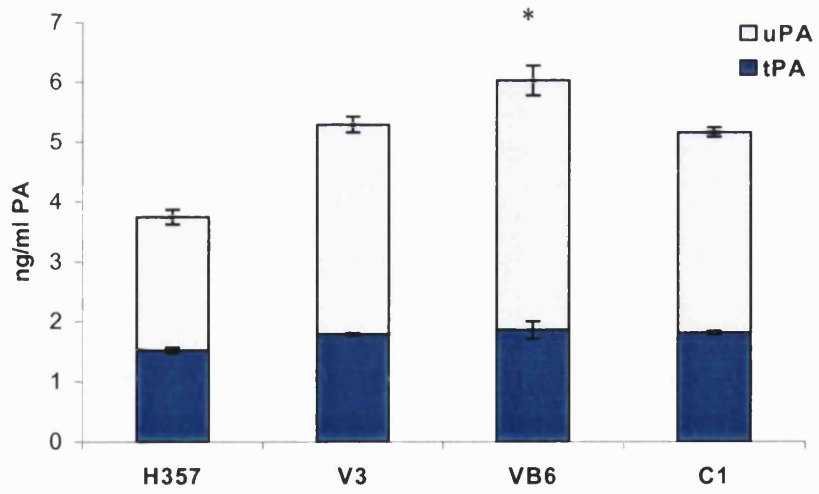


Figure 3.4 ELISA for uPA and tPA

Levels of uPA (grey) and tPA (black) antigen were determined by interpolation from a standard curve using known concentrations of appropriate standards. Figure shows

mean +SD of a representative experiment in triplicate. Levels of tPA are similar between the cell lines but VB6 cells show a significantly greater level of uPA.
In addition to seeding cells onto matrix-coated wells, cells were also incubated with soluble fibronectin. However, no significant difference in secreted uPA activity was seen in cells incubated with soluble fibronectin compared to control cells (figure 3.6).

In order to observe a ligand-dependent effect of the αvβ6 integrin on uPA secretion, an additional method of incubating cells with antibody- or matrix-conjugated latex beads, was carried out (figure 3.7). However, as with the previous experiments, there was no significant difference in the levels of uPA secretion between the treated and control cells.

Inhibition of αvβ6-ligand binding by anti-αvβ6 (10D5) and anti-αv (L230) blocking antibodies also had no effect on uPA secretion, providing further evidence that the αvβ6-associated increase in uPA secretion is ligand-independent (figure 3.8).

These oral carcinoma cells produce their own fibronectin in the time course of the assay, as shown by staining of the cells with anti-human fibronectin antibody, after a 16-hour incubation at 37°C in serum-free medium (figure 3.9). Fibronectin expression can be seen at cell-cell junctions.

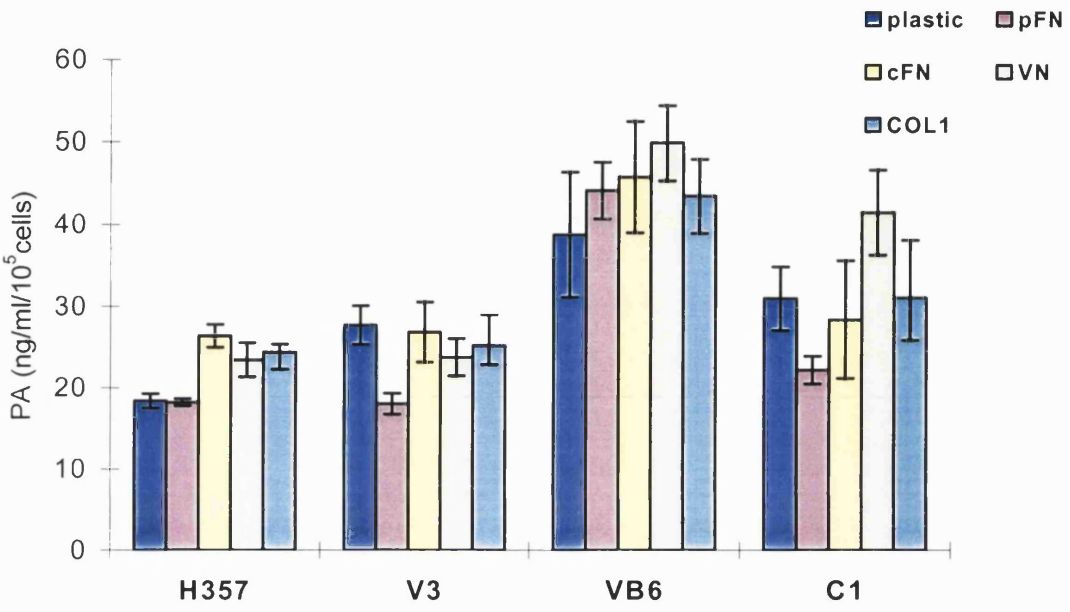


Figure 3.5 Effect of ECM proteins on Secreted PA Activity

Cells were plated on various substrates and cultured supernatants were assayed for uPA activity using CS-2251 and Glu-Plg in excess. Absorbance was measured at 405nm and plasmin generation expressed as ng/ml by comparison with uPA standards. Figure shows mean +SD of a representative experiment in triplicate. There were no significant differences in secreted uPA levels when the Mann Whitney U-test was performed on data from 3 independent experiments in triplicate.

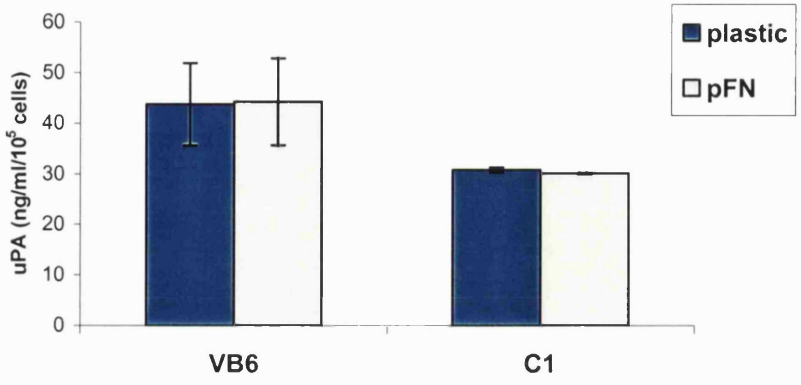


Figure 3.6 Effect of Soluble Fibronectin on Secreted PA Activity

Cells were incubated with soluble plasma fibronectin and cultured supernatants were assayed for PA activity using CS-2251 and Glu-Plg in excess. Absorbance was measured at 405nm and plasmin generation expressed as ng/ml by comparison with uPA standards Figure shows mean +SD of a representative experiment in triplicate.

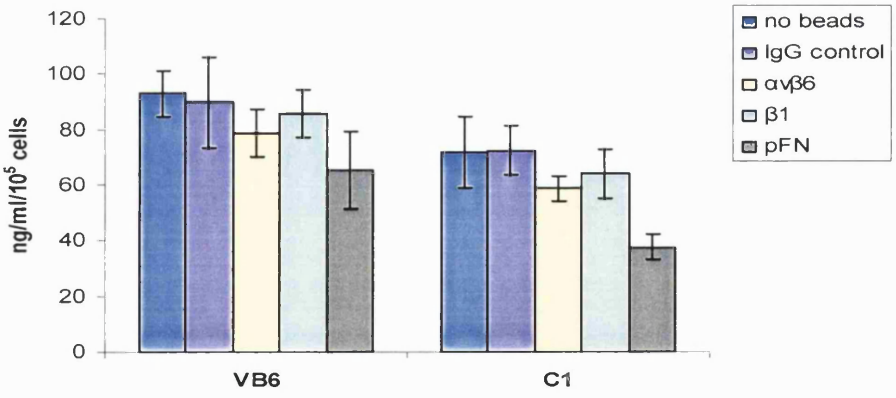


Figure 3.7 Effect of Integrin Clustering on Secreted PA Activity

Cells were treated with antibody- and matrix-conjugated latex beads and cultured supernatants were assayed for PA activity. Figure shows the mean ±SD of a representative experiment in triplicate.

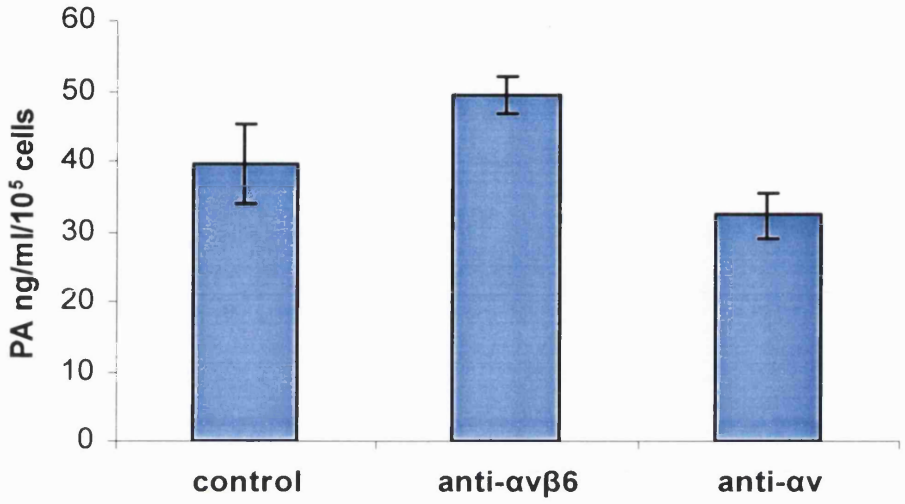


Figure 3.8 Effect of Anti-Integrin Blocking Antibodies on uPA Secretion

VB6 cells were treated with blocking antibodies and plated onto plasma fibronectin. Cultured supernatants were assayed for PA activity. Figure shows the mean ±SD of a representative experiment in triplicate.

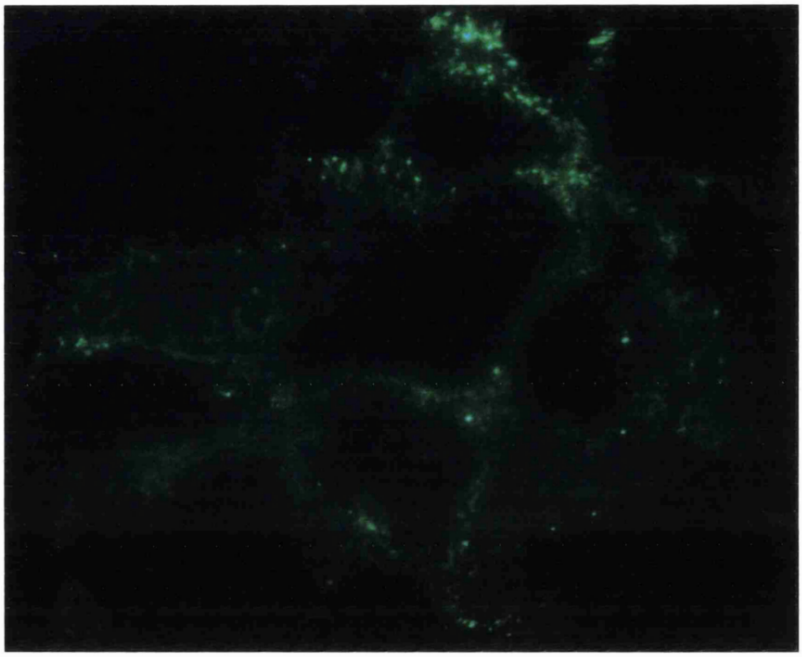


Figure 3.9 Fibronectin expression on VB6 cells

Cells were stained with a monoclonal anti- human fibronectin antibody, followed by a FITC-conjugated secondary antibody and visualised by flurorescence microscopy using X630 magnification.

3.3.3 uPA secretion is mediated by the ERK1/2 branch of the MAP Kinase pathway

Cells were treated with chemical inhibitors of the MAP Kinase and protein kinase C pathways. In order to determine the optimal inhibitory concentration, a range of concentrations was tested (data not shown) as well as using previously recommended concentrations. uPA secretion was inhibited by 75% in all the cell lines, compared to control cells, with 100μM UO126, which inhibits the ERK1/2 branch of the MAP Kinase pathway. However, inhibition of the p38 branch of the MAP Kinase pathway using SB203580 or the protein kinase C pathway, using Calphostin C had no effect on uPA secretion (figure 3.10).

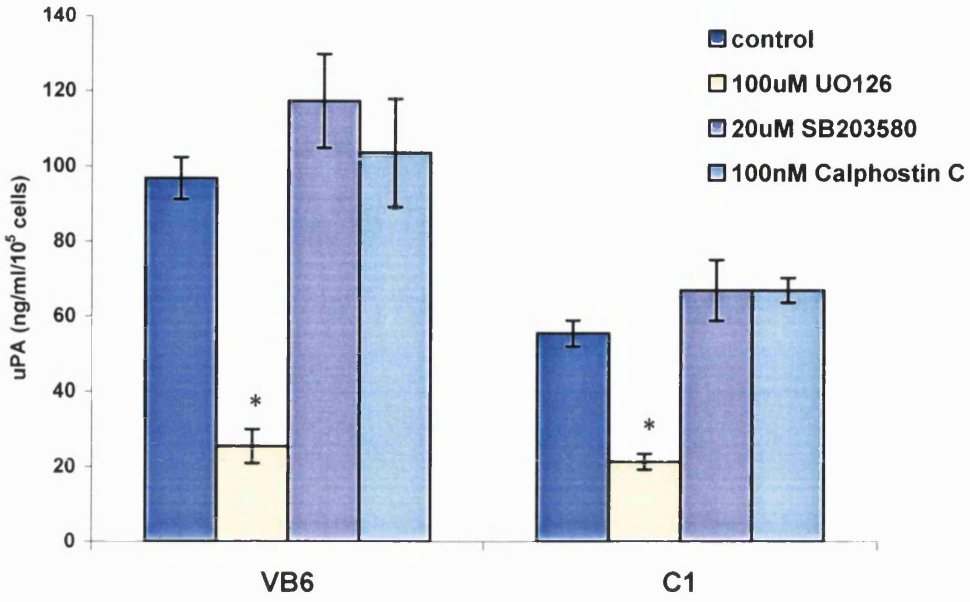


Figure 3.10 PA Secretion Following Treatment with Signalling Pathway Inhibitors

Cells were incubated with chemical inhibitors of ERK1/2 MAPK (UO126), p38 MAPK (SB203580) and protein kinase C (Calphostin C) pathways. Cultured supernatants were assayed for PA activity using CS-2251 and Glu-Plg in excess. Absorbance was measured at 405nm and plasmin generation expressed as ng/ml by comparison with uPA standards Figure shows mean +SD of a representative experiment in triplicate. Inhibition of ERK1/2 significantly decreased PA secretion from all of the cell lines ($p < 0.05$, Mann-Whitney-U-Test).

3.3.4 The 11 amino acid extension of the β6 cytoplasmic tail modulates uPA secretion

The β6 integrin subunit exclusively contains an extra 11 amino acid C-terminal cytoplasmic extension which has previously been shown to mediate cell proliferation and MMP-9 secretion. In this study mutated β6 cDNA lacking this unique sequence and full-length β3 cDNA were also retrovirally infected into the V3 cells (see section 2.1) to create the VB6Δ_{11aa} and V3B3 cell lines. Integrin expression in these cell lines was confirmed by FACS analysis (figure 3.11). αv expression in both cell lines was similar to the control C1 cells with a mean fluorescence intensity of about 150 in all the cell lines. Even though transfected with mutated β6 cDNA, the VB6Δ_{11aa} cells expressed high levels of αvβ6 (geo mean = 146) compared to the C1 and V3B3 cells (geo mean = 105 and 117 respectively). The levels of αv and αvβ6 expression are much higher than in the previous FACS study (figure 3.1), because the RPE (r-phycoerythrin)-conjugated secondary antibody used a much more sensitive filter in the flow cytometer, detecting fluorescence at 567nm. αvβ3 expression in the V3B3 cell line (geo mean = 130) was greatly increased compared to the control C1 cells (geo mean = 10), although the VB6Δ_{11aa} cells did display some αvβ3 (geo mean = 90).

PA activity in the conditioned media of VB6 cells expressing full-length αvβ6 is 60% greater than that of the control, C1 cells. Expression of mutant β6, lacking the 11aa cytoplasmic tail extension (VB6Δ_{11aa}), does not significantly increase PA activity

compared to control cells, indicating that this unique region tail may be responsible for mediating this effect (figure 3.12). Cells over-expressing αvβ3 did not show significantly increased levels of uPA secretion, providing further evidence for a specific β6-mediated effect.

3.3.5 Expression of uPA mRNA

In these cells, the levels of stable uPA mRNA expression are similar between the cell lines, suggesting that αvβ6 does not affect transcription of the uPA gene, therefore is modulating uPA secretion further downstream (figure 3.13).

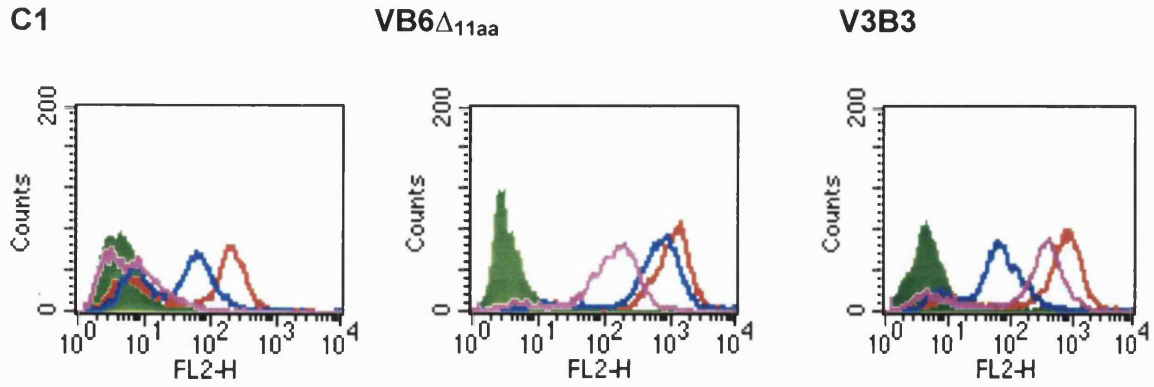


Figure 3.11 Integrin Expression of Transfected Oral Carcinoma Cell Lines

Expression of αv integrin (red), αvβ6 integrin (blue) and αvβ3 integrin (purple). Cells were stained with anti-αv (L230), anti-αvβ6 (10D5) and anti-αvβ3 primary antibodies followed by an RPE-conjugated secondary antibody.

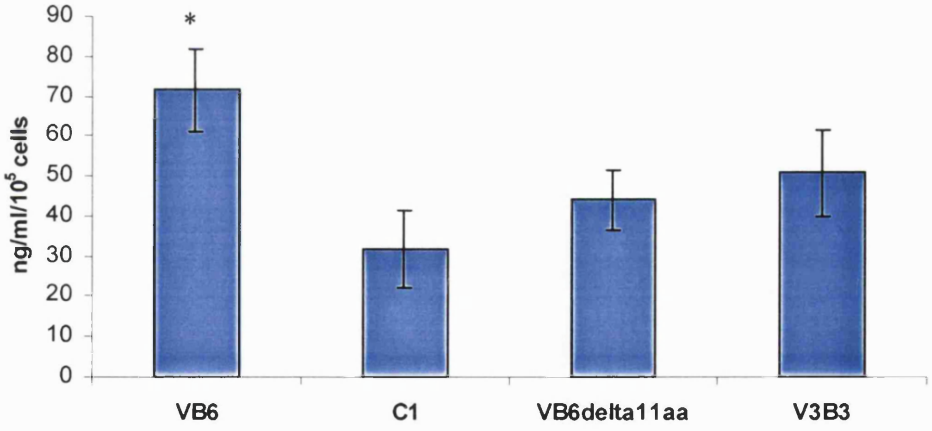


Figure 3.12 Secreted PA Activity of Transfected Oral Carcinoma Cell Lines

uPA secretion is significantly increased from cells expressing full-length αvβ6 but not from cells expressing mutated αvβ6 or αvβ3. Results are expressed as ng/ml uPA secreted per 10^5 cells by comparison with uPA standards. Figure shows mean ±SD of a representative experiment ($p < 0.05$, Mann-Whitney-U-Test).

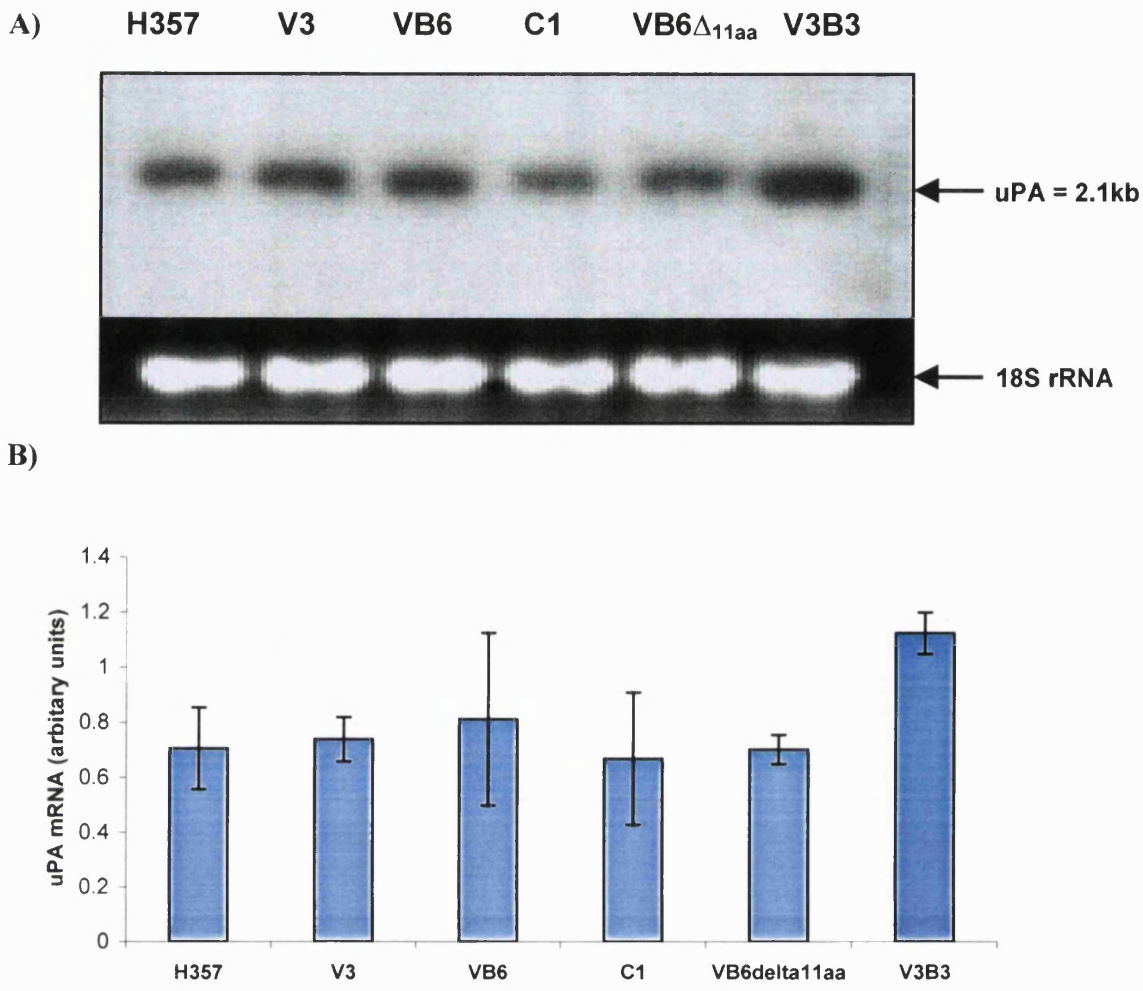


Figure 3.13 Expression of uPA mRNA

uPA mRNA levels are similar in the αvβ6-expressing and control cells. Total cellular RNA was extracted and subjected to Northern blotting. Filters were probed with ^{32}P-labelled full-length uPA cDNA.

(A) Top panel- stable uPA mRNA expression. Bottom panel – Ethidium bromide stain of 18S ribosomal band. A representative result is shown.

(B) Blots were subjected to densitometric analysis using Scion image software, normalized to 18S ribosomal bands and expressed in arbitrary units. Figure shows the combined data of two experiments of 6 samples each.

3.4 DISCUSSION

The plasminogen activation system plays a central role in tumour biology, with evidence being gained from experimental invasion and metastasis models and from expression patterns for components of the uPA system in tumours and normal tissues. It has previously been shown that expression of αvβ6 into the oral carcinoma cells used in this study up-regulates MMP-9 secretion, another protease important in proteolytic matrix degradation and this was functional in mediating cell migration and invasion (Thomas et al, 2001a). Also, in colon carcinoma cells, αvβ6 promoted MMP-9 secretion, through the unique C-terminal cytoplasmic extension (Niu et al, 1998). The effect of αvβ6 integrin on uPA expression was therefore examined in order to further delineate the mechanism by which certain integrins promote the invasive phenotype via control of protease gene expression.

uPA secretion was increased from the V3 cells that had been transfected with αv cDNA, compared to the parental cell line, H357, which completely lacked αv expression. This initial increase is possibly an αv integrin effect or could be due to the small amount of endogenous αvβ6 present in the cells. However, VB6 cells expressing full-length αvβ6 secreted significantly greater levels of uPA than the control C1 cells indicating modulation by the β6 integrin subunit.

Intracellular signals are generated subsequent to ligand binding, enabling ECM-specific information to be provided to the cells (Damsky and Werb, 1992) and

integrin engagement has been shown to regulate expression of numerous gene products (Humphries, 1996 and Boudreau and Jones, 1999), however the contribution of integrins to modulation of uPA expression is inconclusive. Activation of the αvβ6 integrin by plating cells onto fibronectin-coated wells or incubation with plasma fibronectin, an αvβ6 ligand, did not significantly increase uPA secretion in this study, suggesting that αvβ6 modulation of uPA is a ligand-independent process. In previous experiments using the same cell lines as described in this chapter, only the αvβ6-expressing cells significantly up-regulated MMP-9 and MMP-2 secretion as shown by gelatin zymography and ELISA. The integrin dependence of this up-regulation on fibronectin was shown using anti-αv and anti-αvβ6 integrin blocking antibodies. However, the αvβ6-expressing cells also produced increased relative amounts of MMP-9, when plated on plastic that was not inhibited by anti-αvβ6 antibodies, suggesting a degree of ligand independence (Thomas et al, 2001a). In the SW480 colon carcinoma cell line, MMP-9 secretion was induced in cells transfected with the β6-integrin. This increase was maintained in non-adherent cells as well as cells adherent to irrelevant matrix proteins indicating that unligated αvβ6 integrin is capable of inducing MMP-9. No effect on MMP-9 secretion was seen when cells plated on fibronectin were treated with anti-αvβ6 antibody indicating that this MMP-9 up-regulation was through a ligand-independent mechanism (Niu et al, 1998).

In addition to plating cells directly on matrix-coated wells, cells were treated with immobilised integrin antibodies or matrix proteins on latex beads in order to induce integrin clustering. Recently, it was shown that aggregation of α3β1 integrins in oral

keratinocytes, exerts multifunctional control on the uPA system by inducing expression of uPA and PAI-1 as well as regulating the membrane localisation of uPAR (Ghosh et al, 2000). However, in our model system, αvβ6-ligand engagement by any method did not enhance uPA secretion.

Antibody-blocking experiments were performed in order to confirm any ligand-dependent effects of the integrin. Treatment with antibodies blocked the ligand-binding site on the integrin, therefore blocking ligand activation of the integrin. Cells treated with anti-αv antibody slightly inhibited uPA secretion suggesting that the αv subunit is involved in modulating uPA secretion, although this was not to a significant degree. Cells treated with anti-αvβ6 antibody showed a slight increase in uPA secretion but also not to a significant degree. In this case the antibody may actually be stimulating the action of αvβ6. The activated state of integrins for example αIIbβ3, has been shown to be induced by monoclonal antibodies, where even solubilised receptor was activated by antibody (O'Toole et al, 1991). Also, activation of β1 integrin using anti-β1 antibody modulated its function to enhance uPA secretion (Ghosh et al, 2000).

There have been a few studies specifically showing modulation of uPA expression following integrin engagement. In human fibrosarcoma cells, adhesion to FN caused a 2- to 3-fold upregulation of uPA (Ciambrone et al, 1992) and in human keratinocytes, clustering of α3β1 resulted in enhanced uPA secretion (Ghosh et al, 2000). In contrast, it was recently reported that αvβ3 ligation to vitronectin in

ovarian cancer cells, down-regulated uPA expression at the transcriptional level (Hapke et al, 2001a).

uPA gene expression is generally increased in metastatic cells due to a combined increase in gene transcription and mRNA stability (Henderson et al, 1992). The control of uPA expression can occur at both the transcriptional and post-transcriptional levels. The levels of stable uPA mRNA expression are similar between the cell lines, suggesting that the αvβ6-associated increase in uPA secretion in VB6 cells is a result of a possible translational modification to increase protein synthesis from the mRNA transcript.

The cytoplasmic domains of integrin β-subunits interact with many different cytoskeletal and regulatory proteins, which mediate integrin functions (Calderwood et al, 2001). The β6-subunit has a high sequence homology to the β1, β2 and β3 integrin subunits (Sheppard et al, 1990), with highly conserved residues including two NPXY motifs. The sequences in β6 shared by other β-subunit cytoplasmic domains, are sufficient to support functions that are shared with β1 and β3 integrins, i.e. localisation to focal contacts and promotion of cell adhesion and spreading. However, β6 exclusively contains an extra 11 amino acid COOH-terminal cytoplasmic extension not shared by other β integrin subunits (Sheppard et al, 1990). Using cytoplasmic tail mutants of colon carcinoma cells, it was shown that stimulation of cell proliferation, a unique function of the β6 integrin, critically depends on the presence of the unique 11aa extension (Agrez et al, 1994).

Subsequent studies showed that this sequence also regulated MMP-9 secretion through activation of the protein kinase C signalling pathway, suggesting that αvβ6 functions are regulated by this 11-amino acid extension (Agrez et al, 1999, Niu et al, 1998).

Using the mutant VB6Δ_{11aa} cell line, the results demonstrate for the first time that the αvβ6-mediated enhanced uPA secretion is also regulated by this unique 11aa sequence in the β6 cytoplasmic tail. This C-terminal extension, however, does not mediate binding to fibronectin, thus substantiating the observation that αvβ6 binding to its ligand does not affect uPA secretion, a function which is specifically mediated by the β6 tail. The β6 cytoplasmic domain has recently been shown to bind directly to extracellular signal-regulated kinase 2 (ERK2), through a motif on the β6 cytoplasmic domain that is upstream of the 11aa tail (Ahmed et al, 2002b). Therefore, it is likely that β6 modulation of the urokinase system occurs through activation of the MAP Kinase signalling pathway, via the β6 cytoplasmic tail. However in this study, although blocking ERK1/2 inhibited uPA secretion, this was not specific to β6-expressing cells, indicating that this branch of the MAPK pathway is involved in generally mediating uPA secretion from the cells. This is consistent with previous data demonstrating a role of the three MAP Kinase pathways (ERK1/2, JNK/SAPK and p38) in inducing expression of components of the urokinase system (Ossowski and Aguirre-Ghiso, 2000).

CHAPTER 4

αvβ6 MODULATION OF THE UROKINASE RECEPTOR (uPAR) IN ORAL SCC CARCINOMA CELL LINES

4.1 INTRODUCTION

The expression of the urokinase receptor *in vivo* has most extensively been studied in cancer, a situation in which the involvement of proteolytic enzymes is implicated in the invasive process. In all types of human cancers so far studied, uPAR has been found to be consistently present at the invasive foci of the tumours, together with uPA. uPAR expression is generally increased in cancer cells and is widely accepted as an independent prognostic marker (reviewed by Andreasen et al, 1997).

The urokinase receptor, by targeting uPA to the plasma membrane, acts as a focal point for plasmin generation leading to proteolytic degradation of the ECM. uPAR is also able to influence multiple biological events such as cell adhesion, migration and proliferation that are crucial to the processes of tumour invasion and metastasis (Ossowski and Aguirre-Ghiso, 2000), implicating uPAR as a potential signal transducing molecule. The lack of a transmembrane domain in uPAR suggests an association with other adaptor proteins, such as integrins, that are capable of direct interactions with the cell interior (Ossowski and Aguirre-Ghiso, 2000, Preissner et al, 2000, Engelholm et al, 2001, Chapman and Wei, 2001). This leads to an assembly of multimolecular complexes on the cell surface, that are able to dynamically regulate plasmin generation and initiate intracellular signalling events to influence cellular function.

The processes of proteolysis and adhesion, via uPAR and integrins have been linked in a number of recent studies. In addition to the physical association of uPAR with various β-integrin subunits, functional interactions between uPAR and integrins have been shown to influence both integrin and uPAR-directed cell migration (Wei et al, 1999). *In vivo*, there is evidence to suggest that uPAR-integrin complexes are functionally involved in tumour progression (van der Pluijm et al, 2001). Over-expression of the β3- integrin subunit in CHO cells down-regulated uPAR expression at the transcriptional level (Hapke et al, 2001a). However, in melanoma cells αvβ3 integrin over-expression (Nip et al, 1995) and activation (Khatib et al, 2001) was linked to elevated levels of uPAR expression.

4.2 MATERIALS AND METHODS

4.2.1 Cell Lines

The human oral SCC line H357 and its derivatives, which had been created to express different integrin heterodimers: V3, VB6, VB6Δ_{11aa}, V3B3 and C1 were used (see section 2.1).

4.2.2 Flow Cytometry

uPAR expression on the cell surface was determined by flow cytometry (see section 2.3). Cells were labelled with the rabbit polyclonal primary antibody 399R (American Diagnostica, Aris-Shield Diagnostics Ltd., Dundee, UK) at 10μg/ml for 1 hour at 4°C. Cells were then washed and incubated with the secondary antibody: Goat anti-Rabbit FITC-conjugated IgG (DAKO Ltd. Ely, UK) at 50ng/ml for 30 minutes at 4°C.

4.2.3 Western Blotting

Hydrophobic protein and total cell extracts were obtained as previously described (see section 2.4.1.2). The protein concentration was determined using the DC protein assay (Biorad, Hemel Hempstead, UK, see section 2.4.2) and 10μg of protein was electrophoresed on a 10% acrylamide gel and transferred onto nitrocellulose membrane. Soluble uPAR (from M. Ploug, Finsen Laboratory, Copenhagen, Denmark) was loaded as a positive control. This was then probed for uPAR using the monoclonal primary antibody R4 (from G. Hoyer-Hansen, Finsen Laboratory) at a

concentration of 10μg/ml and then horseradish peroxidase conjugated secondary antibody at 50ng/ml (DAKO. Ltd. Ely, UK). Protein was visualised using ECL according to the manufacturers instructions (Amersham Pharmacia, Little Chalfont, UK) followed by autoradiography.

4.2.4 Immunocytochemistry

1 x 10^5 cells were seeded onto sterile glass coverslips placed into 24-well tissue culture plates (Nunc, Invitrogen, Paisley, UK). After overnight incubation at 37°C, cells were stained for uPAR (see section 2.8) using the monoclonal anti-uPAR primary antibody R4 (from G. Hoyer-Hansen, Finsen Laboratory) at 10μg/ml and then with 50ng/ml FITC-conjugated secondary antibody (DAKO. Ltd. Ely, UK). uPAR was visualised using fluorescence microscopy (LEICA, UK).

4.2.5 Cell Surface Plasmin Generation

2.5 x 10^5 cells were seeded in 0.5ml KGM in 48-well plates (Nunc). After 24 hours incubation at 37°C, the rate of cell surface plasminogen activation was determined using endogenously bound uPA as previously described (see section 2.2.5).

In order to determine if uPA secretion was affecting the rate of cell surface plasminogen activation, cells were washed with 1ml warmed serum free media stripped of endogenously-bound pro-uPA by incubating the cell monolayer in 1 ml of a low-pH buffer (50mM Glycine/100mM NaCl/pH3) for 3 minutes at room temperature. The glycine was removed followed by the addition of 1ml of 500mM

HEPES/100mM NaCl/pH7.5 to neutralise (Stoppelli et al, 1986). The HEPES was removed and 1ml of 2% BSA/50mM Tris pH7.4/100mM NaCl was added to each well along with 20μl 100μM stock pro-uPA (M.Ploug, Finsen Laboratory, Denmark). Cells were incubated at 37°C for 20 minutes so that all receptors were saturated with exogenous pro-uPA. This was then removed and cells incubated with substrate and plasminogen and plasmin generation determined as previously described.

Cell surface plasminogen activation was also determined following plating on various matrix proteins. Wells were coated with plasma fibronectin and cellular fibronectin as αvβ6 ligands and vitronectin as a uPAR ligand at a concentration of 10μg/ml in PBS, for 1 hour at 37°C (all from Sigma Chemical Co.). Matrix-coated and uncoated control wells were then blocked in 2% BSA for 1 hour at 37°C. 2×10^5 cells were seeded onto the wells and incubated overnight before being assayed for cell surface plasmin generation as described.

4.2.6 Northern Blotting

The cDNA probe for uPAR was generated as described in section. RNA extracts of all the cell lines were prepared and subjected to Northern blotting (see section 2.3), in order to determine levels of stable uPAR mRNA expression.

4.3 RESULTS

4.3.1 αvβ6 expression is associated with reduced uPAR expression.

Urokinase receptor expression was determined in oral carcinoma keratinocytes, with differing integrin profiles. Cells were stained for anti-uPAR and analysed by flow cytometry (figure 4.1). The αvβ6-expressing, VB6 cells show less uPAR expression than the control C1 cells, however both cell lines showed extremely low expression of uPAR.

uPAR expression was determined by Western blotting of total cell extracts under non-reducing conditions (figure 4.2). Although C1 cells show slightly more expression of uPAR than VB6 cells, this is not as marked a difference, therefore hydrophobic extracts were used to enhance the sensitivity. The αvβ5 expressing V3 and C1 cells show high levels of uPAR protein expression in hydrophobic extracts, compared to the H357 parentals (figure 4.3). However, the VB6 cells expressing high levels of αvβ6 show 50% reduced uPAR levels compared to the control C1 cells. Also, the VB6 cells show a higher proportion of the cleaved, lower molecular weight form of the receptor probably due to the high levels of secreted uPA, which is known to cleave uPAR directly. Cells were also stained for uPAR expression using the anti-uPAR, mAb R4. A FITC-conjugated secondary antibody was used to visualise uPAR by fluorescence microscopy. uPAR expression can be seen on both the cell lines using immunocytochemistry when exposed for the same length of time, although in C1 cells there is more distinct expression at the cell membrane (figure 4.4).

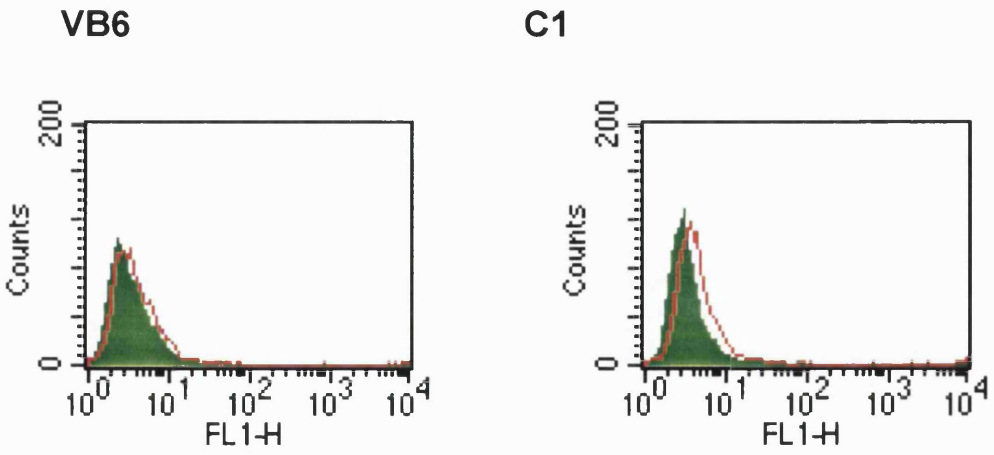


Figure 4.1 uPAR Expression of the Oral Carcinoma Cell Lines

Cells over-expressing αvβ6 (VB6) showed less uPAR expression than control cells with minimal αvβ6 expression (C1). Cells were stained with the polyclonal anti-uPAR antibody 399R, followed by a FITC-conjugated secondary antibody. A representative result is shown.

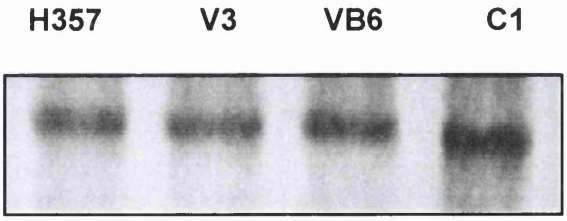


Figure 4.2 uPAR Expression in Total Cell Extracts

10μg protein was electrophoresed on a 10% gel and subjected to Western blotting for uPAR using the mAb R4 at 10μg/ml. A significant difference in uPAR expression between αvβ6-expressing and control cells cannot be seen.

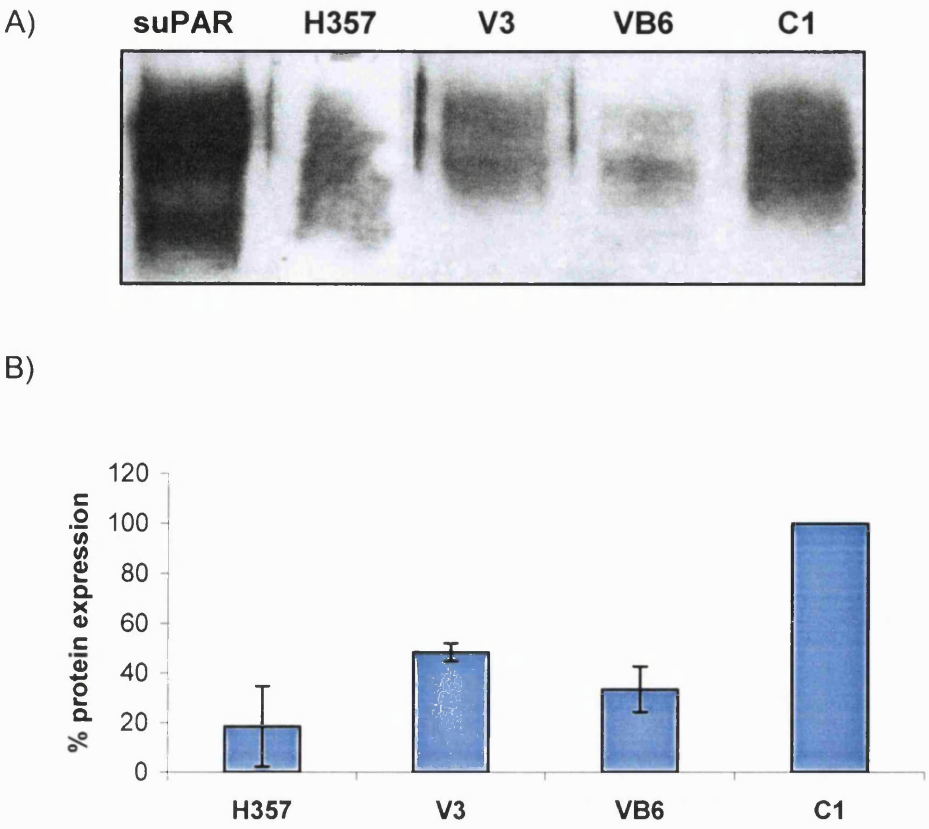


Figure 4.3 uPAR Expression in Cell Membrane Extracts

Expression of full-length αvβ6 in oral keratinocytes decreases uPAR expression.

(A) Western blot for uPAR expression. 10μg protein was electrophoresed under non-reducing conditions and probed for uPAR using 10μg/ml mAb clone R4. soluble uPAR (suPAR) was used as a positive control. Blots were developed using a peroxidase conjugated secondary antibody and ECL. A representative result is shown.

(B) Blots were subjected to densitometric analysis using Scion image software. Results were normalized to the control C1 cells and graph represents the mean (±SEM) % protein expression of three experiments.

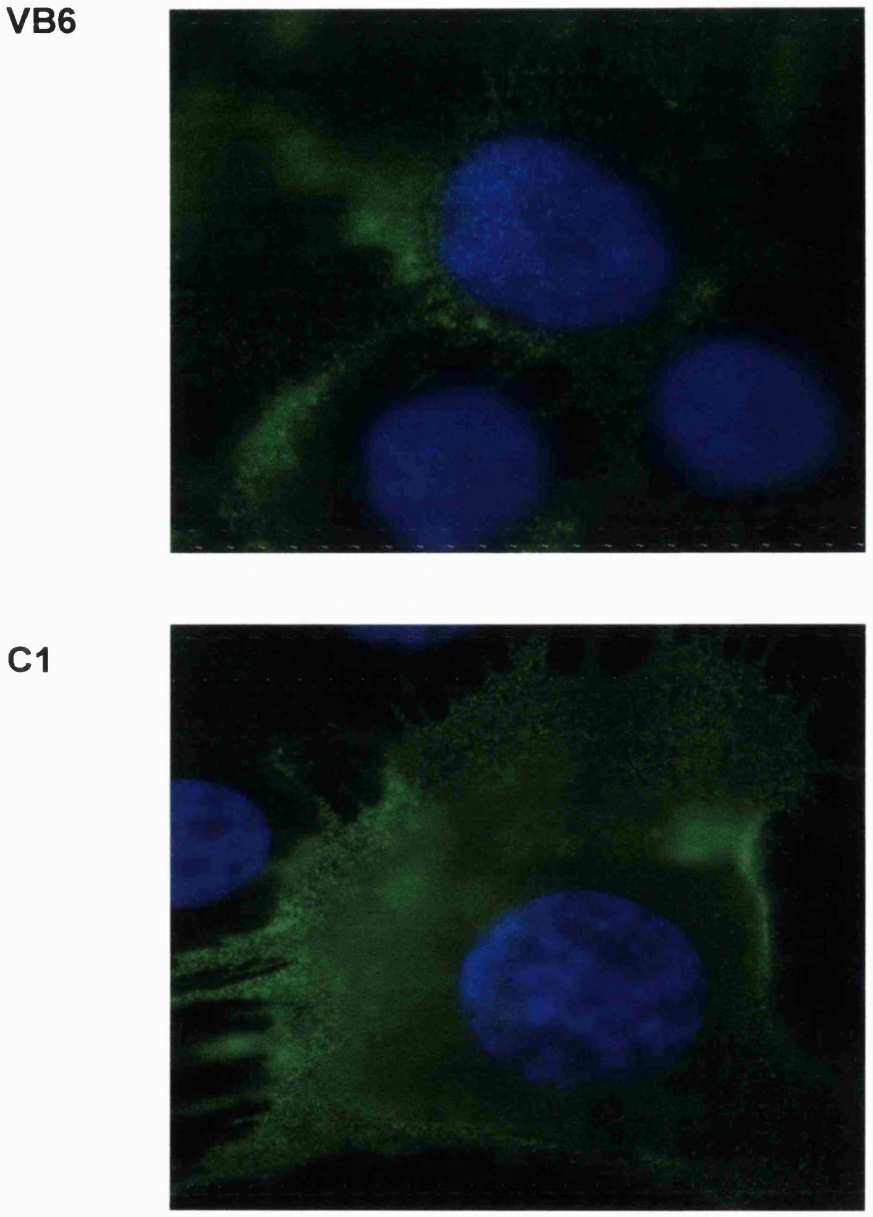


Figure 4.4 Immunofluorescence of uPAR

Cells were stained with the anti-uPAR mAb R4 and a FITC-conjugated secondary antibody. Nuclei were stained with DAPI, then visualised using fluorescence microscopy at X630 magnification.

4.3.2 αvβ6 expression is associated with reduced cell surface plasminogen activation.

Optimal plasminogen activation on the cell surface requires an intact urokinase receptor, with binding sites for both uPA and plasminogen. V3 and C1 cells showed a 2-fold greater rate of cell surface plasminogen activation when compared to H357 and VB6 cells (figure 4.5). The rate of plasmin generation was determined by interpolation from a plasmin standard curve and expressed as nM plasmin/minute: H357 = 0.7nM/min, V3 = 1.5nM/min, VB6 = 0.6nM/min, C1 = 1.4nM/min.

Stripping the cells of endogenously bound uPA by incubation with a low-pH buffer, followed by incubation with exogenous pro-uPA in excess (Stoppelli et al, 1986), so all the receptors are saturated, produced the same results (figure 4.6), indicating that cell surface plasminogen activation in this assay is not influenced by levels of uPA secretion from the cells.

Plasmin generation was also measured following growth of cells on plasma and cellular fibronectin as αvβ6 ligands and vitronectin as a uPAR and αvβ5 ligand (figure 4.7). In both cell lines, levels of cell surface plasminogen activation were not significantly altered following plating on any of the matrix proteins suggesting that the integrin effects on plasmin generation are ligand independent.

