# Functional implications of CD44 splice variant expression in rheumatoid arthritis

by

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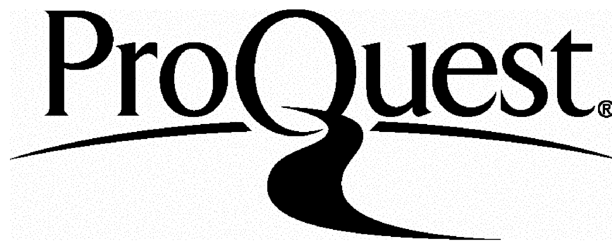

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# Abstract

Fibroblast-like synoviocytes (FLSs) are resident within the synovial membrane. In rheumatoid arthritis (RA), these cells dramatically increase in number and demonstrate a highly aggressive phenotype, characterised by the destruction of bone and cartilage and the release of a variety of cytokines. FLSs from RA patients have been demonstrated in this thesis to constitutively express alternatively spliced CD44 isoforms at the cell surface level, both *in situ* and *in vitro*. These cells show a greater intensity of staining for variant CD44 isoforms containing v3, v6 and v7/8, as compared to those from normal and osteoarthritic patients. This suggests that the expression of CD44 splice variants is closely linked to the inflammatory state of the diseased synovium and may be involved in the pathogenesis of RA. Several cell-based assays have been used to determine whether CD44 splice variants mediate any of the above characteristics. FLSs expressing variant isoforms containing v7/8 exhibit faster proliferation than non-expressing cells. Moreover, treatment with anti-CD44v7/8 monoclonal antibodies (mAbs) inhibits this proliferative advantage. On the other hand, CD44H and variants containing v3 or v6 have no effect on the proliferation of the cells. CD44 isoforms incorporating v3 or v6 are involved in cellular invasion as observed from *in vitro* invasion assays. MAbs against CD44v3 or CD44v6 inhibit invasion of extracellular matrix by FLSs while the anti-CD44v7/8 mAb does not. CD44 isoforms carrying v7/8 also appear to influence IL-6 release of the cultured FLSs as examined by enzyme-linked immunosorbent assays. These results indicate functional implications of particular CD44 splice variants that might allow pharmacological intervention using variant-specific antibodies.

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# Abbreviations

| | |
|---|---|
| A | Absorbance |
| AEC | Aminoethylcarbazole |
| ANOVA | Analysis of variance |
| bFGF | Basic fibroblast growth factor |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CD | Cluster of differentiation |
| CD44E | Epithelial CD44 |
| CD44H, CD44s | CD44 standard form |
| CD44v | CD44 splice variant |
| Cdk | Cyclin-dependent kinase |
| cDNA | Complementary deoxyribonucleic acid |
| CR | Complement receptor |
| DAPI | 4',6-diamidino-2-phenylindole |
| DEPC | Diethyl pyrocarbonate |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Deoxyribonucleic acid |
| ECM | Extracellular matrix |
| ECMR | Extracellular matrix receptor |
| EDTA | Ethylenediamine tetraacetate |
| EGF | Epidermal growth factor |
| ELAM | Endothelial-leukocyte adhesion molecule |
| ELISA | Enzyme-linked immunosorbent assay |

| | |
|---|---|
| ERK | Extracellular signal-regulated kinase |
| ERM | Ezrin-radixin-moesin |
| FBS | Foetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FLS | Fibroblast-like synoviocyte |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GMP | Granule membrane protein |
| gp, GP | Glycoprotein |
| GRB-2 | Growth factor receptor-bound protein 2 |
| GTPase | Guanosine triphosphatase |
| HA | Hyaluronic acid |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| hr | Hour(s) |
| ICAM | Intercellular adhesion molecule |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL-1Ra | Interleukin-1 receptor antagonist |
| INCAM | Inducible cell adhesion molecule |
| LFA | Leukocyte function-associated antigen |
| mAb | Monoclonal antibody |
| MAdCAM | Mucosal addressin cell adhesion molecule |
| MAP | Mitogen-activated protein |

| | |
|---|---|
| MEK | Mitogen-activated protein kinase kinase/ERK kinase |
| MHC | Major histocompatibility complex |
| min | Minute(s) |
| MMP | Matrix metalloproteinase |
| NaOAc | Sodium acetate |
| NCAM | Neural cell adhesion molecule |
| NF | Nuclear factor |
| NF-κB | Nuclear factor for kappa light chain in B cells |
| NFIL-6 | Nuclear factor for interleukin-6 |
| OA | Osteoarthritis |
| P/S | Penicillin-streptomycin solution |
| PBS | Phosphate-buffered saline |
| PCNA | Proliferating cell nuclear antigen |
| PECAM | Platelet-endothelial cell adhesion molecule |
| Pgp | Phagocytic glycoprotein |
| PI | Propidium iodide |
| PKC | Protein kinase C |
| PMA | Phorbol myristate acetate |
| PNAd | Peripheral lymph node vascular addressin |
| proMMP | Precursor of matrix metalloproteinase |
| RA | Rheumatoid arthritis |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| s | Seconds |
| SCID | Severe combined immunodeficiency |
| S.E.M. | Standard error of mean |

| | |
|---|---|
| SH | Src homology |
| sIL-6R | Soluble IL-6 receptor |
| SOD | Superoxide dismutase |
| STAT | Signal transducer and activator of transcription |
| TBE | Tris-borate-EDTA |
| TEMED | N,N,N′,N′-tetramethylethylenediamine |
| TGF | Transforming growth factor |
| TIMP | Tissue inhibitor of metalloproteinases |
| TNF | Tumour necrosis factor |
| TPA | 12-O-tetradecanoyl phorbol-13-acetate |
| TRITC | Tetramethylrhodamine isothiocyanate |
| v/v | Volume per volume |
| VCAM | Vascular cell adhesion molecule |
| w/v | Weight per volume |
| w/w | Weight per weight |

# Chapter 1

# Introduction

The aetiology of rheumatoid arthritis is still not clear and treatment of this disease is far from satisfactory. A good understanding of the factors that maintain the destructive inflammatory environment in the synovial joint is still not complete. This thesis explores the hypothesis that the residential fibroblast of the synovial membrane, the fibroblast-like synoviocyte, plays a role in the orchestration of the chronic inflammatory state of the synovial joint. The recent observation that fibroblasts from arthritic joints, but not from non-inflamed joints, express CD44 splice variants on their surface initiated a study to elaborate the functional implications of their presence. The study described in this thesis demonstrates various implications of CD44 splice variant expression and gives new insights into the behaviour of fibroblast-like synoviocytes. In this first chapter, a broad overview concerning the biology of synovial joints both in normal and in rheumatoid arthritis-induced states will be given. The chapter will also consider the extracellular matrix, inflammatory cytokines, and adhesion molecules involved in the disease, with special emphasis on the CD44 molecule.

## 1.1 Normal human joints

Joints, which are junctions between 2 or more bones or cartilages, are part of the articular system. They can be divided into 3 groups, namely fibrous joints (synarthroses),

cartilaginous joints (symphyses) and synovial joints (diarthroses), according to the structure and degree of movability. Among these, diarthroidal joints are the most highly movable since the adjoining bone ends are coated with smooth cartilage separated by a short tube of strong fibrous tissue, a synovial membrane, and a fluid-filled space (Figure 1.1).

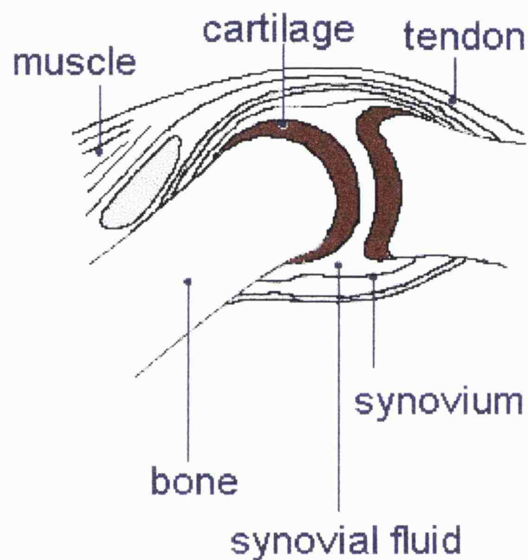



**Figure 1.1.** The structure of a normal diarthrosis.

### 1.1.1 **Synovial membrane**

The synovial lining of normal diarthroidal joints is a superficial layer of 1-3 cells facing the articular cavity and is supported by a loose vascular connective tissue. At least 2 types of the lining cells have been identified: type A synoviocytes and type B synoviocytes. Immunohistochemical examination has indicated a strong expression of Ia

antigens (human leukocyte antigen class II) on most cells. Type A synoviocytes show phagocytic activity and are believed to be of monocytic origin since they express Fc receptors and a number of monocyte lineage differentiation antigens (Burmester *et al.*, 1983). These cells express HLA-DR and -DQ antigens and are also found in the synovium of patients with rheumatoid arthritis and in the almost normal looking synovial membranes obtained from patients with "non-inflammatory" joint diseases (Harding & Knight, 1986).

The other distinguishable cell population, the type B synoviocyte, resembles a fibroblast and is, therefore, often referred to as a fibroblast-like synoviocyte (FLS), a nomenclature I will use throughout the thesis. In contrast to type A synoviocytes, the FLSs lack phagocytic capacity and the monocytic markers (Burmester *et al.*, 1983). They are responsible for the production of the glycosaminoglycan, hyaluronan, which is abundantly present in the synovial fluid. They are Ia-positive cells that express only the DR antigens. They frequently develop a stellate shape, lose the expression of the Ia antigens in tissue culture and can produce large amounts of collagenase (Takasugi & Hollingsworth, 1967). These FLSs demonstrate a potential for proliferation as they predominate in long-term cultures (Burmester *et al.*, 1983).

Cells that exhibit features of both type A and B have sometimes been described as intermediate or type C synoviocytes. They make up approximately one-third of the synovial cells in patients with rheumatoid arthritis but are uncommon in the synovial lining of patients with non-inflammatory diseases. They are classified by a stellate or dendritic morphology with the absence of a phagocytic capacity, IgG Fc receptors,

monocyte lineage antigens, B or T lymphocyte antigens and fibroblast-associated antigens (Burmester *et al.*, 1983). In culture, the intermediate synovial cells progressively lose the expression of Ia antigens and their morphological appearance and become indistinguishable from FLSs. In fact, the FLSs sporadically assume a dendritic appearance (Hendler *et al.*, 1985). There has been a suggestion that these morphologies may reflect different functional states rather than particular cellular origins. This idea is supported by a finding that exposure of subcultured FLSs to interleukin-1 or prostaglandin $E_2$ resulted in a change to dendritic appearance with enhanced production of collagenase, prostaglandin E and nonspecific esterase, as well as an obvious inability to proliferate in culture (Gadher & Woolley, 1987). Similarly, a more recent study showed that FLSs could alter their shape to a dendritic morphology and stop growing. However, when transfected with c-fos DNA, the cells proceeded to grow and repossessed the fibroblastic appearance (Kuroki *et al.*, 1993).

### 1.1.2 Articular cartilage

Articular cartilage is an avascular, alymphatic, aneural tissue that covers the articular ends of bone. It is comprised of cells, which are called chondrocytes, set in a plentiful matrix (see below for details of extracellular matrix). The matrix consists of collagens mainly of type II (Nemeth-Csoka & Meszaros, 1983), large aggregating (aggrecan) (Ilic *et al.*, 1992) and small proteoglycans (Witsch-Prehm *et al.*, 1992), noncollagenous proteins (Chaminade *et al.*, 1982) and other organic and inorganic substances (Reklies & White, 1991; Scotchford & Ali, 1997). The articular cartilage serves to absorb shock, enable movement with little resistance, transmit and distribute weight to underlying bone, and

maintain contact stresses at minimal levels.

### 1.1.3 **Synovial fluid**

Synovial fluid is comprised of several constituents from different origins including blood and the joint itself. Most of the components of plasma, such as glucose and amino acids, appear in the synovial fluid (Blau, 1979) by mechanisms that depend on the size and shape of the molecules. In marked contrast to plasma, normal human synovial fluid has very low concentrations of lipoproteins and apolipoproteins (Prete *et al.*, 1995). Serum proteins and immunoglobulins are also present in low concentrations (Kushner & Somerville, 1971). The main constituent of synovial fluid is hyaluronic acid (Smith *et al.*, 1980), a polysaccharide of high molecular weight, secreted by FLSs. Other macromolecules present in the synovial fluid include fibronectin (Carsons *et al.*, 1983) and the sulphated glycosaminoglycans - chondroitin sulphate (Silpananta *et al.*, 1967) and keratan sulphate (Heimer *et al.*, 1992). Cytokines are hardly detected in the synovial fluid recovered from normal synovial joints. To date, insulin-like growth factor-I seems to be the only growth factor that has been identified in synovial fluid from normal subjects (Schneiderman *et al.*, 1995).

Fluid from normal joints is clear and colourless whereas fluid from patients with a variety of relatively non-inflammatory diseases including osteoarthritis (OA) is usually straw-coloured or yellow but still transparent (Gatter, 1984; Ropes & Bauer, 1953; Schumacher, 1985). However, the clear fluid is not a guarantee that the joint is normal or that the effusion is not part of a systemic inflammatory disease. Normal joint fluid contains only 50-200 white cells/$mm^3$, most being monocytes (Coggeshall *et al.*, 1940; Collins, 1936;

Gatter, 1984; Ropes & Bauer, 1953). Cloudy fluid usually is indicative of increased numbers of leukocytes and thus of an inflammatory process (Schumacher, 1991). Differences in the synovial fluid leukocyte count help to distinguish septic, inflammatory, and "non-inflammatory" effusions (Cohen & Goldenberg, 1985; Krey & Bailen, 1979). The so-called "non-inflammatory" effusions (for example, from OA joints) generally contain between 200 and 2,000 white cells/$mm^3$, suggesting the presence of mild inflammation. Diseases with a strong inflammatory component, such as rheumatoid arthritis, Reiter's syndrome and reactive inflammatory arthritides, demonstrate white blood cell counts ranging from 2,000 to 75,000/$mm^3$ (Cohen & Goldenberg, 1985; Kerolus *et al.*, 1989; Krey & Bailen, 1979; Ropes & Bauer, 1953).

## 1.2 Extracellular matrix

Most cells form an extracellular matrix (ECM) that performs a range of functions. Apart from physical support, ECM components are involved in the regulation of cell division, cell migration, differentiation and rescue from programmed cell death (apoptosis). The components of the ECM consist of 2 structural elements. Fibrous proteins, through the formation of long fibres, make the tissue resistant to tension. The proteoglycans, which form a 3-dimensional network (gels) and which, because of their water retention, make the tissue resistant to compression. In humans, collagen and elastine are the principal tension resistant fibres, and hyaluronan, chondroitin, heparan and keratan sulphates are the compression resistant glycosaminoglycans.

### 1.2.1 **Collagens**

The collagens are glycoproteins made from 3 α-helical polypeptide chains wound together and attached by carbohydrate moities. Each α-chain is identified by a high content of glycine which is repeated at every third position in the amino acid sequence in the pattern $(Gly\text{-}X\text{-}Y)_n$, where X and Y are other amino acids, the most common being proline or lysine in either an unmodified or modified form. The carbohydrate groups consist almost completely of short sugar-galactose-glucose chains (Wolfe, 1995). A lot of different kinds of α-chains with distinct amino acid sequences have been found. These α-chains form combinations to create, to date, 19 types of collagens, designated types I to XIX.

### 1.2.2 **Proteoglycans**

The proteoglycans are a heterogeneous family of a linear polypeptide "core" to which long carbohydrate chains are linked, radiating outward from the core. The primary carbohydrate groups are glycosaminoglycans that consist of unbranched chains assembled from hundreds of repeating 2-sugar units. An amino sugar is always present as one of the 2 sugars in each repeating unit. Many of the amino sugars are further modified by addition of sulphate groups. The polypeptide core may also attach to branched or unbranched glycoproteins other than the glycosaminoglycans (Wolfe, 1995).

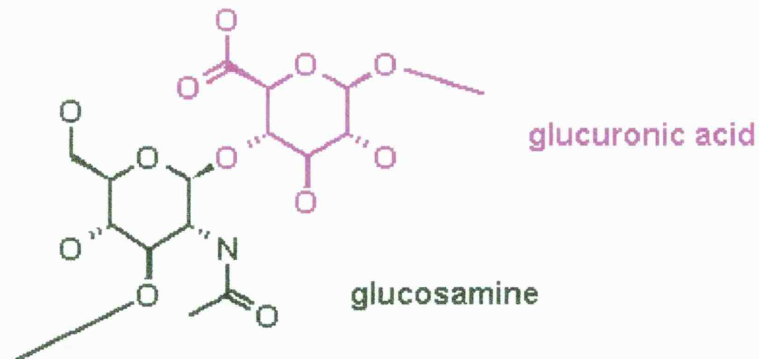
#### 1.2.2.1 **Hyaluronic acid**

In some extracellular structures, particularly in cartilage, a proteoglycan combines further with the unsulphated glycosaminoglycan, hyaluronic acid (HA), to produce a proteoglycan aggregate (Junqueira & Montes, 1983) (Figures 1.2a and 1.2b). An individual HA molecule can be many nanometres in length (Buckwalter *et al.*, 1994). HA-binding proteins, such as CD44, aggrecan and versican, have been related to the ECM structuring by stabilising the large macromolecular aggregates (Bost *et al.*, 1998). They may also play a significant role in cell motility and tumour metastasis (Paulus *et al.*, 1996; Reeder *et al.*, 1998).

## 1.3 **Rheumatoid arthritis**

Rheumatoid arthritis (RA) is a term used to define a multisystem disease where chronic inflammation of many joints is involved in a symmetrical manner. Although it focuses on the joints, it is considered a systemic disease because it involves many extra-articular phenomena. For example, systemic disorders include pulmonary disease, vasculitis, rheumatoid nodules, anaemia, weight loss and an elevated erythrocyte sedimentation rate (Calin & Cormack, 1996; Duthie, 1969). However, the main characteristics of RA are hyperplasia of the synovium, damage of the articular cartilage and bone, and excessive mononuclear cell infiltration.

a




b

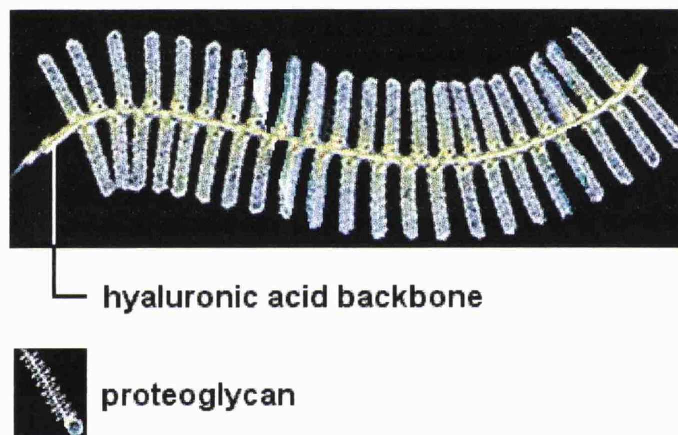



**Figure 1.2.** a) The chemical structure of HA. b) The non-covalent combination between proteoglycan and the HA chain.

The aetiology of RA is not clearly known. However, rheumatoid factor (RF) has been implicated in RA. RF is an antibody generated against autologous immunoglobulin G (IgG). Since approximately 90% of RA patients are seropositive for RF, it has been suggested that the disease may be an autoimmune disorder. However, this is somewhat

controversial since RF is also found in 5-10% of the normal population. In addition, no more than 10% of individuals who are seropositive for rheumatoid factor actually have active RA (Calin & Cormack, 1996). Currently, the disease is postulated to arise from interactions between genetic and environmental factors. The strongest genetic association is with DRB1 genes encoding histocompatibility class II antigens HLA-DR4 and HLA-DR1 (Fugger & Svejgaard, 1997). The environmental factors that are probably involved include infectious agents, metabolic and nutritional abnormalities, psychological make-up of individuals, and the harmful effect of a cold and damp climate on the peripheral circulation (Ghadially, 1983). Among possible infectious agents, Epstein-Barr virus and parvovirus B19 have been focused on. Supporting evidence has indicated a connection between each of these pathogens and RA, but there is a lack of definite proof that they cause RA (Inman, 1991).

### 1.3.1 **Synovial membrane in RA**

In the RA joints, the synovial lining increases to 5-10 cell layers. This marked hyperplasia may be due to proliferation of the resident cells or the influx of mononuclear cells from the blood. Ultrastructural studies, however, show a marked increase in the number of FLSs (Ghadially & Roy, 1967). Moreover, immunohistological and chemical staining has suggested that the active proliferation of fibroblast-like cells may contribute to the hyperplasia observed in the synovial lining in RA (Qu *et al.*, 1994). The mononuclear cells that abundantly infiltrate the synovium are prominently T lymphocytes. T cell-rich regions are detected around small blood vessels that show a similar phenotype to the high endothelial venules of lymphoid organs (Kobayashi & Ziff, 1973). B lymphocytes, plasma cells and cells of the monocyte/macrophage lineage are also found in RA

synovium (Ishikawa & Ziff, 1976; Meijer *et al.*, 1977; Tannenbaum *et al.*, 1975). A study in rabbits suggested that the increase of the lining cells may also be obtained by the migration of active cells, which are deep in the stroma of the synovial tissue, to the synovial lining (Howat, 1987). Under the influence of locally produced cytokines and growth factors, quiescent mesenchymal cell-derived FLSs can change into aggressive, proliferating, invasive cells (Zvaifler & Firestein, 1994). The activated synoviocytes, blood vessels, and the mononuclear cells form into a pannus or "tumour-like" tissue that erodes adjacent cartilage and bone (Figure 1.3).

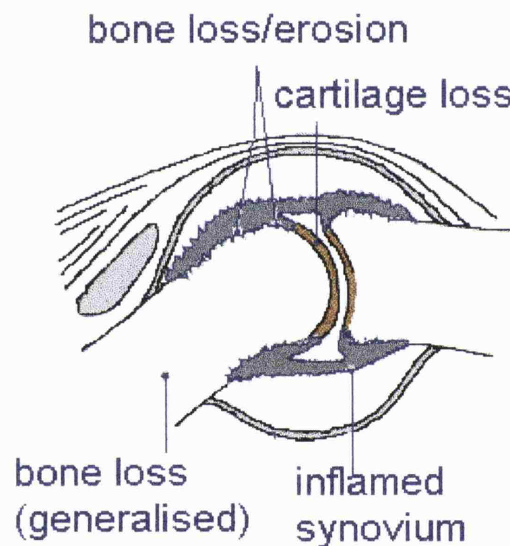



**Figure 1.3.** The structure of a rheumatoid arthritic diarthrosis.

### 1.3.2 **Articular cartilage in RA**

The destruction of the articular cartilage is apparent in arthritis. Changes observed in the matrix include diminished proteoglycan content, fragmentation of collagen and phagocytosis of the fragments by chondrocytes (Mitchell & Shepard, 1978). The process mainly results from an invasive growth of the diseased synovium into the cartilage.

However, since the matrix alteration does not occur only at the cartilage-pannus junction (Mitchell & Shepard, 1978), other mechanisms may also be involved. For instance, the pannus tissue may stimulate the chondrocytes to degrade the matrix (Weyand & Goronzy, 1997).

### 1.3.3 **Synovial fluid in RA**

The components of synovial fluid from RA patients have been noted to be different to that from non-RA patients. RA synovial fluid contains higher levels of apolipoproteins A-I and B, and cholesterol. Analyses of plasma and the synovial fluid apolipoproteins suggest an increased permeability for these constituents across RA synovium (Ananth *et al.*, 1993). A higher concentration of fibronectin (Lu-Steffes *et al.*, 1982), but a lower concentration of keratan sulphate epitope (Pavelka & Seibel, 1989), is present in synovial fluid from RA patients as compared to that from patients with other arthritides.

Large numbers of leukocytes including lymphocytes and neutrophils are found in the synovial fluid. The remaining cells are monocytes, sporadic macrophages and synovial lining cells (Zvaifler, 1973). The activity of lymphocytes and neutrophils in the RA synovial fluid appears to be different from that in peripheral blood (Sebok *et al.*, 1977; Zvaifler, 1973). The mechanisms for this are still to be determined. For T lymphocytes, preferential recruitment of pre-activated cells and induction of activation markers by endothelial contact may play a role in the cell activity (Iannone *et al.*, 1994).

### 1.3.4 **FLSs with an aggressive phenotype in arthritis**

FLSs found in the intimal layer express a number of surface adhesion receptors including intercellular adhesion molecule-1, CD44, $\beta_1$ integrins (such as vascular cell adhesion molecule-1) and $\beta_3$ integrins. Higher levels of expression of adhesion molecules have been found on FLSs in RA tissue than in normal tissue (Johnson *et al.*, 1993; Nikkari *et al.*, 1995; Szekanecz *et al.*, 1994).

As mentioned previously, RA synovium becomes dramatically hyperplastic. Recent evidence has suggested that, at least in part, an increase in the number of resident FLSs accounts for this event. Expression of proliferating cell nuclear antigen and nucleolar organizer region is detected in these cells (Qu *et al.*, 1994). Moreover, various early response genes and oncogenes such as egr-1, c-fos, myc and ras also show considerable up-regulation in RA FLSs both *in situ* and *in vitro* (Müller-Ladner *et al.*, 1995). The expression of c-myc is not unique to cells from patients with RA because equal or higher levels of expression have also been observed in reactive arthritic and OA synovial specimens. The levels of c-myc are also found to be elevated in synovial tissues in animal models of OA (Pelletier *et al.*, 1993; Ritchlin *et al.*, 1994; Roivainen *et al.*, 1995). These findings indicate that the FLSs, as found in diseased tissue, have a transformed phenotype.

The FLSs, under the influence of inflammatory cytokines, produce enormous quantities of enzymes that can degrade ECM. Several groups of enzymes are implicated in this action, including serine proteases, cathepsins (Keyszer *et al.*, 1995) and, perhaps most importantly, the matrix metalloproteinases (MMPs). The family of MMPs comprises at

least 18 members, including collagenases, stromelysins and gelatinases, distributed in various cell types. In RA, the intimal lining is the principal source of MMPs, and *in situ* hybridisation studies localise collagenase and stromelysin messenger RNA (mRNA) almost exclusively to FLSs (Firestein *et al.*, 1991; McCachren *et al.*, 1990). Several MMP proteins, such as MMP-1 (Sorsa *et al.*, 1992), MMP-2 (Okada *et al.*, 1990), MMP-9 (Ahrens *et al.*, 1996) and MMP-13 (Lindy *et al.*, 1997), are, indeed, detected in or released from these cells.

The destructive potential of synovial cells has been demonstrated directly by the model of engraftment of isolated synovial cells or tissue, alone or with pieces of normal cartilage tissue, into severe combined immunodeficiency (SCID) mice (Sack *et al.*, 1996; Müller-Ladner *et al.*, 1996). It is convincing that RA FLSs keep their transformed appearance and maintain cartilage invasive and destructive behaviour over prolonged periods of time in the absence of T cells. These findings and other experimental data (Shiozawa *et al.*, 1992) indicate that by secreting a variety of matrix-degrading enzymes, FLSs play a major role in destruction of bone and cartilage. Their transformed state with, as a consequence, a dramatic increase in their number may in itself be sufficient to explain the ongoing joint destruction.

### 1.4 **Inflammatory cytokines involved in RA**

The word 'cytokine' is a general term for low molecular weight protein mediators involved in cell growth, differentiation, immunity, inflammation and repair. Much evidence clearly indicates that the actions of certain cytokines are responsible for many of the manifestations of RA. Best-known cytokines are interleukins (ILs), tumour necrosis

factor (TNF)-α and interferon (IFN)-γ.

In rheumatoid synovium, in spite of the presence of T cells, there are only low levels of the T cell-derived cytokines, such as IL-2 and IL-4 (Tucci *et al.*, 1997). On the other hand, there are high levels of cytokines derived primarily from synovial macrophages, especially IL-1β, TNF-α and IL-6 (Firestein *et al.*, 1990). These cytokines are pro-inflammatory, affecting FLSs, chondrocytes and lymphocytes; all of which are found in inflamed synovial tissue. IL-4 has some inhibitory effects on generation of IL-1 and TNF-α (D'Andrea *et al.*, 1995), and providing IL-4 as therapeutic agent to RA patients has been considered (Bonder *et al.*, 1999).

Cytokines, especially those derived from macrophages and FLSs, play a role in mediating tissue destruction. To date, 2 of them have met all of four criteria proposed to judge whether cytokines act as mediators of tissue damage in RA.[1] These are IL-1β and TNF-α (Starkebaum, 1998). Their levels are found highly elevated in synovium (Firestein *et al.*, 1990), pannus tissue (Tetlow & Woolley, 1995) and synovial fluid (Hopkins & Meager, 1988; Smith *et al.*, 1989) in patients with RA. *In vitro* culture of a mixture of synovial cells also demonstrates synthesis of IL-1β and TNF-α (Sugiyama *et al.*, 1995). Importantly, the IL-1β and TNF-α were found to influence expression of other cytokines. For instance, a neutralising anti-TNF-α antibody inhibits the production of IL-1β, GM-CSF, IL-6 and IL-8. A neutralising concentration of IL-1 receptor antagonist (IL-1Ra) also reduces IL-6 and IL-8 production. However, IL-1Ra does not reduce TNF-α

[1] Proposed criteria for identifying cytokines as mediators of tissue damage in RA: 1. Cytokines are present at high levels in RA synovium. 2. Recombinant cytokines induce damage to normal cartilage *in vitro* and *in vivo*. 3. Cartilage damage in animals is prevented by specific cytokine inhibitors or antagonists. 4. Progression of tissue damage in patients with RA is prevented by specific cytokine inhibitors.

production, thus indicating that the cytokine interactions are hierarchical (Butler *et al.*, 1995). Il-1β and TNF-α are implicated in cartilage damage since they induce the synthesis and secretion of several MMPs by chondrocytes and FLSs (DiBattista *et al.*, 1994; Shingu *et al.*, 1995; Shinmei *et al.*, 1991). Furthermore, these cytokines inhibit the synthesis of collagen and proteoglycans by articular chondrocytes (Bassleer *et al.*, 1998; Malfait *et al.*, 1994; Reginato *et al.*, 1993) and induce bone resorption through formation of osteoclasts *in vitro* (Pfeilschifter *et al.*, 1989).

Antibodies against certain cytokines have been found to reduce inflammation or cartilage destruction in animal models. These include antibodies against TNF-α, IFN-γ, IL-1, and IL-12 (Boissier *et al.*, 1995; Joosten *et al.*, 1996; Kuiper *et al.*, 1998; Malfait *et al.*, 1998). Similarly, IL-1Ra, which is an endogenous IL-1 inhibitor, suppresses collagen-induced arthritis in mice. Combined anti-TNF-α/anti-IL-1 treatment restrains both inflammation and cartilage damage as seen in locally induced streptococcal cell wall arthritis in mice.

Clinical trials with cytokine antagonists further support the notion that TNF-α and IL-1 are implicated in joint destruction. Treatment of patients with a monoclonal antibody (mAb) to TNF-α, called cA2, resulted in a significant improvement, compared to those given only placebo (Elliott *et al.*, 1994). In addition, the therapeutic value of a TNF-α antagonist has been evaluated. The molecule is a recombinant fusion protein that consists of soluble TNF-α receptor linked to the Fc portion of human $IgG_1$ (TNFR:Fc). Treatment of RA patients with TNFR:Fc led to reductions in the number of tender or swollen joints (Moreland *et al.*, 1997). A trial of recombinant IL-1Ra has also been reported with RA patients (Campion *et al.*, 1996). However, the result was not as striking as the anti-TNF-α studies and no placebo group was included. Although anti-TNF-α therapy appears to be

promising, it remains to be determined whether this therapy will be useful in the long-term management of rheumatoid arthritis and in the achievement of better outcomes of disease. For example, as seen in collagen-induced arthritic mice, anti-TNF-α treatment showed efficacy shortly after onset of the disease but had little effect on fully established arthritis (Joosten *et al.*, 1996). Moreover, because TNF-α production serves a specific function in host defence, the adverse effects of the long-term therapy must be carefully evaluated (Camussi & Lupia, 1998).

The roles of other cytokines present in rheumatoid synovium are being investigated. For instance, transforming growth factor (TGF)-β is abundant in rheumatoid synovium (Taketazu *et al.*, 1994). However, its role is unclear. It stimulates DNA synthesis in FLSs and may lead to hyperplasia of the synovium (Hamilton *et al.*, 1991). In contrast, it stimulates proteoglycan synthesis and restores the proteoglycan content of depleted cartilage (Glansbeek *et al.*, 1998). IL-10 is another important cytokine that has been suggested to have a potential therapeutic role in RA. It inhibits pro-inflammatory cytokine production as well as blocking T-cell responses to specific antigens. A study of human recombinant IL-10 in patients with RA demonstrated that IL-10 is likely to reduce rheumatoid disease activity (reviewed in Keystone *et al.*, 1998). Of increasing interest, IL-15 is a newly discovered cytokine. It is expressed by epithelial cells, endothelial cells, fibroblasts and monocytes/macrophages. An increased expression of IL-15 was found in the synovium of RA patients, compared with those having OA (Thurkow *et al.*, 1997). IL-15 enhances migration of T lymphocytes across human umbilical vein endothelial cells *in vitro* (Borthwick *et al.*, 1997), suggesting that this cytokine may play an important role in the migration of T cells to inflammatory sites. IL-15 has been demonstrated to induce the production in RA of other cytokines including TNF-α (McInnes *et al.*, 1997), IL-8 and

monocyte chemotactic protein 1 (Badolato *et al.*, 1997); all of which are found in rheumatoid synovial fluid (al Mughales *et al.*, 1996). When administered to mice, a soluble fragment of IL-15 receptor $\alpha$ chain suppresses the development of collagen-induced arthritis (Ruchatz *et al.*, 1998). This suggests that IL-15 antagonists may also have therapeutic potential in RA.

