EXPRESSION AND FUNCTION OF INTEGRIN-ASSOCIATED PROTEINS IN HUMAN ARTICULAR CARTILAGE

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PhD THESIS

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To:

my Mum and Dad

for teaching me:
love,
the value of listening & learning
and
for everything...

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DECLARATION

I hereby declare that this thesis has been composed by myself and has neither been presented nor accepted in any previous application for a degree. All work presented in this thesis was, unless acknowledged, carried out by myself. All sources of information have been acknowledged by reference.

Mahmoud Orazizadeh
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ABBREVIATIONS USED IN THIS THESIS

AA	Amino acid
Ab	Antibodies
ABC	Avidin-biotin conjugated
ADAM	Adisintegrin-like and metalloproteinase like domain
APS	Ammonium persulphate
AR	Antigen retrieval
BIT	Brain Ig-like molecule with tyrosine based activation motifs
BSA	Bovine serum albumine
cAMP	Cyclic adenosine monophosphate
CBD	Cell binding domain
CD	Cluster of differentiation
cDNA	complementary DNA
CHO	Chinese hamster ovary
COMP	Cartilage oligomeric matrix protein
CRD	Carbohydrate recognition domain
CTF	Cyclic tensile force
DAB	Diamino benzidine
DAG	Diacylglycerol
DC	Dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid

EC	Endothelial cell
ECL	Enhanced chemiluminesence
ECM	Extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EMMPRIN	Extracellular matrix metalloproteinase
ERK	Extracellular signal-regulated kinase
ESB	Electrophoresis sample buffer
FACS	Fluorescence activated cell sorter
FAK	Focal adhesion kinase
FCS	Fetal calf serum
Fn	Fibronectin
Fs	Fragments
Fn-fs	Fibronectin fragments
GAG	Glycosaminoglycan
Gal-3	Galectin-3
GH	Growth hormone
GP	Glycoprotein
G protein	Guanosine triphosphate-binding protein
GPI	Glycosyl phosphatidyl inositol
HA	Hyaluronic acid, hyaluronan
HAC	Human articular cartilage
HBD	Heparin binding domain
HIV	Human immunodeficiency virus

HP	Hydrostatic pressure
HRP	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cell
Hz	Hertz
IAP	Integrin- associated protein
ICAM	Intracellular adhesion molecule
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IL	Interleukin
JNK	c-jun amino-terminal kinase
KD	Kilo Dalton
LAT	L-type amino acid transporter
LC	Light chain
MAb	Monoclonal antibody
MAP	Mitogen activated protein
MFR	Macrophage fusion receptor
Min	Minute
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
N/A	Not available
N/R	Not recorded

NRS	Normal rabbit serum
OA	Osteoarthritis
Pa	Pascal
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buphered saline
PCM	Pericellular matrix
PG	Proteoglycan
PGE2	Prostaglandin E2
PKC	Protein kinase C
PLC	Phospholipase C
PLL	Poly L-lysine
pp125FAK	Focal adhesion kinase
PMN	Polymorphonuclear
PTX	Pertussiss toxin
PVDF	Polyvinylidene fluoride
RACK	Receptor for activated c kinase
RBC	Red blood cells
RGD	Arginine-glycine-aspartic acid
RPM (rpm)	revolution per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SAC	Stretch-activated ion channel
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate

Sec	Second
SEM	Standard error of the mean
SH	Src homology
SHP	Src homology 2 domain-containing protein tyrosine phosphatase
SHPS-1	SHP substrate
SIRP	Signal regulatory protein
Src	Sarcoma oncogen
SV40	Simian virus 40
Syk	Spleen tyrosine kinase
TBS	Tris buffered saline
TBST	Tris buffered saline +Tween 20
TEMED	N'N'N'N-tetramethyl ethylene diamine
TIMP	Tisse inhibitor of metalloproteinases
TM4SF	Transmembrane-4 superfamily
TNF	Tumor necrosis factor
TSP	Thrombospondin
uPAR	Urokinase plasminogen activator receptor
VCAM	Vascular cell adhesion molecule
Vn	Vitronectin
VSMC	Vascular smooth muscle cells

PUBLICATIONS ASSOCIATED WITH THE WORK IN THIS THESIS

I. Original articles

1. Orazizadeh M, Millward-Sadler J., Wright M.O and Salter DM.
Expression and function of IAP/CD47 in human articular chondrocyte mechanotransduction *(in process).*

2. Orazizadeh M, Millward-Sadler J., Wright M.O and Salter DM
CD98 and its ligands, galectin-3 and CD147, are expressed by human articular chondrocytes but are not involved in the response to mechanical stimulation (*in process*)

II. Abstract

Orazizadeh M, Millward-Sadler J., Wright M.O and Salter DM.

Integrin-associated protein (CD47) is expressed by human articular chondrocytes and regulates cell responses to mechanical stimulation. *Osteoarhtritis and Cartilage, vol. 11 (Suppl. A), 2003, S4, M8*

ABSTRACT

Mechanical stimulation regulates chondrocyte function. In vitro studies show that 0.33 Hz mechanical stimulation of normal articular chondrocytes results in activation of an α5β1 integrin dependent mechanotransduction pathway involving stretch-activated ion channels, actin cytoskeleton, PKC, and tyrosine phosphorylation of focal adhesion complex molecules. Subsequently, there is secretion of IL-4, membrane hyperpolarisation and altered levels of aggrecan and MMP-3 mRNA. In contrast, OA chondrocytes show a membrane depolarisation and no change in aggrecan or MMP3 mRNA levels.

Both normal and OA human articular cartilage use α5β1 integrin as a mechanoreceptor, however, the molecular mechanism of integrin-mediated mechanotransduction events remains obscure. This thesis focuses on the potential roles of integrin-associated molecules, CD47/IAP, CD98 and their ligands, galectin-3, signal regulatory protein α (SIRPα) and thrombospondin (TSP1) in α5β1 integrin dependent chondrocyte mechanotransduction. CD47, CD98 and galectin 3 are shown to be expressed by chondrocytes in both normal and OA cartilage. Using the changes in membrane potential as an indicator of chondrocyte response to mechanical stimulation initial investigations showed CD98 had no identifiable role in chondrocyte mechanotransduction, whereas CD47 and its ligands, TSP1 and SIRPα, showed critical functions. Antibodies to CD47, TSP1 and SIRPα completely inhibited the electrophysiological response of normal and OA chondrocytes to 0.33 Hz cyclical mechanical stimulation.

Further examination of potential roles for CD47 showed that both normal and OA chondrocytes expressed CD47 and its ligands in a similar pattern. In resting chondrocytes, CD47 was coimmunoprecipitated with α5 integrin and TSP1 but not with β1 integrin and SIRPα. Following 1minute mechanical stimulation, chondrocytes showed increased tyrosine phosphorylation of three major proteins of 125, 90 and 65 kD. This tyrosine phosphorylation was inhibited by anti-CD47. PKCα translocation from cytosolic compartment to particulate compartment following 30 seconds mechanical stimulation, in the presence of anti-CD47 was also completely inhibited.

Interactions between CD47, α5 integrin and TSP1 appears to be important in the regulation of the chondrocyte responses to mechanical stimulation, regulating down stream signaling events such as tyrosine phosphorylation and PKCα translocation.

CHAPTER ONE

INTRODUCTION

1.1.0. HUMAN ARTICULAR CARTILAGE (HAC)

Articular cartilage is a highly specialized and uniquely designed biomaterial that forms the smooth, gliding surface of the diarthrodial joints (Muir 1995). Adult articular cartilage is avascular, aneural, and alymphatic, cell nutrition is derived primarily from the synovial fluid. Nutrition in immature cartilage is aided by the presence of vascular canals (Buckwalter and Mankin 1998).

Articular cartilage thickness normally ranges from 1 to 2 mm. This varies in relation to age and the particular joint. In large joints in young people, articular cartilage thickness may reach 5 to 7 mm (Poole et al 2001). The main functions of articular cartilage are related to the mechanical properties of the extra cellular matrix (ECM) (Guilak et al 1999). Articular cartilage is a load-bearing connective tissue, and its mechanical function involves not only transmitting the compressive joint loads to the underlying subchondral bone but also providing a low-friction interface between the contacting cartilage surfaces of the joint (Mankin et al 1994).

1.1.1. Composition of human articular cartilage

Articular cartilage is composed of chondrocytes and ECM. Articular cartilage is synthesized by the sparsely distributed (approximately 2% of the total cartilage) chondrocytes (Muir 1995). The greatest proportion of the ECM is water (65 to 80%). The remainder of the ECM is mostly composed of collagen (10 to 30%) (Kuettner et al 1991), and proteoglycans (PGs) (up to 10%) (Poole 1997). Type II collagen constitutes the major structure component of articular cartilage. Aggrecan is the principal form of proteoglycan molecule found in articular cartilage. The concentration of PGs is inversely proportional to collagen concentration.

1.1.2. Chondrocytes

Chondrocytes are differentiated from mesenchymal cells during embryonic development and represent the only cell type in hyaline cartilage. Individual chondrocytes are located in their lacunae within the matrix, each lacuna containing a single chondrocyte (Sandell and Aigner 2001). Chondrocytes have the full complement of subcellular components. Within the cell, the small nucleus, with dispersed chromatin and basophilic, surrounded by a nuclear envelope, is situated eccentrically inside the cytoplasm. Golgi apparatus, mitochondria, lysosomes, smooth and well-developed rough endoplasmic reticulum are contained within the cytoplasm. These characteristics reflect the active role of chondrocytes in synthesis and maintenance of the cartilage matrix. The phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific ECM molecules, including type II collagen and PGs such as aggrecan (Häuselmann et al 1994).

Chondrocytes in normal adult cartilage maintain a balance between synthesis and degradation of ECM components. These cells are responsible not only for the generation of ECM during growth and development, but also for the maintenance of tissue homeostasis during adult life. The chondrocyte in mature articular cartilage exhibits virtually no mitotic activity and a very low rate of matrix synthesis and degradation (Hedbom and Häuselmann 2002).

Chondrocytes alter their metabolic activity in response to local demands. Chemical signals and mechanical pressure can both result in an increase in production of ECM by chondrocytes (Guilak et al 1995).

1.1.3. Extracellular matrix (ECM) of human articular cartilage

Articular cartilage has unique biomechanical properties, both in terms of stiffness and elasticity (Kerin et al 2002). Articular cartilage matrix consists of two basic components: collagen network and PGs. PGs are composed of the large aggregating PG aggrecan, small PGs, and non-collagenous matrix proteins (**Table 1.1**). In terms of the physical properties of cartilage matrix, tensile strength comes from collagen network (Kempson et al 1973), which hinders expansion of the viscoelastic aggrecan component, providing compressive stiffness (Kempson et al 1970, 1976; Maroudas 1976).

1.1.3.1. ECM Collagens

The endoskeleton of hyaline cartilage composed of collagen fibrils that form an extensive network. These fibrils vary in diameter, from approximately 20 nm in the superficial zone to 70 to 120 nm in the deep zone. The fibrillar network consists mainly of type II collagen, together with other collagens, predominantly types IX, and XI. Several other types of collagen common to other kinds of cell are also present in cartilage (**Table 1.1**), but their function in cartilage is not certain (Bruckner and Rest 1994; Muir 1995; Poole et al 2001). Although collagen types VI, IX, XI, XII and XIV are minor components, they appear to have important structural and functional properties (Goldring 2000).

Type II collagen forms the bulk (approximately 90%) of the collagen fibrils that form an extensive network throughout the matrix. This molecule is composed of three identical chains that form a triple helix form (Poole et al 2001). Type VI collagen forms a highly branched filamentous network (Keene et al 1988). It has only been found in the pericellular matrix (PCM), directly surrounding the chondrocyte (Poole et al 1988,1992).

Type IX collagen is present in the fibril. Its distribution may be limited in the adult to pericellular sites and represents approximately 2% of the total collagen (Poole et al 2001). This collagen type binds covalently to the surface of collagen type II fibres and prevents the addition of more collagen type II molecules. Since collagen type IX allows a

bridging of collagen type II fibres it enhances the mechanical stability of the network (Kuettner 1992; Ostergaard and Salter 1998).

Type XI collagen is present within and on the surface of the fibril. This collagen appears to be located within the interior of collagen type II fibres and may be involved in determining the final diameters of collagen type II fibres (Kuettner 1992; Ostergaard and Salter 1998).

1.1.3.2. ECM proteoglycans

Articular cartilage matrix also contains a large number of other components that are important for matrix cohesion, chondrocyte functions, assembly and repair of cartilage. Articular cartilage proteoglycans are composed of:

1. Large aggregating PG aggrecan and the small non-aggregating PGs (leucin-rich core PGs)
2. The non-collagenous glycoproteins (GPs)

1. The primary PG of articular cartilage matrix, aggrecan, is a very large molecule of high buoyant density that aggregates with hyaluronic acid (HA) and is known as aggrecan. Aggrecan gives the tissue the ability to resist mechanical compression. The core protein of aggrecan is made up of three globular (G) domains. Both G1 and G2, at amino-terminal end of the molecule, are separated by an interglobular domain. G3 domain at the carboxy-terminal end, separated from G2 domain by glycosaminoglycan (GAG), the keratan sulfate and chondroitin sulfate, attachment regions (Ilic et al 1998). The GAG chains, which consist of numerous carboxy and sulfated groups, become negatively charged when dissolved in the interstitial fluid. The presence of these charges gives rise to repulsive forces and osmotic gradients so that a swelling pressure exists within the tissue. This swelling pressure will influence the hydration state of the tissue, as well as the mechanical response to impressive loading or deformation (Guilak et al 1999). Link protein stabilises a noncovalent interaction between aggrecan and HA

through the G1 globular domain of the core protein to form a large macromolecular aggregate (Heinegard and Hascall 1974; Poole et al 1980).

The small non-aggregating PG (leucin-rich core PG) decorin (Yamaguchi et al 1990), biglycan (Scott 1996), versican (Grover and Roughley 1993), fibromodulin (Hedbom and Heinegard 1993), perlecan (Sundarhaj et al 1995), and lumican (Grover et al 1995), have also been detected in articular cartilage matrix (**Table 1.1**).

Decorin (Heinegard and Oldberg 1989) is involved in regulation of important biological functions like matrix organization, cell adhesion, migration and proliferation (Iozzo et al 1999). Decorin is found in the interterritorial matrix (Miosge et al 1994). Biglycan is localized mainly pericellularly and may have important functions in modulating morphogenesis and differentiation (Bock et al 2001). Versican, with 263 kD size, contains G1 and G3 domains (as in aggrecan) that binds to HA through G1 domain (Grover and Roughley 1993). Fibromodulin (Hedbom and Heinegard 1993) might be involved in collagen organization and also in growth factor binding. Perlecan has a core protein of ~260 kD and contains both heparan sulfate and chondroitin sulfate side-chains. Lumican, like fibromodulin, contains keratan sulfate chains and modulates collagen fibril formation.

2. The non-collagenous GPs, include fibronectin (Fn) (Galant et al 1985), chondrocalcin (Poole et al 1984), tenascin (Salter 1993), thrombospondin (TSP)(Miller and McDevitt 1988), cartilage oligomeric matrix protein (COMP) (Hedbom et al 1992), and anchorin C II (Pilar Fernandez et al 1988) (**Table 1.1**).

Fibronectin (Fn) is a GP of several isoforms that is expressed in the PCM of normal articular cartilage (Potts and Campbell 1996). The biosynthesis and accumulation of Fn by normal articular cartilage has also been confirmed by extraction studies (Brown and Jones 1990). Chondrocalcin has an affinity for hydroxyapatite and may be involved with calcification of cartilage matrix (Poole et al 1984; Hinek et al 1987). Tenascin is a large ECM glycoprotein with a hexameric structure, known to have important functions in

processes such as wound repair and embryogenesis during bone and cartilage formation. Immunohistochemical studies have localised tenascin to chondrocytes and ECM of the superficial zone and upper part of the middle zone in normal articular cartilage (Salter 1993). TSP is an ECM protein that displays a complex variety of biological activities (see 1.5.2.6.1 page 63).

COMP belongs to the TSP protein family (Oldberg et al 1992) with a molecular size of ~120 kD and is localized preferentially to the deep zone of articular cartilage (Heinegard et al 1998). This noncollagenous protein, is implicated in the development and proper function of cartilage tissue (Recklies et al 1998). Anchorin CII (cartilage annexin V) contains four internal repeats of 70-80 amino acids (AA), each with shorter, highly conserved 17- AA sequences. Anchorin CII is synthesized and secreted by chondrocytes and binds to the outer surface of cell membrane (Pfaffle et al 1990). A major function of anchorin CII appears to be to bind specially to collagen II fibres, possibly attaching them to the chondrocyte surface. Anchorin CII has the potential for providing a mechanoreceptor to the cells, transmitting signals to chondrocytes (Fernandez et al 1988; Heinegard and Oldenberg 1989; Poole et al 2001).

1.1.3.3. Cell surface proteins

In addition, the presence of some important cell membrane proteins in ECM has been reported. These proteins include syndecan, CD44 and a variety of integrins (α1, 2, 3, 5, 10; β1, 3, 5).

Syndecan is a member of a family of heparan sulfate proteoglycans that are associated with the cell surface. These macromolecules contain a hydrophobic membrane-spanning domain, a short cytosolic domain, and an extracellular domain. Syndecans are known to interact with several matrix molecules, and thus are thought to serve as structural and functional links between the cell surface and the surrounding extracellular matrix. These interactions are required for biological activity, because these factors must first interact with the heparan sulfate chains of the syndecans before they can interact with their high-

affinity signaling receptors. Syndecans seem to play important roles in modulating cellular activities, including cell proliferation and differentiation in a variety of cells including chondrocytes (Bernfield et al 1992; Pfander et al 2001).

CD44 is a single-pass transmembrane receptor expressed by many cell types including chondrocytes (Knudson et al 1996; Chow et al 1995). In chondrocytes, CD44 represents the primary receptor responsible for hyaluronan (HA) binding (Knudson and Knudson 1993; Knudson et al 1996). The binding of HA to CD44 in cartilage facilitates the retention of large, highly hydrated, HA/PG/link protein aggregates at the surface of chondrocytes (Knudson and Loeser 2002).

Table 1.1. Cartilage matrix proteins (Goldring 2000)

Collagens
Types II, VI, IX, XI, XII
Proteoglycans
Aggrecan
Biglycan
Decorin
Fibromodulin
Perlecan
Lumican
Noncollagenous proteins
COMP
Tenascin
Cartilage matrix protein (matrilin)
Anchorin CII
Thrombospondin (TSP)
Chondrocalcin
Fibronectin (Fn)
Membrane proteins*
Syndecan
CD44
Integrins (α1, 2, 3, 5, 10; β1, 3, 5)

-**COMP**, cartilage oligomeric matrix protein

* Some membrane proteins identified in ECM.

1.1.4. Human articular cartilage (HAC) Heterogeneity

Articular cartilage is a remarkably heterogeneous tissue in which the composition of ECM (Bayliss et al 1983; Burkhardt et al 1995), the numbers and morphology of chondrocytes (Mitrovic et al 1983), the thickness of cartilage (Armstrong and Gardner 1977), and the susceptibility to cytokine-induced damage (Häuselmann et al 1996) vary. These are greatly influenced by factors such as zone of depth within section of cartilage, joint type, topography within a joint, as well as age and presence of OA (Ostergaard and Salter 1998).

1.1.4.1. Zonal heterogeneity

The shape, size, and arrangement of the chondrocytes vary according to their location within the articular cartilage. It is established that chondrocytes behave differently, depending on their position within the different zones of the cartilage matrix (Poole 1984; Aydelotte et al 1988; Aydelotte and Kuettner 1988). The matrix surrounding the chondrocytes of articular cartilage varies in its organization and each zone (region) has unique structural and biochemical characteristics (Eggli et al 1988; Zanetti et al 1985; Poole et al 2001).

Articular cartilage can be divided into four separate zones: surface, superficial, middle and deep. The surface, which is bathed by synovial fluid, is composed of about three layers thick chondrocytes that are flattened and aligned parallel to the surface.

The superficial chondrocytes are surrounded by a very polarized organization of thin collagen fibrils that generally run parallel to each other and to the articular surface, providing the highest tensile properties found in articular cartilage (Akizuki et al 1986; Kempson et al 1973). Superficial specialized chondrocytes synthesize a molecule called superficial zone protein (Schumacher et al 1999) that is also known as lubrican, providing almost frictionless articulation provided by articular cartilage (Poole et al 2001).

In the middle zone, chondrocytes appear larger and more round, with an apparently random distribution within the matrix. Extensive ECM rich in the proteoglycan aggrecan and large diameter of collagen fibrils that arranged randomly are other characteristics of the midzone.

Within the deeper zones, the cells form in columns, lying perpendicular to the cartilage surface, as do the collagen fibres. Cell density is at its lowest but aggrecan content and fibril diameter are maximal, although collagen content is minimal (Venn 1979).

Several distinct regions in mid and deep zones, pericellular matrix (PCM), territorial, and interterritorial matrices have been recognized (Poole et al 1984). All chondrocytes are surrounded by a narrow (approximately 2μm wide) pericellular region in which few collagen fibrils are detected. At the ultrastructural level PCM is more amorphous in appearance. Here, numerous molecules are concentrated including type VI collagen (Keene et al 1988) and the proteoglycans decorin (Poole et al 1986) and aggrecan (Poole et al 1980).

A territorial region surrounds this pericellular region, which is present throughout the articular cartilage (Poole et al 1982). In the deep zone, there is a clearly identifiable third region of structure, distinguishable by the ultrastructure of aggregates of proteoglycan (Poole et al 1982). This region is called the interterritorial region. It is the part of the matrix most remote from the chondrocytes. The bulk of the tissue is made up of the interterritorial matrix, which consists primarily of water and electrolytes including Na^+, Cl^-, and Ca^{2+}. The remaining portion is composed of collagen, mainly type II, and the proteoglycan aggrecan, with smaller amounts of other proteins and glycoproteins (Guilak et al 1999). The basophilic ' tidemark ' marks the boundary between the deep zone and the underlying layer of calcified cartilage, which abuts on the bone tissue.

1.1.4.2. Functional heterogeneity in chondrocytes

Since joints experience a wide range of loading conditions, the biomechanical properties of cartilages vary between joints (Froimson et al 1997; Treppo et al 2000; Shepherd and Seedhom 1999) and with position and depth (Athanasiou et al 1995) on the same normal joint surface. It has been shown that chondrocytes from various joints show different response to anabolic and catabolic cartilage mediators (Kang et al 1998; Eger et al 2002; Dang et al 2003).

Eger et al (2002) have shown that PG synthesis by ankle chondrocytes was higher than by the knee chondrocytes. IL-1β was approximately eight times more effective with knee than ankle chondrocytes in reducing PG synthesis. There are also significant differences between knee and ankle chondrocyte response to proteoglycan loss in the presence of a second catabolic mediator, fibronectin-fragment (Fn-fs). It has been previously shown that Fn-fs inhibited PG synthesis in both bovine and human cartilage (Xie et al 1993). The 29 kD Fn-fs causes a greater degree of PG loss (30-50%) from knee cartilage than from ankle cartilage in explant culture. This loss occurs within 7 days in the knee, while PG loss is less evident and less consistent in the ankle even after 28 days of culture (Kang et al 1998). Other reports have shown that primary cultured ankle chondrocytes within alginate beads have a greater ability than knee chondrocytes to synthesize matrix components (Häuselmann et al 1996).

1.2.0. OSTEOARTHRITIS (OA)

Osteoarthritis is a slowly developing joint disease that effects approximately 15% of the world's adult population with pain and disability (Cole and Kuettner 2002). Studies have shown that approximately 6% of the adult population is affected by symptomatic knee OA. In those over the age of 65, this percentage increases to almost 10% (Felson et al 1987).

OA is not a single disease entity, but represents a disease group with rather different underlying pathophysiological mechanisms (Aigner and Mckenna 2002). OA is a result of both mechanical and biologic events that uncouple the normal balance between degradation and synthesis by articular cartilage chondrocytes and ECM (Mollenhauer and Erdman 2002).

OA can be divided into two main classes, primary and secondary OA (Gardner 1992). Primary or idiopathic OA can be further subdivided into localised and generalised forms. These forms of primary OA are characterised by not having known causes, even though the generalised form does carry a heritable predisposition. In contrast, secondary OA is characterised by having a demonstrable cause such as injury, mechanical dysfunction, immunoinflammatory disease, infection, nutritional or metabolic disorder, endocrine disorder or other insult. The distinction between primary and secondary may, however, not always be easy (Ostergaard and Salter 1998).

1.2.1. Cartilage changes in OA

In contrast to normal articular cartilage, which is smooth, with a homogenous shiny surface appearance, OA cartilage is often yellowish or brownish and is typically soft. The surface shows roughening in the early stages and overt fibrillation and matrix loss in the later stages until the eburnated subchondral bone plate is visible (Aigner and Mckenna 2002). A replacement of the normal cartilage by an appearance similar to that of cut velvet is called fibrillation (Byers et al 1970).

A macroscopic pathoanatomical grading system was developed for the cartilage and bone changes of OA joints by Collins (1949). This system consists of 5 grades: Grade 0, normal, smooth articular cartilage; grade I, superficial flaking and surface fibrillation of cartilage in area of pressure and movement; grade II, more extensive destruction of cartilage excluding denuding of bone; grade III, denudation of bone in one or more pressure areas and obvious marginal osteophytes; and grade IV, denudation from large area with eburnation of bone and prominent osteophytes (Collins 1949; Collins and McElligott 1960).

The macroscopic pathoanatomical grading system (Collins and McElligott 1960) was later modified by Collins and Meachim (1961), and Mankin et al (1971). The macroscopic grading system (Collins 1949; Collins and McElligott 1960), which has been used for pathological grading in the present study, is described in chapter 2 (see **Table 2.1**, page 87).

1.2.2. Chondrocyte changes in OA

Chondrocyte changes during the OA disease can be basically summarized in two categories. First, the chondrocytes can undergo cell death, or they can proliferate to compensate for cell loss. Second, chondrocytes activate or deactivate their synthetic-anabolic activity (metabolic activation and hypoanabolism).

1.2.2.1 Cell proliferation and programmed cell death (apoptosis)

Lacuna emptying and cell cloning are typical histological features of OA cartilage (Stockwell 1971). In contrast to normal articular cartilage, which has essentially no proliferation, several studies (Mankin et al 1971; Rothwell and Bentley 1973) have shown that there is a very low proliferatrive activity in OA chondrocytes. The increased proliferative activity of chondrocytes, which is found primarily in the upper cartilage zones, might be due to better access of chondrocytes in these zones to proliferative factors from the synovial fluid due to fissuring or loosening of the collagen network

(Meachim and Collins 1962). Alternatively, damage to the collagen matrix integrity, which is particularly impaired in the upper zones of OA cartilage (Hollander et al 1995), might contribute to the proliferative activity of OA chondrocytes. Chondrocyte proliferation resulting in a higher cellular content could represent a cellular reaction to cartilage destruction, but this is unlikely to represent efficient tissue repair (Aigner and McKenna 2002), as the clusters in the very upper zone do not appear to add significantly to matrix anabolism (Aigner et al 1997). Based on the observation of significant numbers of empty lacunae at the light microscope level and cellular debris-like material at the ultrastructural level in OA cartilage, some authors have suggested that cell death is a central feature in OA cartilage degeneration (Vignon et al 1976; Aigner and McKenna 2002). Aigner et al (1997) have demonstrated that apoptosis occurs in OA cartilage, but at a very low rate. Major cell death would easily lead to a failure in cartilage matrix turnover, because chondrocytes are the only source of matrix components synthesis in articular cartilage (Aigner and Mckenna 2002).

1.2.2.2. Metabolic activation and hypoanabolism

Chondrocytes attempt to repair the damaged matrix by increasing their anabolic activity (Ryu et al 1984; Sandy et al 1984). Despite the increased biosynthetic activity of chondrocytes, a net loss of PG content is one of the hallmarks of all stages of OA cartilage degradation (Collins 1960; Mankin 1972, 1981; Aigner et al 1992). This leads to the assumption that overall enzymatic degradation of matrix components might be the reason for the metabolic imbalance in OA cartilage (Aigner et al 1997). Aigner et al (1997) have demonstrated that hyperactivity of matrix synthesis is restricted to the chondrocytes of the middle and deeper zones of OA cartilage, where the ECM is histochemically still intact and no major loss of proteoglycan is detectable.

OA chondrocytes upregulated the cell adhesion molecules such as CD44 in deep zone (Ostergaard et al 1997b) and β1 expression (Loeser et al 1995). Aberrant expression of α2, α4, β2 integrins, which are not expressed in normal cartilage, is also documented in OA cartilage (Ostergaard et al 1998).

1.2.3. ECM changes in OA

The earliest biochemical change in human OA is an increase in water content due to the altered network of collagen fibers (Bollet 1967). In OA cartilage, a number of biochemical studies have demonstrated enhanced synthesis of ECM components such as fibronectin (Miller et al 1984), type VI collagen (Swoboda et al 1998) and tenascin (Salter 1993).

Degradation processes appear to be specifically prominent in surface zone and around the chondrocytes in osteoarthritic cartilage (Dodge and Poole 1989; Bank et al 1997). Matrix metalloproteinases (MMPs) that degrade articular cartilage matrix molecules have a crucial role in causing progressive joint degeneration. Enhanced levels of many MMPs including MMP-3 (stromelysin) (Goldring 1999, 2000), MMP-7 (matrilysin) (Ohta et al 1998), membrane-type I (MT1)-MMP (Imai 1997), MMP-8 (collagenase-2) (Shlopov et al 1997; Chubinskaya et al 1996), MMP-13 (collagenase-3) (Moldovan et al 1997) and aggrecanase (Lohmander et al 1993) have been reported to accompany the increased matrix degradation in OA cartilage. Active stromelysin (MMP-3) serves as an activator of latent collagenases. The collagenases implicated in type II collagen degradation include collagenase 1,2, and 3(MMPs 1,8, and 13, respectively) and membrane type 1 (MT1)-MMP (MMP-14) (Goldring 2000). MMP-13 (collagenase-3) is a very potent degrader of type II collagen (Reboul et al 1996). The expression of MMP-13 in the deep zone of OA cartilage and its ability to more effectively degrade type II collagen suggest a major role for this enzyme in cartilage degradation (Mitchell et al 1996). While MMP-3, MMP-8, and MT1-MMP all have the capacity to degrade aggrecan (Lark et al 1997; Buttner et al 1998), there is evidence that degradation at the aggrecanase cleavage site is primary event in chondrocyte-mediated catabolism of aggrecan (Lark et al 1997).

Protein and mRNA for ADAM-10 (A Disintegrin-like And Metalloproteinase-like domain) (Chubinskaya et al 1998), and ADAM-15 (Böhm et al 1999) were found in the most fibrillated areas of OA cartilage, especially in the clusters. The levels of the tissue inhibitor of metalloproteinases (TIMP)-1 are also elevated in OA synovial fluid and

correlated with the MMP levels, possibly reflecting chondrocyte attempts to balance excessive proteinase activities (Ishiguro et al 1999; Goldring 2000; Hedbom and Häuselmann 2002).

Catabolic cytokines such as IL-1 and tumour necrosis factor-α (TNF-α) (Lotz et al 1994) are upregulated by OA chondrocytes (Webb et al 1997; Goldring 2000).

Beside changes in the quantitative amounts and in the degradation of cartilage components, alterations of cartilage components or the expression of molecules, which are not present in normal articular cartilage, also appear to be important phenomena. Studies have reported the appearance of molecules in OA cartilage such as collagens type I (Nimni and Deshmukh 1973), IIA (Aigner et al 1999), and III (Aigner et al 1993) which are barely seen or are undetectable in normal articular cartilage.

1.3.0. MECHANOTRANSDUCTION

1.3.1. Mechanical forces and chondrocytes

Physical forces play an important role in modulating cell functions. Mechanotransduction, the process by which cells transduce physical force-induced signals into biochemical responses, is critical for mediating adaptations to mechanical loading (Ko and McKulloch 2001). Chondrocytes in articular cartilage utilize mechanical signals in conjunction with other environmental and genetic factors to regulate their metabolic activity. This capability provides a means by which articular cartilage can alter its structure and composition to meet the physical demands of the body. A number of different approaches have been used to investigate the role of physical stimuli in regulating cartilage and chondrocyte activity, ranging from in vivo studies to in vitro experiments at the cellular and molecular level (Guilak et al 1999). In this regard two things should be noted:

First, physical forces applied to cells should be quantified in specific physical terms such as stress, strain, shear, tension and compression (Liu et al 1999). Second, the physical

response of tissue and/or cell (cartilage, chondrocyte) to loading is complex and involves many factors including tissue and cell deformation, change in hydrostatic pressure, and fluid flow (Hall et al 1991). The relatively small size of an individual cell and the complexity of cellular morphology are such that, in most circumstances, the forces applied to individual cells can not be precisely classified (Liu et al 1999).

1.3.2. Terminology used in mechanical stimulation

Strain is a measure of deformation per unit length of a body. It is a ratio of a deformation and an initial length.

Tensile strain is an increase in length per unit length of material along a given line, while **compressive strain** is the decrease in length per unit length.

Shear strain is the change in angle in the deformed state between two lines that were vertical in the undeformed state (Armstrong et al 1992; Woo et al 1992).

1.3.3. Mechanical loading of articular cartilage

Human articular cartilage experiences wide ranges of force during normal joint loading. Loading of articular cartilage will generate a combination of tensile, compressive and shear stress in the material (Kempson et al 1973; Akizuki et al 1986).

With daily activity, cyclic forces imposed on diarthrodial joints create complex, time-varying stress and strain fields throughout articular cartilage. High contact pressures in the joint cause fluid to be exuded and imbibed cyclically (Mow et al 1984). Most fluid exchange occurs at the superficial layers where flattened chondrocytes predominate due to confined boundary conditions of cartilage and limited permeability (Setton et al 1993). Below the superficial layers, there is less fluid exchange, and the cells in the middle and deep layers of cartilage exhibit a rounded shape that is maintained in the high compressive hydrostatic stresses created in these areas. In some regions (under and near the margins of the joint contact area and near the layer of calcified cartilage [Wong and

Carter 1990a,b]), normal activity creates significant distortional stresses. In these locations, a potential exists for physical damage to the matrix. Distortional stresses are associated with distortional strains that may cause chondrocytes to deform into a more ellipsoidal shape. This distortion, which can occur without fluid exchange, potentially could alter cartilage homeostasis (Giori et al 1993).

The joint forces are transmitted across the joint by the generation of pressure over the contact area of the two opposing cartilage surfaces. In common daily activities (like walking and running) the joint contact area sweeps quickly over some region of the articular surfaces. The magnitudes of the contact pressure at any one location may typically go from zero to a large value and then back to zero in some fraction of a second (Wong and Carter 2003). The joint pressures imposed by these intermittent loads introduce very high cyclic hydrostatic pressure in the bound and unbound intercellular fluid phase of cartilage, known as the interstitial fluid. The loading of the interstitial fluid component of cartilage can ultimately be credited both for providing the excellent lubrication between the two cartilage layers and also for distributing and transmitting the joint forces to the underlying bone tissues in an efficient manner (Ateshian and Wang 1995).

In animal models, immobilization of a limb (Akeson et al 1987; Behrens et al 1989; Langeskiold et al 1979; Smith et al 1992), abnormally high or unusual joint forces (Radin et al 1991), and ligamentous instability (Brandt et al 1990) initiate degradation of cartilage and impair joint function. Preliminary studies have shown that immobilization of one forelimb of canine knees (Palmoski et al 1980) can result in a massive reduction in the rate of cartilage proteoglycan synthesis. Similar observations have been reported in immobilized joints of rabbits (Eronen et al 1978; Akeson et al 1973).

1.3.4. In vitro methods for applying mechanical stimulation to human articular cartilage

Because of the complexity of the in vivo environment, study of cellular responses to mechanical stimulation has relied heavily on the development and use of in vitro systems designed to mimic the in vivo situation. A major goal is establishing in vitro systems reflecting the in vivo differentiation patterns of OA chondrocytes in order to further analyze the disease process and to develop experimental systems to delay, stop, or even reverse it (Aigner and McKenna 2002).

1.3.4.1. Cell and tissue culture methods

There are three main classes of in vitro model systems commonly used in the investigation of mechanical loading of cartilage.

1.Chondrocyte culture of primary cells and cell lines in monolayer (see chapter 2 page 94). Studies relating biological and physical stimuli at the cellular level to the load-induced biological response of chondrocytes, such as altered secretion of matrix macromolecules, could provide key information in understanding cartilage remodelling processes. The frequency, amplitude, and spatially dependent response found in previous studies could be the result of regulatory mechanisms including cell deformation, cell-matrix interactions near the cell membrane, connective transport of soluble mediators, and load-induced electrical fields (Buschmann et al 1999). Phenotypic changes are a central feature of chondrocytes in vivo and in vitro, particularly in monolayer culture (Aigner and Mckenna 2002). In cell culture, artifactual conditions can arise due to flattened cell morphology and the absence of a pericellular matrix (Wong and Carter 2003). A major disadvantage of this approach is that the cells, which are attached to the plastic of the culture dish, usually remain phenotypically stable for only a few weeks (Thonar et al 1986).

2. Three-dimensional (3D) culture: To overcome the difficulties of monolayer culture, many researchers have seeded chondrocytes in three-dimensional (3D) constructs (Lee and Bader 1997; Wong and Carter 2003). In 3D cultures the cells maintain their morphology and phenotype, and will rebuild their matrix with many characteristics of the in vivo cells (Häuselmann et al 1996; Aigner and Mckenna 2002). A unique 3D cell stretching device, originally designed by Skinner et al (1992), was further developed into a computerized Bio-Stretch System by Liu et al (1999).

I. Agarose: To overcome the problems of primary cell and explant culture (see below), different groups have used the 3D model system involving chondrocytes embedded in agarose (Buschmann et al 1995; Lee and Bader 1997; Chowdhury et al 2001). The entrapment of chondrocytes within agarose (Benya et al 1978; Bruckner et al 1989) or on agarose (Archer et al 1990) has been applied in a number of studies. Most of the PGs are retained in the vicinity of the cells and accumulates with time, a matrix that appears to share many of the physicochemical properties of normal cartilage matrix (Buschmann et al 1992). The maintenance of chondrocyte phenotype during long-term culture in agarose gels allowed the development of a mechanically functional cartilage-like matrix (Buschmann et al 1992).

II. Alginate beads: Several studies have shown that chondrocytes also can be entrapped in alginate, a linear polysaccharide (mannuronic acid-guluronic acid)$_n$ which forms a gel in the presence of calcium or other divalent cations (Kupchik et al 1983; Guo et al 1989; Häuselmann et al 1992). The alginate-based culture system appears to offer all of the advantages of the agarose system (Häuselmann et al 1992). However, as the gel can be solubilized by the addition of a chelating agent, it is relatively easy to separate the cells surrounded by a tightly bound cell-associated matrix from the interterrtorial matrix components, which is further-removed from the cells (Häuselmann et al 1992, 1994). In this method the chondrocytes are enzymatically released from their matrix and then resuspended in alginate without allowing the cells to dedifferentiate, in order to increase

cell number. In the alginate beads with 40,000 chondrocytes/ bead, the cells maintain their chondrocyte phenotype (Häuselmann et al 1996).

3. Explant cultures (exvivo): As articular carilage explants can be maintained in steady state for several weeks (Handley et al 1986), this type of culture system has been used not only to examine how newly synthesized radiolabeled molecules are incorporated into the existing cartilage matrix but also how they are turned over (Campbell et al 1984). For example, the average half-life of PGs in explants of articular cartilage from different sources and ages varies from 10 to 25 days (Campbell et al 1984; Handley et al 1986), this average half-life is believed to be shorter than that in vivo (Maroudas and Urban 1980). Thus, this in vitro model system enables the chondrocytes to remain in their native ECM environment. In addition, well-defined and controlled loading conditions can be applied to explants and the resulting effects on cell-mediated synthesis and degradation of ECM can be quantified. Cartilage explants can be subjected to axial compression, resulting in fluid flow and accompanying flow-induced ECM changes, or placed under hydrostatic pressure or pure tissue shear, which can result in essentially no volume change or intra tissue fluid flow. Such mechanical stimuli can be applied to stimulate the ranges of matrix deformation, fluid flow, and pressure that are caused by joint loading. To elucidate the effects of load on biosynthesis and gene expression by chondrocytes, explant culture is frequently used (Palmoski and Brandt 1984; Sah et al 1989, 1994; Kerin et al 2002). A disadvantage of the explant culture system is that the newly synthesized aggregating PG, aggrecan, molecules can be recovered in high yield only by using solvents, which cause the dissociation of the large molecular mass aggregates (Campbell et al 1984).

1.3.4.2. Types and effects of loading on articular cartilage in vitro

1.3.4.2.1. Effect of hydrostatic pressure (HP) on articular cartilage in vitro

The influence of hydrostatic pressure on chondrocyte and cartilage behaviour has been examined in several earlier studies (Veldhuijzen et al 1979,1987; vanKampen et al 1985; Hall et al 1991). Hydrostatic pressure is one of the several physicochemical factors, which operate in articular cartilage subjected to loading. Among these factors only hydrostatic pressure can be studied in a relatively pure form, independent of the others. Hydrostatic pressure induces no fluid flow or tissue deformation, which would bring about the other factors (Parkkinen et al 1993; Hall et al 1991).

A computer-controlled pressure chamber has been designed to subject both primary chondrocyte cell cultures and explants of bovine articular cartilage to cyclic hydrostatic pressure. The sealed petri dishes are placed within specially designed rack into the pressure chamber, located in the upper part of stainless steel cylinder. A hydraulic cylinder is under the pressure chamber. The oil flow generated by the hydraulic pump is conducted into the hydraulic cylinder through hydraulic valves (proportional pressure control and proportional regulator valve). The pressure of the oil is regulated by a proportional pressure valve. Both valves are driven by computer-controlled electronic amplifiers. A peristaltic pump is used to fill the chambers and to circulate the water through the pressure and reference chambers (Parkkinen et al 1993).

Parkkinen et al (1993) have demonstrated that cyclic hydrostatic pressure influences the PG synthesis rate in both culture systems. Both stimulatory and inhibitory responses were induced, depending on the culture conditions and testing regimens. Cyclic HP (5MPa) decreases proteoglycan synthesis in chondrocyte monolayers when applied at 0.0167, 0.05, 0.25, and 0.5 Hz but stimulates proteoglycan synthesis when applied to cartilage explant at 0.5 Hz. At 30 MPa, continuous but not cyclic HP alters chondrocyte Golgi apparatus and decreases PG synthesis.

Smith et al (1996) by using high density primary cultures of bovine chondrocytes, have investigated the effects of hydrostatic pressure (10MPa) on mRNA and extracellular matrix synthesis. The cells were exposed to hydrostatic pressure applied intermittently at 1 Hz or constantly for 4 hours in serum-free medium or in medium containing 1% fetal bovine serum. Signal levels of aggrecan, types I and II collagen, and β-actin mRNA were analyzed by northern blots and quantified by slot blots. PG synthesis was quantified by radiolabled sulfate uptake into cetylpiridinium chloride-precipitable glycosaminoglycans, and cell-associated aggrecan and type II collagen were detected by immunohistochemical techniques. In serum-free medium, intermittent HP increased aggrecan mRNA signal by 14% and constant pressure decreased type II collagen mRNA signal by 16% ($p< 0.05$). In the presence of 1% fetal bovine serum, intermittent pressure increased aggrecan and type II collagen mRNA signals by 31% ($p< 0.01$) and 36% ($p<0.001$), respectively, whereas constant HP had no effect on either mRNA. Intermittent and constant pressure stimulated GAGs synthesis 65% ($p<0.001$) and 32% ($p<0.05$), respectively. Immunohistochemical detection of cell-associated aggrecan and type II collagen was increased in response to both intermittent and constant pressure (Smith et al 1996, 2000).

1.3.4.2.2. Effects of compression on human articular cartilage in vitro

The chambers for applying static and dynamic compression on explant culture have been initially modified by Sah et al (1989). Loading is done within polysulfone compression chambers, housed in a standard incubator and designed to maintain compression rods in an orientation perpendicular to each chamber's base. The compression rods translated relatively large displacements into defined compressive forces that were transmitted to the cartilage disks, which were positioned between two flat-ended, impermeable loading platens fashioned out of polysulfone. By regulating the nature (waveform, frequency, and amplitude) of the external displacements, disks (chondrocyte/agarose (CA) or tissue blocks in explant) were subjected to either static or dynamic compressive loads (Sah et al 1989; Li et al 2001).

A number of groups (Palmoski and Brandt 1984; Sah et al 1989; Larsson et al 1991; Parkkinen et al 1992; Grodzinsky et al 2000) have studied the effects of both static and intermittent compression on full-thickness plugs of articular cartilage. Larsson et al (1991) applied both static and cyclical compression on full thickness explants of bovine articular cartilage at high frequency (2s of load with 2 s intervals of no load) and low frequency (60s of load with 60s intervals of no load), at 1 MPa. High frequency load had a stimulatory effect on protein and PG synthesis while low frequency and static load showed decreased synthesis.

The results from different groups were variable and depended on the specific loading protocol and specimen geometry. Different frequencies of cyclical compression produce differences in metabolic activity within the cells, although the results may vary for various cells.

To use the compression system for 3D cultures, further alterations were applied by Buschmann et al (1995). Several groups have utilised a system involving the culture of isolated chondrocytes embedded in agarose gel (Buschmann et al 1995; Lee and Bader 1997; Lee et al 1998a, Knight et al 1998). The application of static or dynamic compressive loading to the chondrocyte/agarose system results in cell deformation and frequency dependent alterations in cell metabolism.

The effects of static and dynamic loading over a range of frequencies (0.3-3 Hz), all within the physiological range, were investigated by Lee and Bader (1997). Three major markers of chondrocyte metabolism, namely PG synthesis, cell division, and protein synthesis, were assessed. Static and low-frequency strain (0.3 Hz) inhibited the synthesis of GAG, while a frequency of 1 Hz stimulated synthesis. Static compression reduced the level of cell proliferation, whereas dynamic compression at all frequencies induced an increase in chondrocyte proliferation (Lee and Bader 1997).

1.3.4.2.3. Effects of fluid-induced shear stress (FISS) on articular cartilage in vitro

A cone viscometer system (Bussolari et al 1982) has been used to apply fluid-induced shear on primary high density monolayer cultures of adult human and bovine chondrocytes. This system was chosen as the optimal method for applying a uniformly distributed shear stress to all the cells. It was a 145mm diameter cone with a cone angle of 0.5°, was rotated within the cell culture medium at 200 revolution per minute (rpm). The tip of the cone was fixed at 1 mm above the cells. The rate of rotation of the cone was maintained and monitored using an electronic motor controller, the acquisition of final speed was essentially instantaneous. This system applied a uniform level of shear across the plate with little or no turbulence. The level of fluid-induced shear applied to chondrocytes has been calculated to range from 1.6 Pa near the centre of the plate, 2.2 Pa at the edge of cone (Smith et al 1995).

Smith et al (1995) applied the fluid-induced shear for periods of 24, 48 and 72 hours. High density cultures of normal bovine, normal human and human OA chondrocytes were exposed to fluid-induced shear at 0.5 Hz using a cone viscometer. They have reported that chondrocytes subjected to shear stress showed alteration in chondrocytes morphology and orientation, two-fold increased GAG synthesis, large monomers of PGs, increased GAG synthesis, the chain length of GAG, 10-fold increased prostaglandin E2 (PGE2) secretion and 9-fold increased in TIMP-1 mRNA. In contrast, mRNA levels for the neutral metalloproteinases, collagenase, stromelysin, and 72 kD gelatinase, did not show such major changes. In another study, Smith et al (2000) have assessed the cellular response of normal and OA human and normal bovine articular chondrocytes to FISS at 1 Hz. They have reported that IL-6 mRNA expression occurs in chondrocytes from osteoarthritic cartilage but not in normal chondrocytes by using northern blot analysis. Applying FISS at 1 Hz to primary high-density cultures of chondrocytes increased IL-6 mRNA signal 4-fold at 1 hour and 10-15 fold at 48 hours compared with unsheared control cultures.

Studies focused on the mechanisms by which shear stress alters signalling pathways in chondrocytes (Das et al 1997; Smith et al 2000). It has been shown that shear stress increased nitric oxide (NO) production by chondrocytes, and the shear-induced change in matrix macromolecule metabolism was influenced by nitric oxide synthesis, G protein activation, and phospholipase C activation. They have also shown that in cultured OA chondrocytes, in the presence of shear stress the NO levels were increased 4-fold following 24-hour exposure period (Das et al 1997; Smith et al 2000).

1.3.4.2.4. Stretching or cyclic tensile force (CTF) by using Flexercell Strain Unit

A computerized, pressure-operated instrument, Flexercell Strain Unit, has been used to apply mechanical stretching on different cell types. The apparatus (Banes et al 1985; Fukuda et al 1997) consists of computer-controlled vacuum unit and a baseplate to hold the culture dishes. Frequency, stretch rate, and degree of elongation of the substrates are all controlled by computer. According to supplier's manual, there is a linear relation between the vacuum level (kPa) and maximal percentage elongation of cells. When a precise vacuum level is applied to the system, the culture plate bottom is deformed to a known percentage elongation, which is maximal at the edge and decreases to the centre. The strain is translated to cultured cells and causes the deformation of cells. When the vacuum is released, the bottoms of dishes return to their original conformation.

Holmvall et al (1995) by using the Flexer system subjected bovine chondrocytes to 18 kPa stress (15 cycles/min, 2 sec on/ 2 sec off, 24% elongation) for 3 h. Mechanical stimulation of these cells increased the mRNA levels of both collagen type II and aggrecan but not the β1 integrin. They have also applied the same regime on cultured chondrosarcoma cells (105KC) on collagen type-II coated and uncoated dishes (in the presence of serum). They found that the mean mRNA expression of α2-integrin subunit increased in collagen type II coated dishes and α5 increased to a significant level in uncoated dishes.

In another experience, cultured articular chondrocytes (Fukuda et al 1997) on flexible-bottomed culture dishes were exposed to mechanical stretching by the Flexercell technique. They reported that 2 kPa of cyclic mechanical stimulation (3 sec on/ 357 sec off) (0.003 Hz) increases PG synthesis, while 10 kPa of mechanical stimulation (3 sec on/ 3 sec off) (0.17 Hz) decreases PG synthesis.

Fujisawa et al (1999) applied two types of pressure (5 kPa and 15 kPa) and three kinds of frequency (high frequency, 30 cycles/min, 1 sec on/ 1 sec off; middle frequency, 1 cycle/2 min, 1 sec on/119 sec off; and low frequency, 1 cycle/4 min, 1 sec on and 239 sec off) to chondrocytes by using this unit. Five or 15 kPa of high frequency of cyclic tensile force induced the expression of interleukin-1 (IL-1), matrix metalloproteinase (MMP)-2 and –9 mRNA, and increased the production of pro- and active-MMP-9. The degradation of PG induced by high frequency of CTF. Moreover, reducing the frequency of CTF from high to low decreases the inhibition of PG synthesis (Guilak et al 1999).

The results obtained from different groups show variation between experiments. The cell density, force magnitude and frequency, the type of chondrocytes, are some of the variables that could affect the response of cultured cells.

Salter et al (Wright et al 1992, 1997; Millward-Sadler et al 1999, 2000; Lee et al 2000; Salter et al 2001, 2002) using a technique that allows the application of controlled mechanical stimulation induced stretch to cultured chondrocytes (see 2.5 and 2.7, pages 97 and 111), analyzed mechanotransduction pathways in human articular chondrocytes. In these experimental system, chondrocytes derived from normal adult articular cartilage from human knees show numerous reproducible electrophysiologic, molecular, and biochemical responses to 0.33 Hz cyclical mechanical stimulation. These responses include changes in membrane potential (Wright et al 1992, 1996, 1997), activation of stretch-activated ion channels (SAC) (Wright et al 1992, 1997), protein tyrosine phosphorylation of paxillin, pp125FAK and β–catenin (Wright et al 1997; Lee et al 2000), increased sulphate incorporation (Bavington et al 1996), IL-4 release (Millward-Sadler et al 1999), increases in aggrecan mRNA, decreases in MMP3 mRNA (Millward-

Sadler et al 2000) and substance P (SP) release (Millward-Sadler et al 2003). They have also shown that the mechanical-induced signalling pathway involves the actin cytoskeleton, phospholipase C (Salter et al 2001, 2002) and protein kinase C (Lee et al 2002).

1.3.4.3. Tensegrity: A model mechanism for mechanotransduction

Little is known about how cells convert the mechanical signals into an electrophysiological and/or biochemical response. How different mechanotransduction components (e.g, stretch-induced ion channels, signalling molecules, cytoskeleton (CSK), and integrins) within the context of the structural complexity of living cells play their roles is still elusive. Based on tensegrity model, the overview of mechano-transduction will be a process that cells can convert any form of force application to cellular and tissue adaptation by using a hard-wired cellular circuits. When each form of mechanical force is exerted on a cell, three different types of cell sensor will be activated including mechanosensitive ion channels, integrins and CSK and stretched-activated enzymes. Hard-wired circuits link the ECM and CSK to control a series of effector functions. These include reinforcement of cell-cell and cell-matrix connections, cell proliferation and differentiation, ECM remodelling and cell migration (**Figure 1.1**). Thus, any external mechanical force acting on the cells is balanced between tensile actin filaments (from CSK) and the ECM anchoring proteins in the focal adhesion plaques. The CSK may be viewed as mechanical filter, the same chemical or mechanical input will produce a different output (cellular response) depending on the geometry and mechanics of this structural framework (Ingber 1991,1997).

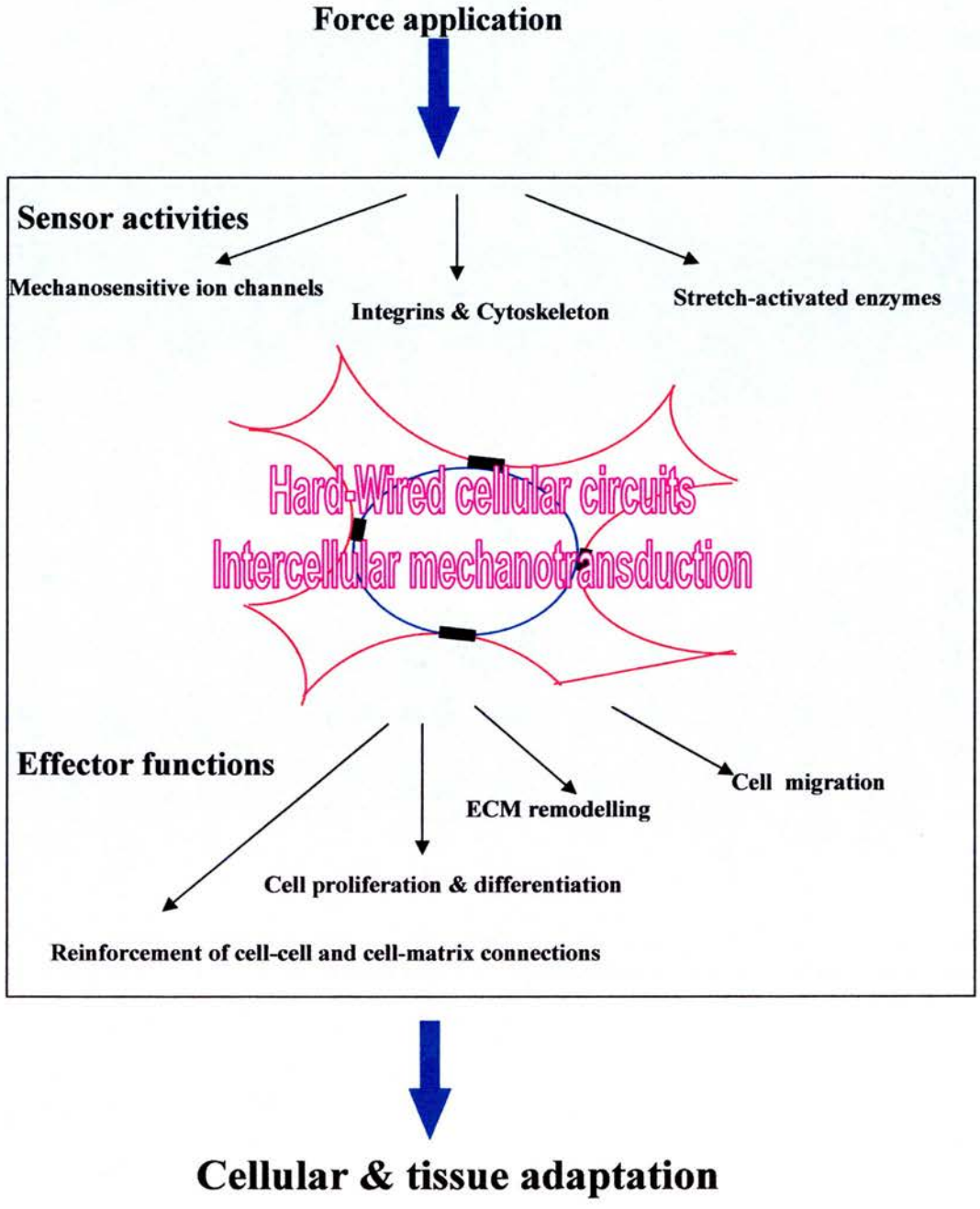

Figure 1.1. Intercellular mechanotransduction mediates cellular and tissue adaptations to force application (Ko and McKulloch 2001).