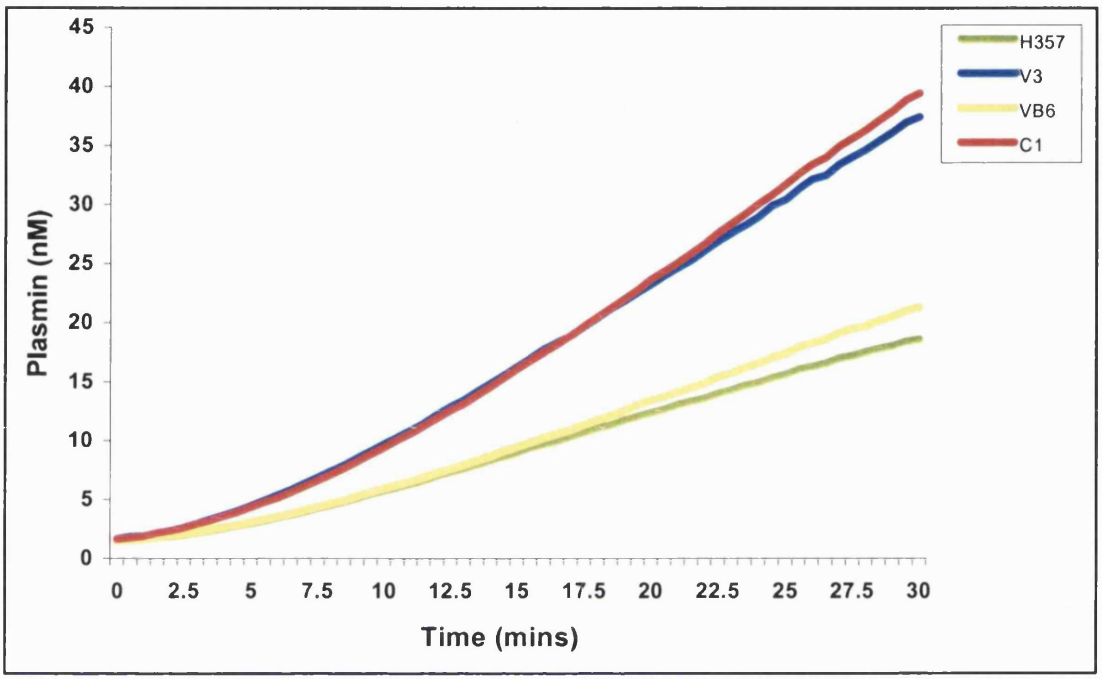


Figure 4.5 Cell Surface Plasmin Generation Using Endogenous uPA

Plasminogen activation on the cell surface was measured kinetically for 30 mins using the fluorogenic plasmin substrate HDVLK-AMC and plasmin generation was determined by interpolation from a plasmin standard curve. Plasmin generation from the V3 and C1 cells is 2-fold greater than that from H357 and VB6 cells. Figure shows the combined results of two experiments in triplicate.

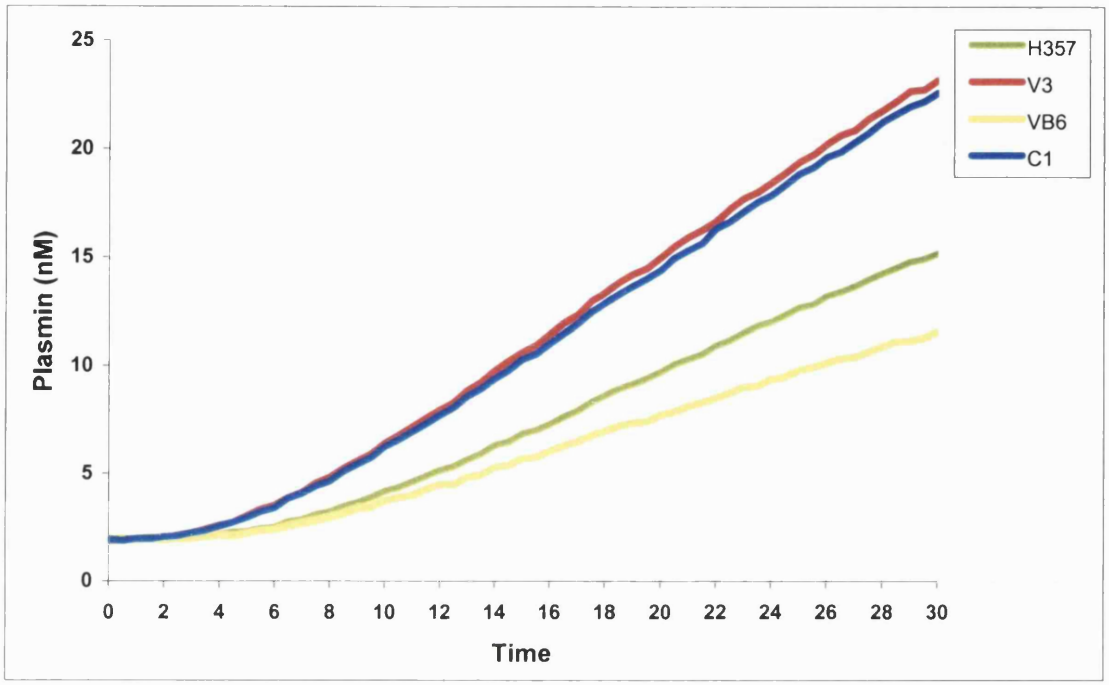
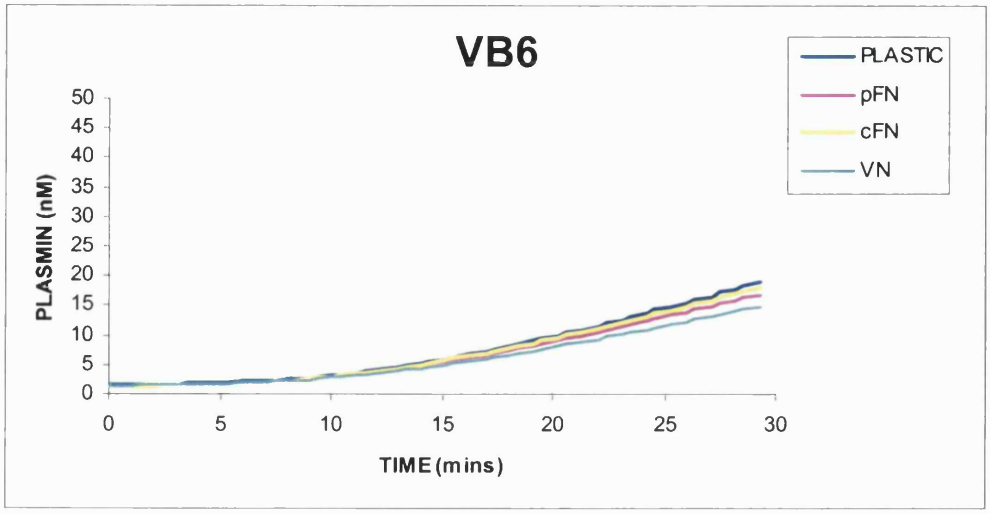


Figure 4.6 Cell surface Plasmin Generation Using Exogenous uPA

Cells were stripped of endogenously-bound uPA then incubated with pro-uPA in excess. Plasminogen activation was measured kinetically for 30 mins using the fluorogenic plasmin substrate HDVLK-AMC and plasmin generation was determined by interpolation from a plasmin standard curve. Plasmin generation from V3 and C1 cells is 2-fold greater than from H357 and VB6 cells. Figure shows a representative experiment in triplicate.



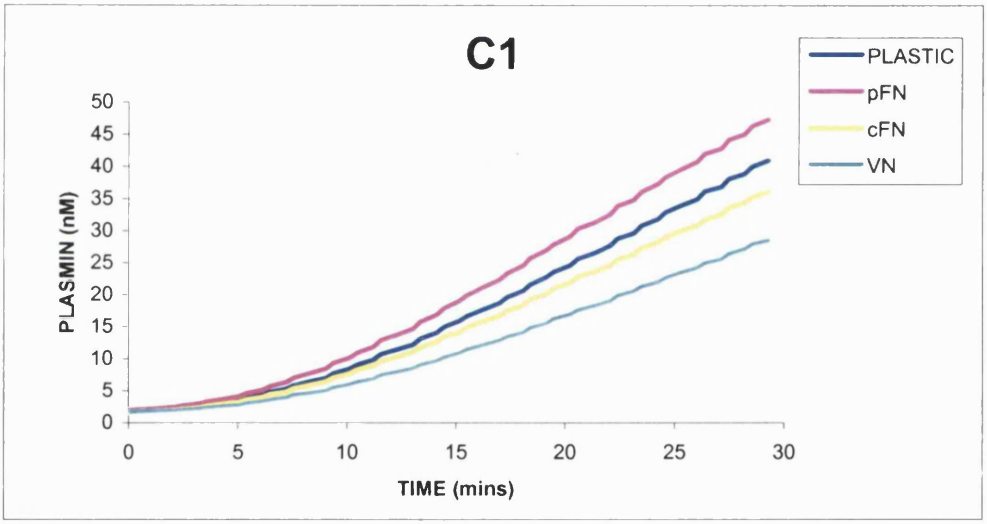


Figure 4.7 Cell Surface Plasminogen Activation on Matrix Proteins

Cells were seeded onto wells coated with various matrix proteins and incubated for 24 hours after which cells were assayed for cell surface plasmin generation. This was measured kinetically for 30 mins using the fluorogenic plasmin substrate HDVLK-AMC and plasmin generation was determined by interpolation from a plasmin standard curve. Figure shows a representative experiment in triplicate.

4.3.3. The β6 cytoplasmic tail modulates the reduced uPAR expression and function.

Expression of mutant αvβ6, lacking the 11aa C-terminal extension (VB6Δ_{11aa}), did not reduce uPAR levels and in fact showed very high expression. Transfection of the β3 subunit, so the cells predominantly expressed the αvβ3 integrin heterodimer, also showed high uPAR expression (figure 4.8), indicating that only the full-length β6 integrin is associated with a decreased uPAR expression.

The pattern of cell surface plasminogen activation between the cell lines, mirrors that of expression of surface uPAR with full-length αvβ6 expressing cells showing an overall 50% reduced plasmin generation compared to the control, mutated β6-expressing and β3-expressing cell lines (figure 4.9). The rate of plasmin generation was determined by interpolation from a plasmin standard curve and expressed as nM plasmin/minute: VB6 = 0.35nM/min, C1 = 0.6nM/min, VB6Δ_{11aa} = 0.58nM/min and V3B3 = 0.65nM/min.

4.3.4 Expression of uPAR mRNA Levels

Stable uPAR mRNA expression in the cell lines was measured by Northern blotting (figure 4.10). Expression of only full-length and not mutated αvβ6 into the cells reduced uPAR mRNA levels by 50% compared to the control C1 cells. This pattern mirrors that of protein expression, suggesting that integrin regulation of uPAR occurs at the transcriptional level. In contrast to αvβ6, expression of αvβ3 into the cells resulted in an up-regulation of uPAR mRNA levels compared to the control cells.

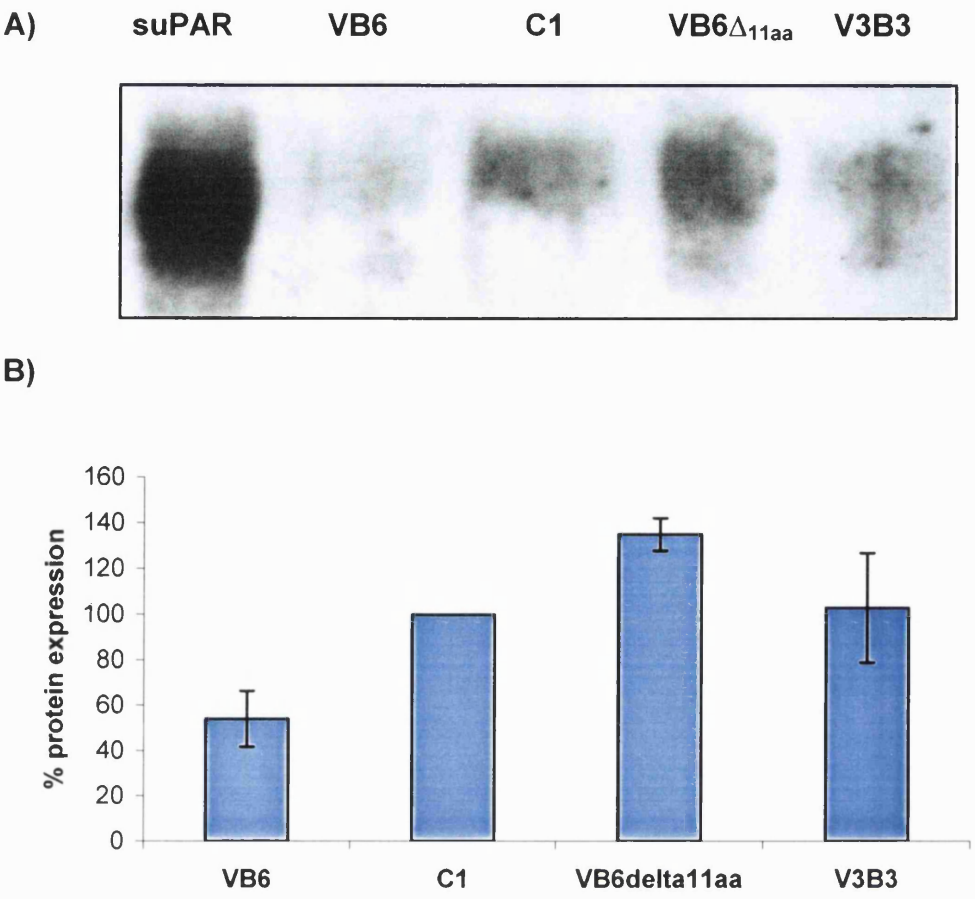


Figure 4.8 Expression of uPAR in Cell Membrane Extracts

Expression of full-length αvβ6 in oral keratinocytes decreases uPAR expression.

(A) Western blot for uPAR expression. 10μg protein was electrophoresed under non-reducing conditions and probed for uPAR using 10μg/ml mAb clone R4. Blots were developed using a peroxidase conjugated secondary antibody and ECL. A representative result is shown.

(B) Blots were subjected to densitometric analysis using Scion image software. Results were normalized to the control C1 cells and graph represents the mean (±SEM) % protein expression of 3 experiments.

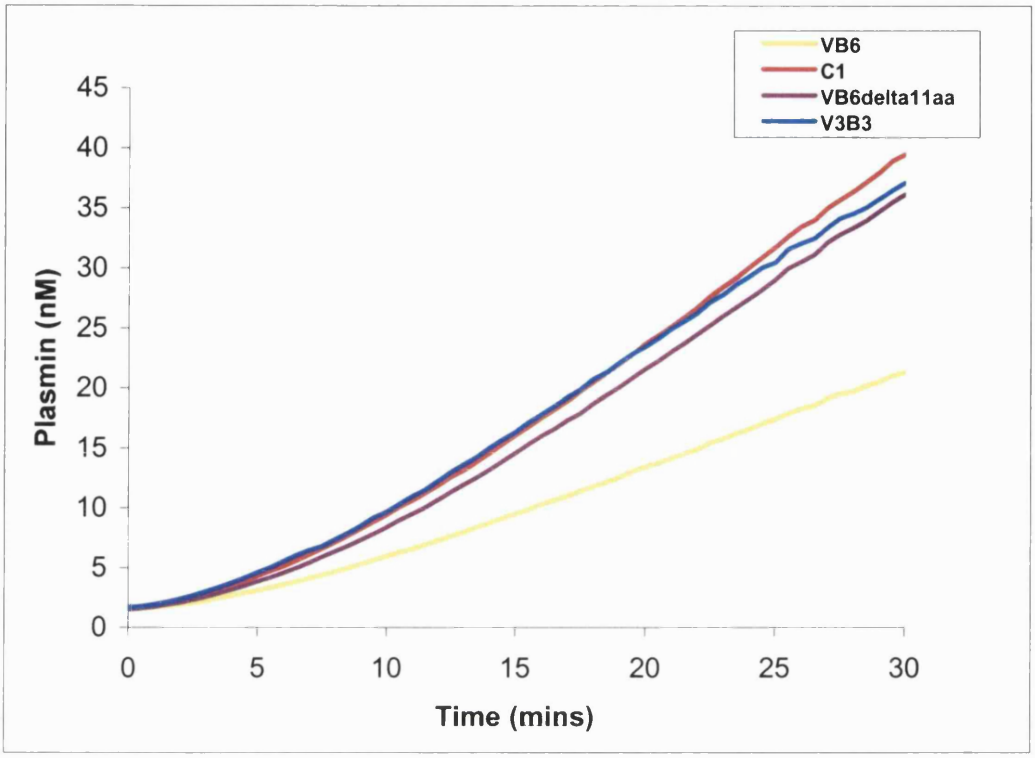


Figure 4.9 Cell Surface Plasmin Generation

Cells expressing full-length αvβ6 show reduced cell surface plasmin generation. This was measured kinetically for 30 mins using a fluorogenic plasmin substrate. Plasmin generation was determined by interpolation from a plasmin standard curve. Figure shows the combined results of two experiments in triplicate.

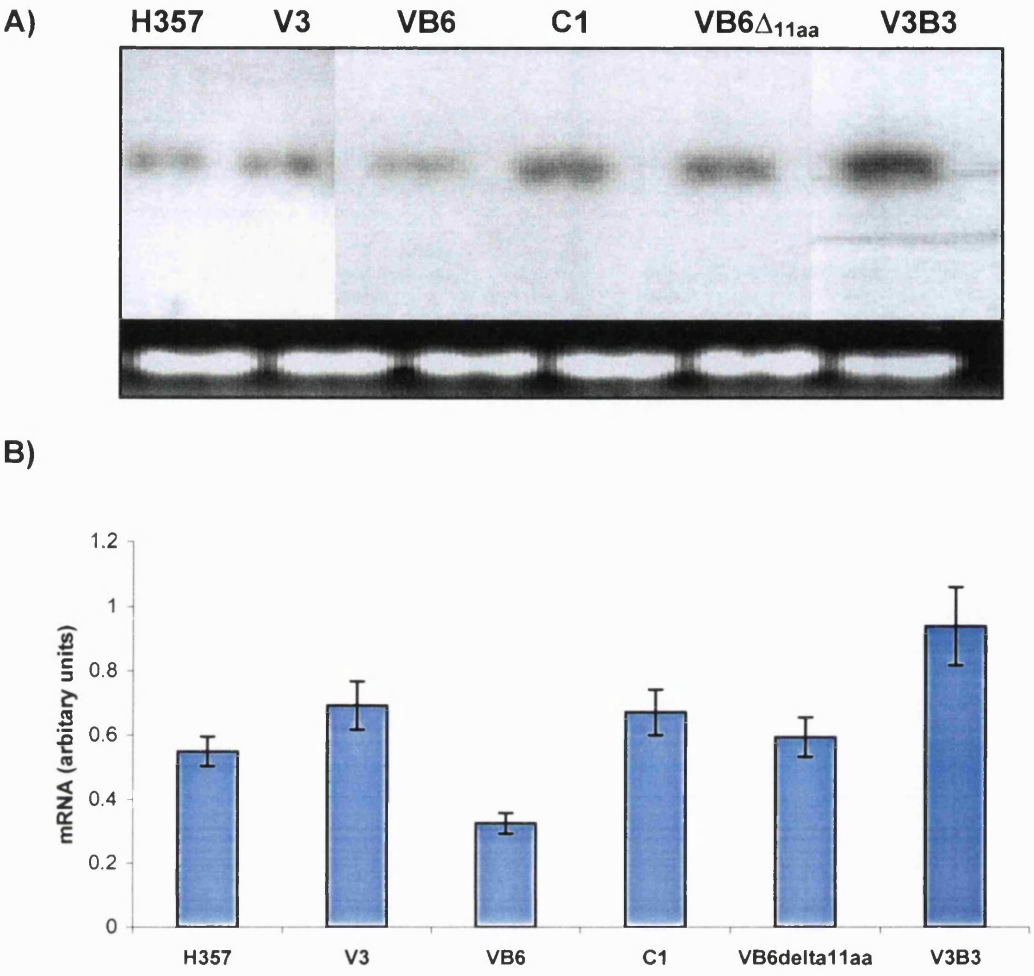


Figure 4.10 Expression of uPAR mRNA

uPAR mRNA levels are reduced in cells expressing full-length αvβ6. Total cellular RNA was extracted and subjected to Northern blotting. Filters were probed with ^{32}P-labelled full-length uPAR cDNA.

(A) Top panel- stable uPAR mRNA expression. Bottom panel – Ethidium bromide stain of 18S ribosomal band. A representative result is shown.

(B) Blots were subjected to densitometric analysis using Scion image software, normalized to 18S ribosomal bands and expressed in arbitrary units. Figure shows the combined data of two experiments of 6 samples each.

4.4 DISCUSSION

Invasion and metastasis of tumour cells require a complex regulation of different cell-surface associated proteins that facilitate proteolysis and adhesion of cells to the basement membrane and ECM. The present study shows that heterologous expression of the αvβ6 integrin in oral carcinoma cells is associated with reduced uPAR expression. This regulation occurs at the transcriptional level, since the αvβ6 expressing cells show 2-fold reduced mRNA expression than the control cells, with negligible αvβ6. This is confirmed at the cell surface with decreased plasminogen activation.

Certain integrins directly affect expression of components of the urokinase system. The data indicates that transfection of the αv integrin subunit into the oral carcinoma keratinocytes (V3 cells) is associated with an increased expression of uPAR. In support of this, a study in metastases-derived melanoma cell lines showed that treatment with αv antisense phosphorothioate oligonucleotides resulted in reduced uPAR expression (Nip et al, 1995). In this same study, the αv subunit was expressed predominantly as the αvβ3 integrin heterodimer and activation of this integrin by ligation with immobilised monoclonal antibodies rapidly increased uPAR expression at the transcriptional level. Contradictory to this, over-expression of the β3- integrin subunit in CHO cells down-regulated uPAR expression at the transcriptional level (Hapke et al, 2001a). However, expression of β3 into these oral carcinoma cells did

not significantly up- or down-regulate uPAR expression, suggesting that αvβ3 is not a crucial integrin in modulating cell function in this particular cell system.

Transfection of the β6 subunit into the cells so they over-express the αvβ6 heterodimer reduces uPAR expression at the transcriptional level. This observation is supported by a study in a murine mammary tumour cell line, where fibronectin, an αvβ6 ligand reduced uPA membrane binding which was associated with a lower expression of uPAR (Urtreger et al, 1999). However, contrary to this, during the course of this study, flow cytometric analysis showed that ovarian carcinoma cells with high expression of αvβ6 integrin also had high uPAR expression (Ahmed et al, 2002b).

The reduced uPAR levels in the αvβ6-expressing cells suggests that precisely controlled levels of proteolysis are required in order to prevent excessive plasmin proteolysis in cancer. In support of this it was found that, PAI-1, the primary physiological inhibitor of uPA, promoted tumour growth and angiogenesis by preventing excessive plasmin formation (Bajou et al, 2001). In addition to this role, PAI is also able to disrupt the interaction between vitronectin, uPAR and integrins, so inhibiting cell adhesion to the extracellular matrix and promoting cell migration (Loskutoff et al, 1999).

Regulation of uPAR expression can be achieved in two ways. PAI-1 can complex with uPAR-uPA-LRP and trigger internalisation/recycling of uPAR, re-exposing free

uPAR at a site where it can bind novel uPA (Cubellis et al, 1990). Also, uPAR can be inactivated by uPA-mediated cleavage between domains D1 and D2 (Hoyer-Hansen et al, 1992). Therefore, the decreased uPAR expression may in part be explained by the cleavage of uPAR mediated by the increased uPA secretion from the VB6 cells. In fact, western blotting of hydrophobic extracts shows the presence of two forms of uPAR; with a greater proportion of the cleaved, lower molecular weight form present in the VB6 cells. However, this does not explain the transcriptional down-regulation of uPAR.

The pattern of uPAR mRNA expression between the cell lines mirrors that of protein levels, suggesting that uPAR expression is regulated at the transcriptional level. A recent study showed that in CHO cells, the β3 integrin down-regulates uPAR expression at the transcriptional level through a PEA3/ets transcriptional silencing element in the uPAR promoter (Hapke et al, 2001a). It is possible that the β6 integrin may also be down-regulating uPAR expression through this same sequence within the uPAR promoter. The down-regulation of uPAR expression and function is only seen in cells expressing full-length αvβ6 and not in the mutated VB6Δ_{11aa} cells, suggesting that the cytoplasmic tail of the β6 integrin is modulating this effect. The cytoplasmic domain of integrins is fundamental for gene expression, cell proliferation and cell cycle regulation. Within this domain, the membrane-distal NXXY motif is highly conserved in β1, β2, β3, β5, β6 and β7 integrins and plays a crucial role in integrin-mediated cell functions (Clark and Brugge, 1995). The down-regulation by β3 was mediated through the (NITY^{759}) motif within the β3 cytoplasmic tail, which is known

to be important for different functions, including cell adhesion, focal contact formation and FAK/paxillin tyrosine phosphorylation (Schaffner-Reckinger et al, 1998). The β3 and β6 cytoplasmic tails have over 60% homology, suggesting that they may perform similar cellular functions (Sheppard et al, 1990). Although this NITY motif is unique to the β3 integrin, the β6 integrin only has a single amino acid substitution, with the NVTY motif at this particular site. However, this site within the β6 integrin does not seem to be crucial in modulating uPAR expression and in fact it is the 11 amino acid cytoplasmic tail extension that is unique to the β6 integrin that is more important. This sequence has been shown to mediate cell proliferation (Agrez et al, 1999) and MMP-9 secretion (Niu et al, 1998). This study demonstrates for the first time that this sequence down-regulates uPAR expression at the transcriptional level.

CHAPTER 5

ANTISENSE TREATMENT OF αVβ6 INTEGRIN USING MORPHOLINO OLIGONUCLEOTIDES

5.1 INTRODUCTION

Antisense oligonucleotides are potentially very useful research tools for investigating gene function both *in vitro* and *in vivo* by down-regulating the expression of targeted genes (Crooke, 1998a, Crooke, 1998b, Gewirtz et al, 1998). They also provide the prospect of safe and effective therapeutics for a wide variety of diseases and have been used in clinical trials (Gewirtz, 1998, Agrawal and Zhao, 1998) and clinical practise (Crooke, 1998b). Oligonucleotides can block the translation of selected messenger RNAs by sequence-specific hybridisation to the mRNA and thus are able to prevent protein synthesis. Antisense oligonucleotides were used in this study in order to investigate the observed effects of the αvβ6 integrin on the urokinase system.

Originally, antisense oligos comprised of natural genetic material with added cross-linking moieties enabling irreversible binding to their targeted genetic sequences, however, these were rapidly degraded in biological systems (Summerton and Bartlett, 1978). Over the last decade, phosphorothioate-linked DNA oligos (SDNAs) had dominated the antisense field. These have 5-membered deoxyribose backbone moieties joined by ionic linkages (Stein and Cohen, 1989). Although these provided much better efficacies than previously used antisense oligos, they still had many drawbacks. These include only a reasonable sequence-specificity within a narrow concentration range, instability, a low affinity to the selected RNA sequence and unpredictable targeting resulting in non-antisense effects. They are also targets for mRNA degradation by RNase H. Due to these drawbacks, the use of antisense

technology in biological research has not progressed rapidly and their full potential has not been realised (Summerton and Weller, 1997).

Recently, however, there have been improvements in the chemical properties of oligonucleotides and in the understanding of their mechanism of action, resulting in the development of morpholino oligos by James Summerton in 1985 (Summerton, 1999, Gene Tools LLC (http ://www.gene-tools.com). These antisense oligos are assembled from four different morpholino subunits. Each subunit contains one of the four genetic bases (Adenine, Cytosine, Guanine, and Thymine) linked to a 6-membered morpholine ring. Eighteen to twenty-five subunits of these four subunit types are joined in a specific order by non-ionic phosphorodiamidate inter-subunit linkages to give a morpholino oligo (figure 5.1).

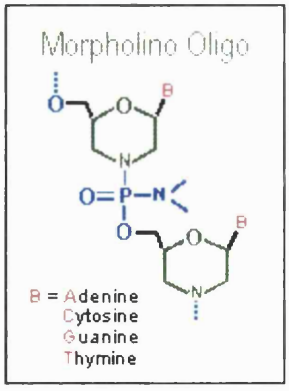


Figure 5.1 Structure of a Morpholino Oligonucleotide

Morpholino oligos have a number of advantages over phosphorothioates. Due to their unnatural structures, they lack anionic sites on the backbone rendering them completely resistant to nucleases so they are highly stable. Therefore, in theory antisense effects may continue indefinitely, not just for hours or days. Morpholinos form RNA-Morpholino hybrids that are not substrates for RNase H therefore the mRNA is not degraded. They have good aqueous solubility and may be delivered into both the cytosol and nuclear compartments. This homogeneous distribution enables access to their target RNAs within the brief time in the nucleus and during their longer presence in the cytosol where translation of the mRNA occurs. They achieve very high inhibition levels with only nanomolar concentrations due to their greater binding affinity and show a more reliable activity in cells (reviewed by Summerton and Weller, 1997).

Targetting of the morpholino oligos is an important consideration, because morpholinos targeted to most of the coding region will be displaced by the ribosome as it translocates along the mRNA, and therefore will be ineffective in preventing translation. Therefore, morpholinos are preferentially targeted to the 5'-untranslated region (UTR) or the start codon. They block translation by steric blocking of the translation initiation complex. This "Translational Start Target" comprises the translational start site and the 22 bases 3' to that site. Blocking these sites prevents the translation machinery from binding, therefore preventing protein synthesis. This sequence has little or no self-complementarity with less than 36% Guanine content (Gene Tools LLC).

The oral carcinoma keratinocyte cell line, VB6, which had been transfected to over-express the αvβ6 integrin was used in this study. The VB6 cells were treated with antisense oligonucleotides targeted to the αv and β6 integrin subunits in order to down-regulate αvβ6 integrin expression. The treated cells would then be used to measure uPA and uPAR expression.

5.2 TRANSFECTION METHODS AND RESULTS

5.2.1 Antisense Oligonucleotide Sequences

Morpholino oligos pre-paired with a partially complementary DNA oligo of the αv and β6 integrin subunits were obtained from Gene Tools (Corvalis, USA). The antisense sequences were designed to target a sequence in the post-spliced mRNA region from the 5' cap to 25 bases 3' to the AUG translational start site. Control oligos with 4 mispairs appropriately distributed along the sequence were also obtained. These mis-paired oligos provided a highly stringent assessment of the true sequence specificity of the antisense oligos.

αv = 5' GCGGAAAAGCCATCGCCGAAGTGCCG 3'

4-mis-αv = 5' GCGcAAAtGCCATCGCCGtAGTcCG 3'

β6 = 5' CAATCCCCATTCGTTTCAGTTCTTG 3'

4-mis-β6 = 5' CAAaCCCgATTCGTTTCAcTTCaTG 3'

300 nanomoles of oligo DNA was reconstituted in dH_2O to provide a stock concentration of 0.5mM or 4.17μg/ml.

5.2.2 Transfection Methods

Transfection of the antisense oligos into the VB6 cells was attempted using a variety of methods in order to optimise delivery of the oligos into the cells to produce the desired antisense effect. Three different transfection reagents were used; these

included the special delivery reagent Ethoxylated Polyethylenimine (EPEI) provided by Gene Tools, Superfect reagent and Effectene reagent (both from Qiagen). Transfection was carried out on cells seeded at different densities in 24-well and 6-well plates, with and without serum, for varying lengths of time of DNA/delivery reagent complex formation, for various incubation periods of cells with transfection reagent and varying incubation times before testing for integrin expression, which reflected integrin turnover time.

5.2.3 Detection Methods

Following transfection, cells were analysed by flow cytometry and Western blotting in order to determine if the antisense oligos delivered into the cells had down-regulated expression of the αvβ6 integrin. Levels of PA activity in the cultured supernatants were also determined.

(I) Flow Cytometry

Cells were trypsinised from the plates and subjected to flow cytometric analysis (see section 2.3). Cells were incubated with primary anti-αvβ6 antibody, E7P6 at 10μg/ml or anti-αv antibody L230 and a FITC-conjugated secondary antibody at 5μg/ml (see Appendix 2).

(II) Western Blotting

Following transfection, cells were lysed using the integrin lysis buffer (Appendix 4). 10μg of protein, as determined by the DC protein assay was electrophoresed on a

10% agarose gel and subjected to Western blotting (see section 2.4). Protein extracted from NIH3T3 cells that had been transfected with β6 cDNA, to create the 3T3β6 cell line was used as a positive control (from J. Marshall, Cancer Research UK, St. Thomas' Hospital, London). The control cells transfected with empty plasmid pBabepuro, was used as a negative control, since 3T3 cells lack any endogenous expression of the αvβ6 integrin. Blots were probed with a rabbit polyclonal anti-β6 primary antibody (C-19): sc-6632 at 2μg/ml, and a swine anti-rabbit HRP conjugated secondary antibody at 0.15μg/ml. Blots were developed by ECL and exposed to autoradiographic film for 15 seconds to 2 minutes.

Some of the blots were re-probed for β1 integrin in order to check for equal loading. Bound antibody was removed by incubation with stripping buffer (Appendix 4) for 30 minutes at 50°C with gentle agitation, followed by three washes for 15 minutes each in PBST. Blots were blocked again in blocking buffer (Appendix 4) and probed for β1 integrin using the monoclonal antibody A11B2 at 10μg/ml. Following incubation with, rabbit anti-mouse HRP-conjugated secondary antibody at 0.15μg/ml, blots were developed again using ECL for about 5-10 minutes. Bands were subjected to densitometric analysis and β6 expression in the cell extracts determined following normalisation to β1 expression.

(III) Plasminogen Activator Activity Assay.

Following transfection in 24-well plates, cells were incubated at 37°C for a further 20 to 48 hours, after which the cells were washed twice with PBS and incubated with

0.5ml SFM (0.1% BSA). After incubation at 37°C for 15 hours, cultured supernatants were collected and assayed for plasminogen activator activity (see section 2.4).

5.2.4 Transfection using the special delivery system of EPEI

Ethoxylated polyethylamine (EPEI) is a weakly basic transfection reagent, specifically designed for fast, easy and non-toxic transfection. It is based on the principle that the non-ionic Morpholino oligo is paired to a complementary DNA, which binds electrostatically to cationic sites of EPEI. This Morpholino/DNA/EPEI complex then binds electrostatically to the anionic cell surfaces leading to endocytosis of the complex. Subsequent acidification within the endosome ionises additional weakly basic moieties of the EPEI, and this more extensively ionised EPEI permeabilises the endosomal membrane causing release of the Morpholino oligo into the cytosol/nuclear compartment of the cell (Qiagen).

Cells were seeded in 24-well and 6-well plates 24 hours prior to transfection. Triplicate wells of 50% confluent cells, were treated with αv, 4-mis-αv, β6 and 4-mis-β6 oligos and just EPEI as a further control. A master mix of each oligo was prepared, where DNA oligo was mixed with ddH_2O, followed by addition of EPEI. This was vortexed and left to stand for 20 minutes at room temperature to allow complex formation. Serum free medium (SFM) was then added to the oligo master mix to generate a complete delivery solution. Following a wash with PBS, complete delivery solution was added to the cells and incubated for 3 hours at 37°C. The oligo media was removed and replaced with fresh KGM to enable optimal cell growth and proliferation. After a further 24-72 hour incubation, cells were harvested and analysed for integrin expression, using different methods.

Analysis by Flow Cytometry

An oligo master mix for triplicate wells was prepared for each DNA oligo: αv, β6 and their appropriate control oligos 4-mis-αv and 4-mis-β6. 16.8μl oligo (1.4μM final concentration or 70μg DNA) was mixed with 566.4μl ddH_2O and 16.8μl EPEI. Following complex formation, this was diluted in 5.4ml SFM and 1.5ml of the complete delivery solution was added to each well of a 6-well plate containing VB6 cells at 60% confluency. After 3 hours incubation at 37°C, the oligo media was removed and replaced with 2ml fresh KGM and incubated for a further 36 hours. Cells were the harvested and analysed by FACS for αv and αvβ6 integrin expression. Treatment with anti-αv or anti-β6 antisense oligos did not affect integrin expression compared to the control treatments (figure 5.2).

Analysis by Western Blotting

VB6 cells at 60% confluency in 6-well plates were treated with the αv and β6 oligos together; control oligos 4mαv and 4mβ6 were also added together. The complete delivery solution for triplicate wells was prepared as described above, except that 8.4μl of each oligo (70μg total DNA) were added together to the oligo mix. Cells were incubated in KGM for a longer period of 48 hours, before being lysed and analysed for αvβ6 expression by Western blotting. There is no difference in αvβ6 expression in cells treated with antisense oligos compared to the control treatments (figure 5.3).

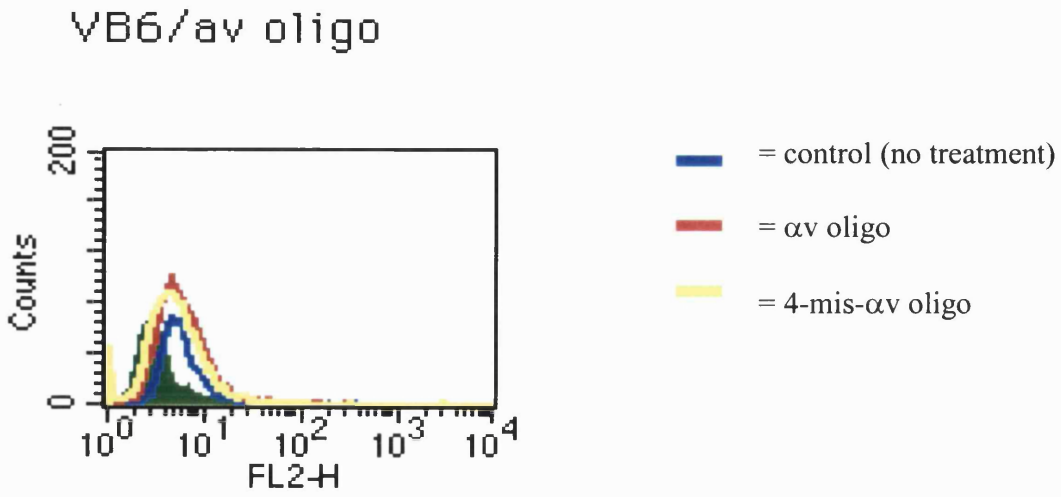


Figure 5.2(A) FACS analysis of αv expression in VB6 cells treated with αv oligo and 4-mis-αv control using EPEI. Cells were stained with the anti αv mAb, L230 at 10μg/ml, followed by a FITC-conjugated secondary antibody.

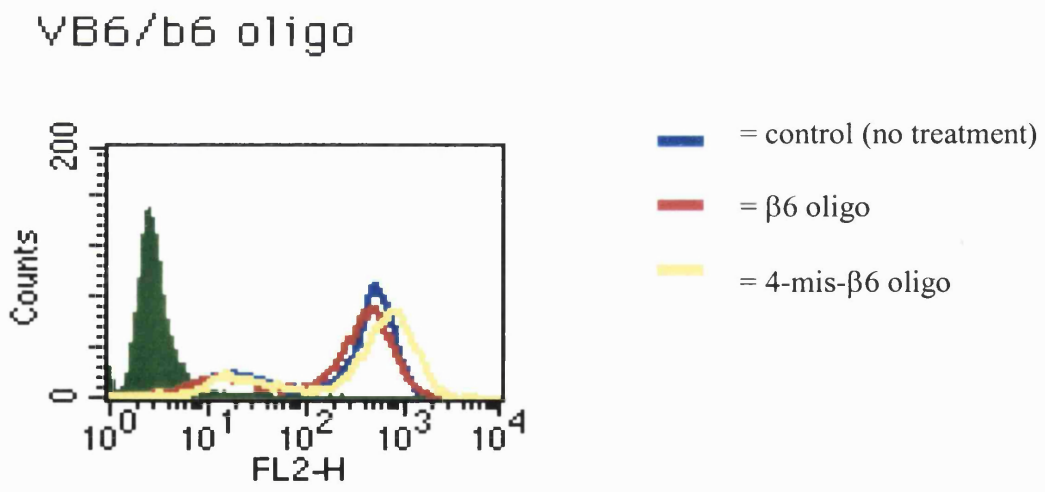


Figure 5.2(B) FACS analysis of αvβ6 expression of VB6 cells treated with β6 oligo or 4-mis-β6 control oligo using EPEI. Cells were stained with the anti-αvβ6 mAb, E7P6 at 10μg/ml and a FITC-conjugated secondary antibody.

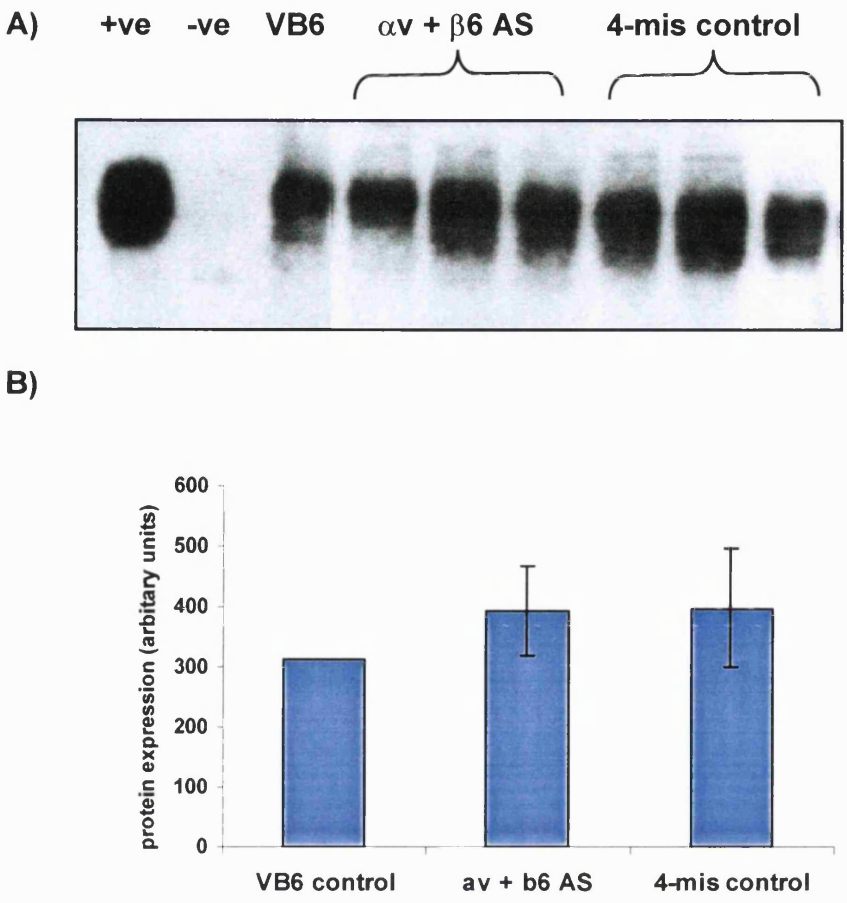


Figure 5.3 Western blot for αvβ6 expression in VB6 cells transfected with antisense oligos using EPEI.

(A) Cell extracts were western blotted for αvβ6 using the anti-β6 Ab, sc-6632 at 2μg/ml. A representative blot is shown.

(B) Blots were subjected to densitometric analysis using Scion image software. Graph shows the mean ±SD of a representative in triplicate.

Analysis by Plasminogen Activator Activity.

VB6 cells at 50% confluency in 24-well plates were transfected with αv, β6 and control oligos separately but the volume of EPEI was increased by 10%, in order to try and optimise delivery. The oligo master mix was prepared for triplicate wells with 188.8μl ddH_2O, 5.6μl DNA (1.4μM final concentration), 6.1μl EPEI and 1.8ml SFM. 0.5ml of the complete delivery solution was added to each well and changed to KGM after 3 hours. The medium was changed to serum free after 24 hours and the supernatants collected after a further 16-hour incubation and assayed for PA activity (figure 5.4). There was no difference in the levels of uPA secretion from cells treated with antisense oligos compared to the control treatments.

Transfection of antisense oligos, using EPEI was attempted a number of times and αvβ6 expression was checked by both FACS analysis and Western blotting; uPA secretion following treatment was also measured. However, no effect on αvβ6 expression or uPA secretion was observed following treatment with antisense oligos compared to the control treatments.

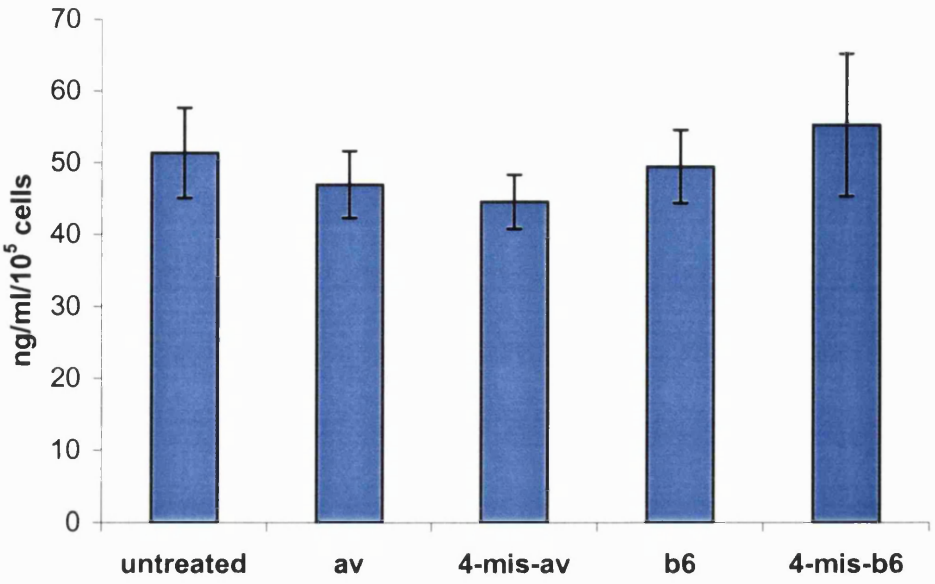


Figure 5.4 Secreted PA activity from VB6 cells transfected with αv and β6 antisense oligos using EPEI.

Cultured supernatants were assayed for PA activity using a chromogenic plasmin substrate and uPA activity determined by comparison with uPA standards.

5.2.5 Transfection Using Scrape-Load Delivery

Scrape-load delivery is a fast, easy and cheap method of transfection. The method entails adding oligos to adherent cells, scraping the cells from the plate and transferring the scraped cells to another plate where they are allowed to re-adhere. When the cell is scraped from the plate, desmosomes connecting the cell to the plate are pulled out of the cell membrane, leaving transient holes in the cytoplasmic membrane. This allows oligos to freely enter the cytoplasm for about a minute before the membrane is resealed (Partridge et al, 1996).

Cells at 60% confluency in 24-well plates were used for transfection. The medium was replaced with fresh KGM and 10μM (20μl) of each oligo: αv and β6 was added to each of triplicate wells. Control cells were treated with the same concentration of the 4-mis oligos as well as with no DNA added. The cells were gently scraped with a 500μl pipette tip, transferred to separate wells and allowed to re-adhere. After 24 hours, the cell debris was removed and the medium replaced with fresh KGM. Cells were allowed to grow for 72 hours before being lysed for western blot analysis of integrin expression (figure 5.5).

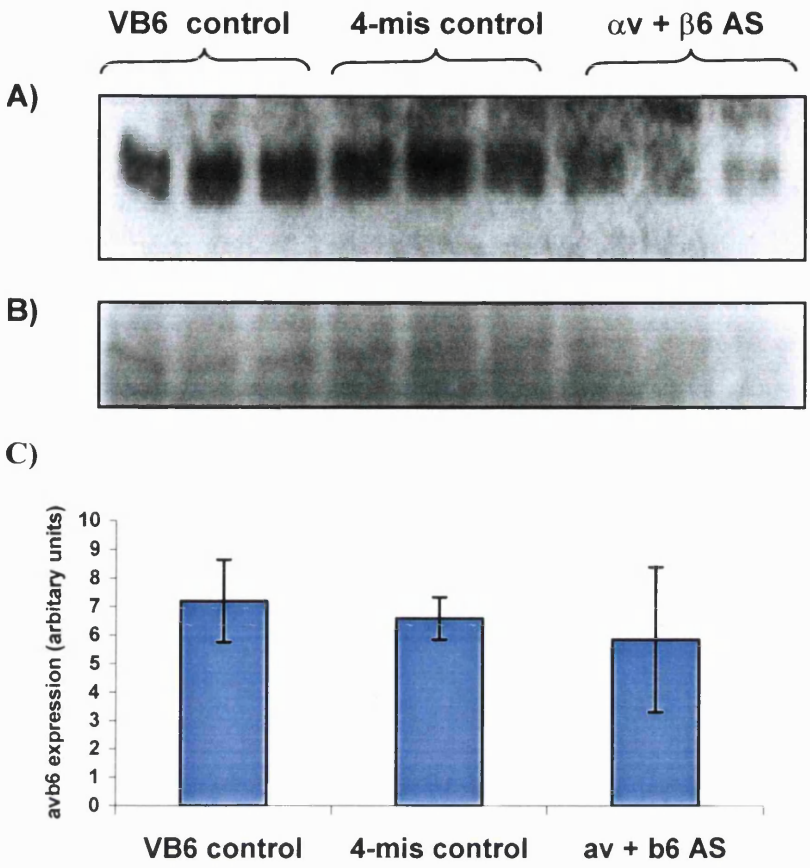


Figure 5.5 Integrin expression in VB6 cells treated with antisense oligos using scrape-load delivery.

A) αvβ6 expression – Cell extracts were subjected to Western blotting and probed with anti-β6 C-terminal goat polyclonal antibody, sc-6632 at 2μg/ml. A representative blot is shown.

