INTEGRIN-MEDIATED MECHANOTRANSDUCTION IN HUMAN ARTICULAR CHONDROCYTES

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PhD THESIS

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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.

Herng-Sheng Lee

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Abbreviations Used in Thesis

APC	adenomatous polyposis coli
APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cGMP	cyclic guanosine monophosphate
cPLA2	cytoplasmic phospholipase A2
Csk	C-terminal Src kinase
DAG	diacyl glycerol
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorter
FAK	focal adhesion kinase
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Fgr	feline sarcoma virus (Strain Gardner-Rasheed)
GDP	guanosine diphosphate
G protein	guanosine triphosphate-binding protein
Grb	growth factor receptor-bound protein
GRGDSP	glycine-arginine-glycine-aspartic acid-serine-proline
GRADSP	glycine-arginine-alanine-aspartic acid-serine-proline
GTP	guanosine triphosphate

HRP	horse radish peroxidase
HSP	heat shock protein
HUVEC	human umbilical vein endothelial cell
Hz	hertz
IAP	integrin-associated protein
ICAM	intercellular adhesion molecule
IL	interleukin
ILK	integrin-linked kinase
INF	interferon
IP3	inositol 1,4,5-triphosphate
JAK	Janus tyrosine kinase
kD	kilo Dalton
LEF	lymphoid enhancer factor
mAb	monoclonal antibody
MAP	mitogen-activated protein
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
mSOS	mammalian homology of the Drosophila son of sevenless
NG2	human melanoma proteoglycan (HMPG)
OA	osteoarthritic
Pa	pascal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PGE2	prostaglandin E2
PI 3-kinase	phosphatidylinositol 3-kinase
PIP	phosphatidylinositol 4-phosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PIS	pressure-induced strain
PKC	protein kinase C
PLC	phospholipase C

PTB	phosphotyrosine binding
RACK	receptor for activated C-kinase
RGD	arginine-glycine-aspartic acid
RTPCR	reverse transcriptase-polymerase chain reaction
SAC	stretch-activated ion channels
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SH	Src homology
SK	small conductance calcium-activated potassium ion channels
Src	sarcoma oncogene
STAT	signal transducer and activator of transcription
SV40	simian virus 40
Syk	spleen tyrosine kinase
TBS	tris buffered saline
TBST	Tween20 in TBS
TEMED	N'N'N'N-tetramethyl ethylene diamino
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor
TRITC	tetramethyl rhodamine isothiocyanate
VCAM	vascular cell adhesion molecule
WD	tryptophan-aspartic acid

Publications Associated with This Thesis:

Original articles:

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Millward-Sadler SJ, Wright MO, **Lee HS**, Caldwell H, Nuki G, Salter DM 2000 Altered electrophysiological responses to mechanical stimulation and abnormal signalling through α5β1 integrin in chondrocytes from osteoarthritic cartilage. *Osteoarthritis Cartilage* 8:272-278.

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Abstracts:

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Lee HS, Millward-Sadler SJ, Wright MO, Nuki G, Salter DM 2000 β-catenin is tyrosine-phosphorylated via integrins in mechanically stimulated human articular chondrocytes. *J Pathol* 190(suppl):21A.

Robinson HE, Lin H, Millward-Sadler SJ, **Lee HS**, Salter DM, Wright MO, Goldring M, Nuki G 2000 Phosphorylation of p38 and JNK1 MAP kinases following cyclical strain in immortalized human articular chondrocytes. *Rheumatology* 39(suppl 1):115.

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Afsari F, Millward-Sadler SJ, **Lee HS**, Salter DM, Goldring M, Nuki G 2000 β-catenin and cadherin expression in SV-40 transformed human chondrocytes. *Rheumatology* 39(Suppl 1):116.

Robinson HE, Lin H, Lucking A, Millward-Sadler SJ, **Lee HS**, Salter DM, Wright MO, Goldring M, Nuki G 2000 Upregulation of both p38 and JNK1 MAP kinases in immortalized human articular chondrocytes following cyclical strain. *Bone* 26(suppl):22S.

Lee HS, Millward-Sadler SJ, Wright MO, Nuki G, Salter DM 1998 Tyrosine phosphorylation in articular chondrocytes is induced by mechanical stimulation. *J Pathol* 186(suppl):35A.

ABSTRACT

Mechanical forces influence chondrocyte metabolism and function. It has been shown that 0.33 Hz cyclical mechanical stimulation results in membrane hyperpolarisation of normal human articular chondrocytes by activation of Ca^{2+}-dependent K^{+} (SK) channels. Using pharmacological inhibitors of intracellular signalling molecules, stretch-activated ion channels (SAC) and specific antibodies against integrins, integrin-associated protein, cytokines and cytokine receptors the data show that membrane hyperpolarisation is the result of mechanically-induced secretion of IL-4. Secreted IL-4 binds to type II IL-4 receptors and stimulates a signal cascade involving PLC and PKC leading to activation of SK channels. Specific blockade of α5β1 integrins, SAC and tyrosine kinases block mechanically-induced membrane hyperpolarisation but have no effect on the hyperpolarisation of exogenously added IL-4 consistent with a role for these molecules upstream of IL-4 release in the mechanotransduction pathway.