1.4.0. INTEGRINS

1.4.1. Integrin family

Integrins are a family of cell-surface GPs that act as receptors for ECM proteins, or for membrane-bound counter-receptors on other cells (Aplin et al 1998). The name of integrins refers to their function of integrating the cells' ECM to the cells' interior (cytoskeleton) (vanderFlier and Sannenberg 2001). Each integrin is made up of α and β subunit. Both the α and β subunits are type I transmembrane glycoproteins. In mammals 18 α and 8 β subunits have been shown to combine into 24 different dimers (Plow et al 2000).

Integrins are asymmetric molecules comprised of a ligand binding globular, ~ 10nm diameter head, and two extended tails that contain carboxy terminal portions of α and β subunits and their transmembrane domains (Springer 1997). Most integrins have a large extracellular domain (approximately 1200 amino acids in α subunits and 800 amino acids in β subunits) and a much shorter cytoplasmic domain with 50 residues or less (vanderFlier and Sannenberg 2001). The exception is the β4 subunit that has a cytoplasmic domain of over 1000 residues (Suzuki and Naitoh 1990). Certain β subunits, β1, β3 and β4, can undergo alternative splicing of the cytoplasmic domain and this may play a role in the regulation of integrin signalling. The β4 cytoplasmic domain also undergoes proteolytic processing. The small cytoplasmic domains of integrins have no intrinsic catalytic capacity (Damsky and Werb 1992; Hynes 1992; Ruoslahti 1991).

Integrin cytoplasmic domains are key nexus of interaction between the extracellular environment and intracellular structures and signalling cascades. Both α and β subunit cytoplasmic domains make important contributions to various aspects of overall integrin function including cytoskeletal organization, cell motility, signal transduction, and modulation of integrin affinity for ligands (activation) (Green et al 1998; Aplin et al 1998).

Several cytoplasmic proteins including talin, α–actinin, and possibly focal adhesion kinase (FAK) bind directly to the β1 cytoplasmic domain and contribute to integrin-cytoskeletal interactions (Burridge and Chrzanowska-wodnika 1996; Yamada and Geiger 1997). The β cytoplasmic domain is also important in signal transduction, particularly integrin activation of FAK (Bauer et al 1993; Tahiliani et al 1997). β cytoplasmic domains have played a critical role in a variety of cellular processes including endocytosis (VanNhieu et al 1996), cross talk between integrins (Blystone et al 1995), assembly of fibronectin fibrils (Wu et al 1995), and cell motility (Pasqualini and Hemler 1994).

The α subunit cytoplasmic domain inhibits certain functions of the β subunit. Binding of a ligand to the integrin relieves this inhibition, possibly by allowing the subunits to swing apart like a hinge (Burridge and Chrzanowska-Wodnicka 1996; Hughes et al 1996). The α cytoplasmic domains also can strongly influence cell motility (Bauer et al 1993; Chan et al 1992).

1.4.2. Integrin signalling

Since ligand binding is regulated by signals from within the cell and also triggers cellular responses, mechanisms must exist to propagate information back and forth between the cytoplasmic tails and the globular heads. This overall process is referred to as integrin signalling. A didactic distinction is often made between inside-out and outside-in signalling denoting those reactions initiated by the binding of one or more agonists to their membrane receptors, leading to the conversion of integrin from low affinity/avidity receptor to a high affinity/avidity receptor. Inside-out signalling can have two distinct components that are often difficult to distinguish in practice:

1) affinity modulation, which implies a structural change intrinsic to the heterodimer that results in a greater strength of ligand binding, and

2) avidity modulation, which implies a change in the functional affinity of the interaction between receptor and ligand due to chelate or rebinding effects (Neri et al 1996).

Outside-in signalling denotes reactions initiated by integrin ligation and clustering, and these must be co-ordinated with signals emanating from other plasma membrane receptors (eg, growth factors, cytokines, and G-protein-linked receptors) (Sastry and Horwitz 1996; Juliano 1996). Integrin signals help to regulate a host of postligand binding events, the particular pattern varying with the cell and the integrin (Shattil et al 1998).

Integrins do not have intrinsic kinase activity but instead they initiate cell signalling through the recruitment of adapter proteins including paxillin, talin, vinculin, and caveolin (Hynes 1992; Miyamoto et al 1995; Burridge et al 1996; Aplin et al 1999; Giancotti 2000). These proteins and others such as focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (pyk2) interact with the integrin cytoplasmic tails when integrins are bound to ECM ligands which induce a conformational change in the receptor subunits. The interaction of integrins with ECM molecules results in the clustering of integrins and the formation of focal adhesion complexes, where integrins link the ECM and cytoskeleton (Howe et al 1998; Hughes and Pfaff 1998). The formation of these integrin-associated signalling complexes is closely linked to the cytoskeleton. Many of the signalling proteins recruited into the complex are activated through tyrosine phosphorylation (Loeser 2002).

1.4.3. Integrin ligand binding

Various cell types use the affinity and avidity modulation to regulate ligand binding to integrins, and the relative contribution of each varies with the integrin and the cell type (Diamond and Springer 1994; vanKooyk and Figdor 1997; Aplin et al 1998). The ligand binding regions of integrins have been explored with chemical cross-linking, monoclonal antibodies, mutation, and most recently, molecular modelling and X-ray crystallography (Loftus et al 1994; Qu and Leahy 1995; Springer et al 1997). Three regions are particularly important:

I) A series of several repeats of approximately 60 amino acids in the N-terminal portion of the α chain, each containing a putative Ca^{2+} binding site

II) An inserted domain (I- domain) of approximately 200 amino acids found in several α chains and containing a nucleotide binding fold and a divalent cation coordination site
III) An I-domain like region of approximately 250 amino acids found in the N-terminal region of the β subunit. Less information is available about the I-domain-like region in the β subunit, however, molecular modelling and mutagenesis support its ligand-binding role (Loftus and Liddington 1997).

Integrins likely undergo dynamic structural changes as part of the ligand binding process including relative movements of subunits and of domains, and conformational changes within domains. Integrins can exist in various affinity states for their ligands, structural changes that occur in moving from low- to high-affinity states (or vice versa) can be detected by certain monoclonal antibodies (Loftus and Liddington 1997).

The α/β pairings specify the ligand-binding abilities of the integrin heterodimers. Although the ligands for integrins are often large ECM proteins such as collagen, laminin, vitronectin, or fibronectin, some integrins recognize rather short peptide sequences within the larger protein, for example, the RGD (Arg-Gly-Asp) sequence found in fibronectin and vitronectin. Because of this, there has been considerable interest in the pharmaceutical industry in developing short peptides or peptidomimetics that can interdict integrin functions in a variety of disease processes including coagulation disorders, inflammation, and cancer (Ruoslahti 1996).

1.4.4. Expression of integrins in human articular cartilage

Initial studies were performed to characterize which integrins were expressed by adult articular chondrocytes and which cartilage ECM proteins served as chondrocyte integrin ligands. Work from several laboratories has revealed that chondrocytes express a variety of integrins (Salter et al 1992, 1995; Woods et al 1994; Loeser et al 1995, 2000; Loeser 2002; Knudson and Loeser 2002) (**Figure 1.2**). Using immunohistochemical staining of adult human articular cartilage, Salter et al (1992) noted that the α5β1 integrin was a prominent chondrocyte integrin with variable and weaker expression of α1β1 and

α3β1.Using a combination of immunofluorescence, immunoprecipitation, and FACS (Fluorescence activated cell sorter) analysis, Woods et al (1994) demonstrated that adult human chondrocytes express α1β1, α5β1, and αVβ5 integrins accompanied by weak expressed of α3β1 and αVβ3. Relative to adult chondrocytes, fetal chondrocytes and chondrosarcoma cells have higher levels of α2β1 and α6β1 intergins (Holmvall et al 1995; Salter et al 1995). Loeser et al (2000, 2002) noted that primary adult chondrocytes do express α2 at low levels but expression is stimulated in immortalized and fetal cells.

Compared to histologically normal cartilage, increased immunostaining for chondrocyte integrins has been observed in OA cartilage, with a particular increase noted in α1β1 in monkey cartilage with OA-like changes (Loeser et al 1995) and the appearance of α2β1noted in OA cartilage in human femoral heads (Ostergaard et al 1998).

The α1β1,α2β1 (Durr et al 1993; Enomoto et al 1993; Loeser et al 1995, 2000) and α10β1 (Camper et al 1998) integrins can all serve as receptors for type II collagen. Although the α1β1 integrin partially mediates chondrocyte adhesion to type II collagen, the recent discovery of α10β1 by affinity chromatography using type II collagen suggests that this integrin may be a key receptor for type II collagen (Camper et al 1998). The α1β1 integrin also mediates adhesion of chondrocytes to type VI collagen (Loeser et al 2000) and to cartilage matrix protein (matrilin-1) (Makihira et al 1999). In addition to collagen, α2β1can mediate binding to chondroadherin (Camper et al 1997).

The α5β1integrin serves as the primary chondrocyte Fn receptor (Loeser et al 1995) while αV-containing integrins bind to vitronectin and osteopontin (Loeser 1993) and may serve as alternative Fn receptors. It is likely that not all of the integrins expressed by chondrocytes have been described and the potential exists for additional integrin-matrix protein interactions to be discovered (Knudson and Loeser 2002).

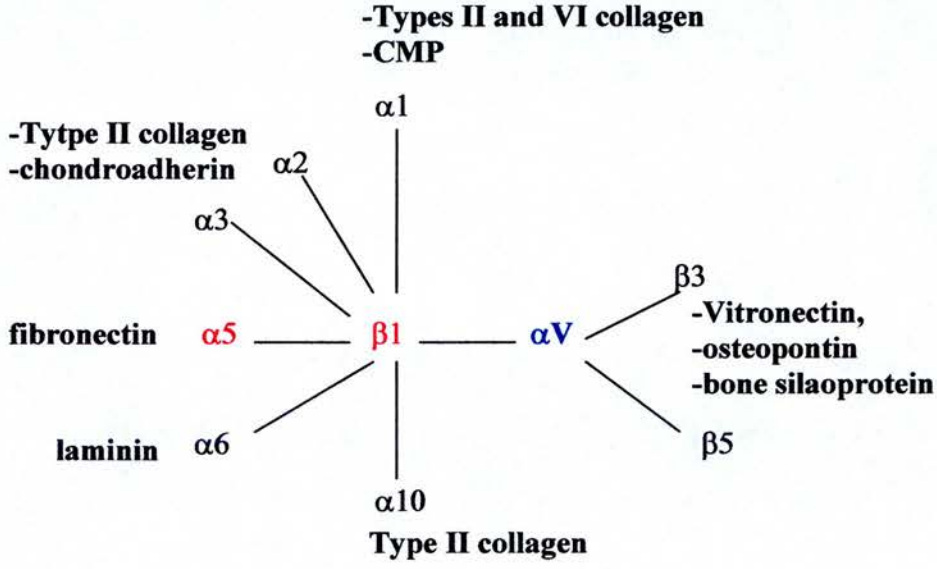

Figure 1.2. Chondrocyte integrins and their lignads. The members of the β1 and αV integrin subfamilies reported to be expressed by chondrocytes are shown along with the ECM proteins which have been demonstrated to bind to the specific integrin subunits. (**CMP**, cartilage matrix protein; **ECM**, extracellular matrix) (Knudson and Loeser 2002).

1.4.5. Evidence for involvement of α5β1 integrin in chondrocyte mechanotransduction

In addition to mediating adhesive interactions, integrins play a further important role on mechanical stress-induced signals. Specifically, it has been shown that integrins like α5β1 and αVβ3 are essential for mechanotransduction of hemodynamic forces into biochemical signals (Urbich et al 2002).

Wright et al (1997) have shown that the membrane potential response of both normal and osteoarthritic chondrocytes to 0.33 Hz cyclic mechanical stimulation was significantly reduced when cells were preincubated with anti-β1 (P4C10) and anti-α5 integrin (BIIG2) antibodies. In contrast antibodies against other subunits had no effect on the membrane response. These observations are consistent with a role for α5β1 integrin, the classical fibronectin receptor, in the mechanotransduction process, potentially as a mechanoreceptor. This idea supported by the observations that RGD containing peptides, which block integrin signalling, prevent the changes in aggrecan and MMP3 mRNA levels that occur following mechanical stimulation of normal chondrocytes (Millward-Sadler et al 2000; Salter et al 2002). These data have been suggested that α5β1 integrin acts as a chondrocyte mechanoreceptor in both normal and osteoarthritic cartilage (Salter et al 2001).

1.4.6. Integrin-mediated signalling pathways

The signalling pathways activated by integrins have been identified through the analysis of biochemical events that are triggered by integrin engagement, and by the identification of proteins that associate with focal adhesion complexes. Some of the critical integrin-mediated signalling pathways can involve protein tyrosine phosphorylation, SH2-SH3 signalling molecules, serine-threonine kinase activation and calcium concentration.

1.4.6.1. Protein tyrosine phosphorylation

Protein tyrosine phosphorylation is one of the earliest events detected in response to integrin activation. Studies in platelets provide the first evidence that integrin receptors can regulate agonist-induced tyrosine phosphorylation (Clark et al 1994, 1995). Subsequently, tyrosine phosphorylation has been shown to be a common and perhaps ubiquitous response to integrin engagement in many cell types including fibroblast, carcinoma cells, and leukocytes (Juliano and Haskill 1993; Hynes et al 1992; Arroyo et al 1994). The ability of tyrosine kinase inhibitors to inhibit the formation of focal adhesions suggests a role for tyrosine phosphorylation in the signalling pathways linked to integrin receptors (Burridge et al 1992). As integrins have no intrinsic enzymatic activity of their own, they must interact with other proteins to generate signals (Damsky and Werb 1992). Several protein tyrosine kinases have been implicated in integrin signalling events by virtue of their integrin-dependent activation or their localization to focal contacts. The focal adhesion kinase (FAK) appears to play a central role in integrin-mediated signal transduction. This kinase is tyrosine phosphorylated, and its tyrosine kinase activity enhanced, upon integrin engagement (Schaller and Parsons 1994). Integrin-induced phosphorylation of FAK requires the cytoplasmic domain of the β integrin subunit, and clustering of chimeric integrin receptors expressing several β cytoplasmic domains is sufficient to induce FAK phosphorylation (Schaller and Parsons 1994; Lukashev et al 1994). It has been shown that FAK can bind to peptides from the β1, β2, and β3 subunits. However, FAK cannot be coimmunoprecipitated with

these subunits, suggesting that either the interaction is weak or there is no direct association during the localization of FAK to focal adhesion plaques (Dedhar and Hannigan 1996). In addition, calcium transients and protein kinase C (PKC) activity may be required as costimulatory events for FAK phosphorylation (Vuori and Ruoslahti 1993; Pelletier et al 1992; Shattil et al 1994). The targeting of FAK to focal adhesions appears to involve multiple binding interactions. A COOH-proximal focal adhesion targeting (FAT) sequence is necessary and sufficient to localize FAK or a FAT-containing chimeric protein to focal adhesions (Hidebrand et al 1993), whereas NH_2-proximal sequences of FAK can bind to synthetic peptides derived from several β cytoplasmic domains(Schaller, unpublished data). The cytoskeletal protein paxillin also associates with FAK through a COOH-terminal sequence of FAK distinct from the FAT domains (Hidebrand, unpublished data). FAK tyrosine phosphorylation sites can serve as binding sites to couple FAK with cellular proteins that contain SH2 domains (Schlaepfer et al 1994; Pawson 1995).

1.4.6.2. SH2-SH3 adapter proteins

Several proteins that associate with integrin-nucleated protein complexes contain modular domains, termed Src homology 2 (SH2) and 3 (SH3) that specifically mediate protein-protein coupling. SH2 domains bind to proteins through interactions with specific peptide motifs containing phosphotyrosine, whereas SH3 domains bind to short proline-rich peptide motifs on their protein targets (Pawson 1995). These domains play critical roles in intermolecular interactions that couple proteins associated with membrane receptor complexes. Members of the Src family, which contain an SH3 and SH2 domain, have also been implicated in integrin signalling events. Activated Src associates with integrin-dependent cytoskeletal complexes in platelets and fibroblasts (Clark et al 1994; Schlaepfer et al 1994). This localisation appears to be dependent on the Src SH3 domain (Kaplan et al 1994), possibly through interaction with proline-rich sequences with paxillin (Weng et al 1993). The localisation of Src family kinases to focal adhesions may also be mediated by their SH2 domains, which bind to the FAK autophosphorylation site

(Okamura and Resh 1994). One such protein, growth factor receptor-bound protein 2 (Grb2), links activated receptor tyrosine kinases, is associated with FAK in an integrin-dependent manner (Clark and Brugge 1995).

Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2) is a nontransmembrane protein tyrosine phosphatase (PTP) that is involved in integrin-mediated signalling pathways such as tyrosine phosphorylation. SHP-2 plays a critical role in growth factor and cytokine signalling pathways (Oh et al 1999). It has been shown that a fraction of SHP-2 moves to focal contacts upon integrin engagement and that binds to SHP substrate-1 (SHPS-1), a transmembrane GP with adhesion molecule characteristics. This protein is also known as signal regulatory protein-α (SIRP-α) (Fujioka et al 1996) (see 1.5.2.6.2, page 68). Oh et al (1999) have shown that SHP-2, acting via SHPS-1/SIRPα, acts as a critical positive component in integrin signaling.

1.4.6.3. Serine-threonine kinase families

Serine-threonine kinase families such as protein kinase C (PKC) and mitogen-activated protein (MAP) kinases are also activated upon integrin stimulation, and inhibitors of PKC block cell attachment and spreading in certain cell systems (Vuori and Ruoslahti 1993; Nakamura and Nishizuka 1994; Schlaepfer et al 1994).

The PKC isoenzymes can be classified into three groups:
I) The conventional PKCs (cPKCs) α, βI, βII, γ are activated by negatively charged phospholipids, diacylglycerol (DAG) or phorbol ester, in a Calcium-dependent manner.
II) The novel PKCs (nPKCs) δ, ε, θ, η/L and μ also require negatively charged phospholipids, but not Calcium for activation.
III) The atypical PKCs (aPKCs) λ/ι and ζ do not require Calcium, DAG, or phorbol ester, but only negatively charged phospholipids.

The isoenzymes are characterized by differences in the various domains, called C1-C4. C1 domain forms the DAG binding site. This domain is immediately preceded by an

autoinhibitory pseudosubstrate sequence. The C2 domain contains the recognition site for acidic lipids and, in some isozymes, the Ca^{2+}-binding site. The C3 and C4 domains form the ATP binding and substrate-binding sites.

In comparison to cPKCs, the Calcium binding domain (C2) is lacking in nPKCs and aPKCs. cPKCs and nPKCs contain two zinc fingers in the phorbol ester binding site (C1), aPKCs are characterized by a single zinc finger. In PKCμ, the pseudosubstrate domain is lacking.

The different PKC isoenzymes are expressed in a tissue-dependent fashion and most cells express multiple isoforms (Dekker and Parker 1994). PKC isoforms are targeted to different cellular compartments on cell stimulation (Haller et al 1995). They also bind to cytoskeletal proteins and to other subcellular compartments (Kiley and Parker 1995) and phosphorylate different substrates (Hofmann 1997). PKCs are implicated in various biological functions including signal transduction, cell proliferation and differentiation.

Various groups have shown that PKC may be activated by integrin- mediated signalling (Vuori and Rouslahti, 1993; Chun et al 1996; Lewis et al 1996; Haller et al 1998; Wrenn and Herman 1995; Ng et al 1999). A unique feature of PKC regulation is that prior to stimulation of the cell, the inactive form of PKC is thought to be diffusely distributed throughout the cytosol, or may be localized to specific regions or structures of the cell. Following stimulation, PKC isoforms are observed to translocate from inactive pools to their active cell loci such as cell membrane where it associates with membrane phospholipids (Haller et al 1990). Several cell membrane proteins have been recognized that bind only activated PKC isozymes termed receptors for activated C-kinase (RACKs) (Mochly-Rosen et al 1991).

Several reports have indicated that the PKCα is translocated from the cytosol (cytosolic compartment) to the cell membrane (particulate compartment) after activation (Khalil et al 1994; Jensen et al 1996; Haller et al 1998b; Li et al 2002; Lee et al 2002).

Lee et al (2002) have recently reported that within 30 sec mechanical stimulation at 0.33 Hz rapidly activates PKCα in cultured articular chondrocytes by translocating the PKCα from the cytosol to the particulate fraction. They have also demonstrated that mechanotransduction via integrins is necessary for association between RACK1/PKCα and β1 integrin following 0.33 Hz cyclical mechanical stimulation.

PKC seems also to play a role in the formation of focal adhesions (Jaken et al 1989; Lewis et al 1996). Because both PKC and pp125FAK localize to the plaques, a relation between these enzymes with various experiments has been investigated. Different studies in Chinese hamster ovary (CHO) cells (Vuori and Ruoslahti 1993), fibroblast cells (Woods and Couchman 1992), platelets (Haimovich et al 1996) and muscle cells (Disatnik and Rando 1999) have reported the association between PKC activation and FAK tyrosine phosphorylation.

1.4.6.4. Intracellular Calcium regulation

Integrin engagement can also induce an increase in intracellular Calcium concentration ($[Ca^{2+}]i$), a key regulator of intracellular signalling via multiple integrins, though the exact response appears to be specific to the integrin, ligand, and cell type (Juliano et al 1993; Schwartz et al 1994). $[Ca^{2+}]i$ acts as a regulator of different cellular processes including gene expression and cell proliferation (Berridge 1993).

1.5.0. CELL MEMBRANE INTEGRIN-ASSOCIATED PROTEINS

1.5.1. Interaction between integrins and other cell membrane proteins

In addition to interacting with molecules on neighboring cell or ECM (trans), integrins can also form associations with other receptors on the same cells (cis) to form multireceptor complexes. These complexes recruit signalling molecules to sites of cell-cell or cell-matrix adhesion, such as focal complexes or focal adhesions. So far, there is little evidence that integrins can signal through exclusive, integrin specific pathways. Instead, they appear to cooperate with other cell surface receptors to influence a variety of signalling pathways. These complexes of integrins and partner receptors can be formed either in response to or independent of integrin activation and ligation. The classes of receptors known to be associated both physically and functionally with integrins are shown in **Table 1.2**. Most of the associations involve extracellular domains (Porter and Hogg 1998).

Table 1.2. Cell membrane proteins associated with integrins (Shattil et al 1998; Porter and Hogg 1998)

Cell membrane protein	Associated integrin	Reference(s)
CD47/IAP	$\alpha_{IIb}\beta3$, $\alpha V\beta3$, $\alpha2\beta1$	Lindberg et al 1993, 1994 Gao et al 1996
CD98	$\alpha_{IIb}\beta3$, $\beta1$	Fenczik et al 1997
Tetraspanins (TM4SF)		
CD9	$\alpha_{IIb}\beta3$, $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$ $\alpha6\beta1$	Brisson et al 1997 Maecker et al 1997
CD63	$\alpha3\beta1$	Berditchevski et al 1996
CD81, NAG-2	$\alpha3\beta1$, $\alpha6\beta1$	Tachibana et al 1997
CD151	$\alpha5\beta1$	Sincock et al 1997
GPI-linked receptors		
CD87 (uPAR)	$\alpha_M\beta2$, $\alpha_V\beta1$, $\alpha_V\beta5$, $\alpha3\beta1$ $\alpha5\beta1$, $\alpha6\beta1$	Wei et al 1996 Xue et al 1994, 1997
CD16 (FcγRIIIB)	$\alpha_M\beta2$	Xue et al 1994
Others		
EMMPRIN (CD147)	$\alpha3\beta1$, $\alpha6\beta1$	Berditchevski et al 1996
Caveolin	$\alpha1\beta1$	Wary et al 1996

Abbreviations: **IAP**, integrin-associated protein; **GPI**, glycosylphosphatidylinositol; **TM4SF**, transmembrane-4 superfamily; **uPAR**, urokinase plasminogen activator receptor; **EMMPRIN**, extracellular matrix metalloproteinase inducer.

1.5.2. Recognition and expression of integrin-associated protein (IAP)/ CD47

Initially, B6H12 protein was purified from placental membranes and platelet (Gresham et al 1989; Brown et al 1990). The molecular mass of the B6H12 protein on SDS-PAGE was 50 -55 kD under reducing conditions. Campbell et al (1992) defined B6H12 protein as ovarian tumor-specific antigen 3 (OA3) for ovarian carcinoma cell line OVM1.

To recognize this molecule, a number of monoclonal antibodies (mAbs) and polyclonal Abs prepared using the purified placental protein as immunogen (Brown et al 1990; Rosales et al 1992; Mawby et al 1994; Lindberg et al 1994). Brown et al (1990) produced a panel of mAbs and one polyclonal Ab and compared their function with B6H12. Fifty kD glycoprotein was first described as a molecule associated with β3 integrin chain on placenta and platelets and called integrin associated protein (IAP) (Brown et al 1990). IAP was widely expressed in hematopoietic cells (erythrocytes, lymphocytes, platelets, monocytes, and neutrophils) and promonocytic and promyelocytic cell lines (U937 and HL60). Other studies reported (Rosales et al 1992; Mawby et al 1994) that tissues such as placenta, surface epithelia, liver, brain, and even cells that did not express integrins, including erythrocytes, showed highly expressed level of IAP. Antibodies that recognised OA3, B6H12 and IAP, were later shown to recognize CD47 (Lindberg et al 1994).

1.5.2.1. Structure and in vivo alternatively spliced forms of CD47

CD47 is a unique Ig superfamily member with an amino-terminal Ig variable (IgV) extracellular domain, a carboxy-terminal five membrane-spanning domain, and a short cytoplasmic tail with four alternatively spliced forms (Lindberg et al 1993; Reinhold et al 1995) (**Figure 1.3**). The IgV homology suggested the disulfide linkage. The extracellular domain composed of about 120 amino acids (AA). The carboxy terminus is located on the inside of the cell membrane and consists of about 152 AA. Four isoforms of mRNA derived by alternative splicing at the intracytoplasmic tail (~30 AA).

Human CD47 has five potential N-glycosylation sites in the EC domain, three of these sites are glycosylated in erythrocytes, leading to its broad migration at 45-60 kD on SDS-PAGE. However, in leukocytes and platelets, CD47 runs as a band of 47-55 kD (Yuan et al 1996). These results suggest type-specific cell glycosylation may be responsible for the differences in the apparent molecular weight.

Four distinct complementary DNAs (cDNAs) for OV-3 antigen were first reported in ovarian tumor cells (Campbell et al 1992) and the identical results obtained in human and murine tissues (Lindberg et al 1993). The cDNAs differ from each other by increasingly long extensions at the 3′ end of the coding region. Since the carboxy terminus of the protein is intracytoplasmic (Lindberg et al 1993) each of these cDNAs would code for protein with a different length intracytoplasmic tail, which are termed forms 1-4, based on increasing length. Each longer cytoplasmic tail came from the addition of a short peptide sequence to the extreme carboxy terminus of the protein. Each of these additional peptide sequences was encoded by a short exon. The three exons encoding the additional extensions of forms 2-4 were 32bp, 25bp, and 36bp, respectively (Lindberg et al 1993). Each of these exons and the one encoding the form 1 cytoplasmic tail ended in G. The common exon encoding the 3′ UT, which is identical for each form, began AATAAC. For forms 1,3, and 4, the splice with the 3′ G of the preceding exon led to a glutamate as the carboxy-terminal amino acid of the predicted protein, followed by a stop (TAA or TAG) codon. For form 2, the frame was shifted and the final AA, NN, were encoded in the same exon as the 3′ UT sequences prior to a new stop codon. Because the cDNAs all contained identical 3′ UT, the different coding regions represented alternative splicing of the IAP gene (Reinhold et al 1995).

Four isoforms of mRNA derived by alternative splicing at the intracytoplasmic tail (~30 AA). It has been shown that IAP form 1 has a 4 AA intracytoplasmic extension, encoded on the same exon as the final transmembrane domain, form 2 a 16 AA extension, form 3 a 23 AA extension, and form 4 a 34 AA extension (**Figure 1.3**)(Lindberg et al 1993; Reinhold et al 1995).

All 4 isoforms of IAP mRNA are expressed at varying levels in a variety of cells and tissues. RT-PCR and immunohistochemical studies have shown tissue-specific expression of different IAP isoforms in human and murine tissues. Thymus and spleen expressed predominantly form 2 mRNA, while brain expressed from 4 to a much greater degree than any other form. Form 2 and form 4 are the most abundant IAP splices variants made in vivo (Reinhold et al 1995).

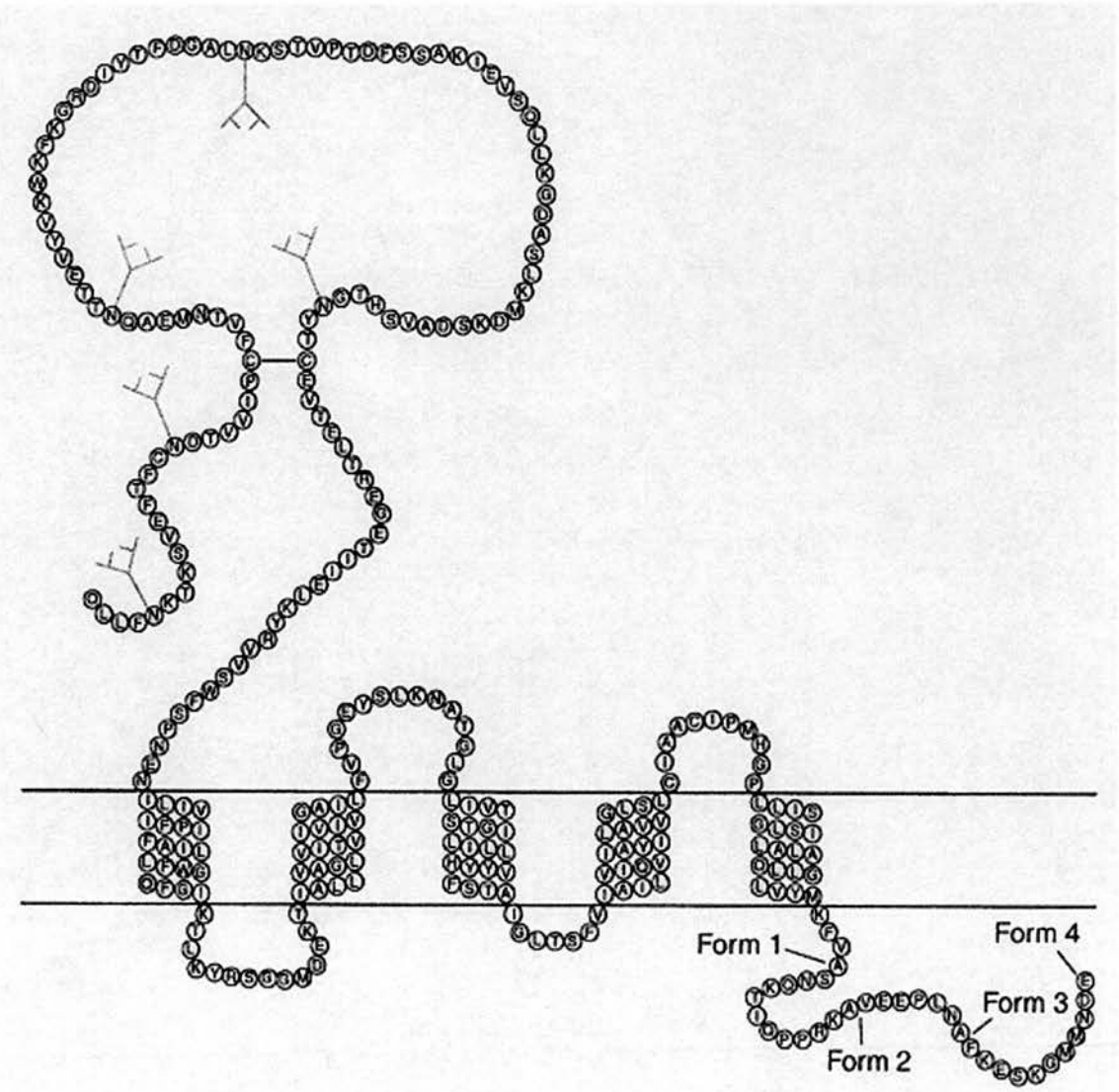

Figure 1.3. Structural model for CD47. The model is based on predictions from the primary sequence, as derived from cDNA sequence, as well as homology to known members of the Ig superfamily (IgSF). The extracellular N-linked glycosylation sites are shown. The disulfide linkage (c-c) is indicated. The Ig variable (IgV) extracellular domain is followed by five transmembrane segments terminating in a cytoplasmic tail that is alternatively spliced, giving rise to four isoforms, the longest of which, form 4, is shown in its entirely (Lindberg et al 1993; Brown and Frazier 2001).

1.5.2.2. CD47 functions

Since IAP is ubiquitously expressed (Brown et al 1990; Rosales et al 1992; Reinhold et al 1995), its function has been inferred from the effect of antibody inhibition, in a number of cells mainly in myeloid and endothelial cells. IAP was first identified as the target of antibodies that block ECM-induced activation of neutrophils (PMN) and monocytes (Brown et al 1990). Anti-IAP mAb B6H12 inhibited PMN transendothelial migration, PMN and endothelial cell chemotaxis to RGD-containing proteins, and PMN adhesion to entactin (Cooper et al 1995). In addition, anti-IAP mAbs inhibit an increase intracellular calcium concentration seen when endothelial cells spread on vitronectin (Vn) in an αV-dependent manner (Schwartz et al 1993). Further, anti-CD47 mAbs can block integrin-stimulated phagocytosis (Blystone et al 1995) and transepithelial (Parkos et al 1996) migration of polymorphonuclears. CD47 knockout mice have a severe host defense defect (Lindberg et al 1996). The cross-linking of CD47 with two mAbs anti-CD47, namely B6H12 and Bric126, showed a strong comitogenic effect on the proliferation of human T lymphocytes stimulated through a specific pathway, but another anti-CD47 mAb, namely 2D3, was ineffective (Ticchioni et al 1997). Salter et al (1997) have shown that anti-CD47 Bric 126 inhibited the hyperpolarisation response of human bone cells (HBC) following 0.33 Hz cyclical mechanical stimulation but had no effect on the response of bone cells at 0.104 Hz stimulation. Ticchioni et al (1997) have shown that signal delivered by CD47 within T cells, induces the phosphorylation on tyrosine of several proteins. CD47 is involved in a number of cellular and molecular functions, some of which are listed in **Tables 1.3** and **1.4**.

1.5.2.3. CD47-integrin interactions and signalling

CD47 forms a physical complex with certain integrins (Brown et al 1990; Gao et al 1996a,b; Wang and Frazier 1998), and blockade of IAP with mAbs inhibits some aspects of integrin signalling (Lindberg et al 1996). Within the plasma membrane of platelets and most cell types, CD47 can associate with and modulate the activity of several families of integrins. Some studies of platelet activation and spreading and chemotaxis of nucleated cells indicated that CD47 initiates a signalling pathway to upregulate integrin function, that is, inside out signalling (Blystone et al 1995; Gao et al 1996a; Chung et al 1997, 1999; Wang and Frazier 1998).

The ligation of CD47 with cell binding domain (CBD) domain of thrombospondin (TSP) causes an increase in the affinity and/or avidity of αVβ3 integrin. The presence of TSP or its CD47 agonist peptide in medium of cultured C32 melanoma cells causes rapid and intense tyrosine phosphorylation (Gao et al 1996; Frazier et al 1999; Green et al 1999). CD47 binds specifically to its agonist peptide from TSP, activates the platelet integrin $\alpha_{IIb}\beta3$, resulting in platelet spreading on immobilized fibrinogen, stimulation of platelet aggregation, and enhanced tyrosine phosphorylation of focal adhesion kinase (Chung et al 1997). Recently Lagadec et al (2003) have shown interaction between platelet CD47 and endothelial TSP1 under flow activates $\alpha_{IIb}\beta3$ integrin and promotes platelet-EC adhesion. The effect of α2β1-mediated chemotaxis requires CD47 and its ligand TSP. CD47 may alter the affinity state of α2β1, either by direct physical interaction (Wang and Frazier, 1998) or via inside out signaling (Wang et al 1999; Chung et al 1999).

Recently it has been shown that CD47 and α4β1 integrin are colocalized on microvilli of K562 erythroleukemia cells (Abitorabi et al 1997), and CD47-dependent arrest of T cells on inflammatory endothelium could be blocked by antibodies that prevent α4β1 integrin binding to vascular cell adhesion molecule (VCAM-1) (Ticchioni et al 2001). Moreover, it has been shown that CD47 expression is required for stimulation of T cell motility and expression of MMP-2 stimulated by α4β1 integrin ligand. An antibody to CD47 also blocked the motility response to an α4β1 integrin ligand (Li et al 2002). Therefore, at

least CD47 and α4β1 integrin could be functionally associated in T cell chemotaxis (Barazi et al 2002).

It has been shown that in human erythroleukemia cell line K562, α5β1 integrin-mediated phagocytosis, was inhibited in the presence of function blocking mAb anti-CD47 (B6H12). This modulation of α5β1integrin phagocytosis was affected by αVβ3 signal transduction (Blystone et al 1995). In addition, Barazi et al (2002) have recently shown that B6H12 physically associated with α4β1 integrin and functionally has inhibitory effect on α5β1integrin role in integrin-mediated adhesion in Jurkat T cells. Therefore, they reclassified B6H12 as a function-modifying Ab for CD47 (Barazi et al 2002).

Fujimoto et al (2003) have recently reported a novel mechanism of affinity modulation of integrin. They have provided evidence that the extracellular Ig domain of CD47 when it binds to TSP-1 can interact with $\alpha_{IIb}\beta_3$ integrin and change $\alpha_{IIb}\beta_3$ to a high affinity state without the requirement of intracellular signalling.

Graf et al (2002, 2003) have recently demonstrated that different types of cells use a common molecular machinery for the mechanosensitive regulation. They also have shown that different external forces induce the same biochemical response in each cell. The proteins involved in this process are, a member of integrin family (such as αVβ3 in fibroblast and endothelial cells), IAP/CD47 and TSP-1.

1.5.2.4. CD47-integrin complex signals via heterotrimeric Gi protein

A relatively stable complex containing CD47 and heterotrimeric Gi proteins is observed in C32 melanoma cells and platelets. It has been shown that different integrin αβ heterodimers associating with CD47 can couple to Gi protein- dependent pathways. Treatment of cells with pertussis toxin (PTX) eliminated the co-immunoprecipitation of CD47 and Gi (Frazier et al 1999). Aside from the physical association of integrins, CD47, and Gi proteins, other factors support the concept of a functional association in which CD47 communicates directly with Gi. This complex integrity is absolutely dependent on cholesterol (Green et al 1999). Incubation of the αVβ3-CD47-Gi complex with β–methyl-cyclodextrin to remove cholesterol causes complete disruption of the protein complex.

Treatment of platelets with TSP peptide initiates a precipitous drop in intraplatelet cAMP levels. This signalling event is common to all of the agonists that activate platelets through G protein-coupled receptors (Shattil et al 1994). A similar fall observed in intracellular cAMP in other cell types that respond to TSP-1 stimulation with integrin activation (Adams 2001). Since most other receptors that activate heterotrimeric G proteins are members of the heptaspanin family (7TMS model), one appealing model for activation by CD47/integrin holds that the five membrane-spanning segments of CD47 and two membrane-spanning domains of the heterodimeric integrin form a seven-transmembrane receptor. Ligation of this complex with an adhesive ligand or TSP could activate GTPase activity in a manner analogous to that of conventional heptaspanins (Gao et al 1996; Frazier et al 1999; Brown and Frazier 2001).

1.5.2.5. Integrin-independent CD47 signalling

Although, as mentioned above, association of CD47 with certain integrins was found to modulate their function, some signal transduction through CD47 is integrin-independent (Cooper et al 1995; Frazier et al 1999; Ticchioni et al 1997; Reinhold et al 1997;Waclavicek et al 1997; Wu et al 1999; Liu et al 2001). Most of these reports are related to T cell costimulation. Ticchioni et al (1997) have shown that cross-linking of CD47 with two mAbs, namely B6H12 and Bric126, has a strong effect on proliferation of peripheral T lymphocytes, and CD47 is not associated with integrins. Reinhold et al (1997) have reported that T cell activation by CD47 is independent of integrins. They showed that in Jurkat cells, ligation of IAP costimulated IL-2 production, and this effect was not mimicked by ligation of any integrin on the cell.

Liu et al (2001) have recently reported that the mechanism of CD47-mediated regulation of PMN transmigration is integrin-independent. They have used a panel of functionally inhibitory mAbs against the specific PMN integrins and did not observe any significant effect on PMN transepithelial migration. In addition, no physical association has been found between CD47 and PMN integrins.

Table 1.3. CD47 functions in various cell types

Cell or tissue type	Biological function/s	Reference/s
Placenta, PMN, platelet	Cell adhesion Phagocytosis/ chemotaxis	Brown et al 1990; Senior et al 1992
Ovarian tumor cells	Ovarian tumor marker	Campbell et al 1992
Endothelial cells	Calcium influx	Schwartz et al 1993
Neutrophil	Neutrophil migration	Cooper et al 1995
Melanoma	Tyrosine- phosphorylation	Gao et al 1996 a, b
Platelet	Aggregation, Spreading, Tyrosine- phosphorylation	Chung et al 1997 Lagadec et al 2003
Bone cells	Mechanical stimulation	Salter et al 1997
Jurkat cells and 3.L2 cells	IL-2 production Co-stimulated proliferation	Reinhold et al 1997
T cells	Comitogenic effect Tyrosine phosphorylation	Ticchioni et al 1997
VSMC	Chemotaxis	Wang et al 1998, 1998
Rat brain	Synaptogenesis	Jiang et al 1999
Rat brain	Synaptogenesis	Jiang et al 1999
Mouse splenic red pulp	Phagocytosis inhibition	Oldenborg et al 2000,2001
Memory T cells	Downregulation of proliferation	Brooke et al 1998 Seiffert et al 2001
Dendritic cells	cytokine production	Latuor et al 2001
Macrophage	Cell fusion	Saginario et al 1998; Han et al 2000
1. Murine Pro-B cell (Ba/F3)	Cell aggregation	Babic et al 2000
T cell	Cell accumulation	Ticchioni et al 2001
PMN (Neutrophil)	Tyrosine phophorylation	Liu et al 2001, 2002

1.5.2.6. CD47 ligands

So far, two important natural ligands of CD47, thrombospondin-1 (TSP-1) and signal regulatory protein-α (SIRPα), have been described.

1.5.2.6.1. Thrombospondin-1 (TSP1)

Thrombospondins (TSPs) comprise a family of five genes encoding proteins designated TSP-1 through TSP-5. Platelet thrombospondin, or TSP-1 as it is known, is the prototypic member of this family. The complete cDNA sequence of TSP-1 provided the basis for detail studies of the structure-function relationships of this complex and interesting molecules.

Thrombospondin-1 (TSP-1) is a trimeric glycoprotein consisting of three identical monomer chains. Molecular cloning of TSP-1 revealed that each subunit of the trimer consists of multiple domains: N- and C-terminal globular domains, a region of sequence with similarity to procollagen, and three types of repeated motifs, designated type I, type II, and type III repeats (Lawler 1986). Molecular cloning of TSP-2, -3, -4 and cartilage oligomeric matrix protein (COMP or TSP-5) indicates that the TSPs can be divided into two subgroups on the basis of their molecular architecture (Adams 2001).

TSP-1 and –2 (subgroup A) have the same set of structural domains. By contrast, TSP-3, -4 and –5 (subgroup B) lack the type I repeats and the region of homology with procollagen, but contain an additional type II repeat. TSP-5 also lacks the N-terminal globular domain. Subgroup A proteins are trimeric and have identical domain structures, while subgroup B are pentameric.

TSP1, ~450 kD trimeric matrix glycoprotein, was first described as an α-granular protein secreted by platelets upon thrombin activation. TSP1 is also synthesized by a number of cells including smooth muscle cells (Wang and Frazier 1998), endothelial cells (EC) (Sheibani and Frazier 1995) and articular chondrocytes (Miller and Mc Devitt 1988; DiCesare et al 1994). TSP1 binds to a large number and wide variety of receptors.

Proteolytic digestion of TSP1 and expression of domains are recombinant or synthetic peptides has enabled identification of several sequences that support these interactions. There are at least six distinct receptors for TSP. These receptors include:

1. The heparin-binding domain (HBD) in the N-terminal domain, which binds to PGs and sulfatides (Gartner et al 1984)
2. Procollagen domain (PC) (Lawler et al 1988)
3. A region within the type I repeats, which binds to CD36 (Asch et al 1992)
4. A region within the type II repeats
5. The Arg-Gly-Asp (RGD) sequence within the last of the type III repeats, calcium-binding domain, which bind to integrins such as $\alpha V\beta 3$ and $\alpha_{IIb}\beta 3$ (Lawler et al 1988; Karczewski et al 1989)
6. Carboxy-terminal domain, named the C-terminal cell-binding domain (CBD), including a minimal sequence (Arg-Phe-Tyr-Val-Val-Met-Trp-Lys), named 4N1-1, and more soluble peptide (Lys-Arg-Phe-Tyr-Val-Val-Met-Lys-Lys), named 4N1K, both contain VVM motif that bind to IAP or CD47 (Kosfeld and Frazier 1993; Gao et al 1994, 1996).

A major cell attachment site was identified in the extreme C-terminal domain ''cell-binding domain" (CBD) of TSP-1 (Kosfeld and Frazier 1992). Using synthetic peptides, the cell-binding activity was localized to two sequences both containing the unlikely adhesion motif VVM. One of these peptides, 4N1 or RFYVVMWK, is highly conserved in all species and isoforms of TSP (Kosfeld and Frazier 1993). It has been shown that CD47 acts as a receptor for 4N1K motif of TSP. The CD47-binding sequence is well conserved in all TSP isoforms, suggesting that all TSP family use CD47 as a receptor. The interaction of CD47 and TSP-1 has been shown to trigger downstream intracellular signalling pathways including caspase-independent cell death in chronic lymphatic B cell leukocyte clones (Mateo et al 1999), rapid decrease in intacellular cAMP level (Frazier et al 1999; Wang et al 1999) and inhibition of ERK activities in smooth muscle cells (Wang

et al 1999). However, Tulasne et al (2001) have recently shown that CD47-null platelets can be stimulated to aggregate by 4N1K.

TSP-1 is a ligand for several integrins, including αVβ3 and $\alpha_{IIb}\beta_3$ on platelets (Chung et al 1997), α3β1 on neurons (DeFreitas et al 1995), α4β1 and α5β1 on T lymphocytes (Yabkowitz et al 1993a), α3β1and α4β1 on breast carcinoma cells (Chandrasekaran et al 1999; Krutzsch et al 1999). TSP-1-integrin interactions appear to mediate numerous TSP-1 functions (**Table 1.4**).

TSP1 mediates activation-dependent T cell adhesion through binding to α4β1 and α5β1 integrins (Yabkowitz et al 1993a). When C32 human melanoma cells attach to a sparsely coated vitronectin substratum, TSP and 4N1K dramatically stimulate the rate of αVβ3-dependent cell spreading (Gao et al 1996b). Further, 4N1K peptide is a chemoattractant of ECs migrating on a gelatin/RGD-containing matrix to which they attach via αVβ3 (Gao et al 1996a).

In several biological settings, the binding of CD47 to TSP1, its cell binding domain, or the agonist peptide 4N1K can stimulate the activation of integrins to higher affinity/ avidity state. For example, 4N1K peptide stimulates spreading on fibrinogen-coated surfaces and induces aggregation of platelets via activation of the integrin $\alpha_{IIb}\beta3$ as judged by the enhanced binding of the ligand mimetic mAb PAC-1 (Chung et al 1997).

The chemotaxis of vascular smooth muscle cells (VSMC) toward soluble collagen is also stimulated by 4N1K. In this case, α2β1, a collagen receptor, is modulated by CD47 (Wang et al 1998). Recently it has been shown that the extracellular Ig domain of CD47 when binds to TSP-1 can interact with $\alpha_{IIb}\beta_3$ integrin and change $\alpha_{IIb}\beta_3$ to a high affinity state without requirement of intracellular signalling (Fujimoto et al 2003). However, Tulasne et al have previously shown that CD47-null platelets can be stimulated to aggregate by 4N1K (Tulasne et al 2001).

TSP1 is known to be present in articular cartilage (Miller and Mc Devitt, 1988; DiCesare et al 1994). Using immunohistochemistry and mRNA expression, the pattern of TSP1expression in human normal and osteoarthritic cartilage has been reported (Pfander et al 2000). They have reported that TSP-1 is predominantly expressed in middle zone chondrocytes in normal and mild OA cartilage. In severe OA reduced matrix staining of TSP1 and significant reduction in TSP-1 mRNA compared with mild OA are demonstrated.

TSPs are implicated in the regulation of the motility, proliferation, and differentiation of many cell types (Frazier 1991; Bornstein and Sage 1994). For example, TSP-1 blocks angiogenesis by inhibiting the chemotaxis and proliferation of EC and promoting their differentiation into capillaries (Frazier 1991; Sheibani and Frazier 1995).

Table 1.4. Summary of functional properties attributed to the TSP-CD47 interaction

Cell type	**Effect**	**References**
Fibroblast	Calcium mobilization	Tsao and Mousa 1995
Melanoma cells	Spreading, Tyrosine phophorylation	Gao et al 1996 a, b
Platelets	Aggregation	Chung et al 1997
VSMC	Spreading	Wang et al 1998
Granulosa cells (GC) Luteal cells (LC)	Autocrine fashion	Higuchi et al1999
T-Lymphoma cells	Activation of Ras and MAP kinase	Wilson et al 1999
Monocytes	Inhibition of IL-12 production	Armant et al 1999
Human melanoma cell line C32 (ATCC, CRL 1585)	Decrease intracellular cAMP	Frazier et al 1999
Smooth muscle cells (SMC)	Inhibition of ERK	Wang et al 1999
B lymphocyte	Apoptosis	Mateo et al 1999
T Lymphocyte	TCR activation	Li et al 2001
Platelet	Platelet adhesion	Lagadec et al 2003
Platelet	Platelet aggregation	Fujimoto et al 2003
Fibroblast and Endothelial cell	Mechanosensitive function	Graf et al 2002, 2003

1.5.2.6.2. Signal regulatory protein α (SIRP α) (CD172a)

Signal regulatory proteins (SIRPs) belong to IgSF and relate to integrin signalling cascades. One of the SIRPs (Fujioka et al 1996; Yamao et al 1997), Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1), is also known as SIRPα1 (Kharitonenkov et al 1997), BIT (brain Ig-like molecule with tyrosine-based activation motifs) (Sano et al 1997), P84 (Comu et al 1997), MFR (macrophage fusion receptor) (Saginario et al 1998) MyD-1 (Brooke et al 1998) and CD172a (Kharitonenkov et al 1997).

SIRPs appear ubiquitously expressed as Ig-like transmembrane glycoproteins composed of two subgroups, SIRPα and SIRPβ, that overall constitute a subfamily within the Ig SF. Among SIRPs, SHPS-1 was first identified as a novel tyrosine phosphorylated protein that binds to Src homology 2 (SH2)-containing protein tyrosine phosphatases, SHP-1 and SHP-2, in v-Src-transformed fibroblasts (SR-3Y1 cells) (Fujioka et al 1996). Various mitogens such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) induce the tyrosine phosphorylation of SHPS-1 to recruit the tyrosine phosphatases and several SH2-containing adapters to the plasma membrane (Kharitonenkov et al 1997).

A protein of the SIRP family, like other IgSF members, consists of three domains: The extracellular domain, the transmembrane domain and the intracellular domain. The extracellular domain is further divided into three Ig- like regions, an amino-terminal Ig variable (V) region and two Ig constant (C) regions. The second and third IgC can be removed by alternative splicing. At least 15 human members (Kharitonenkov et al 1997), varying in the form of subtle amino acid differences in the IgV region, have been identified in the SIRP family. Two subfamilies termed SIRPα and SIRPβ are distinguished by the length of the cytoplasmic region.

The cytoplasmic region of the SIRPα subfamily proteins contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and a proline-rich region near the carboxy-

terminus, which represent a binding site for Src homology 3 (SH3) domain-containing molecules (Kharitonenkov et al 1997; Saginario et al 1998). Phosphorylation of the ITIM-associated tyrosine residues enables recruitment of SHP-1, SHP-2 and SH2 domain-containing inositol phosphatase, which in turn dephosphorylates specific protein substrates involved in mediating various physiological effects.

SHPS-1, has relative molecular weight of 90-120 kD and is differentially glycosylated in a species and tissue specific manner (Kharitonenkov et al 1997). SHPS-1 contains potential N-glycosylation sites and a large number of serines and threonines that can serve as O-glycosylation sites (Fujioka et al 1996; Yamao et al 1997 et al Saginario, 1998 et al Adams, 1998). Because the differential pattern of SHPS-1 glycosylation, the species-specific difference of molecular weight may be due to the number of potential N-glycosylation sites that are found: 15 times in rats (Fujioka et al 1996), 17 times in mice (Yamao et al 1997; Comu et al 1997) and 5 times in humans (Yamao et al 1997). For example the molecular weight (MW) of SIRPα in myeloid cells, 110 kD and in neuronal cells, 85-90 kD has been reported. The tissue-specific difference of glycosylation may be due to the galactosylation capacity. It has been shown that the differential galactosylation of SHPS-1 determines its cellular binding specificity (Van den Nieuwenhof et al 2001).

SHPS-1 is ubiquitously expressed in all tissues examined (heart, brain, spleen, lung, liver, muscles, kidney, and testis), being most abundant in the brain and the spleen (Kharitonenkov et al 1997). Using immunohistochemistry with a specific anti-SHPS-1 antibody, it has been shown that SHPS-1 is strongly expressed in myeloid cells (macrophages, monocytes, granulocytes, dendritic cells) and neurons (Adams et al 1998).