B) Blot was re-probed for β1-integrin expression using anti-β1 mAb, A11B2 at 10μg/ml.

Blots were subjected to densitometric analysis: αvβ6 expression was normalised to β1 expression and expressed in arbitary units. Figure shows mean ±SD of a representative experiment in triplicate.

5.2.6 Transfection Using Effectene® Reagent

Effectene Transfection Reagent (Qiagen) is a non-liposomal lipid formulation designed to achieve high transfection efficiencies. Initially, the DNA is condensed followed by the formation of Effectene-DNA complexes, which are then mixed with medium and directly added to cells. Effectene Reagent should provide excellent reproducibility of transfection complex formation, since it forms micelle structures that show no size or batch variation. (Qiagen).

In order to optimise transfection efficiency using Effectene, anti-MMP-9 FITC conjugated oligos, which are 30 bases long, were used (Biognostik, Gottingen, Germany). VB6 cells at 50% confluency in 6-well plates were treated with a range of DNA concentrations, with varying volumes of Enhancer and Effectene reagent as described in the selector protocol (Qiagen). Firstly, a DNA/Enhancer/EC Buffer master mix was prepared (400µl total), which was vortexed and incubated at room temperature for 5 minutes. Varying volumes of Effectene reagent were added to 100µl of oligo mix and incubated for 10 minutes at room temperature to allow complex formation. During the incubation, the cells were washed with PBS, followed by the addition of 1600µl KGM. 600µl KGM was added to each tube of complete oligo mix and then gently distributed onto the cells. This was incubated at 37°C and the presence of FITC oligos in the cells was checked at 24 hours and 48 hours post-transfection by fluorescence microscopy. Well 9 showed the best expression of the FITC oligos into the cytosol and nuclear compartments (figure 5.6). Here cells were treated with 0.8µg DNA + 6.4µl enhancer + 40µl effectene, i.e. the

highest recommended DNA and effectene concentration at a DNA:Effectene ratio of 1:50.

Treatment 1

In 24-well plates, cells were transfected at 50% confluency using the DNA and effectene concentrations that showed optimal incorporation of the FITC oligos. Triplicate wells were treated with 0.8μg of total oligo DNA per well: 0.4μg of αv + 0.4μg of β6 oligo and 0.4μg of each control oligo. Therefore, 7.6μl of oligo, diluted 1:10 in dH_2O, was added to 25.6μl enhancer and 366.7μl EC buffer, vortexed and incubated at room temperature for 5 minutes. 100μl of this was aliquotted to each of 3 tubes, into which 40μl effectene reagent was added (DNA:Effectene ratio of 1:50) and then allowed to complex at room temperature for 10 minutes. As a control, cells were treated with 100μl EC buffer and 10μl effectene reagent. During complex formation, cells were washed with PBS and incubated with 1600μl of PBS. 600μl KGM was added to each tube, and this was pipetted into the appropriate wells. Cells were incubated at 37°C without a change of medium, so the transfection mix was on cells throughout the whole incubation time. After 48 hours, cells were harvested for western blot analysis of integrin expression (figure 5.7).

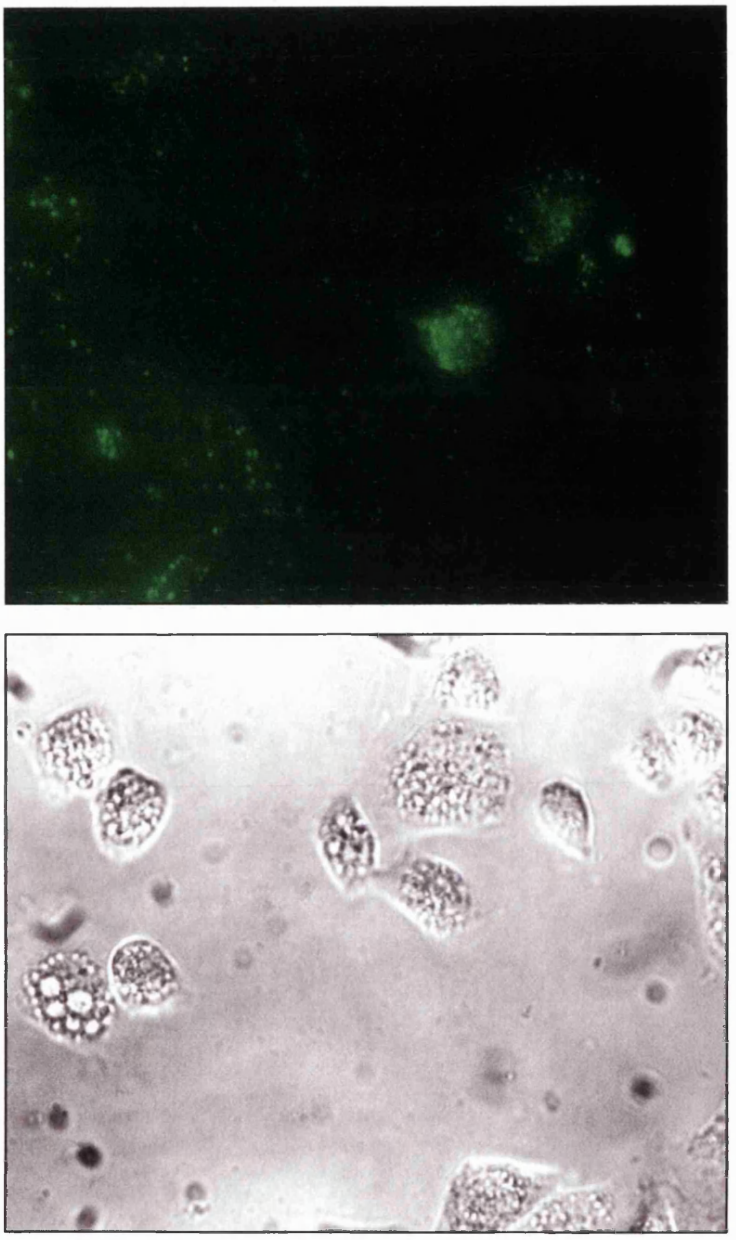


Figure 5.6 Expression of anti-MMP-9 FITC-oligos

VB6 cells were transfected using Effectene, at a DNA:Effectene ratio of 1:50. 24-hours post-transfection, expression of the FITC-oligos into the cells was shown under X630 magnification. Lower panel shows a phase contrast image of the cells.

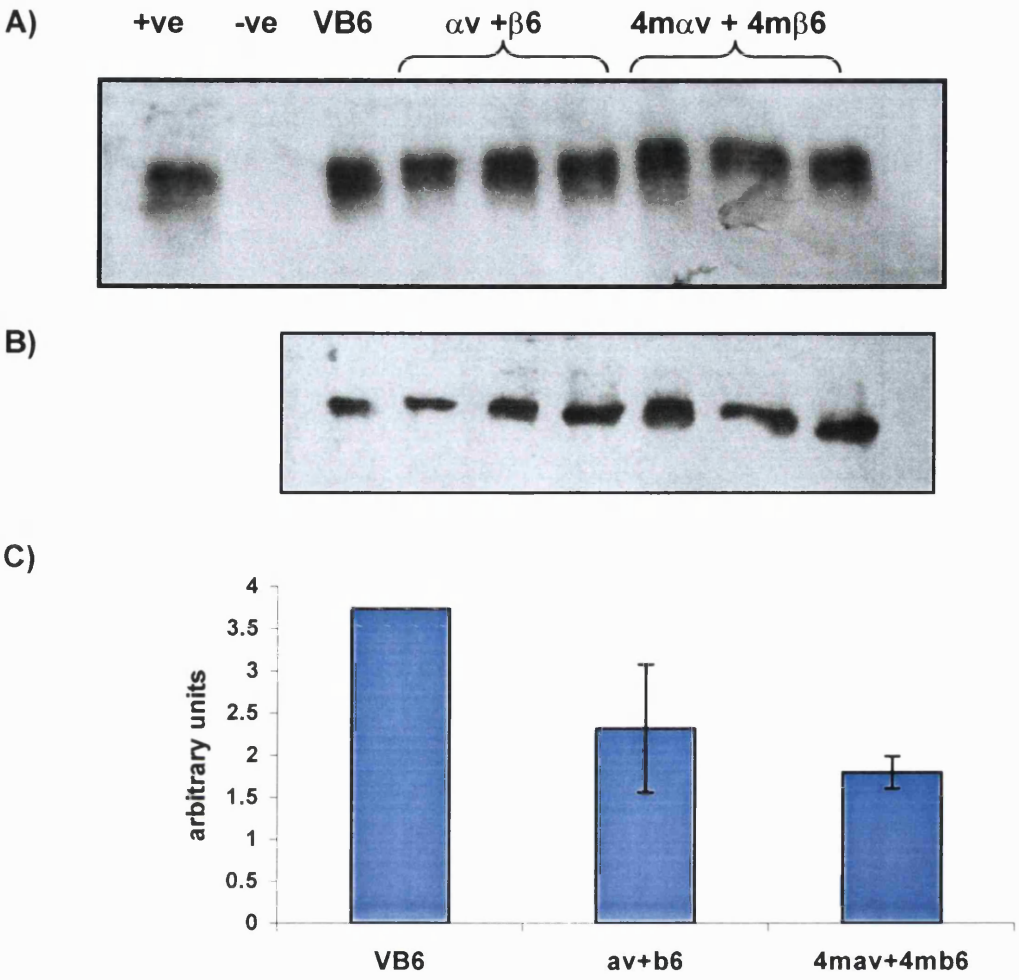


Figure 5.7 Integrin expression in VB6 cells transfected with antisense oligos using Effectene Reagent

A) Cell extracts were subjected to Western blotting for αvβ6 using anti-β6 C-terminal goat polyclonal antibody, sc-6632 at 2μg/ml. A representative blot is shown.

B) The blot was re-probed for β1-integrin expression using anti-β1 antibody mAb, A11B2 at 10μg/ml.

C) Blots were subjected to densitometric analysis using Scion image software and αvβ6 expression was normalised to β1 levels and expresses in arbitary units. Graph shows mean ±SD of a representative experiment in triplicate.

Treatment 2

Since no downregulation of αvβ6 expression following treatment with integrin antisense oligos compared to the control oligos was observed, a much higher concentration of DNA oligo also used to transfect each well at a much higher DNA:Effectene ratio of 1:2. Cells at 50% confluency in 24-well plates were used for transfection. 1μl αv oligo and 1μl β6 oligo (8μg total DNA) was added to 180μl DNA condensation buffer and 4.8μl of enhancer, vortexed and incubated at room temperature for 5 minutes. 15μl effectene reagent was added to this, vortexed and incubated at room temperature for 10 minutes to enable complex formation. Cells were washed with PBS incubated with 350μl KGM. 1050μl KGM was added to the complex mixture of which 350μl was added to each well. Cells were also treated with the control oligos in the same manner. After an 18-hour incubation at 37°C, the medium was changed to fresh KGM and incubated for a further 48 hours, after which the medium was changed to serum free. Cells were harvested for FACS analysis of αvβ6 expression (figure 5.8A) and supernatants were collected and assayed for secreted PA activity (figure 5.8B). Treatment with antisense oligos did not affect either the levels of αvβ6 expression or levels of uPA secretion compared to the control treatments.

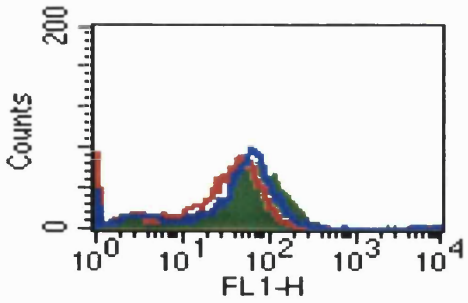


Figure 5.8(A) FACS analysis of αvβ6 expression in VB6 cells transfected with antisense oligos using Effectene

= αvβ6 expression in control VB6 cells

= αvβ6 expression following treatment with αv + β6 antisense oligos.

= αvβ6 expression following treatment with 4-mis-αv + 4-mis-β6 oligos.

Cells were stained with the anti-αvβ6 mAb, E7P6 at 10μg/ml and a FITC-conjugated secondary Ab. Figure shows the results of a representative experiment in triplicate.

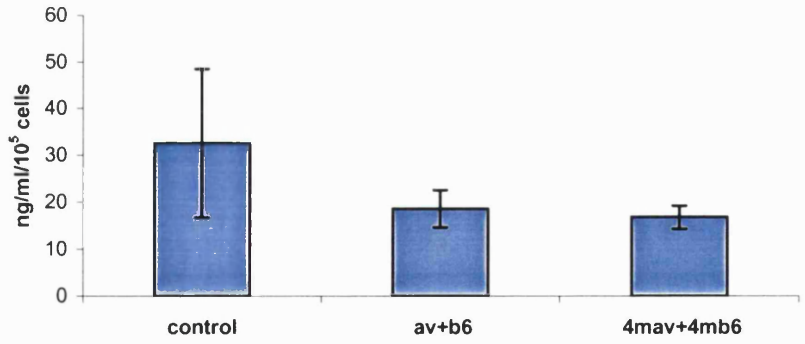
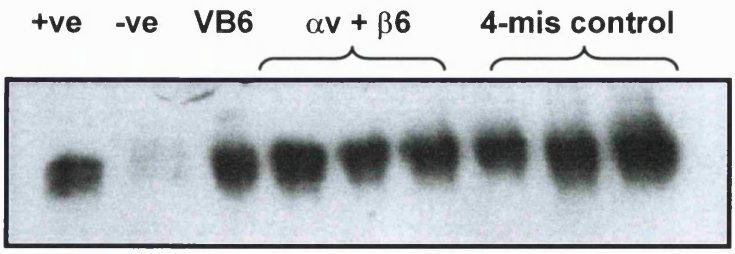


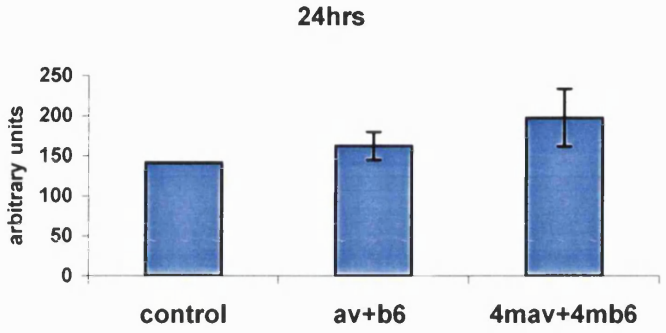
Figure 8(B) Secreted PA activity from VB6 cells transfected with antisense oligos using Effectene. Cultured supernatants were assayed for PA activity using a plasmin-specific chromogenic substrate and uPA secretion determined by comparison with uPA standards. Figure shows mean ±SD of a representative experiment in triplicate.

Treatment 3

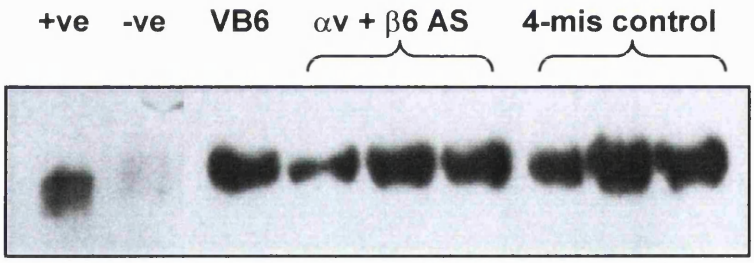
A further method of Effectene treatment was based on a previously described protocol (Qiagen), for transfection of antisense oliogonucleotides was also carried out. Cells were seeded at 250 000 cells per well in 6-well plates and transfected using Effectene and 1μg oligo per 250 000 cells. Therefore, for triplicate wells, 3.6μl of each oligo, αv + β6 (diluted 1:10) was added to 450μl EC buffer and left for 3 minutes at room temperature, after which 24μl enhancer was added and incubated for a further 3 minutes. 75μl of Effectene reagent and 950μl PBS was then added to the oligo master mix, to give a DNA:Effectene ratio of 1:25. The conditioned medium was removed from the cells and 500μl of fresh KGM and 500μl of the PBS/oligo mix was added to each well. Cells were also transfected with the control oligos and as a further control, were treated with just the Effectene, EC buffer and enhancer. Cells were incubated with oligo treatments at 37°C and lysed for western blot analysis after 24 and 48 hours (figure 5.9A and 5.9B). Cells were also harvested after a 72-hour incubation (figure 5.9C), thus giving more time for expression of new proteins and depletion of existing αvβ6 integrin.

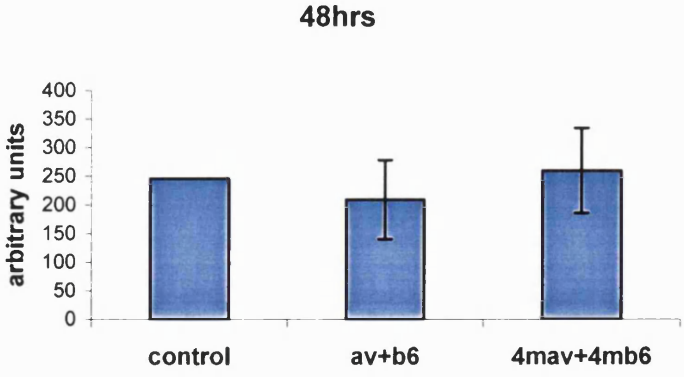
5.9A) Effectene – 24hrs





5.9B) Effectene – 48hrs





5.9C) Effectene –72 hours

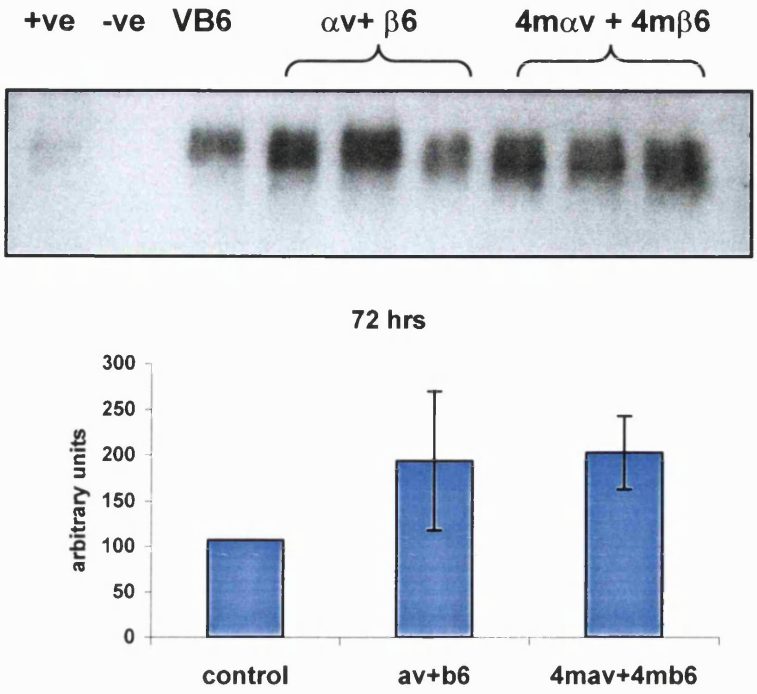


Figure 5.9 αvβ6 expression in VB6 cells treated with antisense oligos using Effectene. Total cell extracts were subjected to Western blotting and probed for αvβ6 using the mAb, sc-6632 at 2μg/ml. Integrin expression was determined after (A) 24 hours (B) 48 hours and (C) 72 hours incubation with antisense oligos. Representative blots are shown. These were subjected to densitometric analysis using scion image software and graphs show mean ±SD of a representative experiment in triplicate.

Summary of Effectene Treatment

Transfection of antisense oligos into VB6 cells was attempted using Effectene by 3 methods with a range of DNA:Effectene ratios. However, none of the methods produced a significant down-regulation of αvβ6 integrin expression following treatment with antisense oligonucleotides compared to the control treatments.

DETECTION SYSTEM	**[DNA]**	**DNA:EFFECTENE RATIO**	**TIME**	**αvβ6 EXPRESSION**	**uPA EXPRESSION**
Western Blot	0.8μg/600μl	1:50	48 hrs	No significant difference between αv + β6 AS and 4-mis control AS treated cells.	Not tested
FACS PAA Assay	8μg/700μl	1:2	64hrs	No significant difference between αv + β6 AS and 4-mis control AS treated cells.	No significant difference between αv + β6 AS and 4-mis control AS treated cells.
Western Blot	1μg/ml	1:25	24hrs 48hrs 72hrs	No significant difference between αv + β6 AS and 4-mis control AS treated cells.	Not tested

Table 5.1 Summary of Antisense Oligo Treatment Using Effectene Reagent

5.2.7 Transfection Using Superfect Reagent

Superfect is an activated-dendrimer transfection reagent, which possesses a defined spherical architecture, with branches radiating from a central core and terminating at charged amino groups. Superfect reagent assembles DNA into compact structures, optimising the entry of DNA into the cell. The net positive charge of the Superfect-DNA complexes allows them to bind to negatively charged receptors on the surface of eukaryotic cells. Once inside the cell, the superfect reagent buffers the lysosome after it has fused with the endosome, leading to pH inhibition of lysosomal nucleases. This ensures stability of Superfect-DNA complexes and the transport of intact DNA to the nucleus (Qiagen). The DNA:transfection reagent ratio is an important factor that determines transfection efficiency. Optimal binding of transfection reagent-DNA complexes to the negatively charged groups on the cell surface requires a slightly net positive charge. The overall charge of the transfection reagent-DNA complex is determined by the ratio of transfection reagent to DNA.

Treatment 1

Cells at 50% confluency in 6-well plates were transfected using Superfect reagent at a DNA:Superfect ratio of 1:2. Each well was treated with 5μg (1.2μl) of each oligo, αv and β6, a total of 10μg DNA per well. Therefore, for triplicate wells 3.6μl (15μg) of each oligo (αv + β6) was diluted in 900μl SFM. 60μl Superfect (1:2 ratio) was added to this and allowed to complex at room temperature for 15 minutes. 5ml KGM was added to the complex mix, since serum aids transfection at this stage. Following a wash with PBS, the cells were incubated with 2ml of the final oligo mix for 24 hours

before being changed to serum free medium. Cells were also transfected with control oligos and as a further control with only Superfect reagent. After a further 16 hours, cells were harvested for analysis of αvβ6 expression by FACS (figure 5.10A) and supernatants were collected for measurement of secreted PA activity (figure 5.10B). Treatment with antisense oligos had no effect on the levels of αvβ6 expression or uPA secretion compared to the control treatments.

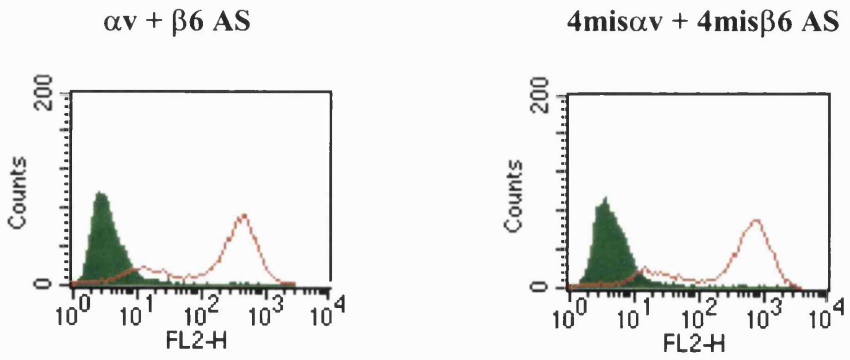


Figure 5.10(A) αvβ6 expression in cells transfected with antisense oligos using Superfect Reagent.

VB6 cells were stained with the anti-αvβ6 mAb, E7P6 at 10μg/ml and a FITC-conjugated secondary antibody. Figure shows the results of a representative experiment in triplicate.

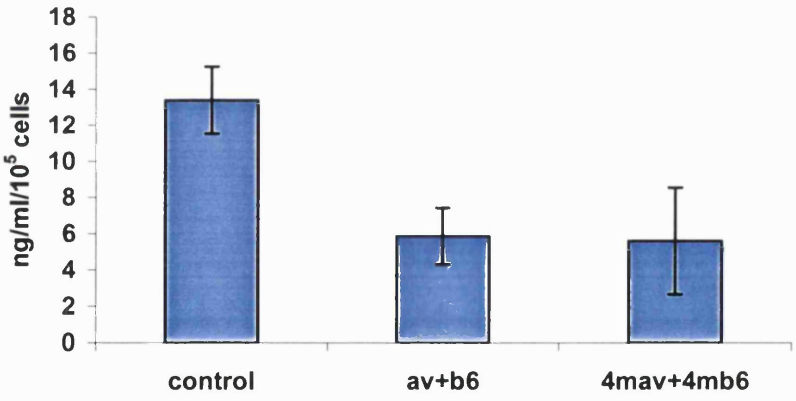


Figure 5.10(B) Secreted PA activity from cells transfected with antisense oligos using Superfect Reagent.

Cultured supernatants were assayed for PA activity using a plasmin-specific chromogenic substrate and PA secretion determined by comparison with uPA standards. Figure shows mean ±SD of a representative experiment in triplicate.

Treatment 2

Cells were transfected at 50% confluency in 24-well plates using a DNA:Superfect ratio of 1:1. 2μl of αv oligo and 2μl β6 oligo, a total of 14μg DNA, was added to 14μl superfect reagent and allowed to complex at room temperature for 15 minutes. 6μl of this complex mix was then added to 0.5ml of KGM on the cell monolayer, so each well was treated with 4.2μg of DNA. After 24 hours, the medium was changed to fresh KGM and incubated for a further 48 hours, before being changed to serum free medium. Following a 16-hour incubation, cells were harvested for analysis of αvβ6 expression by FACS (figure 5.11A) and supernatants were collected and assayed for secreted PA activity (figure 5.11B). Treatment of antisense oligos in a 1:1 ratio with Superfect Reagent also had no effect on αvβ6 expression or uPA secretion.

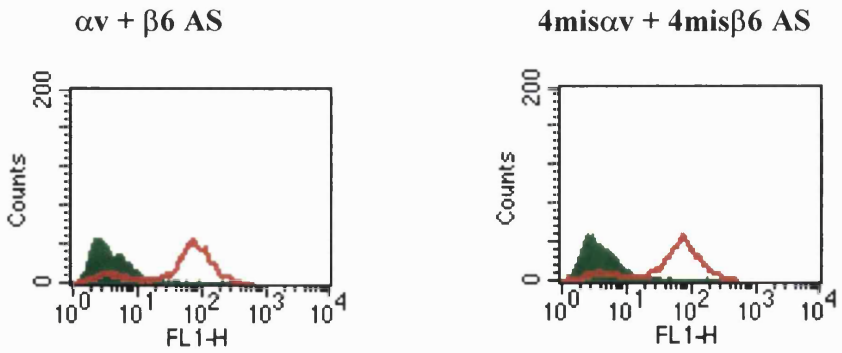


Figure 5.11(A) αvβ6 expression in cells transfected with antisense oligos using Superfect Reagent.

VB6 cells were stained with the anti-αvβ6 mAb, E7P6 at 10μg/ml and a FITC-conjugated secondary antibody. Figure shows the results of a representative experiment in triplicate.

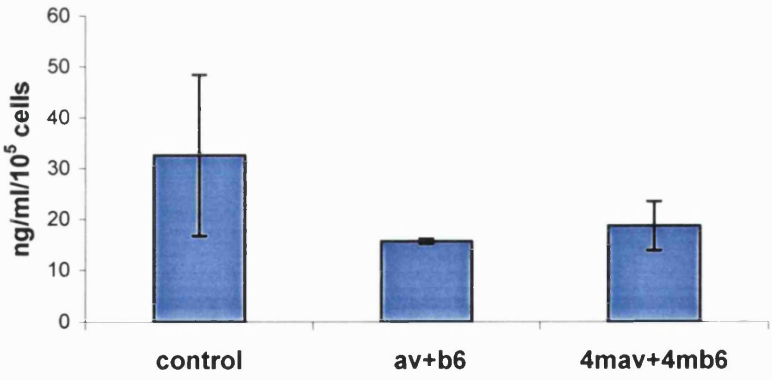


Figure 5.11(B) Secreted PA activity from cells transfected with antisense oligos using Superfect Reagent.

Cultured supernatants were assayed for PA activity using a plasmin-specific chromogenic substrate and PA secretion determined by comparison with uPA standards. Figure shows mean ±SD of a representative experiment in triplicate.

Treatment 3

A further treatment based on a previously described protocol for the transfection of antisense oligonucleotides using Superfect was also attempted (Qiagen). Cells at 50% confluency in 6-well plates, were transfected with 400nM total oligo using a Superfect:DNA ratio of 6:1. Therefore, for triplicate wells, 2.4μl or 10μg DNA, (1.2μl or 5μg of each oligo) was diluted in 297.6μl DMEM. 60μl Superfect reagent (6μl Superfect / 1μg DNA) was added to this, vortexed and allowed to complex at room temperature for 10 minutes. 2640μl DMEM + 10% FCS was added to the complex mix and 1ml of this was added to each well of cells that had been washed in PBS and incubated at 37°C. After 12 hours, the oligo media was removed and replaced with fresh KGM. Cells were harvested for Western blot analysis for αvβ6 expression 48 hours post-transfection (figure 5.12). The cultured supernatants of these were collected and analysed for uPA by ELISA (Biopool, UK) as the presence of protease inhibitors in serum prevents the use of the PA activity assay (figure 5.13). The results show a 50% down-regulation of αvβ6 expression in antisense treated cells compared to cells treated with the control oligos. However, there was no difference in the levels of uPA secretion. The antisense effect on αvβ6 expression was not reproducible to the same degree in an exact repeat of this protocol.

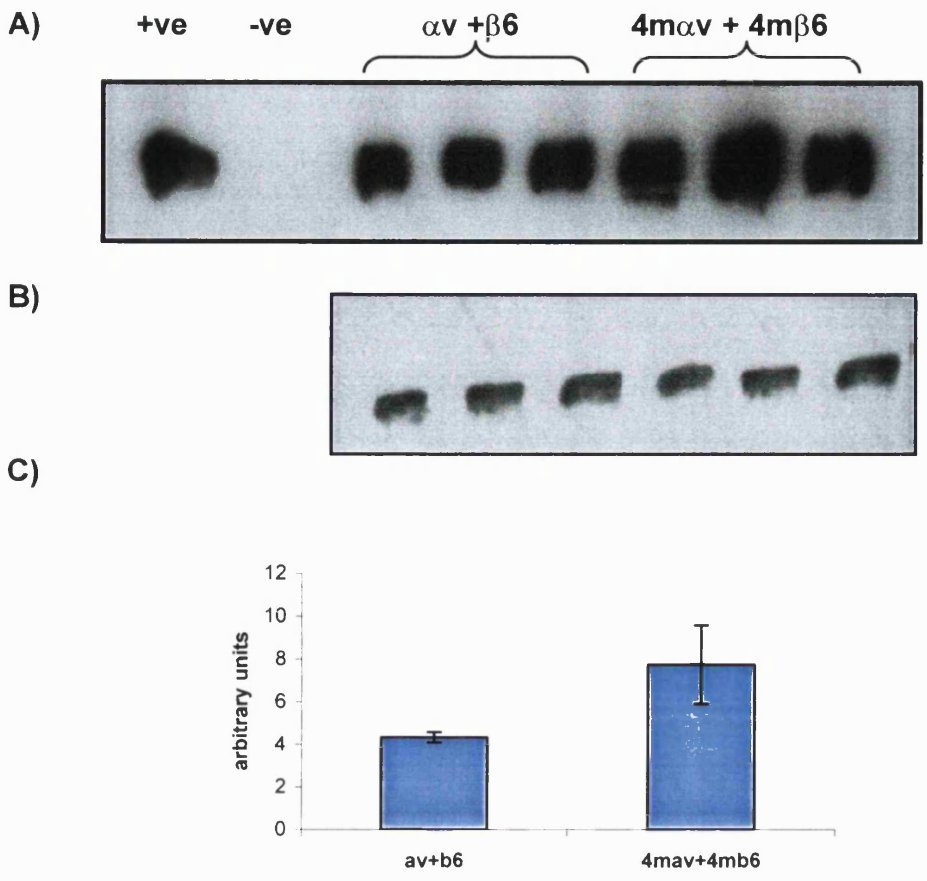


Figure 5.12 Integrin expression in VB6 cells transfected with antisense oligos using Superfect.

A) 10μg of total cell extract was subjected to Western blotting for αvβ6 probed the anti-β6 C-terminal goat polyclonal antibody, sc-6632 at 2μg/ml. Figure shows a representative blot.

B) The blot was re-probed for β1-integrin expression using the mAb, A11B2 at 10μg/ml.

C) Blots were subjected to densitometric analysis using Scion image software and αvβ6 expression was normalised to β1 levels and expressed in arbitary units. Graph shows mean +SD of a representative experiment in triplicate.

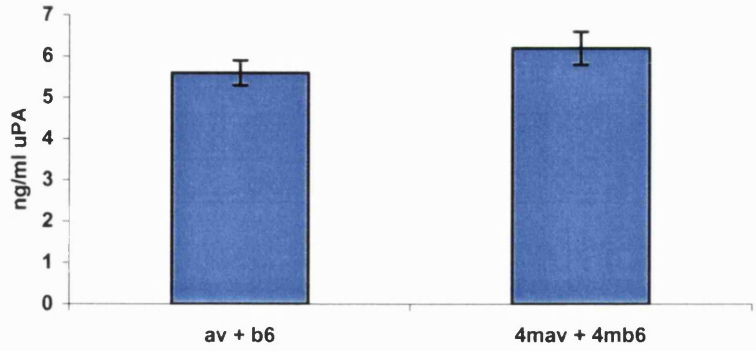


Figure 5.13 Levels of uPA secretion from VB6 cells transfected with antisense oligos using Superfect.

Cultured supernatants were subjected to ELISA for uPA. Samples were diluted 1:100 and 50μl was assayed. Absorbance values were converted to ng/ml of plasminogen activator using a standard curve. Values are expressed as mean ±SD of triplicate samples of a single experiment.

Summary of Superfect Treatment

DETECTION SYSTEM	[DNA]	DNA: EFFECTENE RATIO	TIME	αvβ6 EXPRESSION	uPA EXPRESSION
FACS PAA Assay	10μg/ 2ml	1:2	40 hrs	No significant difference between αv + β6 AS and 4-mis control AS treated cells.	No significant difference between αv + β6 AS and 4-mis control AS treated cells.
FACS PAA Assay	4.2μg/ 500μl	1:1	88hrs	No significant difference between αv + β6 AS and 4-mis control AS treated cells.	No significant difference between αv + β6 AS and 4-mis control AS treated cells.
Western Blot ELISA	3μg/ ml	1:6	60hrs	A 45% inhibition in the αv + β6 AS treated cells.	No significant difference in uPA secretion between αv + β6 AS and 4-mis control AS treated cells.

Table 5.2 Summary of Antisense Oligo Treatment using Superfect Reagent

5.3 DISCUSSION

Oral SCC keratinocytes expressing the αvβ6 integrin were transfected with morpholino antisense oligonucleotides targeted against the αv and β6 integrin subunits in order to down-regulate αvβ6 integrin expression. Delivery of oligos into the cells was attempted using the scrape-load delivery method as well as the transfection reagents EPEI (Gene Tools), Effectene and Superfect (Qiagen). Numerous concentrations, DNA:transfection reagent ratios and incubation times were attempted. Using scrape-load delivery, EPEI and Effectene, no significant down-regulation of αvβ6 expression was observed in cells treated with αv + β6 antisense oligos compared to the cells treated with the control 4-mis oligos. Transfection using Superfect at a DNA:Superfect ratio of 1:6 showed a 45% inhibition of αvβ6 expression in αv + β6 antisense treated cells compared to control cells (figure 5.12). However, this was not reproducible to the same degree in subsequent experiments (data not shown).

Scrape-load delivery is theoretically the quickest and easiest method of delivering the oligos into the cells (Partridge et al, 1996). Following the scraping procedure, there was great cell death with as little as 20% surviving the scraping procedure, however αvβ6 integrin expression was not decreased in the remaining cells (figure 5.5). A major problem in treatment with antisense oligos is their efficient delivery into the cells. Since they are designed against an mRNA target sequence, they represent a predominantly cytoplasmic target. Oligos delivered into cells by free cell uptake

through osmosis or by scrape-delivery, have a greater chance of being sequestered in endosomal vesicles in the cytoplasm, thus limiting their access to targeted mRNA, even though the neutral backbone of morpholinos should have facilitated oligo transfer through the cell membrane and endosomal lipid bilayers (Summerton and Weller, 1997).

Oligonucleotides delivered by cationic lipids or other cationic agents are directed to the nucleus. Therefore, transfection was also attempted using the special cationic lipid formulation EPEI, specifically designed by Gene Tools for transfecting morpholino oligos. FACS analysis shows no difference in expression of αv or β6 subunits in cells transfected with antisense oligo, control 4-mis oligo or the control with no treatment (figure 5.2). Even by Western blot analysis, there was no difference in the levels of β6 expression in cells treated with αv + β6 antisense oligos compared to control oligos. Cells treated with oligos showed a higher level of expression than that of untreated cells, however this was not a significant trend that was repeatable throughout all of the experiments (figure 5.3). This lack of inhibition of integrin expression was reflected in no effect on the levels on PA secretion (figure 5.4).

Transfection was also attempted using Effectene reagent, which is a unique non-liposomal lipid formulation to produce condensed Effectene-DNA complexes, which are used to transfer DNA into eukaryotic cells. Effectene reagent was used since it has been shown to yield significantly better transfection results in a wide variety of

cell lines than many widely used transfection reagents (Qiagen). Effectene reagent can also be used to transfect cells in the presence of serum, thereby reducing stress on the cells and lowering cytotoxicity as well as requiring very low concentrations of DNA. Antisense oligos were transfected into cells using Effectene reagent at DNA:Effectene ratios of 1:2, 25, and 50 and integrin expression was analysed between 24 and 72 hours. However, as shown by FACS and Western blot analysis, there were no differences in the levels of αvβ6 expression in cells treated with antisense oligos compared to those treated with 4-mis control oligos (figures 5.7, 5.8A and 5.9). This was reflected in no change in the levels of PA secretion (figure 5.8B). Secreted PA levels from cells treated with antisense or control oligos were lower than that from control cells with no treatment. This was possibly due to a slight toxic effect leading to reduced cell numbers following treatment with oligo.

Superfect, which is an activated-dendrimer transfection reagent that assembles DNA into compact structures, optimising the entry of DNA into the cell was also used for transfection of the morpholino oligos, which gave more promising results. A 45% inhibition of integrin expression was observed 60 hours post-transfection (figure 5.12), although in the corresponding supernatants there were no significant differences in the levels of secreted PA (figure 5.13). This was possibly because the αvβ6-mediated PA secretion from cells was still abundant before the down-regulation of αvβ6 integrin expression seen at 60-hours post-transfection. However, using a slightly different protocol for transfection using Superfect, secreted PA levels from

antisense treated cells were slightly lower than from cells treated with control oligos (figure 5.11B).

There are a number of potential reasons why the use of morpholino antisense oligonucleotides was not particularly effective at down-regulating integrin expression. Initially, they may not have reached the target site due to a problem in delivery of the oligos. Optimising the delivery of the antisense oligos was difficult since the transfection efficiency could not be determined, as they were not conjugated with a marker (such as a fluorochrome). Certain transfection reagents are better for DNA delivery into particular cell lines, however there is a little literature demonstrating transfection of any type of antisense oligonucleotides into keratinocytes, so it was difficult to determine which was the best method. In general the introduction of foreign genes into keratinocytes is difficult, since the transfection efficiency is low (Jiang et al, 1991, Ohba et al, 1998). Other possible reasons were that the oligos may not have reached the target site could be due to instability of the oligos or problems of hybridisation to the target mRNA sequence. It is also possible that the antisense oligos were only targeted to the low levels of endogenous αv and β6 and not the αvβ6 that had been transfected into the cells. In fact, very few studies have demonstrated the use of antisense oligonucleotides in down-regulating integrin expression and function.

An additional problem with this study was determining the optimal transfection time before analysis of integrin expression. In order to see a significant antisense effect,

the existing integrins on the cell surface needed to be degraded, so that any inhibition of protein expression could be clearly seen. In this study, integrin expression was checked at times ranging between 24 and 72 hours post-transfection with antisense oligos, since previous studies had shown down-regulation at 36 and 72 hours (Chen et al, 2001, Kronenwett et al, 2002). However, there may still have been existing integrins present on the cell surface thus obscuring any antisense effect. This is a general problem with antisense technology where a total inhibition of protein expression is rarely seen. Therefore, it is possible that the morpholino antisense oligos did efficiently reach the target site, but may not have been able to significantly down-regulate integrin expression. The factors affecting this are, rate of integrin turnover, the time of incubation with antisense oligo before testing for expression. In addition, the oligos may have exerted non-specific effects therefore not targeting αvβ6 directly.

Tranfection using Superfect reagent down-regulated αvβ6 expression by 45%, however a concurrent decrease in uPA secretion was not seen. This may be due to the lack of complete inhibition of integrin expression, leading to continued stimulation of uPA secretion by the remaining αvβ6. In conclusion, transfection of morpholino antisense oligos targeted to the αv and β6 integrin subunits did not result in a consistently significant down-regulation of αvβ6 expression therefore the effect of this integrin on the urokinase system could not be confirmed with any accuracy.

CHAPTER 6

THE ROLE OF αVβ6 AND UPA ON CELLULAR FUNCTION

6.1 INTRODUCTION

Tumour cell invasiveness is a multi-step process that involves cell adhesion, proteolytic degradation of tissue barriers and migration of cells through the disrupted matrix. During invasion, cells pass through physical barriers in a three-dimensional matrix. This process involves altered cell-cell and cell-matrix interactions, through altered expression of adhesion molecules, such as integrins as well as acquiring a loss of growth control. Cell migration is a complex process that comprises a repeated cycle of distinct steps of adhesion and de-adhesion to the extracellular matrix. This is mediated primarily through integrin interactions with their specific ligands and dynamic re-organisation of the actin cytoskeleton. By linking the cell to ECM ligands, integrins through their cytoplasmic domains are able to transmit forces and signals necessary for locomotion.

αvβ6 and migration/invasion

αvβ6 is neo-expressed by keratinocytes in both cutaneous and oral wounds (Breuss et al, 1993, Clark et al, 1996) and is preferentially expressed on cells at the leading edge of cutaneous wounds and at the interface between tumour cells and the adjacent stroma (Haapasalmi et al, 1996, Breuss et al 1995). This suggests that the αvβ6 integrin heterodimer could have an active role in cell migration. Further studies on keratinocytes from β6 knockout mice showed a greatly reduced migration on fibronectin and vitronectin, even in the presence of α5β1, suggesting a crucial role for αvβ6 (Zambruno et al, 1995). Several studies have shown that there is *de novo*

expression of αvβ6 in epithelial carcinomas (Breuss et al, 1995, Jones et al, 1997), as well as expression in a number of pre-malignant oral epithelial dysplasias (Hamidi et al, 2000). This suggests that this integrin may be involved in cancer cell invasion and malignant transformation.

uPA/uPAR and migration/invasion

A large body of evidence has implicated components of the urokinase-type plasminogen activator system in the regulation of cell motility. The simultaneous expression of uPA and its receptor has been associated with localised plasminogen activation and pericellular matrix degradation during directed cell migration of normal and tumour cells (Kjoller et al, 2002). Receptor-bound uPA has been associated with neuronal cell migration, keratinocyte migration and endothelial cell migration during tissue remodelling, wound healing and angiogenesis, respectively (Pepper et al, 1993, Jensen and Rodeck, 1993, Dent et al, 1993). In addition to a proteolytic role of the urokinase system, there has been evidence to suggest migration may be stimulated via uPAR mediated signal transduction (Kjoller et al, 2002). The role of αvβ6 and the plasminogen activation system in keratinocytes in cell motility was investigated using various functional assays, including haptotactic cell migration assays, wound assays and invasion assays.

6.2 MATERIALS AND METHODS

6.2.1 Migration Assays

Haptotactic cell migration towards fibronectin was measured using polycarbonate filters as previously described (see section 2.6). The underside of the filters were coated with 250µl of plasma fibronectin (Sigma Chemical Co.), diluted in PBS to give a final concentration of 10µg/ml, for 1 hour at 37°C. Both sides of the filters were then blocked with migration buffer (DMEM + 0.5% BSA) for 30 minutes at 37°C. Meanwhile, cells were trypsinised and washed in the migration buffer in order to remove any traces of serum in the KGM. Before seeding, cells were pre-treated with various antibodies and inhibitors.

Amiloride treatment – the optimal concentration of amiloride was determined by dose-response curves using a PAA assay (Appendix 5). Cells were treated with 10µg/ml amiloride (Sigma Chemical Co.,), or the corresponding volume of DMSO as the controls for 30 minutes at room temperature.

Aprotinin treatment – cells were incubated with aprotinin (Sigma Chemical Co., Poole, UK) at 10µg/ml for 30 minutes at room temperature.

Anti-uPA antibody: mAb394 (American Diagnostica) – this mouse monoclonal antibody binds to the B-chain of the uPA molecule to inhibit uPA activity. Cells were pre-treated with mAb394 at 10µg/ml for 20 minutes on ice.

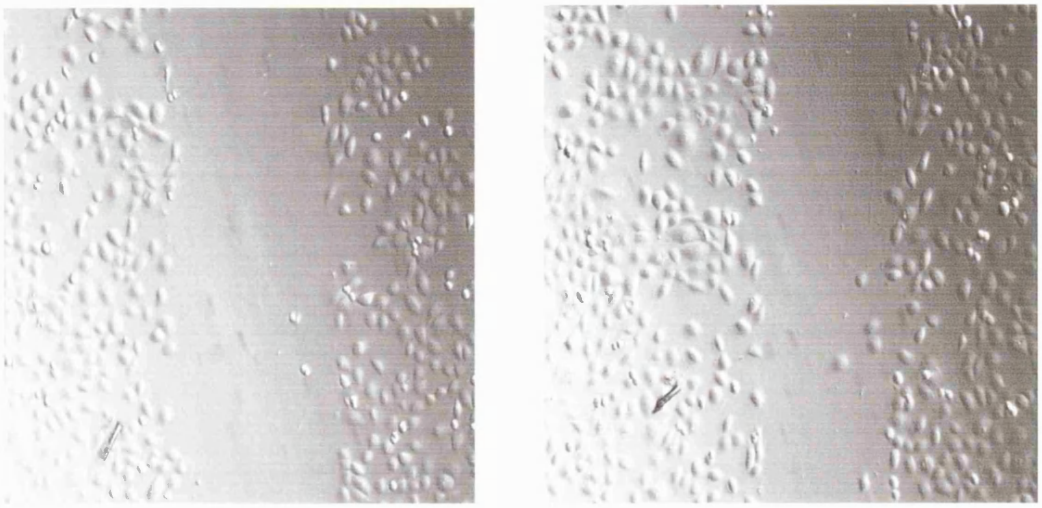
Anti-uPA antibody: mAb3689 – this mouse monoclonal antibody also binds to the B-chain of uPA, but does not inhibit uPA activity. Cells were pre-treated with mAb3689 at 10µg/ml for 20 minutes on ice.

Anti-uPA antibody: mAb Clone 5 – this mouse monoclonal antibody (a gift from Dr Vince Ellis, UEA) is effective at inhibiting uPA proteolytic activity. The optimal concentration for uPA inhibitory activity was determined using a PAA assay (Appendix 6). Cells were pre-treated with Clone 5 at 10μg/ml for 20 minutes on ice.

Following the above treatments, $1X10^5$ cells were placed into the upper chamber of each insert and the whole transwell insert placed into a well containing 600μl of migration buffer. Inhibitors and antibodies at the appropriate concentrations were also added to this, to enable maximum inhibitory activity. Cells were allowed to migrate towards fibronectin for 4 hours before being fixed, stained and counted as previously described (see section 2.6).

6.2.2 Wound Assays

In order to determine the rate of cell motility between the cell lines, wound assays were carried out as previously described (see section 2.5). Cell motility was determined by measurement of the width of the denuded area (figure 6.1), before and after a 6-hour incubation.



Time 0 **6 hours**

Figure 6.1 Wounded Images of Oral SCC Keratinocytes

A confluent monolayer of cells was wounded, and then incubated at 37°C. Figures represent images taken at Time 0 and 4 hours post-wounding under X200 magnification. The width of the denuded area was measured using Scion image software.

6.2.3 Immunocytochemistry

Cells plated in a 24-well plate were wounded with 200µl pipette tip. After 12 hours incubation at 37°C, cells were stained for integrins as previously described (see section 2.8). Cells were stained with mouse monoclonal primary antibodies for integrin αvβ5, with P1F6 at 5µg/ml and integrin αvβ6, with E7P6 at 10µg/ml. Stained integrins were visualised using a rabbit anti-mouse FITC-conjugated secondary antibody at a final concentration of 1µg/ml.

6.2.4 Invasion Assays

Invasion assays were previously carried out as described (see section 2.7), using two concentrations of matrigel (1:40 dilution) and a (1:14 dilution) to provide a more substantial barrier of matrix proteins. In some experiments 0.15µM Lys-plasminogen (Enzyme Research Labs, Swansea, UK) was added to the upper chamber. Cellular invasion through the barrier was determined as previously described.