## 1.5 Adhesion molecules involved in RA

Adhesion molecules play 2 important roles in inflammation. First, they allow extravasation of leukocytes into the affected tissue. Second, they play a role in the retention and maturation of these infiltrated cells, which involves interactions between the resident tissue cells and the infiltrating cells and between both cell groups and ECM. An understanding of the characteristics of adhesion molecules and their roles in the pathogenesis of arthritis should provide pharmacological targets to control chronic inflammation more effectively.

Adhesion molecules of the cadherin, immunoglobulin, selectin and integrin superfamilies facilitate many of the adhesive interactions. Most of the adhesion receptors are transmembrane proteins. They bind at least one counter-molecule, which may be either another receptor on a different cell or a component of the ECM. Certain pro-inflammatory mediators can regulate expression of these adhesion molecules (Croft *et al.*, 1999; Defilippi *et al.*, 1991; Jakob & Udey, 1998; van Setten *et al.*, 1997).

### 1.5.1 **Cadherins**

Cadherins are a family of glycoproteins that mediate the adhesion between cells. They play an important role in a wide variety of tissues. The expression of cadherins is involved in embryonic development. They also help preserve tissue phenotype and integrity in adult animals as part of big junctional complexes linked to the cytoskeleton (Behrens, 1994-95). Alterations in cadherin function, in particular a change in its lateral localisation, have been implicated in tumour progression in a number of carcinomas (Carrato *et al.*, 1998; Washington *et al.*, 1998). This may result from loss of cell adhesion and a contribution to invasive or metastatic potential in the carcinomas.

Numerous different cadherins exist, the principal ones being epithelial (E)-cadherin, neuronal (N)-cadherin and placental (P)-cadherin. They are found in distinct tissues. E-cadherin is primarily expressed on non-neural epithelial tissue (Shimoyama *et al.*, 1989). N-cadherin is the dominant cadherin of neural tissues (Roark *et al.*, 1992). It has also been discovered as a soluble form (Paradies and Grunwald, 1993). P-cadherin is expressed in epidermis, mesothelium and corneal epithelium (Nose & Takeichi, 1986).

E-cadherin is the one that has been identified in the RA synovial tissue (Trollmo *et al.*, 1996). It is a ligand for the integrin $\alpha E\beta_7$, which is involved in lymphocyte binding to the RA synovial tissue (Jorgensen *et al.*, 1996).

### 1.5.2 **Immunoglobulin (Ig) superfamily**

The Ig superfamily is the most divergent group of receptors known. Their common structure is characterised by the presence of Ig homology domains (Buck, 1992). Despite similarity in the Ig fold, the adhesion molecules of this superfamily differ in their distribution and functions. They include CD3, CD4, CD8, class I and class II major histocompatibility complex (MHC) molecules, intercellular adhesion molecule (ICAM)-1, ICAM-2, ICAM-3, leukocyte function-associated antigen (LFA)-3, mucosal addressin cell adhesion molecule (MAdCAM)-1, neural cell adhesion molecule (NCAM), platelet-endothelial cell adhesion molecule (PECAM)-1, T cell receptor and vascular adhesion molecule (VCAM)-1 (Table 1.1).

Members of the group that are important for the extravasation of blood-borne cells into inflamed tissues are ICAM-1 and VCAM-1. ICAM-1 is constitutively expressed on the surface of many cell types, including endothelial cells, but this is increased following exposure to pro-inflammatory cytokines, such as IL-1, TNF-$\alpha$ and IFN-$\gamma$ (Dustin and Springer, 1988; Voraberger *et al.*, 1991). The role for ICAM-1 in extravasation was discovered through the use of a mAb to ICAM-1 that blocked the trans-endothelial migration of both lymphocytes and neutrophils *in vitro* (Greenwood *et al.*, 1995; Luscinskas *et al.*, 1991). With respect to arthritis, mice deficient in ICAM-1 demonstrate a reduced incidence of collagen-induced arthritis, suggesting that naturally occurring genetic variation in the expression of ICAM-1 might influence susceptibility to RA in human (Bullard *et al.*, 1996).

| Receptor | Distribution | Other name(s) | Ligand(s) |
|---|---|---|---|
| CD3 | T lymphocytes | | |
| CD4 | Helper/inducer T lymphocytes, monocytes, macrophages | | MHC class II molecule |
| CD8 | Cytotoxic/suppressor T lymphocytes, natural killer cells | | MHC class I molecule |
| ICAM-1 | Endothelial cells, germinal centre dendritic cells, epithelial cells, activated lymphocytes, synovial fibroblasts, synovial macrophages | CD54 | LFA-1, Mac-1, leukosialin (CD43) |
| ICAM-2 | Endothelial cells, hematopoietic cell lines | CD102 | LFA-1 |
| ICAM-3 | Lymphocytes, monocytes, neutrophils, epidermal Langerhans cells | CD50 | LFA-1 |
| LFA-3 | Endothelial cells, epithelial cells, leukocytes | CD58 | CD2 |
| MAdCAM-1 | Mucosal endothelial cells | | $\alpha_4\beta_7$ integrin, L-selectin |

**Table 1.1.** Members of the Ig superfamily of receptors.

| Receptor | Distribution | Other name(s) | Ligand(s) |
|---|---|---|---|
| MHC class I molecule | Many cells throughout the body except sensory neurons | | T cell receptor, in the presence of peptide fragments; leukocyte Ig-like receptors |
| MHC class II molecule | B lymphocytes, macrophages, dendritic cells, synovial fibroblasts | | T cell receptor, in the presence of peptide fragments |
| NCAM | Neural cells | CD56 | Heparan sulphate, |
| PECAM-1 | Endothelial cells, platelets, smooth muscle cells, leukocytes, neutrophils | CD31 | $\alpha V\beta_3$ integrin, PECAM-1 |
| T cell receptor | T lymphocytes | | Peptide fragments associated with MHC class I or class II molecule |
| VCAM-1 | Endothelial cells, bone marrow fibroblasts, synovial lining cells, synovial macrophages, germinal centre dendritic cells | CD106, INCAM-110 | Very late antigen (VLA)-4 |

**Table 1.1.** Members of the Ig superfamily of receptors (continued).

VCAM-1 is another member of the Ig superfamily that has alternatively been called INCAM-110 (inducible cell adhesion molecule with a molecular weight of 110 kDa) (Osborn *et al.*, 1989; Rice *et al.*, 1990). It is a counter-receptor for the $\beta_1$ integrin VLA-4 (Elices *et al.*, 1990). Unstimulated endothelial cells express very little, or no, VCAM-1 on their cell surface. However, upon exposure of endothelial cells to the cytokines TNF-α, IL-1 or IL-4, levels increase dramatically (Carlos *et al.*, 1990; Masinovsky *et al.*, 1990; Osborn *et al.*, 1989; Rice *et al.*, 1990). This process requires protein synthesis and, thus, does not reflect redistribution of preformed molecules (Masinovsky *et al.*, 1990).

The mechanism by which VCAM-1 is involved in the infiltration of lymphocytes into inflammatory synovium appears to be different from ICAM-1. VCAM-1 is involved in binding to endothelium, in particular in the presence of inflammatory mediators, but it does not function during trans-endothelial migration while ICAM-1 does (Oppenheimer-Marks *et al.*, 1991). Several other studies have similarly suggested that VCAM-1 is important in the accumulation of T cells at sites of inflammation but not directly in mediating migration into the extravascular compartment.

FLSs in RA express elevated levels of VCAM-1, as compared with those from normal synovium (Croft *et al.*, 1999; Morales-Ducret *et al.*, 1992). The expression can be augmented by interactions between the FLSs with either T cells (Bombara *et al.*, 1993) or monocytes (Blue *et al.*, 1993; Chen *et al.*, 1998). Cytokines including TNF-α and ILs also enhance the expression of VCAM-1. A recent study showed that the expression was rapidly but only transiently up-regulated by TNF-α and IL-1β whilst sustained elevated levels were obtained with IL-4 or IL-13. Combination of either IL-4 or IL-13 and TNF-α

resulted in a highly elevated and sustained expression (Croft *et al.*, 1999). It has been suggested that VCAM-1 expressed on FLSs plays a role in the T lymphocyte mitogenic response, a phenomenon that could contribute to the increased cellularity observed in the rheumatoid synovial membrane (Postigo *et al.*, 1993).

### 1.5.3 **Selectins**

There are 3 members of the selectin family; each of which is important for the extravasation of leukocytes into inflammatory sites. They are endothelial (E)-selectin (ELAM-1), leukocyte (L)-selectin, and platelet (P)-selectin (GMP-140). All members of this family have, at their amino termini, a lectin domain, at which most of the binding activity occurs (Bevilacqua *et al.*, 1989; Johnston *et al.*, 1989; Lasky *et al.*, 1989). E-selectin and P-selectin bind charged oligosaccharides including a sialylated structure, sialyl Lewis X, found on neutrophils, monocytes, and tumour cells (Phillips *et al.*, 1990; Zhou *et al.*, 1991). L-selectin expressed by neutrophils contains the sialyl Lewis X sequence and serves as one of the counter-receptors for E- and P-selectins (Picker *et al.*, 1991). L-selectin on neutrophils and lymphocytes also binds to peripheral lymph node vascular addressin (PNAd) (Berg *et al.*, 1991; Lawrence *et al.*, 1995).

The expression of E-selectin is restricted to endothelial cells, and it is found at sites of chronic inflammation including rheumatoid synovium (Koch *et al.*, 1991). Normal, unstimulated endothelial cells do not express E-selectin, but it is induced by IL-1 and TNF (Bevilacqua *et al.*, 1987). The augmented expression of E-selectin in RA synovial endothelium may facilitate the recruitment of leukocytes to this site. L-selectin is

expressed by resting neutrophils, lymphocytes and monocytes (Lewinsohn *et al.*, 1987). As mentioned above, the L-selectin expressed by neutrophils serves as a counter-receptor for E-selectin. It participates in the earliest interactions of neutrophils with inflamed venules (Picker *et al.*, 1991).

P-selectin is a membrane glycoprotein found in human platelets and endothelial cells (Larsen *et al.*, 1989). Induction of this molecule and platelet-activating factor by histamine, thrombin and leukotriene $C_4$ contribute to the acute rolling of polymorphonuclear cells on endothelial surface (Leirisalo-Repo, 1994). In addition, a study in RA synovial tissues showed that mAbs specific to P-selectin blocked adhesion of monocytes to venules by more than 90%, suggesting a prominent role for P-selectin in monocyte recruitment *in vivo* (Grober *et al.*, 1993).

### 1.5.4 **Integrins**

Integrins are widely distributed on all nucleated cells. They mediate many cell-cell and cell-ECM adhesive interactions by binding to specific cell surface counter-receptors and/or to components of ECM (Table 1.2). Many physiological processes including embryological development, haemostasis, thrombosis, wound healing, immune and non-immune defence mechanisms, and oncogenic transformation have been linked to integrins (reviewed in Albelda & Buck, 1990; Hynes, 1987). All members of the integrin family are transmembrane glycoproteins composed of 2 noncovalently linked heterodimers: the $\alpha$ and $\beta$ subunits. There are at least 8 identified $\beta$ subunits and 15 identified $\alpha$ subunits that dimerise to produce more than 20 different receptors. The ability of integrins to

mediate interactions with ligands requires the activity of the cytoskeleton. Interactions with actin-containing microfilaments have been analysed and they occur indirectly by association of the cytoplasmic domains of the receptors with actin binding proteins, such as talin and vinculin (Burn *et al.*, 1988; Burridge *et al.*, 1988).

The activity of many members of the integrin family of adhesion molecules is essential for the infiltration of leukocytes into inflammatory sites. On blood-borne cells, integrins are in an inactive state and require stimulation by chemokines through binding to their G-protein coupled receptors. As mentioned previously, VLA-4 plays a crucial role in the binding of lymphocytes to activated endothelium whereas LFA-1 plays a central role in the subsequent transmigration through the endothelial cell monolayer (Oppenheimer-Marks & Lipsky, 1994). LFA-1 is also crucial in neutrophil transmigration, but, in the case of monocytes, this role seems to be reserved for the $\beta_2$ integrin Mac-1 (Meerschaert & Furie, 1995).

A subset of circulating T cells from RA patients is spontaneously competent to bind ICAM-1. It implies that this subset of circulating T cells expresses LFA-1 in an activated form. The expression of an activated form of LFA-1 may be another feature related to the overall increased activation state of circulating T cells in RA patients (Oppenheimer-Marks & Lipsky, 1994) and may account for their increased accumulation within the rheumatoid synovium, which is found to express ICAM-1 (Hale *et al.*, 1989). A high proportion of T cells from RA synovial tissue and fluid also express activated VLA-4, compared with peripheral blood T cells from the same patients (Laffon *et al.*, 1991).

| Receptor | Subunits | Distribution | Other name(s) | Ligand(s) |
|---|---|---|---|---|
| VLA-1 | $\alpha_1\beta_1$ | Activated T cells, fibroblasts, mesangial cells, liver sinusoids, some cell lines | CD49a/CD29 | Laminin, collagen |
| VLA-2 | $\alpha_2\beta_1$ | Activated T cells, endothelial cells, platelets | CD49b/CD29, glycoprotein Ia/IIa (gpIa/IIa), ECM receptor II (ECMRII) | Collagen, laminin |
| VLA-3 | $\alpha_3\beta_1$ | B cells | CD49c/CD29, ECMRI | Fibronectin, laminin, collagen |
| VLA-4 | $\alpha_4\beta_1$ | Lymphocytes, thymus cells, monocytes, hematopoietic cell lines | CD49d/CD29 | VCAM-1, fibronectin |
| VLA-5 | $\alpha_5\beta_1$ | Lymphocytes, thymus cells, monocytes, fibroblasts, endothelial cells | CD49e/CD29, gpIc/IIa, ECMRVI | Fibronectin |
| VLA-6 | $\alpha_6\beta_1$ | Lymphocytes, platelets | CD49f/CD29 | Laminin |

**Table 1.2.** Members of the integrin family of receptors.

| Receptor | Subunits | Distribution | Other name(s) | Ligand(s) |
|---|---|---|---|---|
| LFA-1 | $\alpha L\beta_2$ | All immune cells | CD11a/CD18, p177/95 | ICAM-1, ICAM-2, ICAM-3 |
| Mac-1 | $\alpha M\beta_2$ | Monocytes, some B cells, granulocytes, large granular lymphocytes, macrophages | CD11b/CD18, complement receptor 3 (CR3), p165/95 | ICAM-1, iC3b, fibrinogen, factor X, endotoxin |
| p150/95 | $\alpha X\beta_2$ | Monocytes, some B cells, granulocytes, large granular lymphocytes, macrophages, hairy cell leukemia cells | CD11c/CD18 | CD23 |
| CD41/ CD61 | $\alpha IIb\beta_3$ | Platelets | gpIIb/IIIa | Fibronectin, von Willebrand factor, fibrinogen |
| CD51/ CD61 | $\alpha V\beta_3$ | Mast cells, B lymphoblastoid cell line | | Vitronectin, fibrinogen, thrombospondin, von Willebrand factor |

**Table 1.2.** Members of the integrin family of receptors (continued).

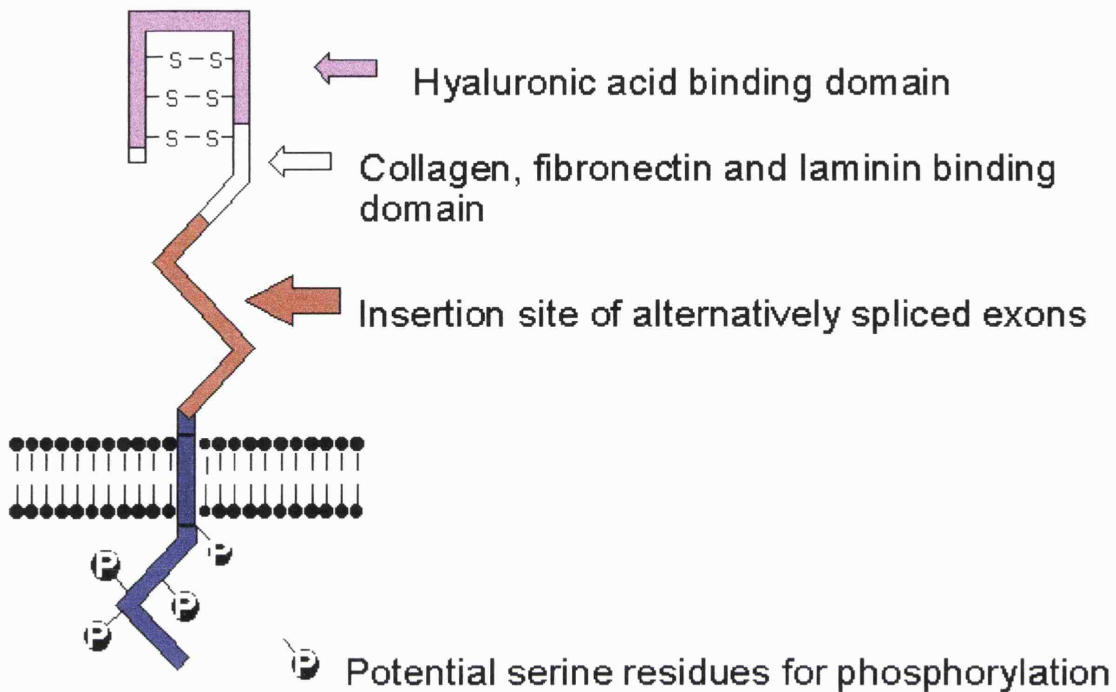
| Receptor | Subunits | Distribution | Other name(s) | Ligand(s) |
|---|---|---|---|---|
| $\alpha_4\beta_7$ | $\alpha_4\beta_7$ | Band T cells, macrophages | | MAdCAM-1, VCAM-1, fibronectin |
| $\alpha E\beta_7$ | $\alpha E\beta_7$ | Intraepithelial lymphocytes | | E-cadherin |

**Table 1.2.** Members of the integrin family of receptors (continued).

### 1.5.5 **CD44 and its isoforms**

CD44, which is also known as phagocytic glycoprotein-1 (Pgp-1), GP90 Hermes, and ECMRIII (Shimizu *et al.*, 1989), is another adhesion molecule, which is not related to the superfamilies described above. It is a widely distributed cell surface receptor. The CD44 glycoprotein family is fairly heterogeneous in size ranging from 85 to >200 kDa (Bartolazzi *et al.*, 1996). It is composed of 3 major domains: extracellular, transmembrane, and cytoplasmic domains (Figures 1.4a and 1.4b). The CD44 gene comprises 20 exons; only 10 of which are normally expressed (Stauder & Günthert, 1995) and encode the common or standard form (CD44H or CD44s; 85-95 kDa) present on most cell types. The additional 10 exons, encoding part of the extracellular region, can be alternatively spliced into mRNA to generate a variety of larger variant isoforms (CD44v). It should be noted that exon v1 is not expressed in the human CD44 (Screaton *et al.*, 1993). Modification by N-glycosylation, O-glycosylation, and glycosaminoglycanation (by heparan sulphate or chondroitin sulphate) further increases the number of CD44 isoforms (Brown *et al.*, 1991; Camp *et al*, 1991; Dasgupta *et al.*, 1996).

a




b

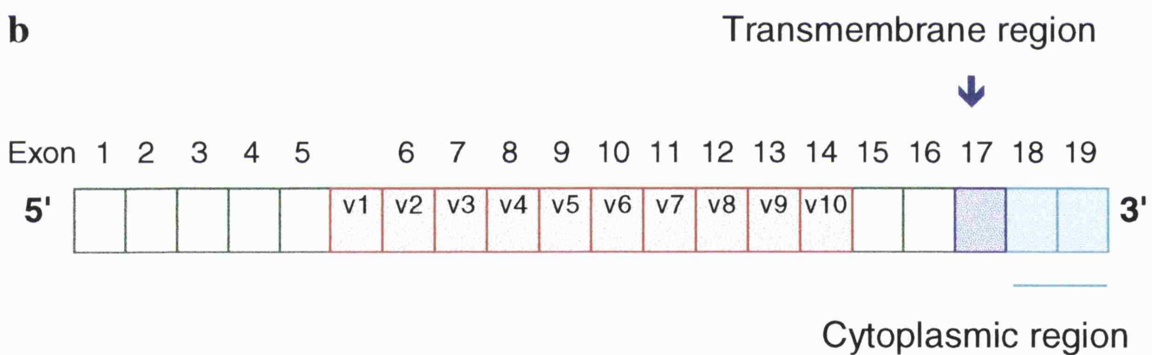


**Figure 1.4.** CD44 molecule and its splice variants. a) Structural features of CD44. The standard protein is described with various domains (Sherman *et al.*, 1994). b) The exon map of CD44 (Wilson, 1996). Variant exons (v1-v10), which are alternatively spliced, are shown.

CD44 is involved in several physiological functions by serving as a cell-ECM receptor. The main ligand for CD44 is HA (Aruffo *et al.*, 1990). Other ECM ligands for this receptor are fibronectin, collagen, laminin and sulphated proteoglycan (Jalkanen & Jalkanen, 1992; Radotra *et al.*, 1994; Toyama-Sorimachi & Miyasaka, 1994). CD44 also participates in the uptake and intracellular degradation of HA and hence may be important during tissue morphogenesis and cell migration (Culty *et al.*, 1992).

Additional important functions of CD44 include T cell signalling and activation, lymphocyte recirculation and cell-cell interactions. CD44 seems to be involved in HA-mediated signal transduction since pretreatment of cells with an anti-CD44 mAb directed against the HA-binding domain of CD44 can inhibit calcium mobilisation in T cells (Galluzzo *et al.*, 1995). Cross-linking of CD44 induces a signal transduction pathway involving tyrosine kinase activation and modulation of intracellular calcium levels (Palmieri *et al.*, 1996; Taher *et al.*, 1996). Indeed, the cross-linking of this surface molecule was found to deliver either a positive or a negative signal in murine antigen-specific T cell hybridomas (Guo *et al.*, 1993). Soluble human CD44 was found to delay reappearance of T lymphocytes in peripheral blood in anti-Thy-1-treated mice, reflecting that the soluble receptors interfere with lymphocyte migration and regulate trafficking of these cells (Guo *et al.*, 1994). The function of CD44-mediated cell-cell interactions can be seen in several circumstances. Interaction between T cells and CD44 is able to induce DNA synthesis of airway smooth muscle cells and may be responsible for hyperplasia of these cells in asthma (Lazaar *et al.*, 1994). Lymphocytes treated with anti-CD44 mAb showed reduced adherence to an endothelial cell line (Brennan *et al.*, 1997), implying the importance of this molecule in adhesion between cells. CD44 also has the capacity to up-regulate the cytotoxic activity of natural killer cells (Palmieri *et al.*, 1996). Moreover,

CD44 takes part in embryogenesis (Wheatley *et al.*, 1993) and transmission of signals mediating hematopoiesis (Kensas *et al.*, 1990) and apoptosis (Ayroldi *et al.*, 1996). A number of studies have shown that the CD44 cytoplasmic tail may have a role in its interaction with cytoskeleton-related components such as actin, ankyrin (Bourguignon *et al.*, 1992; Lokeshwar *et al.*, 1996), or members of the ezrin-radixin-moesin (ERM) family (Nakamura & Ozawa, 1996; Tsukita *et al.*, 1994).

Many pathological cell activities relate to CD44; an example of which is inflammation. In chronic adult periodontitis, CD44 expressed on lymphocytes participates in the adhesive interactions between these cells and human gingival fibroblasts and may lead to the lymphocyte accumulation in periodontal lesions (Murakami *et al.*, 1997). Similarly, the CD44-HA interaction mediates lymphocyte adhesion to white matter in the central nervous system and thus may be involved in the pathogenesis of the central nervous system inflammations (Aho *et al.*, 1994). Up-regulation of the CD44 gene was, furthermore, noticed in salivary glands from mice with experimental autoimmune sialadenitis (Hayashi *et al.*, 1996). Most importantly, antibodies against CD44 have been shown to reduce the incidence and delay the onset of arthritis in a number of animal models (Verdrengh *et al.*, 1995; Zeidler *et al.*, 1995) as well as abolishing tissue swelling and leukocyte infiltration in murine arthritis (Mikecz *et al.*, 1995).

Increased expression of CD44 is found on some carcinomas (Mathew *et al.*, 1996; Stamenkovic *et al.*, 1989). The transition of tumour cell lines from non-metastatic to metastatic may be associated with CD44H expression (Kawasaki *et al.*, 1996) and with changes in the expression of CD44 variants (Welsh *et al.*, 1995).

Isoforms of CD44 have been found to play a number of important roles. For instance, regulation of clustering of CD44, mediated by factors including the presence of variant exons and glycosylation, may be vital in allowing cells to regulate their HA binding properties (Sleeman *et al.*, 1996). It should be noted that single cells can express multiple variants. Examples of this are epithelial cells that utilise exons v8, v9 and v10 of the variable region to generate CD44v8-10 isoform (Stamenkovic *et al.*, 1991) and keratinocytes that express the longest CD44 isoform, CD44v3-10 (Hofmann *et al.*, 1991). Isoforms obtained from the alternative splicing have also been extensively studied in relation to tumours and inflammatory diseases. They are discussed in detail in the following sections.

#### 1.5.5.1 **CD44v2**

Only recently, special interest has been shown in CD44 splice variants containing v2. To date, this variant isoform has been significantly correlated with vessel invasion and decreased overall survival of pancreatic cancer patients (Gotoda *et al.*, 1998), and with shorter survival times of breast cancer patients, as compared with those having v2-negative tumours (Tokue *et al.*, 1998).

#### 1.5.5.2 **CD44v3**

CD44 isoforms containing the v3 are the isoforms associated with both chondroitin and heparan sulphate modifications (Jackson *et al.*, 1995). Heparan sulphate-modified CD44v3 binds to the heparin-binding epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Bennett *et al.*, 1995), which is a growth factor that is associated

with synovial hyperplasia in rheumatoid joints (Qu *et al.*, 1995). CD44 isoforms containing the v3 have been detected in malignant non-Hodgkin's lymphomas (Terpe *et al.*, 1994) and in colon biopsy samples recovered from patients with ulcerative colitis (Rosenberg *et al.*, 1995). Overexpression of CD44v3 is also correlated with a reduced overall survival rate for patients with vulvar carcinoma (Tempfer *et al.*, 1996).

#### 1.5.5.3 **CD44v4 and v5**

A study revealed that CD44 splice variants containing v4 and v5 sequences were present in addition to CD44H epitope in various types of brain tumours (Kaaijk *et al.*, 1995). Moreover, CD44v5 was found in increased levels in biopsies recovered from patients with breast cancer having axillary lymph node metastasis (Tempfer *et al.*, 1996) and with primary squamous-cell carcinomas of the cervix with pelvic lymph node metastases (Kainz *et al.*, 1996).

#### 1.5.5.4 **CD44v6**

CD44v6 and CD44H are involved in hematopoiesis in animals but fulfil distinct functions. Whereas CD44H facilitates homing and proliferation of progenitor cells, CD44v6 is involved in the differentiation processes, particularly of lymphoid progenitor cells (Khaldoyanidi *et al.*, 1997). Exon v6 also plays a role in adhesion to HA, as it was shown in human activated T cells that combined expression of CD44v6 and v9 is necessary for binding (Galluzzo *et al.*, 1995). CD44v6 expression may also be related to the differentiation of monocytes to tissue macrophages in inflammatory sites (Levesque & Haynes, 1996).

The finding that rat pancreatic tumour cells metastasised simply through the expression of CD44v4-7 (also termed pMeta-1) (Günthert *et al.*, 1991) and the subsequent finding that antibodies against v6 could abrogate this capacity (Seiter *et al.*, 1993) instigated the search for a role for CD44v6. It was found that the CD44v6 epitope is commonly associated with tumour progression and metastasis. Its expression has been correlated with infiltrative tumour growth, depth of invasion, lymph node involvement and a higher incidence of distant metastasis in certain cancers (Dammrich *et al.*, 1995; Hong *et al.*, 1995a). Patients with carcinomas over-expressing CD44v6 show a shorter relapse-free survival and overall survival compared to patients with tumours lacking CD44v6 over-expression (Tempfer *et al.*, 1996). Despite a large amount of evidence supporting the role of CD44v6 in particular tumours, a variety of tumours express little or no CD44v6 (Kaaijk *et al.*, 1995; Sliutz *et al.*, 1995). This variant can also be found in a variety of normal cells (Gotley *et al.*, 1996; Hong *et al.*, 1995b). In addition, in laryngeal squamous cell carcinoma, increased CD44v6 expression was related to a longer survival time (Spafford *et al.*, 1996).

#### 1.5.5.5 **CD44v7**

CD44v7 has been identified, using immunohistochemistry, on stratum corneum and on the acinar cells of sebaceous and eccrine sweat gland (Seiter *et al.*, 1996). This variant may be correlated with metastasis or dissemination in malignant pleural effusions as it was expressed in most malignant pleural effusions but was found in only a few cases of benign pleural effusions (Tojo *et al.*, 1996). Recently, mAb to CD44v7 appeared to be an efficient and specific therapeutic reagent in an inflammatory disease; that is, chronic colitis (Wittig *et al.*, 1998).

#### 1.5.5.6 **CD44v9**

CD44v9 has been correlated with a range of cell pathology. Similar to CD44v6, CD44v9 may be correlated with the differentiation of monocytes to tissue macrophages in inflammatory situations (Levesque & Haynes, 1996). With regard to tumours, expression of CD44v9 is positively associated with prostate adenocarcinoma differentiation (Takahashi *et al.*, 1998) and with the recurrence of and mortality from primary gastric tumours (Mayer *et al.*, 1993).

#### 1.5.5.7 **CD44v10**

CD44v10, which is identical to adhesion molecule GP116, is expressed on all endothelial cells from different origins. It has been shown to generate a mitogenic response in endothelial cells. The molecule contains approximately 8 N- and 11 O-linked oligosaccharide chains but lacks glycosaminoglycans. It interacts directly with the cytoskeleton protein, ankyrin (Lokeshwar *et al.*, 1996). Currently, Rosel *et al.* (1998) have demonstrated that CD44v10 is transiently expressed on activated T cells, B cells and monocytes as well as on a subpopulation of bone marrow cells. It seems to be important in a B cell-monocyte interaction. Activation of B cells by engagement both of the B cell receptor and of mitogen receptors is, furthermore, inhibited by anti-CD44v10. Like other variants, CD44v10 has been related to tumours and inflammatory diseases. For instance, it was detected in cutaneous lymphomas and reactive skin lymphocytes (Wagner *et al.*, 1998). Co-expression of both CD44H and CD44v10 can change cellular properties including the promotion of tumourigenesis in breast epithelial cells (Iida & Bourguignon, 1997).

#### 1.5.5.8 **Splicing combinations**

The CD44v7/8 is recognised by mAbs that recognise an epitope shared between v7 and v8. It is expressed in several types of cancers but is not, however, always associated with progression or poor prognosis of the cancers. Among the malignant tumours screened, oral squamous cell carcinoma has a lower positive staining of CD44v7/8 than normal buccal mucosa. This indicates that loss of CD44v7/8 expression may be a valuable factor for determining prognosis in patients with this cancer (Kuo *et al.*, 1998). In cases of cervical cancers, expression of this splice variant is correlated with a significantly poorer prognosis (Kainz *et al.*, 1995).

A CD44 isoform holding the last three exon products of the alterable region (v8-v10) is expressed on epithelial cells and is thus called epithelial CD44 or CD44E. It does not display the same HA binding pattern as CD44H. The CD44-negative B-cell lymphoma line Namalwa transfected with CD44E cannot bind to either immobilised or soluble HA while CD44H is able to, although this is not true in every cell type (van der Voort *et al.*, 1995). Furthermore, for HIV-1 infection, human T-lymphoblast cells transfected with cDNA for the CD44H were susceptible to infection with monocytotropic strains, but those transfected with CD44E could be infected with only the lymphocytotropic strain (Dukes *et al.*, 1995).

In conclusion, CD44 splice variants have been widely correlated with altered prognosis in cancer. However, none of the studies with human cells have shown a direct functional implication for splice variants in cell proliferation or tissue invasion. That is to say, masking splice variant epitopes with variant-specific antibodies has not been able to alter

human cell behaviour.

## 1.6 CD44 and RA

Highly differentiated T lymphocytes that are selectively recruited to the affected joints of RA patients express an increased level of this molecule compared with resting T lymphocytes (Borthwick *et al.*, 1997). CD44 has been found to be expressed to a greater extent on RA or OA macrophages, lining cells and fibroblasts, as compared with those from normal subjects (Johnson *et al.*, 1993). In contrast, another study revealed that cultured rheumatoid synovial cells showed a reduced level of CD44, as compared with normal cells, but this difference was suggested to be involved in altered morphology of the cells in RA patients (Henderson *et al.*, 1994). In an *in vitro* study, lymphocytes treated with anti-CD44 mAb IM7 showed reduced adherence to RA FLSs (Brennan *et al.*, 1997). Furthermore, the FLSs themselves, which also express CD44, can mediate cartilage degradation, and antibodies to CD44 markedly inhibit this destruction (Scott *et al.*, 1997).

A recent study employing the reverse transcriptase polymerase chain reaction (RT-PCR) technique, which compared cultured FLSs from normal tissues with those from RA or OA tissues, reported that the cells from the diseased tissues express CD44 splice variants. Interestingly, the FLSs from RA synovium always express CD44 splice variants in a large percentage of cells whereas those from OA synovium demonstrate great variation. Some OA patients have cells expressing splice variant whilst others have almost none and closely resemble control, non-inflamed joints (Croft *et al.*, 1997). These findings clearly indicated that the VCAM-1-positive fibroblast is not a homogenous cell population but can be further subdivided in terms of expression of certain CD44 splicing combinations.