Studies of OA chondrocytes demonstrate that unlike normal chondrocytes, these cells show a membrane depolarisation response to 0.33 Hz mechanical stimulation as a result of activation of tetrodotoxin-sensitive Na^{+} channels. The mechanotransduction pathway involves α5β1 integrins, SAC, tyrosine kinases, PLC, PI 3-kinase and cytokine secretion but the actin cytoskeleton and PKC, which are important in the membrane hyperpolarisation response in normal chondrocytes, are not necessary for membrane depolarisation in OA chondrocytes following mechanical stimulation.

The tyrosine phosphorylation events in the mechanotransduction pathway have been investigated in detail. The results show tyrosine phosphorylation of three major proteins, p125, p90, and p70 within 1 min of onset of mechanical stimulation. Immunoblotting and immunoprecipitation show these to be FAK, β-catenin, and paxillin, respectively. Tyrosine phosphorylation of all three proteins is inhibited by RGD containing oligopeptides and gadolinium, which is known to block SAC. β-catenin coimmunoprecipitates with FAK and is colocalized with α5 integrin and FAK. These results indicate a previously unrecognized role for an integrin-β-catenin signalling pathway in human articular chondrocyte responses to mechanical stimulation.

0.33 Hz cyclical mechanical stimulation also increases associations between RACK1/PKCα and β1 integrin in normal chondrocytes but not in OA chondrocytes. Those increased associations are prevented by incubation with the PKC inhibitor chelerythrine chloride and mechanotransduction via integrins is necessary for association between RACK1/PKCα and β1 integrin following 0.33 Hz mechanical stimulation.

Dysregulation of the mechanotransduction pathway may be important in osteoarthritis.

CHAPTER 1. INTRODUCTION

1.1 Normal Articular Cartilage

Articular cartilage enables low-friction and high-velocity movement between bones and protects the underlying bone against shearing and compressive forces. Normal articular cartilage is hyaline with a glassy appearance when cut. Mature hyaline cartilage is characterised by small aggregations of chondrocytes embedded in an amorphous matrix of ground substance reinforced by collagen fibers and is also devoid of vascular and nervous tissue and lymphatic vessels. No mitotic division occurs in healthy mature chondrocytes (Young and Heath 2000).

1.1.1 Organization of Articular Cartilage

Articular cartilage is composed of water (65-80%), collagen (10-30%), proteoglycans (5-10%) and chondrocytes (0.4-2%) (Walsh et al 1997). It can be subdivided (between the joint space and the subchondral bone) into a superficial zone (tangential zone), a middle zone (intermediate or transitional zone), a deep zone (the radial or radiate zone), and a calcified zone. Between the zones, there are differences in chondrocyte size and shape, collagen fibril orientation, water content, and proteoglycan content (Mitrovic et al 1983). The cells in the superficial zone are flattened and their density is highest. The collagen fibrils in this zone are arranged parallel to the articular surface. In the middle zone, the cells are rounded and surrounded by an extensive extracellular matrix (ECM). The cells of the deep zone are grouped in clusters and have the lowest cell volume. This zone has territorial, interterritorial, and pericellular regions. The ultrastructural organisation of these regions is different. The pericellular region consists of a high concentration of aggrecan, the proteoglycan decorin, and type VI collagen (Poole et al 1982). The boundary between the deep zone and the calcified zone is the tidemark. The calcified layer contains the hypertrophic chondrocyte. This layer could act as an important mechanical intermediate zone between the uncalcified

cartilage and the subchondral bone and could also provide a barrier to vascular invasion as a consequence of the continued presence of anti-angiogenic materials such as the tissue inhibitor of metalloproteinases (Moses et al 1990). In adult human articular cartilage, water content is maximal in the superficial zone and progressively declines with the depth from the articular surface. The water content is extremely important in maintaining its resiliency and providing a hydrodynamic lubrication system (Brocklehurst et al 1984).

1.1.2. Chondrocytes

Chondrocytes fully occupy the lacunae in the matrix, each lacuna containing a single chondrocyte. Mature chondrocytes are characterised by small nuclei with dispersed chromatin and basophilic, granular cytoplasm reflecting a well developed rough endoplasmic reticulum and abundant glycogen. These characteristics reflect the active role of chondrocytes in synthesis and maintenance of the cartilage matrix (Young and Heath 2000). The chondrocyte is subjected to repeated and abrupt force changes and modifies its metabolic function in response to such mechanical stimuli. For example, proteoglycan synthesis can be upregulated by compression force in a frequency-dependent manner (Urban 1994). In addition to responding to mechanical stimuli, chondrocyte metabolism may be regulated by a variety of cytokines and growth factors. For example, interleukin-1β (IL-1β) stimulates the generation of other proinflammatory factors by chondrocytes, including monocyte chemoattractants, nitric oxide and metalloproteases (Villiger et al 1992; Murrell et al 1995). Transforming growth factor-β (TGF-β) and insulin-like growth factor-I (IGF-I) stimulate chondrocyte differentiation and the synthesis of cartilage matrix components, such as proteoglycans and type II collagen (Peracchia et al 1991; Osborne et al 1989; Centrella et al 1988). These factors are also important in cartilage maintenance and repair in response to traumatic and inflammatory insults (Walsh et al 1997).