6.3 RESULTS

6.3.1 αvβ6 integrin promotes migration towards plasma fibronectin

The role of αvβ6 integrin in cell migration towards fibronectin was determined using haptotactic migration assays (figure 6.2). Migration of the VB6 cells was 4-fold greater than the control C1 cells, indicating that the αvβ6 integrin is functional in promoting migration to one of it's specific ligands, fibronectin. This was previously shown using αvβ6 and α5β1 integrin-blocking antibodies, confirming the αvβ6-mediated increase in migration (Thomas et al, 2001b). Cells expressing the mutant β6 integrin, lacking the unique 11aa cytoplasmic tail, VB6Δ_{11aa} migrated to the same degree as the VB6 cells, which express full-length β6, indicating that this unique sequence is not essential for controlling migration.

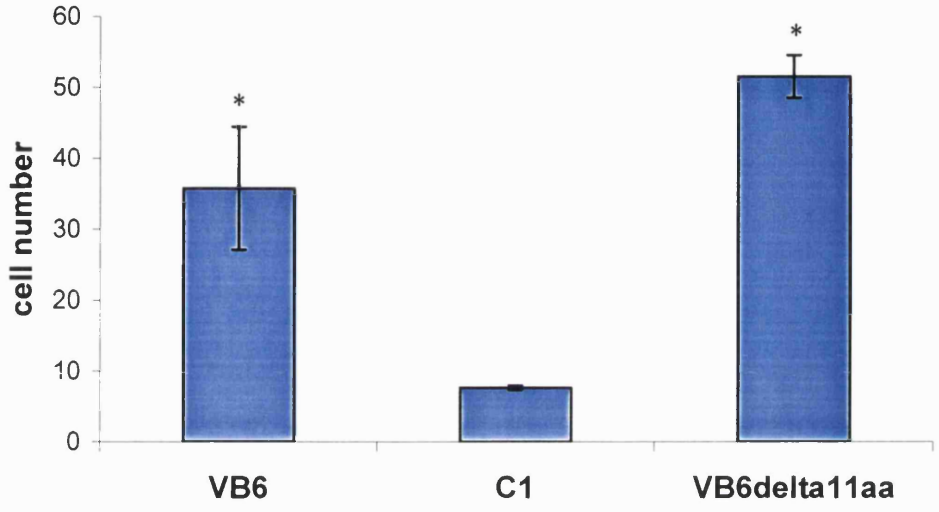


Figure 6.2 Migration on Plasma Fibronectin

Cells were allowed to migrate through fibronectin-coated polycarbonate filters for 4 hours. Cells migrated through to the underside of the filter were fixed with 10% formalin, stained with crystal violet and counted under a light microscope at X200 magnification. Each bar represents the mean count of five random fields of triplicate filters. Full-length (VB6) and mutated (VB6Δ11aa) β6 integrin expressing cells show a 60% and 70% increase in migration towards plasma fibronectin than the control (C1) cells ($p<0.05$, Mann-Whitney-U-Test).

6.3.2 The αvβ6-mediated increase in migration towards fibronectin is not uPA-dependent.

In order to see if uPA played a role in this αvβ6-mediated increased migration, cell migration assays towards fibronectin were performed in the presence of uPA inhibitors. A uPA-specific chemical inhibitor, Amiloride inhibited migration towards fibronectin by 60%, suggesting a role for uPA in the migratory process (figure 6.3). Since Amiloride is known to have effects on cells other than uPA inhibition, migration assays were also performed in the presence of three different uPA inhibitory antibodies. The monoclonal antibody 394 is targeted against the human uPA B-chain and it inhibits the activity of uPA. The monoclonal antibody 3689 is also targeted to the human urokinase B-chain, but does not inhibit uPA activity. The uPA inhibitory antibodies have been shown to completely block uPA activity using the chromogenic plasminogen activator activity assay (Appendix 6). Treatment of the cells with uPA inhibitory antibodies did not affect migration, however, treatment of cells with the mAb 3689 stimulated only VB6 cell migration towards fibronection by 2-fold (figure 6.4). Migration assays were also carried out on cells in the presence of another monoclonal anti-uPA antibody (Clone 5). This was the most effective at completely abolishing uPA proteolytic activity (Appendix 6). VB6 cell migration was not significantly reduced following treatment with Clone 5 antibody (figure 6.5). These data suggest that inhibition of uPA activity does not significantly affect cell migration. However, antibody stimulation of uPA activity significantly increases αvβ6-mediated migration.

Aprotinin is a broad-spectrum serine protease inhibitor that inhibits plasmin, trypsin, chymotrypsin and kallikrein. It was used in this assay to determine if plasmin activity is required for cell migration. Only VB6 cell migration was inhibited by 60% with aprotinin, suggesting that the αvβ6-mediated migration is plasmin-dependent (figure 6.6). Together these data indicate that the αvβ6-mediated increased migration towards fibronectin is dependent on plasmin but not uPA activity.

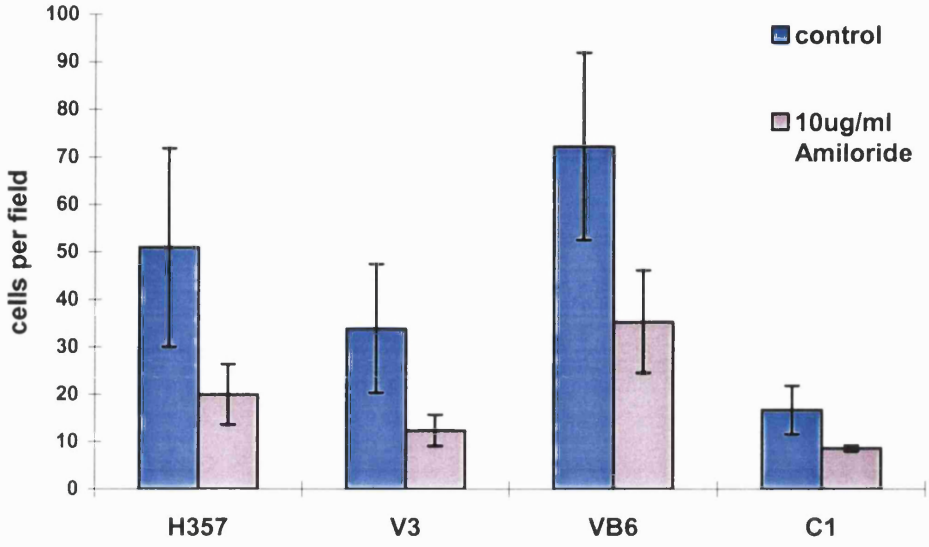


Figure 6.3 Migration on Plasma Fibronectin in the Presence of Amiloride

Cells were treated with 10μg/ml Amiloride or DMSO as the control and allowed to migrate towards plasma fibronectin for 4 hours. Cells migrated to the underside of the polycarbonate filter were stained with crystal violet and five random field on each filter were counted. Figure represents the combined mean ±SD of two experiments in triplicate. Amiloride inhibted migration by 50% in all of the cell lines.

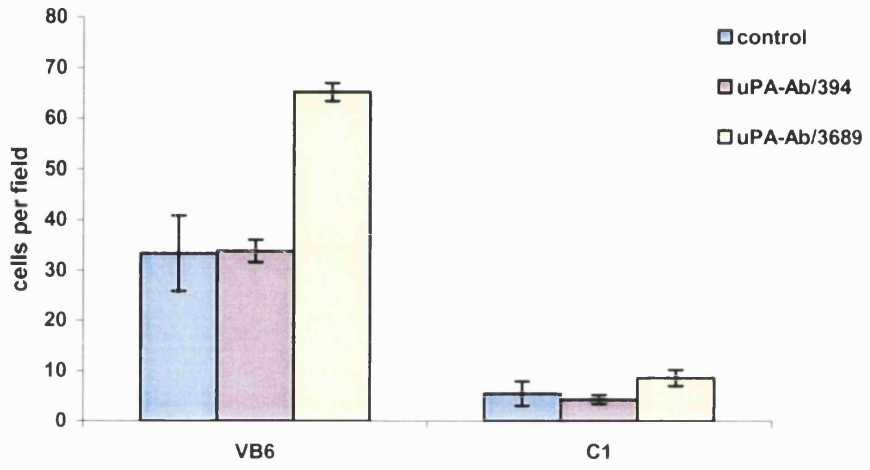


Figure 6.4 Migration on plasma fibronectin in the Presence of uPA Inhibitory Antibodies

Cells were treated with an inhibitory anti-uPA mAb 394 at 10μg/ml and a non-inhibitory anti-uPA mAb 3689 at 10μg/ml and allowed to migrate towards plasma fibronectin for 4 hours. Cells migrated to the underside of the polycarbonate filter were stained with crystal violet and five random fields on each filter were counted. A representative experiment is shown.

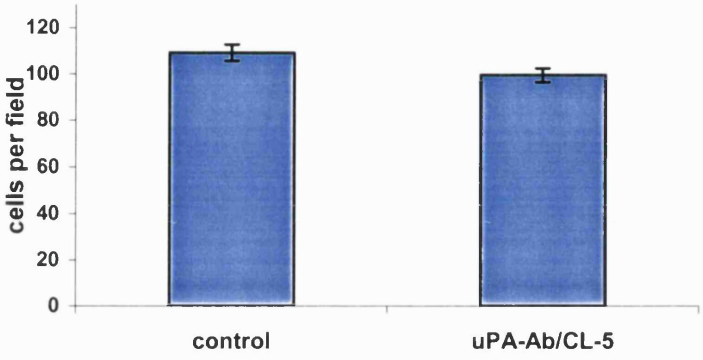


Figure 6.5 VB6 Cell Migration on Plasma Fibronectin in the Presence of the anti-uPA Antibody (Clone 5)

Cells were incubated with Clone 5, which has maximal anti-proteolytic activity for 20 minutes prior to seeding and cells allowed to migrate in the presence of excess antibody for 4 hours. Cells migrated to the underside of the filter were stained with crystal violet and five random fields of triplicate filters were counted.

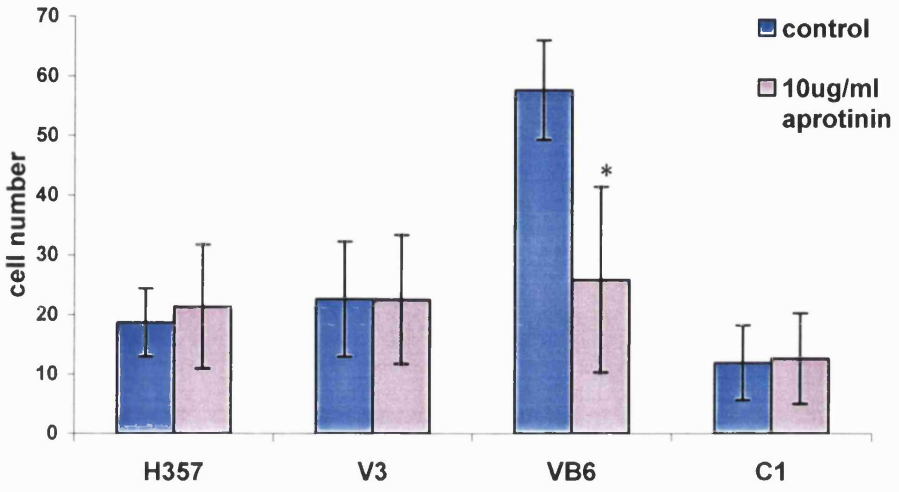


Figure 6.6 Migration on Plasma Fibronectin in the Presence of Aprotinin

Cells were treated with 10μg/ml Aprotinin and allowed to migrate towards plasma fibronectin for 4 hours. Cells migrated to the underside of the polycarbonate filter were stained with crystal violet and five random field on each filter were counted. Figure represents the combined mean ±SD of two experiments in triplicate. Only VB6 cell migration was inhibited by 50% in the presence of aprotinin ($p<0.05$, Mann-Whitney-U-Test).

6.3.3 Cell movement following wounding is not αvβ6-dependent.

Wound assays were carried out in order to establish any differences in cell motility between the cell lines (figure 6.7). The distance migrated was similar between all the cell lines, in contrast to increased migration of the VB6 cells towards fibronectin seen in the Boyden chamber assays. The fact that αvβ6 seemed to be non-functional in cell migration in the wound assays was also highlighted by immunocytochemical staining of the wound edge. In VB6 cells, the αvβ5 integrin rather than the αvβ6 integrin was localised to the leading edge of migrating cells (figure 6.8).

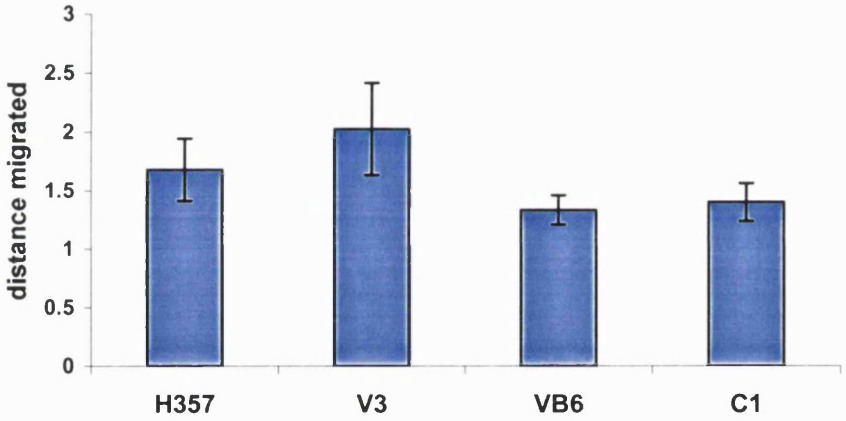
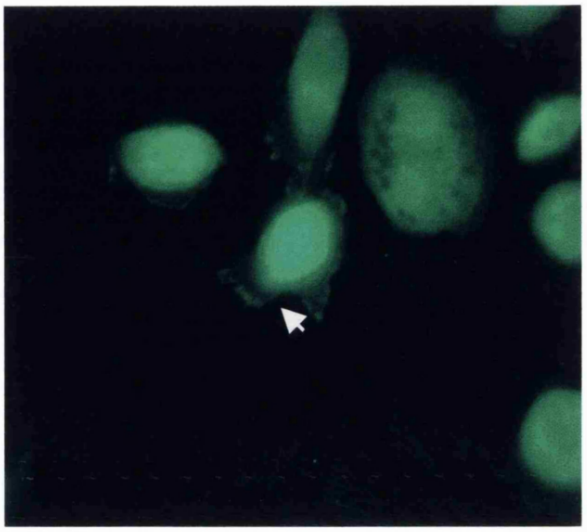


Figure 6.7 Wound Assay

Confluent cell monolayers were wounded and migration of cells into the denuded area was measured. The width of the wound was measured immediately and following a 6 hour incubation. The distance migrated of the cells was determined by the difference between the wound widths of the two time points. Each wound width was measured at four different points and averaged. Figure shows the mean ±SD of a representative experiment in triplicate.

A)



B)

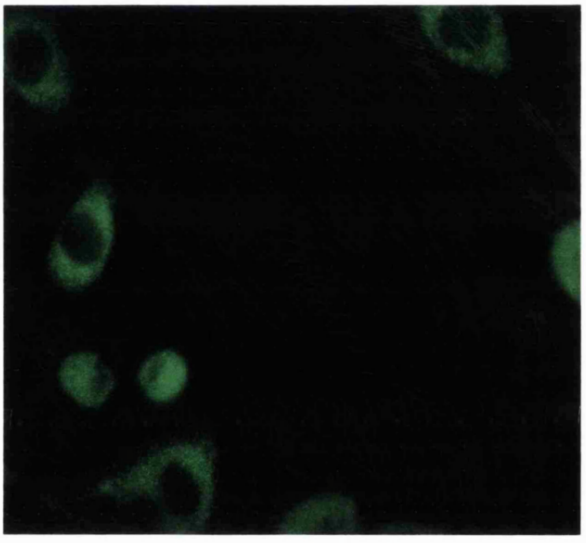


Figure 6.8 Integrin Expression at Wound Edge

A) Cells stained for integrin αvβ5, using mAb P1F6 (1:50).

B) Cells stained for integrin αvβ6, using mAb E7P6 (1:100).

αvβ5 expression is more prominent at the leading edge of migrating cells.

6.3.4 Cell migration involves the ERK1/2 branch of the MAP Kinase pathway

In order to further investigate the mechanism of migration and the signalling molecules involved, haptotactic migration assays were carried out in the presence of the chemical inhibitor, UO126 (Ge et al, 2002), which inhibits ERK1 and ERK2 in the MAP Kinase pathway. The results demonstrate that migration towards fibronectin was inhibited by about 50% in all the cell lines in the presence of this inhibitor (figure 6.9).

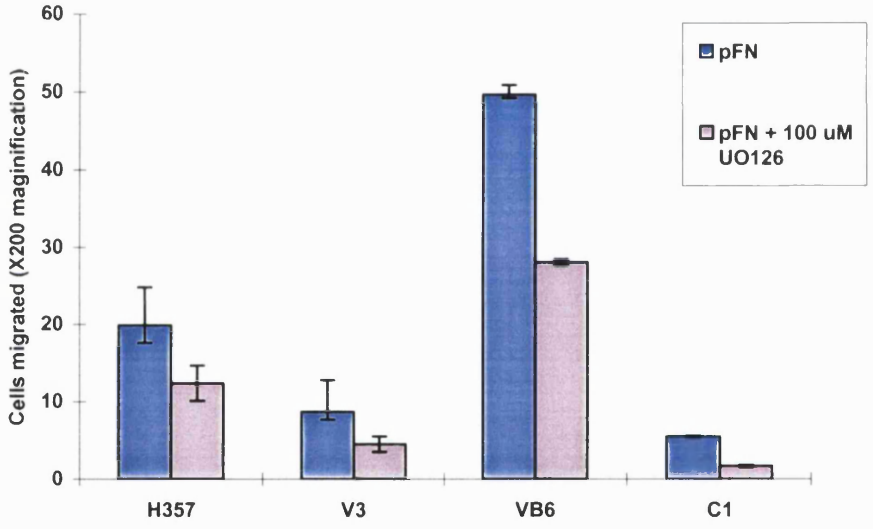


Figure 6.9 Migration on Plasma Fibronectin in the Presence of the MAP Kinase Inhibitor, UO126

Migration towards plasma fibronectin involves the MAP Kinase pathway. Cells were pre-treated with 100μM UO126 and allowed to migrate for 4 hours. Cells migrated to the underside of the filter were stained with crystal violet and five random fields of triplicate filters were counted. A representative experiment is shown. Migration is inhibited following treatment with UO126, by 50% in all the cell lines.

6.3.5 αvβ6 integrin does not promote cell invasion

In the keratinocytes used in this study, there was no difference in the invasive ability of the cell lines. Cells were allowed to invade through a thin layer of matrigel as well as through a thicker, more concentrated layer of matrigel (figure 6.10). Cells over-expressing αvβ6 did not exhibit a more invasive phenotype than the control cells through either matrigel composition. Invasion assays were carried out in the presence of plasminogen in order to optimise the proteolytic breakdown of components of the matrigel layer, however no difference was observed (figure 6.11).

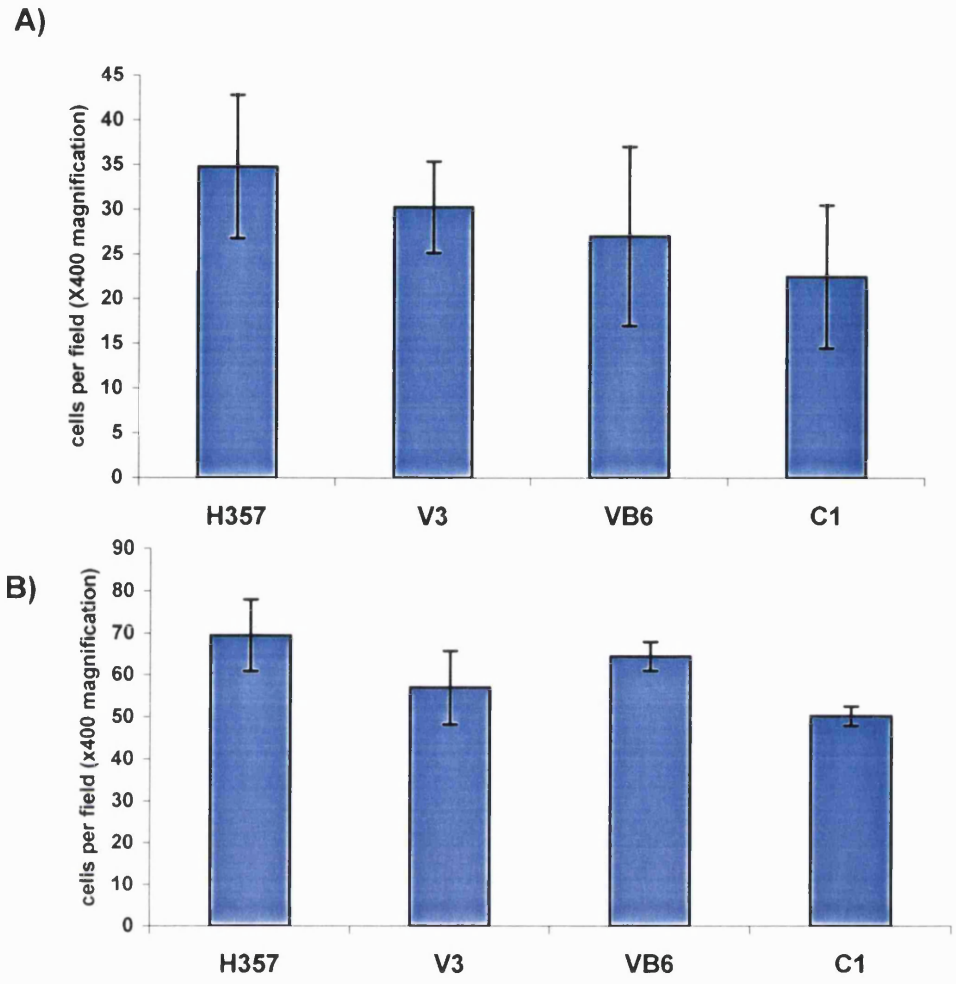


Figure 6.10 Invasion Assays

Transwell filters were coated with matrigel at concentrations of (A) 1:14 and (B) 1:40. Cells were left to invade through the coated filters for 48 hours at 37°C. Cells invaded through to the underside of the filter were fixed in 10% formalin, stained with crystal violet and counted under a high power light microscope. Each bar represents the mean count of five random fields of triplicate filters. Figure shows a representative experiment. There is no difference in invasion levels between the cell lines at both Matrigel concentrations.

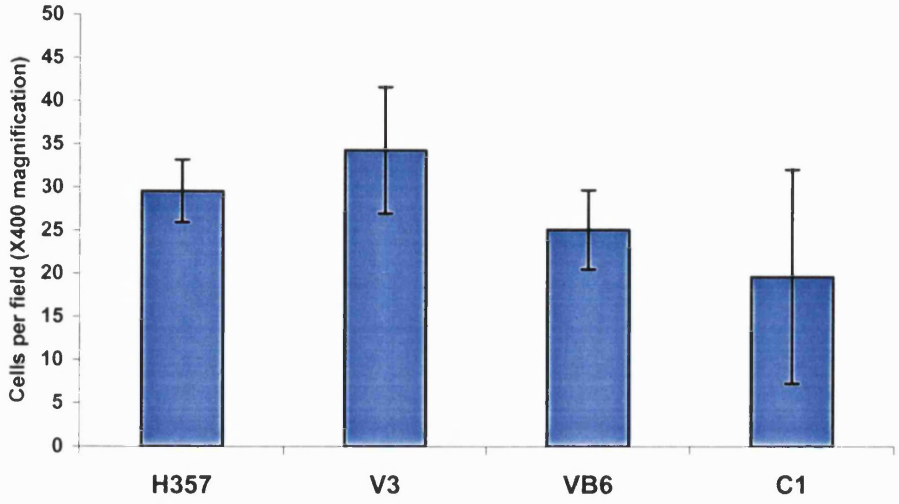


Figure 6.11 Invasion Assay in the Presence of Plasminogen

Transwell filters were coated with matrigel at a concentration of 1:14. Cells were left to invade through the coated filters for 48 hours at 37°C in the presence of 0.15μM plasminogen. Cells invaded through to the underside of the filter were fixed in 10% formalin, stained with crystal violet and counted under a high power light microscope. Each bar represents the mean count of five random fields of triplicate filters. Figure shows a representative experiment. There is no difference in invasion levels between the cell lines even in the presence of plasminogen.

6.4 DISCUSSION

Migration of cells is mediated by adhesion receptors, such as integrins, that link the cell to extracellular matrix ligands, transmitting forces and signals necessary for locomotion (Hynes, 1992, Schwartz et al, 1995, Lauffenburger and Horwitz, 1996). The results show that expression of full-length αvβ6 integrin into an oral SCC cell line promotes migration towards fibronectin, an αvβ6 ligand. Previous data have confirmed the role of the αvβ6 integrin in this migration using antibody-blocking experiments (Thomas et al, 2001b). Cells expressing mutant αvβ6 were also more migratory towards fibronectin compared to the control cells indicating that the 11 amino acid cytoplasmic extension of the β6 subunit is not required for the αvβ6-directed migration towards fibronectin. This suggests that migration may be promoted through a purely physical interaction, where only attachment of the αvβ6 integrin to fibronectin is required. Alternatively, migration may have been stimulated via signalling events through other parts of the cytoplasmic tail of the integrin.

Previous studies have demonstrated a role for αvβ6 in promoting cell migration towards fibronectin both *in vitro* and *in vivo* (Xue et al, 2001). However, in physiological and pathological situations of cellular migration, it is likely that multiple integrins co-operate to promote migration towards a certain matrix ligand. Studies in β6 knockout mice suggested an important role of αvβ6 in cell migration, however murine keratinocytes lacking αvβ6 were still able to migrate on fibronectin, although to a significantly lesser degree, indicating that migration requires the co-

operation of more than one integrin (Huang et al, 1998). In a study of oral SCC lines, the cells co-operatively used αvβ6 as well as α5β1 and αvβ1 integrin receptors for interaction with the fibronectin matrix (Koivisto et al, 2000). Also both αvβ6 and α5β1 integrins appear to be expressed in primary SCC tumours (Jones et al, 1997, Jones et al, 1993). This suggests that integrins are able to adapt to situations in which one receptor is non-functional by switching to another receptor of the same ligand as well as suggesting a co-operative role of integrins in migration.

Haptotactic migration assays are a widely used method for determining cell migration, however this assay is dependent on initial cell adhesion to the matrix proteins via integrins. However, the cell migratory processes involved in cancer are comparable to those seen in wound healing, so *in vitro* assays of wound healing are often used as models of tumour metastasis. Wound assays were carried out in order to determine random cell migration. This is more a measurement of cell motility on plastic into a wounded area, which is composed of a less defined mixed matrix, including fibronectin that is secreted from the cells and that present in the serum along with other matrix proteins. Therefore, this is not a measure of specific integrin-ligand dependent cell migration. Another difference between these assays is that cells are trypsinised prior to migration using Boyden chambers, however, in the wound assays, there is simply a physical disruption of a confluent cell monolayer before migration is measured. The fact that the cells were treated differently, i.e. chemical / physical disruption between the two assays, may have affected integrin expression and therefore function.

The contribution of αvβ6 to migration depends on the assay conditions and the two types of migration assay used in this study demonstrated this. In the Boyden chamber assays, a specific αvβ6-ligand directed cell migration is seen, however, in the wound assays, αvβ6 does not promote cell migration into the wounded area. This suggests that the increased migratory potential of αvβ6-expressing cells is dependent on activation of the integrin by its specific ligand alone. In fact, immunofluorescent staining of the wound edge shows a more prominent expression of the αvβ5 integrin at the leading edge of cell membranes, indicating that in the wound assays, the αvβ6 integrin may not be functioning in a pro-migratory role. A number of studies have shown that expression of αv integrins is induced in wound keratinocytes. Depending on the model of wound healing, the αvβ5 integrin (Gailit et al, 1994, DeLuca et al, 1994) or the αvβ6 integrin (Haapasalmi et al, 1996) is prominently expressed in wound keratinocytes, therefore it is not surprising to see αvβ5 expressed at focal points in the wound edge of the VB6 cells, indicating a role for αvβ5 in cell migration and invasion. In fact, the wound assays show increased cell migration in the V3 cells compared to the VB6 cells, suggesting that high levels of αvβ5 expression promote wound closure, which may possibly be due to the presence of more ligand for αvβ5 in the denuded area. In keratinocytes αvβ5 is expressed during the early stages of wound healing and integrin expression is switched to αvβ6 during late wound healing (Clark et al, 1996). Therefore, in the wound assays performed in this study, αvβ5 may have played a more prominent role in cell migration. In support

of this, uPA secretion was increased from the V3 cells over-expressing αvβ5 (see chapter 3) suggesting that αvβ5 is functional in these cells.

While it is evident that uPA/uPAR proteolytic activity can promote migration, enzymatic activity is not always required and cell migration can occur through direct signalling-mediated events. uPA/uPAR regulation of cell motility through signalling events may occur independently but previous evidence has suggested a functional co-operation between uPA/uPAR and integrins in regulating cell migration. There is a co-operation between integrins and proteases to co-ordinate focal extracellular matrix attachments and detachments central to cell migration (reviewed by Chapman and Wei, 2001).

In the VB6 cells used in this study, the αvβ6-mediated up-regulation of migration on fibronectin was shown to be dependent on MMP-9 activity (Thomas et al, 2001b). Previous studies have suggested that MMP-9 may be activated through an enzymatic cascade involving the generation of plasmin by uPA (Ramos-DeSimone et al, 1999), therefore a role for uPA in this αvβ6-mediated increase in migration on fibronectin was investigated.

Haptotactic cell migration assays were carried out in the presence of Amiloride, which competitively inhibits soluble uPA (not receptor-bound uPA), with a Ki of 7μM and an IC50 of approximately 100μM (Vassali and Belin, 1987). It selectively inhibits uPA over tPA and has been used to implicate uPA in processes such as

tumour metastasis and angiogenesis (Evans et al, 2000, Ray et al, 1998). Amiloride has also been shown to modulate urokinase gene expression at both the transcription and post-transcription levels in human colon cancer cells (Wang et al, 1995). In addition to inhibiting uPA, Amiloride has a variety of biological activities, for instance it has been used widely as a probe for Na+-coupled transport processes and is known to inhibit other transport systems and enzymes in addition to uPA (Kleyman and Cragoe, 1988). Amiloride, inhibited migration in all of the cell lines to the same degree, suggesting that the αvβ6-dependent migration towards fibronectin is not specifically due to uPA.

Inhibition of uPA activity by antibody blocking did not affect migration in these keratinocytes, however, this may have been because optimal inhibitory activity was not achieved in order to see an effect, or a compensatory process may be operating whereby MMP-9 plays a more prominent role in migration when uPA is inhibited. However, a non-inhibitory uPA antibody significantly increased only the αvβ6-dependent migration, suggesting that uPA stimulation in the VB6 cells increased migration. Specifically this increased migration of the VB6 cells was inhibited by aprotinin, indicating a role for plasmin. Since previous data has shown that MMP-9 specific inhibitors could reduce the increased migration of the VB6 cells (Thomas et al, 2001b), the results combined suggest that αvβ6 promotes migration on fibronectin through plasmin-mediated activation of MMP-9. The MMP-9 may play a proteolytic role or promote migration through signalling events either independently or linked to the αvβ6 integrin. Even though uPA does not appear to play a direct role in cell

migration, it may be indirectly required for the generation of plasmin but is just not highlighted in this particular assay system. The role of tPA in cell migration was not investigated but it is possible that this is required for the plasmin-mediated increase in VB6 cell migration.

Both αvβ6 and uPA have been implicated in promoting cancer invasion independently. In these keratinocytes, over-expression of the αvβ6 integrin did not promote invasion. This is contrast to published data on these cells, where VB6 cells were shown to be 2-fold more invasive than control C1 cells. This increased invasion was inhibited by anti-αv and anti-αvβ6 integrin blocking antibodies (Thomas et al, 2001b). However, in this study this pattern of invasion was not reproducible, even in an exact repeat of the published protocol (data not shown). The constitution of the matrigel may have contributed to the lack of function of the αvβ6 integrin. Matrigel is composed of a number of different proteins present within the basement membrane, providing many ligands for activation of integrins on the cells to promote invasion. The role of αvβ6 in promoting migration is more apparent due to the activation of the integrin by its specific ligand, fibronectin, whereas in the invasion assays, αvβ6 is not specifically targeted. The fundamental difference between the invasion and migration assays is the matrix proteins that stimulate cell movement. In invasion assays the cells have to physically move through a thick layer of matrix proteins whereas, in migration assays cells are stimulated to move towards a specific matrix protein. Also, invasion assays were performed over a much longer time period, 48 hours as opposed to a 4-hour migration; therefore cell proliferation may have masked any differences

between the cell lines. A degree of redundancy with regard to integrin function is possibly occurring where the absence of one integrin results in a compensatory amplification of function of another integrin. For example, αvβ6 may be the major integrin in mediating invasion in the VB6 cells however, in the C1 cells, the αvβ5 integrin may promote invasion, therefore any differences in invasion between the cell lines may not be apparent.

Migration of all the cell lines is partly mediated through the ERK1/2 branch of the MAP Kinase pathway, which is in agreement with much literature demonstrating a role of this signalling pathway in integrin-mediated migration (reviewed by Holly et al, 2000).

In conclusion, expression of the αvβ6 integrin in keratinocytes promotes haptotactic cell migration towards its specific ligand, fibronectin. However, the 11 amino acid extension of the β6 cytoplasmic tail is not required for this, suggesting that the β6 integrin is promoting migration either by a physical interaction or by signalling through other parts of the integrin. The αvβ6-mediated increase in migration was shown to be dependent on plasmin activity but not due to uPA directly, although uPA activity was required for migration to a certain degree in all the cell lines. *In vitro*, αvβ6 was only functional in promoting cell migration when it is specifically activated by its ligand, fibronectin and invasion was not enhanced in αvβ6-expressing cells.

CHAPTER 7

αvβ6 MODULATION OF THE UROKINASE SYSTEM IN MELANOMA CELLS

7.1 INTRODUCTION

Cutaneous malignant melanoma is one of the most rapidly increasing cancer groups worldwide (Mackie, 1998). All malignant tumours have the propensity to metastasise, which involves cell-ECM and cell-cell interactions, a process in which integrins and their ligands play a major role. During the progression from benign melanocytic disease to metastatic malignant melanoma, melanocytes undergo a series of changes in the expression of cell-surface molecules.

The αv integrin heterodimer has been implicated in melanoma progression. Loss of the αv chain in an experimental cell line leads to a delay in tumour growth *in vivo* compared with the parental cells (Felding-Habermann et al, 1992; Mitjans et al, 1995). Conversely, re-expression of the αv subunit in αv-defective cells restored tumourigenicity *in vivo* (Felding-Habermann et al, 1992) and survival *in vitro* (Montgomery et al, 1994). The vitronectin receptor, αvβ3 integrin in particular is up-regulated in association with melanoma progression (Albelda et al, 1990). The αvβ3 integrin plays a direct role in progression from non-invasive to the tumourigenic and highly metastatic melanoma (Hsu et al, 1998). De novo β3-expressing non-invasive cells deeply invaded the dermis without undergoing apoptosis, in contrast to control cells; which remained in the epidermis and entered apoptosis (Hsu et al, 1998). Also, αvβ3 plays a critical role in melanoma cell survival within human skin, and blockade of αvβ3 triggers apoptosis of melanoma cells, which ultimately blocks tumour growth (Petitclerc et al, 1999). This integrin therefore represents a potential therapeutic target.

Integrin expression and function are altered in malignant cells, although no specific integrin has been implicated in the transformation to the malignant phenotype and changes in integrin expression vary both between and different tumour types. The over-expression of the αv-subunit is common to most tumour types, however it is the αvβ3 heterodimer that is up-regulated in malignant melanomas but the αvβ6 heterodimer which is neo-expressed in epithelial carcinomas. Therefore, it is probable that integrins are cell and tissue specific in their ability to regulate behaviour. It is not clear as to what mechanism controls cell specific integrin expression.

In order to further delineate whether integrin functions are cell-type specific, the αvβ6 integrin, which is only expressed on epithelial cells (Breuss et al, 1993) was neo-expressed into non-epithelial human melanoma cell lines. αvβ6 function in these cells was then investigated in order to see if the observed effects of the αvβ6 integrin on the urokinase system is epithelial-cell specific.

7.2 MATERIALS AND METHODS

7.2.1 Transfections

Three human melanoma cell lines (A375P, DX3 and MeWo) were retrovirally infected with full-length β6 cDNA in the plasmid pBabepuro. These transfections were carried out by John Marshall (Richard Dimbleby Department, Cancer Research UK, St Thomas's Hospital, London). Following transfection, cells were grown in antibiotic selective medium, DMEM + 10% FCS, 10IU/ml Penicillin/Streptomycin and 1μg/ml Puromycin, since the pBabepuro vector harbours the antibiotic resistance gene. Cells expressing αvβ6 were selected for using antibody-conjugated magnetic bead sorting.

7.2.2 Magnetic Bead Sorting

Two rounds of magnetic bead sorting were carried out on each cell line, resulting in high β6-expressing cell populations. One 125cm^2 confluent flask of cells was trypsinised and washed once in serum free medium (SFM). Cells were incubated on ice with anti-αvβ6 monoclonal primary antibodies, E7P6 and 10D5 both at 10μg/ml in 500μl SFM, for20 mins. They were then washed three times and re-suspended in 500μl SFM. 70μl of anti-mouse secondary antibody conjugated to magnetic beads was added to the cells and incubated on ice for 20 minutes, mixing every few minutes. The cells were then sorted by placing the eppendorff tube in a magnetic holder (DAKO, Ltd, Eley, UK). The cells expressing αvβ6 which are conjugated to magnetic beads were attracted and concentrated to one side of the tube. The rest of the cells in medium were carefully removed and the cells remaining in the tube were re-suspended in SFM, mixed and sorted again as before.

This was repeated three times in total so that only αvβ6-expressing cells remained in the tube. These were then transferred to a 25cm^2 tissue culture flask and allowed to grow.

7.2.3 Flow Cytometry

FACS analysis (see section 2.3) was used to determine the dual expression of αvβ6 and uPAR expression on the β6-transfected melanoma cells. Following trypsinisation cells were washed three times with PBS + 10%FCS, then incubated with the primary anti-αvβ6 mAb, E7P6 at 10μg/ml, followed an anti-mouse RPE-conjugated secondary antibody at 1:50 dilution. The cells were then stained with a rabbit polyclonal anti-uPAR antibody, 399R (American Diagnostica) at 10μg/ml, followed by an anti-rabbit FITC-conjugated secondary antibody. In between each antibody incubation step cells were washed in PBS + 10%FCS. Cells were then analysed for expression of αvβ6 and uPAR.

7.2.4 Plasminogen Activator Activity Assay

Cells were seeded at 2 X 10^5/ml in 0.5ml SFM in 24-well plates. After 20-hour incubation at 37°C, supernatants were collected and assayed for PA activity (see section 2.4.2).

7.2.5 Cell Surface Plasminogen Activation

Cells were seeded at 0.4 X 10^6/ml in 0.5ml complete medium in 48-well plates. After overnight incubation cells were assayed for endogenous cell surface plasminogen activation. Cells were also stripped of endogenous uPA and incubated with exogenous

pro-uPA, so that all uPAR was saturated with uPA and expression of uPA would not be a rate-limiting step (see section 2.2.5).

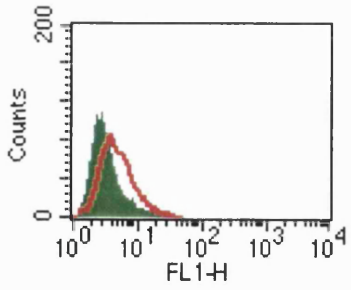
7.2.6 Migration Assays

Haptotactic migration towards plasma fibronectin was carried in Transwell migration assay chambers as described in section 2.6.

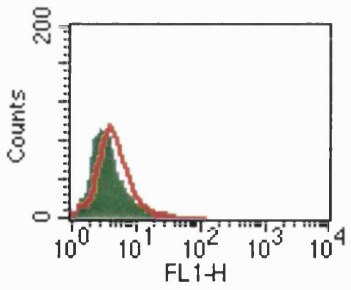
7.3 RESULTS

7.3.1 uPA and uPAR Expression in a Panel of Human Melanoma Cell Lines.

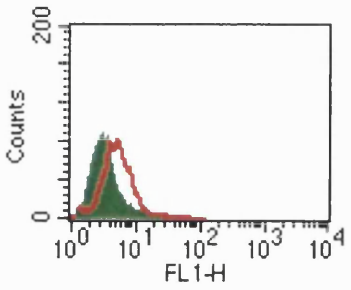
To determine whether αvβ6 could up-regulate uPA secretion and down-regulate uPAR expression and function in non-epithelial cells, αvβ6-negative human melanoma cell lines were transduced with β6 cDNA. In order to determine which melanoma cell lines were to be transfected to neo-express αvβ6 integrin, a panel of five human melanoma cell lines were tested for uPA and uPAR expression. This study demonstrates that αvβ6 over-expressed in epithelial cells up-regulates uPA secretion but down-regulates uPAR expression. Therefore, in order to see if these same effects are observed in melanoma cells, the cell lines initially need to have quite high levels of uPAR expression but show low levels of uPA secretion. uPAR expression was determined by FACS analysis (figure 7.1). The DX3, A357P and MEL 8 cell lines showed higher uPAR expression than the other two cell lines MeWo and HMB2, which had negligible uPAR expression. Secreted uPA activity was also measured from the cell lines (figure 7.2). MeWo and Mel 8 secreted very low levels of PA, with the DX3 cell line secreting very high levels. From these data it was concluded that the MeWo, DX3 and A375P cell lines would be transfected with β6. Since MeWo shows low secreted uPA levels and DX3 expresses high uPAR levels, these were thought to be the ideal cell lines to show an effect of the αvβ6 integrin on the urokinase system. The A375P cell line was also transfected since it secretes uPA and expresses uPAR at moderate levels, therefore any effect would also be seen in this cell line. These cell lines were then transduced with pBabe retroviruses encoding puromycin-resistance alone (to create DX3puro, A375puro, MeWopuro).



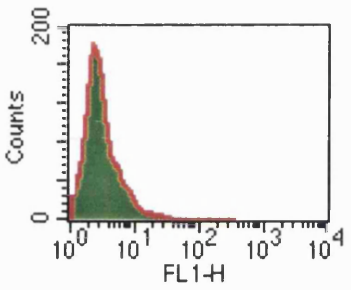
A375P / uPAR



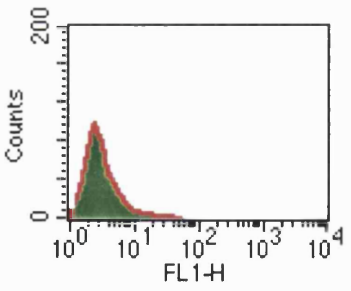
HMB2 / uPAR



DX3 / uPAR



Mel 8 / uPAR



MeWo / uPAR

Figure 7.1 uPAR expression in Melanoma Cell Lines

Cells were stained with anti-uPAR primary antibody, 399R at 10μg/ml, then with a FITC-conjugated secondary antibody. Expression of cell-surface uPAR was determined by FACS analysis. Figure shows a representative result.

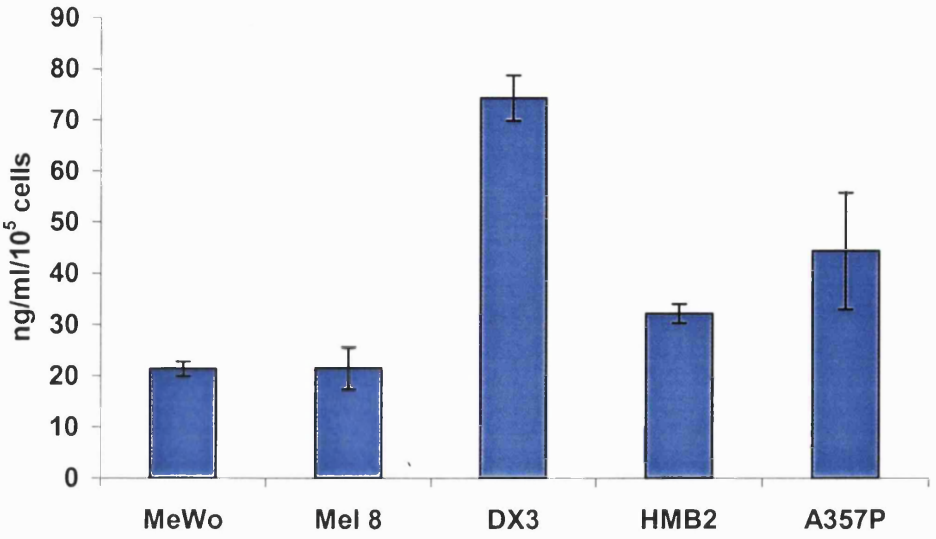


Figure 7.2 Secreted uPA Activity from Melanoma Cell Lines

Cells were seeded into 24-well plates in SFM and incubated for 20 hours. Cultured supernatants were collected and assayed for PA activity using a plasmin specific chromogenic substrate, S2251 and Human Glu-Plasminogen in excess. PA activity was determined by comparison with uPA standards. Figure represents the combined results of 2 experiments in triplicate.

7.3.2 Secreted Plasminogen Activator Activity is not affected in β6-Expressing Melanoma Cells

In order to see if the observed αvβ6-mediated effects on the plasminogen activation system were specific to epithelial cells or general cancer cell effect, the panel of newly created melanoma cell lines were used. Secreted PA activity in the cultured supernatants was not influenced by αvβ6, since there was no difference between the control and β6-expressing cells for each cell line (Figure 7.3). DX3 cells secrete 2-fold more uPA than A375P cells, with MeWo cells secreting very little uPA. This data shows that the observed αvβ6-mediated up-regulation of uPA in the epithelial cells is not repeatable in melanoma cells, suggesting that integrin effects on the plasminogen activation system are cell-type specific.

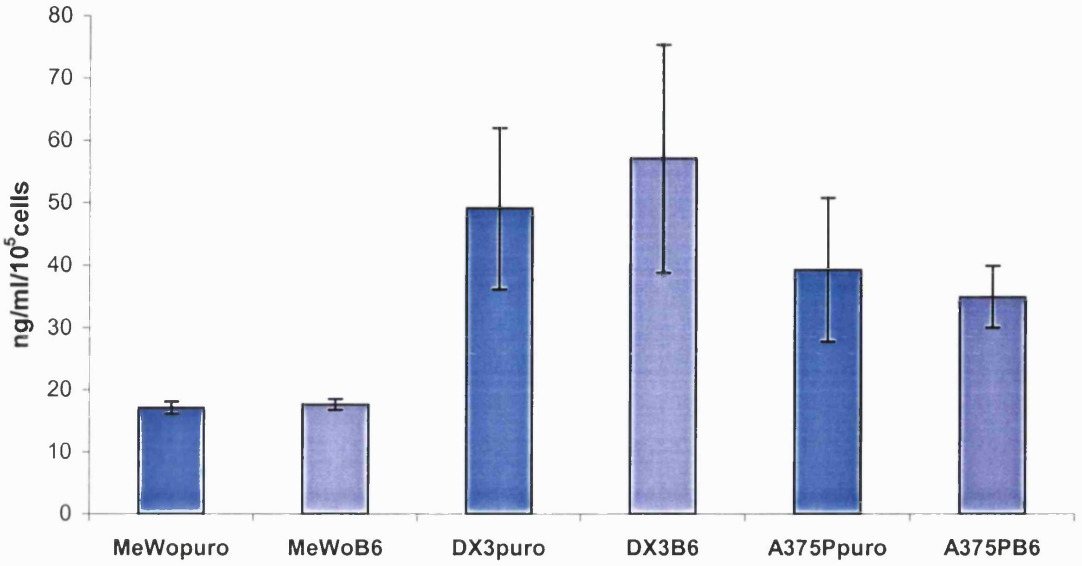


Figure 7.3 Secreted uPA Activity from Melanoma Cell Lines

Cells were seeded into 24-well plates in SFM and incubated for 20 hours. Cultured supernatants were collected and assayed for PA activity using a plasmin specific chromogenic substrate, S2251 and Human Glu-Plasminogen in excess. PA activity was determined by comparison with uPA standards. Figure represents the combined results of 2 experiments in triplicate.

7.3.3 αvβ6 and uPAR expression in β6-transfected cells.

β6-transfected (β6) and control (puro) cells of each of the three melanoma cell lines were co-stained for αvβ6 and uPAR and expression levels determined by FACS analysis (figure 7.4). The expression was quantified using the geometric means of the populations (figure 7.5). The controls of all three cell lines show no αvβ6 expression comparable to the negative control, however the β6-transfected cells show a marked increase in the levels of αvβ6, indicating that the transfections have worked efficiently with neo-expression of αvβ6 in the melanoma cells. The mean fluorescence intensity of αvβ6 on DX3β6, A375Pβ6 and MeWoβ6 is not that dissimilar for the levels of αvβ6 expressed by VB6. However, uPAR expression was not affected to any significant degree between β6-positive and β6-negative cells.