Tsuda et al (1998) have shown indirectly the interaction between integrin engagement by ECM proteins and induction tyrosine phosphorylation of SIRPα and suggested that SIRPα might be a member of the group of proteins that are at focal adhesion contacts and undergo tyrosine phosphorylation in response to the interaction of integrins with the ECM. Oh et al (1999) have reported that SHP-2/ SIRPα complex can act as a critical positive component in integrin signalling. Inagaki et al (2000) have demonstrated that

SIRPα plays crucial roles in integrin-mediated cytoskeletal reorganization, cell motility and the regulation of Rho, and that it also negatively modulates growth factor-induced activation of MAP kinases.

SHPS-1 can act as a ligand for CD47 in various biological functions. Jiang et al (1999) have first discovered in mouse neuronal cells, that SHPS-1 and CD47 form a heterophilic binding pair that was involved in bi-directional signalling at the synapse. Studies by Seiffert et al (1999) revealed that the extracellular regions of human SIRPα adhere to a number of primary hematopoietic cells and cell lines and they also identified human CD47 as a prominent extracellular ligand.

The Ig domain of CD47 and the amino-terminal Ig domain of SHPS-1 can bind directly to each other and binding is sufficient to mediate transcellular bi-directional signalling via respective cytoplasmic regions (Lienard et al 1999; Oldenborg et al 2000; Han et al 2000; Babic et al 2000; Vernon-Wilson et al 2000; Latour et al 2001). The SIRPα/CD47 interaction was first described in mouse where the binding of recombinant mouse SIRP to cells could be blocked by a CD47 mAb (Jiang et al 1999) and later shown to be conserved in rat and human (Vernon-Wilson et al 2000). The SIRPα/CD47 pair represents a novel IgSF interaction between the three-IgSF domain SIRP and CD47 with its single IgSF domain. Most of the sequence variation among SIRPα members lies in their IgV domain and this difference may account for the varied binding affinity of SIRPα proteins to CD47. However, there may be additional ligands for a single receptor or group of receptors of SIRPα . SIRPβ does not bind to CD47 (Seiffert et al 2001).

On normal red blood cells, CD47-SHPS-1 complex appears important in preventing excess blood cell clearance or autoimmune hemolytic anemia (Oldenborg et al 2000; 2001). SIRPα was involved in a variety of cellular functions in different cell types. Some of these are shown in **Table 1.5.**

Table 1.5. SIRP α functions in different cells

Cell type	Effect	References
Fibroblast NIH3T3	MAPK activation	Kharitonenkov et al 1997
Rat alveolar macrophage	NO synthesis inhibition (NO production)	Adams et al 1998
Memory T cell	T cell proliferation	Brook et al 1998
NIH3T3	MAPK activation	Takada et al 1998
Myoblast C2C12	Myogenesis	Stofega et al 1998
Fibroblastic cells	Integrin-mediated tyrosine phosphorylation	Tsuda et al 1998
Macrophage	Scaffold protein	Timms et al 1999
Mast cell activation	Inhibition of cytokine synthesis	Lienard et al 1999
Neuron (Rat brain)	Synaptogenesis	Jianget al 1999
Rat cerebral cortical neuron	Neural survival	Araki et al 2000
Mouse splenic red pulp macrophage	Inhibition of phagocytosis, red blood cell self-recognition	Oldenborg et al 2000, 2001
Pro-B cells (Ba/F3)	Cell aggregation	Babic et al 2000
Myoblast	Differentiation	Kontaridis et al 2001
T cell	T cell activation	Seiffert et al 2001
Dendritic cells (DC)	Cytokine production	Latour et al 2001
Murine macrophage	Cell fusion	Saginario et al 1998; Han et al 2000; Vignery et al 2000
B Lymphocytes	B Lymphocyte-EC adhesion	Yoshida et al 2002
Neutrophil	PMN transmigration	Liu et al 2002

1.5.3.0. CD98, 4F2 antigen, Fusion Regulatory Protein-1 (FRP-1)

1.5.3.1. Structural features of CD98

Early studies of peripheral blood T lymphocytes implicated CD98 (4F2 antigen) in the regulation of cellular activation but did not define a specific function for this antigen (Haynes et al 1981). CD98 is a disulfide-linked ~125 kD heterodimer composed of a glycosylated ~85-kD heavy chain (HC) (designated CD98) and a nonglycosylated 40-kD light chain (LC). The 4F2 Ab recognized an antigenic determinant on the polypeptide backbone of the HC. The cDNA for the HC of human CD98 was cloned (Lumadue et al 1987; Quackenbush et al 1987; Teixeira et al 1987). The predicted protein encodes a glycosylated protein of 529 AA containing a single transmembrane segment (amino acids 83-106). The protein has an extracellular carboxyl terminus and an internal amino terminus and has been classified as a type II transmembrane GP (Warren et al 1996).

So far, six alternative cDNAs encoding associated light chains have been identified. Four of which are associated with L-type amino acid transport activity (LAT) (Verrey et al 1999). The light chains are able to associate to the heavy chain through a disulfide bond, giving rise to different heterodimers (Mastroberardino et al 1998). The light chains are markedly hydrophobic proteins of 502-535 AA and contain 12 putative transmembrane domains. They are linked to CD98 HC through an extracellular cysteine that participates in disulfide bound formation (Pfeiffer et al 1998). All light chains identified function as AA transporters when associated with the HC and the substrate specifity of the heterodimer depends on the nature of the LC. The molecular features, tissue distributions and functional properties of the six LCs, LAT-1 (Mastroberardino et al 1998); LAT-2 (Pineda et al 1999; Segawa et al 1999); y+LAT-1, y+LAT-2 (Torrents et al 1998); xCT (Nakamura et al 1999) and SPRMI (Mastroberardino et al 1998) have been demonstrated (Deves and Boyd 2000).

1.5.3.2. CD98 expression

CD98 is expressed ubiquitously and highly conserved between species. Although it is expressed at low levels on the surface of quiescent cells, CD98 expression is rapidly up-regulated after cellular activation (Azzarone et al 1985; Suomalainen 1986; Parmacek et al 1989). For instance, CD98 is strongly expressed on human embryonic and newborn fibroblasts but expression gradually diminishes from 100 to 1% on fibroblasts from normal adults (Azzarone et al 1985). CD98 is reconstituted to high levels on many tumor cell types (Dixon et al 1990) and furthermore, overexpression of CD98 on NIH3T3 cells has been shown to result in cellular transformation (Hara et al 1999). CD98 is also expressed on normal proliferating tissue such as the basal layer of squamous epithelia (Patterson et al 1984) and on cells having secretion or transport functions (Tabata et al 1994).

1.5.3.3. CD98 functions

Previous studies suggested that an intercellular signalling pathway mediates the action of CD98 (Warren et al 1996; Okamoto et al 1997; Tabata et al 1997). Tyrosine kinase inhibitors inhibit CD98 activity in hematopoietic cells, suggesting that tyrosine kinase activity may be an early signal transduction pathway activated by CD98 (Warren et al 1996). There is also evidence that CD98 is involved in the regulation of intracellular calcium concentration through the Na^{+}/ Ca^{2+} exchanger, although its effect seems to be cell type specific (Michalak et al 1986; Posillico et al 1987; Freidman et al 1994).

CD98 HC appears to be correlated to cell proliferation in several different tissues such as skin, on a variety of different cell types (Gottlieb et al 1985), and intestinal epithelium (Azzarone et al 1986). CD98 HC has also been suggested to play a role in cell differentiation in hemato-lymphopoietic cell (Warren et al 1996). It has been demonstrated that HC acts as unique and highly specific regulator of integrin affinity (Fenczik et al 1997).

Anti-CD98 promotes monocyte-monocyte interactions that ultimately lead to polykaryon (multinucleated giant cell) formation (FRP-1), a phenotype associated with chronic inflammatory conditions (Ohta et al 1994; Ohgimoto et al 1996). Compelling evidence also exists for connection between CD98 and virus-induced cell fusion. Antibodies to HC of CD98 promote cell fusion induced by Newcastle disease virus and by human immunodeficiency virus (HIV) (Ohgimoto et al 1995).

The dimers, obtained from linking of CD98 HC with five different LCs, are the minimal functional units for transport activity. All the CD98 HC linked transporters (except rat LAT-2 which is able to perform net transport) appear to function as highly coupled amino acid exchangers, but the specificity depends on the associated light chain (Pineda et al 1999).

Rintoul et al (2002) have recently shown that cross-linking CD98 stimulates PI3-kinase, as well as its product PI (3,4,5) P3, and its downstream effector, PKB. The finding that treatment with 4F2 activated PI 3-kinase, PI (3,4,5) P3, and PKB in a PI3-kinase-dependent manner suggests that cross-linking CD98 promotes integrin-like intracellular signalling.

1.5.3.4. CD98-integrin interactions

Several lines of evidence suggest that CD98 may modulate the functions of integrins. For example, the β1 integrins may act together during T cell co-stimulation (Warren et al 2000). A genetic screen revealed that CD98 may indirectly influence integrin affinity for ligands. CD98 HC was shown to rescue a dominant suppression of integrin function, which results from overexpression of a fragment of the β subunit. The experimental strategy used a Chinese hamster ovary (CHO) cell line that expresses a chimeric integrin, which is constitutively active. The chimera contains an extracellular and transmembrane domains of $\alpha_{IIb}\beta_3$ integrin fused to the cytoplasmic domains of α6Aβ1 integrin; it thus has the ligand binding properties of $\alpha_{IIb}\beta_3$ and is activated through α6Aβ1 cytoplasmic binding domain. Overexpression of the β1 cytoplasmic domain in

the form of Tac-chimera resulted in suppression of integrin signalling. Suppression was interpreted as resulting from the titration by the overexpressed β 1 subunit of proteins forming an " integrin activation complex". Consistently, expression of Tac-β1 in fibroblast cell lines interfered with cell spreading, migration, fibronectin matrix assembly and integrin activation. Cotransfection of the cells containing the chimeric integrin with CHO cell cDNA expression library and Tac-β1 showed that CD98 HC was able to complement the suppression and suggested that CD98 is a regulator of integrin function (Fenczik et al 1997).

It has been shown that anti-CD98 mAb appeared to modulate integrin-dependent adhesion (Fenczik et al 1997; Chandrasekaran et al 1999; Deves et al 2000). In addition, Abs to both CD98 and α3β1 integrin prompted cell fusion (Ohta et al 1994) and Abs to β1 and β2 integrins blocked monocyte cell-cell function and aggregation functions induced by anti-CD98 mAb (Tabata et al 1994).

It has been demonstrated that like CD98, the α3β1 integrin has been implicated in amino acid transport (McCormack et al 1995). Kolesnikova et al (2001) have demonstrated for the first time that CD98 constitutively and specifically associates with intact β1 heterodimers. Specific CD98-integrin interaction occurs in the context of low density protein-lipid microdomains, possibly resembling lipid rafts (Simons and Ikonen 1997; Brown and London 1998).

It has also been reported that the pro-adhesive and chemotactic activities of the extracellular matrix protein, TSP-1, for breast carcinoma cells are mediated by α3β1 integrin and regulated by CD98 HC and insulin-growth factor (Chandrasekaran et al 1999). Integrin α3β1 is maintained in an inactive or partially active state in these cell lines, but can be activated by exogenous stimuli including serum, insulin, and ligation of CD98 HC by divalent Abs. The effect of CD98 HC clustering on integrin activation may be due to clustering of associated integrin or may involve specific signal transduction from CD98 HC. Although a functional association between CD98 and β1

integrin in the cell membrane has been described previously (Fenczik et al 1997; Warren et al 2000), until recently there was no clear data showing physiological interaction between CD98 and β1 integrin. Recently, Zent et al (2000) have shown that CD98 can associate with isolated cytoplasmic portions of some β1 integrin isoforms. Studies from other groups showed the similar results in different cell lines and chimeric β1integrins (Merlin et al 2001; Kolesinkova et al 2001; Rintoul et al 2002). Very recently, Miyamoto et al (2003) have provided the first evidence of a specific association between CD98 and β1integrin (e.g α4β1) in human T lymphocytes by using immunofluorescence and immunoprecipitation.

1.5.3.5.0. CD98 ligands

In addition to integrins, Galectin-3 and CD147 have been demonstrated as ligands for CD98.

1.5.3.5.1. Galectin-3, Mac-2

The galectin family of proteins consists of β-galactoside binding lectins containing homologous carbohydrate recognition domains (CRDs). Membership in this family required fulfilment of two key criteria: (a) binding affinity for β-galactosides, and (b) conserved sequence elements in the carbohydrate-binding site. To date, 14 mammalian galectin family members have been identified (Barondes et al 1994 a, b; Hughes 2001), with the most extensively studied members being the MW 14 kD (galectin-1) and the MW 30 kD (galectin-3) proteins. Galectin-3 has previously been designated variously as Mac-2, a macrophage surface antigen (Ho and Springer 1982), IgE-binding protein (ε BP), for its IgE-binding activity (Liu et al 1985; Albrandt et al 1987; Robertson et al 1990), CBP35 (carbohydrate-binding protein 35)(Roff and Wang 1983), CBP30 (Mehul et al 1994), L-29 (Leffler et al 1989), and L-34 (Raz et al 1989, 1991).

Galectin-3 consists of two distinct structural domains: an amino-terminal half is made of a small N-terminal domain consisting of proline- and glycine-rich repeats, and the

carboxyl-terminal half with a globular structure encompassing the carbohydrate-binding site (Hermann et al 1993). Galectin-3 is a non-glycosylated protein and, as a member of lectins, is specific for β-galactosides, to which they bind in a Ca^{2+} independent manner (Barondes et al 1994a). Like other members of this family, galectin-3 lacks a signal sequence, and is secreted via a pathway independent of the Golgi apparatus (Lindstedt et al 1993; Sato et al 1993). In addition to the characteristic carbohydrate recognition domain, galectin-3 has a unique amino-terminal domain, which can be digested by collagenase and matrix metalloproteinases-2 and –9 (MMP-2 and MMP-9) in vitro (Hsu et al 1992; Ochieng et al 1994, 1998). The amino-terminal domain of galectin-3 is thought to be important for self-oligomerization, and hence for its functional multivalency (Massa et al 1993).

Galectin-3 normally distributes in epithelia of many organs and various inflammatory cells, including macrophages, as well as dendritic cells and Kupffer cells (Flotte et al 1983). Galectin-3 is expressed in numerous cell compartments of the eukaryotic cells, and appears to play specific functions at each of these locations. Galectin-3 has been found in the cytosolic compartment (Gritzmacher et al 1988), where it participates in the control of apoptosis, possibly through an interaction with the Bcl-2 protein (Akahani et al 1997). Depending on cell types and proliferative states, a significant amount of this lectin can also be detected in the nucleus, where its expression is correlated with cell proliferation (Moutsatsos et al 1987). In addition, one study has demonstrated that galectin-3 could facilitate splicing of pre-mRNA (Dagher et al 1995). Galectin-3 is also associated with the plasma membrane (Sato and Hughes 1994), and can be secreted extracellularly (Sato et al 1993,1994; Lindstedt et al 1993). At these locations, it mediates cell-cell and cell-matrix interactions through its ability to bind to a variety of glycoconjugates (Ochieng et al 1992; Sato and Hughes 1993; Inohara and Raz 1995, Inohara et al 1996; Warfield et al 1997).

The implication of galectin-3 during malignancy progression has been first indirectly suggested by numerous studies focusing on the expression of this molecule in several

cancers, such as colon (Lotz et al 1993), breast (Castronovo et al 1996), ovarian (Van den Brule et al 1994), and uterine (Van den Brule et al 1996) carcinomas. Contradictory observations have been reported (Castronovo et al 1999). Recent studies indicate that these discrepancies could be explained by a more rigorous analysis of galectin-3 expression and subcellular localization, as particularly described in the case of colon cancer. Preliminary data indicated that decreased (Lotz et al 1993) or increased (Irimura et al 1991; Schoeppner et al 1995) expression of galectin-3 in colon carcinoma cells correlates with disease progression. A recent study demonstrated initial down-regulation, with increased cytoplasmic expression of galectin-3 at more advanced stages (Sanjuan et al 1997). Additionally, Lotz et al (1993) reported that exclusion of galactin-3 from the nucleus correlates with progression from normal mucosa to adenoma and carcinoma.

It has been previously shown that in the midgestation mouse embryo, the major site of galectin-3 expression is the cartilage (Fowlis et al 1995). Subcellular distribution of the protein in growth plate chondrocytes of fetal and neonatal mice is mainly cytoplasmic, with high amounts detectable in mature and early hypertrophic cells (Colnot et al 1999). These results suggested an intracellular function for galectin-3 in terminal differentiation of chondrocytes. Galectin-3 is recognized in the cytoplasm of proliferative, mature, and hypertrophic chondrocytes in the growth plate cartilage of developing long bones (Colnot et al 1999). Colnot et al (1998, 2001) have also described several defects in chondrocyte differentiation in the long bones of galectin-3 null mutant embryos, both at the cellular and at the molecular level. The most striking abnormalities were observed at chondrovascular junction that indicated an acceleration of cell death in the absence of coordinated angiogenesis.

Several specific cell surface receptors including integrins and other cell adhesion molecules and matrix components including certain glycoforms of laminin and fibronectin have been shown to bind galectin-3 in vitro (**Table 1.6**), supporting the idea that galectins binding to such receptors in vivo have diverse and important extracellular functions (Liu et al 1993; Barondes et al 1994; Kasai and Hirabayashi 1996).

Galectin-3 binds to CD98 HC in both a human T cell line (Jurkat) and in murine macrophages (WEHI-3) (Dong and Hughes 1996), but a direct link between galectin-3 binding to this surface antigen and its biological effect has not been demonstrated. They suggested that galectin-3 may play a role in cell-cell adhesion (Bao and Hughes 1995; Dong and Hughes 1996). The same authors have used a mutant epithelial cell line and shown that galectin-3 binding, but not the binding of other lectins tested, is thus specifically abolished (Bao and Hughes 1999). In these mutant epithelial cells, growth is markedly aberrant, suggesting that one role of the normal galectin-3 receptor, at least in epithelia, is to transduce signals following lectin binding that act as negative regulators of cell growth thus allowing orderly elongation and branching morphogenesis of the epithelium in culture (Bao and Hughes 1999).

Table 1.6. Ligands for galectin-3 (Hughes 1999)

	Ligand	Source/cells	Reference/s
Matrix	Laminin-1, 5, 10/11?	Placenta	Ochieng et al 1995
	Fibronectin	Fetal	Sato et al 1992
	Tenascin-C, R	Brain	Probstmeir et al 1995
	Mac-2BP	Brain, breast tumors	Rosenberg et al 1991; Koths et al 1993
Integrins	α1β1	Adenocarcinoma	Ochieng et al 1998
	αMβ2 (CD11b/18)	Macrophage	Dong and Hughes 1997
Membrane	N-CAM, L1, MAG	Mouse Brain	Probstmeir et al 1995
	LAMP-1, 2, Mac-3	Ubiquitous	Dong and Hughes 1997
	CEA	Tumor antigen	Ohannesian et al 1995
	NCA-160	Neutrophils	Yamaoka et al 1995
	CD98 HC	Macrophages, Jurkat	Dong and Hughes 1997
Others	Mucin	Colon cancer	Bresalier et al 1996
	AGE	Ubiquitous	Vlassara et al 1995
	IgE	Mast cells	Liu et al 1993
	IgE receptors		
	Fc receptors	Various	Liu et al 1993
	LPS	Endotoxins	Mey et al 1996

N-CAM and **L1**, neural adhesion molecules; **MAG**, myelin associated glycoprotein; **LAMP 1, 2**, lysosomal associated-membrane proteins; **Mac-3**, a mouse macrophage differentiation antigen; **CEA**, carcinoembryonic antigen; **NCA-160**, A neutrophil receptor and human carcinoembryonic antigen-related glycoprotein; **AGE**, Advanced glycation end (AGE) products, the reactive derivatives of neo-enzymatic glucose-protein condensation reactions.

1.5.3.5.2. CD147, Extracellular Matrix Metallo Proteinase Inducer (EMMPRIN)

EMMPRIN activity was initially discovered in a series of studies performed by Dr Biswas et al (1984, 1985) in which they demonstrated stimulation of interstitial collagenase (MMP-1) production in cultures of tumor cells and fibroblasts. These studies led to identification of a factor (TCSF, tumor cell-derived collagenase stimulatory factor) that is associated with the surface of tumor cells and that stimulates synthesis in human fibroblasts (Biswas and Nugent 1987). In various species GPs homologous to CD147 have been identified (**Table 1.7**) including basigin and M6. It was proposed that M6 antigen might stimulate MMP production in arthritis as well as cancer (Biswas et al 1995).

CD147 is a highly glycosylated transmembrane GP of 50-60 kD having typical features of a type integral membrane protein of the IgSF. It contains two extra-cellular Ig domains, a transmembrane domain, and a 39-amino acid cytoplasmic domain (Biswas et al 1995). Within 246 amino acid residues deduced from cDNA cloning five potential N-glycosylation sites have been identified. The protein belongs to the IgSF having two Ig domains with characteristic sandwich β–sheets stabilized by disulfide bridges. Within the postulated single transmembrane region three leucines are repeated every seventh amino acid residue. This sequence is characteristic of leucine zippers found in DNA binding proteins and has been identified in the glucose transporter and ion channels (McCormak et al 1989; White et al 1989). In addition, the presence of a glutamic acid residue in the transmembrane region suggested that the protein might functionally interact with other membrane proteins (William et al 1988).

Structural analyses have demonstrated that the transmembrane and cytoplasmic domains of EMMPRIN are highly conserved among species, suggesting that these regions are of functional importance. The conserved properties of the trans-membrane region, i.e., a central charged residue and a leucine zipper motif, suggested that intramembrane interactions with other proteins are likely to occur (Fossum et al 1991; Kasinrek et al 1992; Miyauchi et al 1991).

Accordingly, interaction of EMMPRIN with several other components of cell membranes has been demonstrated. Recent reports have identified some striking parallels between CD147 and CD98. CD147 associates physically with β1 integrins in the membrane (Berditchevski et al 1997), as does CD98 with isolated cytoplasmic β1 domains (Zent et al 2000). Moreover CD147 was recently found to co-immunoprecipitates with β1 integrins (α3β1 and α6β1), and co-localize with these integrins in areas of cell-cell contact, within the plasma membrane of HT1080 fibrosarcoma cells (Berditchevski et al 1997).

CD147 molecule is broadly expressed on human peripheral blood cells, endothelial cells, and cultured cells of hemopoietic and non-hemopoietic origin. In T cells, its expression level is dependent on the differentiation state. Thymocytes are strongly expressed CD147 (Kirsch et al 1997). Significant expression of CD147 has also been reported in neoplasms of the bladder, liver, and lung (Muraoka et al 1993).

CD147/EMMPRIN was initially identified on the surface of human cancer cells and shown to stimulate adjacent stromal cells to produce and activate several MMPs (Guo et al 1998; Guo et al 1997; Sun and Hemler 2001), including MMP-1, MMP-2, MMP-3, membrane type 1MMP (MT1-MMP), and MT-2-MMP (Guo et al 1997; Sameshima et al 2000).

MMP synthesis in fibroblasts in response to stimulation by EMMPRIN is a relatively slow process, taking 24-48 h to reach maximum (Lim et al 1998). It has been noted that different fibroblast populations differ widely in their response to EMMPRIN (Guo et al 1997); this difference may be is related to varying degrees of expression of a putative receptor.

Treatment of human lung fibroblasts with purified EMMPRIN leads to phosphorylation of the mitogen activated protein (MAP) kinase p38, and induction of MMP-1 synthesis is partially dependent on activity of this kinase but not extracellular signal-regulated kinase-1/2 (ERK1/2) or stress activated protein kinase/c-jun aminoterminal kinase

(SAPK/JNK) (Lim et al 1998). EMMPRIN stimulation of MMP production in fibroblasts is also dependent on N-glycosylation of its extracellular domains. Since partially glycosylated EMMPRIN is relatively inactive, it is likely that all three glycosylation sites in the two Ig domains must be occupied for full activity (Guo et al 1997; Sun and Hemler 2001). EMMPRIN also acts as a chaperone for assembly of lactate transporters in the plasma membrane (Kirk et al 2000; Wilson et al 2002).

A knock out has been produced in which basigin, the murine homolog of EMMPRIN, is lacking (Igakura et al 1998). Basigin-null embryos exhibit numerous defects, including inefficient implantation. Since MMPs are known to be involved in implantation (Alexander et al 1996; Vu and Werb 2000), defective implantation may result from misregulation of MMP production due to lack of basigin stimulation.

Many physiological functions in which EMMPRIN may participate have also been suggested by other studies. Systems in which EMMPRIN is likely to be important include chaperone functions (Kirk et al 2000), calcium transport (Jiang et al 2001), neutrophil chemotaxis (Yurchenko et al 2002), and blood brain barrier development (Schlosshauer 1993). A likely molecular function for EMMPRIN, other than induction of MMP production, is mediation of adhesive cell interactions (Toole 2003).

Recent work has shown that annexin II acts as a binding partner for CD147 (Guo and Toole unpublished results; Toole 2003). Annexin II is a Ca^{2+}-dependent, lipid-binding protein that is commonly found intracellularly but, under some circumstances, is also a prominent cell surface component, e.g on endothelial cells (Hajjar et al 1996), neurons (Jacovina et al 2001), and cancer cells (Tressler et al 1993). Annexin II usually occurs as a tetramer containing two subunits of a 36-kD heavy chain and two subunits of an 11 kD light chain.

Table 1. 7. Alternative names for CD147 (EMMPRIN)

Name	References
Tumor cell-derived collagenase stimulatory factor (TCSF)	Kataoka et al 1993 Nabeshima et al 1991
5A11 (chick)	Fadool and Linser 1993b
HT7 (chicken)	Seulberger et al 1990, 1992
Neurothelin (chicken)	Schlosshauer and Herzog 1990
Basigin (mouse)	Miyauchi et al 1991
MRC OX-47 (rat)	Fossum et al 1991
M6 (Human leukocyte activation antigen)	Kasinrek et al 1992
EMMPRIN (Extracellular Matrix Metalloproteinase Inducer) (human)	Biswas et al 1995
CE9 (rat)	Nehme et al 1993
gp42 (mouse)	Altruda et al 1989
Hab 18G (Human hepatoma)	Jiang et al 2001

1.6.0. HYPOTHESIS AND AIMS

Articular chondrocytes are subjected in vivo, to frequent and repetitive mechanical loads (Knudson and Loeser 2002). Chondrocyte mechanotransduction is activated in normal human articular chondrocytes by 0.33 Hz cyclical mechanical stimulation, which results in membrane hyperpolarisation and changes in gene expression, has been partially elucidated. The chondrocyte mechanotransduction is integrin-mediated and involves tyrosine kinases, PKC activation, IL-4 secretion and substance P secretion.

It has been shown that $\alpha 5\beta 1$ integrin has a critical role in chondrocyte mechanotransduction and may act as a chondrocyte mechanoreceptor (Wright et al 1997; Salter et al 2001). Integrins are able to link the ECM with the cytoskeleton and cell signalling proteins (Hynes 1992; Shyy and Chien 1997). Since integrins, generally lack intrinsic enzymatic activity of their own, therefore they must interact with other proteins to generate signals (Damsky and Werb 1992). Evidence suggests that signal transduction through integrins depends on both integrins and integrin-associated proteins (Wright et al 1996; Hogg and Porter 1998; Shattil et al 1998). There are a variety of cell membrane molecules such as CD47 and CD98 that can interact with different types of integrins in each cell type and are potentially involved in regulating integrin-mediated mechanotransduction (Hogg and porter 1998; Shattil et al 1998).

Hypothesis

CD47 and CD98 and their ligands have role(s) in chondrocyte mechanotransduction

Aims

The aims of this work were:

I) To establish in vivo expression of CD47, CD98 and galectin-3 in normal and OA articular cartilage (chapter 3).

II) To identify potential roles for CD47, CD98 and ligands in chondrocyte mechanotransduction (chapters 4 and 5).

CHAPTER TWO

2.0. MATERIALS AND METHODS

2.1. Tissue sources and handling

All human articular tissues were obtained during arthroplasty from patients who were undergoing knee and hip joint surgery and also from ankle joints from below knee amputation for peripheral vascular disease. The tissues were handled in a class I tissue culture hood. Laboratory coat, gloves and oversleeves were worn when handling biological tissue. If a given tissue was found to be infected, the joint was incinerated and hood fumigated.

2.2. Assessment of cartilage for osteoarthritis (OA)

The articular surface was assessed and graded macroscopically for the presence or absence of osteoarthritis using the Collins/McElligott system (Collins McElligott 1960; Midwood and Salter 1998) (**Table 2.1**). Two sets of full thickness pieces of cartilage were taken for histological confirmation of normal cartilage or OA changes. One set was snap frozen in liquid nitrogen immediately and stored in cryovials at –80°C for frozen sections. Another set was fixed in 4% formalin for embedding in paraffin wax.

Table 2.1. Collins macroscopic grading of osteoarthritis (OA)

COLLINS GRADE	CRITERIA
0	No cartilage degeneration
I	Limited Patches of fibrillation or softening in central area of articular cartilage No area of whole thickness cartilage loss No recognisable marginal osteophytosis No detectable synovial inflammation
II	Large areas of fibrillation and fissuring without denuding bone. Early marginal chondro-osteophytosis
III	At least one area (30% or less) of whole thickness cartilage loss with extensive fibrillation, fissuring and bone exposure. More generalized synovial disease and obvious marginal osteophytosis
IV	Extensive cartilage loss and bone exposure Eburnation and bone grooving, destruction of intra-articular ligaments, and fibrosis or atrophy of synovial fringes.

2.3.0. IMMUNOHISTOCHEMISTRY (IHC)

2.3.1. Avidin-Biotin-Conjugated (ABC) immunoperoxidase method with frozen sections

Cryostat sections (4-6 μm) were cut using a Bright cryostat and mounted on poly L-lysine (PLL) coated glass slides. Then the sections were allowed to come to room temperature and fixed with acetone for 10 minutes. The sections were kept at –20 °C before use. Before staining, the sections were left in room temperature for 30 minutes and washed by phosphate buffered saline (PBS) for 10 minutes. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in distilled water for 10 minutes, followed by washing in running tap water.

To avoid non-specific binding of antibodies, sections were incubated with normal rabbit serum (NRS) (Diagnostics Scotland) diluted 1 in 5 in PBS (NRS/PBS, 1:5) for 30 minutes (100μl per section). The primary antibody was diluted in NRS/PBS at the indicated concentration. Sections were incubated with diluted antibody (50μl per section) for 60 minutes at room temperature. After twice washing with PBS for 5 minutes each, sections were then incubated with NRS/PBS for 30 minutes. Diluted appropriate secondary biotinylated antibody (DAKO) in NRS/PBS (1:300) was then added for one hour at room temperature and followed by a further two washes with PBS. Sections were then incubated with avidin-biotin-conjugated (ABC) (3 drops on each slide) for 30 minutes at room temperature. Slides were then washed twice with PBS and antibody binding visualized using diaminobenzidene (DAB) with 0.1% hydrogen peroxide (H_2O_2) as the peroxidase substrate for 3 min. The sections were then washed with running tap water, counterstained with Harris's hematoxyllin for 30 sec, made blue by dipping in Scott's water (STWS), dehydrated in a graded ethanol series, cleared in xylene, and mounted. All steps were performed at room temperature. The procedure was carried out using a Shandon Sequenza (Thermo Shandon coverplate™) to achieve consistency of staining quality and to decrease sections from floating off.

2.3.2. Paraffin sections

Before staining, paraffin sections of normal and OA cartilage were deparaffinized in xylene for 5 minutes and rehydrated in descending grades of alcohol to water. To block endogenous peroxidase activity sections were incubated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 15 minutes. Then sections were rinsed in tap water and placed in a vertical staining rack in a water container to prevent sections from drying out.

2.3.2.1. Antigen Retrieval (AR)

The metal rack containing dewaxed, rehydrated sections was placed over the top of the 50x10 mm magnetic bead in 2-litre glass beaker of the Jenway hot plate stirrer. It was filled with 1 litre 1 mM EDTA buffer solution containing 0.1% Tween 20 (Invitrogen Life technologies). After leaving the buffer on stirrer for 30 minutes the pH was adjusted to 8.00 by adding 1 M NaOH. Then the heater was turned on and temperature set to 65°C for overnight incubation. The next day, the rack containing sections was placed in a water tap container until the automatic immunostaining instrument was ready.

2.3.2.2. Immunostaining

The immunostaining method was applied using DAKO ChemMate ^{TM} Detection Kit, Peroxidase/DAB, and Rabbit/Mouse with the DAKO automatic immunostaining on the TechMate^{TM} instruments. Stained sections were washed in tap water, dehydrated in a graded ethanol series, cleared in xylene, and mounted. All steps were performed at room temperature.

2.3.3. Procedure controls

The specificities of the staining were confirmed by controls. In all experiments of IHC a negative control was used. Negative controls were provided by omitting the primary antibody by substituting NRS/PBS or non-immune immunoglobulin Gs (IgG) from the

same species for the primary antibody. Cryostat or paraffin sections of normal human tonsil were used as positive controls.

2.3.4. Optimal dilution of primary antibody

For each antibody preliminary studies were performed to optimise staining procedures, and find the standard working titre of antibody. In subsequent studies, sections were treated with the dilution of antibody that maximised specific staining and minimized non-specific reactivity. Various dilutions of each antibody (1:10, 1:50, 1:100, 1:500, and 1:5000) were applied and compared. Antibodies and the optimal dilutions used in immunohistochemistry experiments are shown in **Table 2.3**.

2.3.5. Positive staining

Positive staining was recognized as a dark brown colour associated with the cytoplasmic membrane or cytoplasm. As chondrocytes were either strongly positive or negative no grading of the positivity was made. Areas that showed folding of the cartilage or exhibited non-specific background staining were not scored.

2.3.6. Zones of full thickness articular cartilage and microscopic evaluation

For immunohistochemical evaluation, the normal and OA cartilage were classified in three groups including, normal (Collins grade 0), smooth intact cartilage; mild OA (Collins grade I and II); and severe OA (Collins grade III and IV). To evaluate the results immunoreactivity was assessed by the relative numbers of positive and negative chondrocytes in different zones of cartilage including, surface zone (3 cell thick layer), superficial zone (top 25%), middle zone (50% thick between superficial and deep) and deep zone (bottom 25%). Because in severe OA, the surface is substituted by fibrillation and clustering chondrocytes, the immunoreactivity of clones was assessed.

Immunoreactivity was scored on a five-point scale. In this scoring system, 1, no staining; 2, more cells negative than positive; 3, equal positive and negative; 4, more cells positive than negative; and 5, all cells positive. This scale was applied for each zone separately. The criteria were used for immunohistochemical evaluation of sections are summarized in **Table 2.2.**

Only sections of intact cartilage that clearly displayed the correct vertical orientation and thus contained various zones were considered for further examination. Sections were excluded for evaluation because of artefact alterations or because of excessive non-specific background staining. Evaluation was carried out three times for each section. The cases studied are shown in **Table 2.3.**

Table 2.2. The histological/histochemical system was used to assess the immunohistochemical staining of normal and OA sections

1. Classification of healthy and diseased joints	
A. Normal	Smooth intact cartilage (Collins grade 0)
B. Mild OA	Roughening and cartilage loss (Collins grades I & II)
C. Severe OA	Areas of complete cartilage loss and bone exposure (Collins grades III& IV)
2. Zonation of intact cartilage with no degeneration	
a. Surface	Three cell thick layer
b. Superficial	Top 25%
c. Mid	Middle 50%
d. Deep	Bottom 25%
3. Zonation of cartilage with degenerative changes	
a. Assessment degenerative surface	Clones immunostaining
b. Superficial	Top 25%
c. Mid	Middle 50%
d. Deep	Bottom 25%
4. Scoring scale	
Criteria	Grade
A. No staining	1
B. More cells negative than positive	2
C. Positive cells = Negative cells	3
D. More cells positive than negative	4
E. All cells positive	5

Table 2.3. Cases studied and antibodies used for IHC assessment of CD47, CD98 and galectin-3 in human articular cartilage

	CD47	**CD98**	**Galectin-3**
Antibody/ Dilution	Bric 126, mouse mAb, 1:5000	4F2, mouse mAb 1:50	9C4, mouse mAb 1:400
No. of cases (sex)	22 (11M/9F)*	20 (10M/9F)*	27 (13M/9F)*
Knee joint	13(7M/4F)*	11 (6M/4F)*	9 (4M/2F)*
Hip joint	9(4M/5F)	9 (4M/5F)	14 (6M/6F)*
Ankle joint	-	-	4 (3M/1F)
Age range (mean)	Knee: 44-82 (66) Hip: 74-90 (81)	Knee: 39-85 (67) Hip: 73-90 (81)	Knee: 57-78 (77) Hip: 67-92 (78) Ankle: 71-80 (76)

*, more information not recorded .

M, male; **F**, Female

2.4. CHONDROCYTE CULTURE

2.4.1. Primary articular chondrocytes

Isolated articular cartilage from hip joint, different anatomical regions of the knee joint and ankle were pooled (**Table 2.4**). Normal and OA cartilage were kept separately. Cartilage was cut into small pieces with scalpel and incubated in anti-microbial solution (see appendix II) for 1 hour at room temperature (20° C). Then the cartilage fragments were washed twice with sterile PBS and sequentially digested by 0.25% trypsin (Gibco, Paisley, UK) for 30 minutes and 3 mg/ml collagenase type H (Sigma) for up to 48 hours or until digestion was complete in 95% air/ 5% CO_2 incubator at 37° C.

Extracted chondrocytes were collected using a sterile pastette and strained through a sterile strainer to remove undigested cartilage fragments. Then the cell suspension was centrifuged at 1000 revolution per minute (rpm) for 10 minutes, the supernatant discarded and the pellet resuspended in PBS. This was repeated twice more to give three PBS washes. The cells were then resuspended in 10ml Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Sigma), 100 I.U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco). Suspended cells were then filtered through a 70 micron cell sieve (Falcon, Becton Dickinson UK) to remove large cell clumps and pieces of debris. The cells were counted using a hematocytometer. Cells were seeded in complete media at a density of $1x10^4$ cells/ml (for electrophysiology) or $2x10^5$ cells/ml (for protein extraction) in 58mm petridishes (Nunc, USA) and cultured in a humidified 5% CO_2 atmosphere at 37° C. Primary, non-confluent, 1-2 week cultures of chondrocytes from normal or osteoarthritic articular cartilage were used in all experiments. Morphologically, the cells studied were typically flattened with a polygonal cell shape and did not show the fibroblastic appearance. The day (16-20 hrs) before experiments were carried out, the culture medium containing serum was replaced by serum-free medium.

Table 2.4. Different joints, their anatomical regions and abbreviations used in this study

Joint/Anatomical region	Normal	OA
A. Knee joint		
1. Femoral condyle	FN	FA
2. Tibial plateaux	TN	TA
3. Patellofemoral	PN	PA
B. Ankle joint		
1.Tibia	Tib	-
2.Tallus	Tal	-
3. Fibula	Fib	-
C. Hip joint		
1. Femoral head	F/H, N	F/H, OA

2.4.2. Immortalised human chondrocyte cell line

The human chondrocyte cell line was generously provided by Dr Goldring (Beth Israel Deaconess Medical centre, New England Baptist Bone and Joint Institute, and Harvard Medical School, Boston, Massachusetts). C20/A4 cells were established by transfection of primary cultures of juvenile costal chondrocytes with vectors encoding Simian Virus 40 large T antigen and selection in suspension culture over agarose (Goldring 1994). Stable cell lines were generated that exhibited chondrocyte phenotype, continuous proliferative capacity in monolayer culture in serum-containing medium, and expression of mRNAs encoding chondrocyte-specific collagen II, IX, and XI and proteoglycans when cultured in an insulin-containing serum substitute (1% v/v, Nutridoma-SP, Boehringer Mannheim Biochemicals, Germany).

Using a sterile needle with syringe, different cryopreserved passages (P7, P8, P10) of C20/A4 cells in freezing medium (see below) were suspended in 45 ml Dulbecco's modified Eagle's medium (DMEM) (Sigma) in a large culture flask (Nunc). The medium was supplemented with 10% fetal calf serum (Sigma), 100 I.U/ml penicillin and 100 μg/ml streptomycin (Gibco). Then the cultured cells were grown in a 95% air and 5% CO_2 at 37°C. Every 3 days the medium was changed (feeding).

2.4.2.1. Splitting of adherent cells for seeding or storage cells

To seed at a defined density or cryopreserve cells, the medium was poured off and adherent cells were washed with sterile PBS. Then the cells were incubated with 2 ml trypsin (Gibco, Paisley, UK) for 5 minutes or 10 ml 0.02% EDTA for 10 minutes at 37°C. The flask was tapped to release cells and detachment or rounding up was checked using phase contrast microscope. The floating cells were then resuspended in complete media and centrifuged at 1000 rpm for 10 minutes at 10°C. The supernatant was discarded and the cell pellet resuspended in sterile PBS and centrifugation was repeated.

For seeding, the final cell pellet was resuspended in 5-10 ml media and filtered through 70-micron cell sieve to remove clumps. The cells were counted using a haematocytometer and then seeded at $2x10^5$ cells/ml (for protein extraction) in 58mm petridishes (Nunc, USA) in complete media.

To cryopreserve cells, the cell pellet was resuspended in 1 ml (1ml per flask) freezing medium, consisting of 50% DMEM, 40% FCS and 10% DMSO (dimethyl sulfoxide) (Sigma). Suspended cells in freezing media were immediately aliquoted into labeled cryogenic storage vials (Nunc). To start freezing slowly, the cryovials were wrapped in blue roll and frozen in –80 $^{\circ}$C freezer overnight. The next day, vials were transferred to liquid nitrogen storage.

2.5.0. Induction of cyclical mechanical stimulation

The system used to mechanically stimulate the cells (**Figure 2.1** and **2.2**) is a modification of that described previously (Wright et al 1992, 1996). Flexible, plastic 58-mm tissue culture dishes (Nunc, USA) were placed in a sealed polypropylene stimulation chambers with inlet and outlet ports. The culture dish was supported on six horizontal pins inserted into a cylinder attached to the base of the pressure vessel (**Figure 2.1** and **2.2**). The culture dish made a tight fit with the top of the cylinder, the fit being further enhanced by the presence of a rubber O – ring. An 8 ml volume space (chamber B) existed between the base of the culture dish and the base of the chamber. Gas entered the space from the main chamber (chamber A, volume 89ml) via 18 holes each 2.2 mm in diameter in the cylinder. The chamber was pressurized with Nitrogen (or Helium) gas from a cylinder, the frequency being dictated by an electronic time controlling the inlet and outlet valves.

In this system, the space above the dish is larger than the space below the dish. When pressure is applied to the system, the base of the culture dish becomes distorted because the time constant for equilibration of pressure above and below the dish is different because of differences in volumes of the spaces involved and the relative inaccessibility

of the space below the dish for entering gas. The cells on the petri dish are subjected to both an increase in pressure and an increase in stretch as a result of deformation of the base of the dish (Salter et al 2000). Cyclical mechanical stimulation of this system induces deformation and strain on the base of plastic tissue culture dish and its adherent cells due to a differential rate of rise of pressure above and below the culture dish. In this system, a pressure of 1 Bar above atmospheric pressure, which results in approximately 4000 microstrain (μstrain) on the base of the dish, was used. The standard stimulation regime used was a frequency of 0.33 Hz (2 seconds on, 1 second off) at 37°C (Wright et al 1992).

To assess intracellular signalling following mechanical stimulation, for defined periods (0, 30 sec, 1min, 5min, 10min) cultured chondrocytes were subjected to cyclical mechanical stimulation (see 2.6.2 and 2.6.3). Each experiment was carried out with chondrocytes from at least 3 different donors.

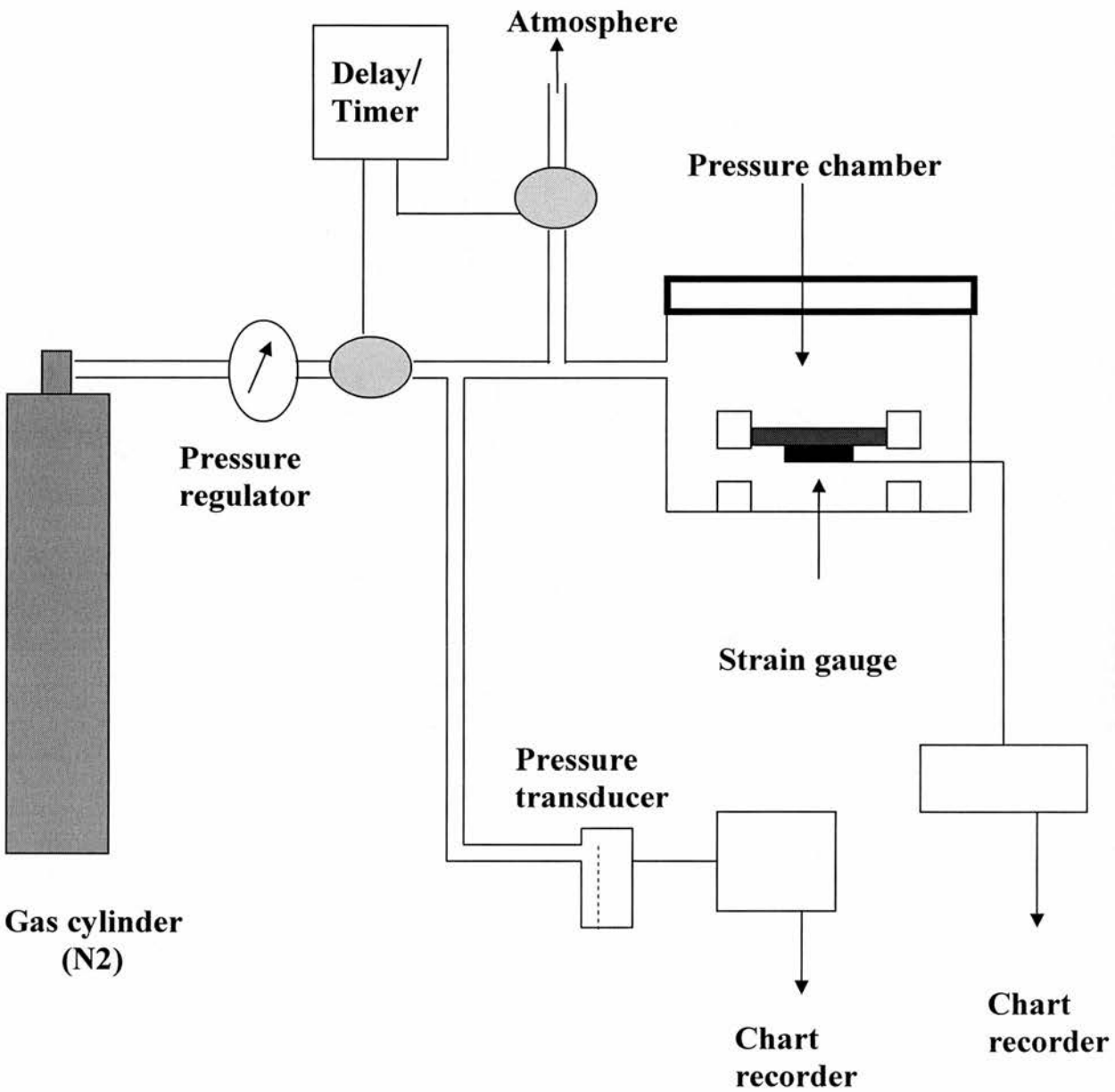

Figure 2.1. Apparatus for the induction of cyclic mechanical stimulation. Nitrogen gas entered the system from a nitrogen cylinder, via a pressure regulator, which controlled the inlet and outlet valves. A delay/timer allowed variation in duration of pressure pulse and time between pulses. The pressure in the system was monitored by a digital monometer (Wright et al 1992).

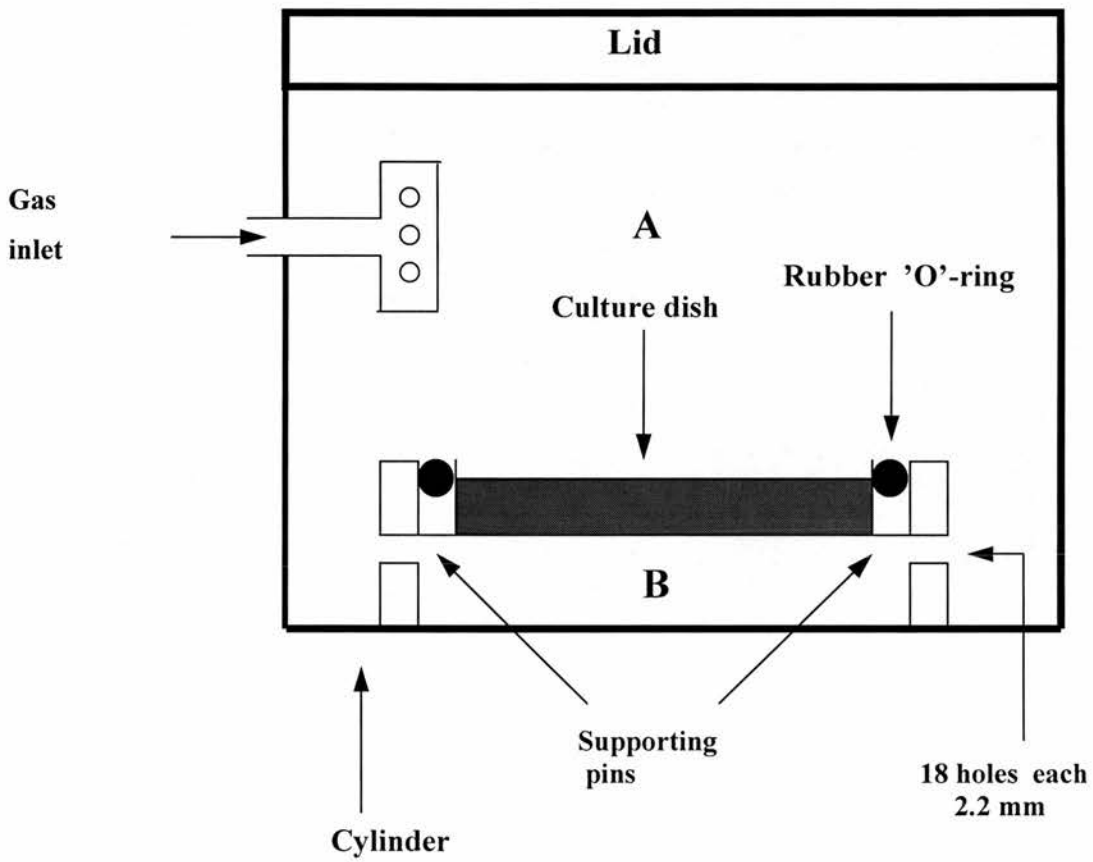

Figure 2.2. Details of pressurisation chamber (Wright et al 1992)

2.6.0. WESTERN BLOTTING

2.6.1. Protein extraction

After indicated mechanical stimulation and/or required conditions, the medium was poured off and cells were immediately washed twice with 10 ml ice-cold PBS containing 1mM Na_3Vo_4 (Sigma). Cells were immediately lysed in situ with 500 µl ice-cold lysis buffer at 4°C for 15 min. Lysis buffer contained 1mM Na_3Vo_4 (Sigma) in PBS, 1% Igepal (Sigma), and protease inhibitor cocktail tablet (Boehringer Mannheim). Lysates were collected by scraping the base of the culture dish with a cell scraper (Grenier bio-one) at 4°C. Supernatants were collected after centrifugation at 13000 rpm for 15 min. The whole cell lysates were stored at –80°C for future use, including separate storage of a 20 µl aliquot of the lysate for protein determination. The protein concentration was determined using Follin-Lowry assay method (Lowry et al 1951) (see 2.6.4).

2.6.2. Assessment of tyrosine phosphorylation

Following mechanical stimulation at 0.33 Hz for defined periods (1minute and 5 minutes), protein extraction was carried out. To assess the role of CD47 in the induction of protein tyrosine phosphorylation, in separate experiments chondrocytes were incubated with 1µg/ml anti-CD47 (Bric 126) for 10 min prior to mechanical stimulation. To maximise the inhibition effect of sodium orthovanadate on protein phosphotyrosyl-phosphatases, activation of sodium orthovanadate protocol (Upstate procedure: http:// www.upstate.com/misc/protocols.asp?prot=activation) was applied. 200 mM solution of sodium orthovanadate was prepared and pH adjusted to 10 using 1N NaOH and/or 1N HCl until the solution was yellow. Then the yellow solution was boiled for approximately 10 min until it turned colourless left at room temperature and again the pH adjusted to 10 until became colourless. This solution was used to make 1mM sodium orthovanadate in PBS.

2.6.3. Assessment of PKCα activation

To assess the role of CD47 in PKCα activation, cultured chondrocytes were incubated with 1μg/ml anti-CD47 (Bric 126) for 10 min prior to mechanical stimulation. Immediately after defined periods (30 sec, 1 min, 5 min, and 10 min) of mechanical stimulation, the cells were washed once with ice cold PBS. Then cells were scraped into buffer A containing 20 mM Tris-HCl, PH 7.6, 2 mM EGTA, 2 mM EDTA, 0.33 M sucrose, 100 μM Na_3VO_4, 20 mM NaF and protease inhibitors cocktail. Extracts were then sonicated on ice 3-5 times for 5 seconds each time at 5 sec intervals. Then sonicated lysates were centrifuged at 13000 rpm for 30 min at 4°C. The supernatants were collected as cytosolic fractions and the pellets (particulate fraction) were re-suspended in buffer B containing 20 mM tris-HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 0.1% Triton X-100, 100 μM Na_3VO_4, 20 mM NaF and protease inhibitors cocktail. Equivalent amount (40 μg) of cell extracts were separated on a 10% polyacrylamide gel under reducing conditions.

2.6.4 Lowry determination of protein concentration

Bovine serum albumin (BSA) (Sigma) standards increasing in concentration from 0 to 200 μg/ml (0, 10, 25, 50, 75, 100, 150, and 200) were set up in triplicate and vortexed. Sample tubes were set up in triplicate by adding 5 μl sample to 195 μl 0.1N NaOH. Blank tubes containing 200 μl 0.1 N NaOH were also set up in triplicate. The tubes were vortexed to mix the sample. To all tubes, 1 ml of alkaline carbonate solution was added, the tubes were then vortexed and allowed to stand at room temperature for 10 minutes. To each tube 10 μl Folin's reagent (Sigma) was added, the tubes were vortexed and allowed to stand at room temperature for 30 minutes. An aliquot of 200 μl of each sample was transferred to a 96 well tissue culture microplate (IWAKI, Japan) and the absorbance was read on a Dynatech MR 500 microplate reader at 570 nm. The software BioLinx 2.20 using a standard curve of BSA protein concentration calculated the average concentration for each triplicate sample (Lowry et al 1951).

2.6.5. IMMUNOPRECIPITATION

2.6.5.1. Immunoprecipitation for tyrosine phosphorylation

Appropriate protein amounts (1mg protein for primary cells and 500μg protein for C20/A4 cell line) from whole cell extracts were made up to 1ml with fresh ice-cold lysis buffer (Appendix II). Then 25 μl anti-phosphotyrosine-agarose (Sigma) was added to each tube. The tubes were incubated at 4°C for at least four hours on a rotating device. The tubes were then centrifuged at 13000 rpm for 15 minutes at 4°C and the supernatants were discarded. The beads, containing immunoprecipitated complex, were washed twice with ice-cold lysis buffer and once with 1ml ice-cold PBS containing 1mM Na_3Vo_4, centrifuged and supernatants discarded. Then, the immunoprecipitated beads were solubilized in equal amounts (25μl) electrophoresis sample buffer 2x (ESB2x) (see Appendix II) and 10% SDS (Sodium Dodecyl Sulphate), boiled in water bath for 5 minutes, and loaded onto 8% reducing acrylamide gel for western blotting analysis (see Appendix II). The blots were immunoblotted with anti-phosphotyrosine-HRP conjugated (Amersham Life Science) (1:1000) or anti-FAK (Santa Cruz) (1:500).