1.1.3. Cartilage Matrix

The major components of the ECM include highly cross-linked fibrils of triple helical type II collagen molecules that interact with other cartilage-specific collagens, including collagen types IX and XI, the large aggregating proteoglycan aggrecan, small proteoglycans, and other collagenous and noncollagenous matrix proteins (**Table 1.1**) (Mayne and Brewton 1993; Poole 1997; Goldring 2000). Collagen is the major matrix structural component of articular cartilage, where it forms a fibrillar network that is found throughout the entire ECM (Ross et al 1995; Young and Heath 2000). Collagen fibril provides the strength of the tissue by resisting shear and tensile forces (Kempson et al 1973). Although collagen types VI, IX, XI, XII and XIV are minor components, they appear to have important structural and functional properties (Goldring 2000). Human aggrecan structure is composed of a core protein with covalently attached glycosaminoglycan side chains of chondroitin sulfate and keratan sulfate (Doege et al 1991). Link protein stabilises a noncovalent interaction between aggrecan and polymeric hyaluronic acid through the G1 globular domain of the core protein to form a large macromolecular aggregate (**Fig. 1.1**) (Heinegard and Hascall 1974; Poole et al 1980; Paulsson et al 1987; Morgelin et al 1988; Fosang and Hardingham 1989; Doege et al 1987, 1991; Hardingham and Fosang 1992). The glycosaminoglycan molecules consist of long-chain, unbranched, repeating dimeric polysaccharides containing both carboxyl and sulfate groups to provide a highly negatively charged aggregate (Muir and Hardingham 1975; Hardingham et al 1986; Hounsell et al 1986; Phillips et al 1990). This high fixed charged density can create a highly hydrated matrix in cartilage, but hydration and swelling is limited by the collagen fibrillar network (Maroudas 1980; Poole 1986). This property endows cartilage with its compressive stiffness and ability to resist deformation and dissipate load (Poole 1997).

Several other matrix proteins are not unique to cartilage, but have important roles in cartilage structure and function. Biglycan, decorin, fibromodulin and lumican are small leucine-rich proteoglycans. They help to maintain cartilage structure by

interacting with the collagen network, contributing to the fixed charge density and binding growth factors such as TGF-β (Heinegard et al 1999). Noncollagenous proteins also play a role in cartilage. For example, fibronectin is distributed throughout the matrix (Burton-Wurster et al 1988) and promotes attachment of chondrocytes via RGD sequence (Hakomori et al 1984). Chondroadherin and proline/arginine-rich end leucine-rich repeat protein (PRELP) are also leucine-rich proteins and may be involved in mediating interaction between the chondrocyte and its pericellular matrix by binding to the chondrocyte through membrane proteins such as syndecan and integrin (Akesson 1999).

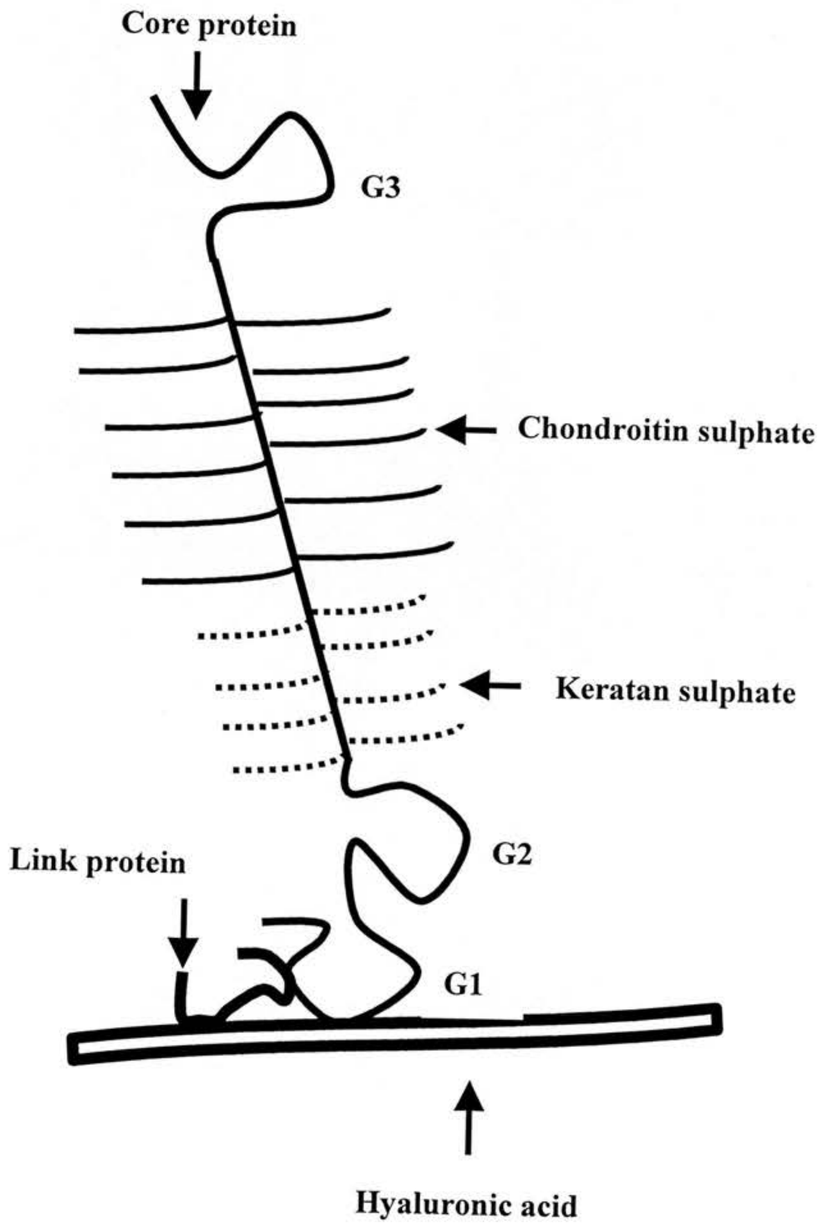
1.1.4. Chondrocyte-Extracellular Matrix Interaction