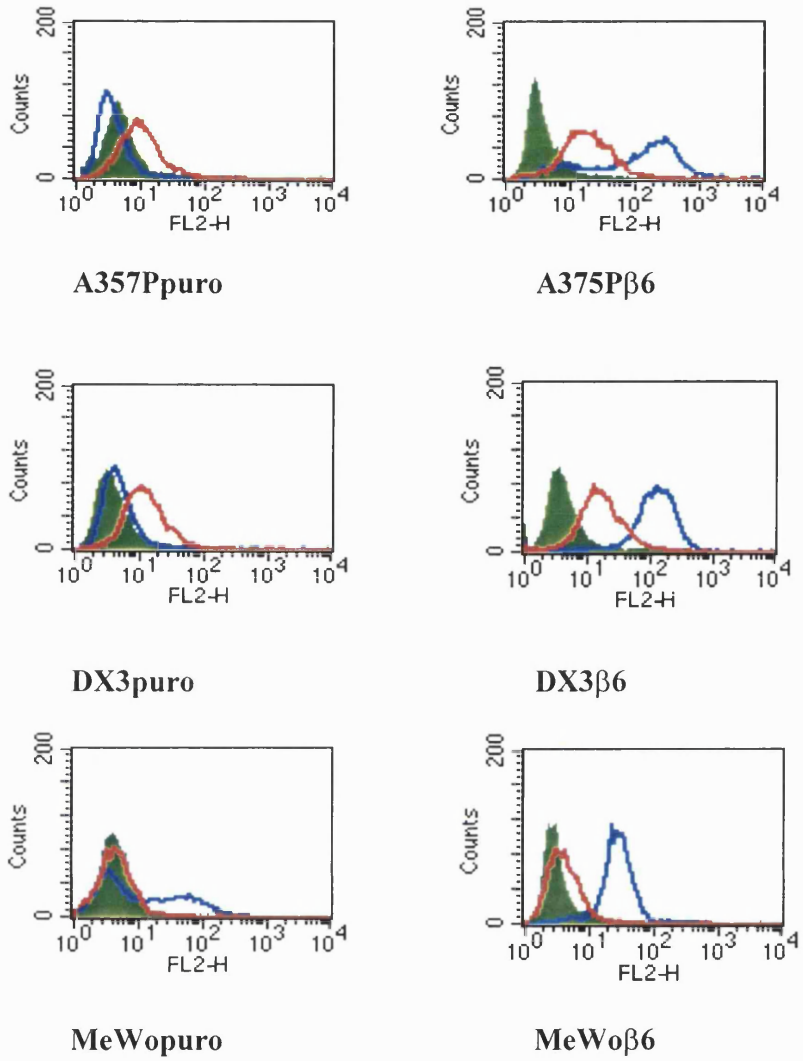
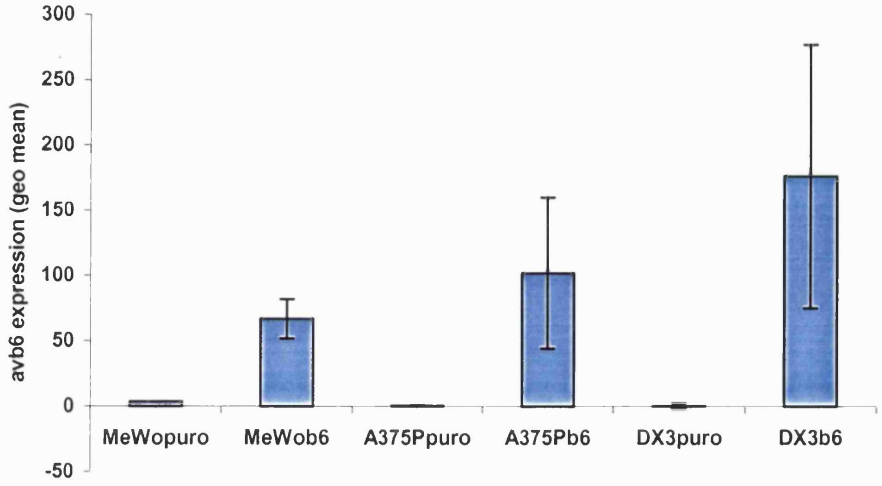


Figure 7.4 Double Stain FACS Analysis for αvβ6 and uPAR Expression in β6-Transfected Melanoma Cells

Cells were stained with the anti-αvβ6 mAb E7P6 at 10μg/ml, followed by an RPE-conjugated secondary antibody. Cells were washed and then incubated with the anti-uPAR primary antibody, 399R at 10μg/ml, then with a FITC-conjugated secondary antibody. Expression of cell-surface αvβ6 (blue) and uPAR (red) was determined by FACS analysis. Figure 7.4A shows a representative result of three independent experiments in duplicate.

A)



B)

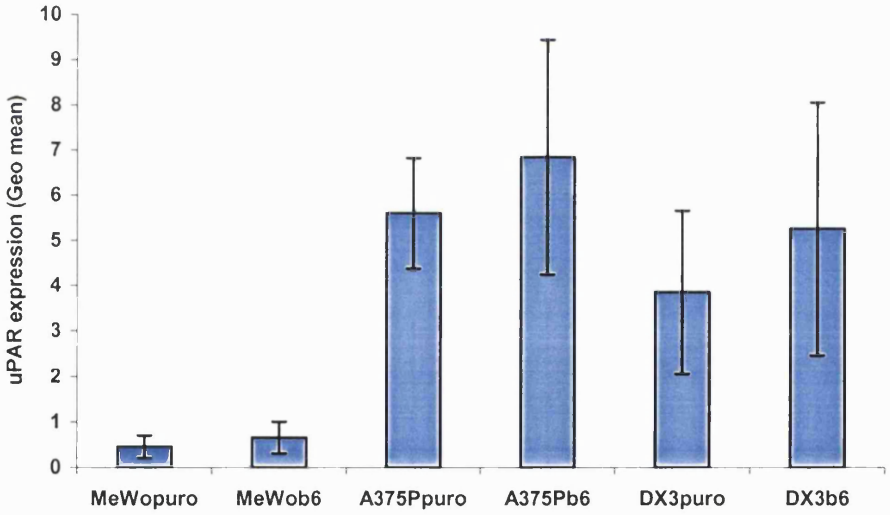


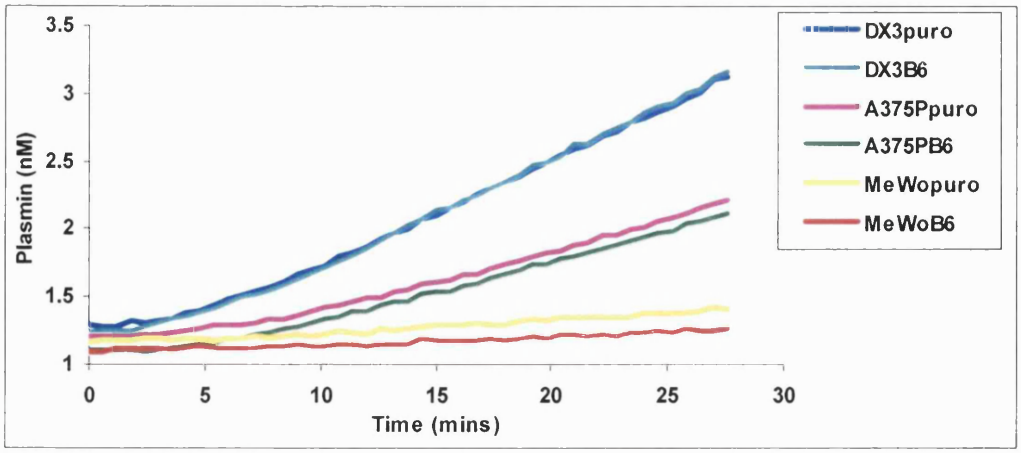
Figure 7.5 αvβ6 and uPAR Expression in the Transfected Melanoma Cell Lines

The flow cytometric data was quantified using the geometric mean values. Figures represent the combined mean ±SD of three independent experiments. (A) αvβ6 and (B) uPAR expression.

7.3.4 Cell Surface Plasminogen Activation is not affected in β6-Expressing Melanoma Cells

In order to confirm that the levels of uPAR were not affected by neo-expression of β6 integrin into the melanoma cells, cell surface plasminogen activation levels were determined, which is a measure of uPAR function. Figure 7.6A shows that in each cell line, the levels of cell surface plasmin generation was similar between the control and β6-expressing cells, confirming that uPAR levels were not affected by expression of the αvβ6 integrin. Cells were saturated with exogenous pro-uPA so that the levels of uPA secretion from the different cell lines was not a rate-limiting factor. Indeed when the assay was carried out with only the endogenous uPA for activity, there were no measurable levels of cell surface plasminogen activation (figure 7.6B). The levels of plasminogen activation between each cell line reflect the expression of surface uPAR, with DX3 cells showing the greatest levels of cell surface plasmin generation and expressing the highest levels of uPAR. Correspondingly MeWo cells show low levels of plasminogen activation and express low levels of surface uPAR.

A)



B)

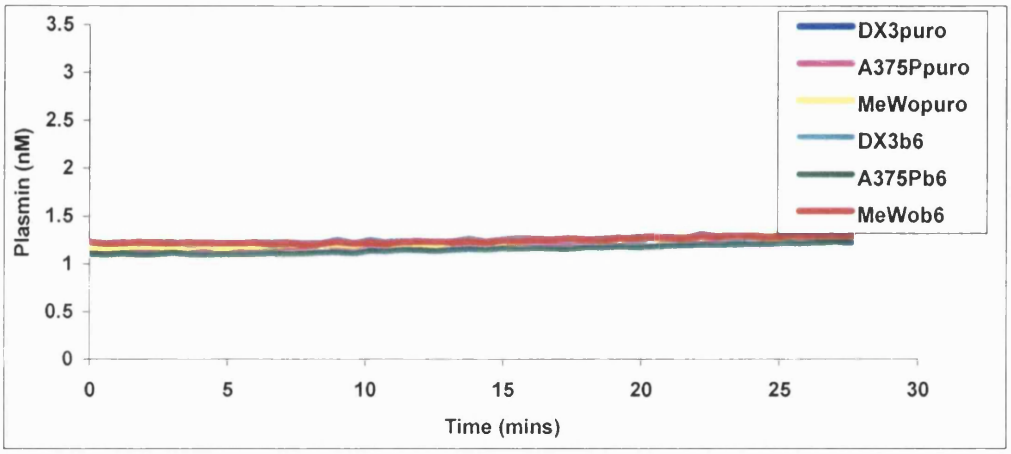


Figure 7.6 Cell Surface Plasmin Generation in Melanoma Cells

Cells were incubated with a fluorogenic plasmin substrate HDVLK-AMC and human Lys-plasminogen both in excess. Plasminogen activation was measured kinetically at 37°C for 30 mins and the plasmin generated was determined by comparison with a plasmin standard curve.

A) αvβ6- and non-αvβ6-expressing cells of each cell line produced the same levels of cell surface plasminogen activation when saturated with exogenous pro-uPA.

B) Plasmin generation in the presence of endogenous uPA secreted by the cells was too low to be detectable in this assay.

7.3.5 αvβ6 is Functional in the Melanoma Cells

This study has shown that de novo expression of αvβ6 into melanoma cells has no effect on the expression or function of components of the urokinase system. Since αvβ6 is not normally expressed in melanoma cells, it may be the case that these cells do not have the capability of exerting αvβ6 function. Therefore, the functionality of αvβ6 was investigated using migration assays. In the epithelial cells, de novo expression of αvβ6 promoted cell migration towards fibronectin; therefore melanoma cell migration towards fibronectin was also determined (figure 7.7). For each of the cell lines, the β6-expressing cells showed increased migration towards plasma fibronectin compared to their control counterparts. This β6-dependent increase in migration toward fibronectin shows that *de novo* expression of αvβ6 on the melanoma cells was functional.

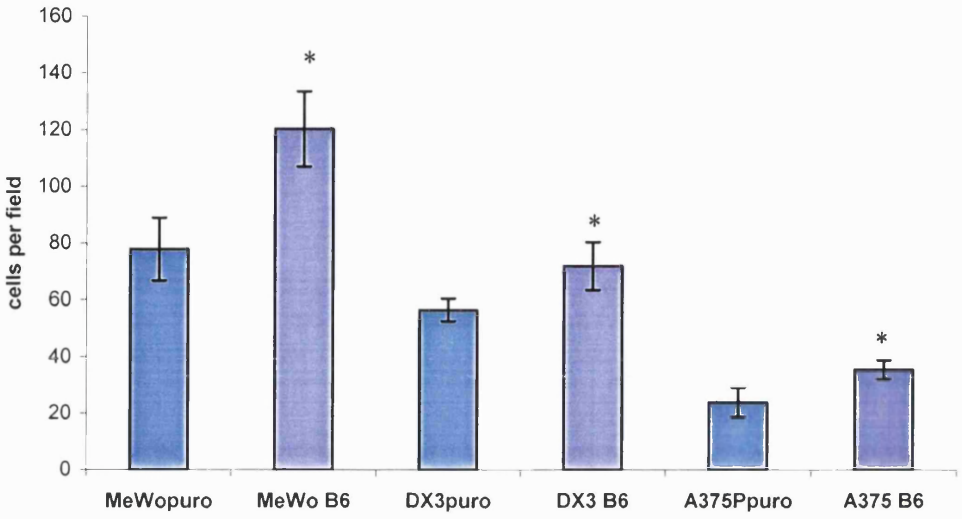


Figure 7.7 Melanoma Cell Migration Towards Fibronectin

In all of the three cell lines, the αvβ6-expressing cells showed a 20-25% increased migration towards fibronectin. Cell migration assays were performed using plasma fibronectin-coated polycarbonate filters. Cells migrated to the underside of the filter after 3 hours were counted using multiple random fields (X 200 magnification). Figure represents the mean ±SD of two experiments in triplicate. β6 expression in all of the celllines significantly increased migration towards fibronectin ($p<0.05$, Mann-Whitney-U-Test).

7.4 DISCUSSION

Integrin expression is altered in malignant cells as compared to their normal counterparts and this appears to be involved in several aspects of tumour growth, invasion and metastasis (Giancotti and Mainiero, 1994 and Juliano and Varner, 1993). However, no specific integrin has been implicated in transformation to the malignant phenotype and changes in integrin expression vary according to the type of tumour (Thomas et al, 1997). Carcinomas exhibit variable loss of expression of α6β4 (a component of hemidesmosomes), α3β1, α2β1 and α5β1 (Pignatelli and Bodmer, 1990, Koretz et al, 1991). However, the laminin receptor α6β1 is overexpressed in highly invasive cells (Dedhar and Saulnier, 1990). Malignant melanomas express the αvβ3 integrin as they enter the vertical growth phase (Albeda et al, 1990) and metastasis of transplantable rhabdomyosarcoma increases with the expression of α2β1 integrin (Chan et al, 1991). The alterations in integrin expression may reflect differing roles on tumour behaviour. There is an inverse correlation between the level of α5β1 fibronectin receptor and tumourigenicity, since expression of this receptor plays a role in inhibiting cell proliferation. Conversely, αvβ6, which also recognises fibronectin as a ligand is neo-expressed epithelial carcinomas such as oral squamous cell carcinoma and colon carcinoma, whereas it is not expressed in normal epithelium (Jones et al, 1997, Breuss et al, 1995). It also appears to enhance cell proliferation and *in vivo* tumour growth in colonic adenocarcinoma cells (Agrez et al, 1994).

In order to establish whether the effects of αvβ6 on the urokinase system are epithelial-cell specific, melanoma cells were transfected with β6 cDNA, which resulted in the

surface expression of the αvβ6 heterodimer. Migration assays confirmed that the αvβ6 integrin was functional, since β6-positive cells showed increased migration towards fibronectin compared to the β6-negative cells. However, there was only a 25% increase in migration in melanoma cells compared to a 65% increase in the β6-expressing keratinocytes, VB6 cells compared to the control, C1 cells (see chapter 6). It is not clear as to why neo-expression of αvβ6 into the epithelial cells has a greater pro-migratory effect compared to that of melanoma cells but may be because this integrin is not naturally expressed in melanoma cells, therefore not substantially affecting migration towards fibronectin. It should be noted that the melanoma cells express other fibronectin-binding integrins, such as β1, however, the roles of these in cell migration needs to be established. *De novo* expression of αvβ6 into the melanoma cells, however did not affect the expression or function of components of the urokinase system. Therefore the observed αvβ6-mediated up-regulation of uPA secretion and down-regulation of uPAR expression in this study is epithelial-cell specific and it may be that in melanoma cells, other factors, such as other integrins, membrane proteins or signalling molecules are more important in regulating the urokinase system.

The αvβ3 integrin in particular is important in melanoma. Increased expression of this receptor in association with the acquisition of a migratory/invasive phenotype has been demonstrated in melanoma (Marshall et al, 1991). It has also been identified as a marker of malignant melanoma progression and been implicated in the regulation of anchorage-independent growth and tumourigenicity (Felding-Haberman et al, 1992). αvβ3 integrin has been shown to regulate the urokinase system in melanoma cells. Suppression of

αvβ3 synthesis by antisense oligodeoxynucleotides in murine mammary tumour cells resulted in a parallel reduction in uPAR mRNA transcript level (Nip et al, 1995). In the same cells receptor ligation by immobilised antibodies induced a rapid increase in uPAR mRNA levels as well as PAI-1 mRNA but not in uPA levels. In addition, ligation of αvβ3 resulted in a significant increase in cell surface-associated plasmin levels, which coincided with a 2- to 3-fold increase in cell invasion (Khatib et al, 2001). The essential role of αvβ3 integrin in the growth of human malignant melanoma has also been shown *in vivo* (Mitjans et al, 2000) and more recently when inhibition of tumour cell αvβ3 function reduced melanoma metastasis significantly and prolonged animal survival (Felding-Habermann et al, 2002). The αvβ3 integrin expressed by malignant melanoma has been shown to modulate expression of proteolytic enzymes by the tumour cells. Stimulating antibodies to αvβ3 in a melanoma cell line caused increased expression of MMP-2 with an enhanced ability to invade the basement membrane (Seftor et al, 1993). Furthermore, this integrin has been shown to co-localise with MMP-2 on the surface of the tumour cell (Brooks et al, 1996). It is also interesting that melanoma cells expressing αvβ3 are protected from apoptosis *in vitro* (Montgomery et al, 1994).

Changes in integrin expression have been shown to be important for the growth and metastatic capacity of melanoma cells. In a study examining the expression of αv integrins in a panel of melanoma cell lines, all of them expressed αvβ5 and αvβ3 but none expressed αvβ6. Analysis of clinical material from cutaneous melanoma showed that although αv expression was increased in 88% of metastases, this could not all be explained by up-regulation of αvβ3 (Marshall et al, 1998). Therefore, although αvβ3

seems to be the most important integrin in melanoma progression, differences in the expression of other integrins, such as α1β1, α2β1 and α6β1 in uveal melanoma have been observed (Baker et al, 2001). Elevated levels of β1 integrins were found in the highly invasive and metastatic melanoma cell lines compared with normal melanocytes and non-metastatic melanoma cell lines, with a corresponding increased adhesion to laminin and collagen in the highly metastatic cell line (van Muijen et al, 1995). Increased expression of α4β1 on melanoma cells *in vivo* may also have a metastasis promoting role (Elices et al, 1990) and it is strongly suggested that α4β1-VCAM1 interactions are important in extravasation of circulating melanoma cells (Okahara et al, 1994). Increased amounts of α3β1 and αvβ3 appear to correlate with progression in melanoma cell lines. However, the αvβ1 heterodimer was observed in one melanoma cell line, suggesting the possibility that heterogeneity of integrin composition could affect the biological behaviour of these tumours (Hart et al, 1991).

Integrins not only act as cell adhesion molecules but also as signalling molecules to control many aspects of cellular behaviour. There are 23 different integrin heterodimers, the expression of which is dependent on the cell type. There may be some redundancy with regard to integrin function, where a particular cell function is mediated by the integrin expressed on that cell and is not specific to the heterodimeric combination. For example, invasion and metastasis is promoted by the αvβ6 integrin in epithelial carcinomas but by the αvβ3 integrin in melanoma.

This study shows that in oral squamous cell carcinoma, αvβ6 modulates components of the urokinase system, through the unique 11 amino acid cytoplasmic tail of the β6 subunit. Increased migration towards plasma fibronectin was shown to be dependent on αvβ6, although not through the unique cytoplasmic tail. *De novo* expression of full-length αvβ6 into melanoma cells also increased migration towards plasma fibronectin, however, there was no effect on the expression of uPA or uPAR. Therefore, suggesting that the role of the β6 cytoplasmic tail in mediating the urokinase system is specific to epithelial cells. This data suggest that there are common signalling pathways between integrins, since forced expression of αvβ6 into melanoma cells is functional even though it is not normally expressed. The αvβ6 integrin, whether it is expressed in melanoma cells or epithelial cells may interact with certain signalling molecules such as those of the MAP Kinase pathway to cause an increase in migration. Previous literature has demonstrated correlations between integrin expression and cellular functions, however this data is novel in demonstrating the cellular specificity of integrin function, since forced expression of a totally foreign integrin into melanoma cells was functional in promoting migration but did not affect expression of components of the urokinase system. Therefore, αvβ6 modulation of the urokinase system is specific to epithelial derived carcinomas.

CHAPTER 8

INDUCIBLE EXPRESSION OF αVβ6 IN ORAL SCC CARCINOMA CELLS

8.1 INTRODUCTION

Precise control of gene expression is an invaluable tool in studying many physiological processes, including cancer. A system of inducible gene expression is capable of controlling translation of a particular gene *in vitro* or *in vivo* by the addition of external factors. Being able to precisely regulate expression of certain genes by exogenous inducers is a major advance in medical research and may prove to be of therapeutic use.

The aim of the experiments described in this chapter were to investigate the expression of the β6 integrin subunit in epithelial carcinomas. Oral SCC keratinocytes expressing the αvβ6 integrin show increased uPA secretion and decreased uPAR expression associated with decreased cell surface plasminogen activation (chapters 3 and 4). The development of a system where β6 gene expression could be specifically induced would provide a means of confirming these observations on the urokinase system. In addition further effects of the αvβ6 integrin in these carcinoma cells could be directly investigated, thus making the inducible system would provide a highly useful research model.

In cultured cells, glucocorticoids and other steroids are commonly used to induce expression of a desired gene. In the past, a tetracycline-regulated system in which gene activity is induced in the absence of antibiotic and is repressed in its presence has been used as a useful research tool (Blau and Rossi, 1999). It has been used in

transgenic mice (Furth et al, 1994) and the system has been improved by the identification of a mutant tetracycline repressor, which acts conversely as an inducible activator (Rossi and Blau, 1998). However, the pharmacokinetics of tetracycline with its slow clearance from cells hinders its use in studying developmental processes when a precise and efficient on-off switch is essential (Gossen et al, 1995). Tetracycline is also known to affect the activity of MMPs (Sadowski and Steinmeyer, 2001); therefore it would not have been suitable in this study where proteinase expression was specifically to be investigated.

In this study, inducible β6 gene expression was attempted using the complete control inducible mammalian expression system (Stratagene). This is a gene transfer system that allows precise control of gene expression in a wide variety of mammalian cell types. It is based on the finding that the insect hormone ecdysone or its analogue ponasterone A (Pon A) can activate transcription in mammalian cells harbouring both the gene for Drosophila melanogaster ecdysone receptor and a promoter containing a binding site for the ecdysone receptor (No et al, 1996). The complete control system has several advantages over other inducible systems. Pon A has no known measurable effect on mammalian physiology. It has a short *in vivo* half-life and its lipophilic nature allows it to efficiently penetrate all tissues, including the brain. This results in a rapid and potent induction of gene expression and rapid clearance. A 1000-fold induction of a reporter gene, with negligible basal expression has been obtained with the complete control system (Wyborski et al, 2001).

8.1.1 System Design

The complete control system is based on the responsiveness of insects to steroid hormones. During metamorphosis of Drosophila melanogaster, a cascade of morphological changes is triggered by the steroid hormone 20-OH ecdysone via the ecdysone receptor (EcR). In insect cells, EcR and the nuclear receptor ultraspiracle (USP) form a promoter-bound heterodimer, which regulates transcription. In the absence of ecdysone, the receptor heterodimer binds to corepressors and tightly represses transcription. When ecdysone binds to the ligand binding domain (LBD) of the EcR, the corepressors are released, coactivators are recruited to the complex, and transcriptional activation is enabled (Chen et al, 1996).

This system was adapted for use in mammalian cells (Stratagene, UK). EcR heterodimerizes with retinoid-X-receptor (RXR), the mammalian homologue of USP, in cells harbouring the EcR gene. The EcR-RXR heterodimer binds to multiple copies of the ecdysone-responsive element (EcRE), and in the absence of ponA, represses transcription of an expression cassette. When ponA binds to the receptor, the receptor complex activates transcription of the gene of interest (figure 8.1). In the development of this system, the EcRE sequence was modified to create a synthetic recognition site that does not bind any endogenous transcription factors and the EcR protein was modified to create a synthetic ecdysone-binding receptor, VgEcR that does not transactivate host genes and exhibits no pleiotropic interactions with endogenous pathways in mammalian host cells. VgEcR is a fusion of the ligand-binding and dimerization domain of the D. melanogaster ecdysone receptor (EcR),

the DNA binding domain of the glucocorticoid receptor (GR), and the transcription activation domain of herpes simplex virus (HSV) VP16. VgEcR and RXR bind as a heterodimer to five copies of the E/GRE recognition sequence (E/GREx5), which are located upstream of a minimal promoter composed of three SP1 binding sites (SP1x3) and the ΔHsp minimal promoter. The E/GRE recognition sequence consists of inverted half-site recognition elements for the RXR and the GR DNA-binding domains (which are separated by one nucleotide). In the absence of ponA (the inducer), the promoter is tightly repressed by corepressors. When ponA binds to VgEcR, the corepressors are released, coactivators are recruited and the complex becomes transcriptionally active.

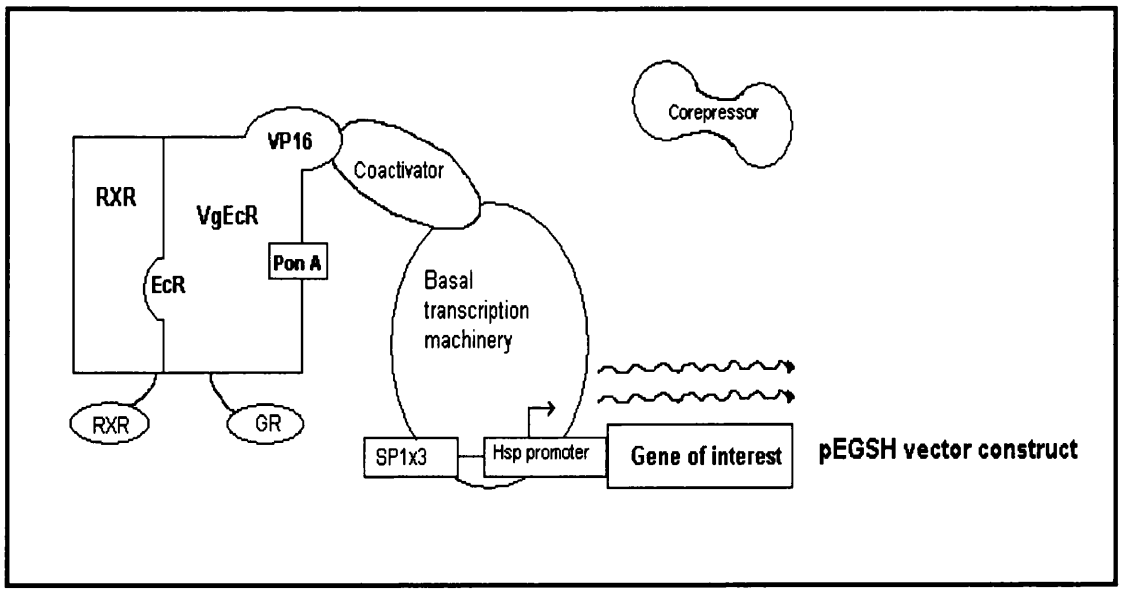


Figure 8.1 Regulation of Transcription by the Complete Control System

8.1.2 Vectors

pERV3 receptor vector

This contains an expression cassette from which the genes for VgEcR and RXR are constitutively expressed from a message transcribed from the CMV promoter. The vector also contains a neomycin-resistance gene so that mammalian cell transfectants can be selected with the antibiotic G418 (Appendix 8).

pEGSH vector

This contains a ponA-inducible expression cassette that includes five copies of the ecdysone-responsive element. It also contains the multiple cloning site (MCS) for inserting the gene of interest (Appendix 9).

The ecdysone regulatory system was chosen to develop an inducible β6 gene expression system since it exhibits lower basal activity and higher inducibility than other methods. Also, in contrast to tetracycline-based systems, the pharmocokinetics of ecdysone allow both fast distribution and clearance of the inducer dosage-specific manner (No et al, 1996).

8.2 METHODS

8.2.1 Overview of the Complete Control Inducible Expression System

The β6 gene was isolated from pcDNA3.1 using EcoRI and the pEGSH vector was digested with Mun1 (ABgene Ltd., Surrey, UK) to produce compatible restriction sites for ligation of the vector to the insert. This pEGSH construct was then transformed into competent bacterial cells for amplification and purified. Co-transfection of this construct with the pERV3 vector into oral SCC cells was attempted so that Hygromycin and G418-resistant stable clones could be generated. Induction of β6 gene expression was attempted by treatment with Ponasterone A.

8.2.2 Creation of the Plasmid Construct

8.2.2.1 Isolating the β6 gene.

The full-length β6 gene was excised from the pcDNA3.1 vector (from Dr Melanie Keppler, St Thomas' Hospital, London, Appendix 10) using the restriction enzyme EcoRI (ABgene Ltd.,UK) (figure 8.2). This produced two fragments, the 3.9kb vector and full-length β6 of 2.3kb. Full-length β6 DNA was recovered from the gel by electroelution (see sections 2.13.1 and 2.13.2).

8.2.2.2 Digestion of the pEGSH vector

The pEGSH vector was digested with the restriction enzyme Mun1 (Abgene Ltd.) and the linearised vector produced a band slightly heavier than the undigested pEGSH vector of 4.8kb (figure 8.3). The digested vector was then recovered from the gel by electroelution (see sections 2.13.1 and 2.13.2).

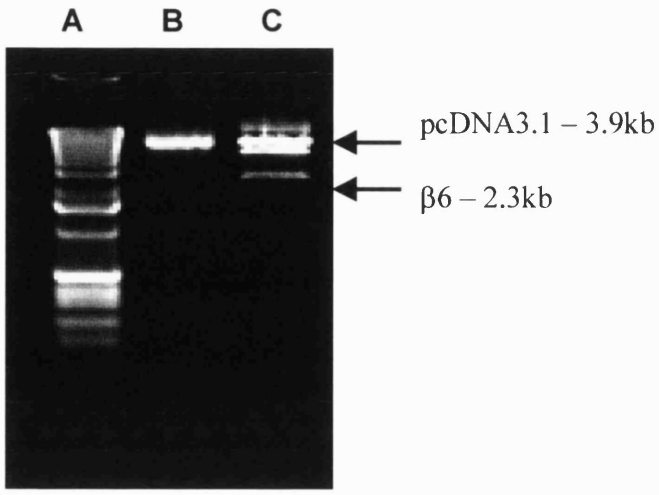


Figure 8.2 Isolation of β6 gene

The restriction digest was separated by electrophoresis on a 1% agarose gel.

A = 1kb DNA ladder, B = pcDNA3.1/β6, C = pcDNA3.1/β6 digested with EcoR1

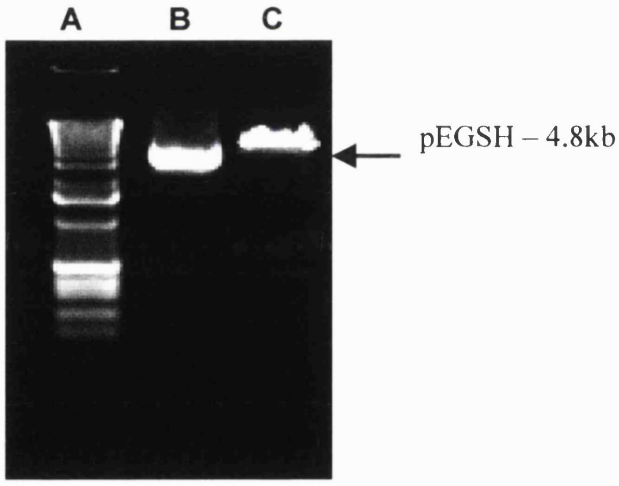


Figure 8.3 Digestion of the pEGSH vector

The restriction digest was separated by electrophoresis on a 1% agarose gel.

A = 1kb DNA ladder, B = pEGSH vector, C = pEGSH vector digested with Mun1

8.2.2.3 Ligation of Vector to Insert

For ligation, the ideal insert-to-vector ratio was determined experimentally. The amount of vector and insert DNA used was calculated as follows:

$$\frac{\text{size of insert (bp) x amount of vector (ng)}}{\text{size of vector (bp)}} \text{ X (ratio desired)} = \text{amount of insert (ng)}$$

The concentration of the DNA was estimated by comparison of the band intensities of the DNA digests to the bands of a 1kb DNA Hyperladder (Bioline, UK; figure 8.4). Therefore, the DNA concentrations were: β6 = 60ng/4µl, pEGSH = 100ng/0.4µl. These concentrations were then used to calculate the volume required for ligation.

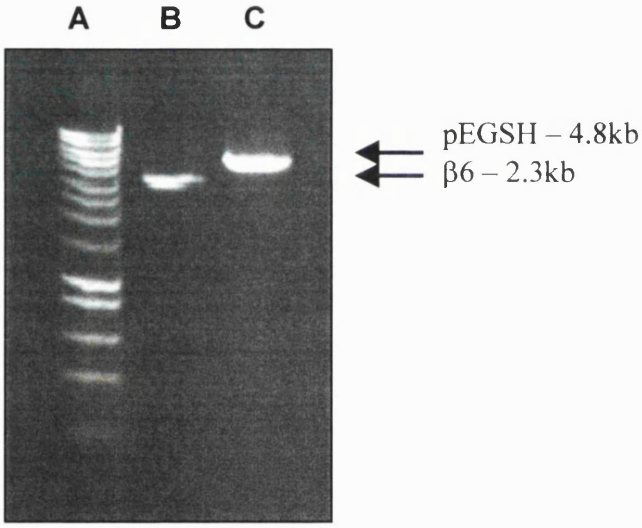


Figure 8.4 Determining DNA Concentrations

The DNA was electropheresed on a 1% agarose gel.

A = 1kb DNA Hyperladder, B = β6 gene, C = pEGSH (linearised vector)

Ligation was attempted using three insert:vector ratios of 1:1, 3:1 and 6:1, the latter proving to be most successful. For a 6:1 ratio the ligation mix consisted of 115ng β6 DNA and 40ng pEGSH. A control ligation of only linearised pEGSH DNA was also carried out.

8.2.2.4 Transformation of Competent E.Coli

The ligations were then transformed into competent bacterial cells (see section 2.13.6) and then selected on tet/amp agar plates. An efficient ligation with no re-circularisation of the vector was confirmed since the control plate had very few colonies (<10 colonies/plate) and the β6/pEGSH ligation produced 10 times more colonies.

8.2.2.5 Screening for the correct pEGSH/β6 construct

Since only one restriction enzyme site was used, β6 may have inserted into the vector in the wrong orientation resulting in three possible plasmids, an empty re-circularised pEGSH vector, pEGSH with β6 in the wrong orientation, or the correct construct of pEGSH with β6 in the correct orientation. Therefore, colonies were screened for plasmids containing the β6 gene in the correct orientation, using two methods.

(i) Restriction digests of miniprep DNA

Bacterial colonies were grown up, the DNA extracted and then digested with the restriction enzyme BamH1 (ABgene Ltd.) that cuts at only one site in the β6 gene in the sequence coding for the cytoplasmic tail, at a site 100bp upstream from the 3'

end. BamH1 also cuts within the pEGSH vector, at one site in the MCS downstream to Mun1 site (see Appendix 8). An empty pEGSH vector was cut at just one site, producing a linearised plasmid of 4.8kb, however when β6 had inserted into this, BamH1 cut at two sites. When β6 was in the correct orientation, digestion produced a band at 7kb and a very small band of 100bp (which is undetectable on the gel). When β6 was inserted in the incorrect orientation, digestion produced 2 bands at 4.9kb and 2.2kb.

(ii) PCR Screening of Plasmid Constructs

This method was highly efficient since many colonies were screened at once. Two sets of primers were designed using Primer 3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/25k).

1. **pEGSH/β6** - the 5' primer was targeted to 100bp from the end of the pEGSH sequence and the 3' primer to 300bp in from the start of the β6 gene to produce a product size of 400bp. This enabled detection of the correct plasmid construct, by encompassing both pEGSH and the β6 gene in the correct orientation.

   5'- TCTACGGAGCGACAATTCAA

   3'- CACAGCCACCTTGTACGTGA

2. **β6 internal primers** - this primer set was targeted to internal sites within the β6 gene to produce a product size of about 400bp.

   5'- ATGGGGATTGAACTGCTTTG

   3'- AAATCCACCGGGTAGTCCTC

Colonies were picked and diluted into 150μl of LB-media containing tet/amp in a 96-well plate and allowed to shake at 37°C. 1μl of this was then used to set up a 20μl PCR reaction mix (see section 2.14). As the positive control, 1μl of the ligation mix was used. A 35-cycle PCR reaction using an annealing temperature of 62°C was then carried out. The PCR products were electrophoresed on a 2% agarose gel (figure 8.5). A band at 400bp by the positive controls indicated that the PCR reaction had worked, and any other bands of this same size suggested a positive result and the correct construct.

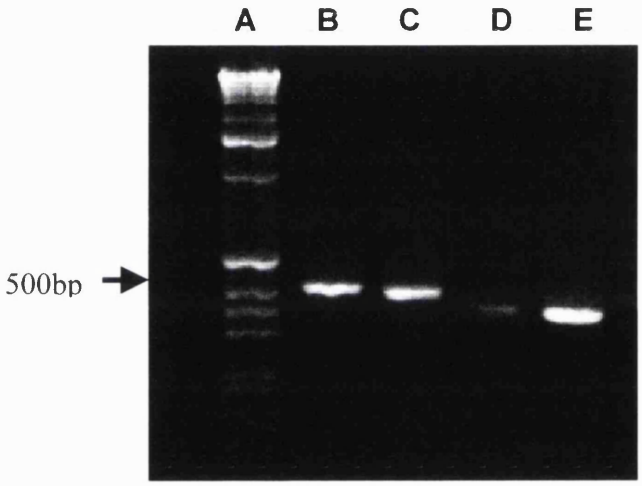


Figure 8.5 PCR for β6/pEGSH Construct

Bacterial colonies were screened for expression of the correct construct by PCR. The PCR products were electrophoresed and visualised on a 2% agarose gel.

A = 1kb DNA ladder

B = positive control – PCR of ligation using β6 internal primers, 400bp

C = PCR of β6/pEGSH construct using β6 internal primers, 400bp

D = positive control – PCR of ligation using pEGSH/β6 primers, 350bp

E = PCR of β6/pEGSH construct using pEGSH/β6 primers, 350bp

The bacterial colonies that produced a positive result were grown up in LB-medium and the plasmid DNA extracted (see section 2.12). To further confirm the correct construct the DNA was digested with BamH1; a band at 7kb indicated that β6 had inserted into the pEGSH vector in the correct orientation (figure 8.6, lane C). The construct was then sequenced using the pEGSH/β6 primer set (Lark Technologies inc., Essex, UK) to confirm the expression of the β6 gene in the pEGSH vector.

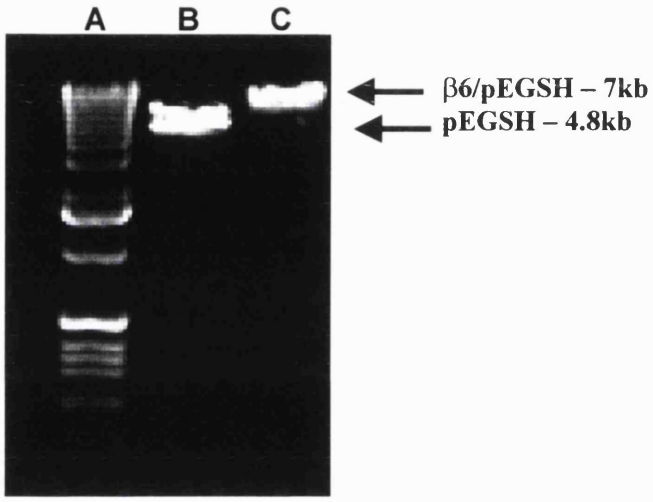


Figure 8.6 Restriction Digest Check for Correct β6/pEGSH Construct

Miniprep DNA of the pEGSH control and the β6/pEGSH construct were digested with BamH1 and electrpohoresed on a 0.8% agarose gel. A = 1kb DNA ladder, B = pEGSH control digested with BamH1, C = β6/pEGSH digested with BamH1

8.2.2.6 Amplification and Purification of Plasmid DNA

Large quantities of purified stocks of the **pEGSH/β6** plasmid construct and the **pEGSH control** plasmid were prepared (see section 2.12.4). **pERV3** vector DNA

(Stratagene) was transformed into E.Coli, a bacterial colony was grown up and plasmid DNA extracted using a maxiprep as previously described. The DNA concentrations were measured using a UV spectrophotometer at wavelengths of 260/280nm:

8.2.3 Creation of a Stable Cell Line

8.2.3.1 Choice of Cell Line

The cell line chosen for expression of the gene of interest was derived from an oral SCC carcinoma, which had not previously been genetically manipulated. A cell line with the characteristics of robust and stable growth was optimally desired however; the most important phenotype was that of the pre-existing integrin expression. A number of oral SCC cell lines (Prime et al, 1990) were screened for αv and αvβ6 integrin expression. The H376 cell line was used since it has very high αv and negligible αvβ6 integrin expression (figure 8.7), which is ideal for the development of an inducible β6 cell line.

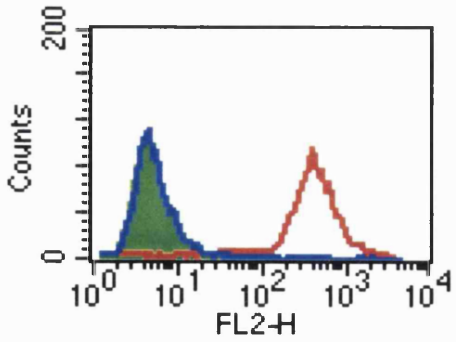


Figure 8.7 Integrin Expression in the H376 Cell Line

Cells were stained for αv using the mAb L230 at 10μg/ml (red) and for αvβ6 using the mAb E7P6 at 10μg/ml (blue), followed by an RPE-conjugated secondary antibody. Fluorescently labelled integrin expression was determined by flow cytometry (see chapter 2).

8.2.3.2 Determining Antibiotic Concentration for Cell Selection

The presence of specific antibiotic resistance genes within the plasmids enables selection of transfected cells. The pERV3 vector was selected with the neomycin analogue G418 (Sigma Chemical Co.) and the pEGSH vector with Hygromycin (Gibco BRL). Antibiotic sensitivity the cells was determined by treating with a range of antibiotic concentrations so that the the minimal lethal concentration (the lowest that killed all of the cells within 10-14 days) could be determined.

H376 cells were seeded at 1 X 10^5 cells per well in 2ml KGM into 6-well plates. After overnight incubation, the cells were replaced with antibiotic selective media: G418 at final concentrations of 200, 400, 500, 700, 900μg/ml, 1 and 1.5mg/ml and

Hygromycin at final concentrations of 50, 100, 200, 500 700μg/ml and 1mg/ml. Cells were grown for 14 days, changing the antibiotic selective media every other day. This was to account for degradation of the antibiotics, so the desired concentration of antibiotic in each well was maintained throughout the 14 days. The lowest concentrations that killed all the cells after 14 days incubation were 1mg/ml G418 and 100μg/ml Hygromycin.

8.2.3.3 Transfection Using Effectene Reagent

Effectene Reagent (Qiagen) was the method of choice for transfection of the oral keratinocytes, since it has shown high transfection efficiencies in a range of mammalian cell lines and exerts minimal toxicity to cells so does not require removal of the transfection complex. H376 cells at 50% confluency in 24-well plates were co-transfected with β6/pEGSH and pERV3. As a control, cells were transfected with the pEGSH control plasmid and pERV3. A further control was also employed where cells were treated with Effectene Reagent but no DNA. Each well was transfected with 0.2μg total DNA (0.1μg of each vector), according to the manufacturer's instructions using an Effectene:DNA ratio of 25:1. During Effectene:DNA complex formation, the cells were washed in PBS and incubated with 350μl KGM. 350μl KGM was also added to the transfection complex mix and added dropwise onto the cells. The cells were incubated with the transfection complexes for 48 hours.

8.3 RESULTS

8.3.1 Transient Transfections

Transient transfections were carried out to determine whether the expression construct could be induced in the cell line. 48-hours post-transfection, cells were treated with 10μM ponasterone A for 20 hours in order to induce β6 expression. The supernatants were collected and the cells lysed and analysed for β6 expression by Western blotting (section 2.7, figure 8.8). However, β6 expression could not be detected in the transfected cells.

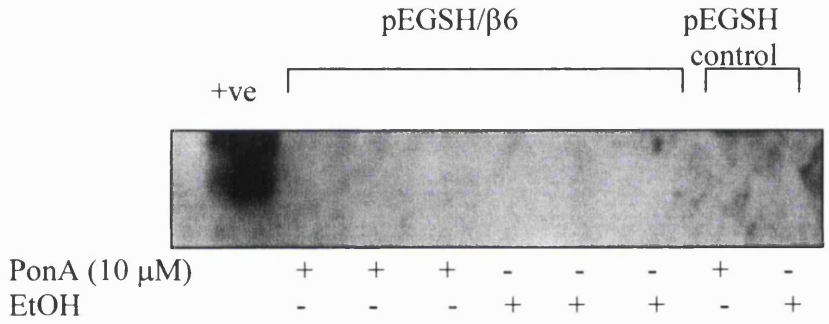


Figure 8.8 αvβ6 Expression in Transiently Transfected Cells

Cells were transiently co-transfected with either the β6/pEGSH construct or the pEGSH control vector along with the pERV3 vector. After 48 hours cells were treated with 10μM Ponasterone A, or ethanol as a control for 20 hours, then harvested for Western blot analysis. 10μg of protein was separated by SDS-PAGE and transferred to nitrocellulose membrane, which was probed with the anti-αvβ6 Ab, sc6632 at 2μg/ml. The positive control is β6-expressing NIH3T3 cell extracts.

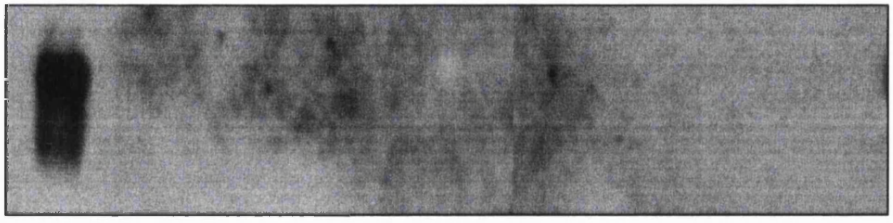
8.3.2 Stable Transfections

In order to establish a stable cell line expressing both plasmids, after 48 hours the cells were selected out by cloning in antibiotic selective medium (KGM with 1mg/ml G418 and 100μg/ml Hygromycin). The transfected cells from a single well were trypsinised and re-suspended to 43.5mls of antibiotic selective medium. This was diluted into 96-well plates, so that 150μl was added to each well of three 96-well plates. This method enabled selection of true clones containing both vectors from a single cell. Cells were grown for 7 days, after which the medium was removed, dislodging any loose cells and replaced with fresh antibiotic selective medium. This was repeated at Day 14 and at Day 21 the cells from a single well were transferred to a well of a 24-well plate, with 1ml antibiotic selective medium and allowed to reach confluence under selection.

8.3.3 Inducing Gene Expression of Stable Clones with Ponasterone A

The cells were then re-seeded into 4 wells of a 24-well plate. Three of the wells were used for analysis of β6 expression and the other well of that particular clone was kept growing. 10 clones of β6/pEGSH and two pEGSH control clones were expanded and tested for β6 expression. β6 gene expression was induced by treating the cells with ponasterone A at final concentrations of 5, 10 or 15μM and then incubated at 37°C for 20 or 24 hours. Cells were both untreated and treated with ethanol as controls. Following induction, cells were washed once with PBS and then harvested using 100μl integrin lysis buffer (Appendix 4). A quantitative DC protein assay (Biorad, UK) was used to determine total protein levels in the samples and 10μg of each

sample was analysed for β6 expression by Western blotting (section 2.7, figure 8.9). However, β6 expression could not be induced in any of the clones with either of the ponA concentrations.



Clone	1	2	3	4	5	6	7	8	9	10	9	10	1
PonA (μM)	5	5	10	10	10	10	15	15	15	15	-	-	15
EtOH (μM equivalent)	-	-	-	-	-	-	-	-	-	-	15	15	-

Figure 8.9 αvβ6 Expression in Stably Transfected Cells

Stable cell clones of cells co-transfected with either the β6/pEGSH construct or the pEGSH control vector along with the pERV3 vector were treated with 5, 10 or 15μM ponasterone A, or with ethanol as a control for 20 hours, then harvested for Western blot analysis. 10μg of protein was separated by SDS-PAGE and transferred to nitrocellulose membrane, which was probed with the anti-αvβ6 Ab, sc6632 at 2μg/ml.