2.6.5.2. Assessment of cell membrane receptors association

To minimize the non-specific background staining and preclearing, protein A/G resin (cytosignal™ research product) was mixed with equal amounts of normal rabbit serum (NRS). The beads obtained from this mixture were resuspended in 1mg protein of cell extract for overnight on a rotating device and then centrifuged at 13000 rpm for 5 min at 4°C. The supernatant was made up to 1ml by adding fresh ice-cold lysis buffer. Indicated amounts of anti-CD47 or anti-β1integrin antibodies (2 μg/ml) and protein A/G resin (30 μl per 1ml lysate) were added to the supernatant and incubated for overnight on a rotating device at 4°C. To immunoprecipitate CD47 and its associated ligands anti-CD47 CC2C6 (25 μl /ml) (mouse Ig G1, kindly provided by Dr. H.J.Büring, University of Tübingen, Germany) or 1F7 (2μg/ml) (mouse IgG1; kindly provided by Frederik P.

Lindberg, Washington University) were applied. Immunoprecipitated β1integrin and its complex were obtained by using 2 μg/ml anti-β1integrin ,clone JB1A (Chemicon). The immunoprecipitated proteins were washed at least 3-5 times with lysis buffer at 4°C. Protein complexes bound to beads were solubilized in equal amounts (25μl) of sample buffer 2x plus 10% SDS, boiled for 5 min in water bath, analysed by appropriate percentage of SDS-PAGE, and the next stages will be discussed in below.

2.6.6. Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE according to the method of Laemmli (Laemmli 1970), using equipment from Bio-Rad. Glass gel plates (12 cm x 10 cm, 12 cm x 9 cm), spacers, clamp assembly, casting stand and grey silicone gaskets were cleaned with 70% industrial methyl alcohol (IMS). Glass plate sandwiches were assembled into the clamp assembly and the unit transferred to the casting stand. A separating gel solution was prepared of appropriate acrylamide (national diagnostics, UK) percentage (6%, 8%, 10%, 15%) (see Appendix II) for the resolution required. The solution was mixed and poured into the gap between the glass plates up to the mark using a Pasteur pipette. The solution was immediately overlaid with distilled water and allowed to polymerise for 45 minutes in a vertical position at room temperature. The overlaid distilled water was drained off and the surface of the gel dried with filter paper. In a sterile bijoux, the 5% stacking gel solution was prepared and poured over the separating gel to the top of the plates. A ten-well comb was placed in the sandwich, a mark made 0.5 cm below the edge of the teeth, and the gel allowed to polymerise at room temperature for 15 minutes. After polymerisation of the stacking gel, the comb was removed, and wells washed with dH_2O, the clamp assemblies removed from the casting stand and attached to the inner cooling core. The electrode buffer was prepared from ten times stock of electrode buffer (see Appendix II).

Samples were prepared by mixing 40μg protein extract from whole cell lysate with equal amount of two times reducing or non-reducing electrophoresis Laemmli sample buffer 2x (ESB2x) and aliquoted. In non-reducing ESB2x, 10% β–mercaptoethanol (β–ME) was replaced by distilled water (see Appendix II). To denature the proteins, aliquots were boiled for 3 minutes and then loaded onto the gel. The inner cooling core was placed into the cell and electrode buffer poured into the lower chamber so that at least 1 cm of gel was covered. Then the electrophoresis apparatus was attached to an electric power supply and the samples were electrophoresed through the gel at a voltage up to 150V and stopped when the blue line of bromophenol blue had reached the bottom of the separating gel.

2.6.7. Electrophoretic transfer of proteins to polyvinylidene fluoride (PVDF) membrane

After running, the gel assembly was removed from the gel apparatus, the glass plates separated and the gel soaked in a jar containing transfer buffer (see Appendix II). For each gel a piece of PVDF membrane and three pieces of 3MM filter paper (Whatman, UK) were cut to a size ~0.5 cm larger than the gel. The PVDF membrane was first soaked in methanol for a few seconds (~15 sec) and transferred to a container of distilled water for two minutes. The PVDF membrane, filter papers and fibre pads were equilibrated in transfer buffer for 5 minutes. The plastic holder for the gel membrane sandwich was opened, a fibre pad was placed onto it followed by a piece of filter paper, then the gel, the PVDF membrane, the two pieces of filter paper and finally the second fibre pad. The holder was closed, squeezed gently to exclude air bubbles and then placed in the transfer apparatus ensuring that the PVDF membrane lies between the cathode and the gel (**Figure 2.3**). The tank was filled with transfer buffer sufficient to cover the gel, membrane sandwich without touching the electrodes and transfer was carried out at 45V overnight in cold room.

2.6.8. Immunoblotting and development of blot using enhanced chemiluminescence (ECL) or ECL plus

Following electrophoretic transfer of proteins to the PVDF membrane, the plastic holder was removed from the blotting apparatus and opened on a flat surface. The PVDF membrane was removed and incubated with 20 ml blocking solution for 1 hour shaking at room temperature. The blocking solution was composed of 2% BSA or 5% dried milk (Marvel), 0.1% sodium azide in TBST (see Appendix II). Then the blot was washed three times quick wash with TBST (see appendix II) and incubated with primary antibody diluted in 5 ml TBST for 1 hour with shaking at room temperature. After three quick washes and two 10 minutes washes with TBST, the blot was incubated with appropriate HRP-conjugated secondary antibody (DAKO) (1:2000) for 1 hour with rotation at room temperature, and then washed as above.

The proteins were then visualised by using enhanced chemiluminescence (ECL) or ECL plus detection kit (Amersham, UK). When ECL detection system was used, a mixture of 1 ml solution A and 1ml of solution B were poured onto one membrane for 1 minute. A mixture of solution A: solution B (1:40) was applied for 5 min when ECL plus was utilised. The fluid was drained off and the membrane wrapped in saran wrap. Any air bubbles were smoothed out. The blot was placed in autoradiography cassette and exposed to ECL hyperfilm for 1 minute. The film was developed by hyperprocessor and the blot re-exposed as required depending on the strength of the signal.

2.6.9. Estimation of protein size

The molecular weight of proteins analysed by SDS-PAGE was determined by comparison with coloured recombinant molecules of defined size (Rainbow RPN 800, Amersham) that were electrophoresed on the same gel. Proteins detected, cases studied and primary antibodies utilised by western blotting are summarised in **Tables 2.5** and **2.6.**

Table 2.5. Cases studied by western blotting analysis to detect CD47 and its ligands, TSP1 and SIRPα, in cultured human articular cartilage (in vitro).

	CD47	**TSP**	**SIRPα**
Antibody/ Dilution	Miap 400.1, mouse mAb, 1: 500	P10, mouse mAb 1:100	Rabbit ploYclonal 1:1000
No. of cases (sex)	20 (14M/5F)*	9 (6M/3F)	13 (8 M/5 F)
Knee joint (Normal/OA)	11 (5 N/6 OA)	3 (1 N/2 OA)	6 (2 N/4 OA)
Hip joint (Normal/OA)	4 (3N/1 OA)	3 (2 N/1 OA)	4 (3 N/1 OA)
Ankle joint§	5	3	3
Age range (Mean)	Knee: 62-87 (73) Hip: 70-83 (79) Ankle: 62-80 (70)	Knee: 62-87 (74) Hip: 70-83 (76) Ankle: 62-72 (67)	Knee: 62-87 (73) Hip: 70-83 (76) Ankle: 69-71 (70)

*, more information was not recorded.

§, All ankle joints were normal.

Table 2.6. Cases studied by western blotting analysis to detect CD98 and its ligands, galectin-3 and CD147 (EMMPRIN) in cultured human articular cartilage (in vitro).

	CD98	**Galectin-3**	**CD147 (EMMPRIN)**
Antibody/ Dilution	Goat polyclonal, 1:500	Mouse mAb 9C4 1:500	Mouse mAb 8G6 1:5000
No. of cases (sex)	19 (14 M/ 4 F)*	17 (11 M/ 6 F)	17 (11 M/ 6 F)
Knee joint (Normal/ OA)	10 (3 N/ 7 OA)	7 (3 N/ 4 OA)	7 (3 N/ 2 OA)*
Hip joint	5 (3 N/ 2 OA)	5 (3 N/ 2 OA)	5 (3 N / 2 OA)
Ankle joint§	4	5	5
Age range (Mean)	Knee: 62-87 (73) Hip: 70-98 (82) Ankle: 69-80 (73)	Knee: 62-87 (74) Hip: 70-98 (83) Ankle: 62-80 (70)	Knee: 62-87 (73) Hip: 70-98 (83) Ankle: 62-80 (70)

*, more information was not recorded.

§, All ankle joints were normal.

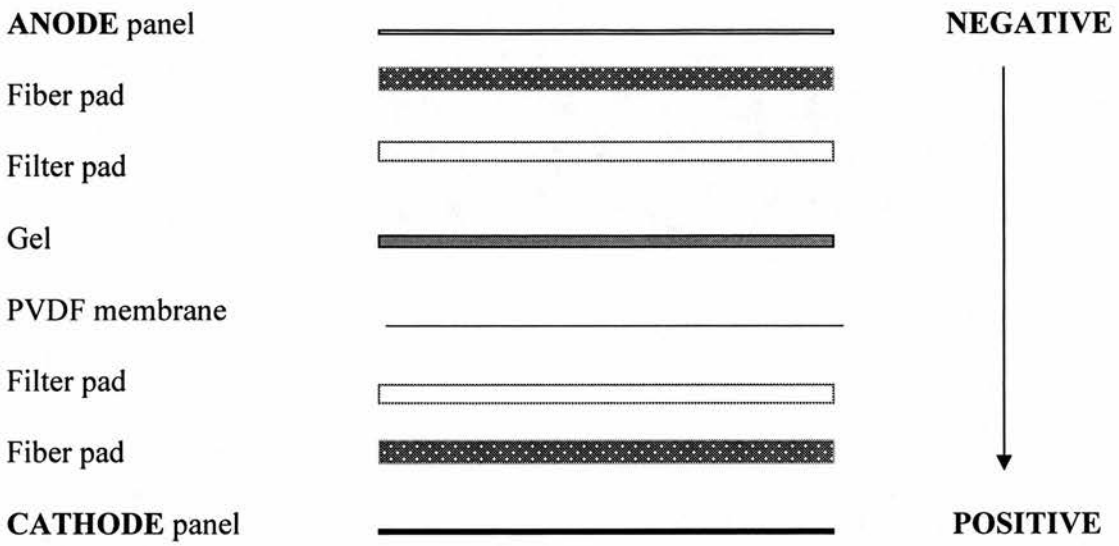

Figure 2. 3. Assembly of tank transfer apparatus

2.6.10. Blot stripping

In some cases, immunoblots were stripped of bound antibodies by incubating the blots with a stripping buffer containing 62.5 mM Tris (PH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol (ME) (see Appendix II) for 30 minutes at 50°C. Then the blots were washed twice with TBST for at least 10 minutes each wash. Blocking of non-specific binding was applied by incubating blots in blocking buffer for one hour. Then blots were reprobed with other primary antibodies and appropriate relevant secondary antibodies and developed.

2.7.0. ELECTROPHYSIOLOGY

2.7.1. Preparation of microelectrodes

Ten-cm borocilicate glass capillaries GC150F-10, 1.5 mm outer diameter (O.D) x 0.86mm inner diameter (I.D), with inner filament (Harvard apparatus, Edenbridge, Kent, UK) were pulled to produce two microelectrodes by using Narishege microelectrode puller (Narishege, Japan). After pulling, a batch of microelectrodes were stored in a clean container with their tips uppermost.

2.7.2. Filling of microelectrodes

Prior to use, the electrodes were filled with 3M potassium chloride solution (3M KCl). A glass tissue culture beaker was filled with 3M KCl and plasticine was pressed firmly around the rim of the beaker. Electrodes were then pressed onto the plasticine so that their tips were submerged in the KCl. The tips filled by capillarity and the shafts of the electrodes were filled using a syringe and lumbar puncture needle. After filling the electrodes were left for at least 3 minutes to allow the tips to fill by capillary action. Electrodes were used when there were no air bubbles visible in either the tip or the shaft (**Figure 2.4**).

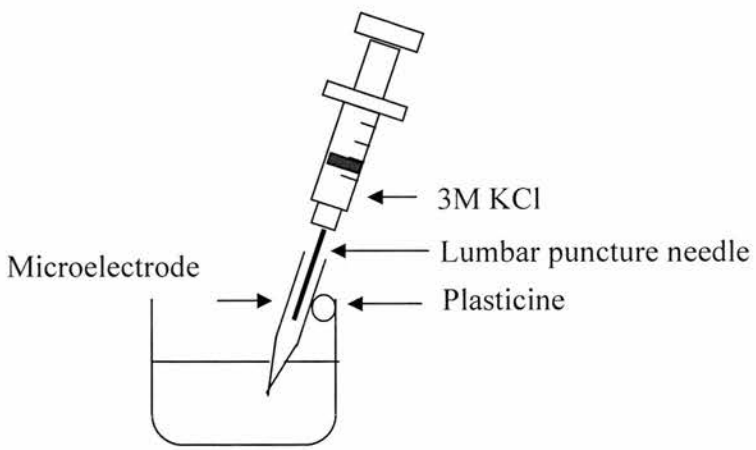

Figure 2.4 - Filling of Microelectrodes with 3M KCl.

2.7.3. Electrophysiological recording

After 20 minutes pressurisation at 0.33 Hz (2 seconds on, 1 second off), the dishes of cells were removed from the pressure chamber immediately (about 2 minutes) and post pressurisation values of membrane potential recorded on the cells over the further 10-12 minutes.

Electrophysiological experiments were performed at room temperature (~ 20°C). Dishes of cultured chondrocytes were placed on the stage of a wild M40 inverted microscope (**Figure 2.5**) and were observed under x200 magnification. A silver/silver chloride (Ag/AgCl) earth wire was placed in the periphery of the culture dish, making contact with the culture medium, to make more stable recording during experiment. The other end of this wire was connected to the ground terminal of the input headstage of the Axoclamp Amplifier. The microelectrode was connected to the headstage via a microelectrode holder, which housed an Ag/AgCl wire. A pre-filled microelectrode was connected to microelectrode holder so that Ag/AgCl wire of holder passed down the shaft of the microelectrode making contact with 3M KCl in shaft of microelectrode.

Microelectrode passed over Ag/AgCl wire into blunt end of microelectrode contacted the recess and pipette seat. The pipette cap was then turned to firmly hold microelectrode in place in the holder (**Figure 2.6**). The central end of microelectrode holder connected to the input of the headstage. Using a Zeiss micromanipulator (**Figure 2.5**), the microelectrode connected to the headstage lowered until its tip was beneath of the surface of culture medium and focused.

Power to the Axoclamp-2B (Microelectrode Clamp, Axon Instruments) was switched on (**Figure 2.7**) and by using the input offset potentiometer any voltage recorded on microelectrode was offset to the zero line on the oscilloscope (Gould Advance type OS 4000/4001). A command current of 5 nA was set with the step command thumbwheel switch. A voltage pulse was observed on the oscilloscope. Bridge dial advanced until this voltage step was eliminated. The bridge was then in balance. The microelectrode tip

resistance was read from the bridge dial and was usually in the range of 25-50 MΩ, which was acceptable for experimentation. Lower tip resistances indicated that there was some damage to the tip or that the electrodes had a large tip diameter. High tip resistances (> 100 MΩ) indicated the probability of air bubbles in the electrode tip, due to inadequate filling by the capillary. Therefore, electrodes with tip resistances <20 MΩ or >100 MΩ were discarded and not used for experimentation. The cells were left at room temperature for 5 min before the first membrane potential measurement was made. Each experiment was performed with chondrocytes from at least 3 different donors.

2.7.4. Electrophysiological Procedure

I. An isolated and polygonal human articular chondrocyte (HAC), with no visible contact with neighbouring cells, was selected for impalement. It has been previously shown that resting membrane potentials are higher in cells, which are in contact with one or more neighbouring cells (Bard and Wright 1974).

II. The voltage dependent oscillator was switched on. This produces a tone, which is dependent on voltage, and changes in accordance with changes in membrane potential. This piece of equipment provides the advantage that the experimenter can observe the microelectrode on cell impalement and not have to continually check the oscilloscope monitor, as a change of note alerts the experimenter that the electrode has entered the cell.

III. The microelectrode tip was positioned over the cell using the micromanipulator.

IV. The electrode tip was then lowered whilst listening to the note of the voltage dependent oscillator. When the electrode tip entered the cell, the note changed suddenly from high to a lower frequency.

V. The digital readout of membrane potential was then observed on the Axoclamp-2B and this was noted. The electrode tip was then removed from the cell using the micromanipulator.

The results from individual cells were accepted, if, upon impalement there was a rapid change in voltage which remained constant for at least 20 seconds. A microelectrode was changed if there was a dramatic increase or decrease in electrode resistance as this usually indicated that the electrode had become blocked with debris, or cellular material or that the electrode had become damaged during cell impalement. Membrane potentials of a minimum of 5 cells were measured before and after addition of each reagent to be tested and again following mechanical stimulation.

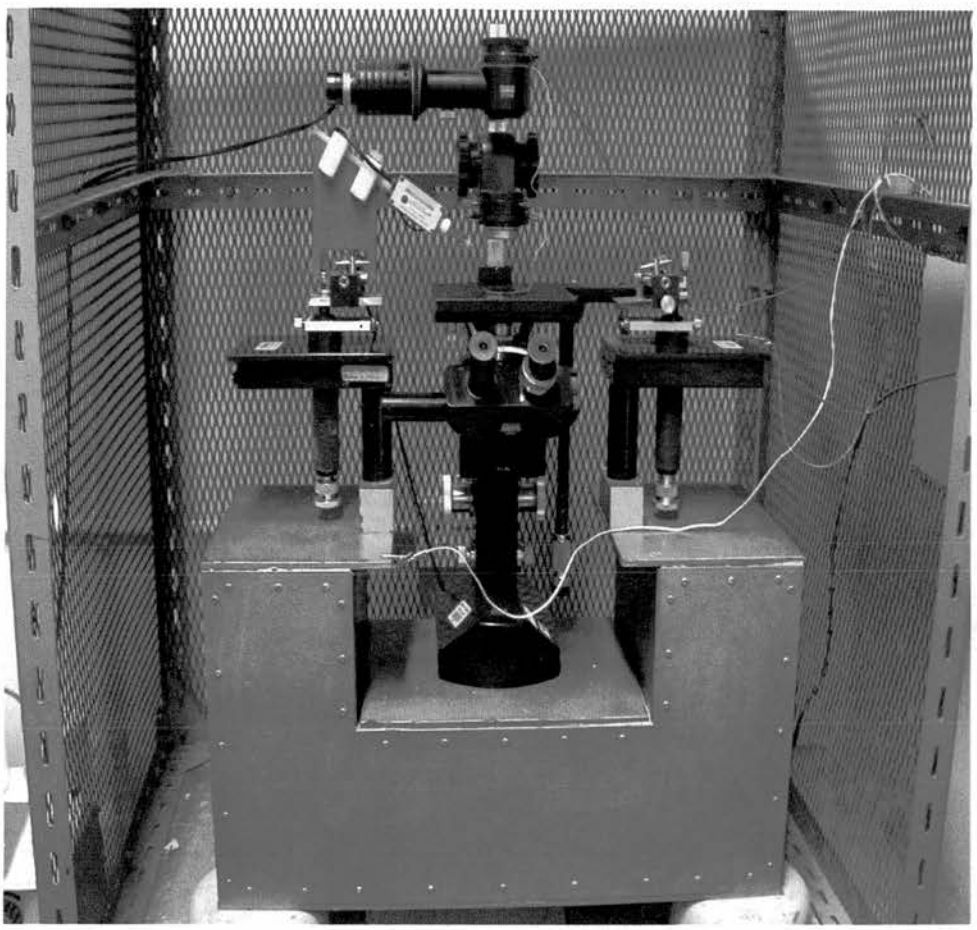

Figure 2. 5. Photograph of Wild M40 inverted microscope and micromanipulation system.

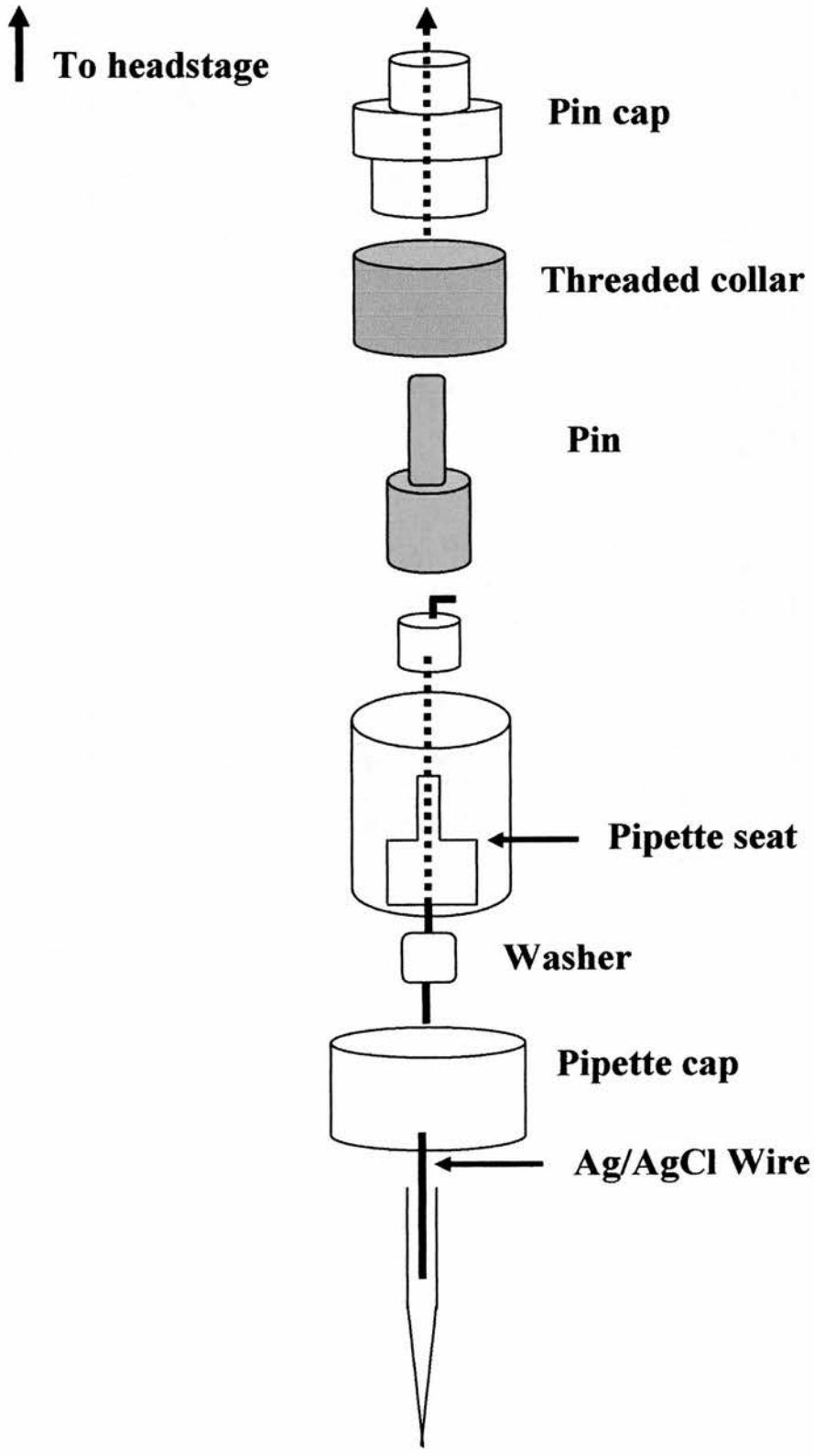

Figure 2.6 - Details of the microelectrode holder

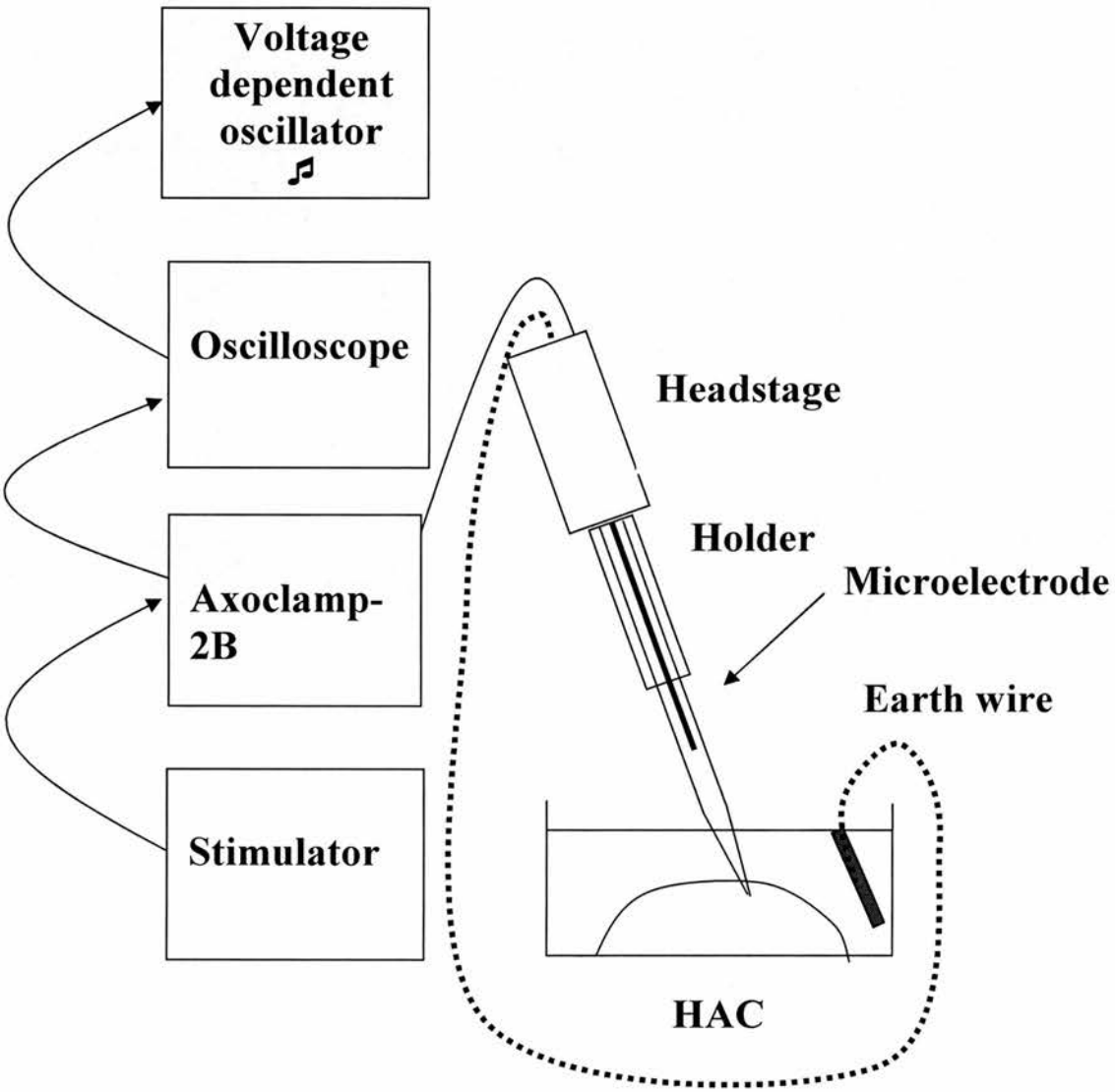

Figure 2.7 – Diagram of Axoclamp-2B system.

2.8. Chemical reagents

To investigate the role of CD47, CD98 and their ligands in the mechanotransduction pathway induced by mechanical stimulation at 0.33 Hz in cultured human articular chondrocytes, a panel of antibodies to CD47, CD98 and their ligands, SIRPα, TSP and CD147, were added separately to cell cultures. Membrane potentials of cultured chondrocytes were measured before (as resting) and 10 minutes after adding Ab. Then the cells were subjected to standardised mechanical stimulation regime and immediately after mechanical stimulation, the post-stimulation membrane potentials of chondrocytes were measured. The Abs and their concentrations utilised are shown in **Table 2.8**.

Table 2.8. Chemical reagents

ANTIBODY	EFFECT	CONCENTRATION	SOURCE
Bric 126	Anti-CD47 mAb*	1 μg/ml	IBGRL***
C-20	Anti-CD98 pAb**	1.5 μg/ml	Sanata Cruz
BU89	Anti-CD98 mAb	1:100	Dr D. Hardie
BU53	Anti-CD98 mAb	1:100	Dr D. Hardie
SE7C2	Anti-SIRPα mAb	1:100	Dr. H.J. Büring, Tübingen University, Germany
SE5A5	Anti-SIRPα mAb	1:100	Dr. H.J. Büring, Tübingen University, Germany
8G6	Anti-CD147 mAb	1.5 μl/ml	Dr M. Hemler
P10	Anti-TSP mAb	1.5 μg/ml	Chemicon International

-Incubation period of all antibodies: 10 min.

*mAb: Monoclonal antibody

**pAb: Polyclonal antibody

***IBGRL: International Blood Group Reference Laboratory (Bristol, UK)

2.9. Statistical analysis

The mean, standard deviation (SD) and standard error of the mean (SEM) were determined in each experiment. For statistical comparisons, when the F-ratio of the two variances reached significance, the non-parametric Mann-Whitney test was used. When the ratio did not reach significance, the Student's *t* test was used.

CHAPTER THREE

3.0. IN VIVO AND IN VITRO EXPRESSION OF INTEGRIN-ASSOCIATED PROTEINS IN NORMAL AND OA HUMAN ARTICULAR CARTILAGE

3.1. IN VIVO AND IN VITRO EVALUATION OF CD47 EXPRESSION IN HUMAN ARTICULAR CARTILAGE

To evaluate in vivo expression of CD47 by normal and OA articular cartilage, a series of immunohistochemical staining was undertaken on frozen sections of articular cartilage from femoral head and different anatomical regions of knee joint (femoral condyle, tibial plateaux or patella). Immunohistochemical assessment of both normal and OA sections was carried out using mouse mAb anti-CD47 Bric 126. The antibody was first tested over a wide range of concentrations. Preliminary studies showed that this mAb worked with frozen sections at 1:5000 but not with formalin fixed, paraffin embedded sections.

The positive immunoreactivity in different sections was graded in a range of 1-5. CD47 staining for each zone was scored as: 1, no staining; 2, more cells negative than positive; 3, equal positive and negative; 4, more cells positive than negative; and 5, all cells positive (see chapter two, 2.3.6, page 90). Each zone of cartilage sections was evaluated and scored separately.

3.1.1. Expression of CD47/IAP in normal human articular cartilage

Twelve sections of normal articular cartilage were obtained from 6 males (age range 62-82, mean age 71), 5 females (age range 74-90, mean age 78) and one case whose age and sex was not recorded. Normal human articular cartilage sections were isolated from 4 femoral heads, 3 females, one male (age range 74-90, mean age 79) (day book numbers

(DB Nos): 13857, 13867, 13836, 13871); 4 femoral condyles, 2 females, 1 male, 1 case whose age and sex was not recorded (age range 64-82, mean age 74) (10052, 13860, 10070,10051); 3 tibial plateaux, 1 male, 1 female, one case whose age and sex was not recorded (age range 64-88, mean age 73)(10070, 13860, 13855) and 1 patella (a case whose age and sex was not recorded)(11829).

Normal articular chondrocytes in all zones exhibited a consistent pattern of CD47 expression (**Figure 3.1**). Chondrocytes in all zones showed very strong expression for CD47. There was no difference in the pattern of CD47 staining with respect to various zones (**Table 3.1**). The median scores for all zones of normal sections were 5. All chondrocytes in different sections from different donors showed similar staining pattern. Strong immunoreactivity of CD47 at very high dilution (1:5000) of mAb Bric 126 was observed, suggesting strong expression of CD47 in normal articular cartilage. Normal articular cartilage sections isolated from femoral head and various anatomical regions of knee joint (femoral condyle, tibial plateaux or patella) showed similar strong immunostaining pattern. Similarly, there was no difference in immunoreactivity of different zones in different joints. There was no difference in immunoreactivity of sections removed from adult donors of different age or sex.

3.1.2. Expression of CD47/IAP in OA human articular cartilage

CD47 expression pattern was assessed in sections from 20 mild OA (7 grade I and 13 grade II) and 12 severe OA (10 grade III and 2 grade IV) of articular cartilage samples. Samples of OA articular cartilage were obtained from 11 males (age range 62-82, mean age 73), 9 females (age range 62-90, mean age 78) and 2 cases whose age and sex were not recorded. Samples of mild OA articular cartilage were obtained from 5 femoral heads, 1 male, 4 females (age range 74-90, mean 78) (13835, 13873, 13871, 13836, 13857); 8 femoral condyles, 4 males, 2 females, 2 cases whose age and sex were not recorded (age range 62-88, mean 74) (10071, 13860, 13855, 11045, 11370, 13853, 10051, 13854); 6 tibial plateaux, 3 males, 1 female, 2 cases whose age and sex were not

recorded (age range 72-88, mean 75) (11045, 13855, 10071, 13853, 13860, 13854) and 1 patella, female (age 67) (10070).

Chondrocytes in all zones showed extensive membrane staining for CD47 (**Figure 3.2**). Articular cartilage from mild OA (grades I and II) exhibited a consistent expression of CD47, which was similar in all zones of cartilage. Strong expression of CD47 was observed in mild OA sections isolated from femoral head and various anatomical regions (femoral condyle, tibial plateau or patella) of knee joint (**Table 3.1**). The median scores for all zones of mild OA sections were 5.

Samples of severe OA articular cartilage were obtained from 6 femoral condyles, 3 males, 1 female, 2 cases whose age and sex were not recorded (age range 62-88, mean 76) (11370, 10071, 13855, 13853, 13860, 13854); 5 tibial plateaux, 3 males, 2 females (age range 62-88) (10052, 10071, 13855, 10070, 10051) and 1 patella, male (age 82) (10071). In severe OA sections (grades III and IV), chondrocytes in all zones showed strong immunoreactivity. Strong expression of CD47 was observed in severe OA sections isolated from femoral head and various anatomical regions (femoral condyle, tibial plateaux or patella) of knee joint. Clusters and areas of excessive surface fibrillation showed extensive anti-CD47 immunoreactivity (**Figure 3.2**). There was no difference in the pattern of CD47 staining with respect to various zones in mild and severe OA sections. The median scores for all zones of severe OA sections were 5 (**Table 3.1**). Different grades of OA articular cartilage did not show difference in the strong CD47 expression pattern (**Table 3.1**).

Like normal articular cartilage sections, both mild and severe OA, showed similar strong immunoreactivity of CD47 at very high dilution (1:5000) of mAb anti-CD47 Bric126, suggesting a similar expression pattern of CD47 in both normal and OA articular cartilage. CD47 positive staining did not show detectable modification in sections removed from adult donors of different age or sex.

Table 3.1. CD47 expression in normal, mild OA and severe OA human articular cartilage. Each zone of articular cartilage from different donors was evaluated and scored separately. In each zone, the results are shown as mean ± SD. Evaluation was performed three times. Normal, mild OA and severe OA showed the similar strong expression pattern in different zones. P value was calculated to compare different zones in both normal and OA articular cartilage sections. In normal and mild OA surface zone compared with all zones and P value was >0.05 (not significant). In severe OA clones grade was compared with the other zones and P value was >0.05 (not significant).

		Immunostaining Score mean ± SEM				
Sample	**n**	**Surface**	**Superficial**	**Clones**	**Middle**	**Deep**
Normal	12	4.9 ± 0.08	5	—	5	5
Mild OA	20	4.7 ± 0.1	5	—	5	5
Severe OA	12	N/A	N/A	4.9 ± 0.07	5	5

*N/A, not available.

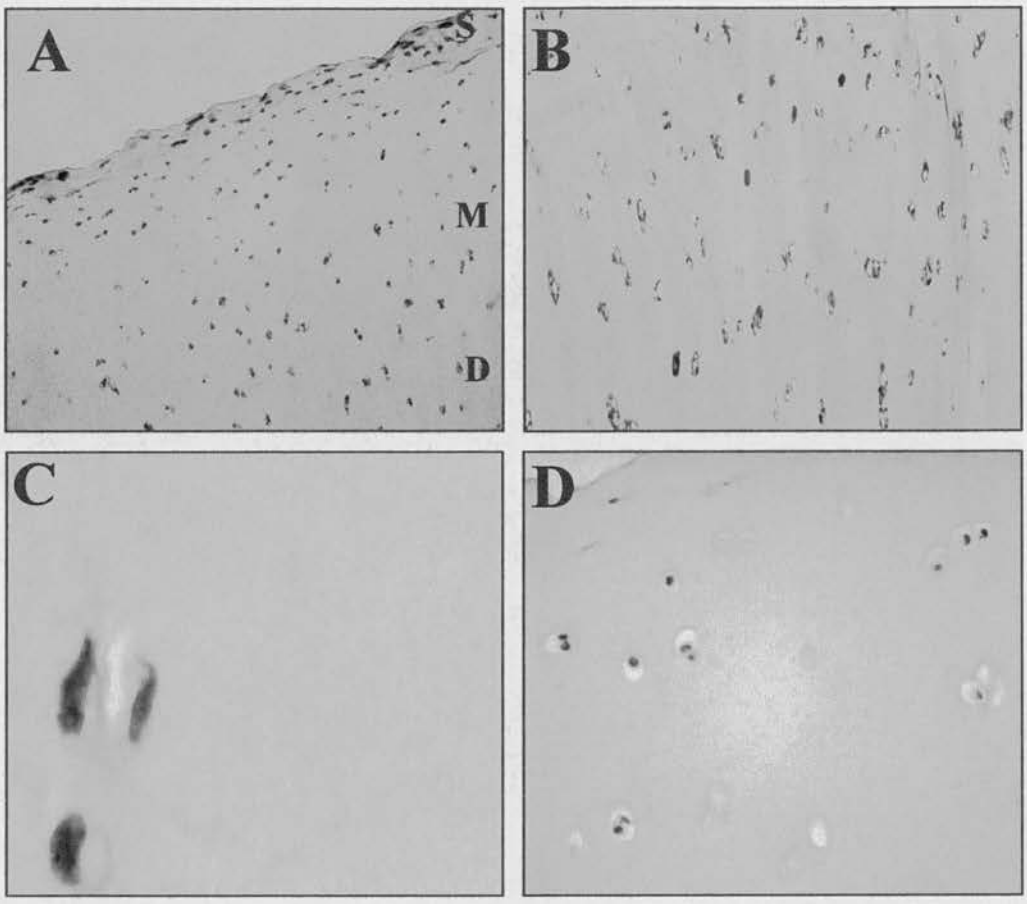

Figure 3.1. Immunoreactivity of mAb anti-CD47, Bric 126 in normal adult human articular cartilage sections. Immunoreactivity was assessed by using mouse anti-CD47 Bric126 at 1:5000. All sections showed strong expression of CD47 in different zones. **A**, Femoral head, normal, superficial (**S**)-deep (**D**), (female, 90 years) (13857), x100. Chondrocytes in all zones showed a similar strong expression pattern of CD47. **B**, Femoral condyle normal (FN), mid –deep zone (female, 88 years) (10051), x100. Chondrocytes in middle and deep zones showed extensive membrane staining for CD47. **C**, Tibial plateaux normal (TN), mid-zone (**M**), (male, 64 years) (13855), x400. High magnification of chondrocytes in mid-zone of TN, with very strong expression of CD47. **D**, Negative control, tibial plateaux normal, mid- zone, (male, 73 years) (11045), x200. No positive signal at all was observed in TN section as negative control.

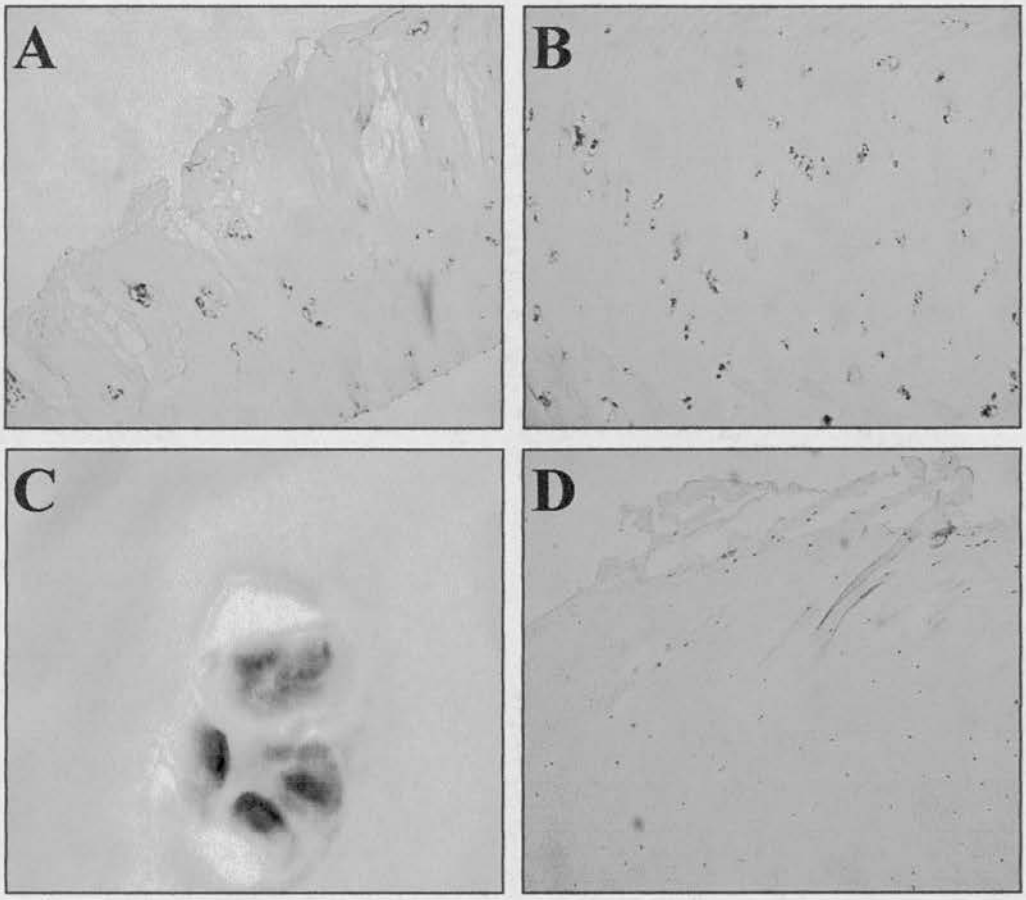

Figure 3.2. Immunoreactivity of mAb anti-CD47, Bric126, in OA human articular cartilage. Immunoreactivity was assessed by using mouse anti-CD47 Bric126 at 1:5000. All sections showed strong expression of CD47 in different zones. Various Collins grades of OA showed similar extensive positive expression pattern. **A**, Femoral head, OAII surface-deep zone, (female, 90years) (13857), x100. From clones towards deep zone of OA femoral head, all chondrocytes showed a similar strong expression pattern for CD47. **B**, Femoral condyle (FA), OA III, superficial-mid zone, (male, 82 years) (10071), x100. No identifiable difference was observed in chondrocytes from superficial and middle zone for CD47 expression. **C,** Tibial plateaux, clone in OAII, (male, 64 years) (13855), x400. High magnification of a clone from TAIII showed very strong expression of CD47. **D,** Negative control, femoral head, OAII, (female, 74) (13836), x100. No positive signal at all was observed in FA II section as negative control.

3.1.3. In vitro evaluation (western blotting analysis) of CD47 expression in cultured human articular chondrocytes

CD47 is an IgSF member of ~ 50 kD MW, which a number of cell types are recognized to express this molecule using western blotting and immunoprecipitation (Brown et al 1990; Mawby et al 1994).

In next step of this study, to confirm the in vivo immunohistochemical results, a series of western blotting experiments was carried out. Chondrocytes were obtained from hip, knee and ankle joints. Chondrocytes were isolated from normal articular cartilage obtained from the tibia and talus (ankle joint), femoral head (hip), tibial plateaux and femoral condyle (knee) of 11 males (age range 69-83, mean 75) and 2 females (age range 71-80, mean 75). OA chondrocytes were isolated from OA articular cartilage obtained from the femoral head, tibia plateaux, femoral condyle and patella of 4 males (age range 68-87, mean 77) and 2 females (age range 62-87, mean 75). Primary chondrocytes were seeded at $2x10^5$ cells/ml concentration. After a period of 10-15 days cultured chondrocytes were lysed and the proteins extracted.

3.1.3.1. Work of antibodies to CD47

To identify the biochemical characterization of CD47 chondrocytes, a panel of mouse mAbs anti-CD47, Bric126, 2B7, miap 400.1, 2E9, 1F7, 2E11 and CC2C6, one rat mAb miap 300.1, and one goat polyclonal Ab (SantaCruz) were applied. Each Ab was tested with three samples, one normal ankle, one normal and one OA sample, at concentrations 1: 500 and 1:1000. Four mAbs, 2B7, miap 400.1, 2E9 and miap 300.1, showed strong band at molecular weight approximately 47-55 kD and one polyclonal Ab (Goat polyclonal, SantaCruz) as well. Four mAbs, Bric 126, 2E11, 1F7 and CC2C6, showed multiple bands, a very faint band or no binding (**Figure 3.3**).

3.1.3.2. Western blotting assessment

Based on the preliminary experiments three Abs, 2 anti-CD47 mouse monoclonal (2B7 and miap 400.1) and one polyclonal antibody (goat polyclonal SantaCruz), were selected for further assessment. Both mAbs showed single band in normal and OA samples and the polyclonal Ab showed two bands. Chondrocytes isolated from normal and OA samples taken from different joints were assessed with these antibodies (**Table 3.2**).

1. Mouse mAb miap 400.1: Cell extracts obtained from two normal ankles, both talus, 1 male (age 71), and another case, male, whose age was not recorded (13915, 13901); 1 normal femoral head, male (age 70) (13822), 1 normal femoral condyle, male (age not recorded) (13949); and 1 OA grade II femoral head, male (age 83) (13828); 1 OA grade III tibia plateaux, male (age 87) (13965), were separated under reducing conditions and probed with mAb miap 400.1 at 1:500 (**Table 3.2**). All showed a similar single band at ~50 kD (**Figure 3.4. A**).

2.Goat polyclonal anti-CD47 (SantaCruz): Cell lysates obtained from two normal ankle, both talus, 1 male (age 71), and another case, male whose age was not recorded (13915, 13901); 1 normal femoral head (male, age 83) (13828); 1 normal tibial plateaux (male, age not recorded) (13949); and one OA grade II femoral head (male, age 83) (13828); 1 OA grade III tibia plateaux (male, age 87) (13965), were separated under reducing conditions and probed with this polyclonal Ab at 1:100 (**Table 3.2**). Two bands, 47 and 55 kD sizes, were detected (**Figure 3.4. B**).

3. Mouse mAb 2B7: Normal samples were obtained from ankle (2 talus, 2 tibia), all male (age range 69-80, mean 73) (13901, 13915, 13920, 13907); 2 femoral condyles, both male (mean 75) (13919, 13939); 1 tibia plateau, male (age not recorded) (13949); 2 femoral heads, one male and one female (mean 75) (13816, 13822). OA samples were obtained from 3 femoral condyles, 2 males, 1 female (age range 69-87, mean 78) (13905, 13964, 13939); 2 tibia plateau, both female (mean 75) (13820, 13965); one patella, male (age 68) (13966). Cell lysates from these samples were analyzed under reducing

conditions and probed with 2B7 mAb at 1:500 (**Table 3.2**). All chondrocytes isolated from normal and OA samples, showed a single band at ~50 kD (**Figure 3.4. C**).

There was no appreciable variation in the intensity of CD47 band between samples from normal and OA cartilage. There was no difference in results from experiments using chondrocytes removed from donors of different age and sex or from femoral head and different areas of knee joint (femoral condyle, tibial plateaux and patella). Compared with chondrocytes isolated from normal and OA hip and knee joints, slightly stronger band of CD47 was observed from chondrocytes isolated from different anatomical areas of ankle (tibia, talus). The intensity of band in ankle chondrocytes, by using different mono and polyclonal antibodies was consistently observed (**Figure 3.4.A, B**).

Table 3.2. Anti-CD47 antibodies and cases studied in western blotting

ANTIBODY	CULTURED CHONDROCYTES (day book No.)	SEX (M/F) MEAN AGE
2B7 (mouse mAb) (1: 500)	**1.Normal**	
	2 talus (ankle) (13901, 13915)	2 M, mean 73
	2 tibial (ankle) (13920, 13915)	2 M, mean 75
	2 femoral condyle (13919,13939)	2 M, mean 75
	1 tibial plateaux (13911)	1 M, not recorded
	2 femoral head (13822, 13828)	1 M, 1 F, mean 75
	2.OA	
	3 femoral condyle (grades I, II and III of OA) (13905, 13964, 13939)	2 M/1 F, mean 78
	2 tibial plateaux (grades I and III)	2F, mean 75
	1 patella (II) (13820, 13965)	M, age 68
Miap 400.1 (mouse mAb) (1:500)	**1.Normal**	
	2 talus (ankle) (13901, 13915)	2 M, age 71, n/r*
	1 femoral condyle (13949)	male, n/r
	1 femoral head (13822)	M, age 70
	2. OA	
	1 tibial plateaux (13965)	F, age 87
	1 femoral head (grade II) (13828)	M, 83
Goat polyclonal (Santa Cruz) (1:100)	**1. Normal**	
	2 talus (ankle) (13915, 13901)	2M, age 71, n/r 2 M, mean 71
	2 tibial plateaux (13941, 13911)	M, age 83
	1 femoral head (13828)	
	2. OA	
	1 tibial plateaux (grade III) (13965)	F, age 87
	1 femoral head (grade II) (13828)	M, 83

*, n/r: more information not recorded.

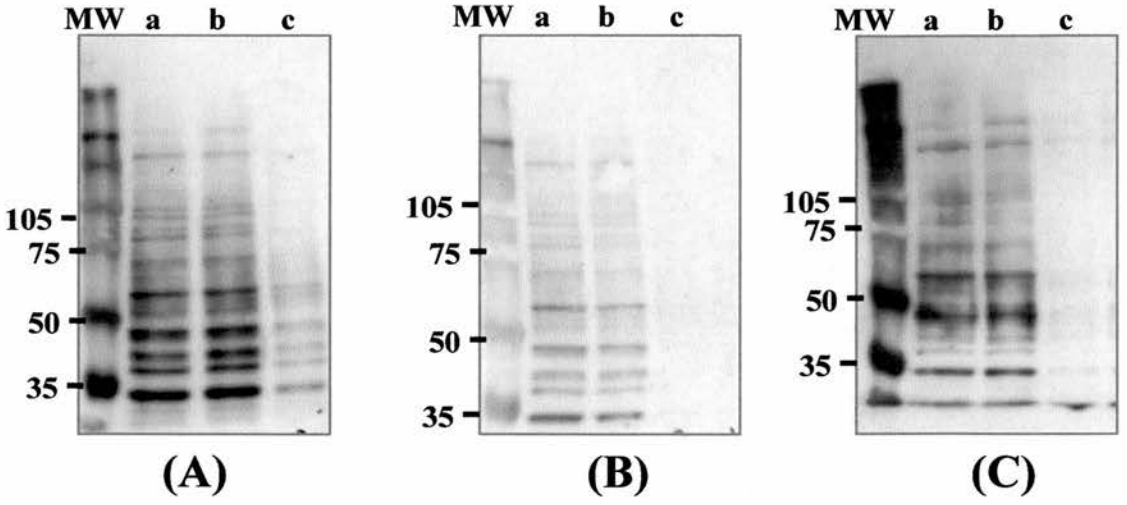

Figure 3.3. A) Work of antibodies to CD47. Equal amounts (40μg/lane) of total extracted proteins obtained from the cultured chondrocytes derived from normal ankle (lanes **a**), normal (lanes **b**) and OA (lanes **c**) knee and femoral head were run in a 10% SDS-PAGE under reducing conditions. Blocking was applied with 5% dry milk (marvel) solution overnight at 4°C. CD47 was detected by probing blots with monoclonal CC2C6 (**A**), 2E11 (**B**) and 1F7 (**C**) anti-CD47 antibodies. **A)** By probing blots with mouse mAb anti-CD47 CC2C6 (1:500), all samples showed multiple bands in a very broad range of molecular weight. **B)** By using anti-CD47 2E11(1:500), all samples showed very faint multiple bands or no binding. The expression did not show consistency in different samples. **C)** By using mouse mAb anti-CD47, 1F7 (1:500), samples showed faint multiple bands or no binding. The blots shown, are representative a series of experiments including at least three different donors (normal and OA). Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

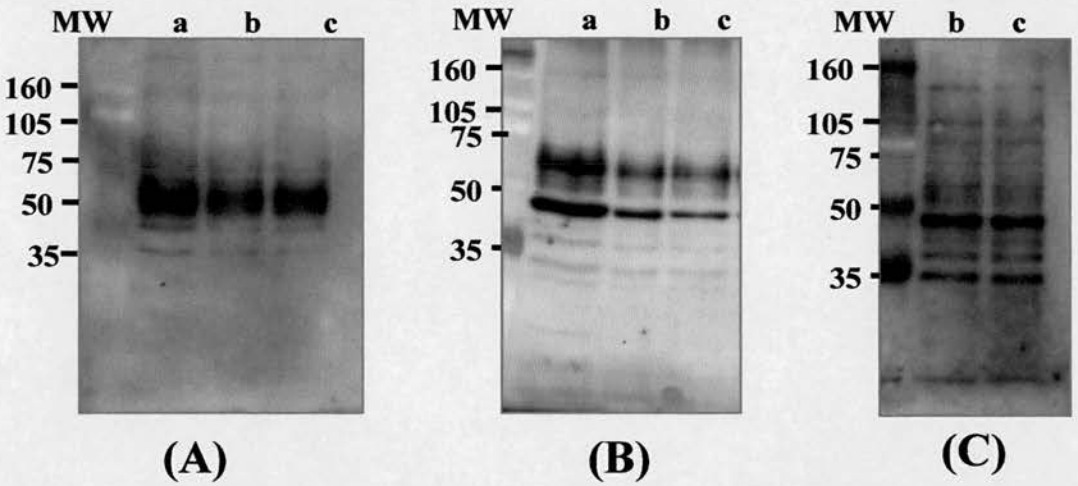

Figure 3.4. A) Detection and comparison of CD47 in normal and OA human articular chondrocytes. Equal amounts (40μg/lane) of total extracted proteins obtained from the cultured chondrocytes derived from normal ankle (lanes **a**), normal (lanes **b**) and OA (lanes **c**) knee and femoral head were run in a 10% SDS-PAGE under reducing conditions. Blocking was applied with 5% dried milk (Marvel) solution overnight at 4°C. CD47 was detected by probing blots with monoclonal miap400.1 **(A),** polyclonal goat (SantaCruz) **(B),** and monoclonal 2B7 **(C)** anti-CD47 antibodies. **A)** By probing blots with mouse mAb anti-CD47 miap 400.1 (1:500), normal ankle (lane **a**, tallus, male, age not recorded) (13901)*, normal (lane **b**, femoral head, male, age 70) (13822) and OA III (lane **c** tibia plateaux, female, age 87) (13965) chondrocytes showed an ~50 kD single band. **B)** By using polyclonal goat anti-CD47 (SantaCruz) (1:100), normal ankle (lane **a**, tallus, male, age not recorded) (13901), normal (lane **b**, tibia plateaux, male, age not recorded) (13949), OA II (lane **c**, femoral head, male, age 83) (13828), two bands with different sizes, 47 and 55 kD sizes, were detected. **C)** By using mouse mAb anti-CD47 2B7 (1:500), normal (lane **b**, femoral head, male, age 70) (13822) and OA III (lane **c**, tibia plateaux, female, age 87) (13965) chondrocytes derived from different joints expressed an ~50 kD single band. The blots shown, are representative a series of experiments including at least three different donors (normal and OA). Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left. *,(day book No.)