Do these subpopulations have other differences, for instance, in the level of expression of MMPs or inflammatory cytokines? Or what is the topography of splice variant expression in the synovial membrane; is it limited to the intimal layer or are splice variants also expressed in deeper synovium? The question also remains as to whether or not expression of these splice variants plays a role in the pathology of the disease. The proliferative and invasive behaviour of FLSs greatly resembles that of transformed cells and expression of CD44 splice variants may play a role in the development of this transformed phenotype in analogy to the rat pancreatic cells mentioned above.

## 1.7 **Aims of the research project**

- To analyse the pattern of CD44 splice variant expression at the protein level in the synovial membrane.
- To describe the consequences, which are involved in FLS behaviours, of CD44 splice variant expression by
    - ❖ determining if CD44 v3, v6 and v7/8 splice variants expressed in FLSs have a role in the regulation of proliferation.
    - ❖ determining if CD44 v3, v6 and v7/8 splice variants expressed in FLSs have a role in the regulation of matrix invasion.
    - ❖ determining if CD44 v3, v6 and v7/8 splice variants expressed in FLSs have a role in the regulation of expression of cytokines and MMPs.

# Chapter 2

# Materials and methods

## 2.1 Materials

The materials used in this study and their sources are listed below.

| Material | Source |
|---|---|
| Antibodies | |
| Anti-human CD44 mAb (5F12 CL4) | NeoMarkers |
| Anti-human CD14 mAb (TÜK4) | Dako |
| Anti-human CD44v3 mAb (3G5) | R&D Systems |
| Anti-human CD44v6 mAb (2F10) | R&D Systems |
| Anti-human CD44v7/8 mAb (VFF-17) | Bender MedSystems |
| Anti-human CD45 mAb (T29/33) | Dako |
| Anti-human CD68 mAb (EBM11) | Dako |
| Anti-human Ki67 mAb (Ki67) | Serotec |
| Anti-human VCAM-1 mAb (BBIG-V1(4B2)) | R&D Systems |
| Anti-human VCAM-1-biotin mAb (1.G11B1) | Serotec |
| Dynabeads M-450 bound to goat anti-mouse IgG | Dynal |
| Goat anti-mouse IgG-biotin | Sigma |
| Rat anti-human CD44 | A gift from Dr. Sirpa Jalkanen (MediCity Research Laboratory, University of Turku, Finland) |

| Material | Source |
|---|---|
| Sheep anti-mouse IgG coupled with fluorescein isothiocyanate (FITC) | Sigma |
| Sheep anti-rat IgG-FITC | Dako |
| Materials for immunostaining | |
| 4-well chamber slides | Nunc |
| Aminoethylcarbazole (AEC staining kit) | Sigma |
| Avidin-peroxidase | Sigma |
| Fluoromount-G | Southern Biotechnology |
| Mayer's hematoxylin | Sigma |
| Tetramethylrhodamine isothiocyanate (TRITC)-conjugated avidin | Sigma |
| Materials for isolation of FLSs and cell culture | |
| Cell dissociation solution | Sigma |
| Collagenese type 2 | Worthington |
| Dulbecco's Modified Eagle Medium (DMEM) | Gibco BRL |
| EDTA | Sigma |
| Foetal bovine serum (FBS) | Gibco BRL |
| Penicillin-streptomycin solution (P/S) | Gibco BRL |
| Phosphate-buffered saline (PBS) | Gibco BRL |
| Tissue culture flasks | Nunc |
| Trypsin-ethylenediamine tetraacetate (EDTA) solution | Gibco BRL |
| Materials for incorporation experiments | |
| $^3$H-thymidine | Amersham |
| UltimaGold | Packard |

| Material | Source |
|---|---|
| Materials for HA binding experiments | |
| 96-well Maxisorb dishes | Nunc |
| HA | Rooster comb, Sigma |
| Rose Bengal | Sigma |
| Materials for isolation and DNase treatment of total RNA | |
| Chloroform | Sigma |
| Concentrated DNase I buffer | ClonTech |
| Deionised water | ClonTech |
| Denaturing solution [2.7 M guanidine thiocyanate; 1.3 M ammonium thiocyanate; 0.1 M sodium acetate (NaOAc) (pH 4.0)] | ClonTech |
| Diethyl pyrocarbonate (DEPC) | Sigma |
| DNase I (1 unit/μl) | ClonTech |
| Ethanol | BDH |
| Isopropanol | Sigma |
| NaOAc solution (2 M) | ClonTech |
| Phenol | Sigma |
| Phenol:chloroform:isoamyl alcohol (25:24:1) | Sigma |
| RNase-free water | ClonTech |
| Saturation buffer [19% glycerol; 0.25 M NaOAc (pH 4.5)] | ClonTech |
| Termination Mix [0.1 M EDTA (pH 8.0); 1 mg/ml glycogen] | ClonTech |
| Materials for detection of cell cycle gene expression | |
| [$\alpha$-$^{32}$P]dATP | Amersham |
| Atlas Human Cell Cycle Array and reagent package | ClonTech |

| Material | Source |
|---|---|
| DNA from salmon testes | Sigma |
| Materials for invasion assays | |
| Growth factor-reduced Matrigel Matrix | Becton Dickinson |
| Transwells (6.5 mm, polycarbonate membrane) | Costar |
| Materials for enzyme-linked immunosorbent assays (ELISAs) | |
| Human IL-6 ELISA kit | Bender MedSystems |
| Human TNF-α ELISA kit | Bender MedSystems |
| Human total MMP-1 ELISA kit | Amersham |
| Human total MMP-9 ELISA kit | R&D Systems |
| Materials for RT-PCR | |
| [$^{33}$P]dATP | Amersham |
| 10× PCR buffer | Perkin-Elmer |
| Amplitaq DNA polymerase | Perkin-Elmer |
| dNTP | Perkin-Elmer |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer | A gift from Dr. Peter Dall (IGEN, Kernforschungzentrum, Karlsruhe, Germany). |
| IL-1β primer | A gift from Dr. Peter Tabona (Department of Maxillofacial Surgery, Eastman Dental Hospital). |
| IL-6 primer | A gift from Dr. Peter Tabona (Department of Maxillofacial Surgery, Eastman Dental Hospital). |
| Ready to Go T-Primed First-Strand kit | Pharmacia Biotech |

| Material | Source |
|---|---|
| TNF-α primer | A gift from Dr. Peter Tabona (Department of Maxillofacial Surgery, Eastman Dental Hospital). |
| Materials for polyacrylamide gel analysis | |
| Acrylamide/Bisacrylamide solution | Bio-Rad |
| Ammonium persulphate | Bio-Rad |
| EDTA solution | Gibco BRL |
| N,N,N′,N′-tetramethylethylenediamine (TEMED) | Bio-Rad |
| Orange/blue dye | Sigma |
| Tris-Base | Sigma |
| Miscellaneous materials | |
| Dynal MPC-E-1 | Dynal |
| Isoton II | Coulter Electronics |
| Microcon-30 | Amicon |

## 2.2 **Synovial tissues**

The non-inflamed synovium was kindly provided by Dr Andrew Pitsillides (Royal Veterinary College, London) and obtained from knee joints of patients undergoing amputation for sarcomata of the lower limb at Whittington Hospital, London. The RA and OA synovium was from patients undergoing total hip or knee joint replacement at Whittington Hospital, London.

## 2.3 **Methods**

The following paragraphs describe all procedures used in this study. Some additional details may be given in the relevant chapters.

### 2.3.1 **Immunohistochemistry of sections of synovial membranes**

The synovium was snap frozen in *n*-hexane and stored at -74°C until use. Ten-micrometre cryostat sections of these tissues were transferred to glass slides and allowed to air dry. The histology of the synovial lining layer was checked by staining the sections with 1% (w/v) toluidine blue in acetate buffer for 5 minutes (min). The slides were washed with running water, mounted with Fluoromount-G, left to dry and examined by light microscopy using an Olympus PM-10AD. Fields of interest were photographed on Fujichrome T 64ASA film. For detection of CD44 splice variant expression, the sections were incubated with 10% (v/v) FBS in PBS to eliminate non-specific background. They were next incubated for 1 hour (hr) at room temperature with mouse monoclonal anti-CD44v3, -CD44v6 or -CD44v7/8 antibodies diluted in PBS containing 10% FBS. Negative controls were incubated with 10% FBS in PBS alone. The sections were washed with PBS 3 times after each of the following steps. Anti-mouse IgG-FITC was next applied and incubated for 30 min at room temperature. Either double staining or counterstaining was accomplished by further treating the sections with anti-VCAM-1-biotin for 1 hr followed by avidin-TRITC for 30 min, or by staining the sections with propidium iodide (PI) for 5 min. The sections were finally washed with water, mounted with Fluoromount-G, covered with glass coverslips, left to dry and examined by a confocal microscope using a Leica TCS4D. Images obtained from the scanning were

processed using the UTHSCSA ImageTool programme. A threshold was set to eliminate all non-specific staining signals seen in the negative controls, and the same threshold was then used with the sections of interest.

### 2.3.2 Isolation of FLSs and cell culture

Synovium was dissected away from the surrounding joint capsule and washed extensively in PBS. Any remaining connective tissue or fat was carefully removed and the synovial membrane chopped into small cubes (1-2 $mm^2$). The cubes were digested in serum-free DMEM containing collagenase (2 mg/ml) and 1% (v/v) P/S at 37°C for 1 hr in a shaking incubator. The cell suspension thus obtained was sheared through 100-μm nylon gauze to remove undigested tissue. Isolated cells were retrieved by centrifugation (150×g; room temperature; 4 min) and washed in PBS. The cells were resuspended in DMEM containing 10% (v/v) FBS and 1% (v/v) P/S and were either seeded on 4-well chamber slides at a ratio of $2\times10^4$ cells/well for further immunocytochemical staining or transferred to tissue culture flasks. The latter were incubated at 37°C in a humidified 5% carbon dioxide atmosphere. Non-adherent cells were removed after 18 hr of incubation by washing 3 times with PBS, and the culture was continued. When confluent, cells were subcultured using trypsin-EDTA solution. After the second subculture, the population was 98% VCAM-1-positive and, therefore, mainly consisted of FLSs. These cells were used for subsequent studies (Anastassiades *et al.*, 1978).

### 2.3.3 **Immunocytochemistry**

The specific protocol for each experiment is described in the relevant chapters, but a number of details are common to all the immunocytochemical staining. Cells seeded on 4-well slides were washed twice with PBS and fixed in ice-cold methanol for 4 min and ice-cold acetone for 1 min. After being left to dry, the slides were washed twice in PBS and then incubated in 10% (v/v) FBS for 20 min at room temperature to saturate non-specific binding sites for antibodies. The slides were washed again with PBS. Hydrogen peroxide (3% w/w) was applied to the cells for 5 min to terminate endogenous peroxidase activity. Antibodies were diluted to their optimal concentration in PBS containing 10% FBS. One hundred microlitres of each primary mAb were applied to each well and incubated for a certain period of time at a specified temperature. As a negative control, certain slides were incubated in PBS containing 10% FBS only. After 3 washes in PBS, all wells were incubated in 100 μl of secondary antibody for 30 min followed by avidin-peroxidase for 30 min, both at room temperature. Substrate, AEC, was freshly prepared according to the manufacturer's instruction and applied to the cells. The reaction was stopped by washing in water. The slides mounted and examined by light microscopy as described above.

### 2.3.4 **Immunofluorescent staining of cultured FLSs**

FLSs in culture were seeded onto glass coverslips (no. 1) at a density of $4 \times 10^4$ cells/coverslip and fixed in methanol/acetone or, depending on further processing, in freshly 4% (w/v) *p*-formaldehyde in PBS for 10 min followed by a 5-min incubation in 0.2% (v/v) Triton X-100 in PBS to dissolve the membrane. After a 20-min incubation in

10% FBS in PBS, the coverslips were incubated in 10% FBS in PBS containing rat anti-human CD44, mouse anti-human CD44v3, CD44v6 or CD44v7/8 mAbs for 1 hr at room temperature. This was followed by anti-mouse IgG-FITC for 30 min and 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 min. Finally, they were mounted on glass slides with Fluoromount-G and left to dry at room temperature in the dark.

### 2.3.5 **Antibody dialysis**

To remove azide, mAbs against CD44, CD44v3, CD44v6 and CD44v7/8 were extensively washed in PBS supplemented with 1% (w/v) BSA, using Microcon-30 according to the manufacturer's instruction.

### 2.3.6 **Selection of CD44v7/8-expressing cells**

Cells expressing CD44v7/8 were selected from a total population of $10^6$ cells in a protocol using Dynabeads M-450 coated with goat anti-mouse IgG according to the manufacturer's protocol. In brief, cultured FLSs were detached from the flask by treatment with cell dissociation solution at 37°C for 10 min. Subsequent steps were performed at 4°C. The cells were incubated with dialysed anti-CD44v7/8 (5 μg/ml) for 1 hr. Dynabeads were added and incubated for 20 min. A magnet (Dynal MPC-E-1) was used to immobilise cells that had bound to the magnetic beads. Negative cells were then transferred to a new container. To remove antibody-coupled beads after selection, suspensions of both positive and negative sub-populations were treated with trypsin-EDTA for 5 min after which FBS was added to a final concentration of 10% (v/v). The cells were washed and cultured in DMEM supplemented with 10% FBS and 1% P/S.

Selection was assessed in an immunocytochemistry protocol prior to using the cells for further experimentation.

### 2.3.7 **Cell proliferation measurement**

FLSs were plated in 24-well plates at $6\times10^3$ cells/well and left to adhere for 4 hr. Either the CD44v7/8-enriched population was compared with negatively selected cells (both of which were used after 4 days of culture) or the effects of dialysed anti-CD44v3, anti-CD44v6, anti-CD44v7/8 (5 μg/ml), or anti-VCAM-1 (10 μg/ml) were assessed in a total population, over a period of 11 days. For counting, cells were harvested and each cell suspension was resuspended in Isoton II buffer. The cell suspension was then measured in Coulter counter type ZBI with 100-μm orifice (Coulter Electronics). Cell numbers are expressed as cells per well or as percentage of control population.

### 2.3.8 **$^3$H-thymidine incorporation**

FLSs were seeded into 96-well plates at $2\times10^3$ cells/well in 100 μl of medium. The cells were allowed to adhere for 4 hr after which dialysed antibodies against CD44, CD44v3, CD44v6, CD44v7/8 at 5 μg/ml, or VCAM-1 at 10 μg/ml were added. Cultures were continued for a further 3 days. $^3$H-thymidine was added at 0.5 μCi/well and left for 16 hr. Cells were then washed in PBS and fixed in 10% (w/v) trichloroacetic acid. DNA was solubilised in 0.25 M NaOH for 1 hr and radioactivity counted in the presence of UltimaGold in a scintillation counter (Beckman).

### 2.3.9 **Total RNA isolation**

FLSs were cultured with or without anti-CD44v7/8 mAb in 80-cm$^2$ flasks until approximately 70% confluent. Total RNA was then isolated. All steps were performed on ice using RNase-free reagents and materials. In brief, the cells were washed twice with PBS and harvested using cell dissociation solution. They were then centrifuged and the supernatant was discarded. An appropriate volume of denaturing solution was added and mixed thoroughly. To remove cellular debris, the cell lysate was centrifuged (12,000×g; 4°C; 10 min) and the supernatant transferred to new tubes. Saturated phenol was added. The suspension was shaken thoroughly and left to stand for 5 min. Chloroform was then added. The samples were shaken vigorously for 1-2 min and left to stand for 5 min. The solution was centrifuged (12,000×g; 4°C; 10 min) to obtain 3 phases: the upper aqueous phase containing the RNA, the white interphase containing DNA, and the lower organic phase. The upper layer was transferred to new tubes and a second round of phenol:chloroform extraction was performed. An appropriate volume of isopropanol was added to the recovered upper phase to precipitate RNA. The solution was mixed well and left to stand for 10 min. The sample tubes were centrifuged (12,000×g; 4°C; 10 min). After removing the supernatant, the RNA pellets were washed twice with 80% (v/v) ethanol in DEPC-treated water. The pellets were allowed to air dry at room temperature. Finally, the RNA was resuspended in 15 µl of RNase-free water and stored at -70°C.

To assess the concentration and purity of RNA, spectrophotometry was utilised. For each sample, 3 µl of the total RNA suspension were mixed with 900 µl of DEPC-treated water. The absorbance (A) was determined at 260 and 280 nm using a Beckman DU 50 spectrophotometer. RNA concentration was calculated using the following calculation.

$$\text{Concentration } (\mu g/ml) = A_{260} \text{ of dilute sample} \times 300 \times 40$$

where $A_{260}$ represents the absorbance at 260 nm; 300 is the dilution factor (3μl in 900 μl); 40 is the extinction coefficient for RNA (1 mg/ml) at 260 nm. RNA purity was then estimated from the $A_{260}/A_{280}$ ratio. Pure RNA exhibited a ratio of 1.9-2.0.

### 2.3.10 **DNase treatment of total RNA**

The isolated total RNA was mixed well with 1 unit/μl DNase, concentrated DNase I buffer and deionised water at a volume ratio of 10:1:2:7, respectively. The reaction was incubated at 37°C in an air incubator for 1 hr. Concentrated Termination Mix was then added. Phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed thoroughly. The mixture was centrifuged for 15 min to separate phases. The aqueous layer was recovered while the interface and lower phase were discarded. This phenol:chloroform:isoamyl alcohol extraction was repeated once. 1/10 volume of 2 M NaOAc and 2.5 volumes of 96% (v/v) ethanol were added to the aqueous phase. The mixture was mixed thoroughly and centrifuged for 20 min. The supernatant was removed, the pellet overlaid with 80% (v/v) ethanol and each sample centrifuged once more for 15 min. The supernatant was removed. The precipitate was air dried to evaporate residual ethanol, finally dissolved in 4 μl of RNase-free water and stored at -70°C.

### 2.3.11 **Cell cycle gene expression analysis**

Analysis of expression of genes implicated in the regulation of the cell cycle was performed using a "Human Cell Cycle" Atlas Array. The procedures were as instructed

by the manufacturer. In brief, $^{32}$P-labelled cDNA was prepared from the DNase-treated total RNA. The cDNA was hybridised to the Atlas Array membranes, and expression levels were visualised and estimated using a PhosphorImager (Molecular Dynamics Storm II).

### 2.3.12 **HA binding**

HA was dissolved in PBS and coated at 0.1 mg/ml onto 96-well Maxisorb dish at 37°C for 1 hr. The plate was washed 3 times with PBS. To assess the contribution of CD44 in cell binding to the dish, a fraction of FLSs were incubated with anti-Hermes-1 antibody or with a dialysed anti-CD44 mAb at 37°C for 20 min. CD44v7/8 positively and negatively selected cells were added to the plate as a 100 µl aliquot containing $5\times10^4$ cells and left to bind at 37°C for 40 min. After 3 washes in PBS, adherent cells were fixed in 100 µl of ice-cold methanol at room temperature for 10 min and washed once in PBS. Adherent cells were stained in 0.5% (w/v) Rose Bengal in PBS for 5 min, washed 3 times with PBS and the dye was dissolved in a 1:1 (v/v) PBS/ethanol solution for 1 hr. Absorbance was read at 540 nm using a Titertek Multiskan MCC/340 (LabSystems).

### 2.3.13 **MMP measurement**

FLSs were trypsinised and seeded onto wells of 24-well plate at a density of $10^4$ cells/well. They were allowed to adhere for 4 hr before mAbs against CD44v3, CD44v6 or VCAM-1 were added to the selected wells. The cells were cultured at 37°C for 4 days. The culture supernatant was collected and stored at -20°C until being analysed for MMP-1 and MMP-9 using MMP ELISA kits according to the manufacturers' instructions.

### 2.3.14 **Cytokine measurement**

FLSs were trypsinised and plated into flasks. They were allowed to adhere to the surface for 4 hr before dialysed antibodies against CD44v3, CD44v6, CD44v7/8 or VCAM-1 were added. After 4 days of culture at 37°C, supernatants were removed and stored at -20°C until analysis. Adherent cells were counted for normalisation of the cytokine concentrations. This exercise is particularly pertinent in case of addition of anti-CD44v7/8, which was shown to considerably inhibit cell division. Levels of IL-6 and TNF-α were measured using commercially available cytokine kits and samples processed according to the manufacturer's instructions. The cytokine levels were expressed as ng/ml per $10^6$ cells. The data presented are an average of 3 experiments performed in duplicate.

### 2.3.15 **Reverse transcription of mRNA**

The total RNA was reverse-transcribed to cDNA using a Ready to Go T-Primed First-Strand kit. This kit utilises the Moloney leukemia virus reverse transcriptase and an oligo ($dT_{18}$) primer to generate first strand cDNA. The reaction mix provided is a room temperature stable preparation containing dATP, dCTP, dGTP, dTTP, murine reverse transcriptase, RNAguard, RNase/DNase-free BSA and *Not* I-d$(T)_{18}$ primer (5´-d[AAC TGG AAG AAT TCG CGG CCG CAG GAA $T_{18}$]-3´). Each RNA (1–2 μg) was made up to a volume of 33 μl with DEPC-treated water. The samples were heated at 65°C for 5 min. Both the RNA solutions and the first-strand reaction tubes were then transferred to a 37°C bath and incubated for a further 5 min. The RNA solutions were transferred to their respective First-Strand Reaction Mix tubes and incubated at 37°C for 5 min. The tubes were vortexed to mix the contents, which were subsequently collected by brief

centrifugation (8000×g; 10 seconds (s)). The reaction was then incubated at 37°C for 1 hr. The completed first-strand reaction mix was heated at 90°C for 5 min in order to inactivate the reverse transcriptase. The reaction mixture was now ready to use for the PCR.

### 2.3.16 **PCR**

The procedure was as previously described by Chen *et al.* (1998). The completed first-strand cDNA was amplified by PCR using specific primers for IL-1β (sense: 5′ TGG AGA ACA CCA CTT GTT GCT CCA; antisense: 5′ AAA CAG ATG AAG TGC TCC TTC CAG C), IL-6 (sense: 5′ GAA GAC CCC TCA GGC TGG ACT G; antisense: 5′ ATG AAC TCC TTC TCC ACA AGC GC), TNF-α (sense: 5′ CAC CAG CTG GTT ATC TCT CAG CTC; antisense: 5′ CGG GAC GTG GAG CTG GCC GAG GAG) and GAPDH (sense: 5′ AAG GTG AAG GTC GGA GTC AAC; antisense: 5′ GGC AGA GAT GAC CTT TTT GGC). Amplification was performed by adding 28.7 μl of sterile DEPC water, 8 μl of 1.25 mM each dNTP mix, 0.25 μl of [$^{33}$P]dATP, 2.5 μl of each 4μM each primer, 5 μl of 10× PCR buffer and 0.25 μl of Amplitaq DNA polymerase at 5 U/ml to 3 μl of each completed first-strand reaction. The reaction tubes were heated to 94°C for 5 min and incubated in a GeneAmp PCR 96000 (Perkin-Elmer) thermal cycler using 28 cycles of 94°C (30 s), 62°C (30 s) and 72°C (1 min). Fragments were finished off by incubating at 72°C for 10 min.

### 2.3.17 **Polyacrylamide gel analysis**

Analysis of the PCR products was performed by gel electrophoresis using 8% polyacrylamide gels (0.75 mm thick) and a Tris-borate-EDTA (TBE) buffer[1] system. DNA samples were mixed with orange/blue dye in the ratio of 5:1 and 10 μl loaded onto the gel. The samples were allowed to run slowly into the gel (50 volts; approximately 2 hr) until the lower blue band migrated off. The gel was carefully removed from the glass plates and fixed for 15 min in a solution of 10% (v/v) acetic acid and 40% (v/v) methanol. After washing in water, the gels were dried under vacuum at 80°C for 40 min using a gel dryer (BioRad 583). The gels were put against a phosphor screen for 2-4 hr and primer-specific bands were subsequently analysed using a PhosphorImager (Fuji BAS1000 BioImager). The amount of radioactivity is presented as arbitrary counts per PCR product.

### 2.3.18 **Statistical analysis**

All values given are mean ± standard error of mean (S.E.M.). Statistical analyses were performed using analysis of variance (ANOVA) or Student's unpaired *t*-test. P values less than 0.05 were considered to be significant.

[1] 1 litre of TBE buffer contains 10.8 g of Tris-Base, 5.5 g of boric acid and 4 ml of 0.5 EDTA (pH 8.0).

# Chapter 3

# Expression of CD44 splice variants in synovial membrane obtained from patients with rheumatoid arthritis or osteoarthritis

## 3.1 Summary

The expression of standard CD44 has been examined in normal and diseased synovium in a number of studies. The genomic structure of CD44 reveals the presence of numerous variably expressed exons. There are, however, few studies concerning CD44 splice variant expression in synovium. This chapter, therefore, deals with their expression, at protein level using exon-specific antibodies, in the synovium of both healthy and diseased synovial joints. Synovium obtained from patients with RA showed high expression of CD44 isoforms including v3 and v7/8 while synovial tissue from OA patients had low staining signals and normal synovium exhibited no expression at all. Similar results were obtained from immunocytochemical studies using cultured FLSs; whereas the cells from RA patients always expressed CD44v3, v6 and v7/8, those from OA patients appeared to possess a great variation in the splice variant expression. CD44v7/8 was present in a higher proportion of cells than CD44v3 and v6, and this was true for both RA and OA. The staining intensity of CD44v7/8 in RA FLSs was stronger in RA than in OA. These findings suggest that the CD44 splice variants may be related to RA. Their actual relationship deserves further study.

## 3.2 **Introduction**

CD44 is an adhesion molecule found on many cell types including lymphocytes, fibroblasts, glial cells, epithelial cells and certain tumour cells (Sneath & Mangham, 1998). It is involved in cell-cell interactions, cell-matrix interactions, cell signalling and activation, lymphocyte recirculation, embryogenesis, hematopoiesis and apoptosis (reviewed in Naor *et al.*, 1997). These multiple functions, together with its various isoforms, make CD44 of notable interest.

CD44 exists in many isoforms resulting from post-translational modifications, which include N-glycosylation, O-glycosylation and glycosaminoglycanation (Brown *et al.*, 1991; Camp *et al*, 1991; Dasgupta *et al.*, 1996), and from alternative splicing of exons. The most abundant form of CD44 is standard or haematopoietic CD44 (CD44s or CD44H, respectively), which does not contain any alternatively spliced components. Certain cells express variant exons either constitutively, like keratinocytes (Brown *et al.*, 1991; reviewed in Sneath & Mangham, 1998) and many tumour cells (Heider *et al.*, 1993), or transiently, like lymphocytes (Arch *et al.*, 1992) and Langerhans cells (Weiss *et al.*, 1997).

Ten such alternatively spliced exons have been described that encode the membrane-proximal extracellular domain of CD44 (designated v1 to v10). Two other alternatively spliced exons are found in the domain that encodes the cytoplasmic tail (exons 18 and 19) (Screaton *et al.*, 1992) (see Figure 1.4b). The variant exon v1 is not expressed in humans (Screaton *et al.*, 1993). Interestingly, expression of individual variant isoforms v2 to v10 and combined expression of several variants have been associated with many

pathophysiological cell activities including inflammation and neoplasia (Wagner *et al.*, 1998).

CD44 is also expressed on FLSs, and several studies have investigated its expression in normal and inflamed synovium. Conflicting data have been published. Certain studies indicate that CD44 is expressed to a greater extent in RA than normal synovium (Haynes *et al.*, 1991; Johnson *et al.*, 1993) whereas another study, which investigated cultured FLSs derived from RA patients, demonstrated reduced expression of CD44 compared with the expression on FLSs from normal synovium (Henderson *et al.*, 1994). FLSs also express CD44 splice variants; uniquely when derived from diseased joints (both RA and OA). The variants are present both in freshly dissociated cells and those kept in culture (Croft *et al.*, 1996). This chapter deals with localisation of CD44 splice variants in synovial membranes obtained from various arthropathies using an immunofluorescent staining protocol. CD44 splice variants were absent in synovium derived from non-diseased joints; CD44v3 and v7/8 were weakly expressed in synovial membranes derived from patients with OA whereas they were highly expressed in synovium derived from RA patients. Expression of VCAM-1 was used as a marker for the presence of FLSs and it was found that CD44 splice variants co-localise with VCAM-1, indicating that part of the CD44 splice variant-positive population in the synovial membrane consists of FLSs.

### 3.3 **Materials and methods**

All materials and methods used in this chapter are as earlier described in Chapter 2. Additions to these methods are described below or are included in the legends to figures.

### 3.3.1 **Immunocytochemistry of cultured FLSs**

Cells seeded on 4-well slides were washed with PBS and fixed in methanol and acetone. They were incubated in FBS to saturate non-specific binding sites for antibodies and treated with hydrogen peroxide to terminate endogenous peroxidase activity. Primary mAb against CD14 (a marker for monocytes and macrophages), CD45 (a marker for leukocytes), CD68 (a marker for monocytes, macrophages and myeloid cells), VCAM-1 (constitutively expressed by FLSs), CD44v3, CD44v6 or CD44v7/8 was applied to the cells and incubated at room temperature for 1 hr. As a negative control, certain slides were incubated in PBS containing 10% FBS only. After washing in PBS, all wells were incubated in anti-mouse IgG-biotin for 30 min followed by avidin-peroxidase for 30 min, both at room temperature. Substrate, AEC, was freshly prepared and applied to the cells. The reaction was stopped by washing in water. The cells were counterstained with Mayer's hematoxylin for 5 min, the slides mounted and examined by light microscopy using an Olympus PM-10AD. Fields of interest were photographed on Fujichrome T 64ASA film.

### 3.3.2 **Detection of levels of expression of CD44 isoforms using flow cytometry**

Cultured FLSs were washed twice with PBS. EDTA (4mM) in PBS was added and incubated at 4°C. After 20 min, the rounded-up cells were removed from the flask by agitation. Aliquots of $10^5$ cells were put in 15-ml tubes and centrifuged (150×g; room temperature; 5 min). The pellets were resuspended in 100 μl of human AB serum (3% v/v) and left at 4°C for 20 min. The aliquots were transferred to a 96-well plate and washed once with 200 μl of PBS containing 1% bovine serum albumin (BSA). They were

next incubated with primary antibodies against CD44, CD44v3, CD44v6 or CD44v7/8, all of which were diluted in 1% BSA in PBS, at 4°C for 45 min. As negative control, certain aliquots were incubated with 1% BSA in PBS alone. After 2 washes in 1% BSA in PBS, a secondary antibody, either anti-rat IgG-FITC or anti-mouse IgG-FITC, diluted in 1% BSA in PBS was added. After a 45-min incubation at 4°C, the cells were washed 3 times in 1% BSA in PBS and finally resuspended in 100 µl of PBS to which 100 µl of a solution containing 2% (w/v) *p*-formaldehyde in PBS was added. The samples were stored in the dark at 4°C (for a maximum of 10 days) before being analysed in a flow cytometer (Becton Dickinson). Antibody binding is presented as mean fluorescence intensity of 5,000 cells.

## 3.4 **Results**

### 3.4.1 **Immunohistological analysis of CD44 splice variants in human synovium**

To verify the integrity of the synovial intimal region, sections of human non-inflamed and RA synovium were stained with toluidine blue. The histology gave a typical picture; in non-diseased synovium, the lining was composed of only 2-3 layers of cells whereas, in synovium from RA, it was increased to numerous layers (Figure 3.1).

An immunofluorescence protocol was next employed to localise CD44 splice variants in synovial tissue sections. Figure 3.2, as an example, shows how CD44v7/8 was originally stained in RA synovium, in comparison with the negative control where anti-CD44v7/8 was not applied and with the CD44v7/8 staining in normal synovium. Comparisons

among RA, OA and non-inflamed synovium of CD44v3, CD44v6 and CD44v7/8 were then made. Expression of epitope CD44v7/8 predominates; CD44v3 was found at lower levels, and expression of CD44v6 could not be detected in the membrane (Figure 3.3). This latter finding was surprising because Croft *et al.* (1997) showed that v6 was present in a number of splicing combinations as determined by RT-PCR. The absence of staining could mean that the levels of expression are too low to be detected by this protocol (see also Hale *et al.*, 1995). Interestingly, staining signals for CD44v3 and CD44v7/8 were much higher in synovium obtained from RA patients than in synovium obtained from OA patients. CD44 splice variant expression was not detected in non-diseased synovial membrane. It is also possible that, at least partly, there was a cross-reactivity in RA synovium due to the presence of rheumatoid factors. To ascertain if FLSs expressed CD44 splice variants, sections were simultaneously stained with anti-CD44v3 or -CD44v7/8 and anti-VCAM-1 antibodies. Additional sections were included by incubating with anti-CD44v3 or anti-CD44v7/8 followed by anti-mouse IgG-FITC and avidin-TRITC as negative controls for VCAM-1 staining and with anti-mouse IgG-FITC, anti-VCAM-1 and avidin-TRITC as negative controls for CD44 variant staining. The staining in sections of interest showed considerable overlap, indicating that part of the CD44v3- and CD44v7/8-positive cells were indeed FLSs (Figure 3.4). This observation confirms the earlier finding that freshly isolated FLSs do express CD44 splice variants (Croft *et al.*, 1997).

### 3.4.2 **FLSs maintain CD44 splice variant expression in culture**

Cultures of cells isolated from synovium were firstly analysed with antibody against leukocyte common antigen, CD45, in order to assess monocyte contamination.

Expression of this marker was followed for a period of 25 days (Figure 3.5a). The percentage of cells expressing CD45 gradually declined with time in culture and was lost by day 25. After the second subculture, cells were also stained with anti-CD14 (Figure 3.5b), anti-CD68 (Figure 3.5c) and anti-VCAM-1 (Figure 3.5d). The results indicate that the cultured cells were dominantly FLSs; monocytes and macrophages were rare.