8.4 DISCUSSION

DNA vector-based systems that allow precise control of gene expression *in vivo* are invaluable for studying gene function in a variety of organisms, particularly when studying developmental and other biological processes for which the temporal profile, as well as the level of gene expression is critical to gene function. Such systems are successfully used to overexpress toxic or disease-causing genes, induce gene targeting, and express antisense RNA. Pharmaceutical companies currently use inducible systems to facilitate screening for inhibitors of clinically relevant biological pathways and to explore potential applications for gene therapy.

In this study the ecdysone inducible gene expression system was used in an attempt to develop a stable cell line in which αvβ6 integrin expression could be precisely regulated and used to accurately study the effects of αvβ6 on the urokinase system in a malignant cell line. However, cloning of full-length β6 cDNA into the pEGSH vector posed a number of problems. Initially, there were problems in creation of the construct since it is generally more difficult to clone large insert sizes; therefore the ligation efficiency of cloning β6, which is 2.4kb, was significantly reduced (Sambrook et al, 1988). The optimal conditions were eventually determined through trial and error, which were to use electroelution of both the pEGSH vector and the β6 insert and to use a insert:vector ratio of 6:1 for optimal ligation.

In this study, the cells were co-transfected with the β6/pEGSH construct and the pERV3 vector. However, following transfection of the cells and induction with Ponasterone A, αvβ6 expression could not be established, which could have been due to a number of reasons. First, the DNA delivery method may not have been optimal. Cells were transfected using Effectene reagent, which is a simple, quick and non-toxic transfection method that requires only small amounts of DNA and had previously been used in the laboratory to successfully transfect oral carcinoma keratinocytes, however another transfection reagent may have been more efficient at transfecting the cells. A few studies have demonstrated that it is generally difficult to transfect keratinocytes (Jiang et al, 1991). In the absence of a marker to monitor the transfected cells, the transfection efficiency could not be measured.Therefore it was difficult to determine the optimal method of using Effectene reagent, i.e. the concentrations of DNA and Effectene, the DNA:Effectene ratio, cell confluency and transfection time. Secondly, gene expression in this system was dependent upon induction with Ponasterone A. Although induction times of 20 and 24 hours and a number of different concentrations of PonA were tested, these may not have been the optimal conditions. It was difficult to determine what these were since the time taken to initiate integrin translation and the turnover rate were not known. At present there is limited understanding of the mechanisms of integin turnover. Recently this area has become the subject of much research, with a vesicular transport system identified within the cell to maintain integrin expression at the cell surface (Ivaska et al, 2002). The main regulators of integrin turnover are believed to be signalling intermediates

such as small GTPases and tyrosine kinases as well as the calcium-dependent protease, calpain and microtubules (Webb et al, 2002).

Expression of β6 may not have been established due to problems with the actual construct, such as the cmv promoter in the pEGSH vector not being able to drive transcription. With more time, further experiments could have been carried out in order to attempt to optimise gene expression.

DISCUSSION

The aims of this study were to further investigate the relationship between integrin expression and the urokinase-type plasminogen activator system in oral squamous cell carcinoma cells, and examine their effects on cell behaviour.

Human oral squamous cell carcinoma (SCC) is the most common malignancy of the oral cavity, causing more deaths than any other oral disease (Jordan and Daley, 1997). It represents a major clinical challenge not only because it is a major world health problem, comprising 40-50% of all malignancies in parts of India and South-East Asia, but also because there has been little improvement in survival over the last 25 years (Thomas et al, 1997). Treatment of advanced oral SCC is associated with high mortality rates and is complicated by disruption of speech and swallowing that accompany surgical resection (Hicks et al, 1997). Invasion and metastasis of oral SCC require multiple cellular events including cytoskeletal alterations, disruption of cell-cell adhesive contacts and basement membrane attachment, matrix protein proteolysis and migration (Liotta et al, 1983). However, the cellular and biochemical factors that underlie regional and distant spread of the disease are still poorly understood. A necessary prerequisite to the development of novel treatment strategies is a more detailed analysis of the molecular events that contribute to oral SCC invasion. This will involve the development of experimental systems in which the biology of oral SCC can be studied in more controlled conditions.

Integrins are involved in cancer metastasis and are implicated in the regulation of tumour cell growth and survival, migration and invasion, angiogenesis and extravasation (Hood and Cheresh, 2002). Alterations in integrin expression are a common feature of tumours, in particular the $\alpha v\beta 6$ integrin, which is either highly upregulated or neo-expressed in

epithelial carcinomas, including oral SCC (Breuss et al, 1995, Jones et al, 1997). The role of uPA in the metastatic behaviour of tumour cells may be dependent upon the origin of the cells studied. It is still unclear as to the precise role of plasminogen activators and matrix metalloproteinases for clinical outcome, growth and metastatic behaviour of head and neck squamous cell carcinoma (SCC). Many *in vitro* studies have demonstrated a correlation between the co-ordinated expression of metalloproteases and plasminogen activators and invasive behaviour (reviewed by Aguirre-Ghiso et al, 1999a, Ellis and Murphy, 2001). However this correlation is not so obvious in carcinoma cells *in vivo*, which may be due to exposure of the cells to various environmental influences such as stromal cells, angiogenic stimuli and oxygen supply (Schmidt et al, 1999).

The model system used in this study originated from a cell line derived from a well-differentiated carcinoma of the tongue (Prime et al, 1990). This cell line, H357 lacks αv integrin, which was re-introduced genetically to produce a cell line that expressed αv, mainly as the αvβ5 heterodimer, the V3 cells (Jones et al, 1996). These were further transfected with β6 cDNA to create a new cell line (VB6) that expressed the αvβ6 integrin heterodimer (Thomas et al, 2001b). The resultant cell lines proved to be of a stable phenotype, with regard to their behaviour, growth and integrin expression, therefore providing a useful model system for the study of integrins in oral SCC behaviour. It was previously shown that the αvβ6-expressing cells show enhanced MMP-9 secretion compared to the control cells. Since urokinase is able to activate pro-MMP-9 and a number of studies have shown increased protease expression in tumours, this study was focussed on identification of the role of the αvβ6 integrin in regulation of

the urokinase system. Integrin activation has been shown to regulate expression of numerous gene products, however, until recently there was little evidence of the contribution of integrins to modulation of the urokinase system.

The present study shows that uPA secretion was increased from the V3 cells that had been transfected with αv cDNA, compared to the parental cell line, H357, which completely lacked αv expression. VB6 cells expressing full-length αvβ6 secreted significantly greater levels of uPA than the control C1 cells. In accordance with this, recent studies in ovarian cancer cells have shown that elevated αvβ6 expression was also associated with uPA expression (Ahmed et al, 2002b). Activation of the αvβ6 integrin by ligand engagement via a number of methods did not significantly increase uPA secretion from the αvβ6-expressing oral carcinoma cells, suggesting that αvβ6 modulation of uPA is a ligand-independent process.

Adhesive properties of cells are influenced by the selective expression of integrins as well as the ability of cells to modulate the binding properties of integrins. Activation of integrins may occur as a result of cell activation due to an increase in intracellular pH and Ca^{2+}, phosphatidylinositol turnover and activation of G-proteins and protein kinases (Hynes et al, 1992). Activation may also occur by binding to their specific matrix ligands, which induces a conformational change of the integrin (Du et al, 1991, Frelinger et al, 1991). Therefore integrin activation and function requires stimulation by various ligands, either matrix proteins or alterations in signalling molecules. In this study, modulation of uPA secretion by the αvβ6 integrin seems to be a ligand-independent

process as others have suggested. It is possible that other factors may mask ligand effects such as the cells secreting their own fibronectin leading to αvβ6 engagement and unresponsiveness to any exogenous ligand. Also, uPA secretion from these cells is already very high; with VB6 cells secreting 50ng uPA / ml / 10^5 cells, so the cells just may not have the capability to secrete much more uPA.

The integrin repertoire of cancer cells is dependent upon the cell type, for example, αvβ6 over-expression is seen in oral and colon carcinomas whereas αvβ3 is associated with advanced stages of ovarian cancer (Liapis et al, 1996). αvβ3 associates with the ECM component vitronectin and alterations in αvβ3 and VN expression influence tumour cell growth and survival. However, the cellular mechanisms promoted via αvβ3 are still rather inconclusive (Carreiras et al, 1999). In contrast to the study in ovarian carcinoma cells, over-expression of the αvβ3 integrin into these oral carcinoma cells (V3B3 cells) slightly increased uPA expression but not to a significant degree, suggesting that in this particular cell type αvβ6 is the most important integrin in modulating the urokinase system. However, uPA expression following αvβ3 ligation to vitronectin was not investigated and any effect may be more prominent following integrin activation.

The uPA gene has been isolated from several mammalian species (Nagamine et al, 1984, Riccio et al, 1985, Degen et al, 1987); it is 6.4 kb long and is organised in 11 exons. It is regulated by a well-defined promoter sequence containing a number of transcription factor target sites. The uPA mRNA is 2.4 kb long with 900 bp of 3'-untranslated region (UTR) conserved in several mammalian species (Nanbu et al, 1994). uPA gene

expression is generally increased in metastatic cells due to a combined increase in gene transcription and mRNA stability (Henderson et al, 1992). It can be regulated by several stimuli, such as the tumour promoter phorbol myristate acetate (PMA), okadaic acid, cytoskeletal reorganisation, growth factors, oncogenes, UV and TNFα (Irigoyen et al, 1999). The control of uPA expression can occur at both the transcriptional and post-transcriptional levels. The levels of stable uPA mRNA expression are similar between the cell lines, suggesting that the αvβ6-associated increase in uPA secretion in VB6 cells is a result of a possible translational modification to increase protein synthesis from the mRNA transcript. Possible mechanisms of how αvβ6 integrin may promote uPA protein production include alterations in protein folding or alterations in the translocation of proteins leading to post/co-translational modifications. Also, translation may be affected by interference with molecules such as calnexin and calreticulin, which act as molecular chaperones in the quality control of protein folding (Ellgaard and Helenius, 2001). In fact it has been shown that anti-α3 integrin antibody induced a transient enhanced interaction of calreticulin with α3 integrin and this enhanced secretion of the protease MMP-2 (Ito et al, 2001). Previously reported mechanisms of post-transcriptional regulation of uPA involve alterations in mRNA stability. In LL-CPK1 cells, mRNA stability was prolonged by PKC downregulation and calcium ions (Ziegler et al, 1991). This was shown to be mediated by protein binding to an AU-rich (ARE) region in the 3'-UTR of uPA mRNA. Regulation of mRNA stability was also shown to involve p38 MAP kinases (Nanbu et al, 1997). Recently, two other 3'-UTR mRNA-binding proteins have been described which are able to stabilise (Shimba et al, 2000) or destabilise (Shetty et al, 1997) uPA mRNA. Therefore it seems likely that there are a number of

mechanisms of post-transcriptional regulation. The αvβ6 integrin may be modulating uPA expression by activation of signalling molecules, which may either directly or indirectly interfere with protein translation. Further investigation is required in order to determine the precise nature of this modulation.

The present study shows that expression of the αvβ6 integrin in oral carcinoma cells is associated with reduced uPAR expression. This regulation occurs at the transcriptional level, since the αvβ6 expressing cells show 2-fold reduced mRNA expression than the control cells, which have negligible αvβ6. This is confirmed at the cell surface with decreased plasminogen activation in the αvβ6-expressing cells.

The processes of proteolysis and adhesion, via uPAR and integrins have been linked in a number of recent studies. uPAR physically interacts on leukocytes with β2-integrin as shown by co-localisation and immunoprecipitation studies (Bohuslav et al, 1995), although the major class of integrins found in stable physical association with uPAR appears to be the β1 integrins. Using immunoprecipitation studies, complexes between uPAR, a β1 integrin and caveolin were demonstrated in uPAR-transfected embryonic kidney 293 cells (Wei et al; 1996). Also, in fibrosarcoma cells, β1 as well as β3 integrins were co-localised with uPAR (Xue et al, 1997). Transfection of the αv integrin into the keratinocytes (V3 cells), so they express αvβ5 increased uPAR expression by 2-fold. It is possible that uPAR may co-localise with αvβ5 in a membrane complex on the cell surface and this may be functional in promoting cell migration. It has been shown in a pancreatic carcinoma cell line that uPA bound to uPAR is required for αvβ5-dependent

migration towards vitronectin (Yebra et al, 1996). Transfection of the β6 integrin into the cells decreases uPAR expression, suggesting that a physical association with αvβ6 is unlikely. It will be interesting to see if there is a physical association of uPAR with other integrin heterodimers in these oral carcinoma cell lines.

In addition to the physical association of uPAR with various integrin β subunits, recent studies have focussed on functional interactions between uPAR and integrins. These interactions are complex with regulation of both integrin and uPAR functions. β1 (Wei et al, (1996) and αvβ5 (Carriero et al, 1999) have been shown to promote uPAR directed cell migration towards vitronectin. Conversely, uPAR has also been shown to influence integrin activity, although this is a complex process with both inhibition (Wei et al, 1996), and promotion (Carriero et al, 1999, Wei et al, 1999, Simon et al, 2000) of β1 integrin function by uPAR. There is even *in vivo* data to suggest that uPAR-integrin complexes are functionally involved in tumour progression (van der Pluijm et al, 2001). It is likely that mechanisms of cross-talk between integrins and proteases exist to promote cell adhesion and migration. This is clearly a complex process, involving not only integrins and uPAR but also other associated molecules, such as caveolin, uPA, tetraspans and other integrin associated proteins, which may be coupled to G-proteins and other intracellular signalling molecules (Kjoller et al, 2002). This suggests that any subtle temporal and spatial relationship between αvβ6 and uPAR as well as individual cell responses may have been overlooked in this study. Any interaction could be further investigated by using time-lapse microscopy and real-time staining of the molecules.

Elevated levels of uPAR are seen in many malignant tumours and are indicative of a poor prognosis (Andreasen et al, 1997, Reuning et al, 1998). Therefore it is surprising that αvβ6, an integrin expressed in invasive cancers should decrease uPAR expression. However, it cannot be assumed that in all situations, high levels of both integrins and uPAR should promote the invasive phenotype. In fact it requires a complex regulation of different cell-surface-associated proteins to provide optimal proteolysis and adhesion. The invasive process starts with the attachment of cells to the ECM, followed by degradation of various ECM components through proteases. Subsequently cells undergo detachment and migration to distant sites, where they proliferate in order to form metastases. Besides its role in proteolysis, uPAR is a multi-functional receptor that is involved in chemotaxis, angiogenesis, signal transduction, migration and adhesion of cells (Dear and Medcalf, 1998). Therefore, reduced uPAR expression in the αvβ6-expressing VB6 cells may in fact promote the cancerous phenotype by enabling cells to proliferate and metastasise as a result of possibly less adhesion, through uPAR to vitronectin in the ECM and reduced proteolysis at the cell surface. In contrast to published data (Thomas et al, 2001b) no difference in invasion was observed between the αvβ6 expressing cells and the control cells (see chapter 6). The VB6 cells are more migratory towards fibronectin, however migration towards vitronectin was not affected. If uPAR was acting as the governing receptor in determining cell migration, then migration towards vitronectin should have decreased in the VB6 cells. Therefore in this model, there seems to be a dominant effect of integrins over uPAR in cell migration and invasion, which may be the case in physiological and pathological situations.

In addition to integrin regulation of the urokinase system, uPA or uPAR may in a reciprocal manner modulate integrin expression or function. For example, the interaction between the vitronectin receptor αvβ5 and uPAR is well documented (Reinartz et al, 1995, Yebra et al, 1996, Carriero et al, 1999) and a positive correlation was shown between uPAR and αvβ5 protein levels in benign and malignant tumour specimens. uPA was shown to promote a physical association between uPAR and αvβ5 in human breast carcinoma cells (Carriero et al, 1999) and upregulate uPAR expression, possibly via activation of the transcriptional factor Sp1 (Zannetti et al, 2000) which are both required for uPA-dependent cell migration. In breast carcinoma cell lines, engaging uPAR with catalytically inactive urokinase upregulates the expression of αv and β5 integrin chains in a time- and concentration-dependent manner, which is dependent on protein kinase C activity and leads to enhanced tumour cell migration and invasion (Silvestri et al, 2002). These data suggest there is a molecular cross-talk between stromal and epithelial cells involving integrins and uPA/uPAR to enhance tumour metastatic capacity, with integrin regulation of the urokinase system and vice versa.

The results show that the effects of the αvβ6 integrin on the urokinase system are mediated through the unique 11 amino acid cytoplasmic tail extension of the β6 subunit, in a ligand-independent manner. In has previously been shown in colon cancer cells using cytoplasmic tail deletion mutants, that αvβ6 induces MMP-9 secretion through the cytoplasmic extension unique to the β6 integrin subunit. This is a ligand-independent event that involves activation of the protein kinase C pathway (Niu et al, 1998). Loss of the 11 amino acid C-terminal extension does not impair the ability of the receptor to bind

to fibronectin, yet abolishes the density-dependent increase in β6 expression seen in cells expressing wild-type β6 (Agrez et al, 1994). This suggests that the 11aa C-terminal extension is essential for the cell-density dependent increase in β6-expression and that this is ligand independent. However, a single point mutation on the extracellular domain that abolishes β6-dependent binding to fibronectin, also inhibited the cell density-dependent increase in β6-expression (Huang et al, 1995). This unique sequence on the cytoplasmic tail is also required for the PKC-mediated β5 expression in αvβ6-expressing colon cancer cells. The cytoplasmic domains of β-integrins mediate downstream signalling events and affect gene regulation and integrins are known to signal through the Ras-MAP Kinase pathway (Chen et al, 1994 and LaFlamme et al, 1997). Loss of the ERK-binding site on β6 markedly impairs cell-density and PKC-dependent expression of β6 in the presence of the 11aa tail or impairs β5 expression in the absence of the 11aa tail. This suggests that β6-ERK2 interaction dominates over PKC-mediated signalling pathways responsible for integrin up-regulation with cell confluence, therefore targeting the β6-ERK2 interaction may be useful as an anticancer target (Niu et al, 2002). Recently, the wild-type β6 integrin subunit has been shown to promote tumour growth *in vivo*. This growth-enhancing effect is regulated by both a MAP kinase binding motif on β6 and the 11aa extension. It was shown in this study that the β6 cytoplasmic domain binds directly to ERK2 (Ahmed et al, 2002a). These data suggest that the effects of the β6 cytoplasmic domain on uPA and uPAR may be mediated through ERK signalling. In fact, in this study the ERK1/2 pathway was shown to be involved in uPA expression. Also, the uPAR promoter has also been shown to be regulated via a MAPK-dependent signal transduction pathway (Gum et al, 1998).

Epithelial wound healing is associated with a specific sequence of changes in the process of cell-matrix adhesion and extracellular matrix degradation, where keratinocytes modify the expression, cell-surface distribution and cytoskeletal association of integrin receptors (Haapasalmi et al, 1996) and also gain the ability to degrade connective tissue (Ravanti and Kahari, 2000). *De novo* expression of the αvβ6 integrin by keratinocytes in both cutaneous and oral wounds has been demonstrated in several studies (Breuss et al, 1993, Clark et al, 1996, Haapasalmi et al, 1996). There is some evidence to suggest that re-epithelialization of epidermal wounds is associated with a switch from αvβ5 expression to αvβ6 in keratinocytes (Clark et al, 1996). This late expression of αvβ6 during wound healing may act as a signal to stop cell migration/invasion to prevent uncontrolled proliferation. Cultured skin keratinocytes have been shown to produce uPA, tPA and the inhibitors PAI-1 and PAI-2, with the relative amounts of uPA and tPA varying with the state of proliferation and differentiation (Jensen et al, 1990). Although total plasminogen activator activity was increased in more differentiated keratinocyte cultures, uPA seemed to be increased during proliferation and decreased upon differentiation (Grimaldi et al, 1986). uPA and tPA were identified in keratinocytes during wound re-epithelialization *in vivo* (Grondahl-Hansen et al, 1988). In wounded cultures of keratinocytes uPA is localised at the leading edge of migrating cells (Morioka et al, 1985, Morioka et al, 1987), cells that are usually proliferating. uPA and tPA were also involved in the migration of keratinocytes and fibroblasts in wounded cultures *in vitro* (Boxman et al, 1995). Since many of the changes seen in wound healing and cancer are similar it suggests that squamous cell carcinoma may represent a process of uncontrolled tissue

remodelling. The increased expression of αvβ6 and uPA seen in wound healing situations are also seen in the squamous cell carcinoma keratinocytes used in this study, further implying similarities in the two processes.

Migration of cells is mediated by adhesion receptors, such as integrins, that link the cell to extracellular matrix ligands, transmitting forces and signals necessary for locomotion (Hynes, 1992, Schwartz et al, 1995, Lauffenburger and Horwitz, 1996). A migrating keratinocyte changes it shape dramatically as it goes from a stationary basal keratinocyte to a migrating cell. The migrating cell becomes flat and elongated with the appearance of cytoplasmic extensions called lamellipodia and the loss of hemidesmosomes and desmosomes with gap junctions becoming more prominent. Correspondingly there is a redistribution of the actin cytoskeleton into lamellipodia (O'Toole, 2001). The ability of the cell to migrate and it's migration speed is dependent on several variables related to integrin-ligand interactions, including ligand levels, integrin levels and integrin-ligand binding affinities (Palecek et al, 1999). Therefore a combination of receptors of different affinities can be beneficial for migration versatility.

Co-operation between integrins and proteases operates at several levels to co-ordinate focal extracellular matrix attachments and detachments central to cell migration (reviewed by Chapman and Wei, 2001). Firstly, integrin clustering and consequent intracellular signalling induces proteolytic enzyme expression. Secondly, proteases localise on the cell surface to sites of integrin clustering and finally as integrins cluster at sites of their matrix ligands the calcium stimulated calpains localise to the cytoplasmic

tails of integrin clusters and regulate cytoskeletal reorganisation important to integrin migration. Thus, proteases surrounding integrins both outside and inside the plasma membrane are important to regulation of integrin function. The presence of uPAR in focal adhesions and its polarisation in migrating cells is thought to be dependent on interactions with other proteins and a number of potential mechanisms for this have emerged involving direct binding of uPA/uPAR to the adhesion protein vitronectin and interaction with integrins. In a human pancreatic carcinoma cell line, induction of cell surface expression of uPA/uPAR by growth factors or phorbol ester was necessary for vitronectin-dependent cell migration, which was mediated by integrin αvβ5. This demonstrated a specific functional co-operation between uPA/uPAR and αvβ5 in regulating cell migration and a requirement for uPA enzymatic activity (Yebra et al, 1996). uPA was also shown to promote migration on vitronectin in an integrin-selective manner, through β1 and αvβ5 in the MCF-7 breast cancer cell line. This occurred by initiating a uPAR-dependent signalling cascade in which Ras, MEK, ERK and MLCK served as essential downstream effectors (Nguyen et al, 1999). The association of uPAR with αvβ5 leads to a functional interaction of these receptors in HT1080 human fibrosarcoma and MCF-7 human breast adenocarcinoma cell lines. Treatment with uPA promoted the physical association of uPAR with αvβ5, and αvβ5 was required for uPAR-directed cell migration on vitronectin (Carriero et al, 1999). Also, uPAR was shown to interact with and modify the normal function of the β1 integrin by promoting adhesion and migration to the uPAR-specific ligand, vitronectin and inhibiting adhesion and migration to the β1 ligand, fibronectin (Wei et al, 1999).

In order to understand the role of αvβ6 modulation of the urokinase system in cancer, functional assays were carried out. In this study and previous studies it has been shown that αvβ6-expressing cells are more migratory towards its specific ligand fibronectin (Thomas et al, 2001b). This increased migration was plasmin-dependent and also partially dependent on urokinase activity. It is clear from this data that uPA activity is required for keratinocyte cell migration, however the β6-mediated increased migration is not specifically dependent on uPA proteolytic activity. Also, cell migration in all of the cell lines is not entirely dependent on uPA proteolytic activity.

While it is evident that uPA/uPAR proteolytic activity can promote migration, it has also been established that uPA enzymatic activity is not always required for cell migration and components of the urokinase system have been implicated in cell migration through purely signalling mediated events (Andreasen et al, 1997, Kjoller, 2002). Binding of uPAR by the enzymatically inactive amino-terminal fragment of uPA was sufficient to promote human epidermal cell motility (Del Rosso et al, 1993). Also, blocking uPA binding with an anti-uPAR monoclonal antibody but not with an antibody that neutralises uPA catalytic activity prevented human monocyte chemotaxis (Gyetko et al, 1994). It has been suggested that ligation of uPAR by uPA leads to enhanced epithelial cell migration as a result of uPAR mediated signal transduction (Busso et al, 1994). It is also possible that adherent cells use activated enzyme to degrade the matrix (endogenous and exogenous) to which they are attached, making cells less adherent and allowing them to move more freely. Palacek et al (1999), demonstrated the role of cell adhesion in migration and showed optimal migration was reached at a point between cells being "too

adherent" and "not adherent enough". uPA/uPAR regulation of cell motility through signalling events may occur independently but previous evidence has suggested a functional co-operation between uPA/uPAR and integrins in regulating cell migration.

The other components of the urokinase system including uPAR and PAI-1 and tPA have been shown to regulate cell migration (Andreasen et al, 1997), therefore these factors as well as other proteolytic enzymes such as MMPs may play a role in cell migration in these cells. However, in this study high uPAR expressing cells were not more migratory towards vitronectin. Although the roles of tPA and PAI-1 were not investigated, the data suggest that they may not play a role. This is because the levels of tPA were similar between the cell lines and although the levels of PAI-1 were not tested, the similar results of ELISA and plasminogen activator activity assays suggest that PAI-1 expression is similar between the cell lines.

Cellular migration was studied using haptotactic cell migration assays and wound assays, but a role for αvβ6 in cell migration was only seen in the former. This observation that the contribution of αvβ6 to cell migration varies depending on assay conditions was also seen in a human oral SCC cell line, HSC-3. Using the Matrigel drop assay, cell migration was strongly supported by αvβ6 but was also shown to be dependent on β1 integrins. Similar results were obtained using standard Boyden chamber assays, although the contribution of αvβ6 to migration was less pronounced (Xue et al, 2001). This highlights the fact that different characteristics are selected for in different assays and suggests that data from different published reports may not be comparable. It seems that

in SCC, cell migration on fibronectin is not exclusively mediated by αvβ6, therefore suggesting that αvβ6 has other roles in cancer progression, such as mediating migration on tenascin-C, latency associated peptide (LAP) or other fibronectin isoforms expressed by SCC tumours or inducing protease expression.

In order to promote the malignant phenotype, cancer cells must exhibit various aspects of cellular behaviour including cell migration, invasion, proliferation and increased angiogenic activity. This study demonstrated that there was no difference in the invasive potential between αvβ6-expressing cells and control cells. uPAR is known to promote cellular invasion either directly by enabling proteolytic activity at the cell surface or indirectly through signalling-mediated events (Andreasen et al, 1997). However, in this study, the levels of uPAR expression in the cell lines did not correlate with invasion. It may be the case that in this situation, αvβ6 may promote the malignant phenotype by influencing other cellular functions such as cell migration. This is in contrast to previously published data showing increased invasion through matrigel of these same αvβ6-expressing oral keratinocytes (Thomas et al, 2001a). *In vitro*, cell migration and cell invasion assays are different, reflecting the differences between these processes in an *in vivo* situation. Although Boyden chambers were used for both, in invasion assays the filters were coated in matrigel, which is composed of various matrix proteins, rather than just fibronectin as used in the migration assays. The cells have to physically pass through matrigel, whereas they simply move towards fibronectin, suggesting that cells with increased proteolytic activity should be more invasive. It is difficult to ascertain whether the αvβ6-expressing cells have more proteolytic activity than control cells because

although they secrete more protease, the levels of plasminogen activation at the cell surface are lower. The matrigel and fibronectin may have induced different integrin-ligand interactions, causing altered cytoskeletal changes within the cell, leading to the activation of intracellular signalling pathways, resulting in an altered migratory or invasive capability of the cell. Also, the assay conditions for migration and invasion were different; invasion was determined over 48-72 hours, whereas migration was measured only after 4 hours incubation. This short time suggests that the migration assay was largely dependent on adhesion.

Although, this study has focussed on fibronectin, in order to see an αvβ6-mediated effect, the role of uPAR in migration and invasion must also be taken into consideration. Vitronectin can act as a uPAR ligand in order to promote cellular migration towards this, although in this study, high-uPAR expressing cells did not show increased migration towards vitronectin. The role of uPAR as a pro-invasive or pro-migratory cell surface molecule may have masked an effect of αvβ6 in invasion.

The αvβ6 integrin is an epithelial cell restricted integrin; therefore in order to see if the integrin-mediated effect on the urokinase system was epithelial-cell specific, αvβ6 was neo-expressed into melanoma cell lines. Melanoma cells do not normally express αvβ6 but have been shown to overexpress αvβ3. This study demonstrates that αvβ6 is functional within the melanoma cells since migration towards plasma fibronectin was promoted in the αvβ6-expressing cells compared to the control cells. However, there was no effect on uPA or uPAR expression, suggesting that integrin-mediated effects are

cell-specific. It may be the case that in melanoma cells no such mechanism exists or that another integrin such as αvβ3 is more important in regulation of the urokinase system. In fact αvβ3 has been shown to affect both uPA and uPAR expression in previous studies. The keratinocytes used in this study do not express αvβ3, therefore suggesting that the αv integrin that is highly expressed, αvβ6 may be fulfilling this role. Also, the αvβ6 and αvβ3 integrins have high sequence homology suggesting that they may have similar functions. These data add further weight to the suggestion of redundancy with regard to integrin expression and function. For example, various integrins may affect cellular behaviour and protein expression in the same manner, but which integrins are specifically involved may be dependent on the cell type, suggesting that integrins may compensate for one another in their functions. However, this is only speculative and further light could be shed on this by investigating the role of αvβ3 in these melanoma cell lines. Firstly by checking the expression levels of αvβ3 and then determining if there is a correlation of this with the levels of uPA and an inverse correlation with the levels of uPAR.

Two strategies were employed in an attempt to confirm the effects of the αvβ6 integrin on the urokinase system. First, was the use of morpholino antisense oligonucleotides in order to down-regulate αvβ6 integrin expression. Transfection of the oligos using Superfect reagent caused a 50% reduction in αvβ6 expression, however, no concurrent effect on the urokinase system was observed. Secondly, the establishment of an inducible gene expression system was attempted, however this approach proved to be unsuccessful due to a number of technical difficulties including the inability to determine

the transfection efficiency to establish the optimal transfection conditions and to establish the optimal induction time and concentration with Ponasterone A, before testing for integrin expression. A significant problem with both the antisense experiments and with the inducible gene expression system was determining integrin turnover time. There are very few studies addressing this aspect of integrin regulation, with many unanswered questions regarding the translation time resulting in integrin expression, how long integrins are present on the cell surface and whether they are recycled back to the cell surface or degraded. It has been hypothesised that membrane internalised from the cell surface is recycled to the leading edge of the migrating cells, providing directionality (Bretscher, 1996). In order to facilitate cell migration, integrin-containing adhesion sites need to be constantly internalised at the rear of the cell and subsequently transported to the leading edge (Palecek et al, 1996). However, little is known about the exact mechanisms regulating this integrin traffic. Integrins have been shown to traffic through a Rab11-positive endocytic recycling compartment (Ng et al, 1999, Roberts et al, 2001). Most recently, PKCε was shown to be a regulator of β-integrin-dependent migration and traffic of these receptors through the cell (Ivaska et al, 2002). Since the expression of integrins on the cell surface may be regulated at different levels, i.e. at the transcriptional or the translational level, these factors must be taken into consideration when trying to influence integrin expression. It has been suggested that the oral carcinoma cells used in this study harbour intracellular pools of the αv integrin subunit (Thomas et al, 2001b). Therefore, even if antisense treatment was effective in blocking synthesis of new proteins, it may not affect the expression of intracellular pools of integrins. It is not known what controls the pairing and expression of heterodimers at the cell surface,

making it difficult to manipulate integrin expression in these particular cells. This probably depends on the activation of intracellular signalling pathways, however further research needs to be done in order to delineate the precise mechanism.

uPA and integrins are known to be implicated in cancer and identification of a link between them may be crucial in controlling epithelial cell behaviour. This study demonstrates role for αvβ6 integrin in regulating the urokinase-type plasminogen activator system. Along with other studies, this suggests that β6 is able to regulate multiple protease systems, providing a key therapeutic target.

FUTURE WORK

In this study αvβ6 overexpression is associated with increased uPA secretion, which is reflected in increased cell-associated uPA levels. However, the levels of uPA mRNA are similar between the cell lines, suggesting that the αvβ6 integrin is enhancing uPA expression at the post-transcriptional level. It is generally unclear as to how integrins may affect the expression of other proteins, therefore further experiments could be carried out in order to delineate the mechanism by which αvβ6 is promoting uPA secretion. This will initially involve determining whether the αvβ6-expressing cells are actually producing more uPA protein or are simply secreting it at a faster rate. There is no evidence to suggest that cells are able to store uPA within internal cytosolic vesicles, however this could be investigated by preparing cytosolic and membrane extracts of cells and then determining the percentage of uPA in cytosol and membrane. This may be further explored to see if uPA in certain integrin-expressing cell lines is more abundant within certain organelles of the cell, such as the Golgi apparatus or endoplasmic reticulum. Possible experiments could be to monitor the trafficking of uPA through the biosynthetic pathway, by co-staining with certain endosomal markers using immunofluoresence. This along with radiolabelling or electron microscopy may help to more accurately identify the distribution of uPA within the cells.

Another method of determining the mechanism of αvβ6 modulation of uPA expression would be to study the effects of the αvβ6 integrin on the uPA promoter.

Every gene that is expressed is under the control of a specific promoter. This is the nucleotide sequence upstream of a gene that acts as a signal for RNA polymerase binding to DNA, so determines the rate at which mRNA is synthesised. Transcriptional regulation of the 2.6kb human uPA gene relies on the 2.1kb uPA promoter sequence, upstream of the transcriptional start site. The promoter sequence contains functional binding sites for the transcription factors AP1, PEA3 and NFαB, which are important for both constitutive and regulated urokinase expression. Expression of the uPA gene appears to be regulated by a large number of growth factors and cytokines; hepatocyte growth factor/scatter factor (HGF/SF) has been shown to upregulate uPA expression by trans-activation of the urokinase promoter (Ried et al, 1999). The proposed model of activation is through the MAP Kinase pathway where HGF/SF binds to the Met receptor, followed by subsequent activation of Grb2/Sos1/Ha-Ras/c-Raf/RhoA/Mek1/Erk2/cJun. cJun may then act on the AP1 and combined PEA3/AP1 enhancer sites, to induce urokinase expression. The MAP Kinase pathway may also be stimulated by cytoskeletal reorganisation followed by FAK and Src, which leads to activation of the uPA gene. Another growth factor implicated in regulating uPA expression is TNFα, which activates binding sites for AP1 and PEA3 transcription factors. c-AMP dependent protein kinase (PKA) also strongly induces the uPA gene, via structural alterations that permit co-operative interactions between the multiple cAMP responsive sites on the uPA promoter.

uPA promoter activity could be measured using the secreted alkaline phosphatase reporter assay (SEAP). The principle is that activation of the uPA promoter will also

simultaneously drive the activation of the reporter gene, which is indirectly measured by the degree of alkaline phosphatase activity in the culture supernatants. This system has advantages over other reporter assays in that the promoter activity can be measured from cells in culture over a prolonged period since the cells do not need to be harvested. This study would confirm whether uPA is regulated by αvβ6 at the post-transcriptional level.

An association between uPAR and αvβ6 integrin could be further studied, by co-staining of these using fluorescent markers and analysed by confocal microscopy, as well as co-immunoprecipitations between uPAR and integrins. In addition, immunohistochemical localisation of uPAR and integrins on tumour sections could be carried out.

This study has primarily focussed on the αvβ6 integrin, however the results suggest that transfection of the αv subunit alone has an effect on the urokinase system. Both uPA and uPAR expression are significantly increased when the cells predominantly express the αvβ5 integrin heterodimer. This substantiates the observations that numerous integrins are able to regulate the urokinase system. Also, overexpression of αvβ3 in the oral SCC cells slightly increased uPA and uPAR expression and function, although not to a significant degree. Further experiments involving activation of the integrins by ligand binding may optimise any effects on the urokinase system.

Since αvβ6 did not affect expression of urokinase components in melanoma cells, the role of other integrins such as αvβ3 may be investigated, initially by determining expression levels and then studying the effects of this integrin on the urokinase system. It may be the case in the melanoma cells that αvβ3 is overriding any αvβ6-mediated effects. Therefore, a possible avenue for further study would be genetic ablation of endogenous αvβ3 and then transfection with β6. In this situation, αvβ6 may be able to modulate protease expression. This would be interesting to see if certain integrins can compensate for one another's functions.

Numerous functional assays were carried out in this study in order to determine the role of the urokinase system in oral SCC cell migration and invasion. Haptotactic cell migration assays showed that αvβ6-expressing cells were more migratory towards fibronectin, although with these assays, integrin-mediated cell adhesion may have also played a role. Wound assays were also carried out in order to determine any differences in cell migration between the cell lines. Wound assays are different from the haptotactic migration assays in that cells are allowed to migrate on a surface of extracellular matrix secreted by the cells, composed of various matrix proteins. Whereas, haptotactic cell migration focussed on specific integrin-mediated migration towards fibronectin. There was no difference in the rate of wound closure between the cell lines. A different method to study cell migration could be employed where the random movement of cells on surfaces coated with different matrix proteins were tracked over time. This may reflect more accurately the *in vivo* situation of tumour cell movement since the surrounding stroma of a tumour will be composed of many

different matrix proteins enabling cancer cell migration towards a number of proteins in various directions. Cell migration on fibronectin has been shown to involve increased AP1 (Cowles et al, 2000) activity and induction of uPA gene expression also requires activation of AP1 site (Nerlov et al, 1991). Therefore it may be possible that αvβ6 is mediating increased migration on fibronectin and uPA secretion seen in this study is linked through activation of the AP1 site.

There are relatively few studies demonstrating knockdown of endogenous integrins within cells suggesting that antisense technology in general is not that successful. The recently developed technique of RNA interference (RNAi), could prove to be a more useful method to down-regulate integrin expression in future studies. It is not known what controls the pairing and expression of heterodimers at the cell surface, making it more difficult in manipulating integrin expression in these particular cells. This probably depends on the activation of intracellular signalling pathways, however further research needs to be done in order to delineate the precise mechanism. The most useful method of completing these studies would be to finish the development of the inducible β6 gene expression system.

SUMMARY

Interactions between specific cell-surface molecules, such as uPAR and integrins, are crucial to processes of tumour invasion and metastasis. Integrin activation has been shown to regulate expression of numerous gene products, however, until recently there was little evidence of the contribution of integrins to modulation of the urokinase system. The present study shows that heterologous expression of the αvβ6 integrin in oral carcinoma cells promotes the up-regulation of uPA secretion from the cells, but downregulates urokinase receptor expression. This regulation of receptor occurs at the transcriptional level, since the αvβ6 expressing cells show 2-fold less uPAR mRNA expression than the control cells, with negligible αvβ6. This is associated with decreased plasminogen activation at the cell surface. Much previous *in vitro* and *in vivo* data suggest that increased expression of both uPA and uPAR, therefore increased proteolytic activity at the cell surface are correlated with promoting the cancerous phenotype, with elevated levels of uPA and uPAR seen in many malignant tumours and indicative of a poor prognosis (Andreasen et al, 1997, Reuning et al, 1998). Although plasminogen activation and proteolytic breakdown of the ECM are associated with tumour progression, this data provides a novel insight into the roles of proteolytic enzymes in cancer suggesting that that precisely controlled levels of proteolysis are required in order to prevent excessive plasmin proteolysis. Excessive degradation of extracellular matrix is incompatible with efficient cellular migration (Montesano et al, 1990, Pepper and Montesano, 1990), since increased plasmin proteolysis may prevent accumulation of ECM components

that stimulate growth and proliferation and provide a scaffold for cell attachment and motility. αvβ6 expression into melanoma cell lines did not affect the expression or function of uPA or uPAR, however these cells showed an increased migration towards fibronectin confirming that the integrin was functional. This demonstrated that modulation of the urokinase system by αvβ6 is cell-type specific.

Due to the heterogeneity of integrin expression it is difficult to assign specific functions to specific integrins, therefore suggesting a general redundancy of integrin expression and function. The aim of this study was to study the role of a specific integrin, αvβ6 in oral carcinoma, specifically by regulation of the urokinase system. So why is it important to study a particular integrin if there is such redundancy? The answer to this question is that in order to understand cancer cell behaviour, the roles of various integrins, along with other cell surface molecules, protease systems and signalling molecules in various model cancer systems need to be explored. This is such a large and generalised research area, that one particular therapy is not feasible, but rather by investigating the molecular nature of different types of cancer, may help to discover new therapeutic targets and strategies. In particular the αvβ6 integrin is able to control cell migration as well as uPA and MMP expression making it a good therapeutic target.

REFERENCES

Agrawal, S. and Q.Zhao. 1998. Antisense therapeutics. *Curr. Opin. Chem. Biol.* 2:519-528.

Agrez, M., A.Chen, R.I.Cone, R.Pytela, and D.Sheppard. 1994. The alpha v beta 6 integrin promotes proliferation of colon carcinoma cells through a unique region of the beta 6 cytoplasmic domain. *J. Cell Biol.* 127:547-556.

Agrez, M., X.Gu, J.Turton, C.Meldrum, J.Niu, T.Antalis, and E.W.Howard. 1999. The alpha v beta 6 integrin induces gelatinase B secretion in colon cancer cells. *Int. J. Cancer* 81:90-97.

Aguirre Ghiso, J.A., D.F.Alonso, E.F.Farias, D.E.Gomez, and E.B.Kier Joffe. 1999a. Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. *Eur. J. Biochem.* 263:295-304.

Aguirre Ghiso, J.A., K.Kovalski, and L.Ossowski. 1999b. Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *J. Cell Biol.* 147:89-104.

Ahmed, N., J.Niu, D.J.Dorahy, X.Gu, S.Andrews, C.J.Meldrum, R.J.Scott, M.S.Baker, I.G.Macreadie, and M.V.Agrez. 2002a. Direct integrin alphavbeta6-ERK binding: implications for tumour growth. *Oncogene* 21:1370-1380.

Ahmed, N., F.Pansino, R.Clyde, P.Murthi, M.A.Quinn, G.E.Rice, M.V.Agrez, S.Mok, and M.S.Baker. 2002b. Overexpression of alpha(v)beta6 integrin in serous epithelial ovarian cancer regulates extracellular matrix degradation via the plasminogen activation cascade. *Carcinogenesis* 23:237-244.

Ahmed, N., C.Riley, G.E.Rice, M.A.Quinn, and M.S.Baker. 2002c. Alpha(v)beta(6) integrin-A marker for the malignant potential of epithelial ovarian cancer. *J. Histochem. Cytochem.* 50:1371-1380.

Albelda, S.M., S.A.Mette, D.E.Elder, R.Stewart, L.Damjanovich, M.Herlyn, and C.A.Buck. 1990. Integrin distribution in malignant melanoma: association of the beta 3 subunit with tumor progression. *Cancer Res.* 50:6757-6764.

Alberts B., D.Bray, J.Lewis, M.Raff, K.Roberts, and J.D.Watson. 1994. Molecular Biology of the Cell. Third Edition. Garland Publishing Inc. New York

Alessandro, R. and E.C.Kohn. 2002. Signal transduction targets in invasion. *Clin. Exp. Metastasis* 19:265-273.

Alonso, D.F., E.F.Farias, V.Ladeda, L.Davel, L.Puricelli, and J.E.Bal De Kier. 1996. Effects of synthetic urokinase inhibitors on local invasion and metastasis in a murine mammary tumor model. *Breast Cancer Res. Treat.* 40:209-223.

Andreasen, P.A., R.Egelund, and H.H.Petersen. 2000. The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell Mol. Life Sci.* 57:25-40.

Andreasen, P.A., L.Kjoller, L.Christensen, and M.J.Duffy. 1997. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int. J. Cancer* 72:1-22.

Andreasen, P.A., A.Riccio, K.G.Welinder, R.Douglas, R.Sartorio, L.S.Nielsen, C.Oppenheimer, F.Blasi, and K.Dano. 1986. Plasminogen activator inhibitor type-1: reactive center and amino-terminal heterogeneity determined by protein and cDNA sequencing. *FEBS Lett.* 209:213-218.

Antalis, T.M., M.La Linn, K.Donnan, L.Mateo, J.Gardner, J.L.Dickinson, K.Buttigieg, and A.Suhrbier. 1998. The serine proteinase inhibitor (serpin) plasminogen activation inhibitor type 2 protects against viral cytopathic effects by constitutive interferon alpha/beta priming. *J. Exp. Med.* 187:1799-1811.

Aota, S., M.Nomizu, and K.M.Yamada. 1994. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. *J. Biol. Chem.* 269:24756-24761.

Aplin, A.E., A.K.Howe, and R.L.Juliano. 1999. Cell adhesion molecules, signal transduction and cell growth. *Curr. Opin. Cell Biol.* 11:737-744.

Arihiro, K., M.Kaneko, S.Fujii, K.Inai, and Y.Yokosaki. 2000. Significance of alpha 9 beta 1 and alpha v beta 6 integrin expression in breast carcinoma. *Breast Cancer* 7:19-26.

Bader, B.L., H.Rayburn, D.Crowley, and R.O.Hynes. 1998. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. *Cell* 95:507-519.

Bajou, K., V.Masson, R.D.Gerard, P.M.Schmitt, V.Albert, M.Praus, L.R.Lund, T.L.Frandsen, N.Brunner, K.Dano, N.E.Fusenig, U.Weidle, G.Carmeliet, D.Loskutoff, D.Collen, P.Carmeliet, J.M.Foidart, and A.Noel. 2001. The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic strategies. *J. Cell Biol.* 152:777-784.

Baker, J.K., S.R.Elshaw, G.E.Mathewman, C.E.Nichols, A.K.Murray, M.A.Parsons, I.G.Rennie, and K.Sisley. 2001. Expression of integrins, degradative enzymes and their inhibitors in uveal melanoma: differences between in vitro and in vivo expression. *Melanoma Res.* 11:265-273.

Baker, W.F., Jr. 2002. Thrombolytic therapy. *Clin. Appl. Thromb. Hemost.* 8:291-314.

Bakhit, C., D.Lewis, R.Billings, and B.Malfroy. 1987. Cellular catabolism of recombinant tissue-type plasminogen activator. Identification and characterization of a novel high affinity uptake system on rat hepatocytes. *J. Biol. Chem.* 262:8716-8720.

Bass, R. and V.Ellis. 2002. Cellular mechanisms regulating non-haemostatic plasmin generation. *Biochem. Soc. Trans.* 30:189-194.

Behrendt N., M.Ploug, E.Ronne, G.Hoyer-Hansen, and K.Dano. 1993. Cellular Receptor for urokinase-type plasminogen activator : protein structure. *Methods Enzymol.* 223:207-222

Bell, W.R. 2002. Present-day thrombolytic therapy: therapeutic agents--pharmacokinetics and pharmacodynamics. *Rev. Cardiovasc. Med.* 3 Suppl 2:S34-S44.

Berditchevski, F., G.Bazzoni, and M.E.Hemler. 1995. Specific association of CD63 with the VLA-3 and VLA-6 integrins. *J. Biol. Chem.* 270:17784-17790.

Berditchevski, F., K.F.Tolias, K.Wong, C.L.Carpenter, and M.E.Hemler. 1997. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J. Biol. Chem.* 272:2595-2598.

Blasi, F. 1997. uPA, uPAR, PAI-1: key intersection of proteolytic, adhesive and chemotactic highways? *Immunol. Today* 18:415-417.

Blasi, F. and P.Carmeliet. 2002. uPAR: a versatile signalling orchestrator. *Nat. Rev. Mol. Cell Biol.* 3:932-943.

Blasi, F., M.P.Stoppelli, and M.V.Cubellis. 1986. The receptor for urokinase-plasminogen activator. *J. Cell Biochem.* 32:179-186.

Blau, H.M. and F.M.Rossi. 1999. Tet B or not tet B: advances in tetracycline-inducible gene expression. *Proc. Natl. Acad. Sci. U. S. A* 96:797-799.

Bloch, W., E.Forsberg, S.Lentini, C.Brakebusch, K.Martin, H.W.Krell, U.H.Weidle, K.Addicks, and R.Fassler. 1997. Beta 1 integrin is essential for teratoma growth and angiogenesis. *J. Cell Biol.* 139:265-278.

Bohuslav, J., V.Horejsi, C.Hansmann, J.Stockl, U.H.Weidle, O.Majdic, I.Bartke, W.Knapp, and H.Stockinger. 1995. Urokinase plasminogen activator receptor, beta 2-integrins, and Src-kinases within a single receptor complex of human monocytes. *J. Exp. Med.* 181:1381-1390.

Boudreau, N.J. and P.L.Jones. 1999. Extracellular matrix and integrin signalling: the shape of things to come. *Biochem. J.* 339 (Pt 3):481-488.

Boxman, I.L., P.H.Quax, C.W.Lowik, S.E.Papapoulos, J.Verheijen, and M.Ponec. 1995. Differential regulation of plasminogen activation in normal keratinocytes and SCC-4 cells by fibroblasts. *J. Invest Dermatol.* 104:374-378.

Bretscher, M.S. 1992. Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. *EMBO J.* 11:405-410.

Bretscher, M.S. 1996. Moving membrane up to the front of migrating cells. *Cell* 85:465-467.