3.2. IN VIVO AND IN VITRO EVALUATION OF CD98 IN HUMAN ARTICULAR CARTILAGE

To evaluate in vivo expression of CD98 in normal articular cartilage, a series of immunohistochemical staining was undertaken on frozen sections of articular cartilage from femoral head and different anatomical regions of knee joint (femoral condyle, tibial plateaux or patella).

CD98 expression on chondrocytes cell membrane was assessed using mouse mAb anti-CD98 4F2. Preliminary studies showed that this mAb worked with frozen sections at 1:50 dilution, but not with formalin fixed, paraffin embedded sections.

The positive immunoreactivity in different sections was graded in a range of 1-5. CD98 staining for each zone was scored as: 1, no staining; 2, more cells negative than positive; 3, equal positive and negative; 4, more cells positive than negative; and 5, all cells positive (see chapter two, 2.3.6, page 90). Each zone of cartilage sections was evaluated and scored separately.

3.2.1. Expression of CD98 in normal human articular cartilage

Sixteen sections of normal articular cartilage were obtained from 4 males (age range 39-73, mean 55) and 7 females (age range 67-83, mean 75). Normal articular cartilage sections were isolated from 5 femoral heads, all female (age range 73-90, mean 79) (13857,13835, 13836, 13818, 13867); 6 femoral condyles, 2 males, 3 females, one case whose age and sex was not recorded (age range 39-74, mean 64) (10070, 10097, 11271, 11045, 11051, 13860); 6 tibial plateaux, 2 males, 3 females, one case whose age and sex was not recorded (age range 44-73, mean 63) (11417, 10097, 10070, 11045, 11329, 13860) and one patella (male, age 39) (11286).

Immunoreactivity was characterized by a heterogeneous staining pattern (**Figure 3.5**). Some cells were positive, others negative. Chondrocytes in all zones of normal articular cartilage sections isolated from different joints showed positive immunoreactivity with

an identical expression pattern. The median scores (ranges) for surface, superficial, mid and deep zones were 4 (2-5), 4 (3-5), 4 (2-4) and 4(2-4), respectively. There was no statistical difference in immunoreactivity of surface and deep zone (**Table 3.3**).
The age and sex of donors had no noticeable effect on the pattern of CD98 expression.

3.2.2. Expression of CD98 in OA human articular cartilage

CD98 expression was assessed in 17 mild (3 grade I and 14 grade II) and 7 severe OA (6 grade III and 1 grade IV) articular cartilage sections. Samples of OA articular cartilage were obtained from 8 males (age range 62-85, mean 74), 9 females (age range 67-90, mean 75) and one case whose age and sex was not recorded. Sections of mild OA articular cartilage were obtained from 5 femoral heads, 1 male, 4 females (age range 73-90, mean 79) (13857, 13836, 13835, 13818, 13871); 4 femoral condyles, 1 male, 2 females, 1 case whose age and sex was not recorded (age range 67-74; mean 71) (10097, 11271, 11104, 13860); 6 tibial plateau, 2 males, 3 females, one case whose age and sex was not recorded (age range 67-85, mean 73) (11417, 11271, 10097, 11045, 11104, 13860); and 3 patellae, all male (age range 64-74, mean 67) (11104, 11051, 9907). Samples of severe OA articular cartilage were obtained from 4 femoral condyles, all male (age range 62-82, mean 72) (11370, 11045, 9907, 13817); 1 tibial plateaux, male (age 62) (13817) and 2 patellae, both male (age range 73-82, mean 78) (10071,11045).

Articular cartilage from mild OA (grades I and II) exhibited a heterogeneous staining pattern in all zones of different joints. The median scores and ranges for surface, superficial, mid and deep zones were 4 (3-5), 4 (3-5), 4 (2-5) and 4(2-5), respectively. In severe OA sections (grades III and IV), chondrocytes in clusters and in areas of surface fibrillation showed the similar heterogeneous expression pattern with other zones (Figure 3.6). The median scores and ranges for clones, superficial, mid and deep zones were 4 (3-5), 4 (3-5), 4 (3-5) and 4(3-5), respectively.
There was no detectable difference in the pattern of CD98 expression between normal and different grades of OA (**Table 3.3**). The age and sex of donors had no noticeable effect on the pattern of CD98 expression.

Table 3.3. Immunoreactivity of CD98 in different normal and OA articular cartilage sections, isolated from hip (femoral head) and knee (tibia, femoral condyle and patella) joints, was assessed. The positive staining was scored, as explained in chapter 2 (see 2.3.6, page 90), in a range of 1-5. In each zone, the mean ± SD of CD98 immunostaining is shown. Each zone of cartilage from different donors was evaluated and scored separately. Evaluation was performed three times. P value was calculated to compare different zones in both normal and OA articular cartilage sections. In normal and mild OA surface zone compared with all zones and P value was >0.05 (not significant). In severe OA clones grade was compared with the other zones and P value was >0.05 (not significant).

		Immunostaining Score (mean ± SEM)				
Sample	**n**	**Surface**	**Superficial**	**Clones**	**Middle**	**Deep**
Normal	16	3.8 ± 0.2	4±0.1	__	3.5±0.1	3.5±0.1
Mild OA	17	4.2 ± 0.1	3.8±0.1	__	3.5±0.12	3.5±0.2
Severe OA	7	N/A	N/A	5	4.1±0.3	4±0.2

N/A; not available.

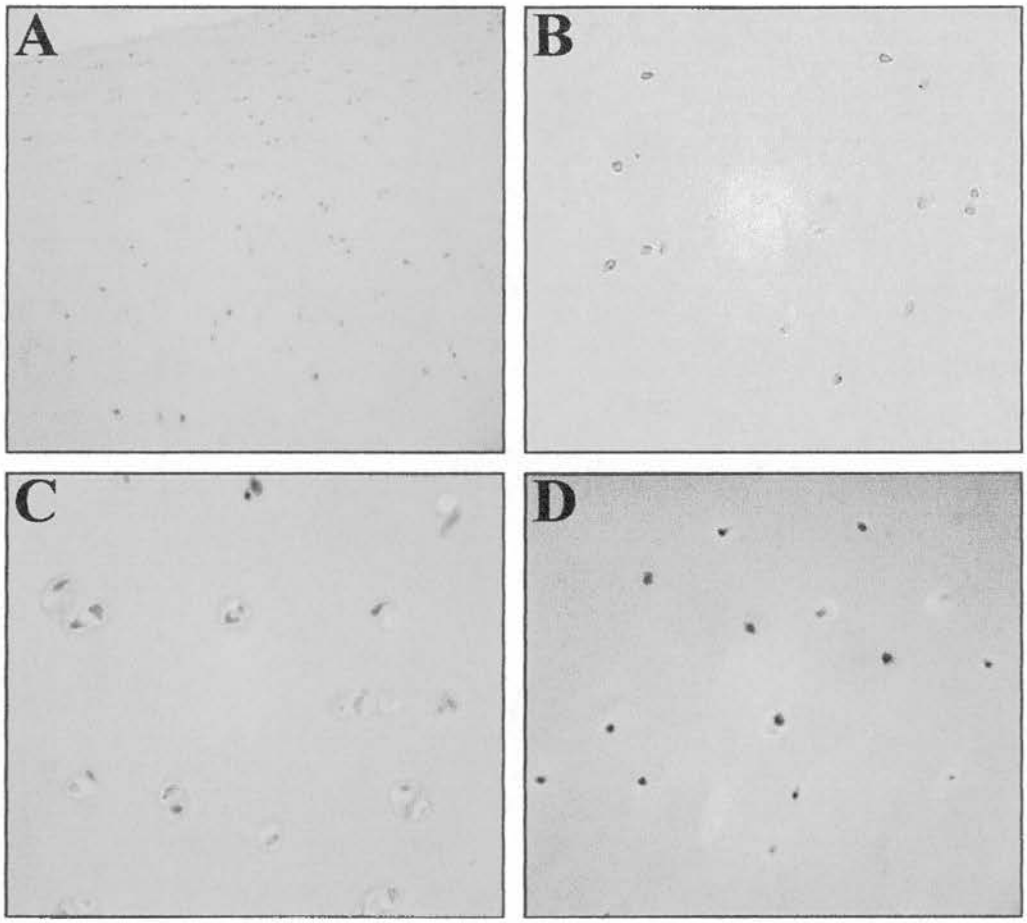

Figure 3.5. Immunoreactivity of mAb anti-CD98 4F2 in normal adult human articular cartilage sections. CD98 positive and negative chodrocytes were assessed in all different zones of normal articular cartilage by using 4F2 mAb at 1:50. **A**, Femoral head, superficial-deep zone, (female, 74) (13836), x100. In all zones from surface towards deep zone, chondrocytes showed a heterogeneous pattern for CD98 expression. **B,** Tibial plateaux, mid-zone, (female, 72) (11417), x200. Chondrocytes in mid zone showed a similar heterogenous pattern with the other zones. **C**, Femoral condyle, normal (FN), mid-zone, (female, 74) (11271), x400. High magnification of chondrocytes in mid-zone of TN, with heterogeneous expression of CD98. **D**, Negative control, femoral head, mid zone (female, 83) (13835), x200. No positive signal at all was observed in F/H section as negative control.

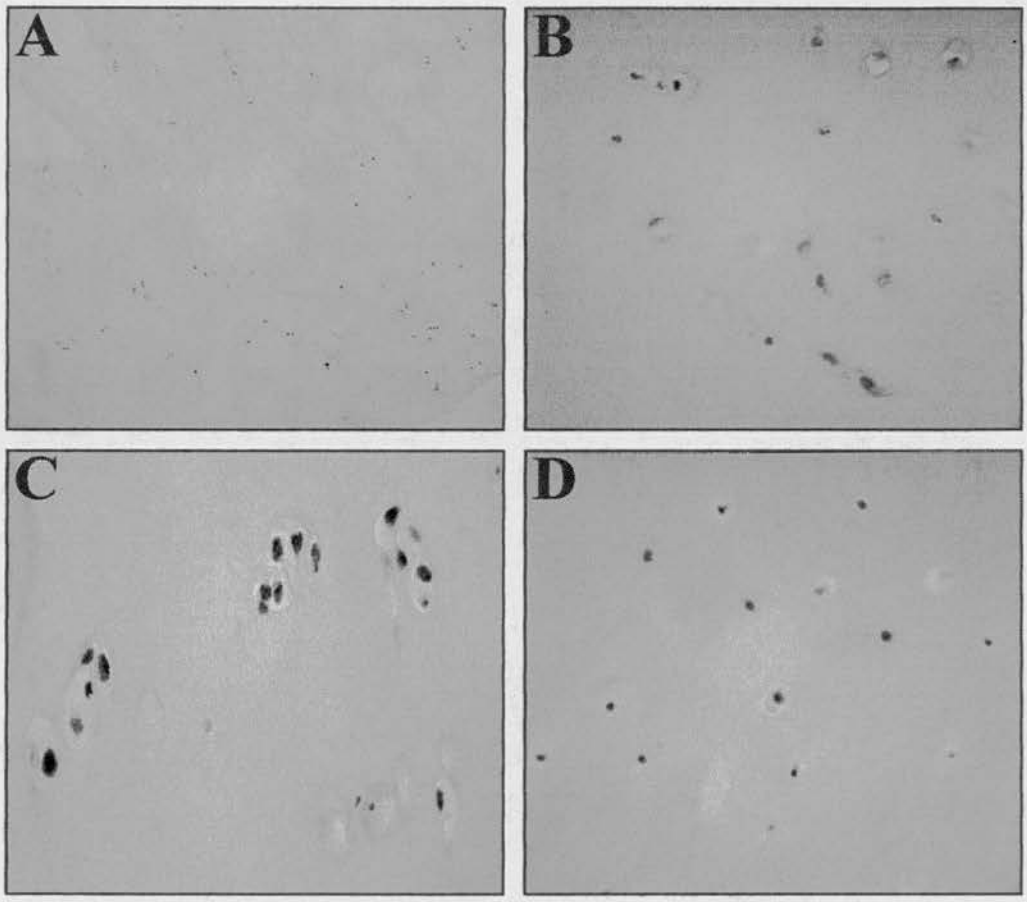

Figure 3.6. Immunoreactivity of mAb anti-CD98 4F2 in OA human articular cartilage sections. CD98 positive and negative chodrocytes were assessed in all different zones of OA articular cartilage by using 4F2 mAb at 1:50. **A,** Femoral condyle, OAIII, (male, age 62) (13817), surface-deep, x100. From clones towards deep zone of OA femoral head, all chondrocytes showed a similar heterogeneous expression pattern for CD98 .**B,** Tibial plateaux, OAII, (female, age 67) (10097), mid-zone, x200. Chondrocyte in mid-zone showed a similar heterogeneous pattern of CD98. **C,** Femoral head, OA II, (female, age 74) (13836), surface-mid zone, x400. High magnification of a clone from FAII showed heterogeneous expression of CD98. **D,** Negative control, femoral head, (female, age 83) (13835), mid-zone, x200. No positive signal at all was observed in F/H section as negative control.

3.2.3. In vitro evaluation (western blotting analysis) of CD98 in human articular chondrocytes

CD98 is a heterodimeric cell membrane glycoprotein consisting of an 85 kD heavy chain (HC) and a 40 kD light chain (LC). CD98 is expressed in various normal cells and tumour cells (Hemler and Strominger 1982). To confirm the in vivo immunohistochemical results, a series of western blot experiments was carried out. Chondrocytes were obtained from hip, knee and ankle joints.

Normal chondrocytes were isolated from 3 talus, 1 tibia (ankle), all male (age range 69-80, mean 73); 2 femoral condyles, both male (mean age 75) (13901, 13907, 13915, 13920); 1 tibial plateaux, male (age 72) (13941); 3 femoral heads, 2 males and one female (age range 70-83, mean 78) (13828, 13816, 13822). OA chondrocytes were isolated from 5 femoral condyles, 1 grade I, 1 grade II, and 3 grade III), 4 males, 1 female (age range 69-87, mean 76) (13964, 13939, 13905, 13918, 13924); 2 tibial plateaux, both grade II and female (age range 68-74, mean 71) (13820, 13938). Primary chondrocytes were seeded at $2x10^5$ cells/ml concentration. After a period of 10-15 days cultured chondrocytes were lysed and the proteins extracted. Extracts from normal and OA chondrocytes taken from different joints were analyzed and probed with one polyclonal goat anti-CD98 (SantaCruz) antibody at 1:500. Cell lysates obtained from cultured chondrocytes, isolated from normal and OA cartilage, were analyzed under reducing conditions and probed with goat polyclonal anti-CD98. A similar single band at ~85 kD was expressed by normal and OA chondrocytes (**Figure 3.7.A**). Cell lysates from normal and OA chondrocytes were also separated under non-reducing conditions and probed with the same polyclonal goat anti-CD98. These samples expressed an approximately 125 kD protein band (**Figure 3.7.B**). The intensity of the CD98 band in normal and OA extracts from different donors with different age and sex under reducing and non-reducing conditions was similar. A stronger band was detected in cell extracts obtained from ankle chondrocytes when compared with normal and OA of the other joints in both reducing and non-reducing conditions (**Figure 3.7. A, B**).

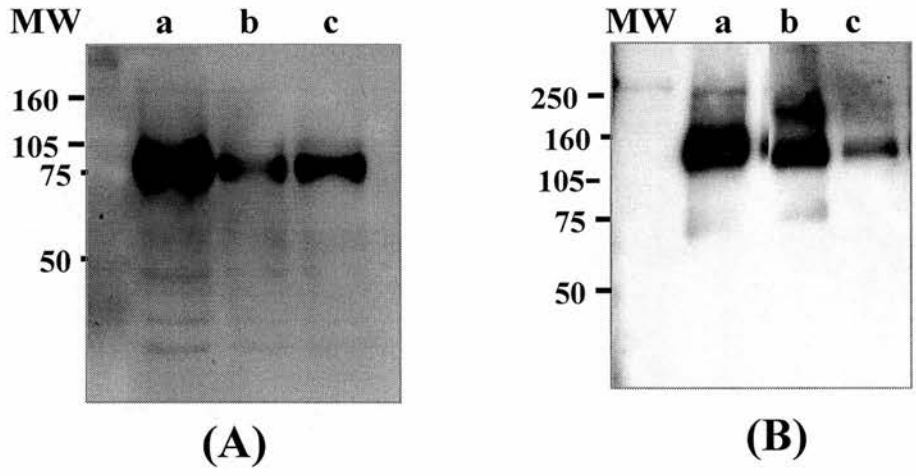

Figure 3. 7. Recognition and comparison of CD98 in normal and OA human articular cartilage. Equal amounts (40μg/lane) of total cellular proteins prepared from the cultured articular chondrocytes derived from normal ankle (lane **a**), normal (lane **b**) and OA (lane **c**) knee and femoral head were run in a 10% SDS-PAGE under reducing **(A)** and non-reducing conditions **(B)**. Goat polyclonal anti-CD98 (1:500) was used in both conditions. Heavy chain of CD98, ~85 kD MW, expressed in chondrocytes isolated from ankle (lane **a**, (tallus), male, age not recorded) (13901), normal femoral head (lane **b**, male, age 70) (13822), and OA (lane **c**, tibial plateaux, female, age 87) (13965) cartilage under reducing conditions **(A).** Total molecule, ~125 kD MW, expressed in chondrocytes isolated from ankle (lane **a**, talus, male, age 69), normal femoral condyle (lane **b**, male, age 80), and OA III femoral condyle (lane **c**, male, age 87) cartilage under non-reducing conditions **(B)**. Stronger band was observed in normal ankle in comparison to normal and OA knee and femoral head. The blots shown are representative a series of experiments including at least three different donors (normal and OA). Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

3.3.0. IN VIVO AND IN VITRO EVALUATION OF GALECTIN-3 IN HUMAN ARTICULAR CARTILAGE

To assess the in vivo expression pattern and determine precise immunolocalization of galectin-3 in normal and OA articular cartilage, paraffin sections isolated from normal ankle, femoral heads, and different anatomical regions of knee (femoral condyle, tibia plateaux and patella) were assessed by using immunohistochemical assessment. Immunohistochemical assessment was performed by using a mouse ani-galectin-3 mAb (9C4) at 1:400. Preliminary studies showed that this mAb was worked very well with paraffin embedded sections.

The positive immunoreactivity in different sections was graded in a range of 1-5. Galectin-3 staining for each zone was scored as: 1, no staining; 2, more cells negative than positive; 3, equal positive and negative; 4, more cells positive than negative; and 5, all cells positive (see chapter two, 2.3.6, page 90). Each zone of cartilage sections was evaluated and scored separately.

3.3.1. Expression of galectin-3 in normal human articular cartilage

Thirty five sections of normal articular cartilage were obtained from 9 males (age range 64-92, mean 76), 6 females (age range 57-90, mean 79) and 2 cases whose age and sex were not recorded.

Normal articular cartilage sections were isolated from 4 tibia, 3 males, 1 female, 4 talus, 2 males, 2 females, 1 fibula, male and 1 calcaneous, female, from 5 normal ankles, 3 males, 2 females (age range 71-80, mean 77) (13901, 13904, 13906, 13915, 13919); 7 femoral heads, 3 males, 4 females, (age range 72-90, mean 82), (13837, 13876, 13822, 13828, 13893, 13857, 13835); 1 femoral condyle, male (age 80) (13919); 4 tibia plateaux, 3 males, 1 female (age range 57-87, mean 70) (13888, 13855, 13905, 13904).

The pattern of expression in all sections showed significant difference between surface and the other zones ($P<0.001$) (**Table 3.4**). The immunoreactivity (cytoplasmic expression of galectin-3) increased progressively from surface towards the deep zone. The median scores and ranges for surface, superficial, mid and deep zones were 3 (2-4), 4 (2-5), 4 (2-5) and 4(3-5), respectively. Articular cartilage isolated from different joints showed a similar expression pattern (**Figure 3.8**). The age and sex of donors had no noticeable effect on the pattern of galectin-3 expression in different joints.

3.3.2. Expression pattern of galectin-3 in OA human articular cartilage

To assess the expression pattern and determine precise immunolocalization of galectin-3 in OA articular cartilage, 11 mild (2 grade I and 9 grade II), and 6 severe OA (5 grade III and 1 grade IV), isolated from femoral heads, and different anatomical regions of knee (femoral condyle, tibia plateaux and patella) were assessed by using immunohistochemical experiments. Samples of OA articular cartilage were obtained from 5 males (age range 64-92, mean 79), 9 females (age range 57-90, mean 79) and one case whose age and sex was not recorded.

Samples of mild OA articular cartilage were obtained from 2 males (age range 64-92, mean 78) and 5 females (age range 57-90, mean 78). These samples were isolated from 7 femoral heads, 4 males, 3 females (age range 71-92, mean 83) (13893, 13857, 13828, 13822, 13876, 13837, 13835); 1 femoral condyle, male (age 64) (13855); 2 tibial plateaux, 1 male, 1 female (age range 57-64, mean 60) (13855, 13904) and 1 patella, male (age 80) (13919). Articular cartilage from mild OA grades I and II exhibited significant difference between expression pattern of surface and the other zones ($P<0.001$). The median scores and ranges for surface, superficial, mid and deep zones were 2 (2-4), 4 (2-4), 4 (2-4) and 4(3-5), respectively. The pattern of galectin-3 expression in mild OA sections was similar to normal articular cartilage sections. In normal and mild OA cartilage sections, mid and deep zone chondrocytes in all samples showed stronger immunoreactivity in comparison to surface zone which fewer cells were positive.

Samples of severe OA articular cartilage were obtained from 3 males (age range 73-87, mean 80), 3 females (age range 71-86, mean 79). These samples were isolated from 4 femoral condyles, all male (age range 73-80, mean 80) (13888, 13919, 13905, 133901); 2 tibial plateaux, both male (age range 64-87, mean 75)(13855, 13905).

Chondrocytes in all zones of severe OA cartilage sections (n= 6) showed a similar strong expression of galectin-3 (**Table 3.4**). Clones of degenerated surface zone showed stronger expression of galectin-3 in comparison to the surface zone of normal and mild OA. The median scores and ranges for clones, superficial, mid and deep zones were 4 (4), 4 (4), 4 (4-5) and 4.5(4-5), respectively. Galectin-3 expression pattern in various zones of severe OA sections was similar ($P>0.05$). In severe OA, chondrocytes of clusters, showed strong immunoreactivity of galectin-3, as did chondrocyte of various zones (**Figure 3.9**).

Table3.4. Immunoreactivity of galectin-3 in cytoplasm of chondrocytes isolated from normal, mild and severe OA articular cartilage sections. Significant difference was observed between surface and the deep zone of normal and mild OA articular cartilage ($P<0.001$). In contrast, different zones of severe OA sections did not show significant difference in galectin-3 expression ($P>0.05$). Chondrocytes in all zones of severe OA sections (grades III and IV) showed strong expression of galectin-3. The positive staining was scored in a range of 1-5. In each zone, the mean ± SEM of galectin-3 immunostaining by chondrocytes is shown. Each zone of cartilage from different donors was evaluated and scored separately. Evaluation was performed three times and then statistically analysed. In normal sections surface zone compared with the other zones and P value was <0.001 (significant). In mild OA surface zone was compared with the other zones :surface and superficial P value >0.05 (NS)*, surface and mid-zone >0.05 (NS) and surface and deep zone P value < 0.001 (significant). Severe OA clones grade was compared with the other zones and P value was >0.05 (not significant). All zones in severe OA sections showed a similar strong expression of galectin-3.

		Immunostaining Score mean ± SEM				
Sample	**n**	**Surface**	**Superficial**	**Clones**	**Middle**	**Deep**
Normal	35	2.8±0.1	3.5 ± 0.1	—	3.7 ± 0.1	4.4 ± 0.1
Mild OA	11	2.7 ± 0.2	3.3 ± 0.2	—	3.4 ± 0.2	4.3 ± 0.1
Severe OA	6	N/A**	N/A	4	4.3 ± 0.2	4.8 ± 0.1

*, NS, not significant

**, N/A, not available.

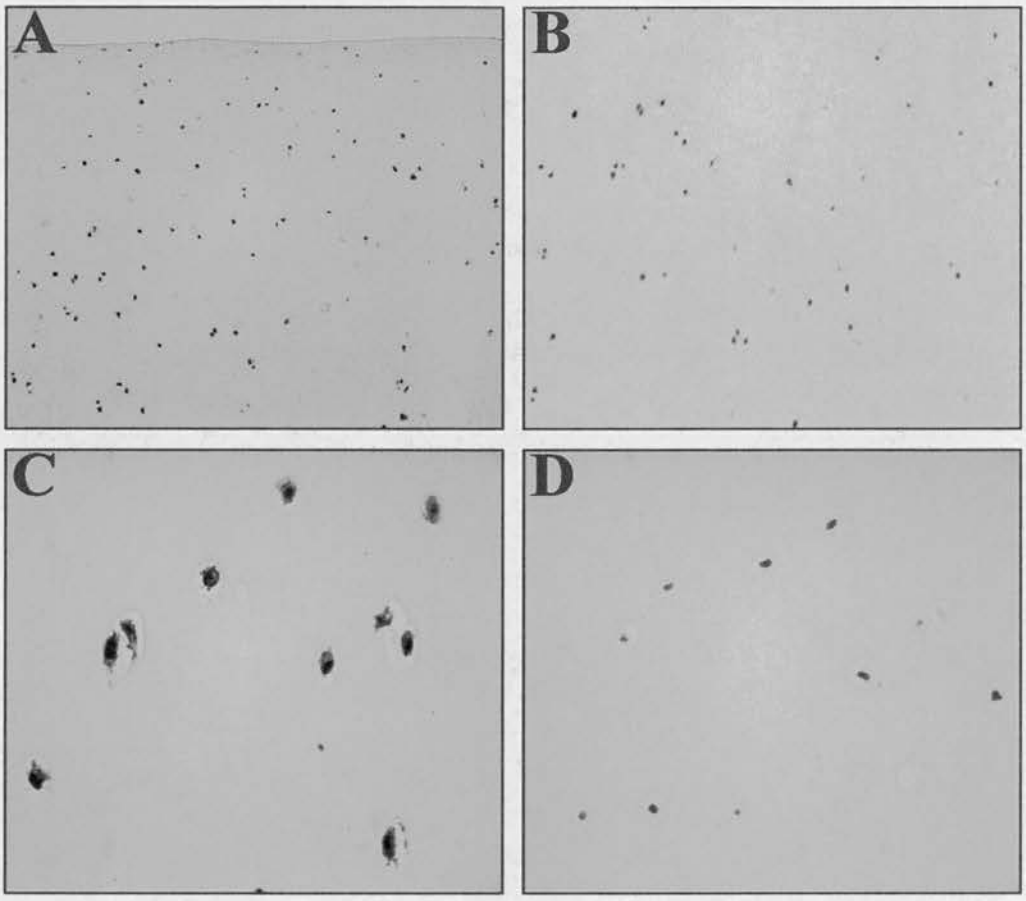

Figure 3.8. Histochemical localization of cytoplasmic expression of galectin-3 in normal human articular cartilage sections. Chondrocytes from normal articular cartilage sections showed positive galectin-3 immunoreactivity in comparison to negative controls. The sections were immunostained by standard ABC method using mouse mAb anti-galectin-3 (9C4) (1:400) and DAKO detection kit. **A**, Femoral condyle, normal (FN) (male, age 80) (13919), superficial-mid zone, x100. Galectin-3 immunoreactivity is increased from surface towards the deep zone. **B**, Tibial plateaux, male, (age 73) (13888), Mid-deep zone, x100. Chondrocytes in middle zone showed strong expression of galectin-3. **C**, Tibia (ankle), (male, 80) (13901), mid-zone, x400. High magnification of chondrocytes in mid-zone of Tibia, with strong expression of galectin-3. **D,** Negative control, talus (ankle) (female, 80) (13908), mid-zone, x100. No positive signal at all was observed in this section as negative control.

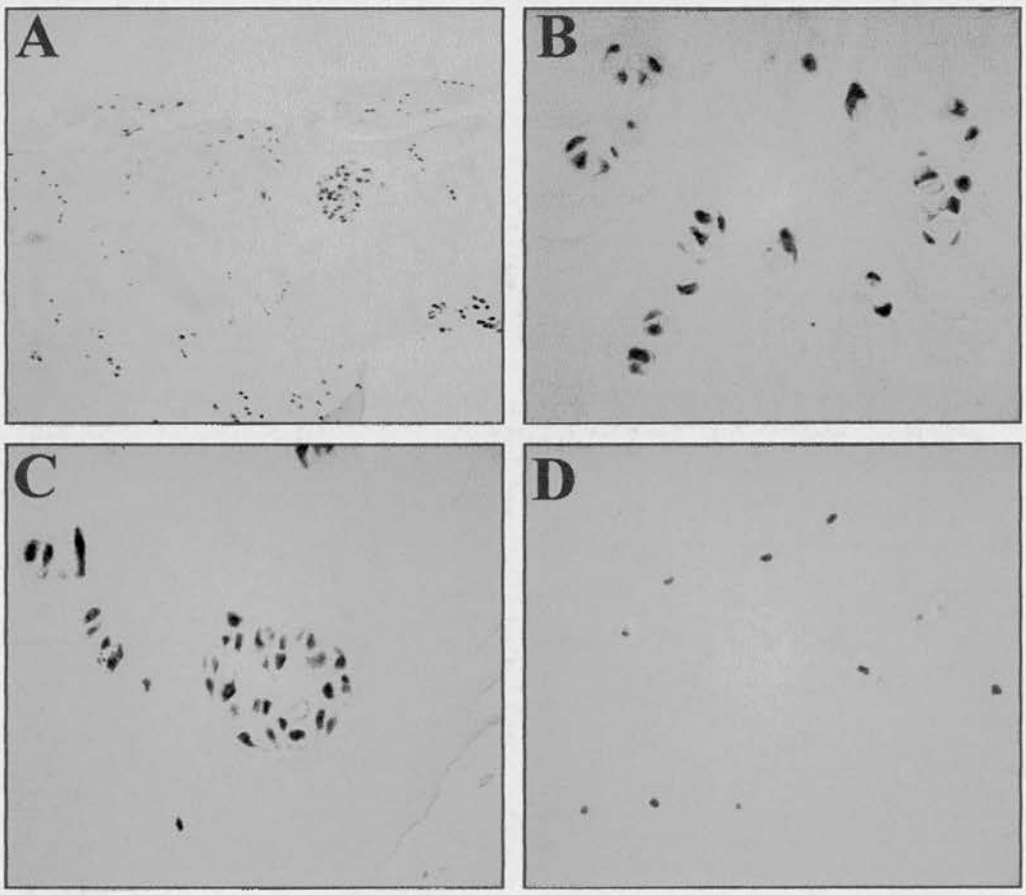

Figure 3.9. Histochemical localization of cytoplasmic expression of galectin-3 in OA human articular cartilage sections. Chondrocytes from OA articular cartilage sections showed positive galectin-3 immunoreactivity in comparison to negative controls. The sections were immunostained by standard method using mouse mAb anti-galectin-3 (9C4)(1:400) and DAKO detection kit. **A**, Tibial plateaux, OAIII, (male, age 87) (13905), surface-deep zone, x100. All zones showed a similar strong expression of galectin-3. **B**, Femoral condyle OA, FAIII, (male, 80) (13901), mid-zone, x200. Chondrocytes in middle zone showed strong cytoplasmic staining for galectin-3. **C**, Clone in tibial plateaux, OAIII, x400 (male, 87) (13905). Clones in severe OA sections showed strong expression of galectin-3. **D**, Negative control, talus (ankle), (female, 80) (13908), mid-zone, x100. No positive signal at all was observed in this section as negative control.

3.3.3. In vitro evaluation (western blotting analysis) of galectin-3 in cultured human articular cartilage

Galectin-3 is a 30 kD carbohydrate-binding protein that plays a role in cell-cell and cell-ECM interactions. To assess in vitro expression of galectin-3 in human articular chondrocytes, analysis of the whole cell lysates obtained from normal and OA cultured human articular chondrocytes was performed.

Normal samples were obtained from ankle (3 talus, 1 tibia), all male (age range 69-80, mean 73) (13901, 13907, 13915, 13920); 2 femoral condyles, both male (mean 71) (13939, 13941); 1 tibial plateaux, male (age not recorded) (13949); 3 femoral heads, 2 males and one female (age range 70-83, mean 78) (13828, 13816, 13822). OA samples were obtained from 1 femoral condyle (grade II), male (age 69) (13939); 3 tibial plateaux, (1 grade II, 2 grade III), all female (mean age 76) (13965, 13938, 13820); 2 femoral heads, both grade II, 1 male, 1 female (age range 87-95, mean age 91) (13828, 13821). Cultured primary chondrocytes at $2x10^5$ cells/ml concentration for a period of 10-15 days, were lysed and their proteins extracted. Extracts from normal and OA cultured chondrocytes taken from different joints were analysed and probed with monoclonal anti-galectin-3 (9C4) antibody at 1:500.

A similar 30 kD band was expressed by normal and OA chondrocytes obtained from knee and femoral head, under reducing conditions (**Figure 3.10. b, c**). The band expressed from normal and OA chondrocytes did not show identifiable modification. All extracts obtained from chondrocytes isolated from mild and severe OA, removed from donors of different age and sex, when separated under reducing conditions and probed with anti-galectin-3 monoclonal (9C4) antibody, showed a single band at ~30 kD (**Figure 3.10**). The intensive of galectin-3 band in normal and OA extracts from different joints or donors with different age and sex under reducing conditions was similar. A stronger band of galectin-3 expression in the same amount of whole cell lysate extracted from normal ankle chondrocytes was detected (**Figure 3.10. a**), when compared with the band obtained from normal and OA cultured chondrocytes from knee and femoral head.

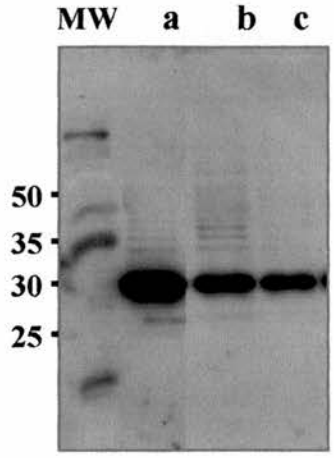

Figure 3.10. Recognition and comparison of galectin-3 expression in normal and OA human articular cultured chondrocytes. Equal amounts (40μg/lane) of total extracted proteins prepared from the cultured chondrocytes derived from normal ankle (lane **a**, tibia, male, age 80) (13920)*, normal (lane **b**, femoral condyle, male, age 69) (13939) and OAIII (lane **c**, tibial plateaux, female, age 79) (13938) were run in a 15% SDS-PAGE under reducing conditions. The detection of galectin-3 was assessed by probing the blots with mouse mAb anti-gal-3 (9C4) at 1:500. The stronger expression of galectin-3 in chondrocytes derived from normal ankle in comparison to knee and femoral head was detected. The blot shown is representative a series of experiments including three different donors (normal and OA). Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

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3.4.0. DISCUSSION

Cell-ECM interactions play a crucial role in cellular functions such as cell adhesion and mechanotransduction. Integrins, heterodimeric molecules, which are type I transmembrane glycoproteins, mediate these interactions. Extracellular domains of integrin subunits constitute the ligand binding domain(s) of these receptors. Cytoplasmic domains of integrins interact with the actin cytoskeleton and membrane ion channels and provide a physical connection between the internal and external environments. Therefore, integrins are signalling receptors that transmit information in both directions across the plasma membrane and provide an intersection where mechanical forces, cytoskeletal organization and biochemical signals meet (Hynes 1992; Aplin et al 1998). It has been previously suggested that α5β1 integrin acts as a mechanoreceptor in human articular cartilage (Wright et al 1997; Salter et al 2001). Since integrins, such as α5β1 integrin have no intrinsic enzymatic activity of their own, and also are not phosphorylated, they must interact with other proteins to generate signals (Damsky and Werb 1992).

In order to find a cell surface accessory molecule(s) for α5β1 integrin, the first step of this investigation was focused on the detection and comparison in vivo expression of three integrin-related proteins, CD47/IAP, CD98 and galectin-3, in normal and OA human articular cartilage. One of the objectives of this study was to determine whether integrin-related proteins CD47, CD98 and galectin-3, are expressed in human articular cartilage and to compare the topographical and zonal distribution of these proteins in normal and OA cartilage. It was noted that alterations in the expression or activity of one or more of the molecules involved in the signal transduction pathway could conceivably modify the response of diseased chondrocytes.

Human articular cartilage is an inhomogeneous tissue, with variation in matrix composition, chondrocyte morphology, and metabolism occurring with depth from the articular surface. Previous studies have demonstrated that cells isolated from different zones of articular cartilage retain their morphological and metabolic characteristics when

maintained in homogeneous cell culture conditions such as monolayer (Aydelotte et al 1988; Lee et al 1993) and agarose culture (Lee and Bader 1995,1997). Variation in the behavior of subpopulations of chondrocytes can be maintained in culture for as long as 3 weeks, demonstrating that intrinsic differences between subpopulations do exist (Lee et al 1998).

3.4.1. In vivo and in vitro expression of CD47 in human articular cartilage

This study showed the presence of IAP/CD47 in normal and OA articular cartilage. CD47 strongly expressed on the plasma membrane of chondrocytes in all zones by human chondrocytes in both normal and OA human articular cartilage. There was no topographical variation in the expression of CD47 in either normal or OA articular cartilage. Different grades of OA did not show any modification in strong expression of CD47 in all zones of articular cartilage. There was no significant alteration in staining pattern between different cases.

Biochemical analysis of both normal and OA cultured chondrocytes obtained from different joints expressed a band at nearly 50 kD as CD47. In vitro results were consistent with in vivo immunohistochemical studies. In addition, in vitro results showed two bands, which could be due to glycosylation of CD47, which is discussed in previous studies (Mawby et al 1994, see below). This supports the extensive expression of CD47 and this cell surface protein might be important in chondrocyte function potentially mechanotransduction.

CD47 is a multiple membrane-spanning IgSF member of ~50 kD molecular weight (MW), and a number of cell types are recognized to express this molecule. Brown et al (1990) have initially demonstrated that CD47 is expressed on the plasma membrane of all hematopoietic cells, such as T cells (Reinhold et al 1997), neutrophils (Parkos et al 1996), mast cells (Ghannadan et al 1998), bone marrow stromal cells and spleen (Furusawa et al 1998) and erythrocytes, which express no known integrins (Brown et al

1990). Non-haematopoietic cells such as mesenchymal cells (Wang et al 1998), epithelial and endothelial cells (Parkos et al 1996), fibroblasts and brain (Mawby et al 1994; Jiang et al 1999) express CD47 as well. Campbell et al (1992) have also shown that the tissue distribution of CD47/IAP is ubiquitous, by examining mRNA (Northern blot studies) from a variety of tissues including heart, skeletal muscle and lung. Mawby et al (1994) have reported a strong expression pattern of CD47 in brain in comparison to variable expression in different layers of epithelia such as skin by using immunohistochemical studies. This is the first study detailing the topographical and zonal distribution of IAP/CD47 in normal and OA human articular cartilage.

IAP is expressed in essentially all tissues, but the four alternatively spliced forms of IAP mRNA have distinct tissue distribution such as forms 1 and 2 in human keratinocytes, only form 2 in human umbilical vein endothelial cells, and form 4 in a nasal epithelial carcinoma cell line (2650). Much of the form 2 found in many tissues arises from endothelium, interstitial fibroblasts, or resident lymphocytes and macrophages. Form 4 is expressed predominantly by neural tissue (Reinhold et al 1995). The two most common CD47 are the isoforms 2 and 4. Variation in the function of CD47 may be due to alternative splicing of mRNA and the production of four isoforms of different molecular weights. The pattern of CD47 expression on cultured chondrocytes, following biochemical analysis, shown in the present study suggests that the molecule may exist in more than one form, possibly due to differential glycosylation or to the variation in existence of four isoforms on different cell types. CD47 has six potential N-glycosylation sites, five of which are in an Ig superfamily domain (Campbell et al 1992; Mawby et al 1994). Mawby et al (1994) have shown that three of these sites carry N-glycans in erythrocytes and deglycosylation of erythrocyte CD47 generated components of 26-26.5 kD, together with a defined band of approximately 30 kD and a mature protein band at 50 kD as well. These studies suggest that the pattern of CD47 glycosylation is varied in different cell types and may change under cellular certain conditions.

Another possible explanation for expression of two different bands with differential molecular weight in a range of 47-55 kD was the presence of two isoforms of CD47 for this protein in chondrocytes. As shown previously, both by RT-PCR and immunohistochemistry, there is cell-type specificity of expression of the different IAP isoforms.

Eight mAbs and one polyclonal Ab were tested to evaluate the biochemical characterization of CD47 molecule on chondrocytes and find out which one works with western blotting. Four antibodies were not suitable for western blotting, which may be because of denaturation or modification of epitopes during the procedure. The presence of specific conformational epitopes on the CD47 molecule may have different functions in antigen presentation and this could be due to the certain conditions of chondrocytes.

Since CD47 and β3 integrin were physically and functionally associated, CD47 was initially called integrin-associated protein (IAP) (Brown et al 1990; Lindberg et al 1994). CD47 has been shown to associate with integrins in multiple cell types (Porter and Hogg 1998) in different cellular functions such as integrin-mediated calcium influx in endothelial cells (Schwartz, 1993), cell adhesion (Lindberg et al 1996) and cell migration (Wang et al 1998). Thus, it is possible that the functions of IAP extend beyond integrin signal transduction. In addition, it has been shown that in human bone cells (HBC), anti-CD47 Bric 126 antibody inhibits the integrin-mediated hyperpolarization response induced by 0.33 Hz mechanical stimulation (Salter et al 1997). Furthermore, recently Graf et al (2003) have demonstrated a common role of CD47/integrin complex in multiple mechanical stimuli and different cell types.

A variety of data suggests that CD47 functions as a signalling molecule. The strong expression of CD47 in normal and OA articular cartilage and the known ability of CD47 to act as an integrin accessory protein suggest that chondrocyte CD47 would have a signalling function. Similarly, strong expression of α1β1, α5β1 and αVβ5 integrins (Woods et al 1994), and especially α5β1 integrin in vivo (Salter et al 1992) and in vitro (Loeser et al 1995) in articular cartilage have been previously

reported. In addition, the critical role of α5β1 integrin in integrin-mediated response of articular chondrocytes to mechanical stimulation and acting as a mechanoreceptor (Wright et al 1997; Salter et al 2001, 2002) suggests a parallel function of CD47 and α5β1 integrin. CD47 showed an expression pattern similar to α5β1 integrin on chondrocytes, and also has a potential to act as an accessory molecule for α5β1 integrin in articular cartilage. These suggest a functional role for CD47 in integrin-mediated cell matrix interactions in human articular cartilage.

It has been previously shown that CD47 is involved in integrin-independent functions in multiple cell types (Cooper et al 1995; Frazier et al 1999; Ticchioni et al 1997; Reinhold et al 1997; Waclavicek et al 1997; Wu et al 1999; Liu et al 2001). CD47 has integrin-dependent and integrin-independent functions. Since IAP/CD47 is expressed on cells independent of integrin expression (e.g., on erythrocytes), it may represent a molecule involved in signal transduction for other receptors as well (Brown et al 1990). Studies of the distribution of possible ligands such as thrombospondin-1 and SIRP-α would suggest potential interaction. It has been previously reported that TSP-1 is expressed in human articular cartilage (Miller and McDevitt 1988; DiCesar et al 1994; Pfander et al 2000). The interaction between TSP and CD47 induces the activation of autoreactive T lymphocytes (Vallejo et al 2000), the spreading of platelets (Chung et al 1999) and melanoma cells on fibrinogen (Gao et al 1996). These data raise the possibility that chondrocyte CD47 recognizes a ligand present in the ECM of articular cartilage. It will be important to establish CD47 ligand (s) in ECM of cartilage and to identify possible functions of this molecule in cell signalling and regulation of chondrocyte activity both in normal cartilage and in diseases such as OA. Thus, possible association between TSP-1 and CD47, suggests a route by which CD47 may influence chondrocyte-matrix interactions and mechanotransduction.

In summary, CD47 is strongly expressed on chondrocytes in normal and OA cartilage, and its expression does not appear to be altered by age, site, or grade/progression of

disease. This suggests that CD47 plays an important role in chondrocyte metabolic functions, such as mechanotransduction.

3.4.2. In vivo and in vitro expression pattern of CD98 in human articular cartilage

This study showed the presence of CD98 in normal and OA articular cartilage. CD98 is expressed on the plasma membrane of chondrocytes in heterogeneous immunoreactivity in both normal and OA adult human articular cartilage. There was no topographical variation in the expression of CD98 in either normal or OA articular cartilage. Different grades of OA did not show any modification in expression pattern of CD98 in all zones of articular cartilage. The staining pattern in different cases did not show significant alterations. Biochemical analysis showed that both normal and OA cultured chondrocytes obtained from different joints expressed CD98. Consistent with in vivo immunohistochemical results, CD98 is expressed by cultured normal and OA articular chondrocytes.

CD98 is a 125 kD disulphide-linked heterodimeric membrane glycoprotein, consisting of an 85 kD glycosylated heavy chain and a 40 kD non-glycosylated light chain (Haynes et al 1981). CD98 (4F2 antigen) was originally detected by a murine mAb raised against the human T cell tumor line HSB-2 (Eisebarth et al 1980; Haynes et al 1981). This antigen is expressed on all human tissue lines, the majority of malignant human cells, and peripheral blood cells (Eisebarth et al 1980; Haynes et al 1981). This antigen has also been detected in breast (Chandrasekaran et al 1999), keratinocytes (skin and hair follicles) (Patterson et al 1984), and embryonic but not adult fibroblasts (Azzarone et al 1984).

The present study for the first time showed the expression of CD98 by human chondrocytes in both normal and OA articular cartilage in a similar heterogeneous pattern. The possible mechanisms for the heterogeneous expression pattern of CD98 in either normal or OA articular cartilage are suggested by the other data. Initial reports on CD98 showed expression to be predominantly restricted to activating T cells. CD98 is

only expressed at low levels on resting T and B lymphocytes, a high level of expression can be induced following stimulation of resting T cells. Thus, CD98 appears to be a marker for cell activation and is found as well on monocytes (Haynes et al 1981; Suomalainen 1986). In this study, variation of CD98 expression in articular chondrocyte may reflect the following possibilities:

1. Chondrocyte heterogeneity

The recognized heterogeneity of chondrocytes is reflected in differences of metabolism and cell shape (Aydelotte et al 1988; Siczowski and Watt 1990). It is also extended to expression of cell receptor molecules, enabling chondrocytes to regulate their interactions with the pericellular environment under certain conditions. Salter et al (1995), using immunohistochemistry technique showed variation of integrin expression by chondrocytes at different sites (articular, epiphyseal, growth plate, and meniscal cartilage) in human fetal knees. Cells from the epiphyseal area showed findings consistent with those obtained by Durr et al (1993). There was no expression of β3, β4, β6, and the α3 subunit by chondrocytes (Durr et al 1993; Salter et al 1995). Salter et al (1992, 1995) also demonstrated that fetal articular chondrocytes expressed α2 and α6 integrin, whereas adult articular chondrocytes did not. This heterogeneity may reflect the variation in morphology and macromolecular content during cartilage development and maturation.

Previous studies have also demonstrated that the expression of cell surface molecules, such as integrins, is preferentially related to their ligand molecule(s) that are appropriately present in the pericellular matrix of human articular cartilage. Studies have shown that annexin V, a receptor for type II collagen (Reid et al 2000), as well as CD44, a receptor for hyaluronan (Aguiar et al 1999), are expressed by chondrocytes and mediate interactions with cartilage matrix components. However, in a study using the same adhesion assays, these molecules did not appear to play a major role in the adhesion of bovine chondrocytes to bovine cartilage (Kurtis et al 2001). Integrin or non-integrin receptors, may also mediate chondrocyte adhesion to cartilage. Nevertheless,

under the certain conditions (Kurtis et al 2003), several integrins were implicated in adhesion of chondrocytes to cartilage, whereas others were not. In addition, Woods et al (1994) have demonstrated that mAbs to integrins, readily detected their target subunits once chondrocyte integrins were freed from interactions with matrix components or ligands. In this respect, this study focused on galectin-3, as a potential ligand for CD98, but was unable to find a parallel pattern of expression of the two molecules in human articular cartilage. The expression of galectin-3 was limited to intracellular in chondrocytes. The zonal variation of galectin-3 immunoreactivity in normal articular cartilage and significant difference between normal and OA cartilage in the expression pattern of galectin-3 were the other dimensions of difference between CD98 and galectin-3 expression. This is the first study that has shown the expression of CD98 and its potential ligand, galectin-3, by chondrocytes and there is no more about the function of this glycoprotein in cartilage.

2. Differential masking of accessibility of the anti-CD98 antibodies to chondrocyte

It is possible that because of CD98 interaction with ECM ligands epitopes recognized by the antibody 4F2 used in this study could be masked. To look at this, It would be possible to pretreat sections with proteases such as chondroitinase which will remove ECM molecules. Alternatively, we could use a panel of antibodies against epitopes or look at expression pattern other subpopulations of chondrocytes from cartilage. Cho et al (2001) have reported that CD98 antibodies are highly heterogeneous both in function and in the ability to bind to CD98 on the U937 cell surface. They have also demonstrated that it seems unlikely that this heterogeneity simply reflects concentration or affinity of the antibodies used, since each antibody was tested over a wide range of concentrations. Thus CD98 molecule has heterogeneous biological characteristics.

In this respect, several groups have focused on the variation of the other cell surface molecules such as integrin expression under some specific circumstances. Salter et al (1995) have shown that α2 integrin immunoreactivity was similar to the distribution of types I and III collagen in human fetal articular chondrocytes (Treilleux et al 1992).

This suggested the possibility that expression of adhesive molecules is dependent in part on the environment in which a chondrocyte finds itself. Both tenascin (Mackie et al 1987) and fibronectin (Dessau et al 1980) are variably expressed in developing limb cartilage. In later stages of limb and joint development, it appears that molecules such as fibronectin isoforms and tenascin may show differential expression in articular cartilage and immature cartilages that will undergo maturation and be replaced by bone (Pacifici et al 1993). Evidence shows that integrins on a number of cells must be activated to function as adhesive receptors (Kieffer and Philips 1990; Philips et al 1991). Examples exist of continued expression of inactive forms of some integrins, such as $\alpha 5\beta 1$ and $\alpha 6\beta 1$, after differentiation of keratinocytes and retinal neurons (Neugebauer and Reichardt 1991; Adams and Watt 1990). Studies have shown poor adherence in vitro of freshly extracted adult articular chondrocytes to a variety of adhesion proteins, including fibronectin and collagen, despite expression of appropriate receptors (Loeser 1993; Salter et al 1995). However, adherence to these substrates was identified after a period in monolayer culture. These findings suggested that mature chondrocytes may express low-affinity receptors which are activated in culture conditions. Similarly, high affinity receptors may be expressed by chondrocytes in vivo only when the appropriate stimuli are present (Salter et al 1995; Loeser et al 1993).

The diversity of responses in which the 85 kD membrane protein, as CD98, has been implicated, in multiple cell types, supports the notion that this molecule probably plays a key role in cell signalling. The functional (Fenczik et al 1997; Warren et al 2000) and physical (Miyamoto et al 2003) association between CD98 and CD29 ($\beta 1$ integrin) in the cell membrane are well documented and has led to the suggestion that CD98 signalling into the cell is in fact mediated via integrin activation. Several lines of evidence suggest that CD98 may modulate the integrin-mediated functions such as T cell co-stimulation (Warren et al 2000), integrin-dependent adhesion in two separate studies (Fenczik et al 1997; Chandrasekaran et al 1999), cell fusion (Ohta et al 1994), cell-cell fusion and aggregation (Tabata et al 1994) and amino acid transport (Pineda et al 1999).

A genetic screen revealed that CD98 might indirectly influence integrin affinity for ligand. In particular, CD98 has been shown to stimulate β1 integrin-mediated cell adhesion to ECM (Fenczik et al 1997). Kolesnikova et al (2001) by using co-immunoprecipitation have recently demonstrated that CD98 constitutively and specifically associates with β1 integrins (α2β1, α3β1, α5β1, α6β1), but minimally with α4β1. Recently, Rintoul et al (2003) have reported that cross-linking CD98 induced the clustering of β1 integrins to form high density complexes and proposed that CD98 acted as a 'molecular facilitator' in the plasma membrane. This results in integrin activation, integrin-like signalling, and anchorage-independent growth.

These findings reveal that CD98 can regulate different types of adhesion molecules including integrins through distinct mechanism, reinforcing the notion that CD98 acts as a critical protein in articular cartilage metabolism and mechanotransduction. Based on these data so far, showing the functional and physical association between CD98 and β1 intgerins, it would be reasonable to test the idea that CD98 in articular cartilage might form a physical or functional complex on the chondrocyte cell membrane that potentially coordinates and regulates integrin-dependent functions and signalling in articular cartilage. However, it is equally possible that CD98 has a dynamic role in chondrocytes metabolic activity such as matrix receptor in cell-matrix interactions and protein transport.

3.4.3. Comparison of the expression pattern of CD98 and Galectin-3 in human articular cartilage sections

Galectin-3 binds to CD98 heavy chain in both a human T cell line (Jurkat) and in murine macrophage (WEHI-3) (Dong and Hughes 1997), but a direct link between galectin-3 binding to this surface antigen and its biological effect has not been demonstrated. This suggested comparing the pattern of expression of CD98 and Galectin-3 in normal and OA cartilage.

An objective of this project was to ascertain and compare the pattern of CD98 and galectin-3 expression in normal and different grades of OA by using immunohistochemical studies. Both CD98 and galectin-3 were expressed in human articular cartilage. CD98 was expressed on cell membrane and galectin-3 expressed in cytosol.

Compared with CD98 expression pattern, galectin-3 immunoreactivity observed in human articular cartilage sections was strikingly different. There was no positive relationship between the immunohistochemical staining score of CD98 and galectin-3. Indeed, CD98 scores in all zones were moderate, and this pattern was similar (consistent) in different grades of normal and OA cartilage sections. In contrast, a statistical trend was observed for galectin-3 scores in various zones of normal and mild OA sections. In addition, an upregulation pattern of staining in severe OA was observed.

Analysis of the data according to immunohistochemistry results revealed that the expression of galectin-3 in human articular cartilage was significantly associated with the zone of cartilage and the grade of OA disease. Whereas, CD98 expression approximately in all zones of normal and OA articular cartilage sections showed a similar heterogeneous pattern.

Thus, no detectable correlation was found between CD98 and galectin-3 expression pattern in normal and OA human articular cartilage.

In summary, although this study was unable to show an association between CD98 and galectin-3 expression pattern in human articular cartilage, it seems that CD98 and galectin-3 have the other roles in chondrocyte physiology.

3.4.4. Role for galectin-3 in human articular cartilage

This study has demonstrated that galectin-3 is expressed by human articular chondrocytes. Galectin-3 is expressed in the cytoplasm of chondrocytes in both normal and OA human articular cartilage. There was topographical variation in the expression of galectin-3 in normal and mild OA articular cartilage. In both normal and mild OA sections the immunoreactivity was increased progressively from surface towards the deep zone. Severe OA did not show any modification in strong expression of galectin-3 in all zones of articular cartilage. The immunohistochemistry results from different cases were consistent. Biochemical analysis showed that galectin-3 is expressed by cultured normal and OA articular chondrocytes obtained from different joints.