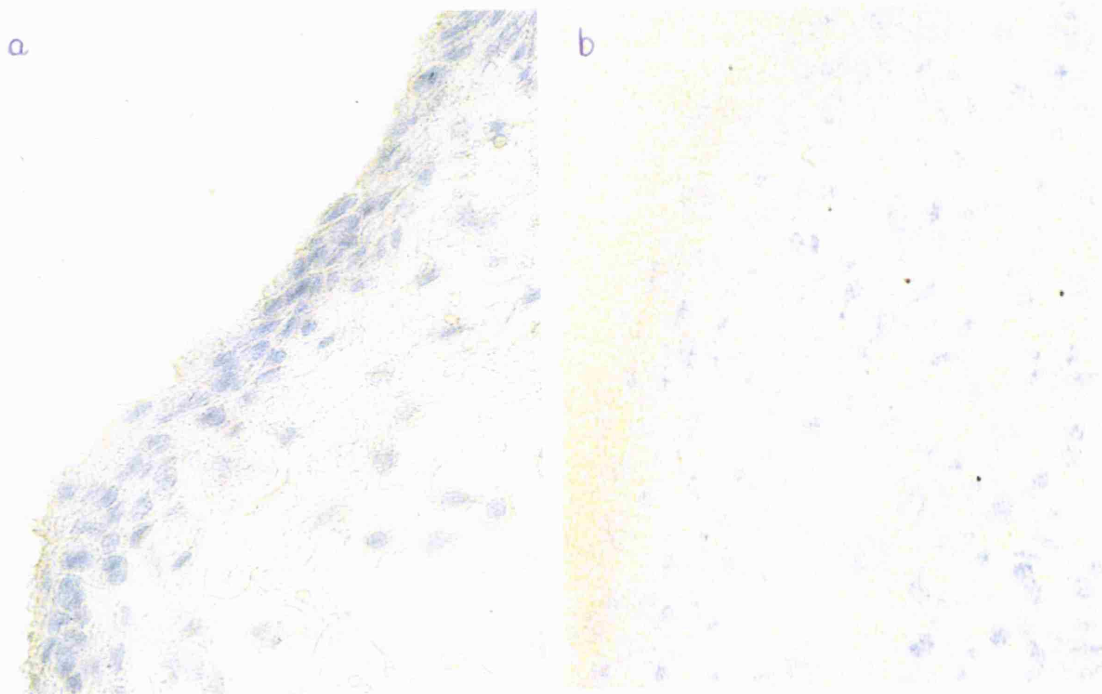



**Figure 3.1.** Histology of human synovial membrane demonstrated by toluidine blue staining of synovial tissue sections. a) Non-inflamed synovium. b) Rheumatoid synovium. Results shown are representative of the staining on synovial tissues obtained from 2 non-inflamed joints and 4 RA joints.

All 3 CD44 epitopes were detected, albeit non-quantitatively, in these cultures, where expression of CD44v7/8 was, again, predominant (Figure 3.6), meaning that it was

detected in a large number of cells. In general, a higher number of cells obtained from synovium from RA patients express CD44v3 and CD44v6, as compared to those obtained from synovium from OA patients. Frequently, CD44v3 and CD44v6 expression was seen in cell clusters. FLSs isolated from patients with OA had a greater variation in percentage of cells expressing the splice variants compared to cells obtained from patients with RA. Immunofluorescent staining was also employed in detecting CD44 splice variant expression on FLSs (Figure 3.7). This method confirmed the maintenance of the CD44 splice variant expression in cell culture, but, unlike the staining of sections of synovial membrane, this time the presence of CD44v6 could be detected.

### 3.4.3 **Staining of CD44 splice variants using different fixation techniques**

Two different procedures of fixation of the cells were employed for the immunofluorescence in order to assess solubility of the CD44 isoforms. FLSs fixed with methanol/acetone showed stronger CD44v3 and CD44v6 expression signals than those fixed with *p*-formaldehyde and permeabilised with 0.2% Triton X-100. On the other hand, CD44v7/8 detection was not different between the 2 techniques. This finding suggests that either CD44 variants containing v3 and v6 are easily extracted by Triton X-100 while the isoforms containing CD44v7/8 are not extracted or that the anti-CD44v3 or v6 epitope is more easily recognised by the antibodies after the treatment with methanol/acetone. That the isoforms containing CD44v7/8 are not extracted suggests that they may have high affinity and are tightly and stably bound to cytoskeletons, namely, ERM family members (Tsukita *et al.*, 1994).

### 3.4.4 **Quantitative levels of expression of CD44 splice variants**

Levels of expression of CD44, CD44v3, CD44v6 and CD44v7/8 on cultured FLSs derived from RA and OA synovium were assessed by flow cytometry. The results showed that CD44 splice variants were expressed at much lower levels than those of the total CD44 (Figure 3.8). The splice variants could not be detected on cells derived from OA synovium.

**Figure 3.2.** The expression of CD44v7/8 in RA synovium, in comparison with the negative control where anti-CD44v7/8 was not applied and with the CD44v7/8 staining in normal synovium. The expression of CD44v7/8, as shown in bright green in the RA section stained with anti-CD44v7/8, is seen in the synovial lining layer. Results shown are representative of the staining performed on synovial tissues recovered from 2 normal patients and 4 RA patients.

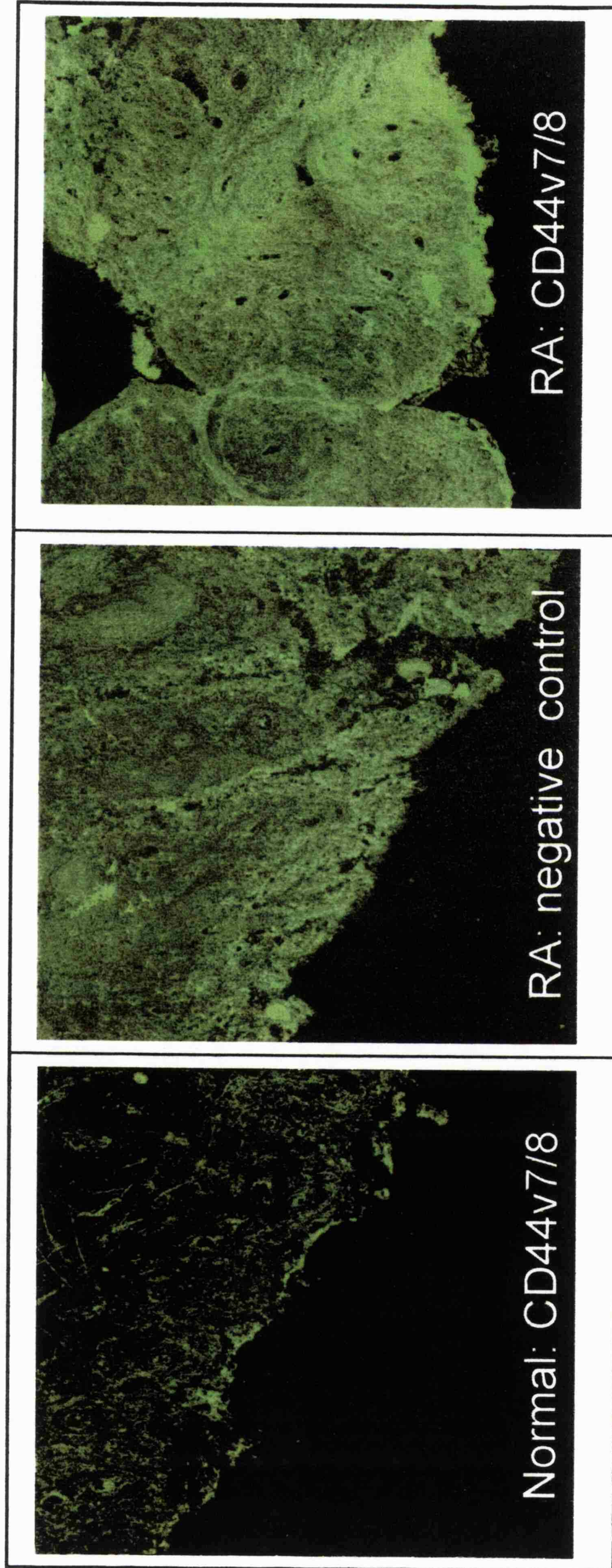
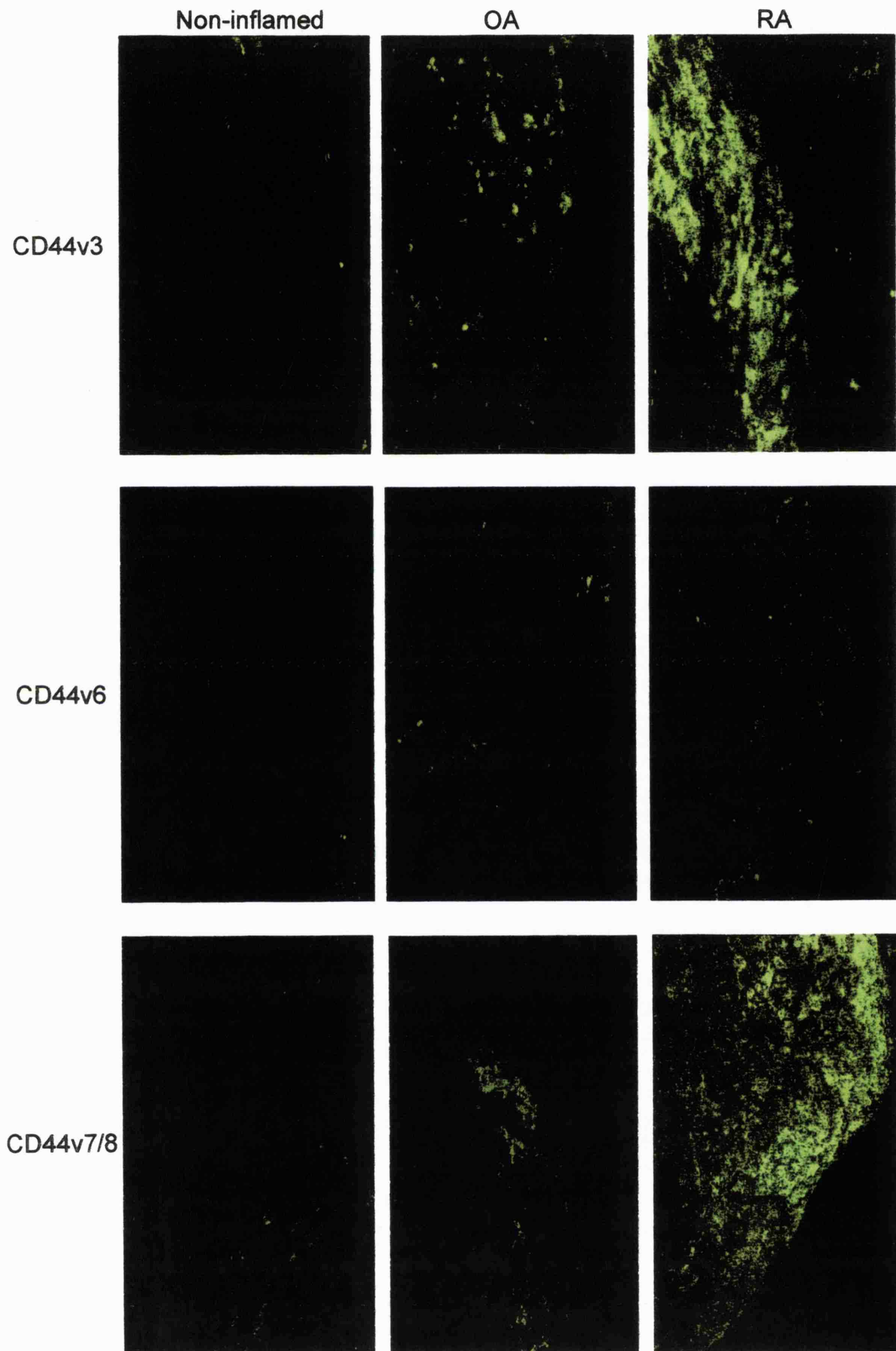

RA: CD44v7/8
RA: negative control
Normal: CD44v7/8

**Figure 3.3.** Expression of CD44v3 (top panel), CD44v6 (middle panel) and CD44v7/8 (bottom panel) in human synovium. Immunofluorescent detection was performed in non-inflamed (left panel), OA (middle panel) and RA (right panel) synovial tissues. Cells stained positive for CD44 splice variants appear green due to the labelled FITC. Distribution of variant-containing isoforms of CD44 seems to be restricted to the intimal layer of the synovial membrane. Results shown are representative of the detection rendered on synovial tissues obtained from 2 normal patients, 6 OA patients and 4 RA patients. In each tissue, 2 sections were stained for CD44v3, 2 for CD44v6 and 2 for CD44v7/8.



Non-inflamed
OA
RA
CD44v3
CD44v6
CD44v7/8

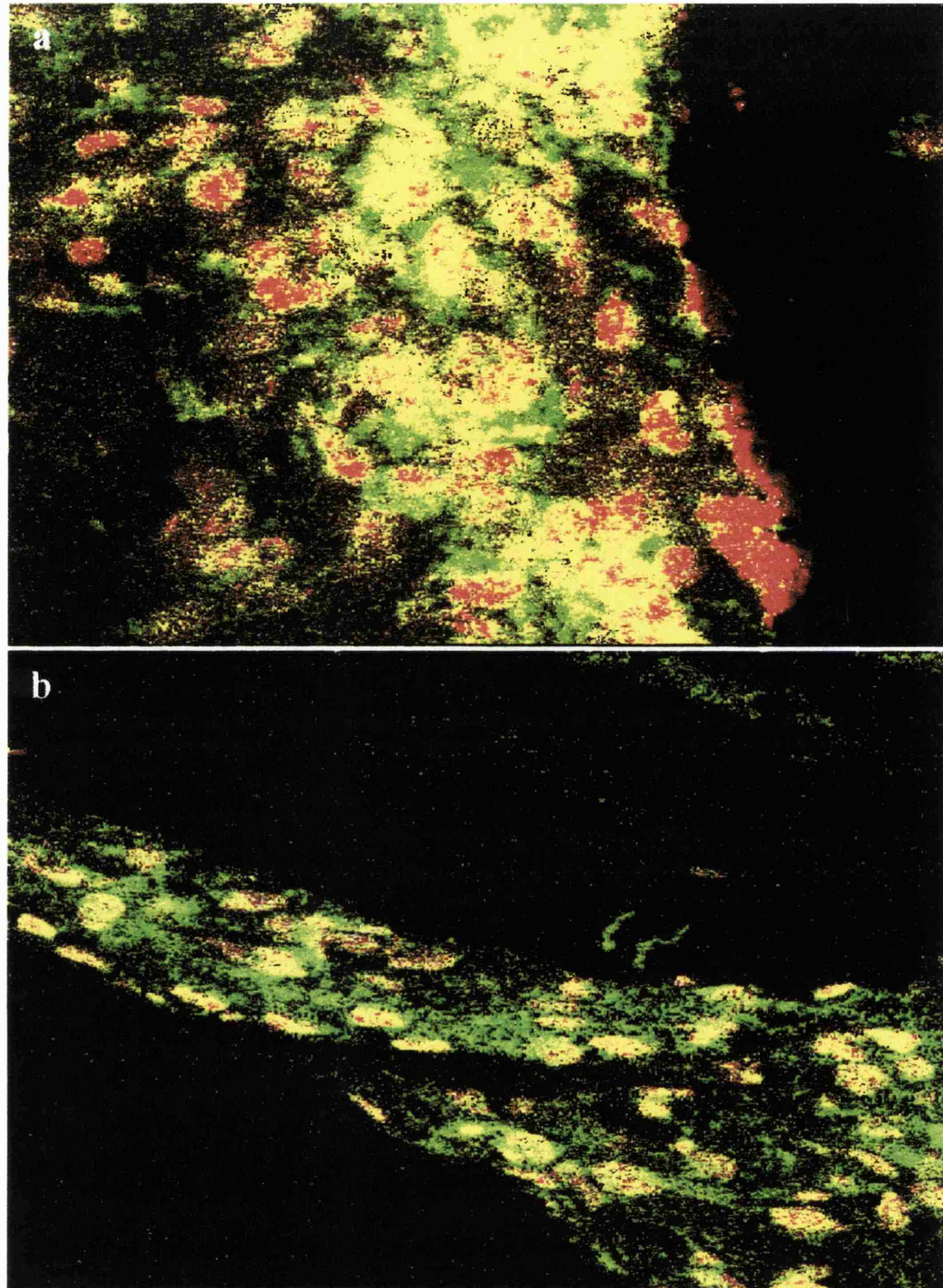



**Figure 3.4.** Double immunofluorescent staining of synovial membrane from patients with RA, using anti-CD44v3 (a) or anti-CD44v7/8 (b) (of which secondary antibody was FITC-conjugated) and anti-VCAM-1 (which subsequently bound to avidin-TRITC). Cells expressing both CD44v3 or CD44v7/8 and VCAM-1 appear yellow. Results shown are representative of the staining on synovial tissues derived from 4 RA patients. In each tissue, 2 sections were double-labelled for CD44v3 and VCAM-1 and 2 for CD44v7/8 and VCAM-1. The expression of CD44v6 is not demonstrated here since the staining signals were too low.

a

b c d

**Figure 3.5.** (a) Time course of CD45 expression on cultured cells isolated from synovium. The cells were treated as described in 'Materials and methods'. Percentage of CD45-positive cells is shown as mean ± S.E.M.. (b) Immunocytochemical staining of CD14 on synovial cells. (c) Immunocytochemical staining of CD68 on synovial cells. (d) Immunocytochemical staining of VCAM-1 on synovial cells.

**Figure 3.6.** Expression of CD44 splice variants as demonstrated by immunocytochemistry. The positive cells appear brownish-red. a) Negative controls were processed normally but without the application of a primary antibody. b) and c) Detection of CD44v3 and CD44v6, respectively, on cells isolated from synovium from patients with RA. d) and e) Detection of CD44v3 and CD44v6, respectively, on cells isolated from synovium from patients with OA. f) and g) Detection of CD44v7/8 on cells isolated from synovium from patients with RA and OA, respectively. Results shown are representative of the immunocytochemistry performed on cells isolated from 18 OA patients and 6 RA patients.

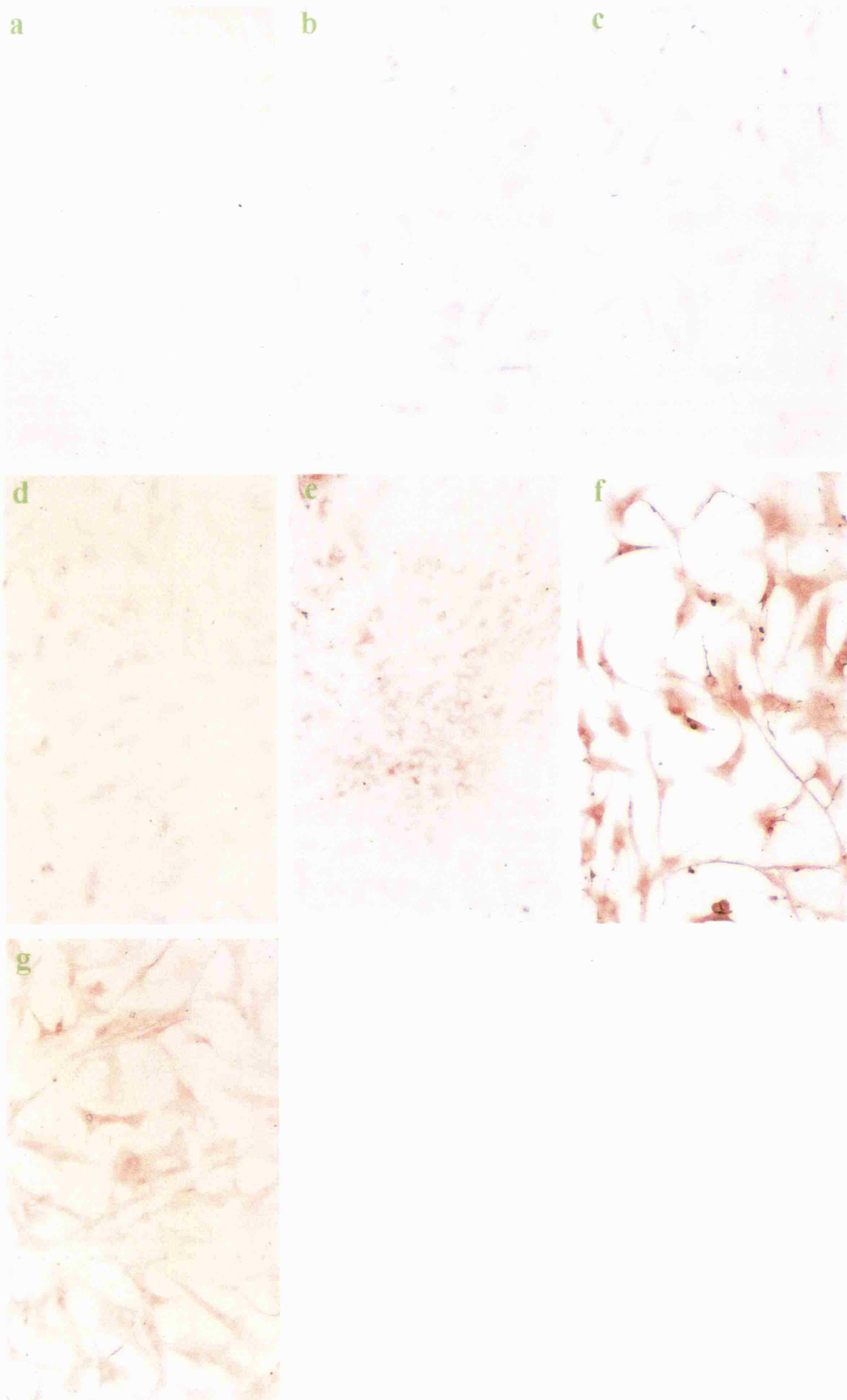
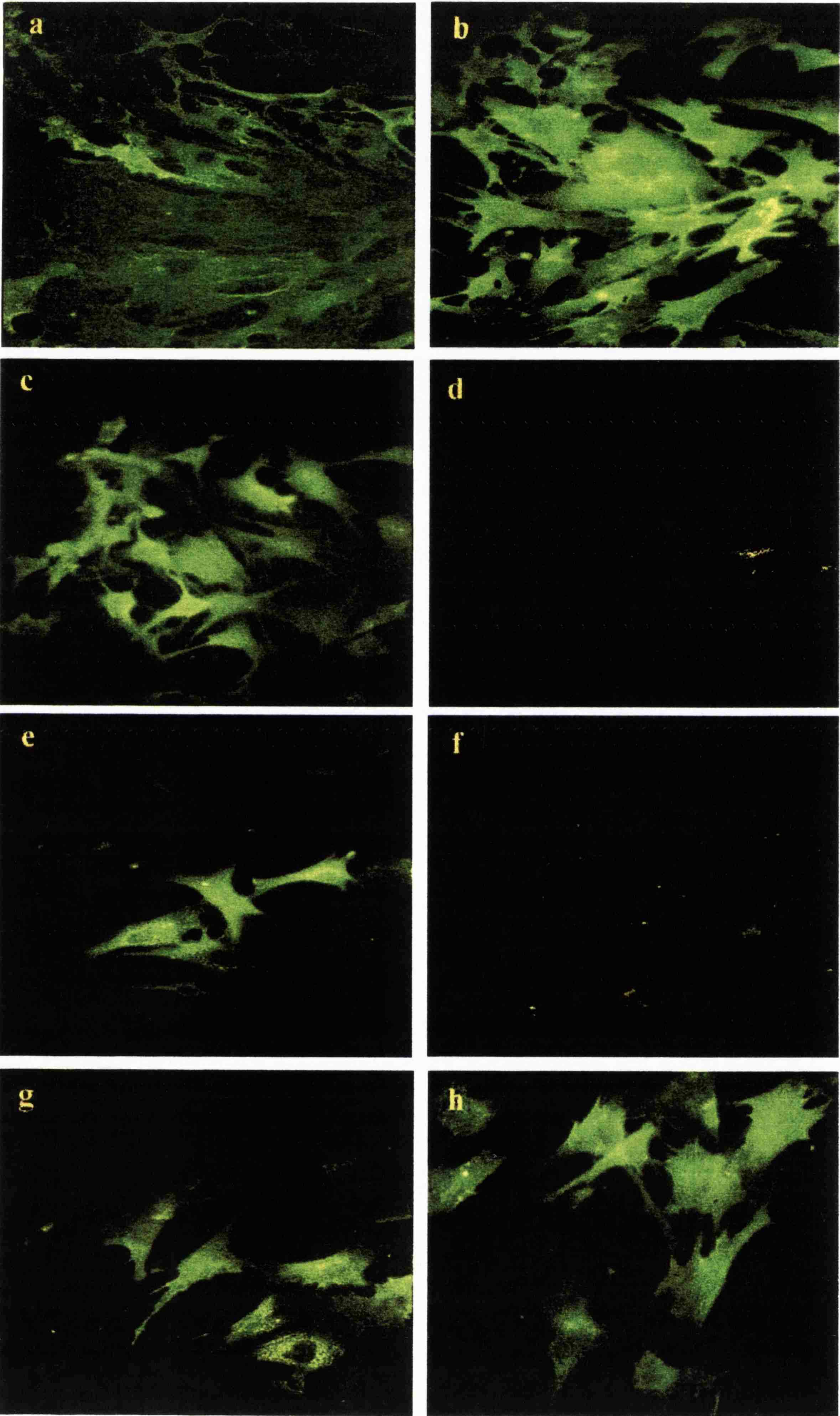

a
b
c
d
e
f
g

**Figure 3.7.** Expression of CD44 splice variants on FLSs in culture using immunofluorescent staining. The cells were fixed with either methanol/acetone (left panel) or 4% *p*-formaldehyde/0.2% Triton X-100 (right panel), and stained with antibodies against CD44 (a and b), CD44v3 (c and d), CD44v6 (e and f) or CD44v7/8 (g and h). In general, splice variant epitopes were best preserved when fixing the cells with methanol/acetone. Results shown are representative of RA FLSs plated on 20 glass coverslips. The cells on 10 of these coverslips were fixed with methanol/acetone and the other 10 were fixed with *p*-formaldehyde/Triton X-100.


a
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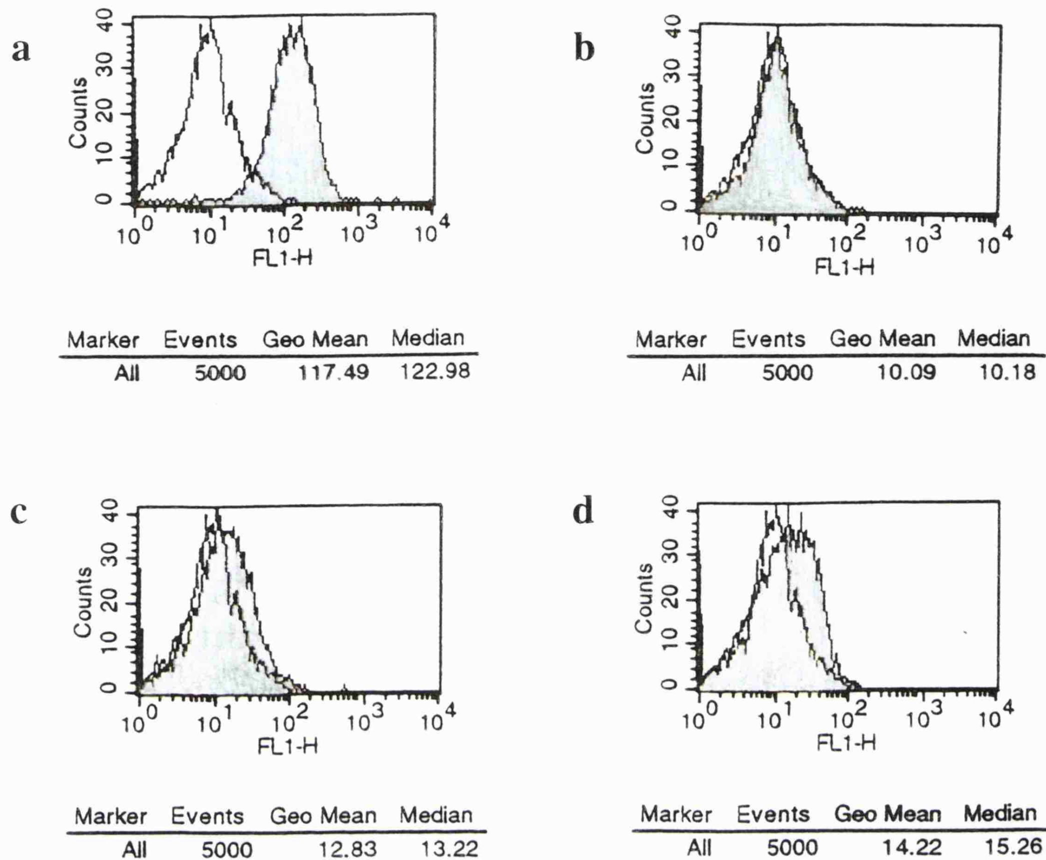



**Figure 3.8.** Flow cytometric analysis of cell surface expression of CD44 (a), CD44v3 (b), CD44v6 (c) and CD44v7/8 (d) on FLSs in culture obtained from synovium from patients with RA. The unshaded histograms represent negative controls, cells stained with FITC-conjugated anti-rat or anti-mouse IgG only. The shaded histograms represent cells incubated with both primary antibodies and FITC-conjugated anti-rat or anti-mouse IgG. The histogram presented is a representative example of 3 experiments performed with cells from 3 patients. Mean fluorescence intensity of the shaded histogram is given underneath each frame. Splice variants could not be detected in cells obtained from synovium from patients with OA, indicating that the overall expression of each splice variants was too low to be detected by flow cytometry.

## 3.5 Discussion

This chapter has shown by a number of methods, using one mAb for each CD44 splice variant, that the variants are expressed in synovium and cultured FLSs derived from diseased joints, both from patients with RA or OA, but are absent from synovium obtained from non-diseased joints. Splice variant expression in the synovial membrane co-localises in part with that of VCAM-1, and it is, therefore, concluded that, amongst other cell types, the splice variants are expressed on FLSs. Expression of the splice variants is maintained in cultures of synovial cells. Synovium from RA patients displays higher levels of expression of CD44v3 and CD44v7/8 than synovium from OA patients. This difference is maintained in cultures of FLSs. A large percentage of cultured cells obtained from synovium from patients with RA expresses CD44v3, CD44v6 and CD44v7/8 whereas this percentage is very variable in cultures of cells obtained from synovium from OA patients. This finding confirms the previous study which showed that, with respect to CD44 splice variant expression, the patients with OA were a much less homogeneous group (Croft *et al.*, 1997). In all cases, expression of CD44 splice variants is a fraction of total CD44 expressed on the cell surface.

Expression of CD44v6 is not detectable in synovial membrane whereas it is detected in cells in culture and by RT-PCR of freshly isolated synovial cells. These data could mean that expression of CD44v6 at the protein level is a consequence of cell culture. There have been reports that show similar findings; for instance, monocytes (Levesque & Haynes, 1996) and smooth muscle cells (Jain *et al.*, 1996) in culture respond by expressing multiple splice variants. However, this explanation is not entirely satisfactory because cells obtained from non-diseased joints never express these splice variants,

neither at the level of mRNA (as detected by RT-PCR) nor at the level of protein (Croft *et al.*, 1997). The point of view here is that expression levels of CD44v6 in the synovial membrane are beyond detection by immunofluorescent staining and that these levels increase when cells are brought in culture. This discrepancy warrants further study, for instance, a detailed time-course of expression from freshly isolated cells up to 3 weeks of cell culture both at the mRNA and protein expression levels.

How expression of CD44 splice variants is regulated is not understood. The majority of studies are mainly concerned with regulation of expression of the CD44s epitope by extracellular stimuli; few of them focus on specific CD44 splice variants (Fichter *et al.*, 1997; Sakai *et al.*, 1996). Among the stimuli tested, cytokines (in particular, TNF-α, IFN-γ, IL-1, IL-2, IL-4 and IL-13) and phorbol ester have received widespread interest. However, the studies have been far from conclusive about the mechanism of regulation of CD44 expression (Benz *et al.*, 1996; Foster *et al.*, 1998; Gross *et al.*, 1995; Han *et al.*, 1996; Hirano *et al.*, 1997; Osada *et al.*, 1995; Patel *et al.*, 1995; Sokoloff *et al.*, 1996; Trejdosiewicz *et al.*, 1998). Interestingly, when cells are treated with phorbol myristate acetate (PMA, also known as 12-O-tetradecanoyl phorbol-13-acetate or TPA), the expression of total CD44 is always enhanced (Atkins *et al.*, 1998; Gross *et al.*, 1995; Hirano *et al.*, 1997; Ladeda *et al.*, 1998). A specific protein kinase C (PKC) inhibitor can reverse the PMA-induced up-regulation, suggesting the involvement of the PKC pathway in CD44 expression. Focusing on CD44 splice variant expression, the influence of several cytokines and PMA has also been investigated. IL-4 can either increase the expression or has no effect (Koopman *et al.*, 1998; Trejdosiewicz *et al.*, 1998). No changes in expression was caused by IL-1β or TNF-α (Hirano *et al.*, 1997; Koopman *et al.*, 1998). Similar to the observations regarding the total CD44, PMA can enhance expression of

CD44E, CD44v6 and/or CD44v7 (Fichter *et al.*, 1997; Kryworuckho *et al.*, 1995). Moreover, a specific inhibitor of PKC was found to block PMA-up-regulated expression of CD44v6 in human neuroblastoma cell line SK-N-SH. Cellular infection by Epstein-Barr virus was shown to increase CD44E expression in B cells. No studies have been done on the elements that may regulate the expression of CD44 splice variants in the synovial membrane. With respect to fibroblast type of cells, while the total CD44 mRNA in human gingival fibroblasts is up-regulated after the treatment with IL-1β, TNF-α or phorbol ester, there is no effect in overall patterns of CD44 isoform expression (Hirano *et al.*, 1997). It suggests that the expression of CD44 splice variants in RA synovial membrane may not be induced by inflammatory cytokines. Since FLSs derived from the diseased synovium maintain the expression of CD44 splice variants in long-term cultures, it is possible that this expression results from cell transformation caused by mutations of certain genes such as APC (adenomatous polyposis coli) gene and K-ras (Kim *et al.*, 1994; Tahara, 1995). However, because the regulation of CD44 splice variants has not been widely studied, final conclusions cannot be drawn.