Breuss, J.M., N.Gillett, L.Lu, D.Sheppard, and R.Pytela. 1993. Restricted distribution of integrin beta 6 mRNA in primate epithelial tissues. *J. Histochem. Cytochem.* 41:1521-1527.

Breuss, J.M., J.Gallo, H.M.DeLisser, I.V.Klimanskaya, H.G.Folkesson, J.F.Pittet, S.L.Nishimura, K.Aldape, D.V.Landers, W.Carpenter, N.Gillet, D.Sheppard, M.A. Matthay, S.M.Albelda, R.H. Kramer and R. Pytela. 1995. Expression of the beta 6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. *J. Cell Sci.* 108 (Pt 6):2241-2251.

Brooks, P.C., R.A.Clark, and D.A.Cheresh. 1994. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 264:569-571.

Brooks, P.C., S.Stromblad, L.C.Sanders, T.L.von Schalscha, R.T.Aimes, W.G.Stetler-Stevenson, J.P.Quigley, and D.A.Cheresh. 1996. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 85:683-693.

Bugge, T.H., K.W.Kombrinck, Q.Xiao, K.Holmback, C.C.Daugherty, D.P.Witte, and J.L.Degen. 1997. Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice. *Blood* 90:4522-4531.

Bugge, T.H., L.R.Lund, K.K.Kombrinck, B.S.Nielsen, K.Holmback, A.F.Drew, M.J.Flick, D.P.Witte, K.Dano, and J.L.Degen. 1998. Reduced metastasis of Polyoma virus middle T antigen-induced mammary cancer in plasminogen-deficient mice. *Oncogene* 16:3097-3104.

Burridge, K. and M.Chrzanowska-Wodnicka. 1996. Focal adhesions, contractility, and signaling. *Annu. Rev. Cell Dev. Biol.* 12:463-518.

Busk, M., R.Pytela, and D.Sheppard. 1992. Characterization of the integrin alpha v beta 6 as a fibronectin-binding protein. *J. Biol. Chem.* 267:5790-5796.

Busso, N., S.K.Masur, D.Lazega, S.Waxman, and L.Ossowski. 1994. Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. *J. Cell Biol.* 126:259-270.

Calderwood, D.A., R.Zent, R.Grant, D.J.Rees, R.O.Hynes, and M.H.Ginsberg. 1999. The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. *J. Biol. Chem.* 274:28071-28074.

Calderwood, D.A., A.Huttenlocher, W.B.Kiosses, D.M.Rose, D.G.Woodside, M.A.Schwartz, and M.H.Ginsberg. 2001. Increased filamin binding to beta-integrin cytoplasmic domains inhibits cell migration. *Nat. Cell Biol.* 3:1060-1068.

Calderwood, D.A., B.Yan, J.M.de Pereda, B.G.Alvarez, Y.Fujioka, R.C.Liddington, and M.H.Ginsberg. 2002. The phosphotyrosine binding-like domain of talin activates integrins. *J. Biol. Chem.* 277:21749-21758.

Carmeliet, P., R.C.Mulligan, and D.Collen. 1994. Transgenic animals as tools for the study of fibrinolysis in vivo. *J. Intern. Med.* 236:455-459.

Carmeliet, P.F. 1995. Physiological consequences of over- or under-expression of fibrinolytic system components in transgenic mice. *Baillieres Clin. Haematol.* 8:391-401.

Carmeliet, P., L.Moons, R.Lijnen, M.Baes, V.Lemaitre, P.Tipping, A.Drew, Y.Eeckhout, S.Shapiro, F.Lupu, and D.Collen. 1997. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat. Genet.* 17:439-444.

Carreiras, F., V.Rigot, S.Cruet, F.Andre, P.Gauduchon, and J.Marvaldi. 1999. Migration properties of the human ovarian adenocarcinoma cell line IGROV1: importance of alpha(v)beta3 integrins and vitronectin. *Int. J. Cancer* 80:285-294.

Carriero, M.V., S.Del Vecchio, M.Capozzoli, P.Franco, L.Fontana, A.Zannetti, G.Botti, G.D'Aiuto, M.Salvatore, and M.P.Stoppelli. 1999. Urokinase receptor interacts with alpha(v)beta5 vitronectin receptor, promoting urokinase-dependent cell migration in breast cancer. *Cancer Res.* 59:5307-5314.

Carroll, P.M., W.G.Richards, A.L.Darrow, J.M.Wells, and S.Strickland. 1993. Preimplantation mouse embryos express a cell surface receptor for tissue-plasminogen activator. *Development* 119:191-198.

Chambers, A.F., A.C.Groom, and I.C.MacDonald. 2002. Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer* 2:563-572.

Chan, B.M., N.Matsuura, Y.Takada, B.R.Zetter, and M.E.Hemler. 1991. In vitro and in vivo consequences of VLA-2 expression on rhabdomyosarcoma cells. *Science* 251:1600-1602.

Chang, D.D., C.Wong, H.Smith, and J.Liu. 1997. ICAP-1, a novel beta1 integrin cytoplasmic domain-associated protein, binds to a conserved and functionally important NPXY sequence motif of beta1 integrin. *J. Cell Biol.* 138:1149-1157.

Chapman, H.A. 1997. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. *Curr. Opin. Cell Biol.* 9:714-724.

Chapman, H.A. and Y.Wei. 2001. Protease crosstalk with integrins: the urokinase receptor paradigm. *Thromb. Haemost.* 86:124-129.

Chen, J., C.Baskerville, Q.Han, Z.K.Pan, and S.Huang. 2001. Alpha(v) integrin, p38 mitogen-activated protein kinase, and urokinase plasminogen activator are functionally linked in invasive breast cancer cells. *J. Biol. Chem.* 276:47901-47905.

Chen, Y.P., T.E.O'Toole, T.Shipley, J.Forsyth, S.E.LaFlamme, K.M.Yamada, S.J.Shattil, and M.H.Ginsberg. 1994. "Inside-out" signal transduction inhibited by isolated integrin cytoplasmic domains. *J. Biol. Chem.* 269:18307-18310.

Chomczynski, P. and N.Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

Chung, J., A.G.Gao, and W.A.Frazier. 1997. Thrombspondin acts via integrin-associated protein to activate the platelet integrin alphaIIbbeta3. *J. Biol. Chem.* 272:14740-14746.

Ciambrone, G.J. and P.J.McKeown-Longo. 1992. Vitronectin regulates the synthesis and localization of urokinase-type plasminogen activator in HT-1080 cells. *J. Biol. Chem.* 267:13617-13622.

Clark, E.A. and J.S.Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science* 268:233-239.

Clark, R.A., G.S.Ashcroft, M.J.Spencer, H.Larjava, and M.W.Ferguson. 1996. Re-epithelialization of normal human excisional wounds is associated with a switch from alpha v beta 5 to alpha v beta 6 integrins. *Br. J. Dermatol.* 135:46-51.

Clowes, A.W., M.M.Clowes, Y.P.Au, M.A.Reidy, and D.Belin. 1990. Smooth muscle cells express urokinase during mitogenesis and tissue-type plasminogen activator during migration in injured rat carotid artery. *Circ. Res.* 67:61-67.

Cohen, S.N., A.C.Chang, and L.Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. *Proc. Natl. Acad. Sci. U. S. A* 69:2110-2114.

Conese, M., A.Nykjaer, C.M.Petersen, O.Cremona, R.Pardi, P.A.Andreasen, J.Gliemann, E.I.Christensen, and F.Blasi. 1995. alpha-2 Macroglobulin receptor/Ldl receptor-related protein(Lrp)-dependent internalization of the urokinase receptor. *J. Cell Biol.* 131:1609-1622.

Cooper, C.R., C.H.Chay, and K.J.Pienta. 2002. The role of alpha(v)beta(3) in prostate cancer progression. *Neoplasia.* 4:191-194.

Coppolino, M., C.Leung-Hagesteijn, S.Dedhar, and J.Wilkins. 1995. Inducible interaction of integrin alpha 2 beta 1 with calreticulin. Dependence on the activation state of the integrin. *J. Biol. Chem.* 270:23132-23138.

Cowles, E.A., L.L.Brailey, and G.A.Gronowicz. 2000. Integrin-mediated signaling regulates AP-1 transcription factors and proliferation in osteoblasts. *J. Biomed. Mater. Res.* 52:725-737.

Crooke, S.T. 1998a. An overview of progress in antisense therapeutics. *Antisense Nucleic Acid Drug Dev.* 8:115-122.

Crooke, S.T. 1998b. Antisense therapeutics. *Biotechnol. Genet. Eng Rev.* 15:121-157.

Crowley, C.W., R.L.Cohen, B.K.Lucas, G.Liu, M.A.Shuman, and A.D.Levinson. 1993. Prevention of metastasis by inhibition of the urokinase receptor. *Proc. Natl. Acad. Sci. U. S. A* 90:5021-5025.

Cubellis, M.V., M.L.Nolli, G.Cassani, and F.Blasi. 1986. Binding of single-chain prourokinase to the urokinase receptor of human U937 cells. *J. Biol. Chem.* 261:15819-15822.

Cubellis, M.V., P.Andreasen, P.Ragno, M.Mayer, K.Dano, and F.Blasi. 1989. Accessibility of receptor-bound urokinase to type-1 plasminogen activator inhibitor. *Proc. Natl. Acad. Sci. U. S. A* 86:4828-4832.

Cubellis, M.V., T.C.Wun, and F.Blasi. 1990. Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1. *EMBO J.* 9:1079-1085.

Damsky, C.H. and Z.Werb. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* 4:772-781.

Dano, K., P.A.Andreasen, J.Grondahl-Hansen, P.Kristensen, L.S.Nielsen, and L.Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* 44:139-266.

Dano, K., J.Romer, B.S.Nielsen, S.Bjorn, C.Pyke, J.Rygaard, and L.R.Lund. 1999. Cancer invasion and tissue remodeling--cooperation of protease systems and cell types. *APMIS* 107:120-127.

De Luca, M., G.Pellegrini, G.Zambruno, and P.C.Marchisio. 1994. Role of integrins in cell adhesion and polarity in normal keratinocytes and human skin pathologies. *J. Dermatol.* 21:821-828.

Dear, A.E. and R.L.Medcalf. 1998. The urokinase-type-plasminogen-activator receptor (CD87) is a pleiotropic molecule. *Eur. J. Biochem.* 252:185-193.

Dedhar, S. and G.E.Hannigan. 1996. Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Curr. Opin. Cell Biol.* 8:657-669.

Dedhar, S. and R.Saulnier. 1990. Alterations in integrin receptor expression on chemically transformed human cells: specific enhancement of laminin and collagen receptor complexes. *J. Cell Biol.* 110:481-489.

Degen, S.J., J.L.Heckel, E.Reich, and J.L.Degen. 1987. The murine urokinase-type plasminogen activator gene. *Biochemistry* 26:8270-8279.

Del Rosso, M., E.Anichini, N.Pedersen, F.Blasi, G.Fibbi, M.Pucci, and M.Ruggiero. 1993. Urokinase-urokinase receptor interaction: non-mitogenic signal transduction in human epidermal cells. *Biochem. Biophys. Res. Commun.* 190:347-352.

Deng, G., G.Royle, D.Seiffert, and D.J.Loskutoff. 1995. The PAI-1/vitronectin interaction: two cats in a bag? *Thromb. Haemost.* 74:66-70.

Dent, M.A., Y.Sumi, R.J.Morris, and P.J.Seeley. 1993. Urokinase-type plasminogen activator expression by neurons and oligodendrocytes during process outgrowth in developing rat brain. *Eur. J. Neurosci.* 5:633-647.

Deutinger, J., J.C.Kirchheimer, A.Reinthaller, G.Christ, G.Tatra, and B.R.Binder. 1988. Elevated tissue type plasminogen activator in human granulosa cells correlates with fertilizing capacity. *Hum. Reprod.* 3:597-599.

Dobrovolsky, A.B. and E.V.Titaeva. 2002. The fibrinolysis system: regulation of activity and physiologic functions of its main components. *Biochemistry (Mosc.)* 67:99-108.

Downer, C.S., F.M.Watt, and P.M.Speight. 1993. Loss of alpha 6 and beta 4 integrin subunits coincides with loss of basement membrane components in oral squamous cell carcinomas. *J. Pathol.* 171:183-190.

Du, X.P., E.F.Plow, A.L.Frelinger, III, T.E.O'Toole, J.C.Loftus, and M.H.Ginsberg. 1991. Ligands "activate" integrin alpha IIb beta 3 (platelet GPIIb-IIIa). *Cell* 65:409-416.

Duffy, M.J. 1993. Cellular oncogenes and suppressor genes as prognostic markers in cancer. *Clin. Biochem.* 26:439-447.

Duffy, M.J. 1995. Can molecular markers now be used for early diagnosis of malignancy? *Clin. Chem.* 41:1410-1413.

Duffy, M.J., C.Duggan, T.Maguire, K.Mulcahy, P.Elvin, E.McDermott, J.J.Fennelly, and N.O'Higgins. 1996. Urokinase plasminogen activator as a predictor of aggressive disease in breast cancer. *Enzyme Protein* 49:85-93.

Duffy, M.J., P.O'Grady, D.Devaney, L.O'Siorain, J.J.Fennelly, and H.J.Lijnen. 1988. Urokinase-plasminogen activator, a marker for aggressive breast carcinomas. Preliminary report. *Cancer* 62:531-533.

Dumler, I., T.Petri, and W.D.Schleuning. 1993. Interaction of urokinase-type plasminogenactivator (u-PA) with its cellular receptor (u-PAR) induces phosphorylation on tyrosine of a 38 kDa protein. *FEBS Lett.* 322:37-40.

Dumler, I., A.Weis, O.A.Mayboroda, C.Maasch, U.Jerke, H.Haller, and D.C.Gulba. 1998. The Jak/Stat pathway and urokinase receptor signaling in human aortic vascular smooth muscle cells. *J. Biol. Chem.* 273:315-321.

Elices, M.J., L.Osborn, Y.Takada, C.Crouse, S.Luhowskyj, M.E.Hemler, and R.R.Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* 60:577-584.

Ellgaard, L. and A.Helenius. 2001. ER quality control: towards an understanding at the molecular level. *Curr. Opin. Cell Biol.* 13:431-437.

Ellis, V., M.F.Scully, and V.V.Kakkar. 1989. Plasminogen activation initiated by single-chain urokinase-type plasminogen activator. Potentiation by U937 monocytes. *J. Biol. Chem.* 264:2185-2188.

Ellis, V., T.C.Wun, N.Behrendt, E.Ronne, and K.Dano. 1990. Inhibition of receptor-bound urokinase by plasminogen-activator inhibitors. *J. Biol. Chem.* 265:9904-9908.

Ellis, V., N.Behrendt, and K.Dano. 1991. Plasminogen activation by receptor-bound urokinase. A kinetic study with both cell-associated and isolated receptor. *J. Biol. Chem.* 266:12752-12758.

Ellis, V. and K.Dano. 1991. Plasminogen activation by receptor-bound urokinase. *Semin. Thromb. Hemost.* 17:194-200.

Ellis, V., C.Pyke, J.Eriksen, H.Solberg, and K.Dano. 1992. The urokinase receptor: involvement in cell surface proteolysis and cancer invasion. *Ann. N. Y. Acad. Sci.* 667:13-31.

Ellis, V. 1996. Functional analysis of the cellular receptor for urokinase in plasminogen activation. Receptor binding has no influence on the zymogenic nature of pro-urokinase. *J. Biol. Chem.* 271:14779-14784.

Ellis, V. and S.A.Whawell. 1997. Vascular smooth muscle cells potentiate plasmin generation by both urokinase and tissue plasminogen activator-dependent mechanisms: evidence for a specific tissue-type plasminogen activator receptor on these cells. *Blood* 90:2312-2322.

Ellis, V., S.A.Whawell, F.Werner, and J.J.Deadman. 1999. Assembly of urokinase receptor-mediated plasminogen activation complexes involves direct, non-active-site interactions between urokinase and plasminogen. *Biochemistry* 38:651-659.

Ellis, V. and G.Murphy. 2001. Cellular strategies for proteolytic targeting during migration and invasion. *FEBS Lett.* 506:1-5.

Engelholm, L.H., B.S.Nielsen, K.Dano, and N.Behrendt. 2001. The urokinase receptor associated protein (uPARAP/endo180): a novel internalization receptor connected to the plasminogen activation system. *Trends Cardiovasc. Med.* 11:7-13.

Evans, D.M. and K.Sloan-Stakleff. 2000. Suppression of the invasive capacity of human breast cancer cells by inhibition of urokinase plasminogen activator via amiloride and B428. *Am. Surg.* 66:460-464.

Fabbri, M., L.Fumagalli, G.Bossi, E.Bianchi, J.R.Bender, and R.Pardi. 1999. A tyrosine-based sorting signal in the beta2 integrin cytoplasmic domain mediates its recycling to the plasma membrane and is required for ligand-supported migration. *EMBO J.* 18:4915-4925.

Fassler, R. and M.Meyer. 1995. Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev.* 9:1896-1908.

Felding-Habermann, B., B.M.Mueller, C.A.Romerdahl, and D.A.Cheresh. 1992. Involvement of integrin alpha V gene expression in human melanoma tumorigenicity. *J. Clin. Invest* 89:2018-2022.

Felding-Habermann, B., T.E.O'Toole, J.W.Smith, E.Fransvea, Z.M.Ruggeri, M.H.Ginsberg, P.E.Hughes, N.Pampori, S.J.Shattil, A.Saven, and B.M.Mueller. 2001. Integrin activation controls metastasis in human breast cancer. *Proc. Natl. Acad. Sci. U. S. A* 98:1853-1858.

Felding-Habermann, B., E.Fransvea, T.E.O'Toole, L.Manzuk, B.Faha, and M.Hensler. 2002. Involvement of tumor cell integrin alpha v beta 3 in hematogenous metastasis of human melanoma cells. *Clin. Exp. Metastasis* 19:427-436.

Felding-Habermann, B. 2003. Integrin adhesion receptors in tumor metastasis. *Clin. Exp. Metastasis* 20:203-213.

Fernandez, C., K.Clark, L.Burrows, N.R.Schofield, and M.J.Humphries. 1998. Regulation of the extracellular ligand binding activity of integrins. *Front Biosci.* 3:D684-D700.

Filardo, E.J., P.C.Brooks, S.L.Deming, C.Damsky, and D.A.Cheresh. 1995. Requirement of the NPXY motif in the integrin beta 3 subunit cytoplasmic tail for melanoma cell migration in vitro and in vivo. *J. Cell Biol.* 130:441-450.

Fisher A. 1925. Beitrag zur Biologie der Gewebezellen. Eine vergleichend-biologische Studie der normalen und malignen Gewebzellen in vitro. *Z Krebsforsch.* 25:89

Folkman, J. and Y.Shing. 1992. Angiogenesis. *J. Biol. Chem.* 267:10931-10934.

Frelinger, A.L., III, X.P.Du, E.F.Plow, and M.H.Ginsberg. 1991. Monoclonal antibodies to ligand-occupied conformers of integrin alpha IIb beta 3 (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function. *J. Biol. Chem.* 266:17106-17111.

Fukao, H., S.Ueshima, T.Takaishi, K.Okada, and O.Matsuo. 1997. Enhancement of tissue-type plasminogen activator (t-PA) activity by purified t-PA receptor expressed in human endothelial cells. *Biochim. Biophys. Acta* 1356:111-120.

Furth, P.A., L.St Onge, H.Boger, P.Gruss, M.Gossen, A.Kistner, H.Bujard, and L.Hennighausen. 1994. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc. Natl. Acad. Sci. U. S. A* 91:9302-9306.

Gailit, J., M.P.Welch, and R.A.Clark. 1994. TGF-beta 1 stimulates expression of keratinocyte integrins during re-epithelialization of cutaneous wounds. *J. Invest Dermatol.* 103:221-227.

Gewirtz, A.M., D.L.Sokol, and M.Z.Ratajczak. 1998. Nucleic acid therapeutics: state of the art and future prospects. *Blood* 92:712-736.

Ghosh, S., R.Brown, J.C.Jones, S.M.Ellerbroek, and M.S.Stack. 2000. Urinary-type plasminogen activator (uPA) expression and uPA receptor localization are regulated by alpha 3beta 1 integrin in oral keratinocytes. *J. Biol. Chem.* 275:23869-23876.

Giancotti, F.G. 2000. Complexity and specificity of integrin signalling. *Nat. Cell Biol.* 2:E13-E14.

Giancotti, F.G. 2003. A structural view of integrin activation and signaling. *Dev. Cell* 4:149-151.

Giancotti, F.G. and F.Mainiero. 1994. Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim. Biophys. Acta* 1198:47-64.

Giancotti, F.G. and E.Ruoslahti. 1999. Integrin signaling. *Science* 285:1028-1032.

Gossen, M. and H.Bujard. 1995. Efficacy of tetracycline-controlled gene expression is influenced by cell type: commentary. *Biotechniques* 19:213-216.

Griffioen, A.W. and G.Molema. 2000. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol. Rev.* 52:237-268.

Grimaldi, G., P.Di Fiore, E.K.Locatelli, J.Falco, and F.Blasi. 1986. Modulation of urokinase plasminogen activator gene expression during the transition from quiescent to proliferative state in normal mouse cells. *EMBO J.* 5:855-861.

Grondahl-Hansen, J., L.R.Lund, E.Ralfkiaer, V.Ottevanger, and K.Dano. 1988. Urokinase- and tissue-type plasminogen activators in keratinocytes during wound reepithelialization in vivo. *J. Invest Dermatol.* 90:790-795.

Grondahl-Hansen, J., E.Ralfkiaer, L.T.Kirkeby, P.Kristensen, L.R.Lund, and K.Dano. 1991. Localization of urokinase-type plasminogen activator in stromal cells in adenocarcinomas of the colon in humans. *Am. J. Pathol.* 138:111-117.

Gum, R., J.Juarez, H.Allgayer, A.Mazar, Y.Wang, and D.Boyd. 1998. Stimulation of urokinase-type plasminogen activator receptor expression by PMA requires JNK1-dependent and -independent signaling modules. *Oncogene* 17:213-225.

Gunzler, W.A., G.J.Steffens, F.Otting, G.Buse, and L.Flohe. 1982. Structural relationship between human high and low molecular mass urokinase. *Hoppe Seylers. Z. Physiol Chem.* 363:133-141.

Gyetko, M.R., R.F.Todd, III, C.C.Wilkinson, and R.G.Sitrin. 1994. The urokinase receptor is required for human monocyte chemotaxis in vitro. *J. Clin. Invest* 93:1380-1387.

Haapasalmi, K., K.Zhang, M.Tonnesen, J.Olerud, D.Sheppard, T.Salo, R.Kramer, R.A.Clark, V.J.Uitto, and H.Larjava. 1996. Keratinocytes in human wounds express alpha v beta 6 integrin. *J. Invest Dermatol.* 106:42-48.

Hagedorn, M. and A.Bikfalvi. 2000. Target molecules for anti-angiogenic therapy: from basic research to clinical trials. *Crit Rev. Oncol. Hematol.* 34:89-110.

Hamidi, S., T.Salo, T.Kainulainen, J.Epstein, K.Lerner, and H.Larjava. 2000. Expression of alpha(v)beta6 integrin in oral leukoplakia. *Br. J. Cancer* 82:1433-1440.

Han, D.C., L.G.Rodriguez, and J.L.Guan. 2001a. Identification of a novel interaction between integrin beta1 and 14-3-3beta. *Oncogene* 20:346-357.

Han, J., S.Liu, D.M.Rose, D.D.Schlaepfer, H.McDonald, and M.H.Ginsberg. 2001b. Phosphorylation of the integrin alpha 4 cytoplasmic domain regulates paxillin binding. *J. Biol. Chem.* 276:40903-40909.

Han, Q., J.Leng, D.Bian, C.Mahanivong, K.A.Carpenter, Z.K.Pan, J.Han, and S.Huang. 2002. Rac1-MKK3-p38-MAPKAPK2 pathway promotes urokinase plasminogen activator mRNA stability in invasive breast cancer cells. *J. Biol. Chem.* 277:48379-48385.

Hanahan, D. and R.A.Weinberg. 2000. The hallmarks of cancer. *Cell* 100:57-70.

Hannigan, G.E., C.Leung-Hagesteijn, L.Fitz-Gibbon, M.G.Coppolino, G.Radeva, J.Filmus, J.C.Bell, and S.Dedhar. 1996. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. *Nature* 379:91-96.

Hapke, S., M.Gawaz, K.Dehne, J.Kohler, J.F.Marshall, H.Graeff, M.Schmitt, U.Reuning, and E.Lengyel. 2001a. beta(3)A-integrin downregulates the urokinase-type plasminogen activator receptor (u-PAR) through a PEA3/ets transcriptional silencing element in the u-PAR promoter. *Mol. Cell Biol.* 21:2118-2132.

Hapke, S., H.Kessler, d.P.Arroyo, A.Benge, M.Schmitt, E.Lengyel, and U.Reuning. 2001b. Integrin alpha(v)beta(3)/vitronectin interaction affects expression of the urokinase system in human ovarian cancer cells. *J. Biol. Chem.* 276:26340-26348.

Hart, I.R., M.Birch, and J.F.Marshall. 1991. Cell adhesion receptor expression during melanoma progression and metastasis. *Cancer Metastasis Rev.* 10:115-128.

Heiss, M.M., H.Allgayer, K.U.Gruetzner, I.Funke, R.Babic, K.W.Jauch, and F.W.Schildberg. 1995. Individual development and uPA-receptor expression of

disseminated tumour cells in bone marrow: a reference to early systemic disease in solid cancer. *Nat. Med.* 1:1035-1039.

Hemler, M.E., B.A.Mannion, and F.Berditchevski. 1996. Association of TM4SF proteins with integrins: relevance to cancer. *Biochim. Biophys. Acta* 1287:67-71.

Henderson, B.R., W.P.Tansey, S.M.Phillips, I.A.Ramshaw, and R.F.Kefford. 1992. Transcriptional and posttranscriptional activation of urokinase plasminogen activator gene expression in metastatic tumor cells. *Cancer Res.* 52:2489-2496.

Hewitt, R. and K.Dano. 1996. Stromal cell expression of components of matrix-degrading protease systems in human cancer. *Enzyme Protein* 49:163-173.

Hewitt, R.E., I.H.Leach, D.G.Powe, I.M.Clark, T.E.Cawston, and D.R.Turner. 1991. Distribution of collagenase and tissue inhibitor of metalloproteinases (TIMP) in colorectal tumours. *Int. J. Cancer* 49:666-672.

Hicks, W.L., Jr., T.R.Loree, R.I.Garcia, S.Maamoun, D.Marshall, J.B.Orner, V.Y.Bakamjian, and D.P.Shedd. 1997. Squamous cell carcinoma of the floor of mouth: a 20-year review. *Head Neck* 19:400-405.

Hill, C.S. and R.Treisman. 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* 80:199-211.

Hodivala-Dilke, K.M., K.P.McHugh, D.A.Tsakiris, H.Rayburn, D.Crowley, M.Ullman-Cullere, F.P.Ross, B.S.Coller, S.Teitelbaum, and R.O.Hynes. 1999. Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J. Clin. Invest* 103:229-238.

Holly, S.P., M.K.Larson, and L.V.Parise. 2000. Multiple roles of integrins in cell motility. *Exp. Cell Res.* 261:69-74.

Hood, J.D. and D.A.Cheresh. 2002. Role of integrins in cell invasion and migration. *Nat. Rev. Cancer* 2:91-100.

Hooper, J.D., J.A.Clements, J.P.Quigley, and T.M.Antalis. 2001. Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. *J. Biol. Chem.* 276:857-860.

Horwitz, A., K.Duggan, C.Buck, M.C.Beckerle, and K.Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin--a transmembrane linkage. *Nature* 320:531-533.

Hoshino, R., Y.Chatani, T.Yamori, T.Tsuruo, H.Oka, O.Yoshida, Y.Shimada, Ari-i S, H.Wada, J.Fujimoto, and M.Kohno. 1999. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* 18:813-822.

Howe, A., A.E.Aplin, S.K.Alahari, and R.L.Juliano. 1998. Integrin signaling and cell growth control. *Curr. Opin. Cell Biol.* 10:220-231.

Hoyer-Hansen, G., E.Ronne, H.Solberg, N.Behrendt, M.Ploug, L.R.Lund, V.Ellis, and K.Dano. 1992b. Urokinase plasminogen activator cleaves its cell surface receptor releasing the ligand-binding domain. *J. Biol. Chem.* 267:18224-18229.

Hoyer-Hansen, G., E.Ronne, H.Solberg, N.Behrendt, M.Ploug, L.R.Lund, V.Ellis, and K.Dano. 1992a. Urokinase plasminogen activator cleaves its cell surface receptor releasing the ligand-binding domain. *J. Biol. Chem.* 267:18224-18229.

Hsu, M.Y., D.T.Shih, F.E.Meier, P.Van Belle, J.Y.Hsu, D.E.Elder, C.A.Buck, and M.Herlyn. 1998. Adenoviral gene transfer of beta3 integrin subunit induces conversion from radial to vertical growth phase in primary human melanoma. *Am. J. Pathol.* 153:1435-1442.

Huang, X., M.Griffiths, J.Wu, R.V.Farese, Jr., and D.Sheppard. 2000. Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. *Mol. Cell Biol.* 20:755-759.

Huang, X., J.Wu, W.Zhu, R.Pytela, and D.Sheppard. 1998. Expression of the human integrin beta6 subunit in alveolar type II cells and bronchiolar epithelial cells reverses lung inflammation in beta6 knockout mice. *Am. J. Respir. Cell Mol. Biol.* 19:636-642.

Huang, X.Z., A.Chen, M.Agrez, and D.Sheppard. 1995. A point mutation in the integrin beta 6 subunit abolishes both alpha v beta 6 binding to fibronectin and receptor localization to focal contacts. *Am. J. Respir. Cell Mol. Biol.* 13:245-251.

Huang, X.Z., J.F.Wu, D.Cass, D.J.Erle, D.Corry, S.G.Young, R.V.Farese, Jr., and D.Sheppard. 1996a. Inactivation of the integrin beta 6 subunit gene reveals a role of

epithelial integrins in regulating inflammation in the lung and skin. *J. Cell Biol.* 133:921-928.

Huang, X.Z., J.F.Wu, D.Cass, D.J.Erle, D.Corry, S.G.Young, R.V.Farese, Jr., and D.Sheppard. 1996b. Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin. *J. Cell Biol.* 133:921-928.

Hughes, P.E. and M.Pfaff. 1998. Integrin affinity modulation. *Trends Cell Biol.* 8:359-364.

Humphries, M.J. 1996. Integrin activation: the link between ligand binding and signal transduction. *Curr. Opin. Cell Biol.* 8:632-640.

Humphries, M.J. 2002. Insights into integrin-ligand binding and activation from the first crystal structure. *Arthritis Res.* 4 Suppl 3:S69-S78.

Hynes, R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.

Hynes, R.O. 1999. Cell adhesion: old and new questions. *Trends Cell Biol.* 9:M33-M37.

Hynes, R.O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell* 110:673-687.

Irigoyen, J.P., P.Munoz-Canoves, L.Montero, M.Koziczak, and Y.Nagamine. 1999. The plasminogen activator system: biology and regulation. *Cell Mol. Life Sci.* 56:104-132.

Irigoyen, J.P. and Y.Nagamine. 1999. Cytoskeletal reorganization leads to induction of the urokinase-type plasminogen activator gene by activating FAK and Src and subsequently the Ras/Erk signaling pathway. *Biochem. Biophys. Res. Commun.* 262:666-670.

Ito, H., Y.Seyama, and S.Kubota. 2001. Calreticulin is directly involved in anti-alpha3 integrin antibody-mediated secretion and activation of matrix metalloprotease-2. *Biochem. Biophys. Res. Commun.* 283:297-302.

Ivaska, J., R.D.Whelan, R.Watson, and P.J.Parker. 2002. PKC epsilon controls the traffic of beta1 integrins in motile cells. *EMBO J.* 21:3608-3619.

Jensen, P.J., M.John, and J.Baird. 1990. Urokinase and tissue type plasminogen activators in human keratinocyte culture. *Exp. Cell Res.* 187:162-169.

Jensen, P.J. and U.Rodeck. 1993. Autocrine/paracrine regulation of keratinocyte urokinase plasminogen activator through the TGF-alpha/EGF receptor. *J. Cell Physiol* 155:333-339.

Jiang, C.K., D.Connolly, and M.Blumenberg. 1991. Comparison of methods for transfection of human epidermal keratinocytes. *J. Invest Dermatol.* 97:969-973.

Johnsen, M., L.R.Lund, J.Romer, K.Almholt, and K.Dano. 1998. Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation. *Curr. Opin. Cell Biol.* 10:667-671.

Jones, J., M.Sugiyama, P.M.Speight, and F.M.Watt. 1996. Restoration of alpha v beta 5 integrin expression in neoplastic keratinocytes results in increased capacity for terminal differentiation and suppression of anchorage-independent growth. *Oncogene* 12:119-126.

Jones, J., M.Sugiyama, F.M.Watt, and P.M.Speight. 1993. Integrin expression in normal, hyperplastic, dysplastic, and malignant oral epithelium. *J. Pathol.* 169:235-243.

Jones, J., F.M.Watt, and P.M.Speight. 1997. Changes in the expression of alpha v integrins in oral squamous cell carcinomas. *J. Oral Pathol. Med.* 26:63-68.

Jordan, R.C. and T.Daley. 1997. Oral squamous cell carcinoma: new insights. *J. Can. Dent. Assoc.* 63:517-5.

Juliano, R.L. and J.A.Varner. 1993. Adhesion molecules in cancer: the role of integrins. *Curr. Opin. Cell Biol.* 5:812-818.

Keski-Oja, J. and A.Vaheri. 1982. The cellular target for the plasminogen activator, urokinase, in human fibroblasts - 66 000 dalton protein. *Biochim. Biophys. Acta* 720:141-146.

Khatib, A.M., J.Nip, L.Fallavollita, M.Lehmann, G.Jensen, and P.Brodt. 2001. Regulation of urokinase plasminogen activator/plasmin-mediated invasion of melanoma cells by the integrin vitronectin receptor alphaVbeta3. *Int. J. Cancer* 91:300-308.

Kindzelskii, A.L., Z.O.Laska, R.F.Todd, III, and H.R.Petty. 1996. Urokinase-type plasminogen activator receptor reversibly dissociates from complement receptor type 3 (alpha M beta 2' CD11b/CD18) during neutrophil polarization. *J. Immunol.* 156:297-309.

Kjoller, L. 2002. The urokinase plasminogen activator receptor in the regulation of the actin cytoskeleton and cell motility. *Biol. Chem.* 383:5-19.

Klemke, R.L., J.Leng, R.Molander, P.C.Brooks, K.Vuori, and D.A.Cheresh. 1998. CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. *J. Cell Biol.* 140:961-972.

Kleyman, T.R. and E.J.Cragoe, Jr. 1988. Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol.* 105:1-21.

Koivisto, L., R.Grenman, J.Heino, and H.Larjava. 2000. Integrins alpha5beta1, alphavbeta1, and alphavbeta6 collaborate in squamous carcinoma cell spreading and migration on fibronectin. *Exp. Cell Res.* 255:10-17.

Kolanus, W., W.Nagel, B.Schiller, L.Zeitlmann, S.Godar, H.Stockinger, and B.Seed. 1996. Alpha L beta 2 integrin/LFA-1 binding to ICAM-1 induced by cytohesin-1, a cytoplasmic regulatory molecule. *Cell* 86:233-242.

Komarov, A., E.Panchenko, A.Dobrovolsky, Y.Karpov, A.Deev, E.Titaeva, K.Davletov, A.Eshkeeva, and L.Markova. 2002. D-dimer and platelet aggregability are related to thrombotic events in patients with peripheral arterial occlusive disease. *Eur. Heart J.* 23:1309-1316.

Konakova, M., F.Hucho, and W.D.Schleuning. 1998. Downstream targets of urokinase-type plasminogen-activator-mediated signal transduction. *Eur. J. Biochem.* 253:421-429.

Kooistra, T., Y.Schrauwen, J.Arts, and J.J.Emeis. 1994. Regulation of endothelial cell t-PA synthesis and release. *Int. J. Hematol.* 59:233-255.

Kook, Y.H., J.Adamski, A.Zelent, and L.Ossowski. 1994. The effect of antisense inhibition of urokinase receptor in human squamous cell carcinoma on malignancy. *EMBO J.* 13:3983-3991.

Koretz, K., P.Schlag, L.Boumsell, and P.Moller. 1991. Expression of VLA-alpha 2, VLA-alpha 6, and VLA-beta 1 chains in normal mucosa and adenomas of the colon, and in colon carcinomas and their liver metastases. *Am. J. Pathol.* 138:741-750.

Koshelnick, Y., M.Ehart, P.Hufnagl, P.C.Heinrich, and B.R.Binder. 1997. Urokinase receptor is associated with the components of the JAK1/STAT1 signaling pathway and leads to activation of this pathway upon receptor clustering in the human kidney epithelial tumor cell line TCL-598. *J. Biol. Chem.* 272:28563-28567.

Kristensen, P., J.Eriksen, F.Blasi, and K.Dano. 1991. Two alternatively spliced mouse urokinase receptor mRNAs with different histological localization in the gastrointestinal tract. *J. Cell Biol.* 115:1763-1771.

Kronenwett, R., T.Graf, W.Nedbal, M.Weber, U.Steidl, U.P.Rohr, T.Mohler, and R.Haas. 2002. Inhibition of angiogenesis in vitro by alphav integrin-directed antisense oligonucleotides. *Cancer Gene Ther.* 9:587-596.

Kruithof, E.K., M.S.Baker, and C.L.Bunn. 1995. Biological and clinical aspects of plasminogen activator inhibitor type 2. *Blood* 86:4007-4024.

Kulkarni, S., T.C.Saido, K.Suzuki, and J.E.Fox. 1999. Calpain mediates integrin-induced signaling at a point upstream of Rho family members. *J. Biol. Chem.* 274:21265-21275.

LaFlamme, S.E., S.M.Homan, A.L.Bodeau, and A.M.Mastrangelo. 1997. Integrin cytoplasmic domains as connectors to the cell's signal transduction apparatus. *Matrix Biol.* 16:153-163.

Larjava, H., K.Haapasalmi, T.Salo, C.Wiebe, and V.J.Uitto. 1996. Keratinocyte integrins in wound healing and chronic inflammation of the human periodontium. *Oral Dis.* 2:77-86.

Larjava, H., T.Salo, K.Haapasalmi, R.H.Kramer, and J.Heino. 1993. Expression of integrins and basement membrane components by wound keratinocytes. *J. Clin. Invest* 92:1425-1435.

Lauffenburger, D.A. and A.F.Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell* 84:359-369.

Lengyel, E., E.Stepp, R.Gum, and D.Boyd. 1995. Involvement of a mitogen-activated protein kinase signaling pathway in the regulation of urokinase promoter activity by c-Ha-ras. *J. Biol. Chem.* 270:23007-23012.

Liapis, H., A.Flath, and S.Kitazawa. 1996. Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. *Diagn. Mol. Pathol.* 5:127-135.

Licato, L.L., T.O.Keku, J.I.Wurzelmann, S.C.Murray, J.T.Woosley, R.S.Sandler, and D.A.Brenner. 1997. In vivo activation of mitogen-activated protein kinases in rat intestinal neoplasia. *Gastroenterology* 113:1589-1598.

Lijnen, H.R. and D.Collen. 1982. Interaction of plasminogen activators and inhibitors with plasminogen and fibrin. *Semin. Thromb. Hemost.* 8:2-10.

Lijnen, H.R., H.B.Van, L.Frederix, M.C.Rio, and D.Collen. 2002a. Adipocyte hypertrophy in stromelysin-3 deficient mice with nutritionally induced obesity. *Thromb. Haemost.* 87:530-535.

Lijnen, H.R., H.B.Van, L.Frederix, M.C.Rio, and D.Collen. 2002b. Adipocyte hypertrophy in stromelysin-3 deficient mice with nutritionally induced obesity. *Thromb. Haemost.* 87:530-535.

Lindberg, F.P., H.D.Gresham, M.I.Reinhold, and E.J.Brown. 1996. Integrin-associated protein immunoglobulin domain is necessary for efficient vitronectin bead binding. *J. Cell Biol.* 134:1313-1322.

Liotta, L.A. and E.C.Kohn. 2001. The microenvironment of the tumour-host interface. *Nature* 411:375-379.

Liotta, L.A., C.N.Rao, and S.H.Barsky. 1983. Tumor invasion and the extracellular matrix. *Lab Invest* 49:636-649.

Liotta, L.A. and W.G.Stetler-Stevenson. 1991. Tumor invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res.* 51:5054s-5059s.

Liu, S., D.A.Calderwood, and M.H.Ginsberg. 2000. Integrin cytoplasmic domain-binding proteins. *J. Cell Sci.* 113 (Pt 20):3563-3571.

Loo, D.T., S.B.Kanner, and A.Aruffo. 1998. Filamin binds to the cytoplasmic domain of the beta1-integrin. Identification of amino acids responsible for this interaction. *J. Biol. Chem.* 273:23304-23312.

Loskutoff, D.J., S.A.Curriden, G.Hu, and G.Deng. 1999. Regulation of cell adhesion by PAI-1. *APMIS* 107:54-61.

Lukes, A., S.Mun-Bryce, M.Lukes, and G.A.Rosenberg. 1999. Extracellular matrix degradation by metalloproteinases and central nervous system diseases. *Mol. Neurobiol.* 19:267-284.

Ma, Z., D.J.Webb, M.Jo, and S.L.Gonias. 2001. Endogenously produced urokinase-type plasminogen activator is a major determinant of the basal level of activated ERK/MAP kinase and prevents apoptosis in MDA-MB-231 breast cancer cells. *J. Cell Sci.* 114:3387-3396.

MacKie, R.M. 1998. Incidence, risk factors and prevention of melanoma. *Eur. J. Cancer* 34 Suppl 3:S3-S6.

Mannion, B.A., F.Berditchevski, S.K.Kraeft, L.B.Chen, and M.E.Hemler. 1996. Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associated with integrin alpha 4 beta 1 (CD49d/CD29). *J. Immunol.* 157:2039-2047.

Mansour, S.J., W.T.Matten, A.S.Hermann, J.M.Candia, S.Rong, K.Fukasawa, G.F.Vande Woude, and N.G.Ahn. 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* 265:966-970.

Markus, G. 1988. The relevance of plasminogen activators to neoplastic growth. A review of recent literature. *Enzyme* 40:158-172.

Mars, W.M., R.Zarnegar, and G.K.Michalopoulos. 1993. Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA. *Am. J. Pathol.* 143:949-958.

Marshall, J.F., S.A.Nesbitt, M.H.Helfrich, M.A.Horton, K.Polakova, and I.R.Hart. 1991. Integrin expression in human melanoma cell lines: heterogeneity of vitronectin receptor composition and function. *Int. J. Cancer* 49:924-931.

Marshall, J.F., D.C.Rutherford, L.Happerfield, A.Hanby, A.C.McCartney, J.Newton-Bishop, and I.R.Hart. 1998. Comparative analysis of integrins in vitro and in vivo in uveal and cutaneous melanomas. *Br. J. Cancer* 77:522-529.

May, A.E., S.M.Kanse, L.R.Lund, R.H.Gisler, B.A.Imhof, and K.T.Preissner. 1998. Urokinase receptor (CD87) regulates leukocyte recruitment via beta 2 integrins in vivo. *J. Exp. Med.* 188:1029-1037.

Mazzieri, R., L.Masiero, L.Zanetta, S.Monea, M.Onisto, S.Garbisa, and P.Mignatti. 1997. Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cell-bound reactants. *EMBO J.* 16:2319-2332.

Mignatti, P. and D.B.Rifkin. 1993. Biology and biochemistry of proteinases in tumor invasion. *Physiol Rev.* 73:161-195.

Miles, L.A., C.M.Dahlberg, and E.F.Plow. 1988. The cell-binding domains of plasminogen and their function in plasma. *J. Biol. Chem.* 263:11928-11934.

Minden, A., A.Lin, M.McMahon, C.Lange-Carter, B.Derijard, R.J.Davis, G.L.Johnson, and M.Karin. 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* 266:1719-1723.

Miralles, F., M.Parra, C.Caelles, Y.Nagamine, J.Felez, and P.Munoz-Canoves. 1998. UV irradiation induces the murine urokinase-type plasminogen activator gene via the c-Jun N-terminal kinase signaling pathway: requirement of an AP1 enhancer element. *Mol. Cell Biol.* 18:4537-4547.

Miranti, C.K. and J.S.Brugge. 2002. Sensing the environment: a historical perspective on integrin signal transduction. *Nat. Cell Biol.* 4:E83-E90.

Mitjans, F., T.Meyer, C.Fittschen, S.Goodman, A.Jonczyk, J.F.Marshall, G.Reyes, and J.Piulats. 2000. In vivo therapy of malignant melanoma by means of antagonists of alphav integrins. *Int. J. Cancer* 87:716-723.

Mitjans, F., D.Sander, J.Adan, A.Sutter, J.M.Martinez, C.S.Jaggle, J.M.Moyano, H.G.Kreysch, J.Piulats, and S.L.Goodman. 1995. An anti-alpha v-integrin antibody that blocks integrin function inhibits the development of a human melanoma in nude mice. *J. Cell Sci.* 108 (Pt 8):2825-2838.

Miyamoto, S., H.Teramoto, J.S.Gutkind, and K.M.Yamada. 1996. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J. Cell Biol.* 135:1633-1642.

Mizejewski, G.J. 1999. Role of integrins in cancer: survey of expression patterns. *Proc. Soc. Exp. Biol. Med.* 222:124-138.

Moller, L.B. 1993. Structure and function of the urokinase receptor. *Blood Coagul. Fibrinolysis* 4:293-303.

Montero, L. and Y.Nagamine. 1999. Regulation by p38 mitogen-activated protein kinase of adenylate- and uridylate-rich element-mediated urokinase-type plasminogen activator (uPA) messenger RNA stability and uPA-dependent in vitro cell invasion. *Cancer Res.* 59:5286-5293.

Montesano, R., M.S.Pepper, U.Mohle-Steinlein, W.Risau, E.F.Wagner, and L.Orci. 1990. Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. *Cell* 62:435-445.

Montesano, R., J.V.Soriano, G.Hosseini, M.S.Pepper, and H.Schramek. 1999. Constitutively active mitogen-activated protein kinase kinase MEK1 disrupts morphogenesis and induces an invasive phenotype in Madin-Darby canine kidney epithelial cells. *Cell Growth Differ.* 10:317-332.

Montgomery, A.M., R.A.Reisfeld, and D.A.Cheresh. 1994. Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen. *Proc. Natl. Acad. Sci. U. S. A* 91:8856-8860.

Montuori, N., G.Rossi, and P.Ragno. 1999. Cleavage of urokinase receptor regulates its interaction with integrins in thyroid cells. *FEBS Lett.* 460:32-36.

Morioka, S., P.J.Jensen, and G.S.Lazarus. 1985. Human epidermal plasminogen activator. Characterization, localization, and modulation. *Exp. Cell Res.* 161:364-372.

Morioka, S., G.S.Lazarus, J.L.Baird, and P.J.Jensen. 1987. Migrating keratinocytes express urokinase-type plasminogen activator. *J. Invest Dermatol.* 88:418-423.

Mueller, B.M. 1996. Different roles for plasminogen activators and metalloproteinases in melanoma metastasis. *Curr. Top. Microbiol. Immunol.* 213 (Pt 1):65-80.

Munger, J.S., X.Huang, H.Kawakatsu, M.J.Griffiths, S.L.Dalton, J.Wu, J.F.Pittet, N.Kaminski, C.Garat, M.A.Matthay, D.B.Rifkin, and D.Sheppard. 1999. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 96:319-328.

Murphy, G., S.Atkinson, R.Ward, J.Gavrilovic, and J.J.Reynolds. 1992. The role of plasminogen activators in the regulation of connective tissue metalloproteinases. *Ann. N. Y. Acad. Sci.* 667:1-12.

Nagamine, Y., D.Pearson, M.S.Altus, and E.Reich. 1984. cDNA and gene nucleotide sequence of porcine plasminogen activator. *Nucleic Acids Res.* 12:9525-9541.

Naik, U.P., P.M.Patel, and L.V.Parise. 1997. Identification of a novel calcium-binding protein that interacts with the integrin alphaIIb cytoplasmic domain. *J. Biol. Chem.* 272:4651-4654.

Naldini, L., L.Tamagnone, E.Vigna, M.Sachs, G.Hartmann, W.Birchmeier, Y.Daikuhara, H.Tsubouchi, F.Blasi, and P.M.Comoglio. 1992. Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. *EMBO J.* 11:4825-4833.

Nanbu, R., P.A.Menoud, and Y.Nagamine. 1994. Multiple instability-regulating sites in the 3' untranslated region of the urokinase-type plasminogen activator mRNA. *Mol. Cell Biol.* 14:4920-4928.

Nanbu, R., L.Montero, D.D'Orazio, and Y.Nagamine. 1997. Enhanced stability of urokinase-type plasminogen activator mRNA in metastatic breast cancer MDA-MB-231 cells and LLC-PK1 cells down-regulated for protein kinase C--correlation with cytoplasmic heterogeneous nuclear ribonucleoprotein C. *Eur. J. Biochem.* 247:169-174.

Newham, P. and M.J.Humphries. 1996. Integrin adhesion receptors: structure, function and implications for biomedicine. *Mol. Med. Today* 2:304-313.

Ng, T., D.Shima, A.Squire, P.I.Bastiaens, S.Gschmeissner, M.J.Humphries, and P.J.Parker. 1999. PKCalpha regulates beta1 integrin-dependent cell motility through association and control of integrin traffic. *EMBO J.* 18:3909-3923.