Interactions between chondrocytes and ECM are important in the regulation of cartilage function including cell anchorage, matrix biosynthesis and matrix degradation (Benya and Shaffer 1982; Lee et al 1993). Cell adhesion molecules are essential mediators in chondrocyte-ECM interactions. Human articular chondrocytes are known to express a range of cell adhesion molecules such as integrins (Woods et al 1994; Salter et al 1992, 1995), CD44 (Salter et al 1996a), intercellular adhesion molecule 1 (ICAM-1) (Davies et al 1991), syndecan (Barre et al 2000) and NG-2 (Midwood and Salter 1998). These molecules orchestrate the multiple and complex adhesive interactions in cartilage. For example, the integrin family can engage in both 'inside-out' and 'outside-in' biochemical signal transduction (Giancotti and Ruoslahti 1999) and also act as the mechanotransducers which transduce the mechanical signalling into biochemical events (Ingber 1991; Wright et al 1997; Salter et al 1997).

Table 1.1 Cartilage matrix proteins

Collagens	
	Type II, VI, IX, X, XI, XII, XIV
Proteoglycans	
	Aggrecan
	Biglycan
	Decorin
	Fibromodulin
	Lumican
Noncollagenous proteins	
	Fibronectin
	Chondroadherin
	Proline/arginine-rich end leucine-rich repeat protein
	Cartilage matrix protein (matrilin 1)
	Anchorin II
	Tenascin
	Thrombomodulin
	Cartilage oligomeric matrix protein
Membrane proteins	
	Syndecan
	Integrins
	CD44
	Intercellular adhesion molecule I
	NG2

Fig. 1.1 Diagrammatic structures of the large proteoglycan aggrecan and link protein, which bind to each other and to hyaluronic acid. Core protein forms three globular domains G1, G2 and G3 (Paulsson et al 1987; Morgelin et al 1988).



1.2. Osteoarthritis

Osteoarthritis (OA) is the most common of the various diseases of articular joints which affect humans (Lawrence et al 1966; Gordon 1968). Because of its high frequency, OA contributes greatly to the cost of health care for ageing populations. The etiopathologic processes involved are very complex and include the effects of ageing, exercise, joint disuse, abnormal mechanical effects, cartilage metabolism, proteases, cytokines, growth factors, etc (Howell et al 1992). Osteoarthritis usually affects large weight-bearing joints and is classified as primary (idiopathic) or secondary (Altman et al 1986). In the absence of any known underlying predisposing factor the disease is classified as primary OA. Secondary OA has clearly defined etiological factors contributing to its development. These conditions include trauma, systemic metabolic disease (e.g. Wilson's disease, ochronosis), endocrine disorders (e.g. hyperparathyroidism, diabetes mellitus, acromegaly), calcium crystal deposition diseases, neuropathic disorders, bone dysplasia, other joint disorders (e.g. avascular necrosis, infection etc) (Moskowitz 1992). The cartilage changes in OA consist of a slowly progressive loss of articular cartilage that is associated biochemically with increased water content, alteration in the collagen fiber network and loss of proteoglycan.

1.2.1 The Macro and Microscopic Changes in Cartilage

There are three patterns of macroscopic alteration involving the cartilage surface and to a variable degree, the underlying cartilage tissue: fibrillation, erosion and cracking (Bullough 1992). A replacement of the normally smooth, shiny surface by an appearance similar to that of cut velvet is called fibrillation (Byers et al 1970). Cartilage erosion is solution of the surface and is characteristic of progressive degenerative change in the joint. Erosion of damaged cartilage tissue may be so extensive as to completely denude the bone surface of its cartilaginous cover, which is called eburnation. Cracking extends vertically deep into the cartilage and microscopically is often found to have a deep horizontal component.

The most widely used system for gross pathological grading of OA is that proposed by Collins and McElligott who recommended the use of four grades of severity (Collins 1949; Collins and McElligott 1960). A widely used system for microscopic surveys has been described by Mankin et al (1971). This histological/histochemical grading system consists of an index ranging from 0 (normal) to 14 (advanced OA). The categories described include structure, cells, Safranin O staining and tidemark integrity (**Table 1.2**). Both macroscopic and microscopic assessments have been suggested to be used in conjunction with one another to allow accurate assessment of osteoarthritic changes in a joint (Ostergaard et al 1997a).

1.2.2 Cartilage Matrix in Osteoarthritis

Osteoarthritis involves cartilage breakdown and progressive destruction of the joint (Gardner 1992). Both chondrocytes and ECM are affected. Protease activity increases, along with a downregulation in the levels of protease inhibitors (Blanco 1999). The extracellular matrix network is, therefore, disorganised and macromolecules are degraded including type II collagen and proteoglycans (Poole 1997). Type II collagen degradation is initially pronounced in the superficial zone and spreads to the middle and deep zones with increasing severity of OA (Pelletier et al 1983). Remodelling by new collagen synthesis occurs; however, its overall tensile strength is reduced. Proteoglycan change also occurs initially at the articular surface, the proteoglycans being chemically different and more swollen (Mankin and Lippiello 1970; Muir 1977; Thompson and Oegema 1979). In advanced OA, the proteoglycans demonstrable by cationic dyes such as safranin O and alcian blue are considerably reduced (Mankin et al 1971). Other changes which have been described in matrix components include increased synthesis of fibronectin (Burton-Wurster et al 1986), type VI collagen (Swoboda et al 1998) and tenascin (Salter 1993).