It would be interesting to study CD44 variant expression in comparison with inflammatory indices such as T cell infiltration and cytokine or MMP expression since that may give a picture of how CD44 variant expression is associated with disease or inflammation states. Unfortunately, the patient information was not revealed to the present study. Alternatives may be to detect the expression of the splice variants and the presence of T cells in the same tissues and to double stain the cells *in situ* for CD44 splice variants and cytokines or MMPs. Examination of the synovium is also useful in diagnosing the disease in patients whom diagnosis is not clear after clinical evaluation and synovial fluid analysis. Synovial membrane findings of villous proliferation,

superficial fibrin, marked lining cell increase, focal necrosis, plasma cells and lymphoid follicles may strongly suggest RA. Moreover, the histology may help distinguish early from late disease. Especially in RA of very recent onset, all the aforementioned findings may not be present, and vascular occlusion or mild vasculitis may be prominent (Schumacher, 1975).

In conclusion, CD44 splice variant expression on FLSs (amongst other cells) is a unique feature of the diseased synovial joint. However, the possibility of the staining artefacts should not be overlooked since no isotype control was used particularly when rheumatoid factors were likely to exist in the RA tissues, and the staining of the tissues and FLSs was not quantitative. The latter problem may be eliminated by analysis of the images using appropriate softwares. The question remains to be answered as to, if the splice variants of CD44 are indeed present on FLSs in RA patients, what are the functional consequences of such expression. The next chapters will deal with this question with respect to its consequence for cell proliferation and cell invasion into ECM.

# Chapter 4

# Expression of CD44v7/8 is implicated in regulation of proliferation of fibroblast-like synoviocytes[1]

## 4.1 Summary

Hyperplasia of the synovial membrane, in particular the FLSs, is a characteristic of RA. When derived from diseased synovial joints, a proportion of these cells express a number of CD44-splicing combinations. The CD44v7/8 epitope is most abundantly expressed. In this chapter, evidence is provided showing that FLSs expressing the CD44v7/8 epitope have a great proliferative advantage compared to FLSs derived from the same joint that does not express it. The addition of antibodies that recognise CD44v7/8 specifically annuls this advantage through induction of the expression of a number of cell cycle inhibitors like p21$^{WAF/CIP}$ and members of the GADD family.

## 4.2 Introduction

One of the main characteristics of RA is hyperplasia of the synovial membrane. It results from an increase in cellularity of the intimal layer due to infiltration of inflammatory cells into synovium and a local proliferation of resident type B cells also known as FLSs (Hogg *et al.*, 1985; Lalor *et al.*, 1987; Qu *et al.*, 1994). The latter cell type found within the

[1] Part of this study was done in conjunction with Dr Daniel Croft, The Institute of Cancer Research, London.

intimal layer and at sites of cartilage and bone erosion has been demonstrated to show signs of transformation (Case *et al.*, 1989; Fassbender, 1983; Müller-Ladner *et al.*, 1995; Shiozawa *et al.*, 1983; Zvaifler & Firestein, 1994). They can, in some circumstances, proliferate in an anchorage-independent manner (Lafyatis *et al.*, 1989). *In situ* and in culture, these cells express several oncogenes that are characteristic of cells that have escaped normal growth-regulatory mechanisms (Firestein, 1996). One of the most important is c-myc, which is a critical signal that initiates cell proliferation (Gay & Gay, 1989). Moreover, while normal cultured FLSs show signs of growth arrest after a certain cell density has been obtained, fibroblasts derived from diseased joints sometimes lose this restriction and continue to proliferate even after cellular contact has occurred. Due to the absence of contact inhibition, cells grow in clusters and pile onto each other.

Another transformation marker linked to the aberrant proliferation of FLSs in RA could be expression of CD44 splice variants. The previous chapter has illustrated that CD44v3, CD44v6 and CD44v7/8 are commonly found in synovium and cultured FLSs isolated from RA. A question remains as to whether these variants are present as an upstream (causally involved) or downstream event (epiphenomenon) to the transformation of FLSs. That CD44 variant expression may be associated with the synovial hyperplasia or proliferation of the synovial cells, as observed in RA, is based on several findings. A number of studies have shown that certain variant isoforms of CD44 are positively correlated with a proliferative advantage in a range of cell types (Cooper *et al.*, 1998; Fromont-Hankard *et al.*, 1998; Yasui *et al.*, 1998). Furthermore, a study on endothelial cells has indicated that the interaction between the CD44 variant, CD44ex14/v10, and either HA or small fragments of HA can induce a mitogenic response (Lokeshwar *et al.*, 1996). Likewise, particular subsets of CD44 splice variants may be directly related to the

proliferation of FLSs seen in RA. The current study has employed various techniques, including immunocytochemistry, cell selection, cell counting and $^{3}H$-thymidine incorporation, to study the role of splice variants in cell proliferation. FLSs expressing the CD44v7/8 epitope have a proliferative advantage. Antibodies recognising CD44v7/8 specifically abrogate this advantage through induction of expression of inhibitors of the cell cycle like $p21^{WAF/CIP}$ and members of the GADD family. These observations make CD44v7/8 a potential target for pharmacological intervention in the treatment of RA.

## 4.3 **Materials and methods**

All materials and methods employed in this chapter are as previously described in Chapter 2. The following paragraphs describe additional protocols for immunocytochemical staining, using anti-Ki67 mAb to determine proliferative capacity of FLSs, and for detection of cell apoptosis.

### 4.3.1 **Immunocytochemistry**

Cultured FLSs plated on 4-well chamber slides was double-stained using the peroxidase system. In brief, the cells were washed with PBS and fixed in methanol and acetone. They were incubated in FBS to saturate non-specific binding sites for antibodies and treated with hydrogen peroxide to terminate endogenous peroxidase activity. The cells were then simultaneously incubated with anti-CD44v3, anti-CD44v6 or anti-CD44v7/8 and anti-Ki67 at 4°C overnight. Negative controls were performed to confirm that there was neither cross-reaction between anti-CD44 splice variants and anti-Ki67 (by incubating additional cells with mAb against each CD44 splice variant alone and with anti-Ki67

alone) nor non-specific binding. After washing in PBS, all wells were incubated in anti-mouse IgG-biotin for 30 min followed by avidin-peroxidase for 30 min, both at room temperature. Substrate, AEC, was freshly prepared and applied to the cells. The reaction was stopped by washing in water. No hematoxylin was applied in the final step. The slides were mounted and examined by light microscopy using an Olympus PM-10AD. Ki67-positive cells were scored as the number of Ki67-positive cells per 100 CD44 splice variant-positive cells viewed. Fields of interest were photographed on Fujichrome T 64ASA film.

In order to determine if CD44 splice variant-positive cells have a growth advantage over CD44 splice variant-negative cells under restrained conditions, cells were cultured in DMEM supplemented with 0.3, 1 and 3% (v/v) FBS for a week before the proliferative capacity was studied as described in the above paragraph.

### 4.3.2 **Detection of apoptosis using flow cytometry**

FLSs were cultured with or without dialysed anti-CD44v7/8 or anti-VCAM-1 mAbs. At day 5, the cell supernatant was collected and the cells harvested by trypsinisation. In order to record DNA histograms, the cells, together with the culture supernatant, were centrifuged (150×g; room temperature; 5 min) and then washed once with PBS. They were fixed in cold 70% (v/v) ethanol in PBS and kept at 4°C until further processing. The cells were washed twice in phosphate-citrate buffer. RNase (100 μg/ml) was added to ensure that only DNA is stained. PI (50 μg/ml) was subsequently added and the suspension analysed for sub-G1 apoptotic cells using a flow cytometer (Becton

Dickinson).

## 4.4 **Results**

### 4.4.1 **Correlation between expression of CD44 splice variants and the cell proliferation marker Ki67**

Co-expression of the splice variants v3, v6 and v7/8 with Ki67, a nuclear antigen present in proliferating cells (Gerdes *et al.*, 1983), was studied using immunocytochemistry. Compared to the CD44v7/8-negative cells, the CD44v7/8-expressing cells had a much higher number of cells co-expressing Ki67 whereas a reverse correlation was observed with CD44v3 and Ki67. In the case of CD44v6, the difference in % Ki67-positive cells between the CD44v6-expressing and non-expressing cells was very little. So it suggests that Ki67 expression does not depend on the expression of CD44v6. Contrastly, that Ki67 was preferentially expressed in CD44v7/8-positive cells implies that Ki67 expression has some correlation with CD44v7/8. (Figures 4.1a and 4.1b). In order to assess whether or not the advantage was maintained under restrained growth conditions, the relationship between CD44 splice variants and Ki67 co-expression with low levels of serum was tested. The results are presented in Figure 4.2. Two-way ANOVA showed that the difference in the co-expression levels between CD44v3, CD44v6 or CD44v7/8 and Ki67 persisted in the presence of lower amount of FBS; that is, a significantly higher percentage of CD44v7/8-expressing cells than CD44v7/8 non-expressing cells co-expressed Ki67. These findings suggest that cells expressing the CD44v7/8 epitope have a proliferative advantage. Interestingly, they also indicate that the cells do not all express the same set of the splice variants. For instance, certain cells must express CD44v3 or

CD44v6 in the absence of CD44v7/8; otherwise the same correlation figure would have been expected for all the splice variants tested.

### 4.4.2 **Proliferation rate of CD44v7/8-selected FLSs**

To further analyse the relation between expression of certain splice variants and cell proliferation, a panning protocol with anti-CD44v7/8 mAb coupled to magnetic beads was employed to select FLSs expressing the CD44v7/8 epitope. Using this method, an almost 100% CD44v7/8-positive population and a negatively selected population of around 20% CD44v7/8-positive cells were routinely obtained (Figure 4.3a). Both the positively and negatively selected populations were analysed in a cell proliferation protocol. FLSs enriched for expression of CD44v7/8 had a higher proliferation rate, with an observed 3.3-fold increase in cell number after 4 days compared to a 2.6-fold increase of low-expressers (n=3) (Figure 4.3b). From these data, an average doubling time of 55 hr (96 × [log 2]/[log 3.3]) for the positive population and an average doubling time of 69 hr (96 × [log 2]/[log 2.6]) for the negatively selected cells were estimated.

### 4.4.3 **Direct functional implication of CD44v7/8 in FLS proliferation**

To determine whether or not expression of the CD44v7/8 epitope is functionally implicated in cell proliferation, cell counting and $^{3}$H-thymidine incorporation experiments with a total population of FLSs in the presence of either dialysed anti-CD44v3, anti-CD44v6, anti-CD44v7/8 or anti-VCAM-1 antibodies were performed. The anti-VCAM-1 was included because FLSs express high levels of this adhesion molecule for which no implication in proliferation had been reported and could, therefore, serve as a negative

control for antibody treatment. Only the presence of anti-CD44v7/8 antibodies reduced cell proliferation (Figure 4.4) and, likewise, reduced $^{3}$H-thymidine incorporation (Figure 4.5). These findings indicate that expression of CD44v7/8 confers a proliferative advantage on FLSs.

### 4.4.4 **Regulation of expression of cell cycle genes by CD44v7/8**

In order to discern a molecular mechanism, through which the CD44v7/8 epitope regulates cell proliferation, a high-density oligonucleotide array was employed to analyse expression of human genes implicated in the regulation of the cell cycle, following the addition of anti-CD44v7/8 over a 5-day time period, which was when the effects of the antibodies on thymidine uptake and cell proliferation were measured. The results are presented in Figure 4.6 and Table 4.1. Expression levels of each sample were normalised for expression of the highly basic protein (HBP). Treatment with anti-CD44v7/8 significantly induced higher levels of expression of the genes encoding cyclin I, proliferating cell nuclear antigen (PCNA), cyclin-dependent kinase (Cdk) inhibitor $p21^{WAF1/CIP1}$, CDC16Hs, growth arrest and DNA-damage-inducible proteins GADD45 and GADD153 (CHOP), growth factor receptor-bound protein 2 (GRB-2), ras-related C3 botulinum toxin substrate 1 (p21rac1) and superoxide dismutase (superoxide dismutase 1 (Cu/Zn); SOD).

Expression of GADD gene products has been associated with either growth arrest or programmed cell death (apoptosis). To determine whether anti-CD44v7/8 antibodies caused cell apoptosis, DNA fragmentation was measured after 5 days of incubation. No increase in the sub-G1 fraction of DNA, as detected by flow cytometry of PI-stained

nuclei, could be observed, and, therefore, it was concluded that there was no sign of DNA fragmentation hence no apoptotic cells. The data are presented in Figures 4.7a and 4.7b.

**Figure 4.1.** Co-expression of CD44 splice variants and the proliferation marker Ki67. The cells were used after the third subculture. a) FLSs were stained for CD44v3, CD44v6 or CD44v7/8 and Ki67. Cells co-expressing CD44 splice variant and Ki67 appear red in cytoplasm and nuclei. b) Ki67-positive cells are shown as the number of Ki67-stained cells per 100 CD44 splice variant-positive cells from 5 fields of view. The percentages shown are mean ± S.E.M. of 3 experiments performed with FLSs isolated from 3 patients. A higher proportion of CD44v7/8-positive cells co-expressing Ki67 means a higher proliferation rate (Student's unpaired t-test, $P < 0.05$).

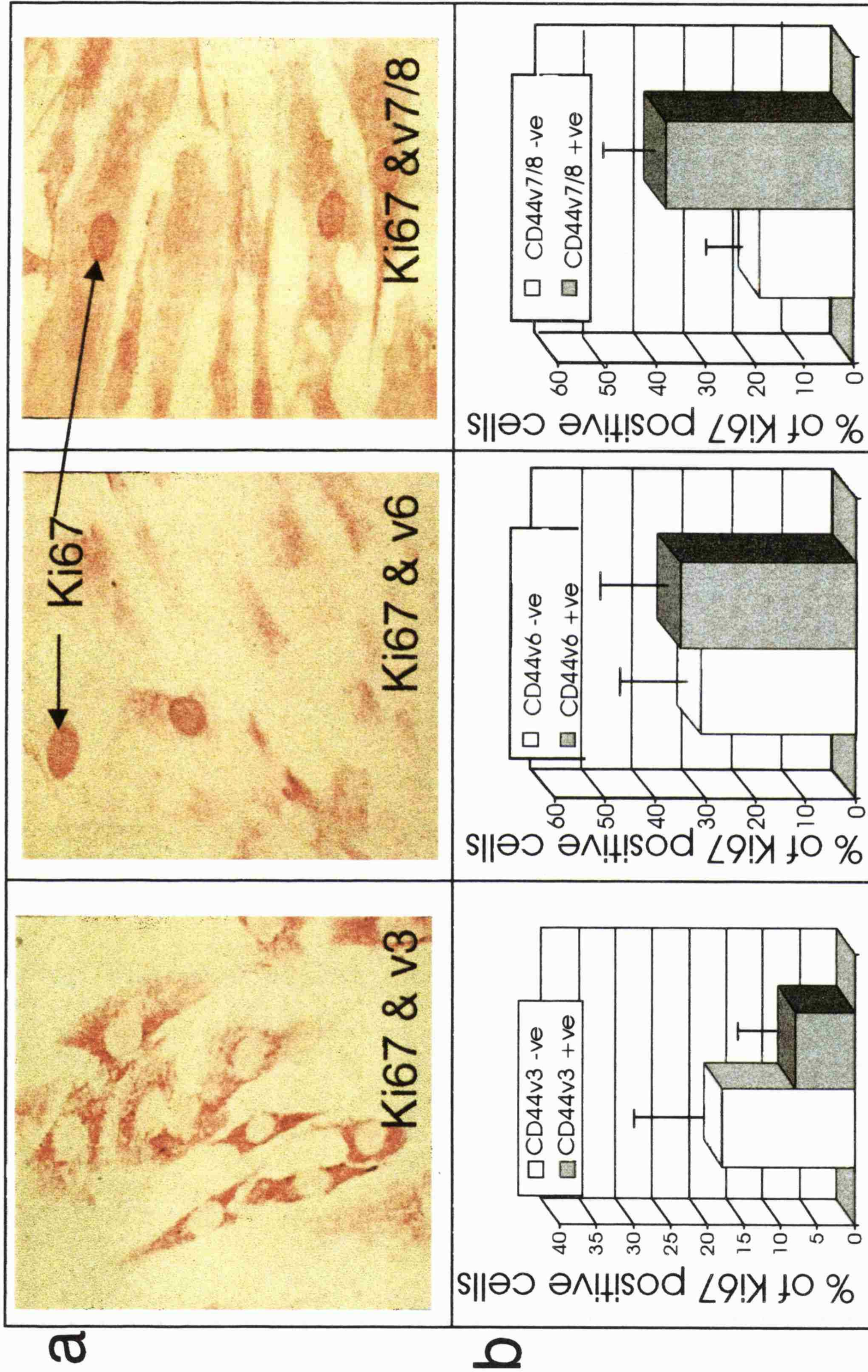
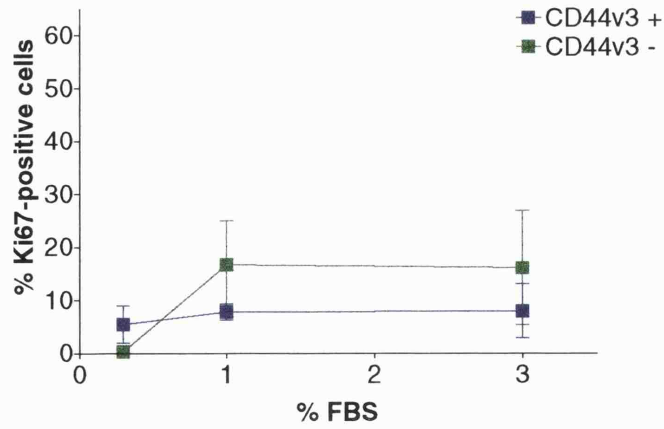
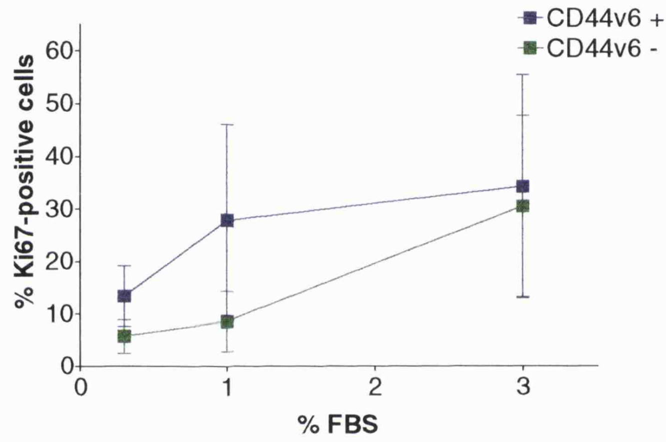
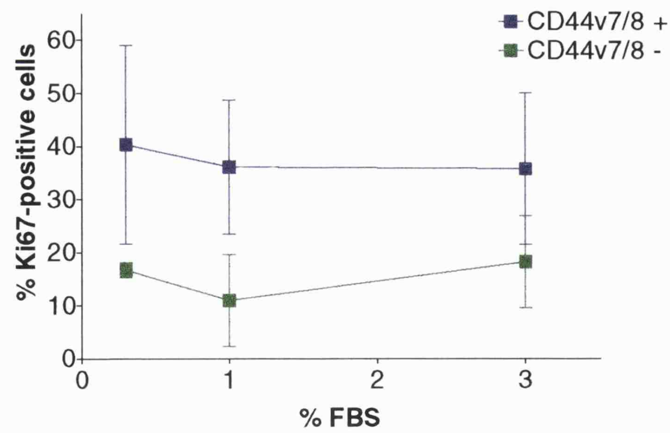

a
b
Ki67 & v3
Ki67 & v6
Ki67 &v7/8
Ki67
% of Ki67 positive cells
CD44v3 -ve
CD44v3 +ve
CD44v6 -ve
CD44v6 +ve
CD44v7/8 -ve
CD44v7/8 +ve
0 5 10 15 20 25 30 35 40
0 10 20 30 40 50 60

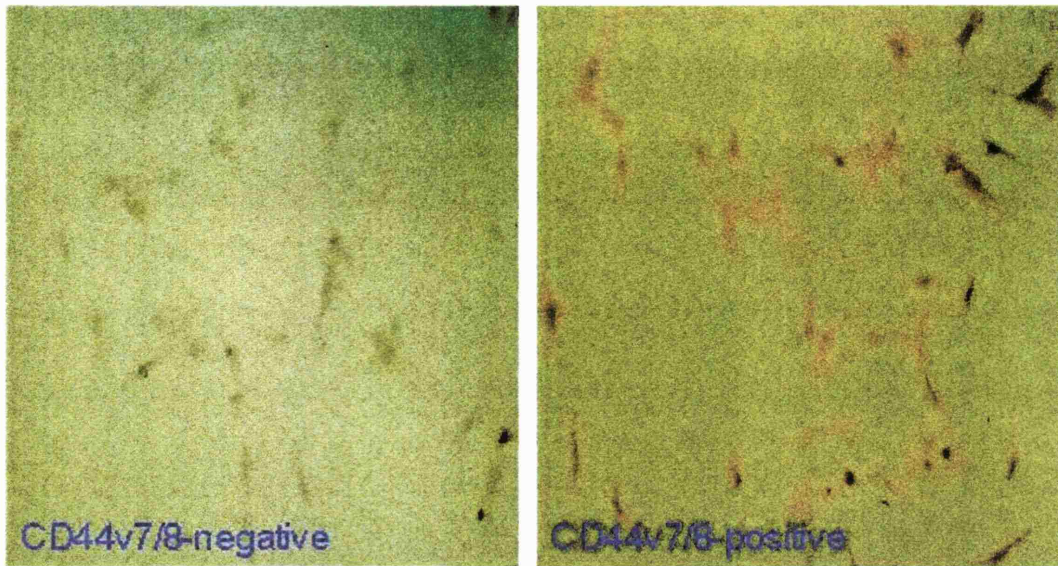
**Figure 4.2.** Co-expression of Ki67 and CD44 splice variants under reduced serum conditions. The values shown are mean ± S.E.M. of the 3 experiments mentioned in Figure 4.1. Two-way ANOVA indicated that, under restricted serum concentrations, only FLSs expressing CD44v7/8 had a significantly higher proliferative capacity than the cells that did not express this variant ($P < 0.05$).


CD44v3
CD44v3 +
CD44v3 -
% Ki67-positive cells
60
50
40
30
20
10
0
0
1
2
3
% FBS


CD44v6
CD44v6 +
CD44v6 -
% Ki67-positive cells
60
50
40
30
20
10
0
0
1
2
3
% FBS


CD44v7/8
CD44v7/8 +
CD44v7/8 -
% Ki67-positive cells
60
50
40
30
20
10
0
0
1
2
3
% FBS

**a**




**b**

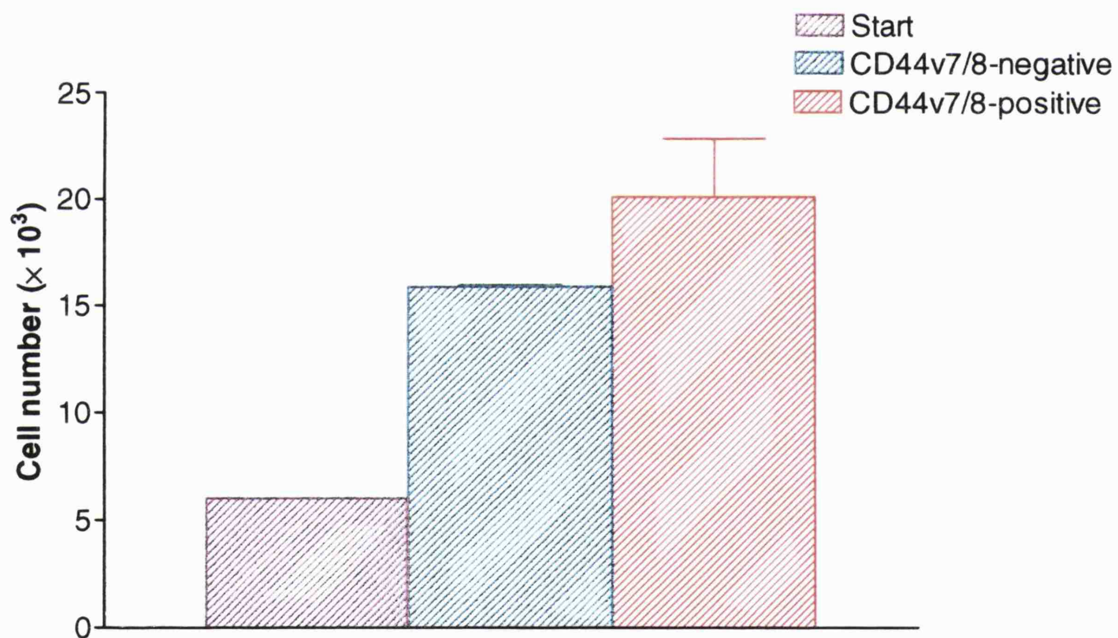



**Figure 4.3.** Proliferation of FLSs positively and negatively selected for expression of CD44v7/8. a) Immunostaining of CD44v7/8-expressing cells after panning with an anti-CD44v7/8 antibody. b) Cell numbers of CD44v7/8 negatively and positively selected FLSs after a period of 4 days (an equal number of cells was seeded at the start of the experiments). The data presented are mean ± S.E.M. of 3 separate experiments, using

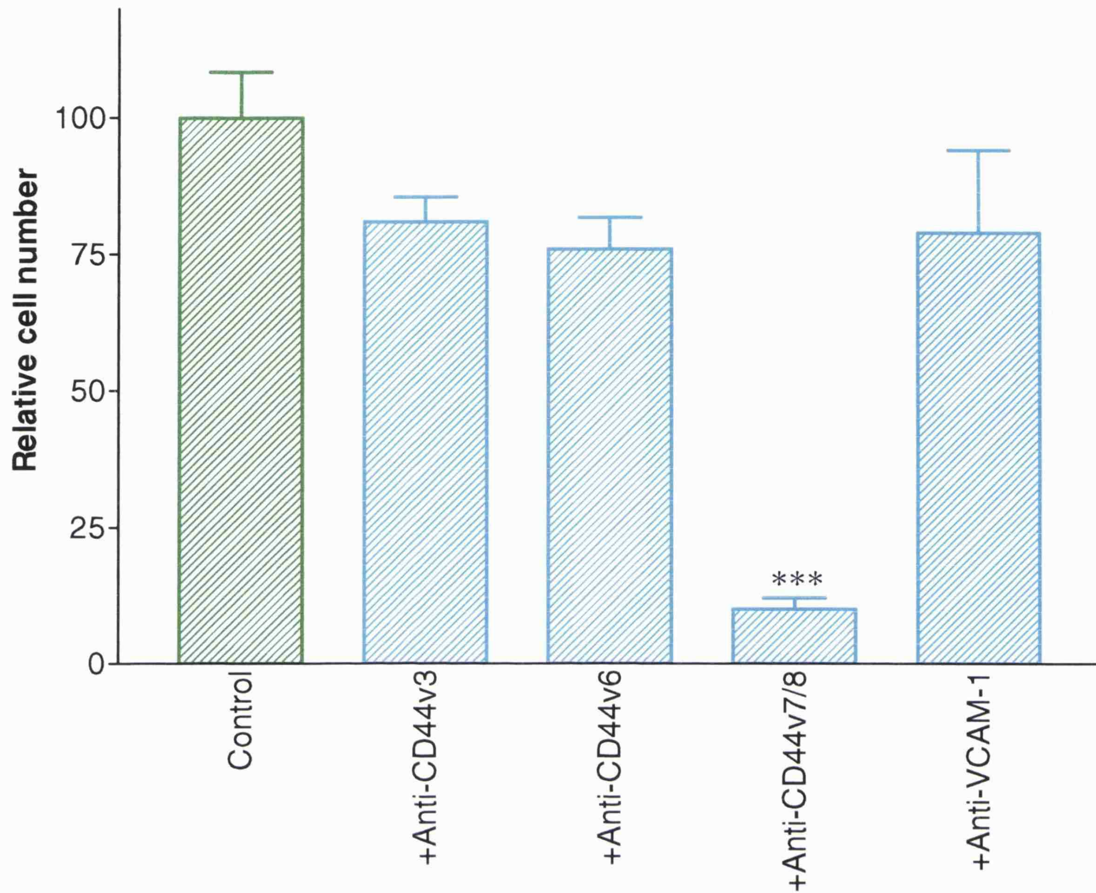



**Figure 4.4.** Relative cell numbers of FLSs after 11 days of cell culture in the absence or presence of dialysed antibodies against CD44v3, v6 or v7/8, or against VCAM-1. Each sample was assayed in triplicate. Cell numbers were determined using a Coulter counter. Anti-CD44v7/8 mAb significantly reduced cell proliferation ($P < 0.0001$). Data were made relative to the value of control (100) and are presented as mean ± S.E.M. of 6 separate experiments.

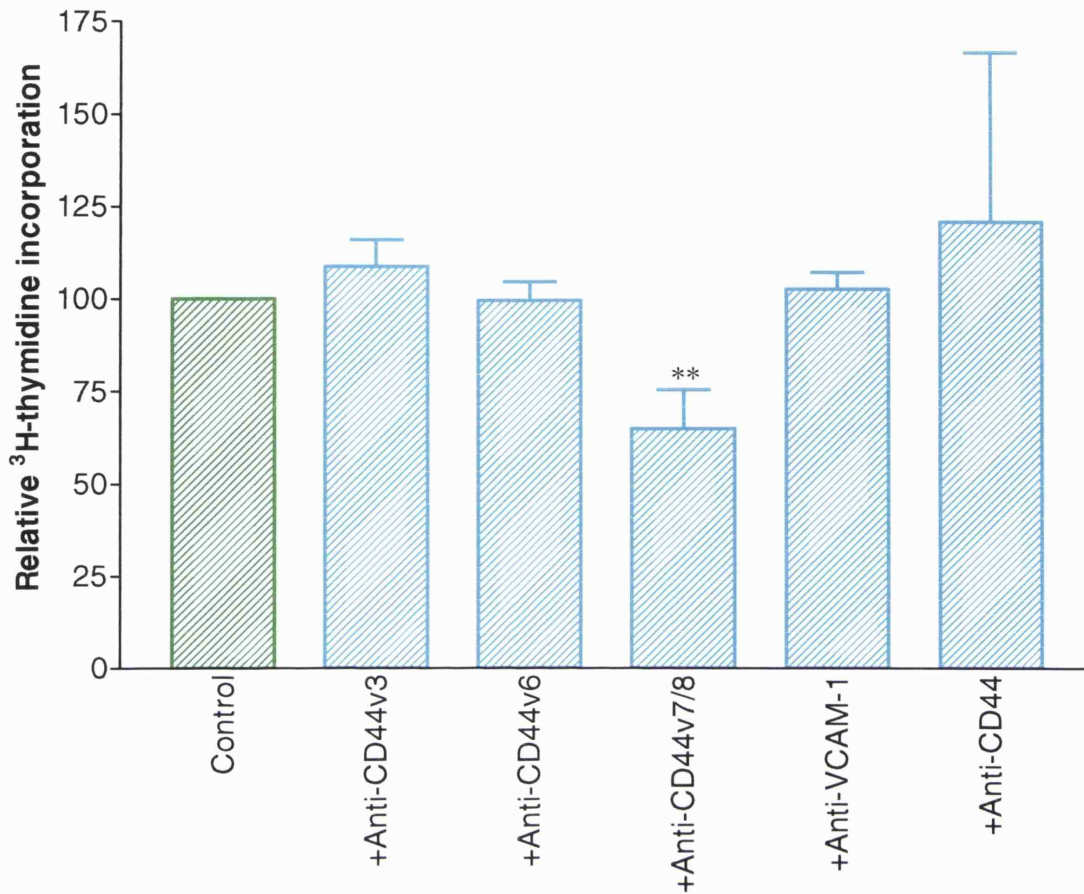
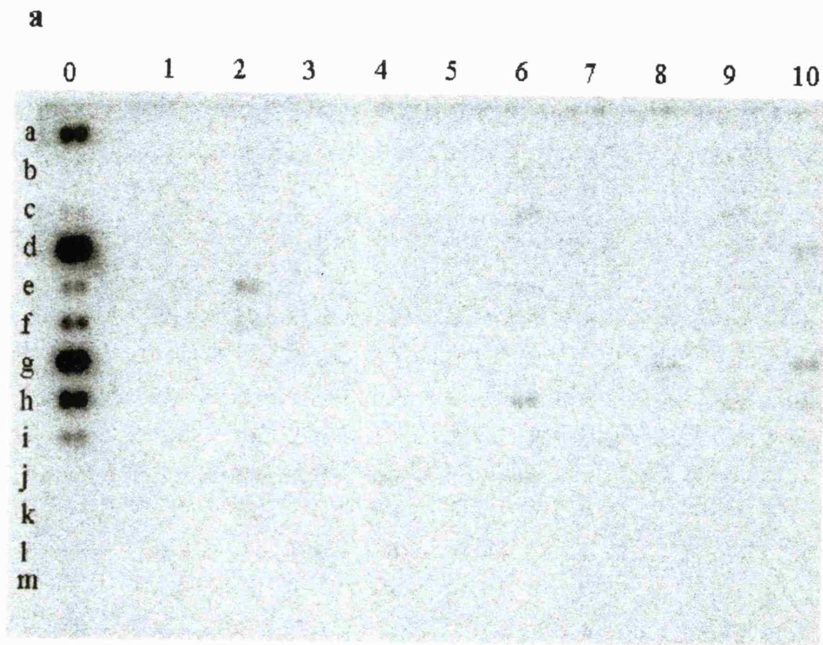



**Figure 4.5.** Relative $^{3}$H-thymidine incorporation of FLSs after 4 days in culture in the absence or presence of dialysed antibodies against CD44, CD44 isoforms containing v3, v6, v7/8 or against VCAM-1. Each sample was assayed in triplicate. Label incorporation was determined using a scintillation counter. Only the addition of anti-CD44v7/8 antibodies significantly reduced thymidine incorporation ($P < 0.01$). Data were made relative to the value of control (100) and are presented as mean ± S.E.M. of 4 separate experiments.