Nguyen, D.H., A.D.Catling, D.J.Webb, M.Sankovic, L.A.Walker, A.V.Somlyo, M.J.Weber, and S.L.Gonias. 1999. Myosin light chain kinase functions downstream of Ras/ERK to promote migration of urokinase-type plasminogen activator-stimulated cells in an integrin-selective manner. *J. Cell Biol.* 146:149-164.

Nguyen, D.H., I.M.Hussaini, and S.L.Gonias. 1998. Binding of urokinase-type plasminogen activator to its receptor in MCF-7 cells activates extracellular signal-regulated kinase 1 and 2 which is required for increased cellular motility. *J. Biol. Chem.* 273:8502-8507.

Nielsen, L.S., P.A.Andreasen, J.Grondahl-Hansen, L.Skriver, and K.Dano. 1986. Plasminogen activators catalyse conversion of inhibitor from fibrosarcoma cells to an inactive form with a lower apparent molecular mass. *FEBS Lett.* 196:269-273.

Nip, J., S.A.Rabbani, H.R.Shibata, and P.Brodt. 1995. Coordinated expression of the vitronectin receptor and the urokinase-type plasminogen activator receptor in metastatic melanoma cells. *J. Clin. Invest* 95:2096-2103.

Niu, J., D.J.Dorahy, X.Gu, R.J.Scott, B.Draganic, N.Ahmed, and M.V.Agrez. 2002. Integrin expression in colon cancer cells is regulated by the cytoplasmic domain of the beta6 integrin subunit. *Int. J. Cancer* 99:529-537.

Niu, J., X.Gu, J.Turton, C.Meldrum, E.W.Howard, and M.Agrez. 1998. Integrin-mediated signalling of gelatinase B secretion in colon cancer cells. *Biochem. Biophys. Res. Commun.* 249:287-291.

No, D., T.P.Yao, and R.M.Evans. 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. U. S. A* 93:3346-3351.

Nykjaer, A., M.Conese, E.I.Christensen, D.Olson, O.Cremona, J.Gliemann, and F.Blasi. 1997. Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes. *EMBO J.* 16:2610-2620.

O'Toole, E.A. 2001. Extracellular matrix and keratinocyte migration. *Clin. Exp. Dermatol.* 26:525-530.

O'Toole, T.E., D.Mandelman, J.Forsyth, S.J.Shattil, E.F.Plow, and M.H.Ginsberg. 1991. Modulation of the affinity of integrin alpha IIb beta 3 (GPIIb-IIIa) by the cytoplasmic domain of alpha IIb. *Science* 254:845-847.

O'Toole, T.E., J.Ylanne, and B.M.Culley. 1995. Regulation of integrin affinity states through an NPXY motif in the beta subunit cytoplasmic domain. *J. Biol. Chem.* 270:8553-8558.

Ohba, M., K.Ishino, M.Kashiwagi, S.Kawabe, K.Chida, N.H.Huh, and T.Kuroki. 1998. Induction of differentiation in normal human keratinocytes by adenovirus-mediated introduction of the eta and delta isoforms of protein kinase C. *Mol. Cell Biol.* 18:5199-5207.

Okahara, H., H.Yagita, K.Miyake, and K.Okumura. 1994. Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor alpha enhancement of experimental metastasis. *Cancer Res.* 54:3233-3236.

Okamoto, C.T. 1998. Endocytosis and transcytosis. *Adv. Drug Deliv. Rev.* 29:215-228.

Olivier, P., G.Bieler, K.M.Muller, D.Hauzenberger, and C.Ruegg. 1999. Urokinase-type plasminogen activator inhibits alpha 4 beta 1 integrin-mediated T lymphocyte adhesion to fibronectin independently of its catalytic activity. *Eur. J. Immunol.* 29:3196-3209.

Ossowski, L. Reich. E. 1983. Antibodies to plasminogen activator inhibit human tumor metastasis.*Cell.* 35(3 Pt 2):611-9.

Ossowski, L. 1988. Plasminogen activator dependent pathways in the dissemination of human tumor cells in the chick embryo.*Cell.* 52(3):321-8.

Ossowski, L. 1992. Invasion of connective tissue by human carcinoma cell lines: requirement for urokinase, urokinase receptor, and interstitial collagenase. *Cancer Res.* 52:6754-6760.

Ossowski, L. and J.A.Aguirre-Ghiso. 2000. Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. *Curr. Opin. Cell Biol.* 12:613-620.

Ossowski, L. and E.Reich. 1983. Changes in malignant phenotype of a human carcinoma conditioned by growth environment. *Cell* 33:323-333.

Ossowski, L., H.Russo-Payne, and E.L.Wilson. 1991. Inhibition of urokinase-type plasminogen activator by antibodies: the effect on dissemination of a human tumor in the nude mouse. *Cancer Res.* 51:274-281.

Otey, C.A., F.M.Pavalko, and K.Burridge. 1990. An interaction between alpha-actinin and the beta 1 integrin subunit in vitro. *J. Cell Biol.* 111:721-729.

Palecek, S.P., A.F.Horwitz, and D.A.Lauffenburger. 1999. Kinetic model for integrin-mediated adhesion release during cell migration. *Ann. Biomed. Eng* 27:219-235.

Palecek, S.P., A.Huttenlocher, A.F.Horwitz, and D.A.Lauffenburger. 1998. Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *J. Cell Sci.* 111 (Pt 7):929-940.

Palecek, S.P., C.E.Schmidt, D.A.Lauffenburger, and A.F.Horwitz. 1996. Integrin dynamics on the tail region of migrating fibroblasts. *J. Cell Sci.* 109 (Pt 5):941-952.

Partridge, M., A.Vincent, P.Matthews, J.Puma, D.Stein, and J.Summerton. 1996. A simple method for delivering morpholino antisense oligos into the cytoplasm of cells. *Antisense Nucleic Acid Drug Dev.* 6:169-175.

Pennica, D., W.E.Holmes, W.J.Kohr, R.N.Harkins, G.A.Vehar, C.A.Ward, W.F.Bennett, E.Yelverton, P.H.Seeburg, H.L.Heyneker, D.V.Goeddel, and D.Collen. 1983. Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. *Nature* 301:214-221.

Pepper, M.S. and R.Montesano. 1990. Proteolytic balance and capillary morphogenesis. *Cell Differ. Dev.* 32:319-327.

Pepper, M.S., A.P.Sappino, R.Stocklin, R.Montesano, L.Orci, and J.D.Vassalli. 1993. Upregulation of urokinase receptor expression on migrating endothelial cells. *J. Cell Biol.* 122:673-684.

Petitclerc, E., S.Stromblad, T.L.von Schalscha, F.Mitjans, J.Piulats, A.M.Montgomery, D.A.Cheresh, and P.C.Brooks. 1999. Integrin alpha(v)beta3

promotes M21 melanoma growth in human skin by regulating tumor cell survival. *Cancer Res.* 59:2724-2730.

Pignatelli, M. and W.F.Bodmer. 1990. Integrin cell adhesion molecules and colorectal cancer. *J. Pathol.* 162:95-97.

Planus, E., G.Barlovatz-Meimon, R.A.Rogers, S.Bonavaud, D.E.Ingber, and N.Wang. 1997. Binding of urokinase to plasminogen activator inhibitor type-1 mediates cell adhesion and spreading. *J. Cell Sci.* 110 (Pt 9):1091-1098.

Ploug, M. and V.Ellis. 1994. Structure-function relationships in the receptor for urokinase-type plasminogen activator. Comparison to other members of the Ly-6 family and snake venom alpha-neurotoxins. *FEBS Lett.* 349:163-168.

Plow, E.F., D.E.Freaney, J.Plescia, and L.A.Miles. 1986. The plasminogen system and cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. *J. Cell Biol.* 103:2411-2420.

Porter, J.C. and N.Hogg. 1998. Integrins take partners: cross-talk between integrins and other membrane receptors. *Trends Cell Biol.* 8:390-396.

Potempa, J., E.Korzus, and J.Travis. 1994. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. *J. Biol. Chem.* 269:15957-15960.

Preissner, K.T., S.M.Kanse, and A.E.May. 2000. Urokinase receptor: a molecular organizer in cellular communication. *Curr. Opin. Cell Biol.* 12:621-628.

Prieto, A.L., G.M.Edelman, and K.L.Crossin. 1993. Multiple integrins mediate cell attachment to cytotactin/tenascin. *Proc. Natl. Acad. Sci. U. S. A* 90:10154-10158.

Prime, S.S., S.V.Nixon, I.J.Crane, A.Stone, J.B.Matthews, N.J.Maitland, L.Remnant, S.K.Powell, S.M.Game, and C.Scully. 1990. The behaviour of human oral squamous cell carcinoma in cell culture. *J. Pathol.* 160:259-269.

Pyke, C., N.Graem, E.Ralfkiaer, E.Ronne, G.Hoyer-Hansen, N.Brunner, and K.Dano. 1993. Receptor for urokinase is present in tumor-associated macrophages in ductal breast carcinoma. *Cancer Res.* 53:1911-1915.

Pyke, C., P.Kristensen, E.Ralfkiaer, J.Grondahl-Hansen, J.Eriksen, F.Blasi, and K.Dano. 1991. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. *Am. J. Pathol.* 138:1059-1067.

Pyke, C., E.Ralfkiaer, E.Ronne, G.Hoyer-Hansen, L.Kirkeby, and K.Dano. 1994. Immunohistochemical detection of the receptor for urokinase plasminogen activator in human colon cancer. *Histopathology* 24:131-138.

Quax, P.H., G.N.van Muijen, E.J.Weening-Verhoeff, L.R.Lund, K.Dano, D.J.Ruiter, and J.H.Verheijen. 1991. Metastatic behavior of human melanoma cell lines in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-mediated matrix degradation. *J. Cell Biol.* 115:191-199.

Ramos-DeSimone, N., E.Hahn-Dantona, J.Sipley, H.Nagase, D.L.French, and J.P.Quigley. 1999. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. *J. Biol. Chem.* 274:13066-13076.

Raucher, D. and M.P.Sheetz. 2000. Cell spreading and lamellipodial extension rate is regulated by membrane tension. *J. Cell Biol.* 148:127-136.

Ravanti, L. and V.M.Kahari. 2000. Matrix metalloproteinases in wound repair (review). *Int. J. Mol. Med.* 6:391-407.

Ray, P., R.Bhatti, J.Gadarowski, N.Bell, and S.Nasruddin. 1998. Inhibitory effect of amiloride on the urokinase plasminogen activators in prostatic cancer. *Tumour. Biol.* 19:60-64.

Reinartz, J., B.Schafer, R.Batrla, C.E.Klein, and M.D.Kramer. 1995. Plasmin abrogates alpha v beta 5-mediated adhesion of a human keratinocyte cell line (HaCaT) to vitronectin. *Exp. Cell Res.* 220:274-282.

Rejante, M.R., I.J.Byeon, and M.Llinas. 1991. Ligand specificity of human plasminogen kringle 4. *Biochemistry* 30:11081-11092.

Reuning, U., V.Magdolen, O.Wilhelm, K.Fischer, V.Lutz, H.Graeff, and M.Schmitt. 1998. Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis (review). *Int. J. Oncol.* 13:893-906.

Reynolds, L.E., L.Wyder, J.C.Lively, D.Taverna, S.D.Robinson, X.Huang, D.Sheppard, R.O.Hynes, and K.M.Hodivala-Dilke. 2002. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. *Nat. Med.* 8:27-34.

Riccio, A., G.Grimaldi, P.Verde, G.Sebastio, S.Boast, and F.Blasi. 1985. The human urokinase-plasminogen activator gene and its promoter. *Nucleic Acids Res.* 13:2759-2771.

Roberts, M., S.Barry, A.Woods, S.P.van der, and J.Norman. 2001. PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early endosomes is necessary for cell adhesion and spreading. *Curr. Biol.* 11:1392-1402.

Romer, J., T.H.Bugge, C.Pyke, L.R.Lund, M.J.Flick, J.L.Degen, and K.Dano. 1996. Impaired wound healing in mice with a disrupted plasminogen gene. *Nat. Med.* 2:287-292.

Romer, J., C.Pyke, L.R.Lund, E.Ralfkiaer, and K.Dano. 2001. Cancer cell expression of urokinase-type plasminogen activator receptor mRNA in squamous cell carcinomas of the skin. *J. Invest Dermatol.* 116:353-358.

Rossi, F.M. and H.M.Blau. 1998. Recent advances in inducible gene expression systems. *Curr. Opin. Biotechnol.* 9:451-456.

Ruoslahti, E. and M.D.Pierschbacher. 1986. Arg-Gly-Asp: a versatile cell recognition signal. *Cell* 44:517-518.

Ruoslahti, E. and M.D.Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science* 238:491-497.

Sadowski, T. and J.Steinmeyer. 2001. Effects of tetracyclines on the production of matrix metalloproteinases and plasminogen activators as well as of their natural inhibitors, tissue inhibitor of metalloproteinases-1 and plasminogen activator inhibitor-1. *Inflamm. Res.* 50:175-182.

Sambrook J., E.F.Fritsch and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. Second Edition. Cold Spring Harbour Laboratory Press. USA.

Santibanez, J.F., M.Iglesias, P.Frontelo, J.Martinez, and M.Quintanilla. 2000. Involvement of the Ras/MAPK signaling pathway in the modulation of urokinase production and cellular invasiveness by transforming growth factor-beta(1) in transformed keratinocytes. *Biochem. Biophys. Res. Commun.* 273:521-527.

Sastry, S.K. and K.Burridge. 2000. Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. *Exp. Cell Res.* 261:25-36.

Schaffner-Reckinger, E., V.Gouon, C.Melchior, S.Plancon, and N.Kieffer. 1998. Distinct involvement of beta3 integrin cytoplasmic domain tyrosine residues 747 and 759 in integrin-mediated cytoskeletal assembly and phosphotyrosine signaling. *J. Biol. Chem.* 273:12623-12632.

Schaller, M.D., C.A.Otey, J.D.Hildebrand, and J.T.Parsons. 1995. Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *J. Cell Biol.* 130:1181-1187.

Schlaepfer, D.D. and T.Hunter. 1998. Integrin signalling and tyrosine phosphorylation: just the FAKs? *Trends Cell Biol.* 8:151-157.

Schmidt, M., C.Polednik, and F.Hoppe. 1999. Proteolytic patterns of head and neck squamous cell carcinoma. *Eur. Arch. Otorhinolaryngol.* 256:346-350.

Schmitt, M., N.Harbeck, C.Thomssen, O.Wilhelm, V.Magdolen, U.Reuning, K.Ulm, H.Hofler, F.Janicke, and H.Graeff. 1997. Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. *Thromb. Haemost.* 78:285-296.

Schneiderman, J., R.Adar, and N.Savion. 1991. Changes in plasmatic tissue-type plasminogen activator and plasminogen activator inhibitor activity during acute arterial occlusion associated with severe ischemia. *Thromb. Res.* 62:401-408.

Schwab, W., J.M.Gavlik, T.Beichler, R.H.Funk, S.Albrecht, V.Magdolen, T.Luther, M.Kasper, and M.Shakibaei. 2001. Expression of the urokinase-type plasminogen activator receptor in human articular chondrocytes: association with caveolin and beta 1-integrin. *Histochem. Cell Biol.* 115:317-323.

Schwartz, M.A. and M.H.Ginsberg. 2002. Networks and crosstalk: integrin signalling spreads. *Nat. Cell Biol.* 4:E65-E68.

Schwartz, M.A., M.D.Schaller, and M.H.Ginsberg. 1995. Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* 11:549-599.

Sebolt-Leopold, J.S., D.T.Dudley, R.Herrera, K.Van Becelaere, A.Wiland, R.C.Gowan, H.Tecle, S.D.Barrett, A.Bridges, S.Przybranowski, W.R.Leopold, and A.R.Saltiel. 1999. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat. Med.* 5:810-816.

Seftor, R.E., E.A.Seftor, W.G.Stetler-Stevenson, and M.J.Hendrix. 1993. The 72 kDa type IV collagenase is modulated via differential expression of alpha v beta 3 and alpha 5 beta 1 integrins during human melanoma cell invasion. *Cancer Res.* 53:3411-3415.

Senior, R.M., N.L.Connolly, J.D.Cury, H.G.Welgus, and E.J.Campbell. 1989. Elastin degradation by human alveolar macrophages. A prominent role of metalloproteinase activity. *Am. Rev. Respir. Dis.* 139:1251-1256.

Shapiro, R.L., J.G.Duquette, D.F.Roses, I.Nunes, M.N.Harris, H.Kamino, E.L.Wilson, and D.B.Rifkin. 1996. Induction of primary cutaneous melanocytic neoplasms in urokinase-type plasminogen activator (uPA)-deficient and wild-type mice: cellular blue nevi invade but do not progress to malignant melanoma in uPA-deficient animals. *Cancer Res.* 56:3597-3604.

Sharma, C.P., R.M.Ezzell, and M.A.Arnaout. 1995. Direct interaction of filamin (ABP-280) with the beta 2-integrin subunit CD18. *J. Immunol.* 154:3461-3470.

Shattil, S.J., T.O'Toole, M.Eigenthaler, V.Thon, M.Williams, B.M.Babior, and M.H.Ginsberg. 1995. Beta 3-endonexin, a novel polypeptide that interacts specifically with the cytoplasmic tail of the integrin beta 3 subunit. *J. Cell Biol.* 131:807-816.

Sheppard, D., C.Rozzo, L.Starr, V.Quaranta, D.J.Erle, and R.Pytela. 1990. Complete amino acid sequence of a novel integrin beta subunit (beta 6) identified in epithelial cells using the polymerase chain reaction. *J. Biol. Chem.* 265:11502-11507.

Shetty, S., A.Kumar, and S.Idell. 1997. Posttranscriptional regulation of urokinase receptor mRNA: identification of a novel urokinase receptor mRNA binding protein in human mesothelioma cells. *Mol. Cell Biol.* 17:1075-1083.

Shimba, S., M.Hayashi, H.Sone, J.Yonemoto, and M.Tezuka. 2000. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces binding of a 50 kDa protein on the 3'

untranslated region of urokinase-type plasminogen activator mRNA. *Biochem. Biophys. Res. Commun.* 272:441-448.

Silvestri, I., C.Longanesi, I, P.Franco, G.Pirozzi, G.Botti, M.P.Stoppelli, and M.V.Carriero. 2002. Engaged urokinase receptors enhance tumor breast cell migration and invasion by upregulating alpha(v)beta5 vitronectin receptor cell surface expression. *Int. J. Cancer* 102:562-571.

Simon, C., H.Goepfert, and D.Boyd. 1998. Inhibition of the p38 mitogen-activated protein kinase by SB 203580 blocks PMA-induced Mr 92,000 type IV collagenase secretion and in vitro invasion. *Cancer Res.* 58:1135-1139.

Simon, C., J.Juarez, G.L.Nicolson, and D.Boyd. 1996a. Effect of PD 098059, a specific inhibitor of mitogen-activated protein kinase kinase, on urokinase expression and in vitro invasion. *Cancer Res.* 56:5369-5374.

Simon, D.I., N.K.Rao, H.Xu, Y.Wei, O.Majdic, E.Ronne, L.Kobzik, and H.A.Chapman. 1996b. Mac-1 (CD11b/CD18) and the urokinase receptor (CD87) form a functional unit on monocytic cells. *Blood* 88:3185-3194.

Simon, D.I., Y.Wei, L.Zhang, N.K.Rao, H.Xu, Z.Chen, Q.Liu, S.Rosenberg, and H.A.Chapman. 2000. Identification of a urokinase receptor-integrin interaction site. Promiscuous regulator of integrin function. *J. Biol. Chem.* 275:10228-10234.

Sitrin, R.G., R.F.Todd, III, E.Albrecht, and M.R.Gyetko. 1996. The urokinase receptor (CD87) facilitates CD11b/CD18-mediated adhesion of human monocytes. *J. Clin. Invest* 97:1942-1951.

Solberg, H., J.Romer, N.Brunner, A.Holm, N.Sidenius, K.Dano, and G.Hoyer-Hansen. 1994. A cleaved form of the receptor for urokinase-type plasminogen activator in invasive transplanted human and murine tumors. *Int. J. Cancer* 58:877-881.

Solomayer, E.F., I.J.Diel, D.Wallwiener, S.Bode, G.Meyberg, M.Sillem, C.Gollan, M.D.Kramer, U.Krainick, and G.Bastert. 1997. Prognostic relevance of urokinase plasminogen activator detection in micrometastatic cells in the bone marrow of patients with primary breast cancer. *Br. J. Cancer* 76:812-818.

Stahl, A. and B.M.Mueller. 1997. Melanoma cell migration on vitronectin: regulation by components of the plasminogen activation system. *Int. J. Cancer* 71:116-122.

Stein, C.A. and J.S.Cohen. 1989. Antisense compounds: potential role in cancer therapy. *Important Adv. Oncol.*79-97.

Stephens, L.E., A.E.Sutherland, I.V.Klimanskaya, A.Andrieux, J.Meneses, R.A.Pedersen, and C.H.Damsky. 1995. Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev.* 9:1883-1895.

Stetler-Stevenson, W.G., S.Aznavoorian, and L.A.Liotta. 1993. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.* 9:541-573.

Stoppelli, M.P., P.Verde, G.Grimaldi, E.K.Locatelli, and F.Blasi. 1986. Increase in urokinase plasminogen activator mRNA synthesis in human carcinoma cells is a primary effect of the potent tumor promoter, phorbol myristate acetate. *J. Cell Biol.* 102:1235-1241.

Sugiyama, M., P.M.Speight, S.S.Prime, and F.M.Watt. 1993. Comparison of integrin expression and terminal differentiation capacity in cell lines derived from oral squamous cell carcinomas. *Carcinogenesis* 14:2171-2176.

Summerton, J. 1999. Morpholino antisense oligomers: the case for an RNase H-independent structural type. *Biochim. Biophys. Acta* 1489:141-158.

Summerton, J. and P.A.Bartlett. 1978. Sequence-specific crosslinking agents for nucleic acids. Use of 6-bromo-5,5-dimethoxyhexanohydrazide for crosslinking cytidine to guanosine and crosslinking RNA to complementary sequences of DNA. *J. Mol. Biol.* 122:145-162.

Summerton, J. and D.Weller. 1997. Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev.* 7:187-195.

Tachibana, I., J.Bodorova, F.Berditchevski, M.M.Zutter, and M.E.Hemler. 1997. NAG-2, a novel transmembrane-4 superfamily (TM4SF) protein that complexes with integrins and other TM4SF proteins. *J. Biol. Chem.* 272:29181-29189.

Tang, H., D.M.Kerins, Q.Hao, T.Inagami, and D.E.Vaughan. 1998. The urokinase-type plasminogen activator receptor mediates tyrosine phosphorylation of focal adhesion proteins and activation of mitogen-activated protein kinase in cultured endothelial cells. *J. Biol. Chem.* 273:18268-18272.

Thomas, G.J., J.Jones, and P.M.Speight. 1997. Integrins and oral cancer. *Oral Oncol.* 33:381-388.

Thomas, G.J., M.P.Lewis, I.R.Hart, J.F.Marshall, and P.M.Speight. 2001a. AlphaVbeta6 integrin promotes invasion of squamous carcinoma cells through up-regulation of matrix metalloproteinase-9. *Int. J. Cancer* 92:641-650.

Thomas, G.J., M.P.Lewis, S.A.Whawell, A.Russell, D.Sheppard, I.R.Hart, P.M.Speight, and J.F.Marshall. 2001b. Expression of the alphavbeta6 integrin promotes migration and invasion in squamous carcinoma cells. *J. Invest Dermatol.* 117:67-73.

Thomas, G.J., S.Poomsawat, M.P.Lewis, I.R.Hart, P.M.Speight, and J.F.Marshall. 2001c. alpha v beta 6 Integrin upregulates matrix metalloproteinase 9 and promotes migration of normal oral keratinocytes. *J. Invest Dermatol.* 116:898-904.

Thomas, G.J. and P.M.Speight. 2001. Cell adhesion molecules and oral cancer. *Crit Rev. Oral Biol. Med.* 12:479-498.

Urtreger, A.J., J.A.Aguirre Ghiso, S.E.Werbajh, L.I.Puricelli, A.F.Muro, and J.E.Bal de Kier. 1999. Involvement of fibronectin in the regulation of urokinase production and binding in murine mammary tumor cells. *Int. J. Cancer* 82:748-753.

van der Flier, F.A. and A.Sonnenberg. 2001. Function and interactions of integrins. *Cell Tissue Res.* 305:285-298.

van der Pluijm, P.G., B.Sijmons, H.Vloedgraven, B.C.van der, J.W.Drijfhout, J.Verheijen, P.Quax, M.Karperien, S.Papapoulos, and C.Lowik. 2001. Urokinase-receptor/integrin complexes are functionally involved in adhesion and progression of human breast cancer in vivo. *Am. J. Pathol.* 159:971-982.

van Muijen, G.N., E.H.Danen, T.J.De Vries, P.H.Quax, J.H.Verheijen, and D.J.Ruiter. 1995. Properties of metastasizing and nonmetastasizing human melanoma cells. *Recent Results Cancer Res.* 139:105-122.

van Leeuwen R.T.J. 1996. Extracellular proteolysis and the migrating vascular smooth muscle cell. Fibrinolysis. 10(2):59-74

van Roozendaal, C.E., B.van Ooijen, J.G.Klijn, C.Claassen, A.M.Eggermont, S.C.Henzen-Logmans, and J.A.Foekens. 1992. Stromal influences on breast cancer cell growth. *Br. J. Cancer* 65:77-81.

Vassalli, J.D. and D.Belin. 1987. Amiloride selectively inhibits the urokinase-type plasminogen activator. *FEBS Lett.* 214:187-191.

Verrall, S. and N.W.Seeds. 1989. Characterization of 125I-tissue plasminogen activator binding to cerebellar granule neurons. *J. Cell Biol.* 109:265-271.

Wang, X.Q. and W.A.Frazier. 1998. The thrombospondin receptor CD47 (IAP) modulates and associates with alpha2 beta1 integrin in vascular smooth muscle cells. *Mol. Biol. Cell* 9:865-874.

Wang, Y., J.Dang, X.Liang, and W.F.Doe. 1995. Amiloride modulates urokinase gene expression at both transcription and post-transcription levels in human colon cancer cells. *Clin. Exp. Metastasis* 13:196-202.

Webb, D.J., J.T.Parsons, and A.F.Horwitz. 2002. Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. *Nat. Cell Biol.* 4:E97-100.

Wei, Y., J.A.Eble, Z.Wang, J.A.Kreidberg, and H.A.Chapman. 2001. Urokinase receptors promote beta1 integrin function through interactions with integrin alpha3beta1. *Mol. Biol. Cell* 12:2975-2986.

Wei, Y., M.Lukashev, D.I.Simon, S.C.Bodary, S.Rosenberg, M.V.Doyle, and H.A.Chapman. 1996. Regulation of integrin function by the urokinase receptor. *Science* 273:1551-1555.

Wei, Y., X.Yang, Q.Liu, J.A.Wilkins, and H.A.Chapman. 1999. A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signaling. *J. Cell Biol.* 144:1285-1294.

Werb, Z. 1997. ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 91:439-442.

Werb, Z., P.M.Tremble, O.Behrendtsen, E.Crowley, and C.H.Damsky. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J. Cell Biol.* 109:877-889.

Wyborski, D.L., J.C.Bauer, and P.Vaillancourt. 2001. Bicistronic expression of ecdysone-inducible receptors in mammalian cells. *Biotechniques* 31:618-20, 622, 624.

Xiong, J.P., T.Stehle, B.Diefenbach, R.Zhang, R.Dunker, D.L.Scott, A.Joachimiak, S.L.Goodman, and M.A.Arnaout. 2001. Crystal structure of the extracellular segment of integrin alpha Vbeta3. *Science* 294:339-345.

Xue, H., A.Atakilit, W.Zhu, X.Li, D.M.Ramos, and R.Pytela. 2001. Role of the alpha(v)beta6 integrin in human oral squamous cell carcinoma growth in vivo and in vitro. *Biochem. Biophys. Res. Commun.* 288:610-618.

Xue, W., A.L.Kindzelskii, R.F.Todd, III, and H.R.Petty. 1994. Physical association of complement receptor type 3 and urokinase-type plasminogen activator receptor in neutrophil membranes. *J. Immunol.* 152:4630-4640.

Xue, W., I.Mizukami, R.F.Todd, III, and H.R.Petty. 1997. Urokinase-type plasminogen activator receptors associate with beta1 and beta3 integrins of fibrosarcoma cells: dependence on extracellular matrix components. *Cancer Res.* 57:1682-1689.

Yanez-Mo, M., A.Alfranca, C.Cabanas, M.Marazuela, R.Tejedor, M.A.Ursa, L.K.Ashman, M.O.de Landazuri, and F.Sanchez-Madrid. 1998. Regulation of endothelial cell motility by complexes of tetraspan molecules CD81/TAPA-1 and CD151/PETA-3 with alpha3 beta1 integrin localized at endothelial lateral junctions. *J. Cell Biol.* 141:791-804.

Yarnold, J.R. 1992. What are cancer genes, and how do they upset cell behaviour? *Eur. J. Cancer* 28:251-255.

Yebra, M., L.Goretzki, M.Pfeifer, and B.M.Mueller. 1999. Urokinase-type plasminogen activator binding to its receptor stimulates tumor cell migration by enhancing integrin-mediated signal transduction. *Exp. Cell Res.* 250:231-240.

Yebra, M., G.C.Parry, S.Stromblad, N.Mackman, S.Rosenberg, B.M.Mueller, and D.A.Cheresh. 1996. Requirement of receptor-bound urokinase-type plasminogen

activator for integrin alphavbeta5-directed cell migration. *J. Biol. Chem.* 271:29393-29399.

Yu, W., J.Kim, and L.Ossowski. 1997. Reduction in surface urokinase receptor forces malignant cells into a protracted state of dormancy. *J. Cell Biol.* 137:767-777.

Zambruno, G., P.C.Marchisio, A.Marconi, C.Vaschieri, A.Melchiori, A.Giannetti, and M.De Luca. 1995. Transforming growth factor-beta 1 modulates beta 1 and beta 5 integrin receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: implications for wound healing. *J. Cell Biol.* 129:853-865.

Zannetti, A., S.Del Vecchio, M.V.Carriero, R.Fonti, P.Franco, G.Botti, G.D'Aiuto, M.P.Stoppelli, and M.Salvatore. 2000. Coordinate up-regulation of Sp1 DNA-binding activity and urokinase receptor expression in breast carcinoma. *Cancer Res.* 60:1546-1551.

Zhang, X.A. and M.E.Hemler. 1999. Interaction of the integrin beta1 cytoplasmic domain with ICAP-1 protein. *J. Biol. Chem.* 274:11-19.

Ziegler, A., J.Knesel, D.Fabbro, and Y.Nagamine. 1991. Protein kinase C down-regulation enhances cAMP-mediated induction of urokinase-type plasminogen activator mRNA in LLC-PK1 cells. *J. Biol. Chem.* 266:21067-21074.

APPENDIX

APPENDIX 1

KERATINOCYTE GROWTH MEDIUM (KGM)

67.5% Dulbeccos Modified Eagles Medium (DMEM)

27.5% HAMS-F12 Nutrient Mixture

10% Foetal Calf Serum (FCS)

10 ng/ml Epidermal Growth Factor (EGF)

0.5 μg/ml Hydrocortisone

5 μg/ml Insulin

0.18 mM Adenine

10^{-10} M Cholera Toxin

100 IU/ml Penicillin

100 μg/ml Streptomycin

2.5 μg/ml Fungizone

APPENDIX 2

ANTIBODIES

FLOW CYTOMETRY

PRIMARY ANTIBODIES

PROTEIN	CLONE	CONCENTRATION USED	SUPPLIER
αv integrin	L230 (monoclonal)	50μg/ml	J.Marshall (ICRF)
αvβ5 integrin	P1F6 (monoclonal)	100μg/ml	Chemicon
αvβ6 integrin	E7P6 (monoclonal) 10D5 (monoclonal)	100μg/ml 100μg/ml	Chemicon
αvβ3 integrin	13C10(monoclonal)	100μg/ml	M.Horton (UCL)
β1 integrin	P1D6 (monoclonal)	25μg/ml	Life Technologies Inc.
uPAR	399R (polyclonal)	100μg/ml	American Diagnostica, Greenwich, UK

SECONDARY ANTIBODIES

SECONDARY ANTIBODY	CONCENTRATION USED	SUPPLIER
FITC-conjugated rabbit anti-mouse	50 ng/ml	DAKO Ltd. Ely, UK
FITC-conjugated goat anti-rabbit	50 ng/ml	DAKO Ltd. Ely, UK
RPE-conjugated goat anti-mouse	50 ng/ml	DAKO Ltd. Ely, UK

APPENDIX 2

WESTERN BLOTTING

PRIMARY ANTIBODIES

PROTEIN	CLONE	CONCENTRATION USED	SUPPLIER
uPAR	R4 (monoclonal)	10μg/ml	G. Hoyer Hansen (Denmark)
β6	scc-62 (c-19) (c-terminal polyclonal)	20μg/ml	Santa Cruz Biotechnology
β1	A11B2 (monoclonal)	10μg/ml	J. Marshall (ICRF)

SECONDARY ANTIBODIES

SECONDARY ANTIBODY	CONCENTRATION USED	SUPPLIER
Rabbit anti-mouse HRP-conjugated	100 ng/ml	DAKO Ltd. Ely, UK
Goat anti-rabbit HRP-conjugated	100 ng/ml	DAKO Ltd. Ely, UK

IMMUNOFLUORESCENCE

PRIMARY ANTIBODIES

PROTEIN	CLONE	CONCENTRATION USED	SUPPLIER
uPAR	R4 (monoclonal)	10μg/ml	G. Hoyer Hansen (Denmark)
Plasma Fibronectin			
αvβ5	P1F6	5μg/ml	Chemicon
αvβ6	E7P6	10μg/ml	Chemicon

SECONDARY ANTIBODIES

SECONDARY ANTIBODY	CONCENTRATION USED	SUPPLIER
FITC-conjugated rabbit anti-mouse	50 ng/ml	DAKO Ltd. Ely, UK
FITC-conjugated goat anti-rabbit	1 μg/ml	DAKO Ltd. Ely, UK

APPENDIX 2

FUNCTIONAL ASSAYS – BLOCKING ANTIBODIES

MIGRATION ASSAYS

PROTEIN	CLONE	CONCENTRATION USED	SUPPLIER
uPA	mAb 394	10 μg/ml	American Diagnostica, Greenwich, UK
	mAb 3689	10μg/ml	American Diagnostica, Greenwich, UK
	mAb clone 5	10μg/ml	V. Ellis (UEA)

PLASMINOGEN ACTIVATOR ACTIVITY ASSAYS

PROTEIN	CLONE	CONCENTRATION USED	SUPPLIER
β1 integrin	P4C10 (monoclonal)	75μg/ml	Life Technologies Inc.
αv integrin	L230 (monoclonal)	10μg/ml	J. Marshall (ICRF)
αvβ6 integrin	10D5 (monoclonal)	75μg/ml	Chemicon
	E7P6 (monoclonal)	10μg/ml	Chemicon

APPENDIX 3

RNA ISOLATION

Denaturing Solution (4M Guanidium Thiocyanate buffer)

4M Guanidium Thiocyanate (Stratagene)

25mM Sodium Citrate in conc. HCl (Sigma Chemical Co.)

0.5% Sarcosyl

7.2 µl/ml β-mercaptoethanol

Made up in DEPC dH_2O

NORTHERN BLOTTING

10X MOPS buffer

0.2M MOPS

0.05M Sodium Acetate

0.01M EDTA

DEPC treat by addition of 0.5ml DEPC to 500ml MOPS buffer. Leave to shake overnight and autoclave twice at 135°C for 1 hour.

APPENDIX 3

RNA Sample Loading Buffer

For a 1.5ml solution:

720µl Deionised Formamide

160µl 10X MOPS buffer

260µl 37% formaldehyde (stock)

150µl dH2O ((DEPC treated)

50µl Ethidium Bromide (10mg/ml)

80µl Glycerol (sterile)

80µl Saturated bromophenol blue

20X SSC

175.3g/l Sodium Chloride

88.2g/l Sodium Citrate

DEPC treat

APPENDIX 4

WESTERN BLOTTING

Protein Extraction

Integrin Lysis Buffer

For total cell lysates but specific for membrane proteins such as integrins:

20mM HEPES

1% NP-40

50mM NaCl

1mM $CaCl_2$

3mM $MgCl_2$

0.3M Sucrose

0.1% Azide

Acid Wash

50mM Glycine Hydrochloride

100mM Sodium Chloride

: pH 3.0

Neutralising buffer : pH 7.5

500mM HEPES

100mM Sodium Chloride

Membrane Lysis Buffer: pH 8.1

For hydrophobic cell membrane extracts for uPAR detection:

0.1M Tris-Hydrochloride

10mM EDTA

10 μg/ml Aprotinin

1% Triton X-114

Gel Electrophoresis

Sample Loading Buffer

Non-reducing

62mM Tris: pH6.8

10% glycerol

2% SDS

0.04% bromophenol blue

Made up in dH_2O

Reducing

As above but add 5% β-mercaptoethanol

Acrylamide Gels

10% Resolving Gel

Gel Components	Component volumes for 10% gel
dH_2O	4ml
30% acrylamide	3.3ml
1.5M Tris; pH 8.8	2.5ml
10% SDS	100µl
10% ammonium persulfate	100µl
TEMED	4µl

4% Stacking Gel

Gel Components	Component volumes for 4% gel
dH_2O	3ml
30% acrylamide	0.7ml
0.5M Tris; pH 6.8	1.25ml
10% SDS	50µl
10% ammonium persulfate	40µl
TEMED	20µl

10X Running Buffer

250mM Tris base

1.9M glycine

1% SDS

: pH 7.6

Membrane Transfer

10X Transfer Buffer

250mM Tris base

1.9M glycine

: pH 7.6

PBST

1X Phosphate Buffered Saline (PBS

0.05% Tween 20

Blocking Buffer

5% non-fat dry milk in 1XPBST

Stripping Buffer

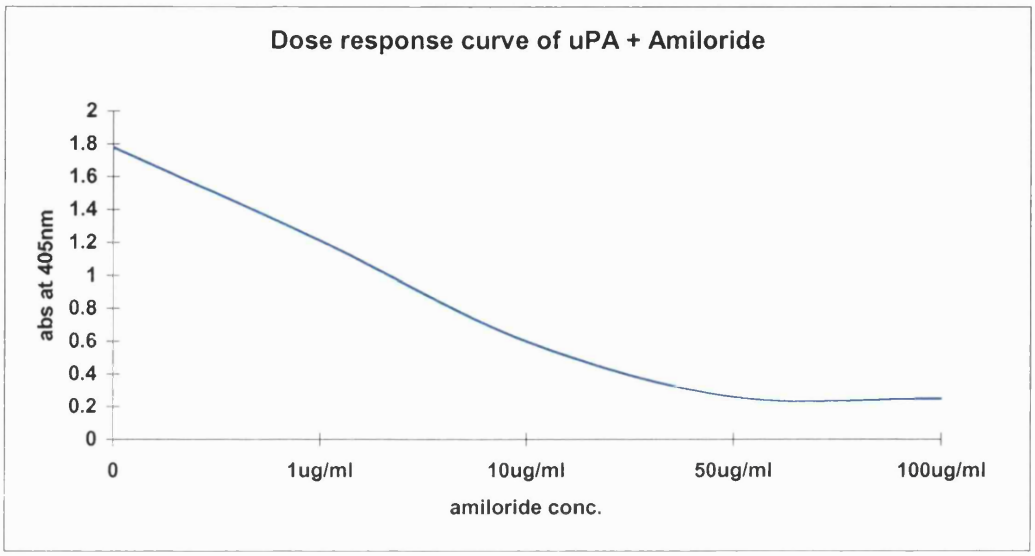
100mM β-mercaptoethanol

2% SDS

62.5mM Tris-HCl

APPENDIX 5

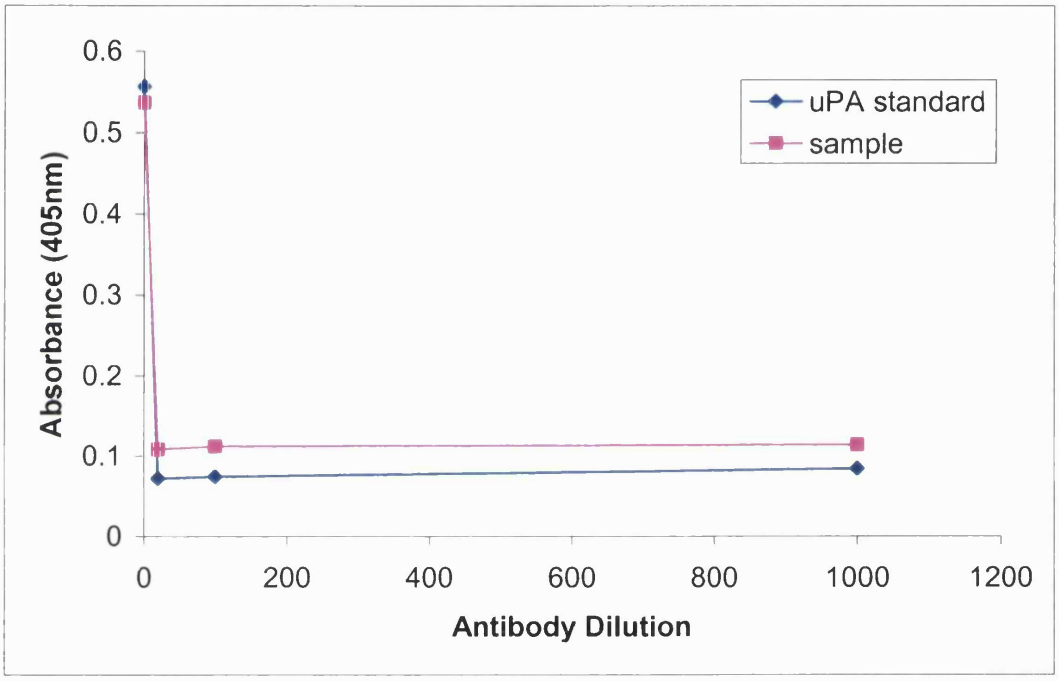
The optimal concentration of Amiloride to inhibit uPA activity was determined by the plasminogen activator activity assay.



1μg/ml urokinase was treated with Amiloride at a number of concentrations for 20 mins at room temperature. The plasminogen activator activity of these samples was determined carried out by the addition of excess Glu-plasminogen and chromogenic substrate. The absorbance was then measured at 405nm.

APPENDIX 6

The optimal concentration of monoclonal anti-uPA antibody, Clone 5 (Dr Vince Ellis, UEA) to inhibit uPA activity was determined by the plasminogen activator activity assay.



uPA standard and a cultured supernatant of oral SCC cells were treated with increasing dilutions (1:20, 1:100 and 1:1000) of clone 5 antibody and incubated at 37°C for 20 minutes. The samples were then treated with Glu-plasminogen and chromogenic substrate in excess and the absorbance measured at 405nm in order to determine plasminogen activator activity. Clone 5 antibody was very efficient in inhibiting uPA activity with total inhibition of activity at a 1 in 20 dilution.

APPENDIX 7

LB-Media

10g Tryptone

10g NaCl

5g yeast extract

1M NaOH

Make up to 1litre with dH_2O and autoclave; pH7.6

SOB Buffer

20g bacto-tryptone

5g bacto-yeast extract

0.5g NaCl

Dissolve in 950ml dH_2O, add 10ml 250mM KCl and adjust to pH7.

Make up to 1 litre with dH_2O and autoclave.

Just before use add 5ml sterile 2M $MgCl_2$ to this.

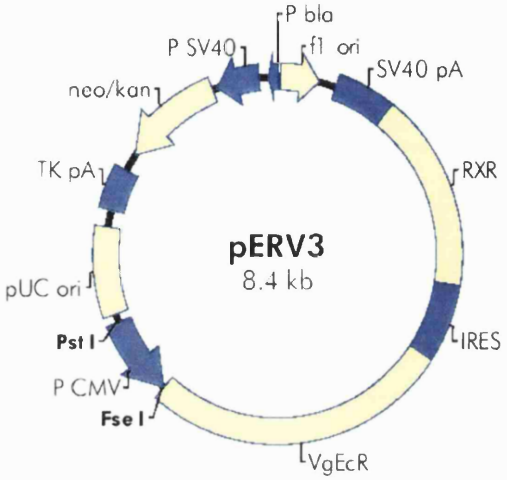
SOC Buffer

Add 1ml of sterile filtered 1M glucose to 50mls SOB buffer to give a final concentration of 20mM.

APPENDIX 8

pERV3 Vector

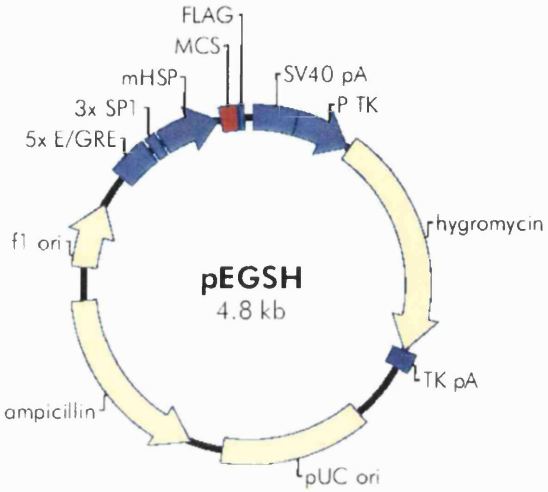
f1 origin 24–330
SV40 polyA 901–463
RXR receptor ORF 902–2333
internal ribosome entry site 2334–2903
VgEcR receptor ORF 2904–5187
Fse I 5187
CMV promoter 5188–5802
Pst I 5802
pUC origin 5869–6536
HSV-TK polyA 6668–6998
neomycin/kanamycin resistance ORF 7121–7915
SV40 promoter 7950–8288
***bla* promoter** 8362–5



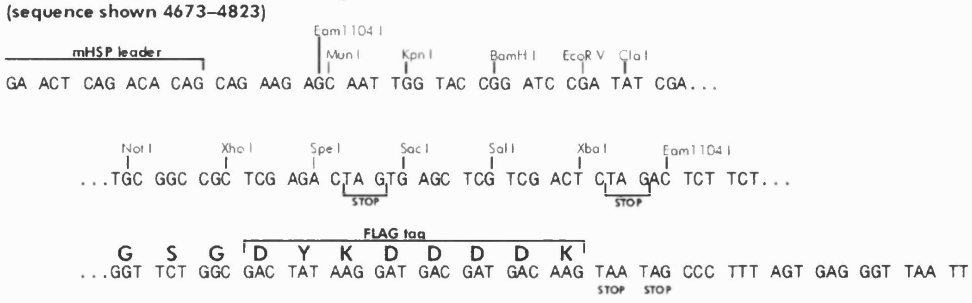
APPENDIX 9

pEGSH Vector

SV40 polyA 8–207
HSV-TK promoter 209–471
hygromycin resistance ORF 480–1517
HSV-TK polyA 1518–1602
pUC origin 1876–2543
ampicillin resistance (*bla*) ORF 2694–3551
f1 origin 3707–4013
5× E/GRE elements 4154–4328
3× Sp1 binding sites 4343–4390
minimal heat shock promoter 4404–4679
multiple cloning site 4689–4767
FLAG tag 4774–4797

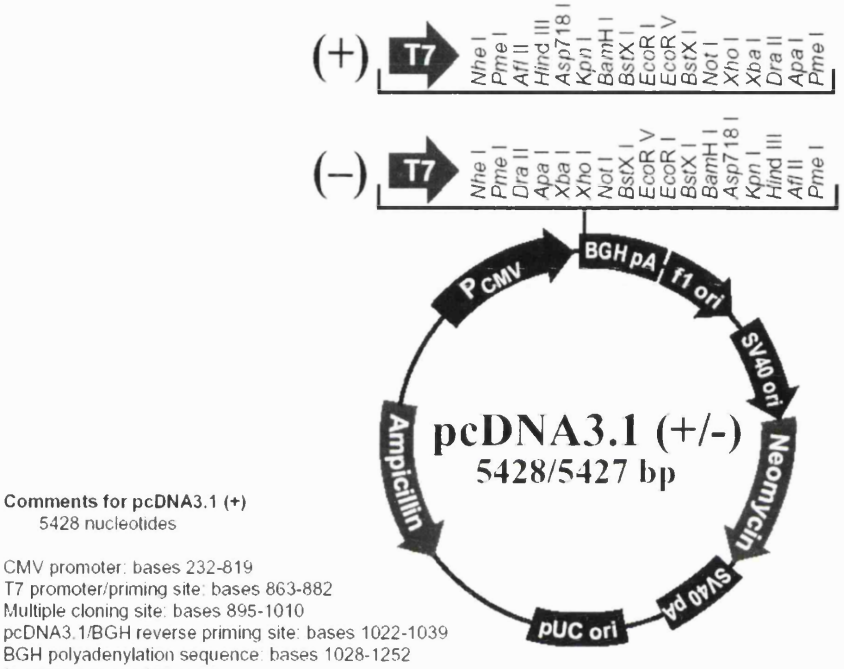


pEGSH Multiple Cloning Site Region
(sequence shown 4673–4823)



APPENDIX 10

pcDNA3.1 Vector



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)