Table 1.2 The histological/histochemical grading system for osteoarthritic cartilage (Mankin et al 1971; Ostergaard et al 1997a)

Category	Subcategory	Score
Structure		
	Normal	0
	Surface irregularities	1
	Pannus and surface irregularities	2
	Clefts to middle zone	3
	Clefts to deep zone	4
	Clefts to calcified zone	5
	Complete disorganization	6
Cells		
	Normal	0
	Diffuse hypercellularity	1
	Cloning	2
	Hypocellularity	3
Safranin O staining		
	Normal	0
	Slight reduction	1
	Moderate reduction	2
	Severe reduction	3
	No dye noted	4
Tidemark integrity		
	Intact	0
	Crossed by blood vessels	1
Total		0-14

1.2.3 Chondrocytes from Osteoarthritic Cartilage

In early OA, the chondrocyte exhibits a transient proliferative response, increased synthesis of cartilage matrix as an early attempt at repair, and increased synthesis of catabolic cytokines and matrix-degrading enzymes (Goldring 2000). The chondrocyte population increases both in size and number with increased rates of DNA synthesis (Poole 1997). Increased synthetic functions of type II collagen and aggrecan occur in early osteoarthritic chondrocytes, but decreased synthetic functions in late OA (Mankin and Lippiello 1970; Thompson and Oegema 1979; Schwartz et al 1981; Teshima et al 1983; Sandy et al 1984; Brocklehurst et al 1984; Poole et al 1991). The enzymes collagenase and aggrecanase which cause the degradation of cartilage collagens and proteoglycans in OA are also increased (Billinghurst et al 1997; Lark et al 1997; Lohmander 1999). The principal cytokines linked to the catabolism of cartilage and to the OA process are IL-1 and tumour necrosis factor (TNF)-α (Lotz et al 1994). Both cytokines are overproduced by osteoarthritic chondrocytes (Towle et al 1997; Webb et al 1997). Increased expression of the IL-1 receptor type I has been detected in osteoarthritic chondrocytes in association with increased matrix metalloproteinase (MMP) production (Martel-Pelletier et al 1992). Expression of the p55 TNF receptor has been localised in chondrocytes at sites of focal loss of cartilage proteoglycans (Webb et al 1997).

Expression of cell adhesion molecules by chondrocytes is also changed in OA. For example, CD44 is upregulated in deep zone OA chondrocytes, compared with normal cartilage (Ostergaard et al 1997b). In vivo and in vitro studies have shown that integrin β1 expression is increased in OA cartilage (Loeser et al 1995). Aberrant expression of integrin α2, α4, and β2, which are not expressed in normal cartilage, is also documented in OA cartilage (Ostergaard et al 1998). These studies indicate that normal chondrocyte-ECM interactions may be perturbed in OA cartilage, and these may play a role in the onset or progression of osteoarthritis.

1.3. Mechanical Forces and Articular Cartilage

1.3.1. Terms Used in Mechanical Studies (Armstrong et al 1992; Woo et al 1992)

In physics, a **force** is the pulling, attracting, or pushing effect that something has on something else (Sinclair et al 1995).

Stress is a measure of the force per unit area acting on a given surface.

Tensile stress causes an increase in length of a body.

Compressive stress causes a reduction in length of a body.

Shear stress results when the applied force lies in the plane of the surface.

The unit of stress is the Newton (N)/m^2 or Pascal (Pa). 1 kPa is equal to 10^3 N/m^2 or 7.5 mmHg.

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 the 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.

The **mechanical properties** of a material include how stiff the material is (how much does it deform under a given stress) and how strong it is (how much stress can the material support before it permanently deforms).

1.3.2. Mechanical Stress of Articular Cartilage

Articular cartilage is exposed to large mechanical forces during joint movement. Mechanical stress has been measured in an instrumented hip prosthesis of a 74-year-old female (Hodge et al 1986). Rising from a chair, pressures in the hip joint cartilage can reach nearly 20 MPa (200 atm) and during walking, pressures cycle

between atmospheric and 3-4 MPa (30-40 atm) at a frequency of around 1 Hz. During walking or running, forces on the joint surface may vary from near zero to several times the whole body weight within a period of 1 second (Seireg and Arvikar 1975; Paul 1980). Loading of articular cartilage will generate a combination of tensile, compressive and shear stress in the material. The tensile modulus (stiffness) of healthy human articular cartilage varies from 5-25 MPa, depending on the site of movement on the joint surface (i.e., high or low weight bearing regions), and the depth and orientation of the test specimen relative to the joint surface (Kempson et al 1973; Akizuki et al 1986). The compressive modulus also has been measured in healthy human articular cartilage. Values vary from 0.4-2.0 MPa (Armstrong and Mow 1982; Athanasiou et al 1994). Articular cartilage responds to shearing forces by both stretching and deformation of the solid matrix. The dynamic shear modulus (a measure of combined elastic and viscous effects when cartilage is subjected to dynamic shearing) are within the range of 0.2-2.0 MPa for healthy bovine or canine cartilage (Zhu et al 1993; Setton et al 1995; Simon et al 1989; Spirt et al 1989). Physiological stress is an important regulator of cartilage metabolism and integrity. Mechanical loading serves to maintain fluid flow and ion phase function (Mow et al 1999).