**Figure 4.6.** Analysis of gene expression of cells cultured in the absence or presence of anti-CD44v7/8 antibodies using an Atlas Human-Cell-Cycle Array. $^{32}P$-labelled cDNA was made from total RNA isolated from cultured FLSs without (a) and with (b) treatment with mAb against CD44v7/8. The cDNA was then hybridised to the membranes according to manufacturer's instructions. Expression was visualised and quantified using a PhosphorImager. The dots in column 0 represent housekeeping genes; the intensities of which can be compared between the 2 samples. Arrows (➨) indicate the genes up-regulated in the presence of anti-CD44v7/8 antibodies. The following is a list of the genes corresponding to the positions on the membranes: 1l - cyclin I; 1m - PCNA; 2e - $p21^{WAF1/CIP1}$; 2f - p57Kip2; 4j - CDC16Hs; 6b - GADD153; 6c - GADD45; 6i - MEK1; 8g - GRB-2; 9f - ras-related C3 botulinum toxin substrate 1; and 10g - SOD. Results shown are representative of 3 separate experiments.

a




b

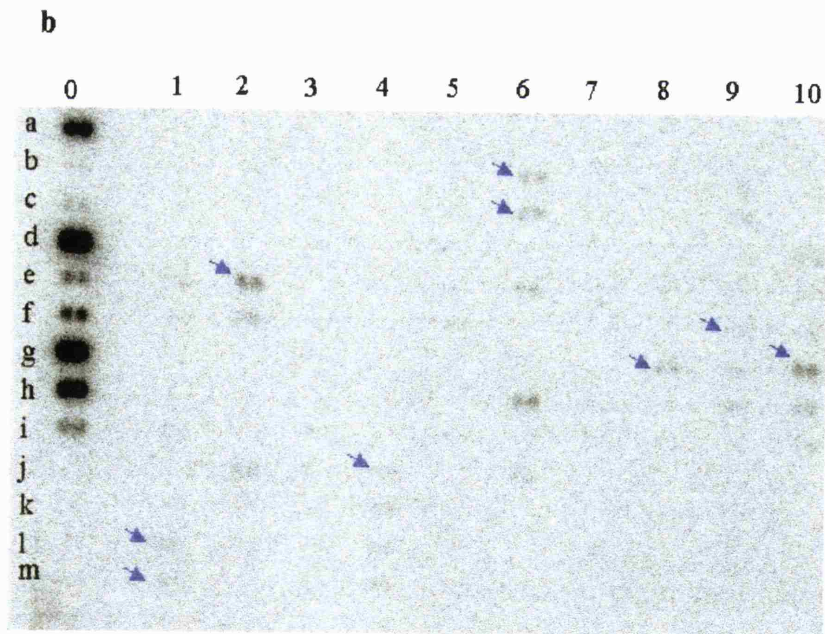


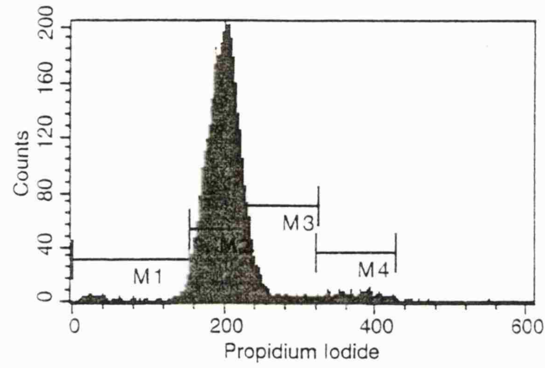
**Table 4.1**. Average normalised intensity values of genes implicated in regulation of the cell cycle as obtained from the Atlas Array hybridisation experiment of cells cultured in the absence or presence of antibodies against CD44v7/8. The mAb significantly up-regulated the expression of several genes, as shown with asterisks (* $P < 0.05$; *** $P < 0.005$).

| GenBank. accession no. | Name of gene/protein | Untreated | | Antibody-treated | |
|---|---|---|---|---|---|
| | | Intensity | Ratio gene:HBP | Intensity | Ratio gene:HBP |
| House keeping genes | | | | | |
| M11886 | HLA class I | 24150±603 | 0.3549 | 25550±580 | 0.3756 |
| X56932 | 23 kDa HBP | 68050±2416 | 1.0000 | 68030±456 | 1.0000 |
| Cell cycle genes | | | | | |
| D50310 | Cyclin I | 381±28 | 0.0056 | 1233±35 | 0.0181*** |
| M15796 | PCNA | 159±106 | 0.0023 | 1166±157 | 0.0171* |
| U09579 | p21Waf1/Cip1 | 4408±280 | 0.0648 | 6707±309 | 0.0986* |
| U22398 | p57Kip2 | 1560±28 | 0.0229 | 2626±442 | 0.0386 |
| U18291 | CDC16Hs | 658±15 | 0.0097 | 1176±78 | 0.0173* |
| M60974 | GADD45 | 152±33 | 0.0022 | 2411±250 | 0.0354* |
| S40706 | GADD153 | 1900±45 | 0.0279 | 2324±61 | 0.0342* |
| L05624 | MEK1 | 392±94 | 0.0058 | 457±34 | 0.0067 |
| L29511 | GRB-2 | 1387±32 | 0.0204 | 2515±217 | 0.0370* |
| M29870 | C3 Botulinum toxin substrate 1 | 25±94 | 0.0004 | 765±156 | 0.0112* |
| HT3218 | Superoxide dismutase (Cu/Zn) | 3674±35 | 0.0540 | 6874±336 | 0.1010* |

**Figure 4.7.** a) DNA size distribution of FLSs cultured for 5 days in the absence or presence of anti-CD44v7/8 or anti-VCAM-1 antibodies. M2, M3 and M4 represent the G1, S and G2 phases of the cell cycle, respectively. M1 represents the sub-G1 fraction that is made up of fragmented DNA, which is an indicator of the presence of apoptotic cells. The anti-VCAM-1 was included to rule out the possibility that the addition of antibody preparations itself might cause apoptosis. The histograms shown are representative of 2 separate experiments. b) Percentages of the apoptotic cells in cultures of FLSs 5 days after the incubation with mAbs against CD44v7/8 or VCAM-1 (n=2).

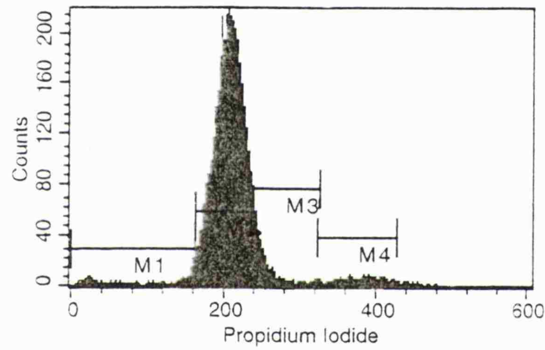
**a**

Control



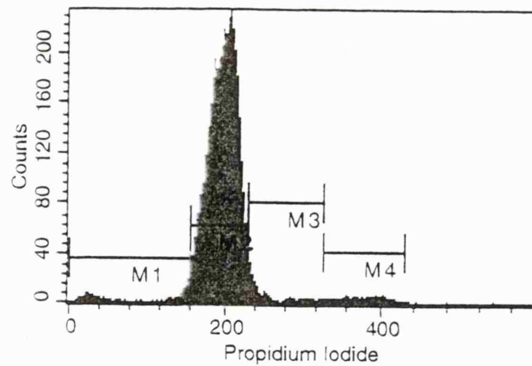

Anti-

CD44v7/8

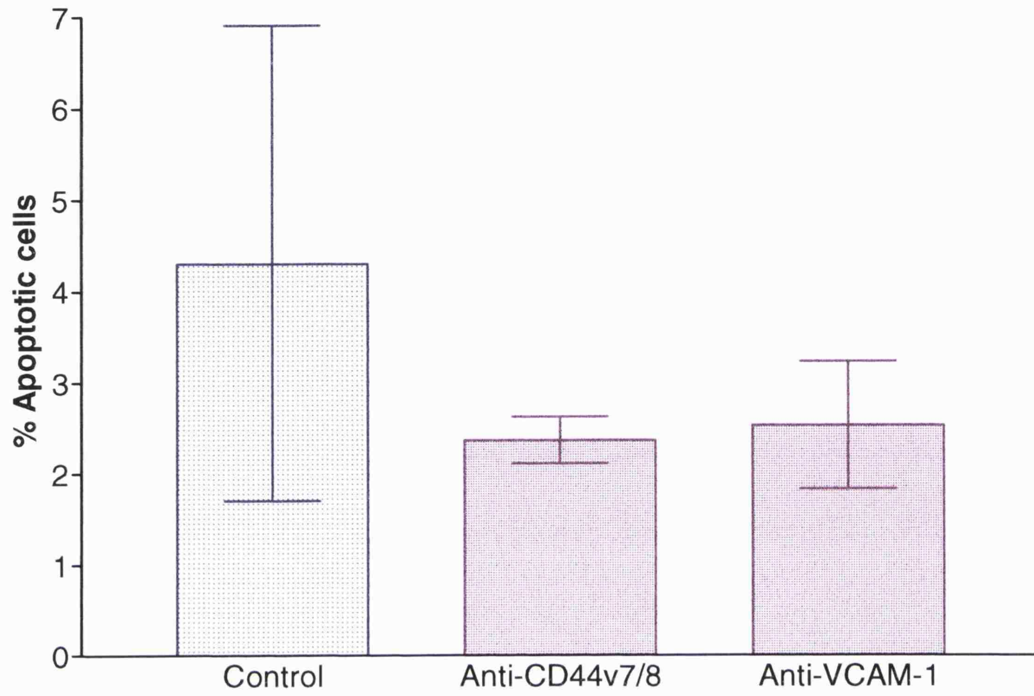



Anti-

VCAM-1



**b**



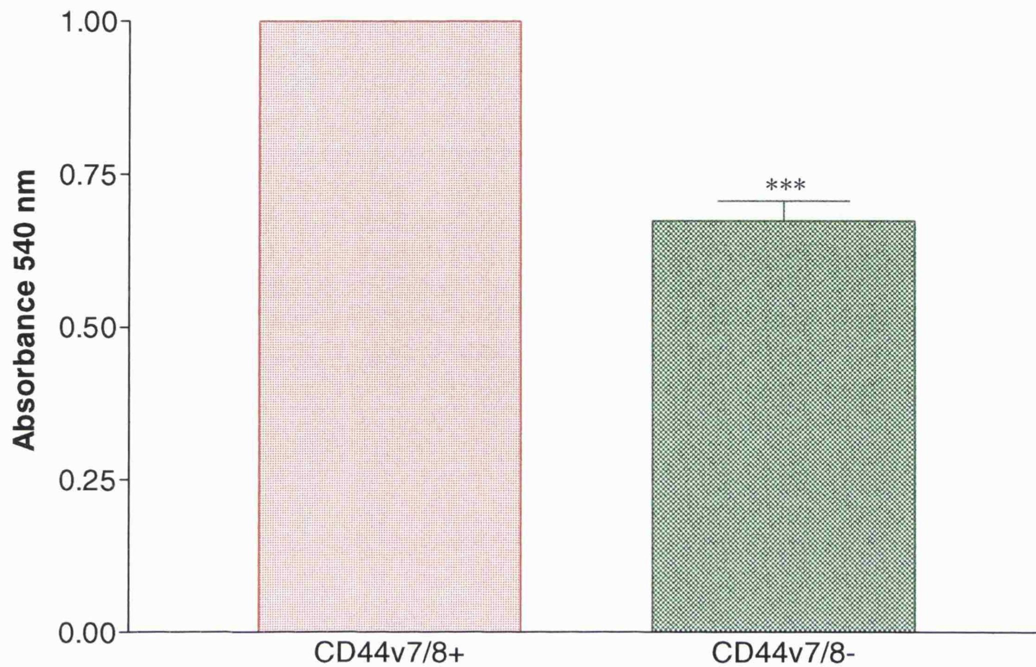



**Figure 4.8.** CD44-dependent HA binding of FLSs selected for expression of CD44v7/8. Results were obtained from the HA-binding assays developed in Dr Kramer's laboratory (University College London, London) as described in Chapter 2. The difference between the 2 populations of cells is significant ($P < 0.001$) ($n=3$).

## 4.5 **Discussion**

This study has advised 2 interesting findings. First, CD44 splice variants containing the v7/8 epitope are associated with faster proliferation rate of FLSs. Second, the expression of the v7/8 epitope may be involved in the regulation of the cell cycle and explain, in part, hyperplasia of the FLSs as observed in RA.

The expression of Ki67 in FLSs derived from RA patients is in favour of previous studies that the hyperplastic synovium, at least partly, results from the active proliferation of FLSs (Mohr *et al.*, 1975; Nykänen *et al.*, 1978; Qu *et al.*, 1994). On the other hand, it contradicts a previous investigation on the co-expression of Ki67 and muramidase, a marker for mononuclear phagocytic cells, which showed that Ki67 was absent in the intimal layer of RA synovium, (Lalor *et al.*, 1987). This is strange since PCNA is expressed *in situ* by proliferating FLSs (Qu *et al.*, 1994), and the presence of PCNA has been found to correlate with other cell proliferation indices including Ki67 immunoreactivity (Hall *et al.*, 1990; Kamel *et al.*, 1989). More studies concerning the expression of Ki67 in synovium should make this clearer. The present study, using serum-restricted cells and 2-way ANOVA, has shown that the cells with CD44v7/8 have a significantly higher proliferative capacity than those that do not express this splice variant, but this capacity is not related to the quantity of serum in medium in which cells are cultured. This suggests that FLSs expressing CD44v7/8 may have an extra self-governing proliferative potential, or they do not require substances in the serum used in the study for proliferation.

CD44 splice variants have gained great attention when it was found that expression of v4-v7 caused metastasis in rat pancreatic tumour cells (Günthert *et al.*, 1991). Dissemination of these cells could be inhibited by the addition of antibodies specific for exon v6, and this finding indicated a possible target for cancer therapy (Seiter *et al.*, 1993). However and unfortunately, although many human tumours express these variable exons and their expression often correlates with an unfavourable prognosis, no functional implication has been found for the expression of any of the splicing combinations (Dall *et al.*, 1994; Driessens *et al.*, 1995; Heider *et al.*, 1993; Koopman *et al.*, 1993; Ristamaki *et al.*, 1995). The current study clearly indicates that expression of the CD44v7/8 epitope in human FLSs has a functional implication; it is associated with enhanced rate of proliferation of these cells. This has been shown in a number of ways. The population of FLSs expressing the CD44v7/8 epitope has a higher percentage of Ki67-positive nuclei. CD44v7/8-enriched population has a higher proliferation rate (average cell doubling time 55 hr compared to 69 hr for the negatively selected population). Addition of antibodies against CD44v7/8, but not antibodies against other CD44 splice variants, reduces cell proliferation and $^{3}$H-thymidine incorporation. Finally, at the molecular level, addition of the antibodies induces expression at the mRNA level of genes that are instrumental in inhibition of the cell cycle, most notably p21$^{WAF1/CIP1}$, GADD45, GADD153 and PCNA (Figure 4.9). The changes in the mRNA are evident after 5 days, meaning that the effect of the antibodies is either delayed or long-lasting since the responses could have occurred within minutes or hours.

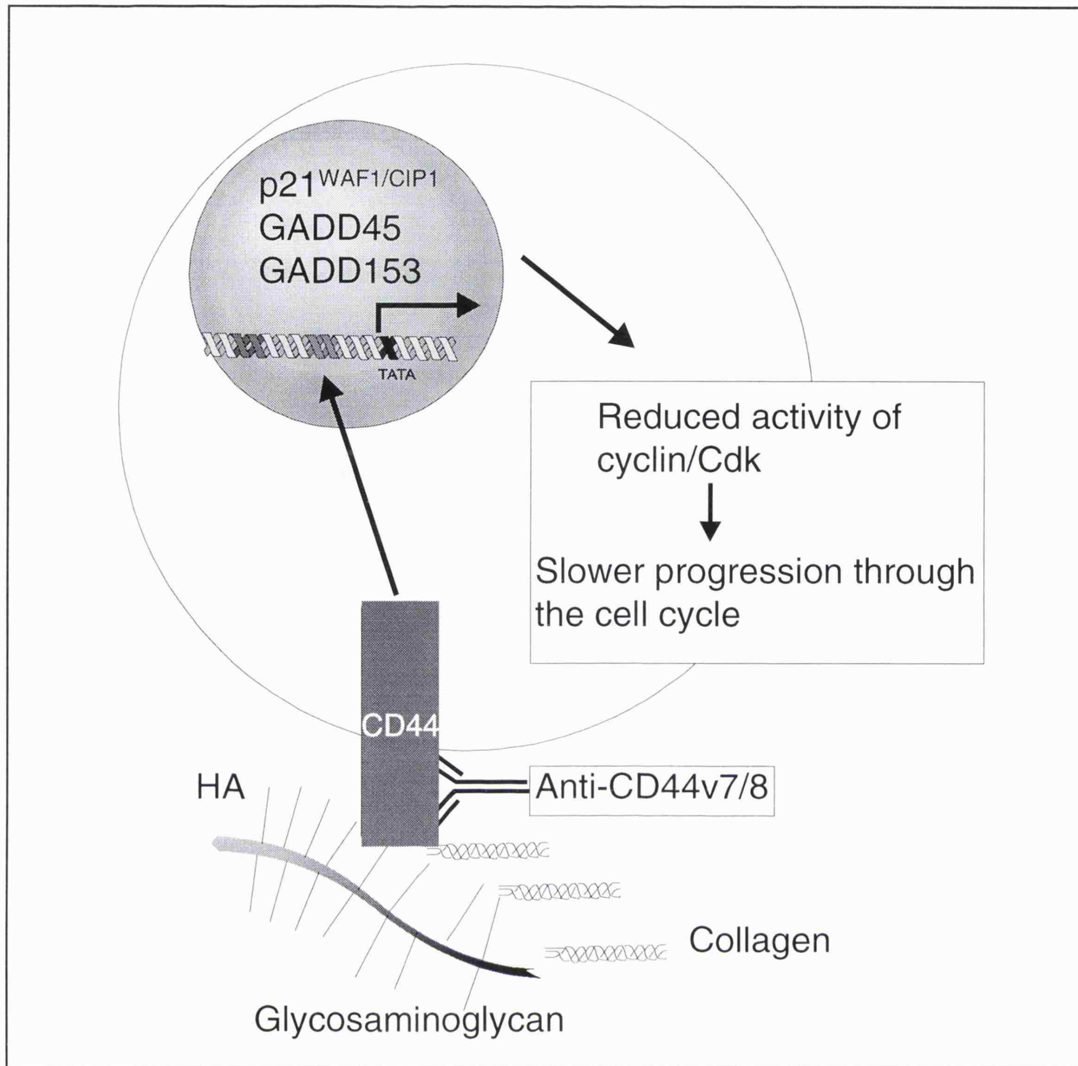



**Figure 4.9.** Schematic model for the regulation of proliferation in CD44v7/8-expressing FLSs. As described in the text, the model illustrates the conclusion drawn from the present study that indicates that addition of anti-CD44v7/8 antibodies inhibit the cell cycle and induces expression of cell cycle inhibitors like $p21^{WAF1/CIP1}$, GADD45 and GADD153. Increased expression of $p21^{WAF1/CIP1}$ means a reduced level of protein kinase activity of cyclin A/Cdk2, cyclin D/Cdk2/4 or cyclin E/Cdk2/4 and thus a slow down in progression from G1 to the S phase of the cell cycle.

The gene product of $p21^{WAF1/CIP1}$ plays a key role in the regulation of cell cycle progression in a number of ways. $p21^{WAF1/CIP1}$ associates with complexes made up of cyclin A/Cdk2, cyclin D/Cdk2/4 or cyclin E/Cdk2/4 (also known as the G1/S Cdk/cyclin complexes) and inhibits their protein kinase activity, thereby preventing phosphorylation of the retinoblastoma gene product and thus inhibiting progression from G1 into the S phase of the cell cycle (Harper *et al.*, 1993; Xiong *et al.*, 1993). In many transformed cells, $p21^{WAF1/CIP1}$ are lost from those multiprotein enzymes (Xiong *et al.*, 1993). In addition, $p21^{WAF1/CIP1}$ is instrumental in inhibition of DNA-replication through activation with, and subsequent inhibition of, the DNA polymerase delta processivity factor also known as PCNA (Luo *et al.*, 1995). The proteins GADD45 and GADD153 have a nuclear localisation and are widely expressed in normal tissues, particularly in quiescent cellular populations. The role of GADD153 is still unclear, but, for GADD45, it was shown that this protein is tightly associated with the Cdk inhibitor $p21^{WAF1/CIP1}$. It is suggested that this interaction is important for the modulation of the cell cycle and for the inhibition of DNA replication (Kearsey *et al.*, 1995). The up-regulation of expression of PCNA comes as a surprise. This protein contains DNA polymerase delta activity and is normally implicated in DNA replication. However, recent studies have shown that PCNA is also up-regulated in cells exposed to ionising radiation (Xu & Morris, 1999) and is, therefore, not a marker of cell proliferation per se. Likewise, GADD45 and GADD153 are also up-regulated in damaged cells, and it has been shown that PCNA and GADD45 can form complexes that are involved in DNA repair (Hall *et al.*, 1995). With this in mind, reduced cell numbers and up-regulation of expression of PCNA and members of GADD family could thus be interpreted as a consequence of anti-CD44v7/8-induced DNA damage; that is, antibodies against this epitope caused cell apoptosis (Orrenius, 1995). However, as shown repeatedly in flow cytometry studies, no DNA fragmentation can be detected at 5

days of incubation with the antibody whereas, at that stage, cell proliferation is already markedly inhibited. It is thus not easily explained why enhanced expression of these genes occurs, and further studies are required to elucidate their mode of action in regulation of the cell cycle.

Up-regulation of cyclin I, a gene that has sequence similarities with cyclin G and E but its expression does not correlate directly to the cell cycle (Nakamura *et al.*, 1995), is also found. Members related to cyclin I were shown to be inducible by p53, giving some indication that they are instrumental in cell cycle arrest. However, its function is unclear at the moment. Lastly, up-regulation of expression is also observed for GRB-2 and C3 Botulinum toxin substrate 1 (also known as rac1), proteins that are involved in signal transduction pathway of a great number of growth factor receptors. GRB-2 is an Src homology (SH) 2- and SH3-containing adaptor protein that couples the growth factor receptor with a ras GTPase crucial in the activation of ras and subsequent activation of the MAP kinase pathway. Rac1 is a GTPase with homology to ras and is implicated in the ras signal transduction pathway. Rac1 plays a role in organisation of the cell cytoskeleton and the formation of lamellipodia, and its presence is required for expression of cyclin D (Page *et al.*, 1999), a cell cyclin which expression is necessary for progression through the G1 phase of the cell cycle (Li *et al.*, 1994). How GRB-2 and rac1 are implicated in the action of the anti-CD44v7/8 antibody is entirely unclear.

CD44 splice variants may alter cell cycle progression through altered outside-in signalling. Outside-in signalling from adhesion molecules has great implications in cell cycle progression as shown in studies with the integrin family of adhesion molecules (Assoian, 1997; Giancotti, 1997). Focal adhesion complexes play an important role in this

outside-in signalling. To date, CD44 has never been shown to exist in these complexes, but it is found in association with members of the ERM family of proteins and components of cytoskeleton (Bourguignon *et al.*, 1992; Legg & Isacke, 1998; Lokeshwar *et al.*, 1996; Tsukita *et al.*, 1994). This and the finding that CD44 can signal through association with tyrosine protein kinases make it more likely that CD44 contributes to the outside-in signalling and may consequently effect cell proliferation (Bourguignon *et al.*, 1997; Ilangumaran *et al.*, 1998; Taher *et al.*, 1996). It is, of course, still unclear how CD44 splice variants containing v7/8 control the expression of cell cycle regulators. Sleeman *et al.* (1997) demonstrated that inclusion of exons v6 and v7, as part of a CD44v4-7 splicing combination, resulted in a stronger binding to HA and permitted CD44 to bind to a larger range of glycosaminoglycans. HA binding of FLSs was tested in the current study, and, compared to negatively selected cells, CD44v7/8-enriched population had an increased CD44-dependent binding capacity (Figure 4.8). Unfortunately, CD44v7/8 expression level was too low to study further glycosaminoglycan binding, and this line of investigation has not been further pursued. From these observations, one may expect that a modified outside-in signalling through either an increased binding to HA or other glycosaminoglycans may account for the proliferative advantage. The CD44 splicing combinations as observed in RA tissues are currently being cloned. Ectopic expression of these clones in fibroblasts will allow further study of their biochemical and functional implications.

A similar growth advantage conferred by CD44v7/8 could play a role in other diseases. For instance, Dall *et al.* (1996) showed that CD44v7/8 expression increased during human cervical carcinogenesis. Subsequently, an immunotherapeutic approach for cervical cancer based on expression of this epitope was developed (Dall *et al.*, 1997).

Unfortunately, this study did not present data on cell doubling times of cells obtained from lesions. CD44 isoforms containing v6 have also been found in various human tumours, but their direct implications have not been verified. The results in this chapter, in particular as seen from the antibody blocking experiments, has suggested that CD44v6 is not likely to directly involved in FLS proliferation while CD44v7/8 is. One may always find that the expression of CD44v6 is high in tumours or proliferating cells, but they should take into account that a subset of CD44v7/8-positive cells may co-express CD44v6 or vice versa, and it may be the CD44v7/8 that participates in cell cycle regulation.

Expression of the CD44v7/8 epitope may explain, at least in part, hyperplasia of the FLSs in RA. It remains to be determined at what stage in the disease CD44 splice variants do appear and whether CD44v7/8 and Ki67 are co-expressed *in situ*. It is possible that the higher level of proliferation explains the higher level of CD44v7/8 expression in RA. Nevertheless, the present study has shown that the expression of this variant is not a passive event; rather, it is implicated in the proliferation of the cells. With respect to RA, the hyperplasia appears to have a role in the development of a chronic erosive environment within the synovial joint, regardless of the role of the immune system therein (Zvaifler & Firestein, 1994). This idea is based on the findings that the degree of synovial hyperplasia correlates with the degree of joint erosion (Rooney *et al.*, 1988) and that H2-c-fos transgenic mice develop destructive arthritis (Shiozawa *et al.*, 1992). Given these considerations, the present observation that antibody against the CD44v7/8 epitope, which presumably acts by inhibiting (via blocking the natural ligands) or reducing (via changing the conformation) the occurrence of CD44 in active state, inhibits FLS proliferation makes this epitope a possible target in RA therapy.

# Chapter 5

# Expression of CD44v3 and CD44v6 is involved in invasive behaviour of fibroblast-like synoviocytes derived from rheumatoid arthritic joints

## 5.1 **Summary**

RA is a disease characterised in part by proliferation and invasion of FLSs into bone and cartilage. The main consequence of the proliferation and invasion is erosion of the joint, resulting in pain and loss of function. FLSs from the diseased joints are distinguished from the cells from healthy joints by the expression of splice variants of a molecule called CD44. One form of CD44, among the many possible CD44-splice variants, namely CD44v4-7, has generated great interest since this variant was shown to be involved in metastatic behaviour of rat pancreatic tumour cells. CD44 splice variants expressed on the FLSs derived from synovial joints from patients with RA are now shown to be involved in the increased capacity of these cells to invade ECM. Antibodies that specifically recognise splice variant v3 or v6 markedly inhibit invasion of ECM by FLSs in an *in vitro* invasion model.

## 5.2 **Introduction**

One of the hallmarks of RA is progressive destruction of bone and cartilage. Growing evidence exists that indicates that FLSs themselves are key players in this destruction process (Firestein, 1996). When derived from RA joints, these cells have a different phenotype and behave differently compared to those obtained from non-diseased joints. For instance, the RA FLSs express elevated levels of the adhesion molecule VCAM-1 and also members of the integrin family, which are receptors for fibronectin, collagen type IV, laminin and tenascin (Rinaldi *et al.*, 1997). Some of these integrins are likely to be involved in adhesion of FLSs, which are normally present in the intimal layer of the synovial membrane, to bone and cartilage: an event that allows invasion of the latter tissues to occur (Müller-Ladner *et al.*, 1998; Wang *et al.*, 1997). FLSs also produce, in excess, certain cytokines and enzymes that promote degradation of the ECM (Ahrens *et al.*, 1996; Chen *et al.*, 1998; Firestein *et al.*, 1991; Harada *et al.*, 1999; Lindy *et al.*, 1997; McCachren *et al.*, 1990; McInnes *et al.*, 1997; Okada *et al.*, 1990; Sorsa *et al.*, 1992; Starkebaum, 1998).

A role for isolated FLSs in the destruction of cartilage was clearly demonstrated in a study with the use of immunodeficient mice. FLSs obtained from patients with RA implanted into these mice, together with pieces of normal human cartilage, were capable of degradative invasion into cartilage devoid of the support of immunocompetent cells like monocytes and lymphocytes (Müller-Ladner *et al.*, 1996). Likewise, when synovial cells from patients with RA were co-cultured with cartilage fragments in a 3-dimensional cell culture system, cartilage erosion occurred. Interestingly, FLSs obtained from patients with OA attached to the cartilage but did not destroy it (Ermis *et al.*, 1998). Shiozawa *et*

*al.* (1992) also showed that joint destruction could be induced in mice even when there were only few infiltrating lymphocytes and that the invading cells in the extensively eroded collagenous tissue had a mesenchymal appearance. These local interactions between the cells and the components of bone and cartilage apparently result from the production of proteases that bear the potential to cleave the ECM. Among various proteases, MMPs and cathepsins may be the most important since they are frequently present at the site or close to the areas of invasion (Ermis *et al.*, 1998; Gay *et al.*, 1993; Müller-Ladner *et al.*, 1996).

Previous chapters have shown that FLSs derived from arthritic joints express splice variants of the adhesion molecule CD44 and that expression of the epitope v7/8 is involved in regulation of cell proliferation. CD44 splice variants, and in particular CD44v4-7 (Günthert *et al.*, 1991), are also expressed in a number of malignant tumours and their presence has been connected with the invasive and metastatic abilities of these tumours. The invasion of bone and cartilage by FLSs, also known as pannus formation, is similar to the behaviour of tumours. In analogy with the rat pancreatic tumour, it is thus possible that CD44 splice variants expressed on FLSs play a similar role in this process. This chapter shows that, indeed, epitopes CD44v3 and v6 are instrumental in the *in vitro* invasion of FLSs into ECM. In addition, the presence of anti-CD44v6 antibodies greatly reduced the release of MMP-1, a protease that plays a role in collagen destruction. This observation suggests one possible mechanism by which inhibition of invasion may be achieved.

## 5.3 **Materials and methods**

The procedures for invasion assays and immunofluorescent staining of the assays are given below. All materials and other methods employed in this chapter are as previously described in Chapter 2.

### 5.3.1. ***In vitro* invasion assay**

The procedure was adapted from that previously described by Hennigan *et al.* (1994). Growth factor-reduced Matrigel was diluted twofold in ice-cold PBS and 120 μl were layered on the top of a 8-μm polycarbonate filter insert in the transwell of a 24-well plate and allowed to solidify at 37°C. FLSs were then trypsinised and resuspended in DMEM supplemented with 10% (v/v) FBS, in the absence or presence of anti-human CD44 or dialysed mAbs against CD44v3, CD44v6, CD44v7/8 or VCAM-1, to $4\times10^5$ cells/ml. The filter inserts were inverted on the 24-well plate top and 100 μl ($4\times10^4$ cells) of the cell suspension were positioned on the filter. The filters were covered with the plate base and placed in the incubator in the inverted position for 1.5 hr. This allowed most of the cells in the sample to adhere firmly to the bottom of the filters. The plate was then placed in an upright position, and cells were washed by transferring the filter inserts sequentially to 2 wells of a 24-well plate each containing 500 μl of 0.5% (v/v) FBS in DMEM. The filter inserts were transferred to another 24 well plate with 500 μl of 0.5% FBS in DMEM in each well. 150 μl of the medium were added above the Matrigel and the system incubated at 37°C for 4 days. The assay was then fixed with 100% methanol at -20°C for 15 min. It was stained with 0.5 μg/ml PI in PBS at room temperature for 15 min, and the inserts were washed 3 times in PBS. They were stored at 4°C in the dark for up to 2 weeks

before being viewed with a Leica TCS4D confocal microscope using a 25× objective. Optical sections were scanned at 10-µm intervals from the filter bottom (Figure 5.1). The number of cells in each optical section was counted on a Dell computer using the UTHSCSA ImageTool programme. To quantitate the assay, the percentage of cells in a given section of the total number of cells in all sections was determined. Since sections at 10-40 and 20-40 µm corresponded to the cells which had migrated through the filters and those which had invaded into the Matrigel, respectively, the number of cells in these section series was summed and expressed as a percentage of the total number of cells in all of the optical sections.