Galectin-3 is a 30 kD galactoside-binding protein belonging to the galectin family. This protein is composed of carboxyl-terminal carbohydrate recognition domain (CRD) and amino-terminal tandem repeats. Galectin-3 normally distributes in epithelia of many organs and various inflammatory cells, including macrophages, as well as dendritic cells and kupffer cells (Flotte et al 1983). The expression of this lectin is up-regulated during inflammation (Flotte et al 1983), cell proliferation (Moutsatsos et al 1987), and cell differentiation (Liu et al 1995; Nangia-Makker et al 1993). Its expression is also affected by neoplastic transformation, up-regulated in certain types of lymphomas (Hsu et al 1996; Konstantinov et al 1996) and thyroid carcinoma (Fernandez et al 1997; Xu et al 1995), while down-regulated in other types of malignancies, such as colon (Lotz et al 1993; Castronovo et al 1992), breast (Castronovo et al 1996), ovarian (vanden Brûle et al 1994), and uterine (vanden Brûle et al 1996) carcinomas. It has been reported that the expression of this lectin has a strong correlation with the grade and malignant potential of primary brain tumors (Bresalier et al 1997), although there are conflicting reports on the role of galectin-3 in cell adhesion and tumor metastasis. Increased galectin-3

expression has also been noted in human atherosclerotic lesions (Nachtigal et al 1998). These findings suggest that galectin-3 expression is affected during these physiological and pathological responses.

Galectin-3 is mainly localized in the cytoplasm, although, depending on cell types and differentiation stage, this protein can also be detected in nucleus, on cell membrane, or extracellularly (Barondes et al 1994). Therefore, it has been reported to function through both intracellular and extracellular actions.

1. Intracellular functions: Galectin-3 has been identified as a component of heterogeneous nuclear ribonuclear protein (hn RNP) (Laing and Wang 1988), a factor in pre-mRNA splicing (Dagher et al 1995), and prevent leukemia T cell apoptosis, that is probably mediated through interaction with the Bcl-2 family members (Yang et al 1996).

2. Extracellular (Exogenous) functions: Galectin-3 has long been suspected of modulating cell to ECM interaction in a novel fashion (Barondes et al 1994; Ochieng et al 1998). In vitro studies have suggested that galectin-3 is involved in a variety of processes, such as cell-cell or cell-ECM interactions, and it could be act as a signalling molecule (Hughes et al 1992, 1994; Mehul et al 1995).

It has been reported that this protein is secreted from monocytes/macrophages (Cherayil et al 1989; Sato and Hughes 1994) and epithelial cells (Sato et al 1993; Lindstedit et al 1993), and demonstrated to function as an extracellular molecule in activating various types of cells, including monocytes/macrophages (Liu et al 1995), lymphocytes (Hsu et al 1996; Dong and Hughes 1996), neutrophil (Yamaoka et al 1995) and mast cells (Frigeri et al 1993; Zuberi et al 1994). Sano et al (2000) have reported that extracellular (exogenously added) galectin-3 acts as a novel chemokinetic for monocytes and macrophages in inflammation process.

Dong and Hughes (1996) have initially shown that galectin-3 stimulated uptake of extracellular Ca^{2+} in human Jurkat T cells. They have also shown that exogenous

galectin-3 binds to surface glycoproteins, including CD98, in the human lymphoblastoid Jurkat T cell line (Dong and Hughes 1995, 1996). It has been recently shown that galectin-3 has a role in recirculation and shuttling of important molecules such as β1 integrin that results in limiting their expression on the cell surface, remodelling the cytoskeleton and modulating cell spreading and adhesion (Furtak et al 2001).

Galectin-3 treated human monocytes upregulate production of IL-1 (Jeng et al 1994). A number of cytokines have been implicated in the pathogenesis of OA, predominantly through regulation of matrix metalloproteinase (MMP) production (see 1.2.3, page 24). Evidence from work in other fields has indicated that galectin-3 may also regulate cytokines production and function (Sano et al 2000). The ability of galectin-3 to enhance the production of IL-1 is of significance, as this cytokine is recognized as mediator of joint destruction.

The data from other studies and the present study suggest the following possible functions of galectin-3 in articular chondrocytes:

1. Anti-apoptotic molecule

Matarrese et al (2000) have shown that galectin-3 overexpression in human breast cancer cells leads to a series of modifications, including cytoskeleton rearrangement of cell spreading as well as surface expression of α4 and β7 integrins, which resulted in increased cell adhesion and prolongs the survival of the cells and confers resistance to cell death by apoptosis. The antiapoptotic effect of galectin-3 appears to result from the ability of this molecule to heterodimerize with Bcl-2 protein, a well known suppressor of apoptosis (Yang et al 1996). Galectin-3 immunoreactivity has never been detected in the extracellular cartilage or bone matrices, suggesting that the protein is not secreted in cartilage and bone (Colnot et al 1999). The present study confirmed that galectin-3 is expressed as a cytoplasmic protein in articular chondrocytes from different joints. Several lines of evidence have previously indicated that galectin-3 is involved in the cascade of cellular events, occurring in chondrocytes, as they enter programmed cell

death in the zone of calcification (Bronckers et al 1996; Ohyama et al 1997; Roach 1997). Galectin-3 is an anti-apoptotic molecule in breast carcinoma cell line BT549 (Akahani et al 1997) and its over production confers resistance to programmed cell death induced by anti-Fas antibody and staurosporine in human leukemia T-cells (Yang et al 1996) and human breast epithelial cells (Matarrese et al 2000). The effect of galectin-3 seems to be mediated via binding to Bcl-2, a proto-oncogen protein suppressor of apoptosis. Bcl-2 is present in chondrocytes of epiphyseal plate cartilage (Wang et al 1997) whereas it may be involved in the survival of early maturing chondrocytes. The spatio-temporal distribution of Bcl-2 and galectin-3 in the plate cartilage are very similar (Colnot et al 1998, 1999).

Colnot et al (2001) have also reported that the galectin-3 null mutation in mouse has several effects on chondrocyte differentiation, most notably premature cell death of chondrocytes without concomitant vascular invasion in the metaphysis during endochondral bone formation. This suggested that there is intracellular regulation of chondrocyte apoptosis by molecules implicated in programmed cell death, like the proto-oncogene Bcl-2 (Alming et al 1997; Wang et al 1997).

2. Chondrocyte differentiation and endochondral ossification

Studies have demonstrated a role for galectin-3 in chondrocyte differentiation (Fowlis et al 1995; Colnot et al 1999, 2001) and endochondral ossification (Colnot et al 1998, 1999, 2001). Fowlis et al (1995) have shown that in the midgestation mouse embryo, the major site of galectin-3 expression is the cartilage. Colnot et al (1999) have shown that subcellular distribution of the protein in growth plate chondrocytes of fetal and neonatal mice is mainly cytoplasmic, with high amounts detectable in mature and early hypertrophic cells. These results suggested an intracellular function for galectin-3 in terminal differentiation of chondrocytes and galectin-3, considered to be a general cytoplasmic marker of the differentiation chondrocytes.

It also could possibly result from a role of galectin-3 as an adhesion or a signalling molecule promoting vascular invasion since it has been shown to induce endothelial cell morphogenesis and angiogenesis in vivo (Nangia-Makker et al 2000). An increase in expression of galectin-3 in advanced stages of OA may occur as a result of the imbalance in chondrocyte homeostasis that occurs in OA cartilage and provides a condition to modify normal chondrocyte to an OA chondrocyte.

Because galectin-3 influences cell growth and repair in peripheral nerve injury in nervous system (Pesheva et al 1998), it may also be important for the growth and injury protection of the cartilage tissue in grades III and IV OA that are advanced stages of OA degradation. Thus, it may contribute to the protection of the surface cartilage against inflammatory attacks.

In order to determine the possible functional significance of the zonal distribution of galectin-3 in normal and OA cartilage, potential ligands of galectin-3 in cartilage must be established and appropriate in vitro studies must be undertaken. A correlation of the zonal distribution of potential ligands of galectin-3 in articular cartilage with the distribution of galectin-3 could indicate a functional relationship. In this study, the main aim of galectin-3 immunohistochemical studies was to determine of one ligand, which in distribution match CD98. It appears that CD98 is unlikely to act as a ligand for galectin-3 in human articular chondrocyte.

Although, this work was unable to disclose a relation between CD98 and galectin-3 expression pattern in human articular cartilage, but the expression pattern of these two molecules in human articular cartilage may clarify more details about the cartilage biology. Further studies are needed to elucidate the precise functional role of this molecule in the healthy and diseased chondrocyte metabolism and possibly in mechanotransduction pathway and progression of OA.

Western blotting analysis did not show difference between normal and OA cartilage in galectin-3 expression pattern. Despite the differential expression of galectin-3 in normal and severe OA cartilage, no identifiable difference was found between normal and OA

cultures chondrocytes. For a precise comparison of the immunohistochemistry results and western blotting analysis, further studies are needed to analyse galectin-3 expression of numerous cultured chondrocytes from different grades of OA cartilage. In addition, it has been shown that in vivo and in vitro conditions affect the expression pattern of different molecules (Salter et al 1995; Loeser et al 1993).

Thus, the picture that has emerged is that intracellular galectin-3 contributes to the essential functions such as terminal differentiation (Colnot et al 1999) and cell survival (Colnot et al 2001) in chondrocytes. In summary, galectin-3 association with some types of integrins in the other cells such as $\alpha 1\beta 1$ in adenocarcinoma cells (Ochieng et al 1998) and $\alpha M\beta 2$ in macrophage (Dong et al 1997) and ECM glycoproteins like fibronectin (Probstmier et al 1995), laminin (Ochieng et al 1995) and tenascin (Rosenberg et al 1991), anti-apoptotic effect (Colnot et al 2001), cell differentiation (Colnot et al 1998, 1999, 2001), effect on IL-1 production (Jeng et al 1994) and also its effect on $[Ca^{2+}]i$ in Jurkat cells (Dong and Hughes 1996), suggest different roles for galectin-3 in human articular cartilage. Further work is needed to determine which proteins interact with galectin-3 in normal and OA articular cartilage, to control chondrocyte metabolism during development and in disease processes such as osteoarthritis.

3.4.5. Comparison of the in vitro expression of various proteins in chondrocytes isolated from different joints

The proteins, CD47, CD98 and galectin-3, studied in this project, appear to be present at greater levels in normal ankle cartilage than in normal and OA cartilage from knee and hip joints. These data, in part, show the biochemical heterogeneity of different joints.

Since the results in several samples obtained from different donors were consistent, the results of this study were not because of artefact or any other technical variation. In addition, blot stripping and reprobing has confirmed the results obtained. The present study would be consistent with published literature confirming cartilage heterogeneity.

Finding out more details of differences between chondrocytes in various joints would provide an insight in the mechanism of articular cartilage disease and elucidate the molecular alterations of primary OA. The goal in OA research is to identify the early stages of disease, develop means of blocking the progression of the disease process, and reversing its effects. A variety of reports have shown biochemical and biomechanical differences of ankle with other joints (Cole and Kuettner 2002). It has been shown that human knee and ankle joint differ in their prevalence of OA or chondrodegeneratoin in vivo (Koepp et al 1999). Approximately 6% of the adult population are affected by symptomatic OA of the knee, this percentage increases to almost 10% in individuals over 65 years of age (Felson et al 1987). Symptomatic OA does develop in the ankle, although rarely ($\leq$1%), and the prevalence does not increase with age (Muehleman et al 1997).

Studies have shown that synthesis of proteoglycans (PGs) (Eger et al 2002) and PG content (Treppo et al 2000) of the ankle are higher than that of the knee. It has also been shown that there are differences in the sensitivity of knee and ankle chondrocytes to their response to catabolic mediators such as IL-1β. Knee chondrocytes responded to lower doses of IL-1β than did ankle chondrocytes. With IL-1β stimulation, there is also an increase in proteolytic activity in both the knee and ankle, as evidenced by increased

neopeptides for both aggrecanase and matrix metalloproteinases (MMPs), the levels of neopeptides were higher in the knee than ankle. Ankle is also able to rebound faster with increased PG synthesis than the knee, when IL-1β is removed (Eger et al 2002). Studies have also shown significant differences between knee and ankle chondrocyte response to proteoglycan loss in the presence of second catabolic mediator, fibronectin-fragment (Fn-fs). The 29 kD Fn-fs causes a greater degree of PG loss (30-50%) from knee cartilage than from ankle cartilage in explant culture (Kang et al 1998).

Chondrocytes within each joint cartilage build a matrix around themselves that confer different properties to their tissues, with the ankle tissue being tougher, more elastic, and more resistant to damage. Even when isolated from that matrix, the cells maintain differences in their responses to stimulation (Cole and Kuettner 2002). In vivo studies (Teppo et al 1998) on cartilage from human knee and ankle pairs have supported the concept that a combination of biochemical and biomechanical factors affects the chondrocytes and their matrix, thus making the ankle tissue more resistant to enzyme damage and disease progression than that of the knee. These works demonstrated that the ankle cartilage appeared denser, with a higher GAG content, lower water content, higher equilibrium modulus and dynamic stiffness and lower hydraulic permeability (Cole and Kuettner 2002).

These differences may provide insight into the pathogenesis of OA and may help in the design of therapeutic strategies for detection and treatment (Huch et al 1997). Additional studies are needed to characterize potential differences between chondrocytes in knee and ankle cartilage in their gene expression and matrix biosynthesis.

CHAPTER FOUR

4.0. ROLE FOR CD47 AND ITS LIGANDS IN CHONDROCYTE MECHANOTRANSDUCTION

4.1. CD47 AND α5β1 INTEGRIN INTERACTIONS

The present study showed strong expression pattern of CD47 by chondrocytes in human articular cartilage. The strong expression of α5β1 integrin in human articular cartilage has been previously demonstrated (Salter et al 1992; Loeser et al 1995). In addition, anti-α5 and anti-β1 antibodies can inhibit chondrocyte electrophysiological responses after 0.33 Hz cyclical mechanical stimulation (Wright et al 1997). It has been suggested that α5β1 integrin may act as a mechanoreceptor in chondrocyte mechanotransduction (Salter et al 2001). Since CD47 has been described to be linked to and to modulate functions of integrins on different cell types, the possibility exists that CD47 may act through α5β1 integrin modulation in chondrocye mechanotransduction. Thus, in this chapter, the physical association of CD47 with α5β1 integrin and ligands in chondrocytes, its effect on electrophysiological response after mechanical stimulation, and its potential role/s in chondrocyte mechanotransduction are investigated.

The physical association of CD47 with α5β1 integrin and recognized CD47 ligands, TSP1 and SIRPα, was investigated by using immunoprecipitation and western blotting. In these experiments primary chondrocytes, isolated from femoral condyle, normal, whose age and sex was not recorded (14013); tibial plateaux, normal, male (age 62) (14016), and cell line C20A4, P8 and P11 were used. Primary chondrocytes were seeded at $2x10^5$ cells/ml concentration. After a period of 10-15 days cultured chondrocytes were lysed and the proteins were extracted. In all experiments whole cell lysates from the same sample were used as positive control.

4.1.1. Assessment the physical association of CD47 with α5 and β1 integrin in resting human articular chondrocytes

Studies have shown that CD47 is functionally associated with α5β1 integrin in the erythroleukemia cell line K562 (Blystone et al 1995) and physically associated with α4 integrin in Jurkat T cells (Barazi et al 2002) and α2 integrin in melanoma cells (Chung et al 1997). Different studies have also shown physical association between CD47 and β integrins including β1 integrin (Gao et al 1996; Chung et al 1997; Wang et al 1998, 1999; Barazi et al 2002).

To analyze potential cross talk between CD47 and α5β1 integrin in articular chondrocytes, in the physical association between CD47 and α5 integrin was assessed. One mg pre-cleared whole cell lysates were immunoprecipitated with mouse mAb anti-CD47 CC2C6 and protein A/G resin. Immunoprecipitated proteins were analyzed by running onto 8% SDS-PAGE under reducing conditions. Separated proteins were immunoblotted with rabbit polyclonal anti-α5 integrin at 1:2000. In both control and immunoprecipitated (IP) samples, a band at ~150-160 kD (**Figure 4.1**) was identified, which is consistent with the known molecular weight of α5 integrin.

Subsequently, immunoprecipitated CD47 and associated proteins were analysed under the same condition and immunoblotted with mouse monoclonal anti-β1 integrin, JB1A, at 1:1000. The immunoprecipitated sample did not show a band at ~125 kD size, the molecular weight of β1integrin, when compared with control (**Figure 4.2.A**).

To assess the possibility of epitope denaturing and antibody interactions during immunoprecipitation and western blotting, the same whole cell lysate was immunoprecipitated with 2 μg/ml mouse mAb anti-β1 integrin, JB1A, and protein A/G resin. The immunoprecipitated proteins were analysed under reducing conditions and immunoblotted with anti-CD47, 2B7 at 1:500. Immunoprecipitated samples did not show ~50 kD band, in comparison with control (**Figure 4.2.B**).

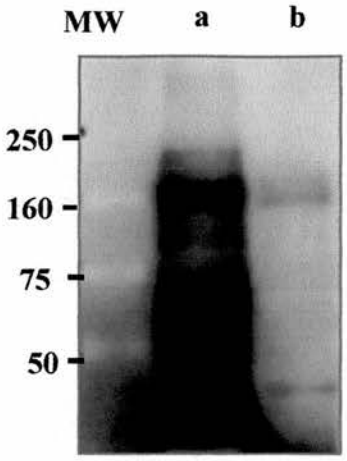

Figure 4.1. Assessment the physical association of CD47 and α5 integrin in resting human articular chondrocytes. One mg cell lysate obtained from normal femoral condyle (14013)* was pre-cleared and then incubated with 25 μl /ml primary mouse mAb anti-CD47, CC2C6, and protein A/G resin overnight. The immunoprecipitated (IP) sample (lane **a**) was suspended in electrophoresis sample buffer 2x plus 10% SDS and run onto 8% reducing gel and blotted with rabbit polyclonal anti-α5 integrin at 1:2000. Total cell lysates were electrophoresed and blotted with anti-α5 integrin as control (lane **b**). As shown above, CD47 is coimmunoprecipitated with α5 integrin in resting primary chondrocytes. The results shown are representative of cultured chondrocytes from three different experiments. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

*, day book number.

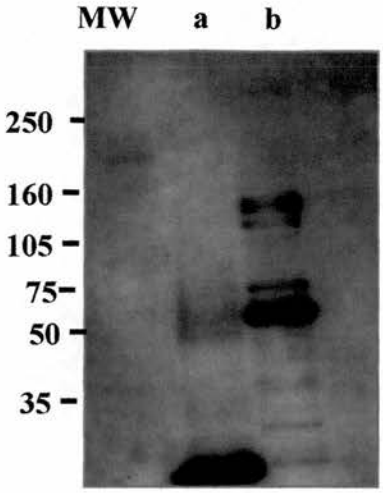

Figure 4.2.A. Assessment of physical association of CD47 and β1integrin in resting articular chondrocytes. One mg normal cell lysate obtained from normal chondrocytes (14013) was pre-cleared and then incubated with 25 μl/ml primary mouse mAb anti-CD47, CC2C6, and protein A/G resin overnight. The immunoprecipitated (IP) sample (lane **a**) was suspended in electrophoresis sample buffer 2x plus 10% SDS and run onto 8% reducing gel and blotted with mouse monoclonal antibody anti–β1integrin JB1A at 1:1000. Total cell lysates (lane **b**) were electrophoresed as control. In IP sample β1integrin is not expressed at ~125 kD and there is no physical association between CD47 and β1integrin in resting chondrocytes. The results shown are representative of cultured chondrocytes from three different experiments. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

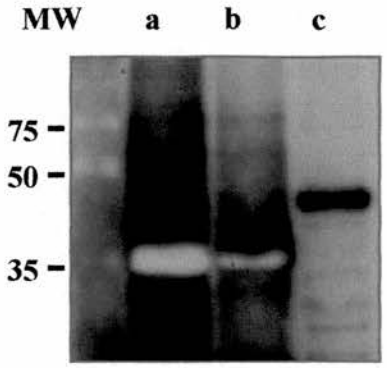

Figure 4.2.B. Assessment of physical association of β1integrin and CD47 in resting articular chondrocytes. One mg cell lysates obtained from normal chondrocytes (14013) (lane **a**) and cell line, P8, (lane **b**) were pre-cleared and immunoprecipitated with 2 μg/ml mouse monoclonal antibody anti–β1 integrin, JB1A, and protein A/G resin overnight. The IP samples were suspended in electrophoresis sample buffer 2x plus 10% SDS and run onto 8% reducing gel and immunoblotted with mouse monoclonal anti-CD47 (2B7) at 1:500. Total cell lysates (lane **c**) were used as control. As shown above, CD47 did not coimmunoprecipitate with β1 integrin in resting chondrocytes. The results shown are representative of cultured chondrocytes from three different experiments. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

4.1.2. Assessment the physical association of CD47 and β1 integrin in the presence of anti-CD47 mAb Bric126 and in stimulated human articular chondrocytes

CD47 was initially identified as an associated protein with β integrins including β3 integrin (Brown et al 1990) and β1 (Gao et al 1996; Chung et al 1997; Wang et al 1998, 1999; Barazi et al 2002). In platelets, CD47 has been described to be physically linked to β1 (Chung et al 1999) and β3 (Chung et al 1997; Brown et al 1990) integrins and to form a signal-transducing complex, allowing their activation to a higher affinity/avidity state after its ligation with the CBD domain of TSP1 in static conditions. Many intracellular proteins have been demonstrated that interact directly with the integrin β subunits including signal transduction proteins such as FAK (Dedhar and Hannigan 1996). To determine whether CD47 is physically associated with β1 integrin in chondrocytes under certain conditions, the interaction either in the presence of anti-CD47 Bric126 or after 0.33 Hz cyclical mechanical stimulation was assessed.

In first experiment, normal cultured chondrocytes were pretreated with anti-CD47 Bric126 for 1 min, 5 min and 10 min. Extracts from these samples were immunprecipitated with anti-CD47 (CC2C6) and analysed by western blotting. Separated proteins immunoblotted with mouse mAb anti-β1 integrin, JB1A, at 1:1000. In comparison to the band obtained from whole cell lysate at ~125 kD, anti-CD47 pretreated chondrocytes did not show a detectable band (**Figure 4.3. A**). Thus, the presence of Bric 126 had no effect on physical association between β1 integrin and CD47. In the second experiment, normal cultured chondrocytes were subjected to 0.33 Hz cyclical mechanical stimulation for 1min and 5 min. Extracts from different resting and stimulated chondrocytes were immunprecipitated with anti-CD47 (CC2C6). Immunoprecipitated CD47 and associated proteins were analysed by western blotting and immunoblotted with mouse mAb anti-β1 integrin, JB1A, at 1:1000. As shown in **Figure 4.3. B** stimulated chondrocytes did not show any modification in the physical association of CD47 and β1 integrin, when compared with unstimulated chondrocytes.

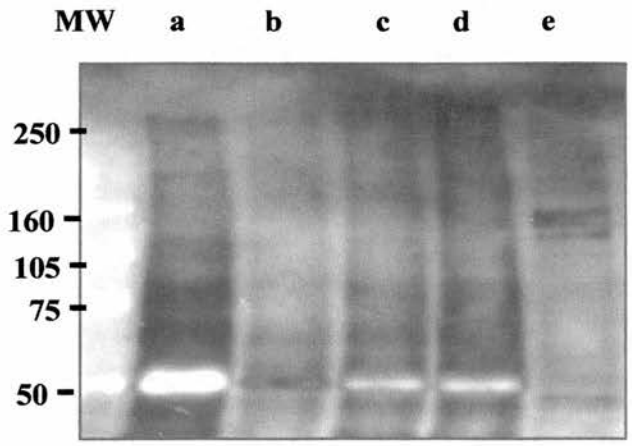

Figure 4.3.A. Assessment of physical association between CD47 and β1integrin in the presence of anti-CD47, Bric126, in cell line C20A4. Non-treated (lane **a**) and pretreated cultured cell line C20A4, P11, with anti-CD47 Bric126 for 1 min (lane **b**), 5 min (lane **c**) and 10 min (lane d) were lysed and whole cell lysates were extracted. The lysates pre-cleared and incubated with primary mouse mAb anti-CD47, CC2C6 (IgG), and protein A/G resin overnight. The IP samples were suspended in electrophoresis sample buffer 2x plus 10% SDS, run onto 8% reducing gel, and immunoblotted with mouse monoclonal antibody anti–β1integrin, JB1A, at 1:1000. As shown above, untreated (lane **a**) and treated lanes (**b-d**) did not show modification in the CD47 and β1 integrin coimmunoprecipitation. Total cell lysates (whole extract, lane **e**) were electrophoresed and immunoblotted with anti-β1 integrin as control. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

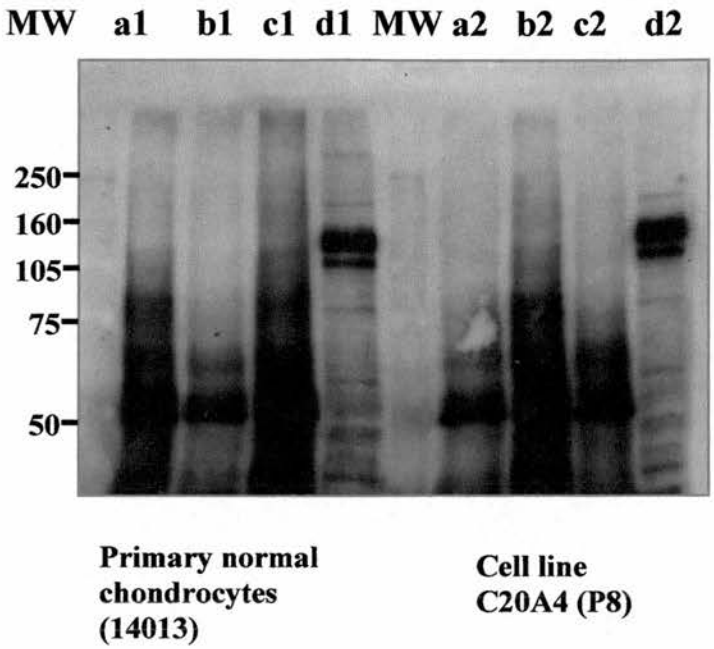

Figure 4.3.B. Assessment of physical association between CD47 and β1 integrin following 0.33 Hz cyclical mechanical stimulation. Normal chondrocytes (14013) and cell line C20A4, P8, were seeded and after 10-15 days were subjected to 0.33 Hz cyclical mechanical stimulation (MS). Resting chondrocytes (lanes **a1** and **a2**) and stimulated chondrocytes at 1 min (lanes **b1** and **b2**), and 5 min (lanes **c1** and **c2**) were lysed. Extracts were immunoprecipitated with anti-CD47 (CC2C6) and immunoblotted with anti-β1integrin, JB1A, at 1:1000. The whole cell lysates obtained from normal primary chondrocytes (14013)(lane **d1**) and cell line C20A4, P8 (lane **d2**) were used as positive control. Resting and stimulated chondrocytes did not show noticeable difference in the CD47 and β1integrin coimmunoprecipitation. **a**, Resting; **b**, 1 min MS; **c**, 5 min MS; **d**, whole cell lysate/ control. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

4.2. EXPRESSION AND INTERACTIONS OF CD47 LIGANDS, TSP1 AND SIRPα

4.2.1. Expression and interactions of TSP1 in human articular chondrocytes

4.2.1.1. Expression of TSP in human articular chondrocytes

TSP1 is ~ 450 kD multifunctional ECM glycoprotein, with three identical subunits and multiple domains, which mediates different cell-matrix interactions. The presence of TSP1 in articular cartilage has been previously reported (Miller and McDevitt 1988; DiCesar et al 1994; Pfander et al 2000). In this study, in vitro expression pattern of TSP-1 by normal and OA articular cartilage was studied.

Normal chondrocytes were isolated from 4 talus (ankle), 3 males, 1 female (age range 62-72, mean age 68)(13901, 13907, 13915, 12820); 1 tibial plateaux, male (age 72) (13941); 2 femoral head, 1 male and 1 female (age range 70-83, mean 78) (13828, 13822). OA chondrocytes were isolated from 1 femoral head (grade II, male, age 83)(13828); and 2 tibial plateaux, grade II and III, both female (age range 62-87 mean 75) (13820, 13965).

Extracts from normal and OA chondrocytes were analyzed using western blotting under reducing conditions and immunoblotted with a mouse monoclonal anti-TSP, P10, (Chemicon) antibody at 1:100. Cell lysates from normal and OA chondrocytes obtained from ankle, knee and femoral head showed similar band of ~160-170 kD which is consistent with the known molecular weight of one subunit of this glycoprotein (**Figure 4.4**).

One normal tibial plateaux, male (age 72), and two OA, both tibial plateaux, grades II and III, both female (age range 62-87 mean 75) showed a faint band. The other samples from different donors showed strong consistent band. The intensity of TSP band in

normal ankle, normal and OA extracts from different donors of different age and sex under reducing conditions was approximately similar (**Figure 4.4**).

4.2.1.2. Interactions between CD47 and TSP1 in articular chondrocytes

Functional and physical interactions between CD47 and C-terminal cell binding domain of TSP1 in a number of cellular activities have been demonstrated (Gao et al 1996; Chung et al 1997; Wang et al 1999; Dorahy et al 1997; Frazier et al 1999; Tulasne et al 2001).

To test the possibility of CD47 and TSP interactions in chondrocyte, one mg pre-cleared whole cell lysates were immunoprecipitated with mouse mAb anti-CD47 CC2C6 and protein A/G resin. Immunoprecipitated CD47 protein and its associated proteins were separated by running onto 8% SDS-PAGE under reducing conditions. Separated proteins were immunoblotted with mouse mAb anti-TSP antibody, P10, at 1:100. As shown in **Figure 4.5** the control and immunoprecipitated (IP) lysates showed a similar size band of ~160-170 kD which is consistent with known molecular size of one subunit of TSP1. CD47 showed physical association with TSP in human articular chondrocytes.

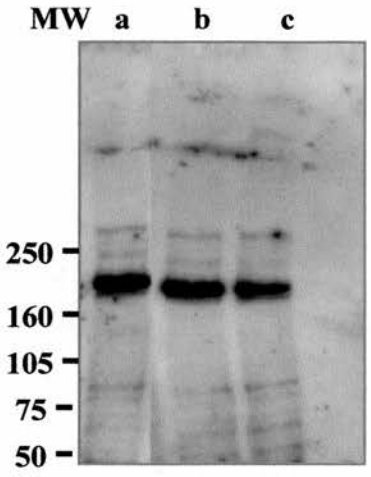

Figure 4.4. Detection and comparison of TSP1 expression in normal and OA human articular cartilage. Equal amounts (80 μg/lane) of total extracted proteins prepared from the cultured chondrocytes derived from normal ankle (lane **a**, talus, male, age 72) (13915), normal (lane **b**, femoral head, male, age 83) (13828) and OA (lane **c**, tibial plateaux, grade III OA, female, age 87) (13965) samples were run onto 6% SDS-PAGE under reducing conditions. The detection of TSP1, was assessed by immunoblotting with mouse mAb anti-TSP, P10, (Chemicon) at 1:100. Normal and OA chondrocytes isolated from different joints, expressed nearly a similar band. The blot shown is representative of three different donors. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

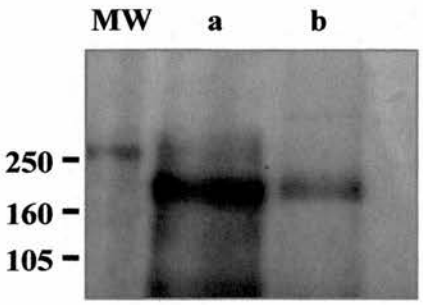

Figure 4.5. Assessment of physical association between CD47 and TSP1 in primary chondrocytes. One mg normal cell lysate obtained from femoral condyle normal (14013) was pre-cleared and then incubated with 25 µl/ml primary mouse mAb anti-CD47, CC2C6 (IgG), and protein A/G resin overnight. The immunoprecipitated proteins were suspended in electrophoresis sample buffer 2x plus 10% SDS and run onto 8% reducing gel and blotted with mouse mAb anti-TSP, P10, at 1:100. As shown above, IP sample (lane **a**) and total cell lysates, as control (lane **b**) expressed TSP at ~160-170 kD. CD47 is coimmunoprecipitated with TSP1 in human articular chondrocyte (lane **a**). Results shown are representative of cultured chondrocytes from three different experiments. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

4.2.1.3. Physical association between TSP and β1integrin in primary chondrocytes and C20/A4 cell line

It has been shown that integrins are important signalling receptors for TSP1 in multiple cell types including T cells (Wilson et al 1999) and platelets (Chung et al 1997). To investigate the physical association between TSP and β1 integrin in human articular chondrocytes, cultured chondrocytes were lysed and the proteins extracted. One mg cell lysate was pre-cleared and incubated with 2 μg/ml primary mouse mAb anti-β1 integrin, JB1A, and protein A/G resin. Immunoprecipitated proteins were separated by running onto 8% reducing gel and immunoblotted with anti-TSP antibody, P10, at 1:100.

In the first experiment, TSP1 was seen to be expressed in both primary chondrocytes (14013) and cell line C20A4 (P8) (**Figure 4.6. A**). To confirm the results, extracts from another primary chondrocytes (14016) and cell line C20A4 (P11) were similarly immunoprecipitated and immunoblotted. These samples did not show band at consistent molecular weight (~160-170 kD), when compared with the whole cell lysates as positive control (**Figure 4.6. B**).

These results suggest a weak, transient physical association between β1 integrin and TSP in human articular chondrocytes.

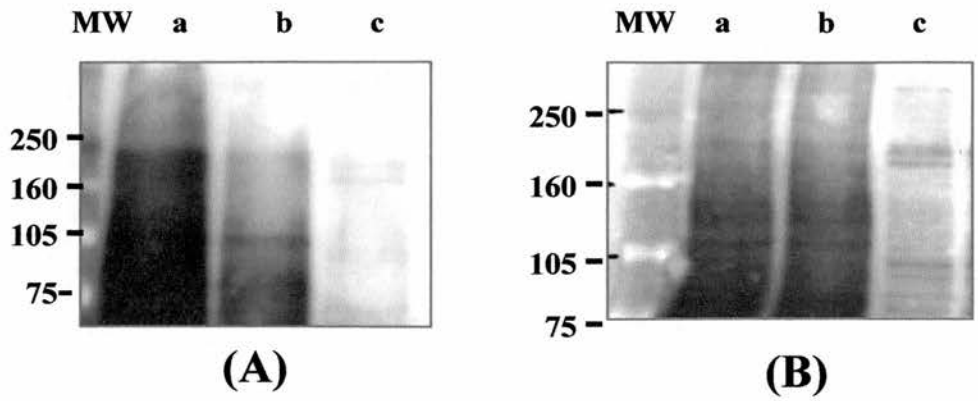

Figure 4.6. Assessment of physical association between β1 integrin and TSP1 in primary human articular chondrocytes (**A**) and cell line C20A4 (**B**). **A**) One mg cell lysate obtained from femoral condyle normal (14013) (lane **a**) and cell line C20A4, P8 (lane **b**) were pre-cleared and then incubated with 2 μg/ml primary mouse mAb anti-β1 integrin, JB1A, (Chemicon), and protein A/G resin overnight. The IP samples were suspended in electrophoresis sample buffer 2x plus 10% SDS and run onto 8% reducing gel and blotted with mouse monoclonal anti-TSP at 1:100. As shown above IP samples, (lanes **a** and **b**), β1 integrin is coimmunoprecipitated with TSP. **B**) The same experiment with another primary normal chondrocytes (14016), (lane **a**) and cell line C20A4, P11 (lane **b**) did not show the physical association between TSP and CD47. Total cell lysates (lane **c**) were electrophoresed and blotted with anti-TSP as control. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

4.2.2. Expression and interactions of SIRPα in human articular chondrocytes

SIRPα is a second natural ligand of CD47. It has been shown that SIRPα is expressed in a variety of cells including myeloid cells and neurons (Adams et al 1998; Oshima et al 2002). In the present study using a panel of anti- SIRPα Abs, immunohistochemistry and western blotting methods, the expression of this IgSF member in articular chondrocytes was investigated.

4.2.2.1. Work of antibodies to SIRPα

To identify whether or not chondrocytes express SIRPα, a panel of mouse mAbs anti-SIRPα antibodies, SE12B6, SE7C2 and SE5A5 and one rabbit polyclonal anti-SIRPα (ABR) were applied. In preliminary studies all antibodies were tested by immunohistochemistry on frozen and paraffin sections. The antibodies did not show positive signal at all on either paraffin or frozen sections at a range of concentrations. To assess the expression of SIRPα molecule by human articular chondrocytes, cell lysates obtained from cultured chondrocytes isolated from normal ankle, normal and OA knee and femoral head, were studied. In vitro expression of SIRPα was assessed by using the same Abs. Each Ab was tested with three samples, one normal ankle, one normal and one OA knee sample, at concentrations of 1:500 and 1:1000 under both reducing and non-reducing conditions. Anti-SIRPα mAbs, SE5A5 and SE7C, showed no binding at all at different concentrations and conditions (**Figure 4.7**). Anti-SIRPα mAb SE12B6 showed a very faint band or no binding under non-reducing conditions (**Figure 4.7. C**). Polyclonal rabbit anti-SIRPα (ABR) showed strong band at molecular weight approximately 70-80 kD, that it was consistent with the range of the molecular weight of this molecule (**Figure 4.8**).

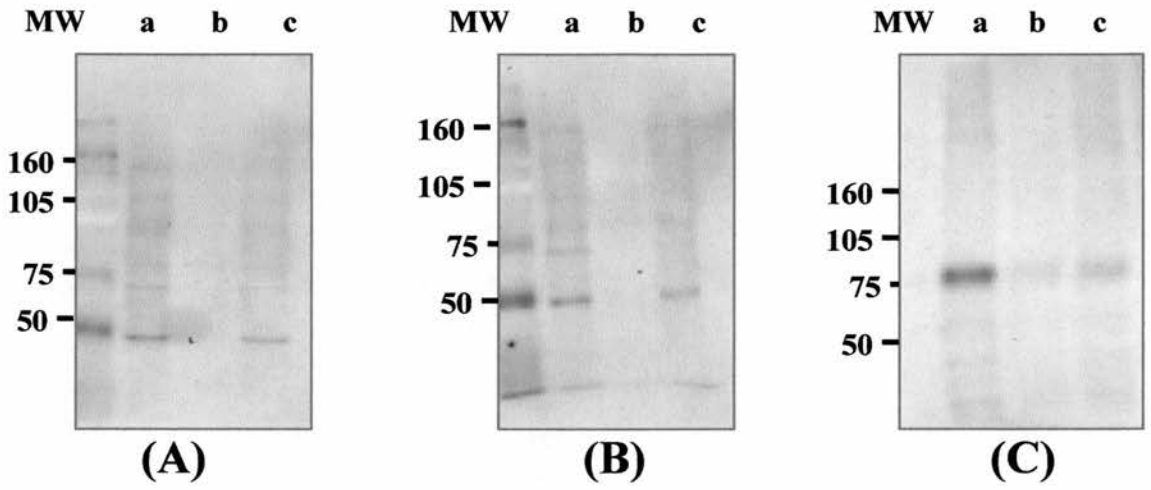

Figure 4.7. Work of mAbs anti-SIRPα, SE12B6 and SE7C2, to SIRPα. Cultured chondrocytes isolated from normal ankle (lane **a**), normal (lane **b**) and OA (lane **c**) articular cartilage were lysed. Extracts were analysed by running onto 8% reducing (**A** and **B**) and non-reducing (**C**) gels. Blot **A** immunoblotted with mAb anti-SIRPα SE7C2 at 1:500, and both blot **B** and **C** immunoblotted with anti-SIRPα ,SE12B6, at 1:500. As shown above, blots **A** and **B** did not show a consistent main single band but blot **C,** anti-SIRPα SE12B6 showed very faint single band at 70-80 kD under non-reducing conditions in some samples and no binding in the other samples. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

4.2.2.2. Expression of SIRPα in human articular chondrocytes

Based on the above preliminary studies rabbit polyclonal anti-SIRPα (ABR) at 1:1000 was selected for further biochemical assessment.

Normal chondrocytes were isolated from 3 talus (ankle), all male (age range 69-71, mean age 70) (13901, 13915, 13907); 1 tibial plateaux, female (age 71) (13911); 3 femoral heads, 2 males and 1 female (age range 70-83, mean 78) (13828, 13822, 13816). OA chondrocytes were isolated from 1 femoral head, grade II, male (age 83) (13828); 2 tibial plateaux, grades II and III, both female (age range 62-87 mean 75) (13820, 13965).

Extracts from normal and OA chondrocytes taken from different joints were analyzed by running onto 8% SDS-PAGE under reducing conditions. Analyzed proteins were immunoblotted with rabbit polyclonal (ABR) anti-SIRPα at 1:1000. Both normal and OA chondrocytes showed a similar band at ~70-80 kD, which is consistent with the known range molecular weight of this receptor. Chondrocytes isolated from different donors did not show detectable difference in the expression pattern of SIRPα in the several experiments (**Figure 4.8**). A slightly stronger band was detected from chondrocytes isolated from normal ankle in comparison to the band visualized from normal and OA chondrocytes isolated from knee and femoral head (**Figure 4. 8**). In cell line C20A4 (P11), SIRPα was expressed at a similar size and similar to expression pattern by primary normal chondrocytes (**Figure 4.8**).

4.2.2.3. Assessment the physical association between CD47 and SIRPα

Recent reports have shown CD47-SIRPα interactions in a number of cellular functions in multiple cell types including fibroblast NIH3T3 (Kharitonenkov et al 1997), rat alveolar macrophage (Adams et al 1998) and T cells (Seiffert et al 2001).

In next step of this part, the physical association between CD47 and SIRPα in resting articular chondrocytes was assessed using immunoprecipitation and western blotting. Cell lysates were pre-cleared and incubated with primary mAb anti-CD47 CC2C6 and

protein A/G resin. Immunoprecipitated proteins were separated by running onto 8% reducing gel and immunoblotted with rabbit polyclonal anti- SIRPα antibody at 1:1000.

Immunoprecipitated proteins did not express a band at the determined molecular weight by positive control. As shown in **Figure 4.9**, in ~70-80 kD molecular weight that SIRPα is expressed in the positive control, this molecule is not expressed in electrophoresed immunoprecipitated sample isolated from primary normal chondrocytes. Human articular chondrocytes do not appear to show physical association between CD47 and SIRPα.

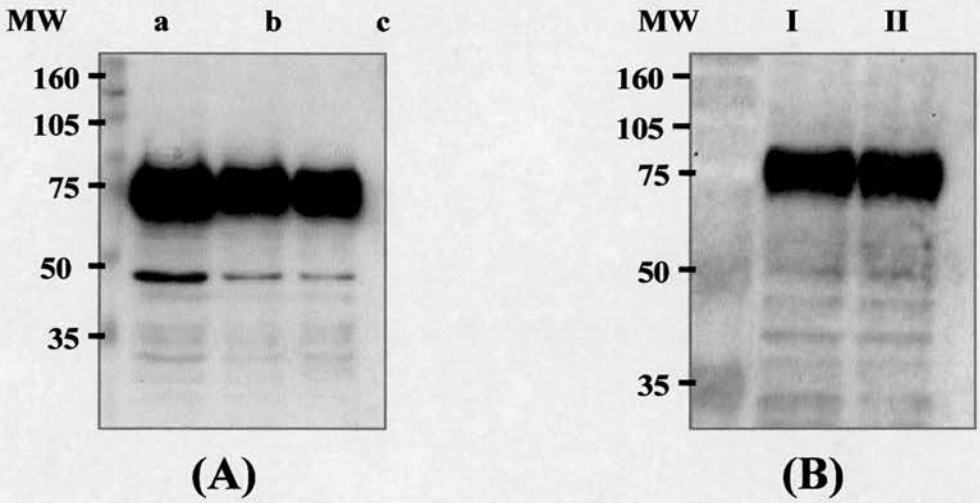

Figure 4.8. Detection and comparison of SIRPα in normal and OA human articular cartilage. Equal amounts (40μg/lane) of total extracted proteins prepared from cultured human articular chondrocytes derived from ankle (lane **a**), normal (lanes **b**, **I**), OA (lane **c**) chondrocytes and cell line C20A4 (lane **II**) were run onto 8% SDS-PAGE under reducing conditions. The detection of SIRPα was assessed by probing the blots with rabbit polyclonal anti-SIRPα (ABR) at 1:1000. **A)** Normal ankle (lane **a**, talus, male, age not recorded) (13901) exhibited stronger band of SIRPα in comparison to normal (lane **b**, femoral head, male, age 70) (13822) and OA (lane **c**, tibia OA grade III, female, age 87) (13965) chondrocytes. Both normal and OA chondrocytes did not show detectable difference in the pattern of SIRPα expression. **B)** Both cell lysates from normal primary chondrocytes from knee (lane **I**, femoral condyle, age and sex were not recorded) (14013) and cell line C20A4, P11 (lane **II**,) expressed SIRPα. All chondrocytes isolated from different joints expressed SIRPα. No detectable difference was observed between normal and OA chondrocytes in the expression of SIRPα. Stronger band of ankle chondrocytes in comparison to femoral head and knee chondrocytes was observed. The blots shown are representative of three different donors (normal and OA). Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

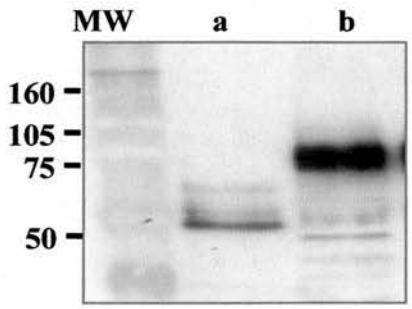

Figure 4.9. Assessment of physical association between CD47 and SIRPα in primary normal chondrocytes. One mg normal cell lysate obtained from femoral condyle normal (age and sex were not recorded)(14013) was pre-cleared and immunoprecipitated with primary mouse mAb anti-CD47, CC2C6 (IgG), and protein A/G resin overnight. The IP sample (lane **a**) was suspended in electrophoresis sample buffer 2x and 10% SDS and run onto 8% reducing gel and immunoblotted with rabbit polyclonal anti-SIRPα at 1:1000. As shown above, CD47 did not coimmunoprecipitate with SIRPα in resting chondrocytes. Total cell lysates (lane **b**) were electrophoresed and immunoblotted with anti-SIRPα as control. The results shown are representative of cultured chondrocytes from three different experiments. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

4.3.0. ROLE FOR CD47 AND ITS LIGANDS IN THE ELECTROPHYSIOLOGICAL RESPONSE OF HUMAN ARTICULAR CHONDROCYTES TO 0.33 Hz MECHANICAL STIMULATION

The full numerical results of individual electrophysiological experiments from which the values given in the Tables in this part are derived are given in Appendix III. The total cases used in this section of the experimental work include 7 females (age range 57-88, mean age 75) and 6 males (age range 66-92 mean 75) (**Table 4.1**). Non-confluent cultures grown in vitro for 10-12 days were used for all experiments. Cells were mechanically stimulated at 0.33 Hz for defined periods. Results shown in this part are from a single representative experiment. Experiments were repeated from human articular chondrocytes derived from at least 3 adult donors and were reproducible between donors.

4.3.1. Effect of function-blocking mAb anti-CD47 Bric 126 on the response of cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

A series of experiments was undertaken to establish whether CD47 is involved in the chondrocyte electrophysiological response to 0.33 Hz (2 sec on, 1 sec off) cyclical mechanical stimulation (MS). Cultured n ormal chondrocytes were used to investigate the role of CD47 in the hyperpolarisation response of normal chondrocytes to 0.33 Hz cyclical mechanical stimulation. Function-blocking mAb anti-CD47 Bric 126 at concentration of 1μg/ml was incubated with monolayer cultured normal chondrocytes at 37° C for 10 minutes prior to 0.33 Hz cyclical mechanical stimulation for 20 minutes. The membrane potentials of 5 cells were recorded prior to and after the addition of Bric126 and also after the standard regime of 0.33 Hz cyclical mechanical stimulation.

In nil dish (as control), chondrocytes were subjected to 0.33 Hz cyclical mechanical stimulation for 20 minutes. Membrane potential of 5 chondrocytes were measured and showed a hyperpolarisation response (**Table 4.2.a**). In test dish, the exposure of the cells to Bric 126 alone produced no significant change in membrane potential. When the normal cells were subjected to cyclical mechanical stimulation in the presence of Bric 126, there was no change in membrane potential (**Table 4.2.a**).

A similar experiment was carried out with OA cultured chondrocytes to investigate whether there was a role for CD47 in the depolarisation response of OA chondrocytes to 0.33 Hz cyclical mechanical stimulation. The membrane potential of OA chondrocytes with antibody only did not show any alteration whereas there was no change in membrane potential which is induced by 0.33 Hz cyclical mechanical stimulation (**Table 4.2.b**).

Bric126 was found to inhibit the electrophysiological response of both normal and osteoarthritic chondrocytes at 0.33 Hz mechanical stimulation (**Table 4.2.a** and **b**). The modification response following mechanical stimulation in both normal and OA cultured chondrocytes significantly abrogated. This suggests the involvement of CD47 in articular chondrocytes mechanotransduction.

Table 4. 1. Normal and OA samples used in electrophysiology experiments.

Normal samples	**OA samples**
A. Femoral head : sex/age (DB No.) 1. M/ 79 (13873) 2. M/ 92 (13876D) 3. F/ 82 (13878)	**A. Femoral head** 1. OAIII, F/88 (13902)
A. Knee joint: Region, sex/age (DB No.) 1. TN, F/57 (13904) 2. FN, M/69 (13939) 3. FN, M/72 (13941) 4. FN, M/- (13949)	**B. Knee joint:** Region, sex/age (DB No.) 1. FA II, F/79 (13938C) 2. FA II, M/66 (13943) 3. FA III, M/- (13949) 4. FA II, F/64 (13959) 5. FA II, F/76 (13962) 6. FA I, N/R (13964) 7. TA II, M/- (13983)

M, male; **F**, female; DB No., day book number. **TN**, tibial plateaux normal; **FN**, femoral condyle normal; **TA**, tibial plateaux OA; **FA**, femoral condyle OA.

Table 4.2.a. Effect of 1 μg/ml anti-CD47 Bric 126 on the membrane hyperpolarisation response of normal cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV) (mean ± SEM)				
Reagent	n	Resting	Bric 126 alone	Bric126 + 0.33 Hz MS	% change	P value
Nil	5	14.8±1.1	-	24±1.9	+62	0.001
Bric126	5	18.6±1	19.4±1	19.4±0.87	+0	NS*

*NS: not significant. Compared with antibody alone

Table 4.2.b. Effect of 1 μg/ml anti-CD47 Bric 126 on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV) (mean ± SEM)				
Reagent	n	Resting	Bric 126 alone	Bric126 + 0.33 Hz MS	% change	P value
Nil	5	46±2.3	-	22.2±2.2	-52 *	0.001
Bric 126	5	40.8±2.6	40.8±1.9	42.2±2	-3	NS*

*NS: not significant. Compared with antibody alone

4.3.2. Effect of mAb anti-TSP, P10, on the depolarisation response of OA chondrocyte to 0.33 Hz cyclical mechanical stimulation

A series of experiments was undertaken to establish whether TSP-1 is involved in the chondrocyte electrophysiological response following 0.33 Hz cyclical mechanical stimulation.

OA chondrocytes were seeded in complete media at a density of $1x10^4$ cells/ml. mAb anti-TSP, P10, at concentration of 1.5 μg /ml was incubated with non-confluent chondrocytes at 37° C for 10 min prior to 0.33 Hz cyclical mechanical stimulation. The membrane potential of suitable chondrocytes were measured at rest, 10 min after incubating with antibody alone and after 0.33 Hz cyclical mechanical stimulation.

The antibody itself was found to have no effect on the membrane potential of chondrocytes. The depolarisation response, seen in osteoarthritic articular chondrocytes subjected to 0.33 Hz cyclical mechanical stimulation, was assessed. This electrophysiological response was significantly inhibited in the presence of anti-thrombospondin at concentration of 1.5μg/ml (**Table 4.3**).

Table 4.3. Effect of 1.5 μg/ml anti-TSP mAb (Chemicon) on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV) (mean ± SEM)				
Reagent	n	Resting	Anti-TSP alone	Anti-TSP + 0.33 Hz MS	% change	P value
Nil	5	30±1.9	-	17.4±1.3	-42	0.0001
Anti-TSP	5	29.6±1.2	29 ± 0.8	29.8 ± 1.6	6	NS*

*NS: not significant. Compared with antibody alone

4.3.3. Effect of anti- SIRPα Abs, SE5A5 and SE7C2, on the OA chondrocyte depolarisation response to 0.33 Hz cyclical mechanical stimulation

A series of experiments was undertaken to establish whether SIRP-α is involved in the chondrocyte electrophysiological response to 0.33 Hz cyclical mechanical stimulation.

Two function-blocking mAbs against SIRPα, SE5A5 and SE7C2, at 1:100, were applied. The monoclonal Abs were incubated with OA cultured chondrocytes in separate experiments for 10 min at 37° C prior to 0.33 Hz cyclical mechanical stimulation for 20 minutes.

In the presence of anti-SIRPα mAb SE7C2, the electrophysiological response of cultured OA human chondrocytes to 0.33 Hz cyclical mechanical stimulation was inhibited, indicating that this protein has a role in chondrocyte mechanotransduction. Another anti-SIRPα mAb, SE5A5, which is known to block the binding of SIRPα to CD47 (Lagadec et al 2003), did not show significant inhibitory effect on depolarisation response of osteoarthritic chondrocytes following 0.33 Hz mechanical stimulation (**Table 4.4**).

Table 4.4. Effect of function blocking anti-SIRPα antibodies, SE5A5 and SE7C2, at 1:1000, on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV) (Mean ± SEM)				
Reagent	n	Resting	Anti-SIRPα alone	Anti-SIRP + 0.33 Hz MS	% change	Pvalue
Nil	5	24.8±0.8	-	16.4±0.9	-33	0.0002
SE5A5	5	29.2±1	29±1.1	23.2±0.8	-20	0.002
SE7C2	5	26.2±0.8	27.4±0.9	28.2±1.3	7	NS*

*NS: not significant. Compared with antibody alone

4.4. CD47 AND INTRACELLULAR SIGNALLING

Previous studies have shown that at least two signals are required for the formation of intracellular integrin-mediated signalling cascades, one involving protein kinase C (PKC) and the other involving tyrosine kinase activation (Burridge et al 1992). Protein tyrosine kinases play a central role in integrin-mediated signalling cascades (Parsons 1996; Kornberg et al 1992; Burridge et al 1992; Schmidt et al 1998). Tyrosine phosphorylation of several cellular proteins appears to play an essential role in integrin-mediated signal transduction (Lin et al 1995). It has been shown that CD47 has a role in protein tyrosine phosphorylation (Gao et al 1996; Chung et al 1997; Frazier et al 1999; Green et al 1999).

Protein kinase C (PKC) appears to be one of the key intermediates in integrin-mediated signalling in many cell types (Clark and Brugge 1995; Juliano and Haskill 1993). In certain cell types, inhibition of PKC activity results in the inhibition of cell attachment and spreading as well as FAK phosphorylation (Woods and Couchman 1992; Vuori and Ruoslahti 1993; Haimovich and Kaneshiki 1996). Activation of PKC can promote the cellular changes mediated by integrin/matrix interactions (Vuori and Ruoslahti 1993; Chen et al 1994; Short et al 1998).