In osteoarthritis, the articular cartilage loses its mechanical function. The tensile modulus has been shown to decrease by as much as 90%, reflecting damage to the cartilage matrix network (Akizuki et al 1986). The compressive modulus also decreases with increasing severity of degeneration as assessed using a histochemical grading scheme or India-ink staining methods (Armstrong and Mow 1982). Other joint tissues (e.g., anterior cruciate ligament) also undergo similar changes of tensile and compressive modulus in an experimental OA model (Setton et al 1999).

Abnormal mechanical loading of joints results in a variety of changes in chondrocyte activity and matrix integrity which lead to cartilage breakdown and the development of OA. Animal studies, for example, have shown that the tensile

modulus of canine knee articular cartilage was reduced after one month of immobilisation (Setton et al 1990). In the dog, severe OA lesions in the knee joint has been produced by treadmill exercise after the limb was immobilised for several weeks (Palmoski and Brandt 1981). These findings suggest that abnormal mechanical forces play a role in the pathogenesis of osteoarthritis.

1.3.3. Articular Cartilage Responses to Mechanical Loading *in vivo*

At the beginning of the last century, the effects of mechanical load bearing on the development and microscopic structure of the articular cartilage in the guinea-pig shoulder joint has been investigated as reported by Helminen et al (1987). Elevated loading of the cartilage increased cartilage thickness, caused hypertrophy of the superficial chondrocytes, and increased intercellular matrix in thickness (Jurvelin et al 1985; Eggli et al 1988; Kiviranta et al 1988; Guilak and Bachrach 1993). The subchondral bone also increased in thickness with load bearing (Radin et al 1984). In normal human joints, load-bearing areas of the cartilage are thicker with a higher proteoglycan concentration and are mechanically stronger than non-load-bearing regions of the same joint (Bjelle 1975; Roberts et al 1986; Slowman and Brandt 1986). Increased weight-bearing of joints in animal models including canine, rabbit, and sheep, leads to an increase of the proteoglycans of articular cartilage (Palmoski et al 1979; Muir and Carney 1987; Kiviranta et al 1988; Eggli et al 1988; Caterson and Lowther 1978). However, non-load-bearing cartilage shows a reduction in proteoglycans (Heilminen et al 1987). Palmoski et al (1979) found that immobilisation of a joint by placing the dog's leg in a cast, leads to cartilage atrophy, loss of Safranin O staining, and a decrease in its uronic acid content. These changes were reversible on remobilization.

Abnormal mechanical loading is associated with osteoarthritis (Woo et al 1992). Most animal models of OA are mechanically induced, for example, by introducing joint instability by anterior cruciate ligament section (Muir and Carney 1987) or by

altering the loading across the joint by menisectomy (Hoch et al 1983). These changes in joint loading affect cartilage structure and chondrocyte activity within days of the procedure, and may eventually result in complete loss of cartilage (Burton-Wurster et al 1993).

These in vivo studies show that increased or decreased functional stress affects the biological properties of cartilage. However in vivo systems are too complicated to study the mechanotransduction pathways involved in the response in any detail: the level of loading over the joint varies and is not reproducibly controlled, growth and ageing complicate interpretation and the whole body effect of exercise on circulating factors (e.g. hormones, cytokines) may affect the cartilage response (Urban 1994). Accordingly, there has been an increasing interest in *in vitro* studies. In vitro experiments allow the study of cellular events involved in the perception of the mechanical stimulus, the intracellular events evoked by mechanical stimuli, and a detailed analysis of the change in matrix components.

1.3.4. Cartilage and Chondrocyte Responses to Mechanical Loading *in vitro*

Articular cartilage is exposed to large mechanical forces during joint movement. For example, peak resultant forces across the human hip and knee joints have been shown to reach 4 and 7 times the body weight, respectively, during normal walking (Seireg and Arvikar 1975; Paul 1980). In vivo, mechanical loading is applied cyclically and chondrocytes are exposed to a composite of radial, tangential and shear stresses (van Kampen and van de Stadt 1987). In vitro systems accordingly are designed to study these effects to gain more insight into the cellular or molecular events evoked by such stimuli.