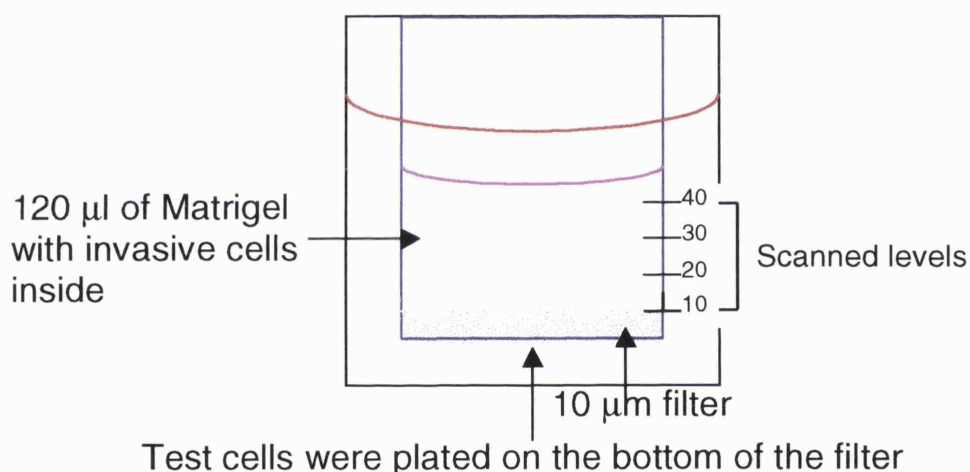



**Figure 5.1.** Schematic presentation of the invasion assay and scanned levels. The cells were seeded onto the bottom of the filter (8-µm pores). Matrigel was placed above the filter. DMEM supplemented with 0.5% FBS was added above the Matrigel. The filter was then placed into a well of a 24-well plate containing the same medium. After 4 days, the cells were stained with PI and visualised by a laser confocal microscope. By taking optical sections, it was possible to observe the cells on the bottom and top of the filter and those that had invaded into the Matrigel.

### 5.3.2 **Immunofluorescence**

After allowing FLSs to migrate through the filters and invade into the Matrigel for 4 days, the cells in the assay were fixed with methanol at -20°C for 10 min followed by ice-cold acetone for 1 min. The assay was incubated in 10% (v/v) FBS in PBS for 20 min. Antibodies against human CD44v3, CD44v6 or CD44v7/8 were applied and incubated for 1 hr. Anti-mouse IgG conjugated with FITC (secondary antibody) was incubated for 30 min after which the cells were stained with 0.5 μg/ml PI in PBS at room temperature for 15 min and washed 3 times in PBS. The cells were observed with a Leica TCS4D confocal microscope using a 25× objective. Optical sections were scanned at 10-μm intervals from the filter bottom. The number of total cells (as estimated by PI stained nuclei) and CD44 splice variant-positive cells (as estimated by FITC stained cells) in each optical section was counted on a computer using the UTHSCSA ImageTool programme. Invasive capacity was defined as the percentage of cells that had entered the Matrigel whereas cell migration was measured as the number of cells that had entered the polycarbonate filter and the Matrigel.

## 5.4. **Results**

### 5.4.1 **FLSs obtained from RA tissue are highly invasive**

Prior to invasion assays, FLSs were screened for their CD44 splice variant expression using an immunocytochemistry protocol. Cultures expressing CD44v3, CD44v6 and CD44v7/8 were chosen to be tested for their invasive capacities using 8-μm porosity polycarbonate filters incorporated in transwells and coated with ECM composite

Matrigel. This assay system is widely used to evaluate the *in vitro* invasive properties of cells from different tumour types (Merzak *et al.*, 1994). The FLSs used in this study were able to invade Matrigel and to migrate to the top of the filter (see, for example, Figure 5.2). Variations existed amongst the various individuals tested. Invasion of Matrigel with FLSs derived from normal synovial specimen could not be detected. In general, FLSs derived from patients with RA were much more invasive than those derived from patients with OA, and, in the following studies, only RA-derived FLSs were used to study the role of CD44 splice variants in matrix invasion.

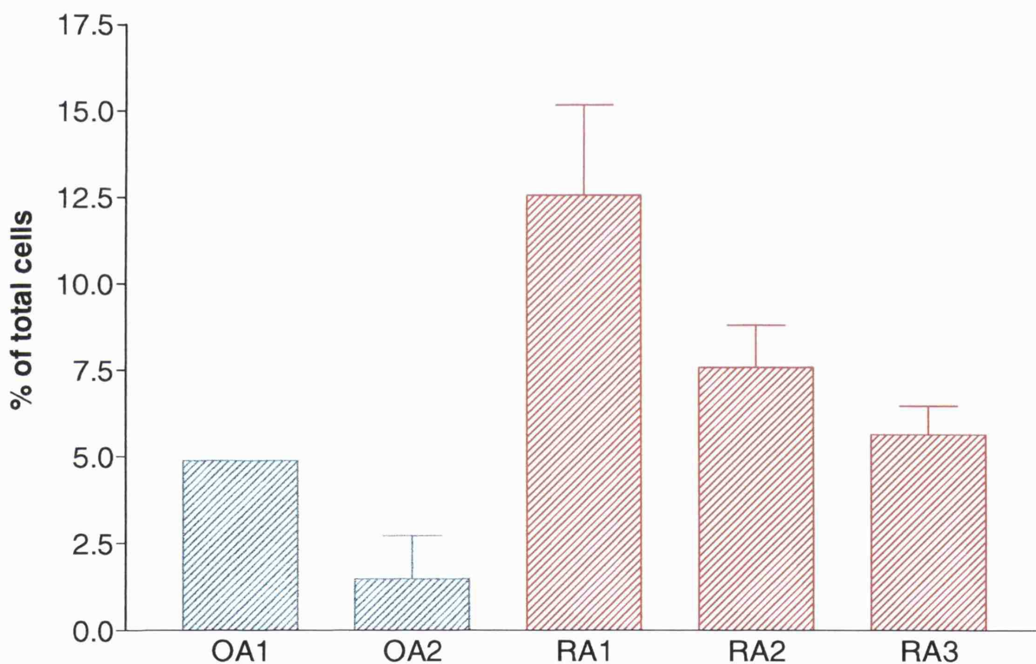



**Figure 5.2.** Quantitative comparison between invasion of FLSs obtained from different OA and RA patients. The data are the percentage of invasive cells in the Matrigel; that is, the percentage of cells present in optical sections from 20 μm distances; and are presented as mean ± S.E.M. of at least 3 optical fields scanned.

### 5.4.2 **Antibodies against CD44v3 or CD44v6 inhibit invasion of Matrigel by FLSs**

In order to study, *in vitro*, the role of CD44 and its splice variants in the characteristic invasive behaviour of human FLSs from patients with RA, the cells were incubated prior to invasion assays in the presence of anti-CD44 or dialysed mAbs directed against CD44v3, CD44v6 or CD44v7/8. Invasion into Matrigel by the cells was inhibited by 62% by anti-CD44v3 and an equal percentage by anti-CD44v6 (Figure 5.3). There was no effect of the addition of anti-CD44 and anti-CD44v7/8. These findings suggest that expression of CD44 splice variants containing v3 or v6 plays a role in the invasion of ECM by FLSs.

The capacity of cells to migrate across the polycarbonate filter was also determined by adding the percentage of cells appearing on top of the filter to the percentage of cells found in the Matrigel. No significant difference in migration was observed among cells treated and untreated with antibodies (Figure 5.4). This finding demonstrates that the inhibition of invasion by anti-CD44v3 and anti-CD44v6, as seen from Figure 5.3, is not a consequence of inhibition of migration since similar percentages of cells had crossed the filter. In other words, the expression of CD44v3 and CD44v6 may promote cell invasion through the mechanism other than by enhancing the cell motility.

### 5.4.3 **FLSs invading Matrigel are enriched for expression of CD44v3 and CD44v6**

If the expression of CD44v3 and v6 favours invasion into Matrigel, then the occurrence of these splice variants must be enriched relative to CD44v7/8. To study possible

enrichment, the cells in the polycarbonate filter/Matrigel after the invasion assay were stained with anti-CD44v3, -CD44v6 or -CD44v7/8 antibodies using an immunofluorescence immunostaining protocol. The polycarbonate filter/Matrigel were subsequently scanned at 10-μm intervals, and the number of splice variant-positive cells was counted as percentage of total cells (as determined by PI staining) in each optical section. Figure 5.5 shows the CD44 splice variant-positive cells stained with FITC in the Matrigel. The percentage of the splice variant-positive cells on the polycarbonate filter was next compared with those present in the Matrigel. As can be seen from Figure 5.6, the percentages of FLSs expressing CD44v3 or CD44v6 were increased in the Matrigel compared with the polycarbonate filter section. In contrast, the percentage of CD44v7/8-expressing cells declined as soon as cells entered the Matrigel. These findings have also been presented as ratios of CD44v3:CD44v7/8 and CD44v6:CD44v7/8 expressing cells in the 2 different sections, the polycarbonate filter versus the Matrigel (see Table 5.1), and a clear shift in favour of expression of CD44v3 or CD44v6 can be discerned. Together with the antibody inhibition experiments, these findings mean that CD44v3- and CD44v6-expressing cells have an invasive advantage.

### 5.4.4 **Anti-CD44v6 inhibits the release of MMP-1**

Since the ability of cells to invade ECM involves the degradation of the ECM components, the levels of matrix degrading enzymes MMP-1 and MMP-9, which were released by FLSs, were investigated. Cells were cultured with or without mAbs against CD44 splice variants for 4 days and the MMP-1 and MMP-9 concentrations in the culture supernatant were analysed using commercially available ELISA kits. The supernatant of the cells treated with anti-CD44v6 mAb contained significantly lower amount of MMP-1

compared to that obtained from untreated cells (Table 5.2). When cells were treated with anti-CD44v3, a lower amount of MMP-1 was also found in the culture supernatant, but it was not significantly different from control cultures. MMP-9 was also released into the medium in small amount, but this was unaffected by incubation of FLSs with anti-CD44 splice variant mAbs.

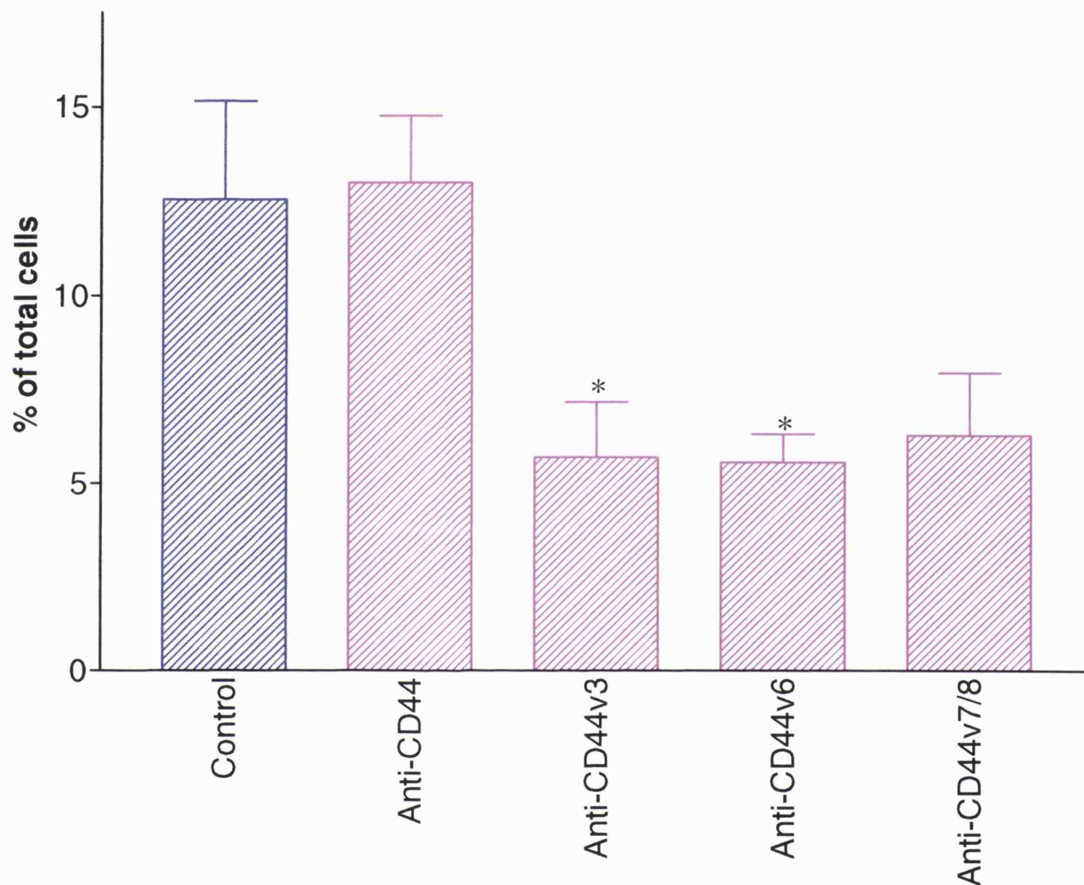



**Figure 5.3.** Inhibition of human FLS invasion *in vitro* by CD44v3- and CD44v6-specific mAbs. The data shown are the percentage of cells that left the filter and invaded the Matrigel and are representative of 3 separate experiments; in each of which, all cell treatments were done in duplicate and a total of 6 optical fields per treatment were scanned. The control is when cells obtained no antibody treatment. * $P < 0.05$.

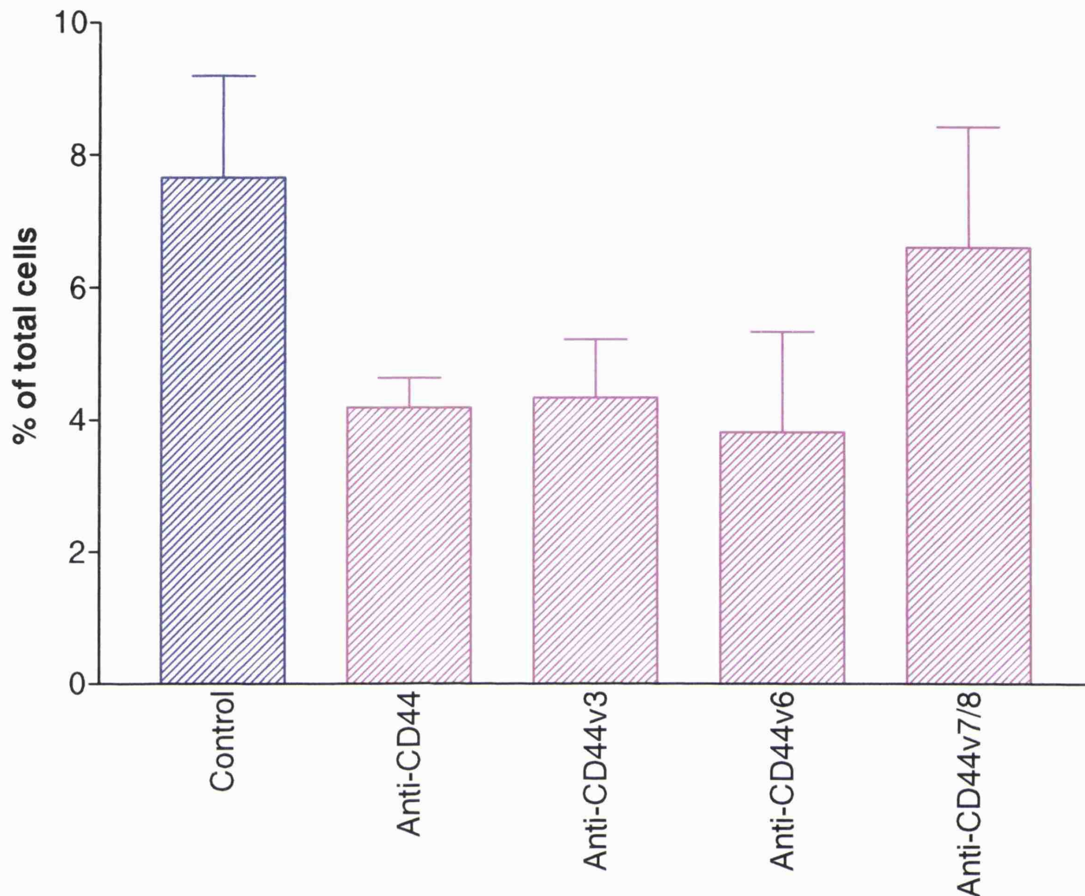



**Figure 5.4.** Analysis of FLS migration in *in vitro* invasion assays. The data are presented as the sum of the percentages of cells in optical sections at the top of the filter (10 μm above the bottom of the filter) and in the Matrigel (20-40 μm above the bottom of the filter). The control, which is not the same control as that in Figure 5.3, is when the cells obtained no antibody treatment. This result is representative of 3 separate experiments; in each of which, all cell treatments were done in duplicate and a total of 6 optical fields per treatment were scanned. No significant difference was found, using Student's unpaired *t*-test analysis.

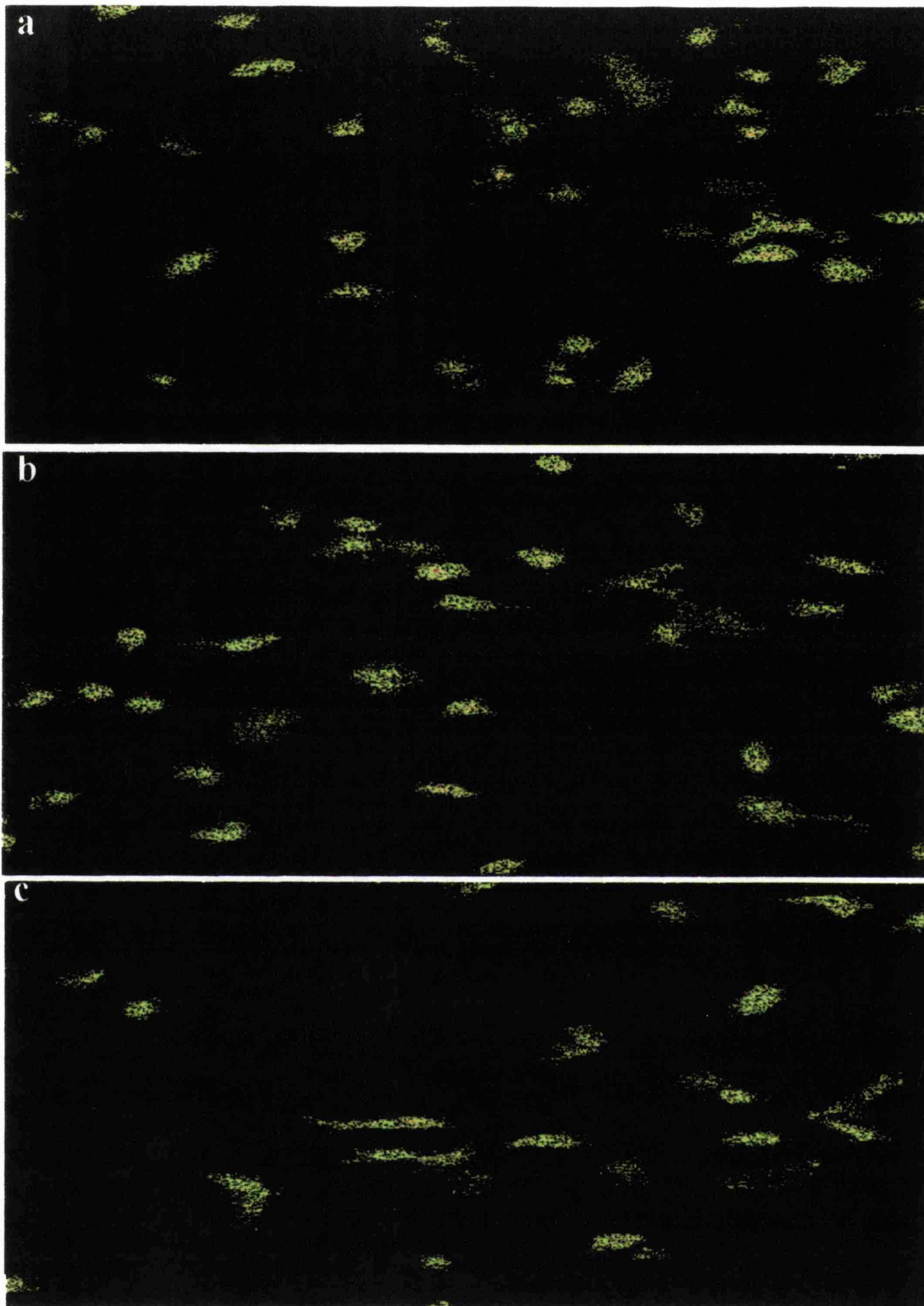



**Figure 5.5.** Immunofluorescence staining for CD44 splice variants showing CD44v3- (a), CD44v6- (b) and CD44v7/8- (c) positive cells in the Matrigel. Results shown are representative of 3 separate experiments.

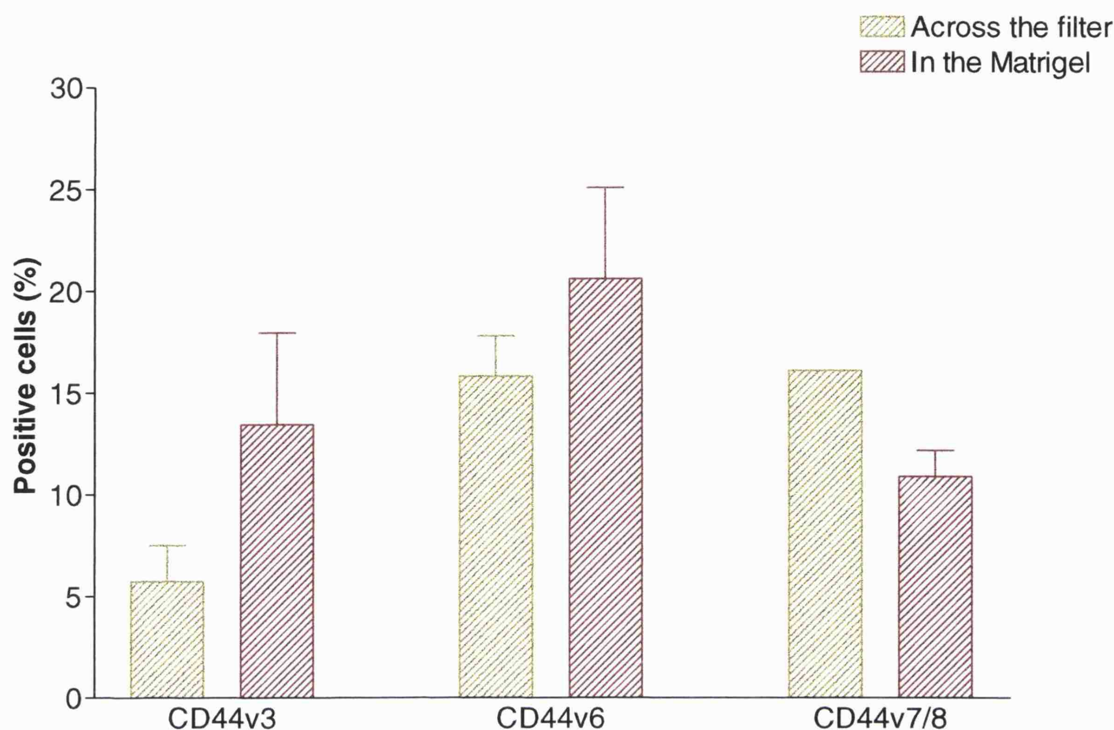



**Figure 5.6.** FLSs expressing CD44v3 and CD44v6 had an invasive advantage over those expressing CD44v7/8. The data are presented as percentages of CD44v3-, CD44v6- or CD44v7/8-positive cells from the total cells seen in the optical sections at 10 μm, where cells had crossed the filter, and at 20-40 μm, where cells were in the Matrigel, and shown as mean ± S.E.M. of 3 separate experiments. They show that the percentage of cells exhibiting CD44v7/8 remarkably dropped while the percentages of CD44v3- or CD44v6-positive cells were increased. This indicates that, after crossing the filter, cells that further invaded into the Matrigel were those with CD44v3 and CD44v6 rather than CD44v7/8.

| | %CD44v3 / %CD44v7/8 | %CD44v6 / %CD44v7/8 |
|---|---|---|
| Across the filter | 0.36 | 0.98 |
| In the Matrigel | 1.24 | 1.90 |

**Table 5.1.** The ratios of % CD44v3- and % CD44v6-expressing cells to % CD44v7/8-expressing cells after they crossed the filter compared to when they were in the Matrigel. The data shown are mean of 3 separate experiments performed on FLSs obtained from 3 RA patients. The result clearly suggests that cells with CD44v3 and CD44v6 have an enhanced invasive capacity.

| Treatment | MMP-1 (ng/ml) | MMP-9 (pg/ml) |
|---|---|---|
| Control (no antibody given) | $5.96 \pm 0.1$ | $22.78 \pm 7.5$ |
| Anti-CD44v3 | $4.27 \pm 0.6$ | $34.30 \pm 4.0$ |
| Anti-CD44v6 | $4.25 \pm 0.1$*** | $46.65 \pm 8.4$ |
| Anti-VCAM-1 | $5.22 \pm 0.3$ | $38.42 \pm 8.1$ |

**Table 5.2.** MMP-1 and MMP-9 release from FLSs in 4 days. The data shown are mean ± S.E.M. of the ELISAs done in duplicate and are representative results from FLSs obtained from 3 patients. The cells from each patient were, in triplicate, treated with or without mAbs against CD44v3, CD44v6 or VCAM-1 and the supernatants collected for the ELISAs. The cells treated with anti-CD44v6 released a significantly low amount of MMP-1 (*** $P < 0.005$).

## 5.5 **Discussion**

These results demonstrate, for the first time, that CD44 splice variants, as expressed in FLSs derived from joints from RA patients, are instrumental in matrix invasion in an *in vitro* assay. The observation that invasion could be inhibited by mAbs against CD44v3 and CD44v6 provides a potential target for treatment of RA, of which distinctive characteristics are invasion and destruction of cartilage and bone.

A role for CD44v3 and v6 in the invasion of ECM

As described in Chapter 4 of this thesis, the evidence indicates that FLSs derived from patients with RA are somehow transformed, and the expression of the CD44v7/8-epitope has been found to contribute to this process by enhancing the proliferation of FLSs. This chapter now concludes that aberrant expression of CD44v3 and v6 may equally contribute to this process by enhancing the cells' capacity to invade ECM. Expression of CD44v6 is associated with a poor prognosis of a number of human tumours, but no clear link with metastasis has been discerned (Gotley *et al.*, 1996) nor are there data published that demonstrate a functional link between invasion and expression of CD44 splice variants. Addition of antibodies against the various variant exons is without any effects. This could be interpreted that, by the time a metastatic tumour is diagnosed, other mechanisms have been put into place by the tumour cells that cause invasion of the ECM and dissemination into other tissues. In other words, the invasive capacity of CD44 splice variants has been made redundant by aberrant expression of other (oncogenic) gene products. With FLSs, this apparently is different; antibodies against v3 and v6 markedly inhibit invasion. It is postulated that FLSs are transformed cells but to a limited extent, and, therefore, the role

of CD44 splice variants has not been obscured by other phenotypic changes. It should be stressed that a total inhibition of invasion with either anti-CD44v3 or –CD44v6 or in the presence of both antibodies is not observed. This could mean that 1) the antibodies are not effectively reaching the cells, 2) the antibodies are internalised with time and lose their inhibitory capacity because new CD44 molecules are presented on the cell surface, or 3) the FLSs have put into place other mechanisms to cause invasion. Invasion has also been correlated with expression of CD44H, independently of the presence of its splice variants. It could, therefore, be possible that FLSs derived from patients with RA are more invasive because they express higher levels of CD44H. This is unlikely to be the case with FLSs for 3 reasons. Firstly, significant differences in expression of CD44H between the various arthropathies were not observed (Croft *et al.*, 1997). Secondly, addition of antibodies against CD44H has no significant effect on invasion, and, lastly, antibodies against specific CD44 splice variants do inhibit invasion. Antibody against CD44v7/8 appears to inhibit the FLS invasion *in vitro* to some extent, but the effect is not significant. More studies are needed to support this observation.

FLSs invading the Matrigel are enriched for the CD44v3 and v6 epitopes and this finding supports the notion that these two variant exons favour an invasive phenotype. However, the percentage of cells expressing CD44v3 (14%) or CD44v6 (22%) in Matrigel is much lower than one would predict on the basis of the inhibition of invasion (62%) (as achieved by either of the antibodies). This apparent discrepancy could be explained by the detection technique not being sensitive enough to identify low expressing cells, that the cells down-regulate expression of CD44v3 or v6 as a consequence of the binding of the antibodies, or that other factors than CD44v3 and CD44v6 are also important in the invasion. It should be noted that the cell selection is based on the presence of only single

marker; so it is possible that cells stained with one splice variant may co-express other variants. Nevertheless, the results have shown that cells invading the Matrigel tend to be those that have included variants v3 and v6 in the CD44 molecule. Undoubtedly, a subset of cells expressing CD44v7/8 can express v3 or v6 as well, and it is, therefore, not necessary that cells selected for CD44v7/8 are all non-invasive.

A role for CD44v6 in the regulation of MMP release

It is now generally accepted that the cytokines TNF-$\alpha$ and IL-1$\beta$ are key mediators in the process of cartilage degradation (Starkebaum, 1998). These two cytokines are derived primarily from synovial macrophages (Firestein *et al.*, 1990) and not from FLSs (see Chapter 6). The presence of TNF-$\alpha$ and IL-1$\beta$ induces the synthesis and secretion of several MMPs by chondrocytes and FLSs (DiBattista *et al.*, 1994; Shingu *et al.*, 1995; Shinmei *et al.*, 1991). Considering that main components of cartilage are collagen type II and proteoglycan (aggrecan), MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3) are very important because they degrade both of these ECM components. Although the inflammatory cytokines play a role, the present studies show that, even in the absence of monocytes/macrophages in cultures and measurable concentrations of these cytokines in cultures, FLSs isolated from RA joints release MMP-1 and MMP-9. Furthermore, the release of MMP-1 is modified by the addition of antibodies specific to CD44v6. This stresses once again the notion that RA is a multifactorial disease; that is, no single event exclusively causes the disease.

Apart from a role in regulation of protease release, CD44 splice variants have been shown to be implicated in localisation and subsequent activation of MMPs. Yu and Stamenkovic

(1999) provided evidence that, in mouse mammary carcinoma cells which constitutively expressed several CD44 isoforms, CD44 served as an MMP-9 docking molecule to retain the MMP-9 activity on the cell surface. The cell surface-bound MMP-9 degrades several substrates including aggrecan and collagen type IV (the latter is a component in the Matrigel used in the invasion assays). If the CD44 splice variants expressed on the surface of FLSs also serve to anchor MMP-9 to the membrane, it is possible that the invasive behaviour of these cells partly results from the activity of this MMP. In addition, it may explain why the levels of MMP-9 in the cell culture supernatant are low, and, therefore, the effects of anti-CD44 splice variants on MMP-9, if any, cannot be detected by analysing the supernatant. MMP-9 release by FLSs has been observed, but the issue of whether or not CD44 splice variants bind this protease remains to be studied. In addition, since there is basal collagenase expression, which would facilitate invasion, and the modulation in the enzyme concentration after the antibody treatment is rather modest, change in levels of TIMPs might also contribute to the altered invasive capacity of the cells. Unfortunately, the TIMP levels have not been measured in this study.

In conclusion, CD44v6 and CD44v3 have been shown in this *in vitro* study to be involved in the invasion of RA FLSs, and antibodies against CD44v6 significantly reduce MMP-1 release. Based on the findings of the current studies, it is tempting to speculate that RA is, in essence, a neoplastic disorder provoking an aberrant immunological response. More studies are, of course, needed to substantiate this point.

# Chapter 6

# Fibroblast-like synoviocytes expressing CD44v7/8 have a reduced expression of IL-6

## 6.1 **Summary**

Elevated levels of the cytokines IL-1β, IL-6 and TNF-α in the synovial joint are eminently linked with RA. These cytokines play a crucial role in the generation of metalloproteinases and toxic oxygen metabolites by infiltrated neutrophils and monocytes. They play a role in the maturation of B cells and in setting the balance between generation and destruction of bone and cartilage. FLSs have been shown to release excessive amounts of IL-6, and this chapter further investigates the role of various CD44 splice variants in the expression and the release of this cytokine. The results presented here confirm earlier data that FLSs in culture express and release IL-6, but not IL-1β or TNF-α. Addition of antibodies specific to CD44v3, CD44v6 or VCAM-1 had no effect whereas antibodies against CD44v7/8 strongly increase the expression of the IL-6 transcript and the release of IL-6 in the medium. When FLSs are selected for CD44v7/8 expression, the positively selected population produces less IL-6 than the negatively selected one. These findings suggest that CD44v7/8 inhibits IL-6 release and that this inhibition is annulled by the presence of anti-CD44v7/8 antibodies.

## 6.2 **Introduction**

Analysis of cytokines present in the synovial joint in RA has given valuable information about the events that underlie joint destruction. Cytokine concentrations in synovial fluid exudates have been estimated by ELISAs or at the levels of mRNA. Cytokines, that appear to play a pivotal role in the pathogenesis of RA, are IL-1β, 1L-6 and TNF-α (reviewed in Feldmann *et al.*, 1990). Both IL-1β and TNF-α have been found to induce cartilage degradation, stimulate synthesis of metalloproteinases by chondrocytes (Schnyder *et al.*, 1987; Shinmei *et al.*, 1989) and synovial cells (Dayer *et al.*, 1985; Dayer *et al.*, 1986). IL-1 and TNF-α also inhibit proteoglycan synthesis in cartilage (Saklatvala *et al.*, 1986).