4.4.1. Effect of anti-CD47 Bric126 on protein tyrosine phosphorylation and PKCα activation in resting articular chondrocytes

To assess CD47 signalling pathway in chondrocyte mechanotransduction, intracellular events, the phosphorylation tyrosine residues of signalling molecules and PKCα activation following cyclical mechanical stimulation in the presence of anti-CD47 Bric126 were assessed. Because studies have shown the effect of some antibodies alone such as CD47 antibodies (Ticchioni et al 1997) on tyrosine phosphorylation, the possibility of the influence anti-CD47 Bric126 alone on tyrosine phosphorylation and PKCα activation was investigated. Cultured cells were incubated with Bric126 at

appropriate time courses and tyrosine phosphorylation and PKCα translocation were assessed.

Cultured primary chondrocytes or cell line C20A4 were preincubated with anti-CD47 Bric126 at 1min, 5 min and 10 min. Extracts were immunoprecipitated with anti-phosphotyrosine, analysed by running onto 8% SDS-PAGE under reducing conditions and immunoblotted with mouse mAb anti-phosphotyrosine-HRP, PY-20, at 1:1000. As shown in **Figure 4.10. A**, the cultured chondrocytes did not show detectable alteration in tyrosine phosphorylation in comparison to the resting cells. In the presence of anti-CD47 Bric 126, no modification in tyrosine phosphorylation was observed, compared with the basal amount in resting cells.

To investigate the possibility of the influence of anti-CD47 Bric 126 on the PKCα translocation, mAb anti-CD47 Bric 126 was incubated with primary chondrocytes at 30 sec, 1 min, 5 min and 10 min. Extracts were fractionated to cytosolic and particulate compartments. Both cytosolic and particulate compartments were analyzed by running onto 10% SDS-PAGE under reducing conditions. Separated proteins were immunoblotted with mouse mAb anti-PKCα, M4 at 1:1000. The presence of anti-CD47 Bric 126 only had no effect on translocation of PKCα from cytosolic compartment to the particulate compartment (**Figure 4.10.B**).

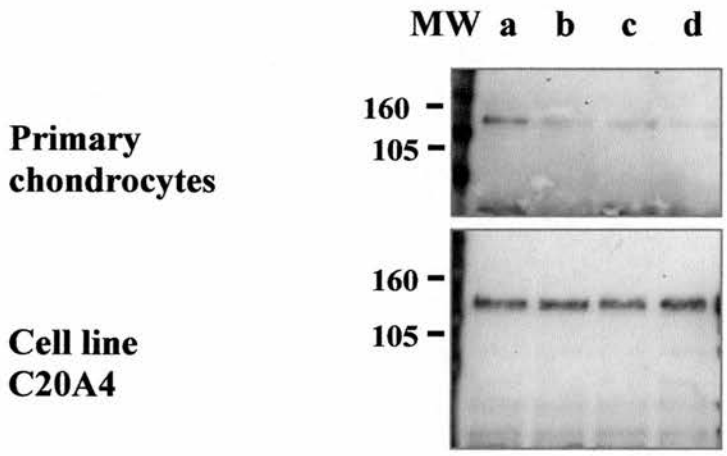

Figure 4.10.A. Effect of anti-CD47, Bric 126 alone on the integrin-mediated pp125FAK tyrosine phosphorylation in primary chondrocytes and cell line C20A4. Cultured chondrocytes preincubated with 1μg/ml anti-CD47 Bric 126 for 1 min (lane **a**); 5 min (lane **b**); and 10 min (lane **c**). Resting cells (nil) (lane **d**) were used as control. Cell lysates obtained from primary chondrocytes (top) (14016) and cell line C20A4, P11 (bottom) were immunoprecipitated with anti-phosphotyrosine, electrophoresed by running onto 8% gel under reducing conditions and immunoblotted with mouse mAb anti-phosphotyrosine-HRP, PY-20 at 1:1000. The presence of antibody alone, at different time courses (**a-c**), did not appear to play a role in the FAK tyrosine phosphorylation. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

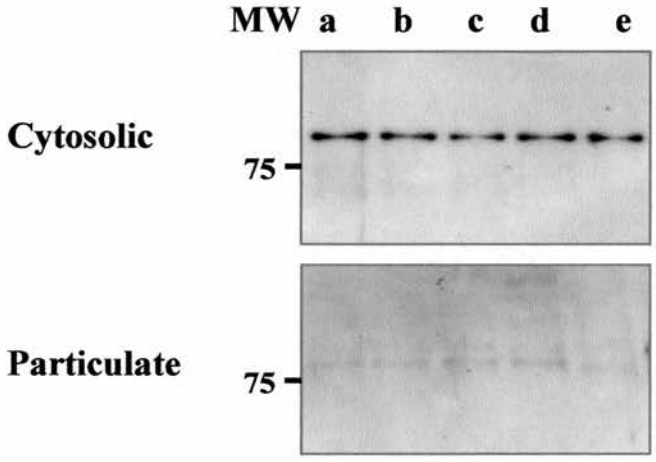

Figure 4.10.B. Effect of anti-CD47, Bric 126 alone on the integrin-mediated PKCα translocation in primary chondrocytes. Cultured chondrocytes were incubated with 1μg/ml anti-CD47 Bric 126 for 30 sec (lane **a**); 1 min (lane **b**); 5 min (lane **c**); and 10 min (lane **d**). Resting (nil) cells dish (lane **e**) was used as control. Whole cell lysates from cultured normal chondrocytes (tibial plateaux, normal, male, age 54 years) (13973) were fractionated to cytosolic and particulate compartments. PKCα expression was assessed by running both compartments onto 10% SDS-PAGE reducing gel and immunoblotted with mouse mAb anti-PKCα, M4, at 1:1000. In the presence of anti-CD47 Bric126 at different time courses, no alterations observed in PKCα translocation from cytosolic to particulate compartment. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

4.4.2. Effect of anti-CD47 on tyrosine phosphorylation and PKCα activation following cyclical mechanical stimulation in articular chondrocytes

Protein tyrosine phosphorylation after mechanical stimulation has now been clearly identified in a number of systems as an important occurrence both early in mechanotransduction and in downstream signalling events that result in regulation of gene expression (Berk et al 1995).

It has been previously revealed that 1min 0.33 Hz cyclical mechanical stimulation results in increased tyrosine phosphorylation of signalling molecules, FAK, β–catenin and paxillin, in both primary and C20A4 cell line cultured chondrocytes (Lee et al 2000). To determine if CD47 is involved in this tyrosine phosphorylation following mechanical stimulation, in the presence of 1μg/ml anti-CD47 (Bric126), the extent of tyrosine phosphorylation of signalling molecules was assessed.

In primary chondrocytes increased tyrosine phosphorylation of FAK within 1 min mechanical stimulation was significantly abolished in the presence of mAb anti-CD47 Bric 126 (**Figure 4.11. A**). Similarly, increased tyrosine phosphorylation of different size molecules including 65, 90 and 125 kD after 1 min mechanical stimulation was observed in cultured cell line C20/A4. This response, in the presence of anti-CD47 Bric 126, was completely blocked. Both primary chondrocytes and C20/A4 cell line showed an increase of FAK tyrosine phosphorylation that in the presence of anti-CD47 was markedly decreased (**Figure 4.11. A**).

It has been shown recently that 0.33 Hz cyclical mechanical stimulation within 30 seconds results in PKCα translocation from cytosolic to particulate compartment (Lee et al 2002). In the present study, the effect of anti-CD47, Bric126, on this translocation was investigated. The results showed that this translocation of PKCα is significantly inhibited (**Figure 4.11. B**).

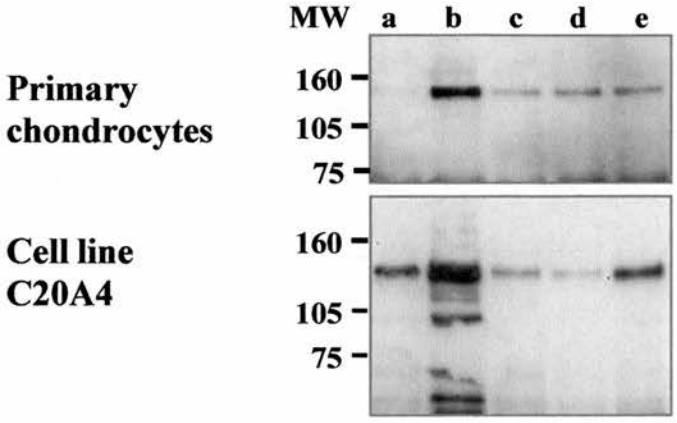

Figure 4.11.A. Effect of anti-CD47, Bric 126, on the pp125FAK tyrosine phosphorylation after 0.33 Hz cyclical mechanical stimulation in primary articular chondrocytes and cell line C20A4. Cell lysates obtained from primary chondrocytes (top) (14016) and cell line C20A4, P11 (bottom) were immunoprecipitated with anti-phosphotyrosine, electrophoresed by running onto 8% gel under reducing conditions and immunoblotted with mouse mAb anti-phosphotyrosine-HRP, PY-20 at 1:1000. Tyrosinre phosphorylation of pp125FAK is increased after 1min mechanical stimulation (lane **b**). This tyrosine phosphorylation in the presence of anti-CD47 was markedly decreased (lane **c**). In 5 min mechanical stimulation alone (lane **d**) and in the presence of anti-CD47 (lane **e**), no significant alteration was observed. In cultured cell line C20/A4, in addition to FAK, the stronger bands of different size molecules including 65 and 90 kD were observed (lane **b**). All bands were considerably blocked in the presence of anti-CD47 (lane **c**). Resting cells (lane **a**); 1 min mechanical stimulation (lane **b**); 1min mechanical stimulation in the presence of anti-CD47 (lane **c**); 5min mechanical stimulation and (lane **d**); and 5 min mechanical stimulation in the presence of anti-CD47 (lane **e**). Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

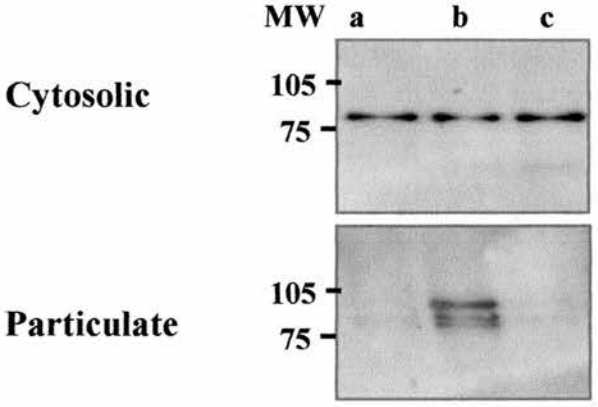

Figure 4.11.B. Effect of anti-CD47, Bric 126, on PKCα translocation after 0.33 Hz cyclical mechanical stimulation in primary articular chondrocytes. Whole cell lysates from cultured normal chondrocytes (tibial plateaux, normal, male, age 54 years) (13973) were fractionated to cytosolic and particulate compartments. PKCα expression was assessed by running both compartments onto 10% SDS-PAGE reducing gel and immunoblotted with mouse mAb anti-PKCα, M4, at 1:1000. PKCα translocation was observed in particulate compartment that subjected to 30 sec 0.33 Hz cyclical mechanical stimulation (lane **b**, particulate). This response was inhibited in the presence of 1μg/ml anti-CD47 Bric 126 (lane **c**, particulate). Resting cells (lane **a**) were used as control. PKCα translocation was assessed in three more donors, but there was technical problems in sonicator and separation of cytosolic and particulate did not occur properly. Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

4.5.0. DISCUSSION

4.5.1. CD47 and natural ligands expression and interactions

Integrin receptors for ECM molecules play central and complex roles in cell interactions. A complex series of steps leads from initial integrin interactions with an extracellular ligand to transmembrane effects on the localization of cytoskeletal molecules or signalling molecules, to the activation of signalling pathways, and to eventual regulation of gene expression. Identifying distinct mechanisms of integrin responses to extracellular stimuli and patterns in the classes of responding molecules will be crucial for understanding how integrins function. It is becoming increasingly apparent that full understanding of integrin functional activities may require understanding of integrin associations with other cell-surface molecules (Miyamoto et al 1995).

It is well established that ligand binding and cell-surface clustering of integrins can lead to the assembly of large multi-component intracellular signalling complexes (Turner et al 1991; Hynes 1992). As integrins including $\alpha 5\beta 1$ have no intrinsic enzymatic activity of their own, they must interact with other proteins to generate signals (Damsky and Werb 1992).

It has been shown that several kinds of proteins, including immunoglobulin superfamily (IgSF) proteins that bind to integrins, may generally function in concert to mediate both cell-cell and cell-ECM interactions, which in turn stimulate cytoskeletal reorganization, migration and a cascade of intracellular signalling pathways.

4.5.1.1. CD47 -integrin associations

The present study showed a physical association between CD47 and $\alpha 5$ integrin in human articular chondrocytes. In vitro binding studies have also demonstrated no physical association between CD47 and $\beta 1$ integrin in unstimulated and stimulated articular chondrocytes. These suggest a role for CD47 in modulation the function of

α5β1 integrin in chondrocyte mechanortransduction. Integrin cytoplasmic domains are a key nexus of interaction between the extracellular environment and intracellular structures and signaling cascades. Both the α and β subunit cytoplasmic domains make important contributions to various aspects of overall integrin function including cytoskeletal organization, cell motility, signal transduction, and modulation of integrin affinity for ligands and activation.

Studies have shown that three regions are implicated in integrin ligand binding: the N-terminal portions of both the α and β subunit and the A (or I) domain in the integrins that contain it (Loftus et al 1994; Cao et al 1998). The ligand binding sites on the β1and β3 subunits are the best understood. RGD-containing peptides cross-link to the N-terminal portion of the β3 subunit (Loftus et al 1990; Takada et al 1994). For integrin α subunits that do not possess an A domain, ligand binding localizes to the N-terminal portion. This region corresponds roughly to the amino-terminal 400 amino acids on several α subunits including α4, α5 and α_{IIb} (Loftus et al 1994). It contains the metal ion-dependent adhesion site (MIDAS) binding sites and is composed of seven homologous, repeated domains, although there are conflicting reports on the role of divalent cations in ligand binding by switching the integrin into an active conformation (Cao et al 1998).

CD47 was initially identified as an associated protein with β integrins including β3 integrin (Brown et al 1990; Lindberg et al 1993; Wu et al 1999) and β1 (Gao et al 1996; Chung et al 1997; Wang et al 1998, 1999; Barazi et al 2002). In platelets, CD47 has been described to be physically linked to β1 (Chung et al 1999) and β3 (Chung et al 1997; Brown et al 1990) integrins and to form a signal-transducing complex, allowing their activation to a higher affinity/avidity state after its ligation with the CBD domain of TSP1 in static conditions. Many intracellular proteins have been demonstrated that interact directly with the integrin β subunits including signal transduction proteins such as FAK (Dedhar and Hannigan 1996). Studies have shown physical associations

between CD47 and the α2 integrin subunit in melanoma cells (Chung et al 1997) and α4 integrin subunit in Jurkat T cells (Barazi et al 2002).

This is the first study that has shown the physical association between CD47 and subunit α5 integrin. There are several reports that have specifically focused on the role of α5 subunit and its importance as a part of an integrin in a variety of cellular functions. Cao et al (1998) have demonstrated that there are domains on the integrin α5 subunit that mediate cell signaling on fibronectin. These include cell adhesion, cell spreading, cell migration, focal adhesion formation, modulation of interactions of focal adhesion components and β subunit cytoplasmic domain, and tyrosine phosphorylation. In addition, it has been previously reported (Loftus et al 1994) that the N-terminal one-third of integrin α and β subunits mediate integrin-ligand recognition. This region is also involved in the interactions between α and β subunits.

Dalton et al (1995) have previously shown that ligand binding of α5β1 integrin alters the integrin life cycle and permits a longer retention of these proteins at the cell surface. Studies have shown that integrin interaction with a ligand is a critical step for signal transduction such as tyrosine phosphorylation (Palecek et al 1997; Koyoma et al 1996; Miyamoto et al 1995; Schmidt et al 1998). Taken together, it is more likely that spatial redistribution of β1 integrin, due to interaction of α5β1 integrin with accessory proteins such as CD47, plays an important role in early signaling events such as tyrosine phosphorylation and cytoskeletal reorganization.

O'Toole et al (1994) have demonstrated previously that affinity modulation and ligand binding of α5β1 integrin are cell type specific, the cell type specific signals that modulate integrin affinity are transmitted through the cytoplasmic domains and both α and β cytoplasmic domains are involved in affinity modulation. They also have shown that the regulation of integrin affinity by the α subunit cytoplasmic domain is α subunit specific. In those studies α_{IIb} and α5 specify different affinity states in CHO cells, α_{IIb} the low and α5 the high affinity state. They concluded that the α subunit

cytoplasmic domain designate integrin-specific affinity differences, whereas β subunit may be permissive for the high affinity state.

It has been shown previously (Blystone et al 1995) that CD47 can regulate the function of α5β1 integrin in erythroleukemia cell line K562. The modulatory effect of CD47 in α5β1–mediated phagocytosis required β3 integrin. Barazi et al (2002) have shown that anti-CD47 B6H12 is physically associated with α4β1 integrin and also has an inhibitory effect on α5β1integrin in integrin-mediated adhesion in Jurkat T cells. Therefore, they reclassified B6H12 as a function-modifying Ab for CD47.

Disatnik and Rando (1999) have shown that outside-in signalling pathways are initiated when muscle cells attach to fibronectin via α5 integrins. It has been shown previously that α5 -deficient cells survive poorly and undergo apoptotic cell death when plated on fibronectin as the sole substrate (Taverna et al 1998). The poor survival of α5 -deficient cells is reflected by the absence of spreading on fibronectin, whereas cells that express α5 integrin attach and spread readily on fibronectin. In many cell types, the process of attachment and spreading is coincident with the formation of focal adhesions and phosphorylation of FAK as well as other proteins that localize to focal adhesions (Schaller et al 1992; Hanks et al 1992). Indeed, α5 expressing cells demonstrate robust FAK phosphorylation when plated on fibronectin. Consistent with their ability to attach and spread, α5 -deficient cells do not manifest any FAK phosphorylation on fibronectin (Disatnik and Rando 1999).

It has been shown previously that integrins play an important role in mediating mechanical stress-induced signals. Specifically, integrins like α5β1 and αVβ3 are essential for the mechanotransduction of hemodynamic forces into biochemical signals (Muller et al 1997; Dimmelers et al 1998; Takahashi and Berk 1996; Traub and Berk 1998; Jalali et al 2001; Urbich et al 2002). A series of studies on α5β1 integrin response to mechanical stimulation in human umbilical vein endothelial cells (HUVEC) has shown that the α5 subunit acts as a mechanoreceptor (Yano et al 1997). They have

demonstrated that α5 subunit acts by sensing and responding to cyclic strain with subsequent transduction of the signal inside the cell through the cytoplasmic domain of β1subunit. It has been also shown previously that in chondrosarcoma cells mechanical strain was found to increase α5 expression when the cells were adherent to plastic (most likely via fibronectin and vitronectin), while cells adherent to collagen type II increased expression of α2 (Holmvall et al 1995). A recent study has shown that mechanical loading induced an increase in the amount of integrin subunit α5 in immature and mature cartilage but not in the integrin subunit β1 content (Lucchnetti et al 2003). In addition, it has been shown that stretching at 0.33 Hz cyclical mechanical stimulation produces hyperpolarisation of cultured chondrocytes, which was found to be inhibited by RGD peptides and antibodies to the α5 and β1 integrin subunits (Wright et al 1997).

There are a number of possible explanations for the lack of association between CD47 and β1 integrin in human articular cartilage. Although, chondrocyte CD47 may function as a β1 integrin binding molecule only under certain transient circumstances.

Miyamoto et al (1995) have demonstrated that extracellular triggering of an integrin response involves a series of specific stages of hierarchies of protein interactions. A substantial number of potential intermolecular interactions are already known, including evidence for the binding of talin (Horwitz et al 1986), α-actinin (Otey et al 1990), and FAK (Schaller and Parsons 1994) to the β1 integrin cytoplasmic domain (Lewis and Schwartz 1995). In addition, there is accumulating evidence to suggest the existence of protein-protein interactions among cytoskeletal proteins and a variety of signalling molecules (Clark and Brugge 1995). Because so many proteins can potentially interact with each other based on in vitro studies, it is important to determine whether there are specific patterns or hierarchies of interactions induced by β1 integrins within cytoplasmic milieu (Miyamoto et al 1995).

Although the results from the present study suggest that there is no physical association between CD47 and β1 integrin, it can not rule out the possibility that the presence of β1 integrin and CD47 interactions. This could be due to several reasons:

1) The biochemical manipulations in the coimmunoprecipitation and western blotting experiments affect the epitope recognized by applied mAbs
2) CD47 ligation by applied mAb affects the interaction between CD47 and β1 integrin
3) CD47-β1 integrin interaction is either weak or that there is no consistent direct association.

In addition to the direct interactions with integrin cytoplasmic domains, a number of cytoskeletal and signalling molecules have been shown to become spatially associated with integrins upon integrin engagement and clustering (Dedhar and Hannigan 1996). Miyamoto et al (1995) have proposed a model of hierarchical association of cytoskeletal and signalling molecules with integrins on the basis both of experiments carried out with immunoprecipitation and immunofluorescence. Some of the interactions have been demonstrated by immunoflurescence alone and showed a spatially localized interaction (spatial colocalization), upon integrin occupation or clustering (Dedhar and Hanningan 1995). It is possible to demonstrate a spatial association of CD47 with β1integrin that can play a role in integrin-mediate chondrocyte mechanotransduction.

Studies have shown that both α and β subunits are involved in FAK tyrosine phosphorylation. For example, Leong et al (1995) have shown that both α_{IIb} and β3 cytoplasmic domains are involved in FAK tyrosine phosphorylation. Evidence shows that both α and β subunit cytoplasmic domains make important contributions to various aspects of overall integrin function including cytoskeletal organization, cell motility, signal tranduction, and modulation of integrin affinity for ligands (Aplin et al 1998).

In summary, the data from this study and other studies suggest that α5-CD47 interaction may play an important role in modulation of α5β1 integrin-mediated signaling pathway in articular chondrocyte. The physical association of CD47 and α5 subunit may influence the β1 subunit and the conformation and activation of total heterodimer molecule α5β1 integrin and produce an important effect on integrin-mediated intracellular signalling.

4.5.1.2. Expression and interactions of TSP1 with CD47 in human articular chondrocytes

The results of this study showed TSP1 is expressed by normal and OA chondrocytes and has physical association with chondrocyte CD47.

4.5.1.2.1. In vitro expression of TSP-1

TSP1, as an important ligand for CD47, possesses a variety of critical structural and functional characteristics. TSP1 is a 450 kD trimeric multifunctional matrix protein composed of several binding sites and differential affinity for a number of cellular and extracellular molecules.

TSP-1 is synthesized by a variety of cells including smooth muscle cells (Wang and Frazier 1998), endothelial cells (Sheibani and Frazier 1995), fibroblasts (Tsao and Mousa 1995) and melanoma cells (Gao et al 1996a, b). TSP-1 has also been shown to be present in human articular cartilage (Miller and McDevitt 1988, 1995; DiCesar et al 1994; Pfander et al 2000). Different studies demonstrated TSP-1 expression and its potential role in the development of embryonic and epiphyseal cartilage (Tucker et al 1997; Iruela-Arispe et al 1993). Miller and McDevitt (1995) proposed that TSP-1 binds to chondrocyte surface receptors by an RGD dependent mechanism.

Pfander et al (2000), using immunohistochemistry and mRNA expression have reported the differential pattern of TSP-1 expression in normal, mild OA and severe OA articular cartilage. They demonstrated TSP-1 expression mainly over mid and deep zone, although TSP-1 mRNA expression was seen mainly in mid-zone chondrocytes. They also reported a slight increase in TSP-1 mRNA expression by middle zone chondrocytes in mild OA cartilage. In their studies, OA cartilage with moderate changes showed an interterritorial reduction in TSP-1 staining. Severe OA cartilage showed a major decrease in TSP-1 protein, and displayed a significant reduction in TSP-1 mRNA expression compared with mild OA cartilage. They concluded that TSP-1 is

predominantly expressed in middle zone chondrocytes in normal and mild/moderate OA cartilage and significantly reduced in severe OA.

In the present study, western blotting analysis both normal and OA cultured chondrocytes from donors showed a little variation in TSP-1 expression. It seems that the biological variance (i.e. age and sex of donor) and/ or cartilage pathology and technical artefact may be the most likely explanations for these differences.

4.5.1.2.2. Physical associations of TSP1 with CD47

This study showed that TSP is physically associated with CD47 in human articular chondrocyte.

Multiple distinct receptors acting together to mediate cell-ECM interactions is a common theme that is likely to be critical in providing various levels by which the strength of the interactions can be regulated. Cartilage homeostasis is regulated, in part, by the interaction of chondrocytes with ECM such as $\alpha 5\beta 1$ integrin with the multidomain ECM protein fibronectin.

TSP1 is a known CD47 ligand, and also it has been shown that TSP1 can regulate, through CD47, the functions of $\alpha_{IIb}\beta 3$ integrin in platelets and $\alpha V\beta 3$ integrin in melanoma cells. A number of cells that have their critical functions regulated by TSP1-CD47 interactions have been reported (see **Table 1.4**, page 67). CD47 and TSP1 physical and functional interactions are involved in a variety of integrin-mediated critical functions including calcium mobilization in fibroblast (Tsao and Mousa 1995), spreading and tyrosine phosphorylation in melanoma cells (Gao et al 1996), platelet aggregation (Chung et al 1997; Dorahy et al 1997; Frazier et al 1999; Tulasne et al 2001), spreading of vascular smooth muscle cells (Wang et al 1998). In addition, recent studies by Graf et al (2002, 2003) have shown that CD47/TSP1 complex with an integrin can act as a common mechanosensitive system in different cell types in response to different mechanical stimuli. They have shown that both endothelial cells and fibroblasts are able to sense both shear stress and mechanical stretch through a common

mechanosensitive molecular mechanism. The decisive proteins involved in this general mechanosensitive regulation are the αvβ3 integrin, IAP/CD47 and TSP1.

These reports and data from the present study, reinforce the hypothesis that TSP1 is involved in integrin-mediated chondrocyte mechanotransduction through binding to CD47. In the present study, the physical and functional association between CD47 and TSP1 in chondrocyte is shown. Like CD47 and α5β1 integrin, TSP is involved in electrophysiological response of chondrocyte following 0.33 Hz cyclical mechanical stimulation. The data suggest that, the CD47/TSP1 complex may modulate α5β1 integrin-mediated signal transduction.

4.5.1.2.3. Physical association of TSP1 and β1 integrin

The present study has shown that TSP1 is coimmunoprecipitated with β1 integrin in human articular chondrocyte.

TSP-1 is a ligand for several integrins, including αVβ3 and $\alpha_{IIb}\beta3$ on platelets (Chung et al 1997), α3β1 on neurons (DeFreitas et al 1995), α4β1 and α5β1 on T lymphocytes (Yabkowitz et al 1993a), α3β1 and α4β1 on breast carcinoma cells (Chandrasekaran et al 1999; Krutzsch et al 1999). TSP-1-integrin interactions appear to mediate numerous TSP-1 functions (see **Table 1.4**, page 67). It has been shown that TSP1 can mediate T cell adhesion to fibronectin through binding to α4β1 and α5β1 integrins (Yabkowitz et al 1993).

This is the first study that has shown the strong indirect interaction of TSP, an ECM multidomain protein, with α5β1 integrin by binding with CD47 and a direct interaction by binding with β1 integrin in resting chondrocytes. It is possible that under certain conditions such as mechanical stimulation and/or alteration of integrin affinity/avidity, this interaction may modulate intracellular signalling. Further studies are needed to determine the precise mechanism of this interaction.

4.5.1.3. SIRPα expression and interaction

4.5.1.3.1. In vitro expression of SIRPα

A panel of antibodies was used to investigate the expression of SIRPα in vivo by using immunohistochemistry. All antibodies didn't show any positive signal. The western blot studies showed that SIRPα is expressed by both normal and OA cultured chondrocytes. No identifiable difference was observed between normal and OA chondrocytes.

This molecule shows heterogeneity in molecular weight (~ 65-120 kD) in various tissues due to differential glycosylation (Kharitonenkov et al 1997) and existence of alternative SIRPα transcripts coding for this molecule (Veillette et al 1998). SIRPα is ubiquitously expressed in all tissues examined (heart, brain, spleen, lung, liver, muscles, kidney, and testis), being most abundant in the brain and the spleen (Kharitonenkov et al 1997). Using immunohistochemistry with a specific anti- SIRPα antibody, it has been shown that SIRPα is strongly expressed in myeloid cells (macrophages, monocytes, granulocytes, dendritic cells) and neurons (Adams et al 1998).

These observations and a similar structure of SIRPα and cell adhesion molecules suggest a role for SIRPα as a signal transducer molecule in various cell types. It has been shown previously that adhesion of cultured fibroblasts to various ECM proteins induces the tyrosine phosphorylation of SIRPα and its association with SHP-2 in a manner dependent on Src family kinases and FAK (Tsuda et al 1998; Oh et al 1999).

This is the first study that showed the expression of SIRPα by both normal and OA cultured articular chondrocytes. No detectable difference was found between the expression of SIRPα by normal and diseased chondrocytes. Like other molecules that were assessed in this study such as CD47, CD98, and galectin-3, ankle chondrocytes showed stronger band of this molecule in comparison to the other normal and OA articular chondrocytes isolated from the other joints.

4.5.1.3.2. Interaction of SIRPα and CD47 in human articular chondrocytes

The present study demonstrated that SIRPα is not physically associated with CD47 in cultured chondrocytes.

Seiffert et al (1999) reported that extracellular domain of SIRPα cannot bind to the CD47-deficient human ovarian carcinoma cell line OV10, but to human CD47-transfected OV10. Yoshida et al (2002) have demonstrated the interaction between Ig domain of CD47 and SIRPα in B lymphocytes. Interestingly, they have noticed that the binding capacity of soluble SIRPα Ig domain varied between B cell lines: pre-B cell lines and myeloma cell lines bound larger amounts of SIRPα Ig than mature B cell lines. They suggested that the affinity/avidity state of CD47 for SIRPα may change during B cell maturation. Thus, the affinity/avidity of CD47 could be modified under certain transient conditions. Latour et al (2001) have demonstrated that CD47/SIRPα in resting state of T cells, is disrupted after an environmental condition, and rapidly restored to down regulate inflammatory response and maintain homeostasis. This group have also proposed theoretically, SIRPα and TSP1 might compete for CD47 on T cells, assuming they would be expressed simultaneously during the immune response. Based on these data, some of the certain transient conditions in the present study such as the presence of physical association of TSP (as counterreceptor) and α5 with CD47, stimulated and unstimulated states and normal and OA chondrocytes may have role in the CD47 and SIRPα physical association.

It has been shown that SIRPα and CD47 are coexpressed by monocytes, immature dendritic cells (iDC) and mature dendritic cells (mDC), and the cross talk between the two molecules on the same cell is under current investigation (Latour et al 2001). In several studies it has been shown that CD47/ SIRPα interaction plays important roles in cell-cell interaction such as B lymphocytes adhesion to endothelial cells (Yoshida et al 2002) and T cell activation (Seiffert et al 2001). Oldenborg et al (2000, 2001) have demonstrated that under normal circumstances, CD47 on RBCs prevents their

elimination by ligating the macrophage receptor SIRPα. Thus, the histological and biomechanical characteristics of cartilage suggest that chondrocyte CD47 interacts with an important extracelluar matrix protein, TSP1. This interaction (ECM-cell interaction) can play a central role in transduction the mechanical stimulation into the intracellular space.

Different studies have shown that SIRPα involves in to function in a variety of cellular signalling systems including integrin-mediated pathways (Inagaki et al 2000) such as MAPK activation and FAK tyrosine phosphorylation (Oh et al 1999).

However, SIRPα is not expressed by chondrocytes in vivo, its consistent expression in vitro and its role in electrophysiological response of cultured chondrocytes are confirmed the presence of SIRPα in articular chondrocyte. Thus biochemical results are not artefact and it seems that the mAbs used in the present study did not work with immunohistochemical experiments.

In conclusion, based on the present results, it appears that SIRPα is involved in chondrocyte mechanotransduction in a CD47-independent pathway, or may be mediated, at least in part, through CD47-dependent under certain conditions. Furthermore, SIRPα may involve in the chondrocyte metabolism functions in an integrin-mediated signalling pathway. Further studies might address this question and provide insight into the role of SIRPα in the chondrocyte mechanotransduction.

4.5.2. The role of CD47 and its ligands, TSP and SIRPα, in the electrophysiological response of chondrocytes to 0.33 Hz cyclical mechanical stimulation

4.5.2.1. Electrophysiological response of normal and OA chondrocytes to 0.33 Hz cyclical mechanical stimulation

Normal and OA chondrocytes showed a consistent and reproducible membrane hyperpolarisation and depolarisation response respectively, following 0.33 Hz cyclical mechanical stimulation.

Biomechanic forces can be transmitted into the cell by a variety of structures: deformation of the cell membrane, stress and strain on the cytoskeleton, deformation of the nucleus, and direct signalling pathways via receptors of the ECM such as integrins (Mollenhauer and Erdmann 2002). Because physical forces are generally applied directly or indirectly to the plasma membrane of cells, it has been noted that mechanical forces can affect the permeability of the cellular membrane to various ions (Sachs 1991; Liu et al 1999). The changes in cell membrane potential have been used as a sensitive bioassay of human articular chondrocyte response to mechanical stimulation and have allowed identification of chondrocyte mechanoreceptors and pathways activated in normal human articular chondrocytes after cyclical mechanical stimulation (Salter et al 2001). Salter et al (Wright et al, 1992; Wright et al, 1996; Salter et al 1997, Wright et al, 1997; Salter et al, 2000; Lee et al, 2000, 2002; Salter et al 2001, 2002) analysed mechanotransduction pathways in chondrocytes from humans by using a technique that allows the application of controlled pressure to cultured cells. The standard regime of cyclical mechanical stimulation used consists of 0.33 Hz (2 seconds on, 1 second off) for 20 minutes at 37°C. In this system that used in the present study as well, cyclical mechanical stimulation has been shown to induce deformation on the base of the plastic tissue culture dish and its adherent cells. A pressure pulse of 50 kPa results in 4000 μstrain on the base of the dish, which is equivalent to 0.40% deformation (Wright et al

1992; Salter et al 2002). Stimulation of human chondrocytes at this regime results in membrane hyperpolarisation, which is attributable to the passage of positive ions potassium from the cell to the exterior through ion channels. In contrast, low frequency stimulation, at 0.104 Hz and continuous stimulation result in membrane depolarisation. This depolarisation is attributable to the entry of positive ions (sodium into the cell) through ion channels, from interstitial fluid. Reagents that block membrane ion channel activation and activity can be used to identify which channels are involved in the production of these membrane responses (Salter et al 2001). It has been shown that cyclical mechanical stimulation at 0.33 Hz cyclical mechanical stimulation, results in membrane hyperpolarisation of normal human articular chondrocytes as a result of small conductance (SK) calcium-dependent K^+ channels (Wright et al 1992).

Previously it has been demonstrated that the apamin-sensitive ion channel is the channel involved in hyperpolarisation response of normal articular chondrocytes (Wright et al 1996), whereas, the depolarisation response is associated with activation of a tetrodotoxin-sensitive Na^+ channel (Millward-Sadler et al 2000a; Salter et al 2002). Although, it has been shown that both normal and OA chondrocytes express small conductance (SK) calcium-dependent K^+ channels (Millward-Sadler et al 2000a), it is likely that the differential electrophysiological response is a result of activation of different signalling pathways, as suggested by the results observed using the chemical inhibitors of intracellular signalling molecules. SK channels play important roles in excitable cells, and generation of long-lasting hyperpolarisation, slow after-polarization is essential for normal neurotransmission (Bond et al 1999).

Chondrocytes in osteoarthritis are known to show significant phenotypic differences from those of normal cartilage (Aigner and Dudhia 1997). These differences in phenotype, including altered expression of integrins, cytokines, and growth factors, as well as production of ECM, are believed to represent a reparative or remodelling response of chondrocytes following damage to cartilage (Ostergaard and Salter 1998). The range of integrin and other adhesion molecules expressed by chondrocytes is modified in OA, increasing the potential for altered cell-matrix interaction (Ostergaard

and Salter 1998; Ostergaard et al 1998; Lapadula et al 1998). There are also changes in the expression of a number of ECM molecules, such as fibronectin (Jones et al 1987), tenascin (Salter et al 1993) and thrombospondin (Pfander et al 2000) which function as potential integrin ligands, and these may also influence integrin-mediated signalling (Probstimer and Pesheva 1999). These differences are reflected in the differences in matrix production and expression of surface receptors for ECM molecules and cytokines (Ostergaard and Salter 1998). The difference in responses to 0.33 Hz cyclical mechanical stimulation is a further example of difference between normal and OA chondrocytes. Such differences may have significant effects on cartilage structure and functions if anabolic mechanical stimuli fail to be recognised or elicit inappropriate response (Salter et al 2002). The mechanotransduction pathway that identified in chondrocytes involves recognition of the mechanical stimulus by integrins and activation of integrin signalling pathway with the generation of a cytokine loop resulting in a second cascade of intracellular signalling events. Normal and OA chondrocytes show differences at multiple stages of the mechanotransduction cascade. Early events are similar, involve $\alpha 5\beta 1$ integrin and stretch activated ion channels and are associated with similar rapid tyrosine phosphorylation events. The actin cytoskeleton and PKC, are required for the integrin-dependent mechanotransduction in normal but not OA chondroccytes. Autocrine/paracrine signalling cascades generated following mechanical stimulation also differ in respect of cytokines and the effect of these cytokines on cell responses. Modified responses of chondrocytes in OA cartilage to mechanical stimulation may be an appropriate, adaptive response to the altered mechanical environment to which the cell is exposed.

The seeding of chondrocytes at very low concentrations ($1x10^4$ cells/ml) and impalement at non-confluent gives the possibility to record the membrane potential of each cell separately. It was noted whilst performing electrophysiological experiments that there was a degree of variation between the baseline resting membrane potential when comparing articular chondrocytes obtained from different donors, although the degree of hyperpolarisation observed following cyclical mechanical stimulation did not differ significantly between donors. Cell confluency is thought to contribute to this, and

although cells were plated at the same density (1 $x10^4$ cells/ml) for every electrophysiological experiment performed, after the 10-12 days incubation period there were noticeable variations in cell confluency between donors. Biological variance (i.e. age and sex of donor) is the most likely explanation for these differences. It has been previously reported that resting membrane potential increases as the density of the culture increases (Bard and Wright 1974) (**Figure 4.12),** and it has been suggested that, as cell confluency increases, there may be alterations in the permeability of the cell membrane to sodium, chloride or potassium, the three ions principally responsible for maintaining and altering membrane potential levels. It has also been shown that the substrate on which cells are cultured can have effects on resting membrane potential level (Bard et al 1974), with fibroblastic cells cultured on collagen having higher resting membrane potentials than the same cells cultured on plastic. Thus, confluent cells may have higher resting membrane potentials, as they are likely to have produced a considerable amount of extracellular matrix which they will have forged strong attachments to.

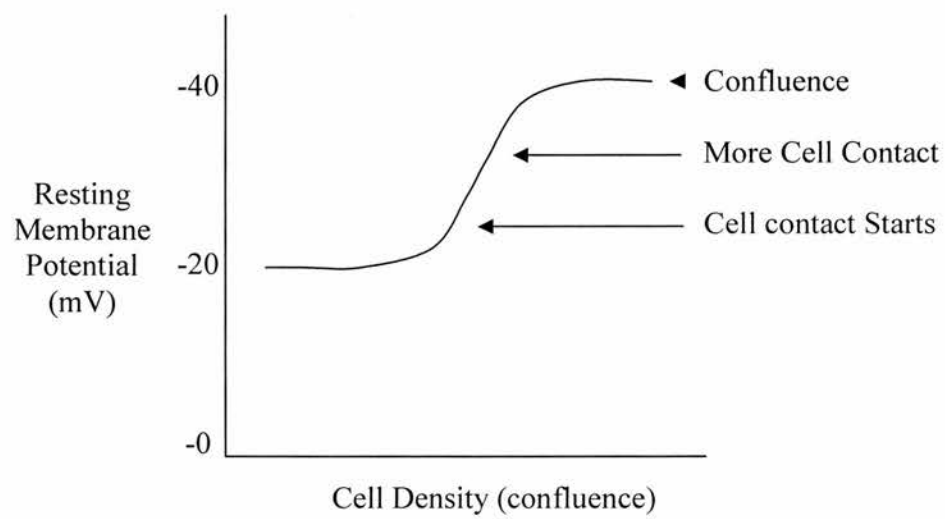

Figure 4.12. Effect of cell confluency on resting membrane potential (Bard and Wright 1974).

4.5.2.2. The role of CD47 in chondrocyte electrophysiological response

This study showed that the electrophysiological response of both normal and OA human articular chondrocytes to 0.33 Hz cyclical mechanical stimulation is inhibited in the presence of anti-CD47, Bric 126.

Integrins act as transmembrane link among the ECM, cytoskeletal proteins, and actin filaments. Mechanical stimuli may activate intracellular signalling as a result of deformation of the ECM and induce changes in the conformation or clustering of integrins following mechanical stimulation (Ingber 1991; Aplin et al 1998). Roles for integrins and integrin-associated signalling molecules in the mechanotransduction pathway activated after 0.33 Hz mechanical stimulation, leading to membrane hyperpolarisation, were shown by the use of function blocking monoclonal antibodies and pharmacologic inhibitors (Wright et al 1996). Exposure of chondrocytes in primary monolayer culture to GRGDSP peptide, which competes for integrin-matrix ligand binding sites, resulted in inhibition of the hyperpolarisation response whereas the control peptide GRADSP had no effect. The hyperpolarisation response was reduced considerably when cells were preincubated with P4C10 (β1) and BIIG2 (α5) anti-integrin antibodies. These data with the other studies have demonstrated that $\alpha5\beta1$ integrin functions as a chondrocyte mechanoreceptor and activates a signal cascade involving the tyrosine protein kinases, the actin cytoskeleton, PLC-associated inositol triphosphate pathway and PKC (Wright et al 1997; Salter et al 2001).

It has been previously shown that $\alpha5\beta1$integrin is strongly expressed by articular cartilage in several studies (Salter et al 1992; Woods et al 1994; Loeser et al 1995) and acts as a mechanoreceptor in mechanotransduction pathway of both normal and osteoarthritic cartilage (Wright et al 1997). Like other types of integrins, $\alpha5\beta1$integrin has no intrinsic enzymatic activity and also is not phosphorylated. Therefore it must interact with other proteins to generate signals (Damsky and Werb 1992; Ishibashi et al 2002). The regulated specific interaction of several individual proteins to form a complex with new functions is a well established principle of signal transduction

(Brown and Frazier, 2001). Thus, to better understand how α5β1 integrin is involved in mechano-induced response of chondrocyte following mechanical stimulation, it is necessary to know which molecule/s in cell membrane or ECM can physically and functionally associate with this integrin.

CD47, integrin-associated protein (IAP), a member of IgSF, ubiquitously 50 kD protein, is a transmembrane glycoprotein that can modulate a range of cellular functions including, cell adhesion (Lindberg et al 1996), cell spreading (Gao et al 1996), and calcium influx (Schwartz et al. 1993). Salter et al (1997) have previously shown that in human bone cells (HBC), anti-CD47 Bric 126 antibody inhibited the hyperpolarisation response induced by 0.33 Hz mechanical stimulation, but had no effect on the depolarisation response induced by 0.104 Hz mechanical stimulation. Interestingly, Graf et al (2003) have recently shown, when fibroblasts are subjected to both laminar flow and pulsatile loading mechanical stimuli, they used a similar mechanosensitive regulation pathway that was composed of CD47/ integrin and TSP. Similarly, when endothelial cells are subjected to the same loading mechanical stimuli, they showed the same mechanosensitive regulation pathway as well. They concluded that a CD47/integrin complex-dependent common mechanism acts as a receptor for TSP1 in the mechanosensitive regulation in different cell types for different mechanical stimuli.

To explain the mechanism of CD47 function in the electrophysiological response after 0.33 Hz cyclical mechanical stimulation the possibilities include 1) modulation of α5β1integrin signalling, 2) integrin-regulated calcium entry, 3) regulation the function of stretch-activated ion channels including Ca^{2+} -activated K^{+} channels and 4) integrin-independent modulation function of CD47.

1. Several reports support the modulation effect of CD47 on integrins in a variety of cells including $\alpha_{IIb}\beta3$ on platelets (Brown et al 1990; Chung et al 1997) , α2β1 on vascular smooth muscle cells (VSMC) (Wang and Frazier, 1999) and αVβ3 on melanoma cells (Gao et al 1996) and may regulate the functions of integrins and act as a transducer element in activation mediated via integrins (Zho and Brown 1993) in various

cell types. Moreover, it has been shown that CD47 can modulate α5β1 integrin functions by affecting other integrins including αvβ3 and α4β1 signaling transduction (Blystone et al 1995; Barazi et al 2002). Evidence suggests that signal transduction through integrins depends on integrins and integrin-associated proteins (Shattil et al 1998; Wright et al 1996; Hogg and Porter 1998). It has been shown that a number of proteins, including IgSF proteins, that bind to integrins, may generally function in concert to mediate both cell-cell and cell-matrix interactions, which in turn stimulate intracellular processes (Oshima et al 2002). Regarding the similar effect of CD47 and α5β1integrin, as mechanoreceptor of chondrocyte, on electrophysiological response of normal and OA chondrocytes, it seems reasonable to suggest the modulation effect of CD47 on α5β1integrin.

2. Wright et al (1992, 1996) have demonstrated that stretch of the base of the culture plate and the attached chondrocytes results in the changes in membrane potential. They have also shown that these changes are mediated by stretch-activated ion channels in chondrocyte plasma membrane. The blockade by quinidine of the hyperpolarisation response to cyclical 0.33 Hz mechanical stimulation (Wright et al 1992), suggested involvement of Ca^{2+}-activated K^+ channels. There is also substantial evidence to suggest that the hyperpolarisation response is dependent on intracellular Ca^{2+} concentrations. The role of calcium influx from outside the cell previously suggested by the L-type calcium channel blocker, verapamil (Wright et al 1992), and confirmed by the use of somatostatin and cadmium ions, which also block the L-type calcium channel (Narahashi et al 1987; Tsunoo et al 1990).

3. Preliminary studies also have shown that CD47 acts as a calcium channel and has a critical function in integrin-mediated calcium influx in endothelial cells (Schwartz et al. 1993). Taken together, suggests that it is likely that CD47 exerts its effect on mechanical stimulation in part by regulation the calcium influx by modulation of the Ca^{2+}-activated K^+ channels.

4. Studies have reported the integrin-independent role of CD47 in some cellular functions (Reinhold et al 1997; Ticchioni et al 1997). Therefore, it is possible that CD47 plays a role in both integrin-dependent and independent of chondrocyte mechanotransduction.

In summary, similar immunohistochemical expression pattern and electrophysiological role of CD47 in both normal and OA cartilage suggest that CD47 plays role/s in early events of mechanical stimulation that is/are similar in both normal and OA cartilage such as integrin-mediated rapid tyrosine phosphorylation events (Salter et al 2002).

4.5.2.3. The role of TSP-1 in OA chondrocytes electrophysiological response

This study showed that antibody to TSP completely inhibited the electrophysiological response of chondrocytes following 0.33 Hz cyclical mechanical stimulation.

TSP-1 is a multifunctional protein, which mediates different cell-matrix interactions. Peptide sequences within the C-terminal domain bind to the CD47 (Gao et al 1996), which has been shown to modulate the integrin function in a variety of cells (Gao et al 1996; Reinhold et al 1997; Ticchioni et al 1997). It is involved in multiple cellular processes including formation of multi-protein complexes on the cell surface and the clustering of receptors that initiate signal transduction. TSP1 dictates the composition of these multiprotein complexes through specific interactions with growth factors, cytokines, other matrix components, and membrane proteins (Chen et al 2000).

TSP1, through association with CD47, influences the integrin functions in a number of cells. For example the interactions of CD47-TSP1 affect the function of $\alpha v\beta 3$ on melanoma cells in integrin-dependent cell spreading (Gao et al 1996a; Frazier et al 1999), $\alpha 2\beta 1$ on vascular smooth muscle cells (VSMC) in chemotactic response and $\alpha V\beta 3$ (Wang and Frazier, 1998;Wang et al 1999; Chung et al 1999), and $\alpha_{IIb}\beta 3$ on platelets (Chung et al 1997). Graf et al (2003) have recently shown that the expression and function of TSP1 is similar with CD47 in a common mechanism for the

mechanosensitive regulation of apoptosis in different cell types and for different mechanical stimuli. In this mechanism TSP1 functions as a ligand and an integrin/ CD47 complex as receptor.

In addition to CD47, several surface receptors, including β1 and β3 integrins (Defrietas et al 1995; Frazier et al 1991; Lawler et al 1993; Yabkowitz et al 1993; Gao et al 1996, Chandrasekaran et al 1999), heparan sulfate proteoglycans (Vischer et al 1997), Ca^{2+} ions (Lawler et al 1982; Miller et al 1995), CD36 (Dawson et al 1997), and Low-density lipoprotein receptor-related protein (Godyna et al 1995) can transduce signal following interaction with TSP1 or specific TSP1 peptides. For example Ca^{2+} ions might be involved in activation of ion channel/s that is shown play important role in chondrocyte mechanotransduction such as SK channels.

Moreover, TSP1 peptides can induce the tyrosine phosphorylation of several proteins, including 70 and 120 kD species (Gao et al 1996; Wilson et al 1999; Chung et al 1999; Ticchioni et al 1997; Tulasne et al 2001). There is also interaction between CD47 and TSP1, via an integrin-independent pathway, that provides costimulatory signals to T lymphocytes (Reinhold et al 1997; Ticchioni et al 1997). Studies have shown the involvement of TSP in cellular functions through a CD47-independent manner (Tulasne et al 2001).

In summary, it is more likely that chondrocytes CD47/TSP1 complex may associate with α5β1 integrin to modulate its function following mechanical stimulation. It has been shown in several settings, the binding agonist peptide 4N1K, or CBD, of TSP1 to CD47 stimulate the activity of integrins to a higher affinity / avidity state (Hughes and Pfaff 1998). Taken together, it appears that CD47-TSP complex affects the affinity / avidity state of α5β1 integrin following mechanical stimulation of human articular chondrocytes.

4.5.2.4. The role of SIRPα in OA chondrocytes electrophysiological response

SIRPα is involved in electrophysiological response following 0.33 Hz cyclical mechanical stimulation.

To establish role/s for SIRPα in chondrocyte mechanotransduction, two recognized function blocking Abs against SIRPα, SE5A5 and SE7C2, in electrophysiology system were tested. The electrophysiological response of chondrocytes in the presence of SE7C2 mAb was significantly inhibited whereas, SE5A5 did not show any modification in this response.

SIRPα is recently known as another important ligand for CD47. Both CD47 and SIRPα belong to Ig superfamily. Like other members of the SIRP family, SIRPα consists of three domains: the extracellular domain, the transmembrane domain and the intracellular domain. The extracellular domain is composed of an amino-terminal Ig variable (IgV) region and two Ig constant (C) regions. In several reports, it has been shown that Ig domain of CD47 and the amino terminal Ig domain of SIRPα can interact directly to each other (Jiang et al 1999; Seiffert et al 1999, Han et al 2000, Vernon-Wilson et al 2000).

Different SIRPα epitopes may have different functional roles, and this could be due to their relative ability to cluster and induce a signalling cascade in chondrocytes. SE5A5 and SE7C2 mAbs were recognized different epitopes on the same antigen (Seiffert et al 1999, 2001) and one of which is involved in chondrocyte mechanotransduction at least in this system. Therefore, both SE7C2 and SE5A5 characterized as function-blocking mAb but have different noncompetitive epitopes. SE7C2 recognizes a site on the SIRPα molecule necessary for chondrocyte response to mechanical stimulation, whereas SE5A5 does not.

It is possible that the EC Ig domain of SIRPα influences the conformation of the integrin/s, CD47 and/or ion channels to promote binding to them and modulate their functions.

Tsuda et al (1998) have shown indirectly the interaction between integrins engagement by ECM proteins and induction tyrosine phosphorylation of SIRPα and suggested that SIRPα might be a member of the group of proteins that are at focal adhesion contacts and undergo tyrosine phosphorylation in response to the interaction of integrins with the ECM. Thus, it seems likely that SIRPα, under specific conditions (circumstances), undergoes a transient state, including interaction with integrins or/and phosphorylation on tyrosine residues and in part participates in mechanical-induced response of articular chondrocytes mechanotransduction. Alternatively, SIRPα could be associated with other proteins, which contribute to the electrophysiological response of chondrocytes such as ion channels. In any case, this study has provided a specific functional role for SIRPα in chondrocyte mechanotransduction. This study demonstrated that SIRPα , a ligand for CD47, is expressed by human articular chondrocytes. Although the results of the present study was unable to show a physical association between CD47 and SIRPα, but indicated that SIRPα is involved in chondrocyte mechanotransduction by inhibiting electrophysiological response following cyclical mechanical stimulation.

The electrophysiology observations, which are obtained from the present study, give evidence that the CD47 and its potential ligands, TSP1 and SIRPα, as components of the mechanotranduction pathway, are also responsive for the regulation of intracellular signalling after mechanical stimulation. In conclusion the results of the present study indicated that both ligands of CD47, TSP1 and SIRPα, are involved in chondrocyte mechanotransduction.

4.5.3. CD47 and intracellular signalling after 0.33 Hz cyclical mechanical stimulation

4.5.3.1. The role of CD47 in chondrocyte protein tyrosine phosphorylation following 0.33 Hz cyclical mechanical stimulation

The present study has demonstrated that CD47 is involved in rapid tyrosine phosphorylation of pp12FAK following 1 minute 0.33 Hz cyclical mechanical stimulation in chondrocyte. Both primary chondrocyte and cell line C20A4 showed the same response.

The intracellular signalling cascades that are activated when integrins bind to their extracellular ligands are varied (Clark and Brugge 1995). Signalling molecules, transmitting information from the extracellular compartment into the cell, so-called outside-in signalling (Hynes 1992; Juliano and Haskill 1993). The specific pathways appear to differ depending on the specific integrin/ligand interaction and the type of cell (Disatnik and Rando 1999). One of the earliest changes initiated by integrin engagement is clustering of integrins at focal adhesions and tyrosine phosphorylation of proteins such as paxillin, talin, and the cytosolic enzyme focal adhesion kinase (FAK) (Schaller et al 1992; Hanks et a1 1992; Kornberg et al 1992; Rozengurt and Fernandez 1 997). Tyrosine phosphorylation of several cellular proteins appears to play an essential role in integrin-mediated signal transductoin because its inhibition blocks gene expression (Lin et al 1995). The phosphorylation state of cellular proteins, which reflects a balance between protein kinases and protein phosphatases activities, could switch on or switch off the biochemical pathways in the cells. FAK phosphorylation is considered to be a critical step in the downstream signalling that promotes a number of cellular functions (Burridge et al 1992).

It has been previously shown that tyrosine phosphorylation in chondrocyte is integrin and stretch-activated ion channels dependent (Lee et al 2000). The mechanism by which integrins activate tyrosine phosphorylation of focal adhesion proteins is poorly

understood. This process may involve engagement of integrins with their ECM ligands leading to conformational change, clustering of integrins and the formation of organized complexes between integrins and cytoskeletal proteins (Howe et al 1998). For example, integrin clustering may lead to the oligomerization of different FAK molecules, FAK then undergoes trans-autophosphorylation and activation (Hotchin and Hall 1995). FAK may also be recruited to nascent focal adhesions because it interacts, either directly or through the cytoskeletal proteins talin and paxillin, with the cytoplasmic tail of integrin β subunit (Giancotti and Ruoslahti 1999). These could be possible mechanisms by which integrins stimulation resulting from mechanical stress leads to tyrosine phosphorylation (Herng-Sheng Lee 2000, PhD Thesis).