1.3.4.1. Hydrostatic or Orientated Force

Rodan et al (1975a) studied the effects of application of compressive forces (80 g/cm^2) to chick tibial epiphyses (16-day-old embryos) in culture and found that glucose consumption reduced to 50% of controls. Twenty four hours after the release of pressure, glucose utilisation again increased, approaching control levels. The same pressure also stimulated thymidine incorporation into DNA. They also found that exposing chick tibial epiphyses to continuous compressive forces (60 g/cm^2, equal to 5.865 kPa) caused a reduction of both cAMP and cGMP (Rodan et al 1975b). An equivalent hydrostatic pressure applied directly to cells isolated from chick tibial epiphyses also affects cyclic nucleotide accumulation (Rodan et al 1975b). The tissue response was uniform throughout the epiphysis, whereas the cell response varied according to the area of origin. Veldhuijen et al (1979) constructed an apparatus that exposed cultured monolayer chondrocytes on the walls of tissue culture tubes to intermittent compressive forces of 12.8 kPa for 6 hours at a frequency of 0.3 Hz. Contrary to the effect of continuous compressive forces, intermittent compressive forces caused a rise in levels of cAMP and a reduction in DNA synthesis. Palmoski and Brandt (1984) studied the effects of both static and intermittent mechanical stress on full-thickness plugs of canine articular cartilage. When the plugs were exposed to compressive force using a regime of 60 sec on/60 sec off, glycosaminoglycan synthesis was reduced to 30-60% of controls. However, when a regime of 4 sec on/11 sec off was employed, the glycosaminoglycan synthesis increased by 34%, although protein synthesis and contents of DNA, uronic acid, and water remained unaltered. This suggested that different frequencies of cyclical strain produce differences in metabolic activity within the cells.

Effects of applied hydrostatic pressure on the transmembrane potentials of human articular chondrocytes have also been investigated (Wright et al 1992, 1996, 1997). Cyclic mechanical strain (0.33 Hz, 120 mmHg for 20 min) caused chondrocyte cell membrane hyperpolarization, whilst continuous stimulation (120 mmHg for 20 min) caused depolarization (Wright et al 1992). The

hyperpolarisation pathway has been shown to involve stretch-activated membrane ion channels, integrin receptors, a range of intracellular signalling molecules including tyrosine kinases, the actin cytoskeleton, phospholipase C, protein kinase C and autocrine/paracrine cytokine secretion (Wright et al 1996, 1997; Millward-Sadler et al 1999).

1.3.4.2. Cell Stretch

Another model designed to test the effects of mechanical force on chondrocytes in vitro involves the use of apparatuses designed to produce cell stretch. The majority of methods employed involve deformation of a cell-laden, flexible membrane and can be categorised according to (1) the method of deformation of the membrane-by control of either the displacement or the force, and (2) the shape and mounting of the deformable membrane-either a circular membrane held at its periphery or a rectangular strip held at the two ends (Schaffer et al 1994). The devices utilised in the production of the displacement include:

(a) a vacuum driven diaphragm (silicone elastomer membrane, 2.5 mm in thickness) (Banes et al 1985; Gilbert et al 1989),
(b) pin shaped displacement (silicone elastomer membrane, 0.254 mm in thickness) (Vandenburgh 1988),
(c) glass dome displacement (polytetrafluoroethylene membrane, 0.025 mm in thickness) (Hasegawa et al 1985; Andersen and Norton 1991),
(d) air or fluid displacement (polyurethane membrane, 0.094 mm in thickness) (Belloli et al 1991; Brighton et al 1991; Williams et al 1992), and
(e) a circular groove displacement (silicone elastomer membrane, 0.076 mm in thickness; polyurethane membrane, 0.094 mm in thickness) (Schaffer et al 1993; Hung and Williams 1994).

The Flexercell^{TM} strain unit (Banes et al 1985) consists of a computer-controlled vacuum unit and a baseplate on which are held the culture dishes. These dishes have a flexible base. A vacuum is applied to the dishes via the baseplate. When a

precise vacuum level is applied to the system, the bases of the culture plates are deformed by known percentage elongation that is maximal at the edge of the culture dish, but decreases towards the centre. Using the system, straining the base of a culture dish leads to strain of the attached cultured cells. When the vacuum is released, the bases of the dishes return to their original conformation.

In experiments utilising cell stretch, it has been shown that by stretching a supportive flexible membrane on which chondrocytes were cultured, Lee et al. (1982) found that a cyclic 10% mechanical stretch for 8 hours increased glycosaminoglycan synthesis and decreased protein and collagen synthesis. DeWitt et al. (1984) showed increased radiosulphate and ^{14}C-glucosamine incorporation into glycosaminoglycans by chick epiphyseal chondrocytes in high density cultures subjected to a 5.5% strain at a frequency of 0.2 Hz. Protein synthesis after 24 hours mechanical strain remained unchanged. Using the Flexercell^{TM} strain system, Fujisawa et al. (1999) investigated the influence of cyclic tension force on the metabolism of cultured chondrocytes. Two levels of force (5 kPa or 15 kPa) and three frequencies 30 cycles/min (1 sec on/1 sec off), 0.5 cycles/min (1 sec on/119 sec off) and 0.25 cycles/ min (1 sec on/239 sec off) were used. Both 5 and 15 kPa of high frequency cyclic mechanical stress for 48 hours significantly inhibited the syntheses of DNA, proteoglycan, collagen, and protein. The expression of interleukin-1, matrix metalloproteinase (MMP)-2 and MMP–9 mRNA were induced by 15 kPa of high frequency force. The production of pro- and active-MMP-9 (resulting in cartilage breakdown) were also increased at this pressure and frequency of stimulation. Reducing in the applied frequency decreased the inhibition of proteoglycan synthesis. Mechanical stretch producing 25% maximal elongation at a frequency of 0.05 Hz for 48 hours also induces the expression of high molecular weight heat shock protein (HSP105) in the human chondrocytic cell line CS-OKB (Chano et al 2000). These findings may suggest that the frequency of cyclic tension force is one of the key determinants of chondrocyte metabolism.