IL-6 is a cytokine that plays a prominent role in the co-ordinated systemic host defense response to injury. It regulates immune and inflammatory responses, hepatic acute-phase protein synthesis, hematopoiesis and bone metabolism. The roles of IL-6 in regulating the defense responses are well supported by studies in IL-6 knockout mice, demonstrating that lack of IL-6 enhances the animal susceptibility to infections (LeBlanc *et al.*, 1999; Williams *et al.*, 1998), impairs sensory functions (Zhong *et al.*, 1999), increases the sensitivity to liver injury by carbon tetrachloride (Katz *et al.*, 1998) and reduces the incidence of fever and anorexia during sepsis (Leon *et al.*, 1998). IL-6 is necessary for IL-1 to inhibit proteoglycan synthesis (Nietfeld 1990), indicating that this cytokine may act as a co-factor in the process of cartilage degradation. IL-6 is implicated in B cell maturation towards plasma cells and subsequent release of IgG and IgM (Burdin *et al.*, 1995) and thus may contribute to the formation of circulating immune complexes known as the rheumatoid factor. These immune complexes are thought to cause inflammation

because they probably become large enough to be phagocytosed and to activate complement (Brown *et al.*, 1982). The phagocytic process itself generates many inflammatory mediators such as lysosomal enzymes, free radicals produced from oxygen metabolism, prostaglandins, leukotrienes, platelet-activating factor, and other substances capable of initiating or augmenting an inflammatory response.

The human IL-6 receptor complex consists of IL-6 and 2 membrane-associated components: the IL-6 receptor (α-subunit) and the high affinity converter and signal transducer, gp-130 (β-subunit) (Crockard *et al.*, 1992; Kishimoto *et al.*, 1992). A soluble form of the IL-6 receptor (sIL-6R) has been detected in serum and urine of healthy individuals and in synovial fluid of RA patients. It interacts with IL-6, and, unlike soluble form of other receptors, the complex is able to bind to gp130 to induce cellular responses (Novick *et al.*, 1989; Tamura *et al.*, 1993; Uson *et al.*, 1997; Yoshida *et al.*, 1994).

The release of IL-1β and TNF-α is predominantly mediated by infiltrated leukocytes in the inflamed synovial joint (Starkebaum, 1998). However, FLSs have been shown to be responsible for the expression of IL-6 because these cells release relatively large quantities of this cytokine when derived from RA joints compared with, for instance, primary cultures of dermal fibroblasts (Chen *et al.*, 1998; Chomarat *et al.*, 1995). There is evidence that the level of IL-6 production in cultured FLSs derived from RA can be enhanced by IL-1β and TNF-α (Donnelly *et al.*, 1993; Takahashi *et al.*, 1998), but what drives the basal level of this cytokine release has not been determined. Antibodies against TNF-α or IL-1β do not affect spontaneous IL-6 release by FLSs. This indicates that the IL-6 gene is constitutively transcribed, perhaps as a result of the spontaneous activation of

nuclear factor (NF)-κB and C-promoter binding factor 1 (Miyazawa *et al.*, 1998).

With respect to CD44, its splice variants and the release of cytokines by FLSs, no clear link has been established. It was shown by Chen *et al.* (1998) that the presence of HA significantly augmented the production of IL-6 in a co-culture of differentiated U937 cells and FLSs. In their study, no detectable levels of IL-1β or TNF-α could be discerned in the medium. Several studies using either HA or cross-linking antibodies have also suggested a role for CD44 and its splice variants in the release of cytokines in various cell types (Galandrini *et al.*, 1996; Haegel-Kronenberger *et al.*, 1997; Kobayashi & Terao, 1997; Webb *et al.*, 1990). To assess whether or not CD44 splice variants contribute to the expression of these cytokines, the cytokine expression (mRNA) and release in medium by FLSs treated with exon-specific antibodies were measured. The cytokines release in a population of CD44v7/8-enriched and CD44v7/8-depleted cells was also compared. The results confirm that FLSs are indeed a rich source of IL-6, and, for all conditions tested, no detectable levels of IL-1β or TNF-α was found. The addition of anti-CD44v7/8, but not antibodies against other splice variants, greatly enhances the level of expression of IL-6. When comparing the two populations, one enriched versus the one depleted for CD44v7/8, a significantly lower level of release was found in the CD44v7/8-enriched population. These findings suggest that the expression of CD44v7/8 somehow blocks the IL-6 release and that antibodies specific for this variant annul this inhibitory effect.

## 6.3 **Materials and methods**

All materials and methods used in this study are as earlier described in Chapter 2.

## 6.4 **Results**

### 6.4.1 **Antibodies against CD44v7/8 increase IL-6 release in culture**

In culture, the average release of IL-6 per $10^6$ cells over a 4-day period of time was $4.6 \pm 1.3$ ng/ml; this is presented as the 1.0 control value in Figure 6.1. In contrast, no detectable levels of TNF-$\alpha$ or IL-1$\beta$ was found. To study the contribution of the various CD44 splice variants, the effects of variant-specific antibodies on IL-6 release were tested over a similar time period. Addition of anti-CD44v3, anti-CD44v6 or anti-VCAM-1 had no significant effect on IL-6 release. By contrast, an 80% increase in release was observed by addition of anti-CD44v7/8 antibodies, and this increase was significant.

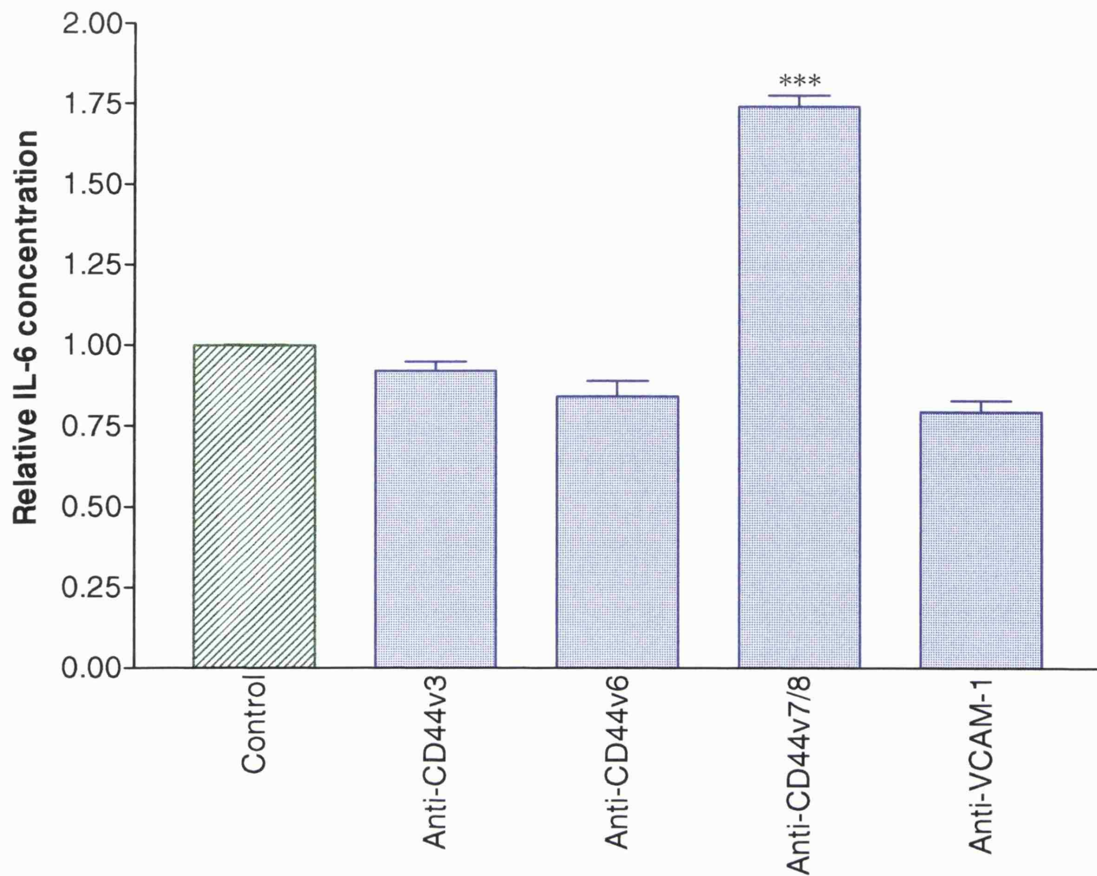



**Figure 6.1.** The effect of anti-CD44v7/8 antibodies on IL-6 release by FLSs in culture. The concentrations shown are from $10^6$ FLSs within 4 days. The result is an average of 3 experiments performed in duplicate. *** $P < 0.005$.

### 6.4.2 **Anti-CD44v7/8 antibodies increase expression of IL-6 mRNA**

To verify the effect of anti-CD44v7/8 antibodies on expression levels of mRNA of IL-1β, IL-6 or TNF-α, the cells were incubated for 4 days in the absence or presence of antibodies. The adherent cells were prepared for RT-PCR using cytokine-specific primers. No transcripts of IL-1β and TNF-α could be detected, confirming earlier data presented by Chen *et al.* (1998). Transcripts of IL-6 were clearly present, and addition of anti-

CD44v7/8 antibodies augmented the expression levels by 2 to 3 folds (Figure 6.2 and Table 6.1).

### 6.4.3 **FLSs enriched for expression of CD44v7/8 show a diminished release of IL-6 compared to a population depleted of CD44v7/8-expressing cells**

The effect of anti-CD44v7/8 could be explained in two ways: it induced a stimulatory signal, or it blocked an inhibitory signal. In the latter case, cells expressing CD44v7/8 should produce less IL-6 because of the presence of an inhibitory signal. Using a magnetic bead panning protocol as described in Chapter 2, the FLS population was separated in two sub-populations, one enriched for CD44v7/8 expression and one depleted of cells expressing this epitope. This way a 100% versus 20% CD44v7/8 populations were routinely obtained. No apparent enrichment of other splice variant (v3 and v6) was evident using this approach. After a week in culture with repeated medium changes to assure removal of any remaining antibodies, both sub-populations were then assessed for IL-6 release over a 4-day time period. The CD44v7/8-enriched population released significantly lower amounts of IL-6 (Figure 6.3). Addition of anti-CD44v7/8 antibodies "restored" the release to levels just above that of the negative population. As expected, the anti-CD44v7/8-restored release of IL-6 was more obvious in a population enriched for CD44v7/8 (from nearly 2-fold increase in a non-selected population to more than 4-fold increase in the selected one).

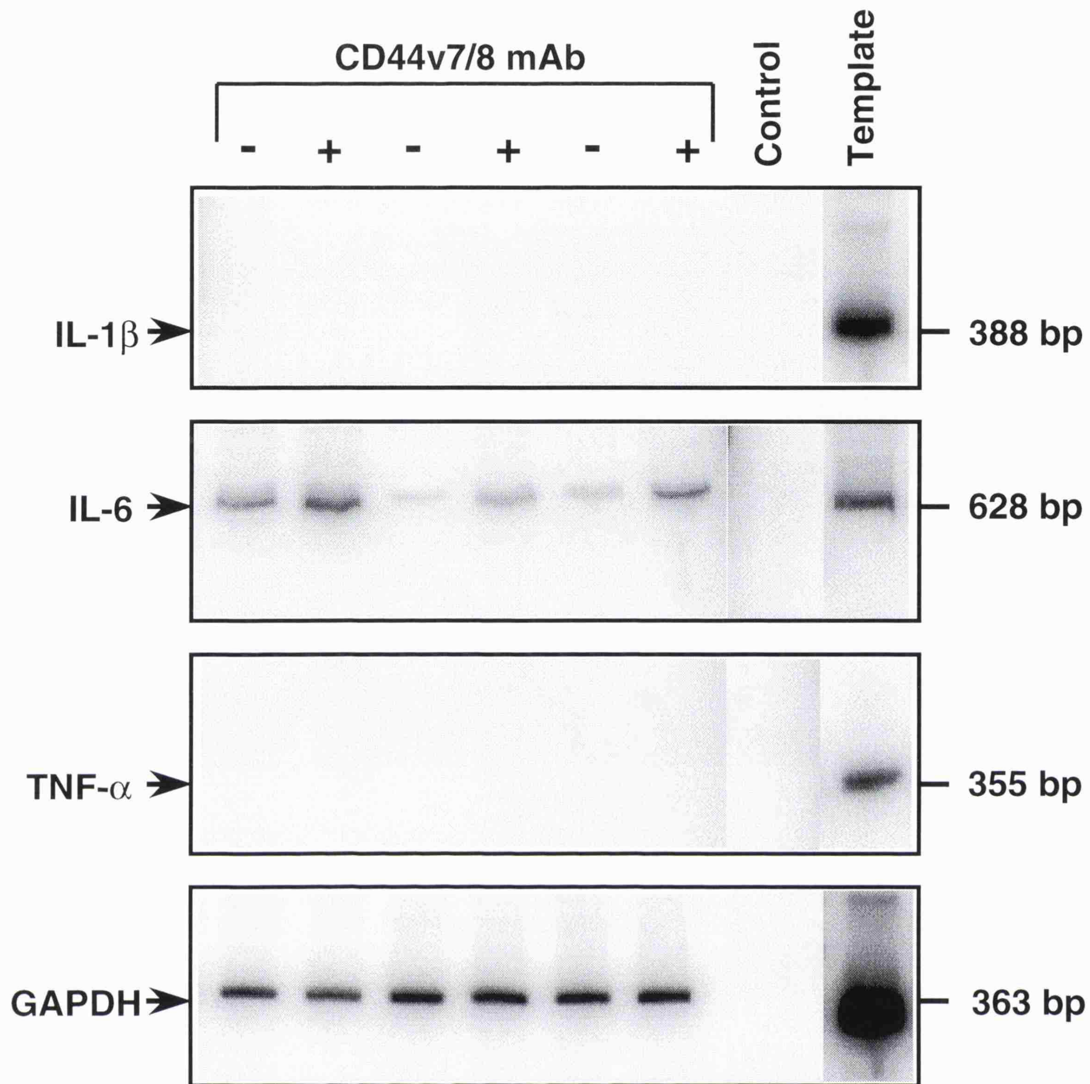



**Figure 6.2.** Analysis of mRNA expression of IL-1β, IL-6 or TNF-α in FLSs. The cells were cultured in the presence (+) or absence (-) of anti-CD44v7/8 antibodies for 4 days. Total RNA was isolated, and semiquantitative RT-PCR was performed using IL-1β-, IL-6-, TNF-α- and GAPDH-specific primers. PCR products were quantified and visualised using a PhosphorImager. Measurement of GAPDH mRNA levels was included for normalisation of the amounts of the cytokine transcripts. The results shown are representative of 3 separate experiments.

| Sample | Untreated (%) | Anti-CD44v7/8-treated (%) |
|---|---|---|
| 1 | 7 | 11 |
| 2 | 1 | 2 |
| 3 | 2 | 6 |

**Table 6.1.** Quantitated and normalised levels of IL-6 transcripts as determined in a semiquantitative RT-PCR protocol. The normalised data are expressed as amount of radioactivity in the IL-6 band relative to that in the respective GAPDH band. The above results are representative of 3 separate experiments.

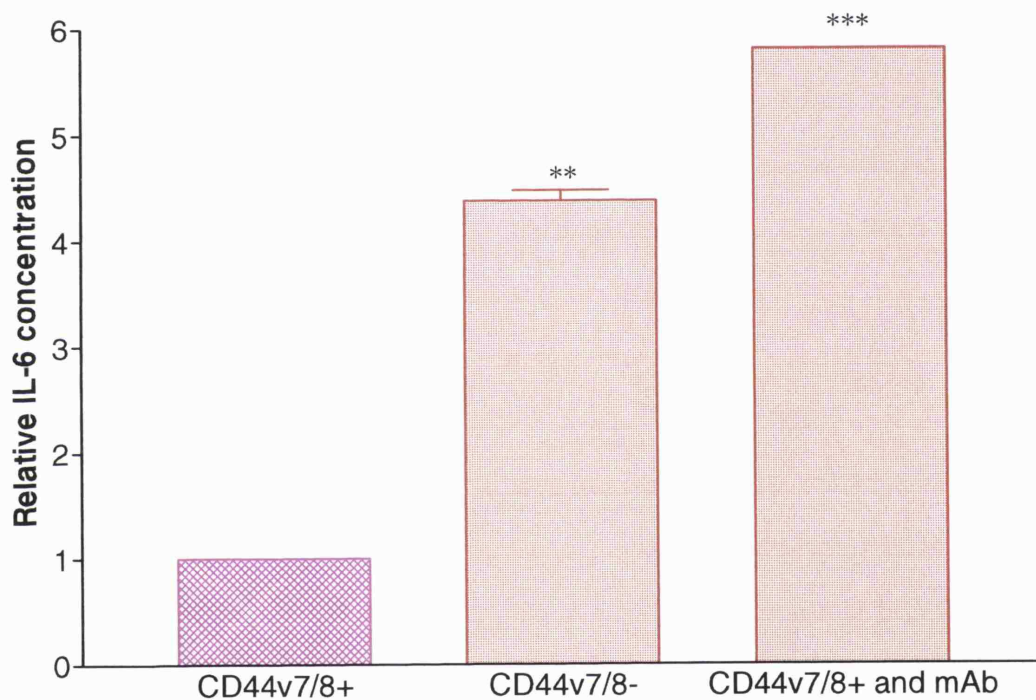



**Figure 6.3.** FLSs enriched for the expression of CD44v7/8 showed diminished IL-6 release compared to a CD44v7/8-depleted population. The cells from 2 patients were selected for CD44v7/8 expression. Both the CD44v7/8-positive and –negative populations from each patient were plated into flasks and, in triplicate, treated with or without mAbs against CD44v7/8. After 4 days, the supernatants were collected for the ELISAs, which were done in duplicate. Asterisks indicate significant differences compared to CD44v7/8-positive population of cells (** $P < 0.01$; *** $P < 0.0001$).

## 6.5 Discussion

The observation that we find, under all the circumstances tested, no expression of TNF-α or IL-1β confirms earlier and similar findings by Chen *et al.* (1998) and Chomarat *et al.* (1995). It stresses the point once more that the presence of these inflammatory cytokines in the synovial joint must be entirely due to its release by infiltrated leukocytes. However, primary cultures of FLSs do release IL-6, and the CD44v7/8 splice variant appears to play an inhibitory role in the regulation of its release. This is concluded on the basis of the observations that 1) the anti-CD44v7/8 antibodies have a stimulatory effect on the transcription of mRNA and the release of IL-6, and 2) cultures enriched for the expression of CD44v7/8 have a reduced expression of IL-6 compared to the negatively selected cells. Thus, CD44 appears to play a role, but the extent to which it regulates IL-6 expression and release by FLSs is moderate compared to, for instance, the effect seen in co-culture of FLSs and monocytes or differentiated U937 cells (Chomorat et al. 1995; Chen et al. 1998). However, the finding does indicate yet another level at which a CD44 splice variant interferes with the metabolism of FLSs.

The effect of IL-6 on cell proliferation varies. This cytokine augments proliferation of many types of cells, including EBV-transformed B cells (Tosato & Pike, 1989), myeloma cells (Kawano *et al.*, 1988) and Kaposi sarcoma cell lines (Miles *et al.*, 1990). In contrast, it possesses growth inhibitory activity, including suppression of breast carcinoma cell lines (Chen *et al.*, 1988; Novick *et al.*, 1992). An increasing number of reports suggests a link between IL-6 and the expression of the cell cycle inhibitor $p21^{WAF/CIP}$ (Bellido et al 1998; Bhattacharya et al 1996; Chin et a 1996; Paulson et al 1999), a gene that is also strongly induced by the addition of anti-CD44v7/8 antibodies (see Chapter 4). IL-6

activates members of signal transducer and activator of transcription (STAT), Stat1 and 3, which bind to the promoter region of $p21^{WAF/CIP}$. Increased expression of $p21^{WAF/CIP}$ reduces the Cdk activity followed by a slowing down of the cell cycle (Sherr & Roberts, 1999). The FLS cell cycle suppression seen in the current study may, therefore, be regulated by IL-6, which makes it possible that this cytokine and the blocking of CD44v7/8 act synergistically on the cell proliferation. Interestingly, a study showed that IL-6 alone did not promote proliferation of FLSs derived from RA patients, but the complex of sIL-6R and IL-6 bound to gp130 and induced FLSs to proliferate (Mihara *et al.*, 1995). A question may rise as to whether the increased IL-6 caused by anti-CD44v7/8 will interact with the sIL-6R *in vivo* and eventually reverse the anti-proliferation of the antibody on FLSs. This may not be the case because the antibody appears to neutralise the IL-6-suppressing effect of CD44v7/8 rather than stimulate the cells to produce extra IL-6. That is, the anti-CD44v7/8 brings the process back to a normal stage as found in the healthy joint. The final outcome of the normal joint, which lacks CD44v7/8 to suppress IL-6 production, is not hyperplasia of the synovium. The use of the anti-CD44v7/8 aiming to stop the FLSs to over-proliferate should, therefore, be reasonable.

How CD44 or its splice variants modify the production of IL-6 in FLSs has not been established. Nuclear factors such as NFIL-6, activator protein-1 (AP-1) (Dendorfer *et al.*, 1994; Ray *et al.*, 1994;) and cAMP response element binding protein (Etienne *et al.*, 1999) have all been shown to bind to and direct activity of the promoter region of the IL-6 gene. These transcription factors apparently play no direct role in the expression of the transcripts of GADD45, GADD153 or $p21^{WAF/CIP}$ genes (Billon *et al.*, 1999; Datto *et al.*, 1995; Moustakas & Kardassis, 1998), which have also been shown in this thesis to be induced by anti-CD44v7/8 antibodies. This information suggests that the transcription of

IL-6 on the one hand and that of GADD45, GADD153 and $p21^{WAF/CIP}$ on the other are unlikely promoted in a similar fashion. As a consequence, the transcription of these genes may occur in a different time scale and a more detailed analysis of the time course of their expression would help in understanding the sequence of events downstream of CD44v7/8. As mentioned in Chapter 4, a number of CD44 splice variants has been cloned, and a future direction for studying their role would be expressing these splicing combinations in FLSs or a fibroblast cell line. Much higher levels of CD44 splice variant expression would then be obtained and would facilitate the studies at the molecular level. Clearly, the current study has highlighted another aspect of FLS functioning that is influenced by a variant-specific antibody. If anything, this and the other findings presented in this thesis have provided excellent tools for further exploration of the molecular mechanisms by which CD44 and its splice variants affects cellular function of human FLSs.

# Chapter 7

# General Discussion

Studies in this thesis have demonstrated the expression of CD44 splice variants in synovial membranes obtained from patients with OA or RA. A strong expression of CD44v7/8, a moderate expression of CD44v3 and a very weak expression of CD44v6 are observed. No expression is seen in tissues from non-inflamed synovial joints. With cells in culture, a fraction of FLSs expresses splice variants, and again the majority being CD44v7/8, a lower percentage for CD44v3 and few CD44v6. This means that the expression of CD44 splice variants is maintained in culture. The studies have then shown that the expression of these splice variants has important phenotypic consequences. The expression of CD44v3 and CD44v6 contributes to the invasive potential as demonstrated in an *in vitro* assay system where invasion can be inhibited by the presence of antibodies specific for CD44v3 and CD44v6. The expression of these splice variants may in part explain the capacity of FLSs to invade bone and cartilage, thus assisting in the formation of pannus. One explanation for the inhibition of invasion is the reduced release of MMP-1 by the cells in the presence of these antibodies. The expression of CD44v7/8 contributes to an increased proliferative capacity of FLSs, and antibodies against an epitope formed by these two exons annul this advantage. Expression of CD44v7/8 may explain in part hyperplasia that is observed in the synovial membrane of the RA joint. Lastly, CD44v7/8 is implicated in the regulation of IL-6 expression as demonstrated by the stimulatory effect of anti-CD44v7/8 antibodies. In short, these findings demonstrate an important fundamental contribution of CD44 splice variants to the transformed phenotype of the

FLSs derived from the RA joint. This is unique with reference to cell transformation because, until now, CD44 splice variants have been observed in a number of human tumours but no functional implications have been found.

**Different functions for different CD44 splicing combinations**

A major topic that emerges from the present studies is that CD44v3 and CD44v6 contribute to functions that are different from those contributed by CD44v7/8. In all experiments, when anti-CD44v3 and –CD44v6 exert an effect, anti-CD44v7/8 is without and vice versa. Surprisingly, addition of an anti-CD44 antibody that blocks HA binding is without significant changes in cellular functioning. This implies that a variable binding efficiency of CD44 splice variants to this ligand cannot explain the differences in cellular effects. Other ligands must thus be involved. Two questions arise. First, if there are specific splicing combinations for specific functions, do they recognise different ligands? Second, how does a single intracellular domain make the distinction between two different functions of the splicing combinations? With respect to ligands, a number of studies has shown a number of ligands for CD44 (and its splice variants), ranging from HA, sulphated proteoglycan, collagen, fibronectin to laminin (Aruffo *et al.*, 1990; Jalkanen & Jalkanen, 1992; reviewed in Naor *et al.*, 1997; Radotra *et al.*, 1994; Toyama-Sorimachi & Miyasaka, 1994). However, because CD44 splicing combinations are numerous, it appears that each different combination will have to be assessed separately for its ligand binding capacity. This aspect is important in order to understand how the different splicing combinations function. For instance, which CD44v7/8-bound ligand promotes proliferation, and which ligand drives invasion?

With respect to signalling, CD44 has been shown to be linked to a family of cytoskeleton linking proteins, ERM, and activates a number of cytoplasmic tyrosine protein kinases (Ilangumaran *et al.*, 1998; Nakamura & Ozawa, 1996; Taher *et al.*, 1996; Tsukita *et al.*, 1994). It is unlikely that CD44 signals as a single molecule or even as a homodimer; rather, it probably forms part of a signalling complex. The alternatively spliced exons in the extracellular domain of CD44 may play a role in the recruitment of these complexes. The recruitment of different components into the signalling complex could nicely explain the disparate functions of the different splicing combinations. The splice variants of CD44 have been reported to form molecular aggregates in the plasma membrane, and the aggregates have different binding affinities. A study in rat pancreatic carcinoma cells showed that the ability of cells to bind to soluble HA was up-regulated by the expression of CD44v4-7 and only when the CD44v4-7 formed intermolecular aggregates (Sleeman *et al.*, 1996). Furthermore, inclusion of exon v3 creates binding sites for bFGF and heparin-binding EGF (Bennett *et al.*, 1995). By binding these growth factors, the different splice variants can recruit growth factor receptors into the receptor signalling complex, which in turn contributes to the "CD44 signal". Importantly, the phenotypic consequences of CD44 splice variants are also cell-type dependent. In other cells, similar CD44 splicing combinations may have totally different effects. One cannot and should not try generalising certain functions for certain splicing combinations.

**Localisation of CD44 splice variants in the synovial membrane in relation to their functional implications**

Does the localisation of FLSs expressing CD44 splice variants in the synovial membrane explain the phenotypes observed in cell culture? CD44v7/8 is most abundantly expressed

in culture and in the synovial membrane, both on VCAM-1-positive cells. It is mainly observed in the intimal layer of the synovial membrane, the site where hyperplasia of these cells is most prominent. It is not excluded that other cells in the membrane, like endothelial cells or infiltrating leukocytes, also express this splice variant. The observation that not all FLSs express splice variants may indicate a process of clonal expansion originating from a small number of CD44v7/8-expressing cells that gradually expand. Evidence of clonal expansion of FLSs has been presented using a restriction fragment length polymorphism analysis of phosphoglycerate kinase 1 gene patterns. The study concluded that synoviocytes with strong growth ability are present in rheumatoid synovium and that these cells expand monoclonally (Imamura *et al.*, 1998).

With CD44v3 and CD44v6, matters are confusing. First of all, very little expression of CD44v6 is found *in situ* whereas some expression is found on cells in culture. This may be because different staining techniques are used (immunofluorescence *in situ* versus peroxidase staining system *in culture*). However, recent collaborative studies performed in Leiden University Medical Center, The Netherlands, have reported the same result using the peroxidase staining system; little or no CD44v6 staining is detected in the synovial membrane. One explanation for this discrepancy is that expression of CD44v6 is a cell culture artefact. An argument against this explanation is the finding that FLSs from control tissues never express any of the splice variants however long they are kept in culture. The staining of CD44v3 is also apparent in the intimal layer of the synovial membrane but less prominent than that of CD44v7/8. One aspect, which has not been addressed but is nevertheless important, is whether CD44v3 and CD44v6 expression is found elsewhere in the synovial joint. If these splice variants are instrumental in the invasion of ECM, then maybe these splice variants in pannus or, even more speculatively,

in cutaneous metaplastic synovial cysts (Bhawan *et al.*, 1990) should be investigated. This thesis has been unable to address this matter because of the difficulties in obtaining sufficient pannus tissues from the hospitals. This aspect is nevertheless of great importance and will require further study.

**RA a neoplastic disease?**

The current studies and those of others have provided evidence that FLSs derived from the RA joint are somehow transformed. Expression of c-myc is enhanced as is the AP-1 activity (Asahara *et al.*, 1997; Müller-Ladner *et al.*, 1995). The cells show loss of function by mutations in the p53 gene (Aupperle et al., 1998), have a proliferative advantage (this thesis and Anastassiades *et al.*, 1978) and cause hyperplasia in the synovial membrane. They grow in SCID mice (Müller-Ladner *et al.*, 1996) and are highly invasive in an *in vitro* assay (this thesis) and *in vivo*, as they are part of the cell mass that forms pannus (Zvaifler & Firestein, 1994). These are characteristics of transformed cells and, with respect to the FLSs, what happens in the diseased joint is the equivalent of a tumour, that is, neoplastic growth and, in an advanced stage, dissemination into other tissues. A number of arguments are in favour of the role of cell transformation in the pathology of RA. First, the degree of hyperplasia correlates with the degree of joint erosion (Rooney *et al.*, 1988). Second, H2-c-fos transgenic mice develop destructive arthritis in the absence of infiltrating lymphocytes (Shiozawa *et al.*, 1992). Third, as many tumours do, the cell transformation may result in the release of pro-angiogenic factors, also known as the angiogenic switch (Czubayko *et al.*, 1997), and angiogenesis in turn facilitates infiltration of immunocompetent cells. Finally, the transformed FLSs express adhesion molecules,

like VCAM-1, ICAM-1 and CD44, which can interact with and activate these infiltrating cells.

It remains to be firmly established whether the inflammatory state triggers the transformation process of the FLSs, or, vice versa, the transformation triggers inflammation and destruction. An example, where inflammation and cell transformation is intimately linked, is a pancreatic adenocarcinoma. This disease starts as a chronic pancreatitis, than gradually transforms into a non-invasive (benign) and finally into an invasive (malignant) pancreatic adenocarcinoma. Interestingly, oncogenic mutations are already observed in the Ki-ras gene during the chronic inflammatory state when an invasive phenotype is not yet discerned (Leathem, 1999). The aspect of cell transformation and joint destruction needs to be explored further and the present findings of the roles of CD44 splice variants in cell transformation have opened a new "port of entry" to this study. One of the next steps is to express these spice variants in primary cultures of FLSs derived from non-diseased joints and to study their potential of cartilage destruction in a SCID mouse model as described by Müller-Ladner *et al.* (1996). In addition, this model is useful to study the inhibitory effect of antibodies. Studies of other forms of synovitis could also make a valuable contribution to understanding the role of cell transformation in the generation of a chronic disease. In this respect, juvenile chronic arthritis is of particular interest. It is an inflammatory joint disease of unknown origin in childhood with a minimal duration of three months. In certain cases, the inflammatory state resolves. The outcome of the disease varies enormously from full recovery to extremely severe crippling polyarthritis (Desaymard *et al.*, 1996). It is of great interest to study phenotypic and genotypic changes that occur during the progression of the disease

and to analyse whether or not a severe destructive outcome can be predicted on the basis of the appearance of these changes.

Lastly, the current findings provide potential pharmacological targets for future treatment of RA, namely the CD44 splice variants. These targets are believed to be valuable for one reason - they address a fundamental change in the FLSs that may lay upstream of other processes like the release of MMPs and inflammatory cytokines.

**Splice variants as targets for pharmacological intervention in RA**

For two reasons, designing a drug that targets the CD44v7/8 seems to be the most promising approach to treat RA. The first reason has already been discussed - targeting this variant, in particular the epitope that is recognised by the anti-CD44v7/8 antibody, inhibits proliferation and, when applied at an early stage of the disease, may prevent hyperplasia. A reduction in hyperplasia may subsequently slow down the destruction of the synovial joint. The second reason is that recent studies have found that targeting CD44v7 reduces the overshooting T lymphocyte response in an inflammatory colitis model (Wittig, 1998). An overshooting T cell response is also observed in RA and regarded as being responsible for joint destruction (Miossec & van den Berg, 1997). In the study by Wittig *et al.* (1998), anti-CD44v7 antibodies prevented death of the mice caused by trinitrobenzenesulfonic acid-induced colitis whereas anti-CD44H or anti-CD44v6 was without effect. Apparently, two aspects fundamental to RA, the hyperplasia and an excessive T cell response, could possibly be kept under control by targeting the variants v7 and v8. Whether such a treatment reverses the chronic inflammatory state and cures and whether or not such a treatment comes with acceptable adverse effects remains, of course, to be established.

# Presentations and publications

WIBULSWAS, A., CROFT, D. & KRAMER, I. (1997). Role of CD44-splice variants in rheumatoid arthritis. Graduate School's Poster Competition, University College London, London.

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WIBULSWAS, A., CROFT, D., BACARESE-HAMILTON, I., MCINTYRE, P., GENOT, E. & KRAMER, I.M. (1999). CD44v7/8 contributes to the transformed phenotype of fibroblast-like synoviocytes in rheumatoid arthritis. Submitted.

# Intellectual property

KRAMER, I.M., DROFT, D. & WIBULSWAS, A. (1998). The use of CD44-splice variants v3, v6, v7 and v8 as exploitable targets for treatment of rheumatoid and osteo arthritis. Applied.

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