As an index of chondrocyte integrin-mediated intracellular signalling, in the present study, the tyrosine phosphorylation status of chondrocytes following 0.33 Hz cyclical mechanical stimulation has been monitored. This process evaluated in the pretreated chondrocytes with function-modifying anti-CD47 Bric 126.

Previous studies have shown an association between CD47 and FAK tyrosine phosphorylation in different cell types. Gao et al (1996) have shown that the presence of TSP or its CD47 agonist peptide in medium of cultured C32 melanoma cells causes rapid and intensive tyrosine phosphorylation (Gao et al 1996; Frazier et al 1999; Green et al 1999). Chung et al (1997) have demonstrated that in platelets, small amounts of FAK are associated with the integrin-IAP complex extracted from resting platelets. FAK association with this complex is rapidly increased upon agonist (4N1K peptide of TSP1) activation. Ticchioni et al (1997) have reported that in T cells, CD47 cross-linking with mAb B6H12, function-modifying mAb, induces the phosphorylation on tyrosine of several proteins. Liu et al (2002) have demonstrated that in neutrophil, tyrosine phosphorylation is a key event occurring down stream of antibody-mediated CD47 ligation. They have particularly shown that involvement of tyrosine kinase linked signalling events in the mechanism of CD47-mediated regulation of PMN migration.

Although ligation of CD47 with its antibody B6H12 is reported to induce tyrosine phosphorylation (Ticchioni et al 1997), but other studies have also shown that the same antibody has opposing effects and can selectively enhance and inhibit the function of different integrins (Barazi et al 2002). Based on these data, B6H12 mAb is recognized as a function-modifying antibody for CD47. In addition, since different antibodies recognize different epitopes on the same protein, the ligation of each antibody with its antigen shows differential effect on the antigen affinity/avidity, and this response is varied in multiple cell types (is cell type specific) (Reinhold et al 1997). In the present study, the presence of antibody alone had no effect on protein tyrosine phosphorylation in chondrocytes. In addition, in electrophysiology experiments and PKCα activation assessment, the presence of antibody only did not show modification in the resting condition of chondrocytes.

The transphosphorylation model has been previously proposed to explain the mechanism of intgerin-mediated FAK phosphorylation (Rodriguez-Fernandes 1999). This model suggests that the aggregation of FAK molecules, which are either directly associated with the β integrin cytoplasmic domain (Otey 1996; Girault et al 1999) or indirectly associated with β integrin through other intermediate molecules would favour the transphosphorylation and activation of FAK in focal contacts (Zheng et al 1998). A similar mechanism of FAK activation could operate in the case of other non-integrin adhesion receptors (Otey 1996). Based on this model CD47 can act as an associated protein in α5β1 integrin-mediated and also non-integrin FAK tyrosine phosphorylation.

It has been shown that pp125FAK is associated with cytoplasmic domain of the integrin β1 subunit and this interaction plays a critical role in pp125FAK activation (Richardson and Parsons 1995). Studies have also demonstrated that although pp125FAK can associate in vitro with β integrins (β1, β2, β3), the precise mechanism may differ among integrins and /or cell types (Kornberg et al 1992; Richardson and Parsons 1994). Thus pp125FAK activation in chondrocyte may result from a change in its conformation, perhaps induced by modulation of affinity/avidity of α5β1 integrin. Affinity/avidity modulation of α5β1 integrin may result in effects of CD47/TSP1

complex on α5β1 integrin due to 0.33 Hz mechanical stimulation. Within 1min 0.33 Hz cyclical mechanical stimulation, CD47/TSP1 complex that is associated with α5β1 integrin through α5 subunit may cause conformational change in α5β1 integrin and result in pp125FAK tyrosine phosphorylation.

In summary, inhibition of the mechanically induced tyrosine phosphorylation of FAK by anti-CD47, coimmunoprecipitation of CD47 with both α5 integrin and TSP1 in chondrocytes as well as coimmunoprecipitation of β1 integrin and TSP1, would suggest a role for CD47/TSP1 complex in integrin-mediated mechanotransduction. As mentioned by Graf et al (2000, 2003), a similar complex in endothelial cells and fibroblasts is shown that responded to different mechanical stimuli.

4.5.3.2. The role of CD47 in PKCα activation following 0.33 Hz cyclical mechanical stimulation in articular chondrocyte

In the present study, PKCα translocation within 30 sec cyclical mechanical stimulation in chondrocytes is inhibited in the presence anti-CD47 mAb Bric 126. This suggests that CD47 is involved in PKCα translocation following 0.33 Hz cyclical mechanical stimulation.

PKC isoforms play an important role in intracellular signalling (Nishizuka 1986; Rasmussen 1986). Since the isoforms are expressed on different genes, have strictly regulated tissue expression, and display biochemical differences, they seem to exert different biological functions (Mischak et al 1993; Ozawa et al 1993;Haller et al 1998). One of the earliest changes initiated by integrin engagement is clustering of integrins at focal adhesions and tyrosine phosphorylation of proteins such as FAK and paxillin (Disatnik and Rando 1999). It has been also demonstrated that PKC is involved in focal adhesion and stress fibre formation in fibroblasts plated on fibronectin (Woods and Couchman 1992). Numerous reports have described the association between integrin signalling and protein kinase C (PKC) isoforms activation (Richardson and Parsons 1995; Gao et al 1996; Woods and Couchman 1992). PKC appears to be one of the key

intermediates in integrin-mediated signalling in many cell types (Clark and Brugge 1995; Juliano and Haskill 1993). In certain cell types inhibition of PKC activity results in the inhibition of cell attachment and spreading as well as FAK phosphorylation (Woods and Couchman 1992; Vuori and Ruoslahti 1993; Haimovich and Kaneshiki 1996). Activation of PKC can promote the cellular changes mediated by integrin/matrix interactions (Vuori and Ruoslahti 1993; Chen et al 1994; Short et al 1998). Using specific PKC inhibitors, Haimovich et al (1996) showed that PKC plays a critical role in integrin signalling and FAK phosphorylation in platelets. Haller et al (1998) showed that PKCα and PKCε translocate to nuclear structures and focal adhesions upon the binding of vascular smooth muscle cells to fibronectin. Disatnik and Rando (1999) have shown that PKC is necessary for outside-in signalling mediated by α5 integrin in muscle cells as a result of cell spreading on fibronectin. These results together demonstrate a specific role of PKC in integrn-mediated signal transductioin.

Previous studies by using PKC inhibitors have shown that PKC activity is important in the signaling pathways activated in human and bovine chondrocytes by mechanical stimulation (Wright et al 1997; Millward-Sadler et al 2000; Han et al 2001), which results in increased proteoglycan production. It has been shown that PKCα is the major isozyme present in human articular cartilage (Zhang et al 1999) and there is no clear difference in PKC isozyme expression in primary cultures of normal and OA chondrocytes (Lee et al 2002). The latter study has also demonstrated increased association of PKCα with RACK1 and β1 integrin in primary human articular chondrocytes. These findings suggested that mechanotranduction via integrins is necessary for association between RACK1/ PKCα and β1 integrin following cyclical mechanical stimulation.

Lee et al (2002) have also reported that within 30 seconds 0.33 Hz cyclical mechanical stimulation, PKCα translocated to particulate compartment. In the present study, this integrin-mediated PKCα translocation is used to indicate the potential role of CD47 in this intra-cellular signalling process. In the presence of function-modifying anti-CD47 Bric 126, translocation of PKCα is inhibited.

The data from this study and other studies suggest that the function of α5β1 integrin and PKCα activation are modified by CD47 function. It appears that CD47-TSP1 complex bound and modified the affinity/avidity of α5β1 integrin and caused rapid activation of PKCα that this in turn activated FAK tyrosine phosphorylation and intracellular cascade.

PKCα is a member of cPKC isoforms that have been found to be activated by a calcium-dependent manner. Maasch et al (2000) have demonstrated that the temporal changes in $[Ca^{2+}]_i$ and the velocity of PKCα translocation in vascular smooth muscle cells are closely correlated. They demonstrated that translocation of PKCα is dependent on a threshold Ca^{2+} concentration (Cannell et al 1994; Maasch et al 2000). Maasch et al (2000) have also demonstrated that in living cells the mode of $[Ca^{2+}]_i$ entry into the cytosol and localization of the $[Ca^{2+}]_i$ rise influence PKCα targeting. The targeting of the enzyme to different sites (nucleus, cytoskeleton, microtubules, contractile fibers and cell membrane) may be regulated by specific binding of PKC to so-called receptors of activated protein kinase C (RACKS). Transmembranous Ca^{2+} influx has previously been suggested to lead to localized PKC activation. These data demonstrate the local increase in $[Ca^{2+}]_i$ increases the affinity of binding sites for PKC at plasma membrane (Maasch et al 2000).

Previous studies in both endothelial cells (Schwartz et al 1993) and fibroblasts (Tsao and Mousa 1995) have shown that IAP inhibition blocks a Calcium transient which may augment PKC activation (Gao et al 1996). A transient increase in intracellular Ca^{2+} concentration as a result of its passage through gadolinium-sensitive ion channels appears to be one of the earliest events involved in the response of chondrocytes to mechanical stimulation and may be necessary for integrin-dependent tyrosine phosphorylation of focal adhesion associated molecules and PKC activation (Guilak et al 1999). These data suggest that CD47 may modulate Ca^{2+} influx and the local fluctuations in Ca^{2+} in turn regulate PKCα activation.

Protein kinase C-α (PKC α) has been detected in focal adhesions (Jaken et al 1989). Preliminary studies have shown that activation of PKC by phorbol ester enhances tyrosine phosphorylation of pp125FAK in CHO cells, while a PKC inhibitor blocks this tyrosine phosphorylation (Vuori and Ruoslahti 1993; Richardson and Parsons 1994).

Since PKC may induce integrin clustering, it is possible that a potential mechanism by which PKC may induce FAK phosphorylation is the promotion of integrin clustering (Rodriguez-Fernandes 1999). Vuori and Ruoslahti (1993) have shown that PKC regulates α5β1integrin-mediated pp125FAK phosphorylation and the spreading of Chinese hamster cells on fibronectin.

In summary, the mechanism of CD47 modulation of PKCα translocation could be exerted by its association with α5β1 integrin and /or its role in Ca^{2+} ion influx.

CHAPTER FIVE

5.0. ROLE OF CD98 AND ITS LIGAND, CD147, IN CHONDROCYTE MECHANOTRANSDUCTION

5.1. ROLE FOR CD98 IN ELECTROPHYSIOLOGICAL RESPONSE OF HUMAN ARTICULAR CHONDROCYTES

5.1.1. Effect of a panel of anti-CD98 antibodies on the cultured chondrocytes response to 0.33 Hz cyclical mechanical stimulation

The results of the present thesis in chapter 3 showed that normal and OA chondrocytes expressed CD98 and its ligands, galectin-3 and CD147. Since studies have shown the interaction between CD98 and integrins in different cell types, a series of experiments were undertaken to investigate the role of CD98 and its ligand, CD147, in chondrocyte mechanotransduction.

In first step, electrophysiological experiments were carried out to establish whether CD98 is involved in chondrocyte response to 0.33 Hz (2 sec on, 1 sec off) cyclical mechanical stimulation. Normal chondrocytes were isolated from 2 femoral condyles, one male (age 72) (13941) and another one, male, whose age was not recorded (13949). OA chondrocytes were isolated from 3 femoral condyles, two grade II and one grade III, 2 females, 1 male (age range 64-79, mean 70) (13938, 13943, 13959) and one tibial plateaux, grade II, male (age not recorded) (13983). Primary chondrocytes, seeded in complete media at a density of $1x10^4$ cells/ml, were used to investigate the role of CD98 in the electrophysiological response of chondrocytes to 0.33 Hz cyclical mechanical stimulation.

To investigate the role of CD98 in chondrocyte mechanotransduction, a panel of anti-CD98 antibodies, polyclonal goat anti-CD98 Ab (Santa Cruz), mouse mAbs, BU89 and BU53, were applied

Polyclonal goat anti-CD98 Ab (Santa Cruz) at 1.5 μg/ml was incubated with normal cultured chondrocytes at 37° C for 10 min prior to 0.33 Hz cyclical mechanical stimulation. The membrane potentials of 5 cells were recorded prior to and after the addition of Ab and also after the period of 0.33 Hz cyclical mechanical stimulation. The exposure of cultured chondrocytes to anti-CD98 polyclonal alone produced no significant change in membrane potential. The Ab had no effect on hyperpolarisation response of normal chondrocytes after 0.33 Hz cyclical mechanical stimulation (**Table 5.1.a**).

A similar experiment was carried out with OA cultured chondrocytes in the presence of polyclonal goat anti-CD98 (Santa Cruz) and two function-blocking mouse mAbs anti-CD98, BU53 and BU89. The membrane potential of OA chondrocytes with antibody only did not show any alteration. The depolarisation response of OA chondrocytes did not show alteration in the presence of polyclonal Ab at 1.5 μg/ml after 0.33 Hz cyclical mechanical stimulation (**Table 5.1.b**). OA chondrocytes in the presence of each monoclonal antibody alone, either BU53 or BU89, at 1:100 did not show alteration in membrane potential. OA cultured chondrocytes were subjected to 0.33 Hz cyclical mechanical stimulation in the presence of each mAb separately. The depolarisation response of OA cultured chondrocytes did not show alteration in the presence of either mAb BU89 or mAb BU53, after 0.33 Hz cyclical mechanical stimulation (**Table 5.1c**).

Table 5.1.a. Effect of polyclonal anti-CD98 antibody (Santa Cruz), at 1.5 μg/ml on the hyperpolarisation response of normal cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV) (mean ± SEM)				
Reagent	n	Resting	Anti-CD98 alone	Anti-CD98 + 0.33 Hz MS	% change	P value
Nil	5	23.6±0.92	-	33.6±1.6	+42	0.0001
Anti-CD98	5	23.4±1.5	23.6±1.3	34±1.3	+45	0.0001

Table 5.1.b. Effect of polyclonal anti-CD98 antibody (SantaCruz), at 1.5 μg/ml on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV) (mean ± SEM)				
Reagent	n	Resting	Anti-CD98 alone	Anti-CD98 + 0.33 Hz MS	% change	P value
Nil	5	33±1.4	-	23±1.5	-30	0.005
Anti-CD98	5	33.2±1.5	31.6 ±1.5	24±1.7	-27	0.002

Table 5.1.c. Effect of function blocking anti-CD98 antibodies, BU89 and BU53 at 1:100, on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV) (mean ± SEM)				
Reagent	n	Resting	Anti-CD98 alone	Anti-CD98 + 0.33 Hz MS	% change	P value
Nil	5	30.6±2.2	-	20±1.5	-35	0.001
BU89	5	35±1.9	31.2±3	24.8±1.5	-29	0.02
BU53	5	26.8±1.2	25.8±1.4	18.6±0.01	-30	0.001

5.2. Expression and function of CD147 (EMMPRIN) in human articular chondrocyte mechanotransduction

5.2.1. In vivo expression of CD147 (EMMPRIN) in normal and OA human articular cartilage

CD147 (EMMPRIN) is a highly glycosylated, ~57 kD, transmembrane glycoprotein that is a member of Ig superfamily (IgSF) located on the surface of human tumor cells and some other cells including normal keratinocytes (Biswas et al 1995). Cho et al (2001) have demonstrated that CD147 acts as a member of CD98 functional unit.

To evaluate in vivo expression of CD147 in normal and OA articular cartilage, a series of immunohistochemical staining was undertaken on frozen sections of articular cartilage from femoral head and different anatomical regions of knee joints (femoral condyle and tibial plateaux). Four sections of normal articular cartilage were obtained from 2 males and 2 females (age range 63-90, mean 75) and one case whose age and sex was not recorded. Normal human articular cartilage sections were isolated from 2 femoral heads, one female, one male (age range 76-90, mean age 83) (13857A, 13871G); 1 femoral condyle, female (age 63) (13867J); 1 tibial plateaux (a case whose age and sex was not recorded) (13860A); and one talus, male (age 69) (13940).

Seven sections of OA articular cartilage were obtained from 1 male (age 76), 3 females (age range 74-90, mean age 81) and one case whose age and sex was not recorded. OA human articular sections were isolated from 3 femoral heads, two females, grades I and II, one male, grade II (age range 74-90, mean age 80) (13857B, 13871G, 13867B); 2 femoral condyles, both grade II, one female (age 78) (13853L) and one whose age and sex not recorded (13860); 2 tibial plateaux, one grade III, female (age 78) (13853J) and one case whose age and sex was not recorded (13860).

CD147 expression was assessed using mouse mAb anti-CD147, 8G6, at 1:5000. This mAb was worked very well with frozen sections. Both normal and OA cartilage showed

a similar strong expression pattern of CD147 (**Figure 5.1**). All chondrocytes in sections from different donors showed a similar strong staining pattern. Chondrocytes in all zones showed a similar expression pattern of CD147 (**Figure 5.1**). Strong immunoreactivity of CD147 at very high dilution (1:5000) of mAb 8G6 was observed, suggesting strong expression of CD147 in normal and OA articular cartilage.

5.2.2. In vitro expression of CD147 (EMMPRIN) in normal and OA human articular chondrocytes

To assess the biochemical characteristics of CD147 in articular cartilage, a series of western blotting experiments was performed. Normal chondrocytes were isolated from 4 talus, 1 tibia (ankle), 4 males, 1 female (age range 69-80, mean age 70) (13920, 13901, 13907, 13915, 13820); 2 tibial plateaux, 1 male (age 72), 1 not recoded (13941, 13949); 1 femoral condyle, male (age 69) (13939); 3 femoral heads, 2 males and 1 female (age range 70-83, mean 78) (13816, 13822, 13828). OA chondrocytes were isolated from 2 femoral heads, both grade II, 1 male, 1 female (age range 83-98, mean 90) (13828, 13821); 3 tibial plateaux, 1 grade II and 2 grade III, all female (age range 62-87 mean 76) (13820, 13865, 13938) and one femoral condyle, grade II, male (age 69) (13939).

In preliminary studies, cell lysates extracted from cultured human articular chondrocytes were run under both reducing (**Figure 5.2. A**) and non-reducing (**Figure 5.2. B**) conditions and probed with mouse anti-CD147, 8G6, mAb at a range of dilutions. Under reducing conditions, some samples showed a band ~50 kD size, but the other samples did not express the same band as a main band and expressed multiple faint bands. The expression pattern in several samples was not consistent. Under non-reducing conditions, all samples showed a broad protein staining band between 35 and 50 kD and this was consistent in all samples (**Figure 5.2.B**). Normal chondrocytes in comparison to OA chondrocytes did not show detectable difference in the expression pattern of CD147. Compared with normal and OA chondrocytes isolated from knee and femoral head, a stronger band of this molecule in chondrocytes obtained from normal ankle (**Figure 5.2. B.** lane **a**) was observed.

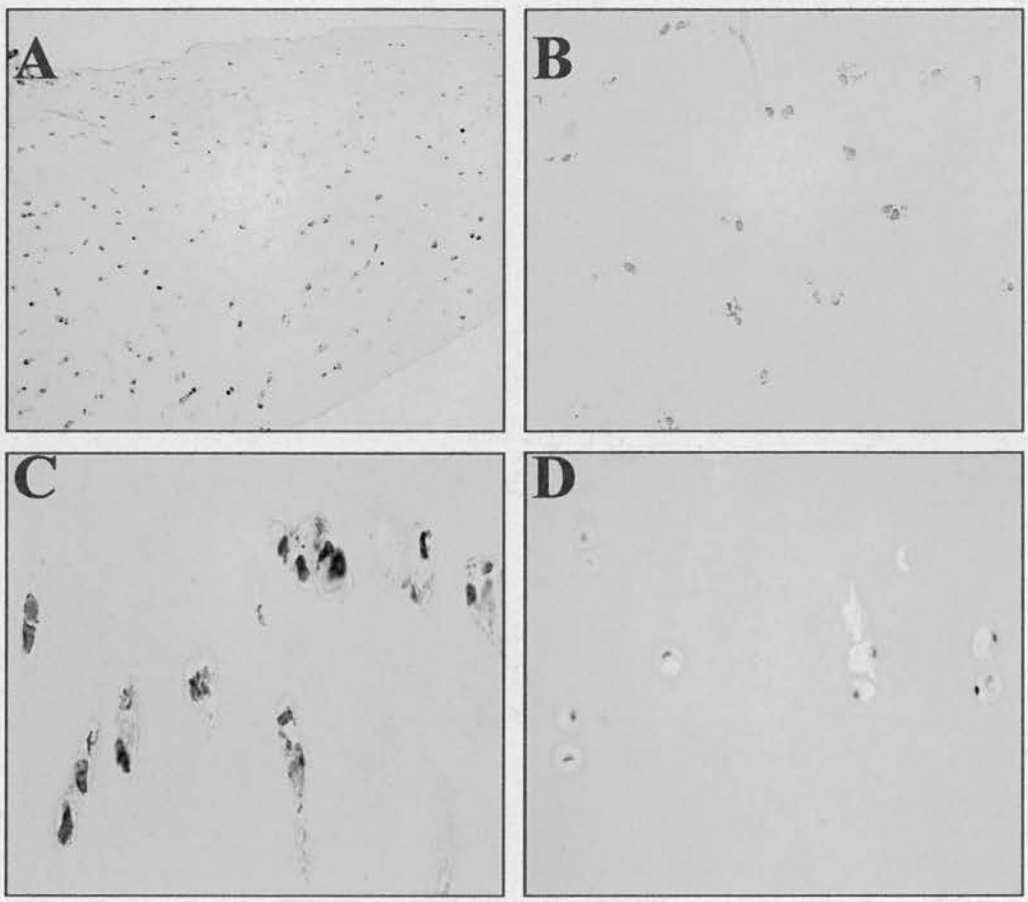

Figure 5.1. Immunoreactivity of mAb anti-CD147, 8G6, in normal and OA human articular cartilage sections. Immunoreactivity was assessed by using mouse anti-C1D47, 8G6, at 1:5000. All sections showed strong expression of C1D47 (EMMPRIN) in different zones. **A**, Tibial plateaux, normal (TN), superficial-deep, (age, sex not recorded), x100 (13860A). Chondrocytes in all zones showed strong expression pattern of CD147. **B**, Femoral head, normal, mid-zone (male, 76 years), x 200 (13871G). Mid-zone chondrocytes showed strong expression pattern for CD147. **C**, Femoral condyle (FA), OA II, mid zone (female, 78 years), x400 (13853J). OA chondrocytes in mid-zone of FAII showed strong expression of CD147. **D**, Femoral condyle, normal (FN), mid zone (female, 63 years), x200 (13967J). In comparison to negative control (**D**), chondrocytes in all zones of normal and OA sections showed a similar strong expression pattern of CD147 immunostaining.

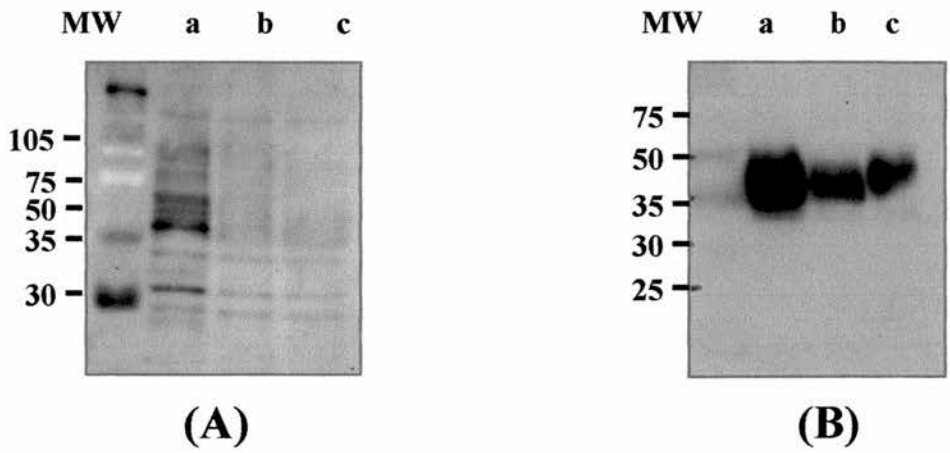

Figure 5.2. Detection and comparison of CD147 (EMMPRIN) in normal and OA human articular cartilage. Equal amounts (40μg/lane) of total extracted proteins prepared from the cultured human articular chondrocytes derived from normal ankle (lane **a**, talus, male, age not recorded) (13901) normal (lane **b**, femoral head, male, age 70) (13822) and OA (lane **c**, tibia plateaux, OAIII, female, age 87) (13965) samples were run in a 10% SDS-PAGE under reducing (**A**) and non-reducing (**B**) conditions. Detection of CD147 was assessed by probing the blots with mouse mAb anti-CD147 8G6 at 1:5000. Under reducing conditions, the samples did not show a consistent expression pattern. Multiple faint bands were observed in some samples and others showed a ~50 kD single band (**A**). All samples, under non-reducing conditions, showed a consistent broad protein staining band between 35 and 50 kD (**B**). No detectable difference was seen in normal and OA cartilage in the expression of CD147. The stronger band of CD147 was detected from normal ankle chondrocytes in comparison to knee and femoral head. The blots shown are representative of three different donors (normal and OA). Molecular weights (**MW**) in kilo Daltons (kD) are indicated on the left.

5.2.3. Effect of anti- CD147 mAb on the OA chondrocyte depolarisation response to 0.33 Hz cyclical mechanical stimulation

To establish whether CD147 would affect the articular chondrocytes electrophysiological response, a series of electrophysiological experiments was carried out. OA chondrocytes were isolated from tibial plateaux, OA grade II, male (whose age was not recorded) (13983). OA chondrocytes were seeded in complete media at a density of $1x10^4$ cells/ml. A function-blocking mAb anti-CD147, 8G6, at 1.5 µl/ml was incubated with OA cultured chondrocytes for 10 min at 37° C prior to 0.33 Hz cyclical mechanical stimulation for 20 minutes.

Antibody alone had no any effect on resting membrane potential. Monoclonal Ab anti-CD147,8G6, had no effect on depolarisation response of OA chondrocytes after 0.33 Hz cyclical mechanical stimulation (**Table 5.2**). Anti-CD147 mAb, 8G6, had no effect on the electrophysiological response of OA chondrocytes after 0.33 Hz cyclical mechanical stimulation.

Table 5.2. Effect of function blocking monclonal anti-CD147 (EMMPRIN) antibody (8G6), at 1.5 µl/ml on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV) (mean ± SEM)				
Reagent	n	Resting	Anti-CD147 alone	Anti-CD147 + 0.33 Hz MS	% change	P value
Nil	5	30 ± 1.9	-	17.4±1.3	-42	0.0001
8G6	5	28.2 ± 0.86	30.4 ± 1.5	17.8±1.15	-36	0.0001

5.3. DISCUSSION

5.3.1 Role for CD98 in human articular chondrocyte

Pretreatment of both normal and OA cultured chondrocytes with a panel of antibodies against CD98 showed that these antibodies had no effect on electrophysiological response chondrocyte to 0.33 Hz cyclical mechanical stimulation.

It has been shown that CD98 to function in a variety of cellular activities including cell fusion, cell aggregation and intracellular calcium concentration (Michalak et al 1986; Posillico et al 1987; Freidman et al 1994). Warren et al (1996) have previously shown that the heavy chain of CD98 (recognized by Juror 177 mAb) can transduce signals into the cell that was manifested in the tyrosine phosphorylation of pp125FAK in hematopoietic cells. Since CD98 HC protein lacks protein tyrosine kinase catalytic sites, they suggested that Joro 177 mAb binding to CD98 stimulates a tyrosine kinase that in turn phosphorylates the pp125FAK protein. CD98 has been characterized as an amino acid transporter and the function and specificity for certain amino acids is determined by the CD98 light chain (LC) (Mastroberardino et al 1998; Verrey et al 1999). Recently, Fenczik et al (2001) demonstrated in a Chinese hamster ovary (CHO) cell model that the two functions of CD98 (regulation of integrin affinity and amino acid transport) are independent of each other.

CD98 physically and functionally interacts with integrin receptors in multiple cell types. Studies have shown that CD98 modulates activity of β1 integrin (Fenczik et al 1997; Chandrasekaran et al 1999; Deves et al 2000). The transmembrane and specific cytoplasmic regions of CD98 HC are required to interact with integrin β1A cytoplasmic domains and this interaction is sufficient for the complementation of dominant suppression of integrins (Fenczik et al 1997) while the extra cellular domains are important for amino acid transport function (Pineda et al 1999).

Further studies are required to investigate the potential physical association of CD98 with chondrocyte integrins including α5β1 integrin and involvement of CD98 in critical chondrocyte intracellular signalling pathways such as tyrosine phosphorylation. In summary, results from this study do not exclude that CD98 might be involved in the other biological functions and/or intracellular signalling events of chondrocytes.

5.3.2. Role for CD147 (EMMPRIN) in human articular chondrocyte

CD147 is expressed by chondrocytes in articular cartilage sections obtained from both normal and OA cartilage. CD147 is also expressed by cultured chondrocytes isolated from normal and OA articular cartilage. Normal and OA cultured chondrocytes did not show detectable differences in the CD147 expression. Cultured chondrocytes isolated from ankle, however expressed significantly stronger band in comparison to normal and OA chondrocytes. The antibody against CD147 did not show any effect on electrophysiological response of OA chondrocytes to mechanical stimulation.

CD147, is a 45-60 kD highly glycosylated transmembrane protein, and also is a cell type-specific molecule, showing differential molecular weight in various cell types (Li et al 2001). These differences in molecular weight of EMMPRIN are mainly due to varying extent of glycosylation since the protein backbone of EMMPRIN corresponds to an approximate molecular weight of 27-32 kD (Biswas et al 1995). Enzymatic deglycosylation by N-glycosidase F produced a polypeptide of ~32 kD in a variety of tissues, indicating that the observed differences in electrophoretic mobility were due to differences in N-linked glycans (Fadool and Linser 1993). Based on these data, the molecular weight of CD147 in chondrocyte is in a broad range of 35-50 kD, which is almost the same as that of EMMPRIN expressed in other human cells such as 46 kD in brain (Fadool and Linser 1993; Sameshima et al 2000).

It has been shown that CD147 is expressed on a variety of cells including endothelial cells, hematopoietic and non-hematopoietic cells. The expression level of this molecule is dependent on the cellular state such as differentiation (Kirsch et al 1997).

Cho et al (2001) have shown for the first time that antibodies to CD147 can neutralize CD98-induced cell aggregation. There are also some striking parallels between CD147 and CD98. CD147 is physically associated with β1 integrin in the cell membrane (Berditchevski et al 1997), as is CD98 with isolated cytoplasmic β1 domains (Zent et al 2000). Antibodies to both molecules can induce aggregation of U937 cells, and the aggregation appears to be mediated in part by β2 integrin activation (Kasinrek et al 1999). Levels of CD98 and CD147 correlate on T cells, with high levels in the thymus, low levels in resting mature T cells, and higher levels on activated mature T cells (Kirsch et al 1997).

Thus, it appears that CD147, like CD98, is acting as a chaperone for multimembrane-spanning transporter molecules. In the case of CD98, these are amino acid transporters (Pfeiffer et al 1999), in the case of CD147, they are the monocarboxylate transporter family of proton-linked monocarboxylic acid transporters (Halestrap and Price 1999). These data have suggested that CD98 forms one component of a ''sensory complex'', containing β1 integrins, CD98, and CD147, together with all their associated molecules (Cho et al 2001).

A possible role/s for CD147 in chondrocytes includes the production and activation of several MMPs including MMP3 (Guo et al 1997; Sun and Hemler 2001; Sameshima et al 2000). The EMMPRIN expression in chondrocytes may play a role in healthy cartilage metabolism under physiologic conditions and progression of degenerating processes under pathologic conditions such as OA and RA. In this regard, Tomita et al (2002), by analysing synovial tissue, have recently shown that EMMPRIN may be one of the important factors in progressive joint destruction in RA.

Second, a recent work by Toole (2003) and his colleagues to identify binding partners for CD147, has shown that annexin II can interact with CD147. It has been demonstrated previously that OA chondrocytes express annexin II and V (Mollenhauer et al 1999; Kirsch et al 2000). Annexin II belongs to the annexin protein family. These proteins enable to form Ca^{2+} channels and influx Ca^{2+} into the chondrocytes, suggesting possible roles in controlling or altering Ca^{2+} homeostasis in cartilage (Kirsch et al 2000). Thus, studying the interactions between CD147 and annexin II during the early events of OA can in part demonstrate the mechanism/s underlying OA cartilage degeneration.

Further studies are required to characterize of CD147 in articular cartilage metabolism and signal transduction.

CHAPTER SIX

CONCLUSIONS

Previous studies have demonstrated that chondrocytes from human articular cartilage show changes in membrane potential following cyclical mechanical stimulation. The hyperpolarisation response of normal chondrocytes to cyclical mechanical stimulation at 0.33 Hz is associated with opening of small conductance (SK) calcium-dependent K^+ channels and mediated via $\alpha5\beta1$ integrin. The signal transduction pathway leading to this response involves cell interactions with ECM ligands via $\alpha5\beta1$ integrin, stretch-activated ion channels (SACs) and a number of integrin-associated signalling molecules including involvement of the actin cytoskeleton and tyrosine phosphorylation of the focal adhesion complex molecules pp125FAK, paxillin and β-catenin. Subsequently, there is secretion of IL-4, increase levels of aggrecan and decrease levels of MMP-3.

In contrast, OA chondrocytes following 0.33 Hz cyclical mechanical stimulation show a membrane depolarisation as a result of tetrodotoxin-sensitive Na^+ channels. This response involves $\alpha5\beta1$ integrin, SAC, tyrosine phosphorylation and cytokine secretion but the actin cytoskeleton and PKC, which are important in membrane hyperpolarisation in normal chondrocytes, are not necessary for depolarisation in OA following mechanical stimulation.

It has been previously suggested that $\alpha5\beta1$ integrin acts as a mechanoreceptor in human articular cartilage (Wright et al 1997; Salter et al 2001). Since integrins, such as $\alpha5\beta1$ integrin have no intrinsic enzymatic activity of their own, and also are not phosphorylated, they must interact with other proteins to generate signals (Damsky and Werb 1992). A well-established principle of signal transduction is the regulated specific interactions of several individual proteins to form a complex with new functions (Brown and Frazier 2001). Thus, to better understand how $\alpha5\beta1$ integrin involves in mechano-induced response of chondrocyte following mechanical stimulation, it is necessary to know which molecules in cell membrane and/or ECM can physically and functionally

associate with this integrin. Therefore, to find partner/s for α5β1integrin in human articular cartilage mechanotransduction, this thesis focused on CD47/IAP, CD98 and galectin-3. The interest in studying these molecules was their association with different integrins in multiple cell types and also their important role in critical cellular functions. CD47, CD98 and galectin 3 are shown, for the first time, to be expressed by chondrocytes in both normal and OA cartilage. Using the changes in membrane potential as an indicator of chondrocyte response to mechanical stimulation initial investigations showed CD98 and EMMPRIN (CD147) had no identifiable role in chondrocyte mechanotransduction, whereas CD47 and its ligands, TSP1 and SIRPα, showed critical functions. Antibodies to CD47, TSP1 and SIRPα completely inhibited the electrophysiological response of normal and OA chondrocytes to 0.33 Hz cyclical mechanical stimulation.

The findings of the present study showed that CD47 expressed in cartilage and is involved in α5β1 integrin-mediated chondrocyte mechanotransduction. First, CD47 expression pattern was similar to α5β1integrin expression in human articular cartilage, showing strong expression in all zones of both normal and OA cartilage. Second, the inhibition of α5β1integrin-mediated electrophysiological response, tyrosine phosphorylation, and PKCα activation by function blocking anti-CD47 mAb Bric126 implies that CD47 induces signalling through a mechanism shared with α5β1integrin. Third, coimmunoprecipitation and western blotting analysis provided evidence for CD47 and α5β1integrin physical association on the chondrocyte cell surface. In addition, an important known ligand for CD47, TSP1, was physically associated with CD47 and β1 integrin and involved in the chondrocyte mechanotransduction.

Several previous studies have shown that CD47-TSP1 complex ligation causes an increase in the affinity /avidity (by clustering) of various integrins. Jalali et al (2001) have also shown that the activation of mechano-sensitive integrins requires the formation of dynamic new connections with ECM ligands. This study has demonstrated that TSP1, an ECM molecule, is involved in integrin-mediated chondrocyte

mechanotransduction and co-immunoprecipitated with both CD47 and β1 integrin. Thus TSP1, both directly (via β1 integrin) and indirectly (via CD47) is associated with α5β1 integrin. These data suggest that TSP1, as an ECM ligand for CD47/α5β1 integrin, may play a critical role in signal transduction from ECM into the intracellular space.

It is shown previously that α5β1 integrin acts as a classic receptor for fibronectin and is physically associated with integrin subunit α5 (Pytela et al 1985). Both TSP1 and fibronectin recognize α5β1 integrin on chondrocytes. Miller and McDevitt (1995) have also previously shown that the mechanism of attachment of TSP-1 to chondrocytes was similar to that of fibronectin and RGD-dependent. The results of this thesis suggest that TSP1 binds to α5β1 integrin to promote the mechanical stimulation through ECM. It seems that α5β1 integrin associates with fibronection to exert structural functions and associates with TSP1 to act as a mechanoreceptor.

Thus, although Fn and TSP1 are associated with α5β1 integrin on chondrocytes, their effects on chondrocyte behaviour differ. In addition, CD47, which is required for chondrocyte mechanotransduction, is a receptor for TSP1 but not for Fn. Additionally, TSP1 has interaction with Ca^{2+} that plays a critical role in cell signalling.

The results of this study suggest that CD47-TSP1 complex may have an auxiliary role by binding to α5 integrin, altering α5β1 integrin conformation in a way that facilitates its activation of the signal transduction cascade. It is shown previously that different integrin αβ heterodimers associating with CD47 can couple to Gi protein- dependent pathways by a 7TMS model. CD47/integrin holds that the five membrane-spanning segments of CD47 and two membrane-spanning domains of the heterodimeric integrin form a seven-transmembrane receptor. Ligation of this complex with an adhesive ligand or TSP could activate GTPase activity in a manner analogous to that of conventional heptaspanins (Gao et al 1996; Frazier et al 1999; Brown and Frazier, 2001). In articular chondrocytes CD47 binding to α5β1 integrin may produce a 7TMS model that promote the signalling_process via a Gi-mediated pathway following 0.33 Hz cycilical mechanical stimulation.

CD47-TSP1 is linked to α5β1-integrin and forms a signal transducing complex, similar to a complex observed in fibroblast and endothelial cells. Graf et al (2002, 2003) have found the molecular mechanism that consists of a CD47 /TSP1 complex which build up together with a member of integrin family such as αVβ3, can act as a mechanosensitive complex and leading intracellular signalling following mechanical stimulation. They have demonstrated that the different external forces induce the same biochemical response inside each cell. They have been suggested that this molecular mechanism is a general part of mechanotransduction in various cell types. The results of the present thesis clearly are consistent with the findings of Jalali et al (2001) and Graf et al (2002, 2003).

In conclusion, this study identified important partner/s for α5β1 integrin in articular chondrocyte mechanotransduction. These data clearly indicated that CD47 and its ligand TSP1 are required for α5β1-integrin mediated chondrocyte mechanotransduction. This function is likely to be through modulation of α5β1 integrin signalling. A complex of TSP1-CD47 and α5β1-integrin is involved in 0.33 Hz cyclical mechanical stimulation of chondrocytes and led to alterations in membrane potential, tyrosine phosphorylation and PKCα translocation. In addition, since CD47 is physically associated with TSP1, it may involve in chondrocyte mechanotransduction in an integrin-independent mechanism as well. More understanding of CD47 functions in chondrocyte biomechanical activities may in the future place it into clinical perspective.

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APPENDIX I

1. Monoclonal antibodies used in this study

ANTIBODY	CLONE	TYPE	SOURCE
Anti-CD47	Bric126	Mouse monoclonal IgG2b	International Blood Group Reference Laboratory
Anti-CD47	CC2C6	Mouse monoclonal (Seiffert et al 1999)	Dr. H.J Büring, University of Tübingen, Germany
Anti-CD47	1F7	Mouse monoclonal IgG1	Frederik P. Lindberg Washington Univ.
Anti-CD47	2B7	Mouse monoclonal IgG2b	Frederik P. Lindberg Washington Univ.
Anti-CD47	3E9	Mouse monoclonal IgG1	Frederik P. Lindberg Washington Univ.
Anti-CD47	2E11	Mouse monoclonal IgG1	Frederik P. Lindberg Washington Univ.
Anti-CD47	Miap400.1	Mouse monoclonal IgG2b	Frederik P. Lindberg Washington Univ.
Anti-CD47	Miap 301	Rat monoclonal IgG2a	Frederik P. Lindberg Washington Univ.
Anti-CD98	4F2	Mouse monoclonal	Serotec
Anti-CD98	BU89	Mouse monoclonal IgG1	Dr D Hardie
Anti-CD98	BU53	Mouse monoclonal IgG2a	Dr D Hardie
Anti-Sirpα	SE5A5	Mouse monoclonal (Seiffert et al 1999)	Dr. H.J Büring, University of Tübingen, Germany
Anti-Sirpα	SE12B6	Mouse monoclonal (Seiffert et al 1999)	Dr. H.J Büring, University of Tübingen, Germany
Anti-Sirpα	SE7C2	Mouse monoclonal (Seiffert et al 1999)	Dr. H.J Büring, University of Tübingen, Germany
Anti-Thrombospondin	P10	Mouse anti-Human Monoclonal IgGικ	Chemicon International
Anti-TSP, Ab-3	C6.7	Mouse monoclonal IgG1	NEOMARKERS

Anti-Galectin-3	9C4	Mouse monoclonal IgG1	Novocastra Laboratories Ltd
Anti-CD147	8G6	Mouse monoclonal (Berditchevski et al 1997)	Dr Martin E. Hemler Harvard Medical School
Anti-β1 integrin	JB1A	Mouse monoclonal, IgG	Chemicon International Inc.
Anti-phosphotyrosine agarose	PT-66	Mouse monclonal IgG1	Sigma, Paisley, UK
Anti-phosphotyrosine HRP conjugated	PY-20	Mouse monoclonal IgG2b	Amersham Life Sciences
Anti-Cellular Fibronectin	FN-3E2	Mouse monoclonal IgM	Sigma
Anti-PKCα	M4	Mouse monoclonal IgG1	Upstate biotechnology

2. Polyclonal antibodies used in this study

ANTIBODY	**TYPE**	**SOURCE**
Anti-CD47	Goat polyclonal	Santa Cruz,USA
Anti-CD98	Goat polyclonal,	Santa Cruz,USA
Anti-Sirpα	Rabbit polyclonal	Affinity bioreagents Inc (ABR)
Anti-integrinα5	Rabbit polyclonal	Chemicon International
Anti-FAK	Rabbit polyclonal	SantaCruz, USA

APPENDIX II

Solutions used in this study

Solution used in immunohistochemistry (IHC)

DAB solution buffer

DAB	0.2ml
DAB buffer	4.8 ml
H_2O_2 0.1%	0.1 ml

DAB buffer

0.2M Tris	12ml
0.1N HCl	19ml
dH_2O	19ml

Imidazole (Sigma) 0.034gr

1M EDTA buffer for antigen retrieval

EDTA	0.372gr
Tween 20	0.1%
dH_2O	1 lit.

pH adjusted to 8.00 with 1N NaOH and 1N HCl

Solutions used in isolation and culture of chondrocytes

Anti-microbial solution

To 100 ml sterile PBS was added 5 ml each of Penicillin-Streptomycin (10000 IU/ml – 10000 μg/ml) (Gibco), L-Glutamine (200 mM) (100X) (Gibco), and Fungizone (250 μg/ml) (Gibco).

Collagenase

Type H collagenase (Sigma): 30 mg per sample was made up in 10 ml of serum free Iscove's modified Dulbecco's medium (Gibco) and filter sterilised.

Solutions used for cell lysis

Lysis buffer (final volume 10 ml)

1 % Igepal (Sigma)

100 μM Na_3VO_4 (Sigma)

one protease inhibitor cocktail tablet (Boehringer Mannheim)

made up in ice-cold sterile PBS

Lysis buffer A for sonication (Buffer A)

20mM Tris-HCl, pH 7.6

2mM EGTA

2mM EDTA

0.33M sucrose

100μM Na_3VO_4

20 mM NaF

Protease inhibitor cocktail tablet (1 tablet/10ml)

Lysis buffer B for sonication (Buffer B)

20mM Tris-HCl, pH 7.6

2mM EGTA

2mM EDTA

0.1% Triton X-100

100μM Na_3VO_4

20mM NaF

Protease inhibitor cocktail tablet (1 tablet/10ml)

Solutions used for Lowry determination of protein concentration

Alkaline carbonate solution

To 5 ml 1% copper sulphate solution was added 5 ml 2% sodium potassium tartrate and 490 ml alkaline carbonate stock solution.

Copper sulphate solution	1 g $CuSO_4$ in 100 ml dH_2O
Sodium potassium tartrate	2 g NaKtartrate in 100 ml dH_2O
Alkaline carbonate stock solution	20 g $NaHCO_3$, 4 g NaOH in 1 litre dH_2O

0.1N NaOH

4 g NaOH in 1 litre dH_2O

Folin's reagent

A mixture of dH_2O : Folin & Ciocalteu's phenol reagent (Sigma) (1:1), solution stored in the dark.

BSA Standards

The stock BSA was diluted with 0.1 N NaOH to give final concentrations.

Stock BSA: 1 mg BSA in 1 ml dH_2O.

BSA standards for Lowry determination of protein concentration

STANDARD NUMBER	**FINAL BSA CONC. (μg/ml).**	**VOL. 1 mg/ml BSA (μl)**	**VOL. 0.1N NaOH (μl)**
S1	0	0	200
S2	10	2	198
S3	25	5	195
S4	50	10	190
S5	75	15	185
S6	100	20	180
S7	150	30	170
S8	200	40	160

Solutions used for SDS PAGE

Separating gel composition (10ml)

Final (%) Acrylamide Conc.	6	8	10	15
dH_2O (ml)	5.3	4.6	4.0	2.3
1.5M tris-HCl pH 8.8 (ml)	2.5	2.5	2.5	2.5
10% (W/V) SDS (µl)	100	100	100	100
30% Acrylamide (ml)	2	2.7	3.3	5.0
10% APS (µl)*	100	100	100	100
TEMED (µl)	8	6	4	4

*Ammonium persulphate (APS) (Amersham UK) was freshly prepared using dH_2O.

Stacking gel composition (5ml)

Final (%) Acrylamide Conc.	5
dH_2O (ml)	3.4
0.5M tris-HCl pH 6.8 (ml)	0.63
10% (W/V) SDS (µl)	50
30% Acrylamide (ml)	0.83
10% APS (µl)	50
TEMED (µl)	5

1.5M Tris-HCl pH 8.8

18.15 g Tris dissolved in 50 ml dH_2O

pH adjusted to 8.8 with 2 N HCl

Make up to 100 ml with dH_2O

0.5M Tris-HCl pH 6.8

6 g Tris in 50 ml dH_2O

pH adjusted to 6.8 with 2 N HCl

Make up to 100 ml dH_2O

10% Sodium Dodecyl Sulphate (SDS)

10 g SDS was dissolved in dH_2O and make up to 100 ml

10X electrode buffer (stock)

30.3 g Tris

144 g Glycine

made up to 1000 ml with dH_2O and stored at 4°C

1X Running electrode buffer

890 ml dH_2O
10 ml 10% SDS
100 ml electrode buffer 10X (stock)

2X Reducing Electrophoresis Sample Buffer (ESB)(for 10 ml)

2.5 ml 0.5 M Tris pH 6.8

0.4 g SDS

1 ml 2-Mercaptoethanol (Sigma)

2.0 ml glycerol

0.01 g (w/v) bromophenol blue

made up to 10 ml with dH_2O

2X Non-reducing Electrophoresis Sample Buffer (ESB)(for 10 ml)

2.5 ml 0.5 M Tris pH 6.8

0.4 g SDS

2.0 ml glycerol

0.02 g (w/v) bromophenol blue

made up to 10 ml with dH_2O

Solutions used for electrophoretic transfer to PVDF membrane

Transfer buffer

made up fresh each time.

700 ml dH_2O

200 ml methanol

100 ml electrode buffer 10X

Solutions used for development of blot

10X TBS (stock)

800 ml dH_2O
Tris 60.7 g

NaCl 87.66

pH adjusted to pH 7.6

made up to 1000 ml with dH_2O

TBS Tween20 (TBST)

900 ml dH_2O

10X TBS 100 ml

Tween20 1ml

Blocking solution

2 % BSA in TBST: 0.2 g BSA was dissolved in 10 ml TBST

5% dried milk (Marvel) in TBST: 0.5g milk in 10 ml TBST

Stripping buffer stock (62.5 mM Tris + 2% SDS, PH 6.7) (1000ml)

dH_2O	900 ml
Tris	7.56 g
SDS	20 g

pH adjusted to 7.6 with concentrated HCl
made up to 1000 ml with dH_2O

Stripping buffer (stripping buffer stock + 100mM 2-mercaptoethanol)

(50 ml per blot)

made up fresh each time.

Stripping buffer stock	50ml
2-mercaptoethanol	390µl

APPENDIX III

Original data from which the results given in the Tables of Chapter 4 and 5 are calculated. The Table number corresponds to that in the results sections.

Table 4.2a – Effect of 1 μg/ml anti-CD47 Bric 126 on the membrane hyperpolarisation response of normal cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV)		
Donor	Regagent	Resting	Antibody alone	Post MS*
F/82 (13878)	Nil	13,17,13, 18,13	-	24,26,20,30,20
	Bric126	16,2018,17,22	17,20,21,22,17	22,18,17,20,20
F/57 (13904)	Nil	35,31,22,30,36	-	57,42,42,43,47
	Bric126	28,37,32,35,36	26,29,26,34,31	29,28,30,37,28
M/92(13876)	Nil	12,11,14,11,15	-	19,16,18,19,24
	Bric126	14,11,13,12,14	15,11,12,14,13	12,15,11,14,13

*, MS: mechanical stimulation

Table 4.2.b. Effect of 1 μg/ml anti-CD47 Bric 126 on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV)		
Donor	Regagent	Resting	Antibody alone	Post MS
F/88 (13902)	Nil	55,42,46,45,42	-	22,30,23,17,19
	Bric126	42,34,36,43,49	35,38,41,45,45	37,47,39,47,41
F/64(13959)	Nil	33,37,24,28,31	-	22,19,20,15,24
	Bric126	31,25,28,24,32	31,33,26,21,29	34,26,28,25,24
N/R(13964)	Nil	32,26,25,23,27	-	15,21,13,20,18
	Bric126	25,24,2625,31	28,23,25,35,27	26,27,24,29,32

Table 4.3. Effect of 1.5 μg/ml anti-TSP mAb (Chemicon) on the depolarization response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV)		
Donor	Regagent	Resting	Antibody alone	Post MS
M/- (13983)	Nil	36,28,26,33,27	-	17,14,22,16,18
	Anti-TSP	27,31,36,29,26	29,27,29,28,32	29,32,27,26,35
F/78(13962)	Nil	27,33,29,35,26	-	22,19,23,21,24
	Anti-TSP	29,37,32,28,27	27,28,31,29,34	33,30,29,36,36
M/-(13983)	Nil	35,29,31,28,29	-	19,21,17,19,22
	Anti-TSP	29,23,27,33,35	28,25,32,33,29	32,29,26,28,34

Table 4.4. Effect of function blocking anti-SIRPα antibodies, SE5A5 and SE7C2, at 1:1000, on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV)		
Donor	Regagent	Resting	Antibody alone	Post MS
F/76(13962)	Nil	22,27,26,25,24	-	16,19,15,18,14
	SE5A5	26,31,29,28,33	29,26,32,31,27	24,21,23,26,22
	SE7C2	22,29,25,24,26	30,25,29,27,26	31,27,25,32,26
M/- (13983)	Nil	25,28,29,31,29	-	19,16,17,14,15
	SE5A5	31,27,35,28,26	26,29,25,27,31	14,17,19,16,22
	SE7C2	30,31,29,26,25	26,32,28,25,33	26,28,31,28,25
N/R (13964)	Nil	24,22,29,31,28	-	17,15,18,14,16
	SE5A5	30,27,26,31,29	31,27,24,36,42	19,22,17,16,24
	SE7C2	40,28,27,25,29	29,26,27,26,24	36,31,29,28,32

Table 5.1.a. Effect of polyclonal anti-CD98 antibody (Santa Cruz), at 1.5 μg/ml on the hyperpolarisation response of normal cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV)		
Donor	Regagent	Resting	Antibody alone	Post MS
M/72(13941)	Nil	24,25,22,26,21	-	35,34,36,27,36
	Anti-CD98	25,28,19,24,21	28,24,21,20,23	38,34,30,35,33
M/- (13949)	Nil	22,25,23,21,20	-	36,33,38,31,33
	Anti-CD98	25,21,27,23,19	21,23,25,20,26	35,37,33,29,31
F/57 (13904)	Nil	22,19,25,23,24	-	37,29,31,33,38
	Anti-CD98	21,25,19,20,23	23,18,21,24,25	33,29,31,37,35

Table 5.1.b. Effect of polyclonal anti-CD98 antibody (SantaCruz), at 1.5 μg/ml on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV)		
Donor	Regagent	Resting	Antibody alone	Post MS
M/-(13949)	Nil	37,33,35,31,29	-	21,25,22,19,28
	Anti-CD98	36,33,29,37,31	29,31,28,37,33	21,27,25,28,19
M/-(13983)	Nil	36,31,29,28,32	-	25,18,24,16,21
	Anti-CD98	37,27,33,32,38	28,27,33,31,36	25,19,18,22,23
M/66(13943)	Nil	40,43,34,35,42	-	22,24,26,23,25
	Anti-CD98	35,31,43,37,32	32,28,38,37,47	23,27,24,25,22

Table 5.1.c. Effect of function blocking anti-CD98 antibodies, BU89 and BU53 at 1:100, on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV)		
Donor	Regagent	Resting	Antibody alone	Post MS
F/64(13959)	Nil	33,37,24,28,31	-	22,19,20,15,24
	BU89	28,35,36,40,36	27,33,35,22,39	25,30,22,21,26
	BU53	26,28,24,25,31	24,27,28,21,29	16,18,19,18,22
N/R(13964)	Nil	32,26,25,23,27	-	15,21,13,20,18
	BU89	29,23,25,26,35	31,33,26,27,25	18,19,19,16,20
	BU53	25,24,27,29,31	32,28,25,28,21	21,17,18,15,13
M/-(13983)	Nil	35,29,31,28,29	-	19,2117,19,22
	BU89	36,33,27,32,31	38,33,39,29,34	22,19,24,23,18
	BU53	27,32,29,36,33	29,24,27,31,33	23,21,18,24,17

Table 5.2. Effect of function blocking monclonal anti-CD147 (EMMPRIN) antibody (8G6), at 1.5 μl/ml on the depolarisation response of OA cultured chondrocytes to 0.33 Hz cyclical mechanical stimulation

		Membrane potential (-mV)		
Donor	Regagent	Resting	Antibody alone	Post MS
M/-(13983)	Nil	36,28,26,33,27	-	17,14,22,16,18
	8G6	27,31,36,29,26	29,27,29,28,32	29,32,27,26,35