Differences in response of chondrocytes to mechanical stimulation have been found, using the Flexercell^{TM} strain unit. Holmvall et al. (1995) demonstrated that 18 kPa of stress at a frequency of 0.25 Hz increases the mRNA levels of ECM components such as type II collagen and aggrecan. However, Fujisawa et al (1999) showed no significant changes of the mRNA levels of type II collagen and aggrecan when 5 or 15 kPa force was applied at a frequency of 0.5 Hz. Furthermore, Fukuda et al. (1997) reported that 2 kPa of cyclic mechanical stress (0.003 Hz) increases proteoglycan synthesis, while 10 kPa of stress (0.17 Hz) decreases proteoglycan synthesis. Fujisawa et al (1999) showed that proteoglycan synthesis was significantly inhibited by cyclic tension force, regardless of the magnitude or frequency. These differences in results obtained may result from differences in the magnitude and frequency of the applied force, or the type of chondrocytes utilised in the studies e.g. chondrosarcoma cell lines or primary chondrocyte cultures.

Using confocal microscopy and fluorescent techniques, intracellular calcium ion concentration was monitored in isolated articular chondrocytes subjected to controlled deformation with the edge of a glass micropipette (Guilak et al 1999). Intracellular calcium ion concentration reached peak within 5 sec following 25% deformation of the cells and returned to baseline levels in 3-5 minutes. The immediate and transient increase of intracellular calcium waves was abolished by removing Ca^{2+} from the culture medium and was significantly reduced by the presence of gadolinium and amiloride, agents known to block mechanosensitive ion channels (Jorgensen and Ohmori 1988; Yang and Sachs 1989; Hamill et al 1992; Wright et al 1996). Inhibitors of intracellular Ca^{2+} release or agents known to cause cytoskeletal disruption including cytochalasin D and colchicine had no significant effect on the Ca^{2+} waves. The results indicate that mechanosensitive ion channels are upstream in the mechanotransduction pathway, which is consistent with the results obtained using electrophysiological parameters in the assessment of the cell response to cyclical mechanical strain (Wright et al 1996).

1.3.4.3. Fluid-Induced Shear Stress

The effects of fluid-induced shear stress on articular chondrocyte morphology and metabolism in vitro have been investigated by Smith et al. (1995). Fluid-induced shear stress (1.6 Pa = 16 dynes/cm^2) was applied by cone viscometer to both normal human and bovine articular chondrocytes. Shear stress for 48 and 72 hours caused individual chondrocytes to elongate and align tangential to the direction of cone rotation. Glycosaminoglycan synthesis was increased 2-fold. After 48 hours of shear stress, the release of prostaglandin E2 was increased 10-20-fold. In human articular chondrocytes, mRNA levels for tissue inhibitor of metalloproteinase (TIMP) increased 9-fold in response to shear stress compared to controls. In contrast, mRNA levels for the neutral metalloproteinases, collagenase, stromelysin, and gelatinase, did not show significant changes. These results indicate that articular chondrocyte metabolism responds directly to mechanical stimulation (Smith et al 1995).

In general, the evidence from in vitro experiments using cartilage tissue or cultured chondrocytes suggest that mechanical loading may be a major factor in control of articular cartilage matrix synthesis and cartilage homeostasis. A detailed analysis of the metabolic response to mechanical stress needs to be further investigated.

1.4. Integrins

1.4.1. Integrin Family and Structure

Integrins were first isolated, characterized, and sequenced from chick embryo fibroblast cDNA clones which encoded one subunit of the complex of membrane glycoproteins (Tamkun et al 1986). The name 'integrin' was proposed as the consequence of its role as an integral membrane complex involved in the transmembrane association between the ECM and the cytoskeleton. Integrins are a large family of α/β heterodimeric cell surface adhesion receptors that can bind a wide variety of ECM and cell surface ligands (**Fig. 1.2**) (Albelda and Buck 1990; Arnaout 1990; Hemler 1990; Springer 1990a, 1990b; Ruoslahti 1991; Aplin et al 1998). The α subunits (120-180 kD) are each noncovalently associated with a β subunit (90-110 kD) (Hynes 1992). Most integrins are expressed on a wide range of cells, and most cells express several integrins. Thus far, 17 α and 8 β subunits have been identified, which can associate in a restricted manner to form at least 23 different combinations (**Table 1.3**) (Curley et al 1999). Alternative splicing of the α and β subunits ($\alpha3$, $\alpha6$, $\alpha7$, $\beta1$, $\beta3$, and $\beta4$) adds additional complexity (Fornaro and Languino 1997). For example, four $\beta1$ isoforms with variant cytoplasmic domains have been described: $\beta1A$ (Argraves et al 1987), $\beta1B$ (Altruda et al 1990), $\beta1C$ (Languino and Ruoslahti 1992) and $\beta1D$ (van der Flier et al 1995; Zhidkova et al 1995; Belkin et al 1996). Each subunit is composed of a large extracellular domain (typically 1000-1150 amino acids for the α integrin and 740-780 amino acids for the β integrin), a single transmembrane domain of about 20 amino acids and a short cytoplasmic domain of 40-50 amino acids (Curley et al 1999). An exception to this is the $\beta4$ integrin which has an extended cytoplasmic domain of approximately 1000 amino acids (Suzuki and Naitoh 1990).

The α subunit has seven repeated sequences of 60-70 residues in its N-terminal (Springer 1997). The repeats have been predicted to fold into a β-propeller

structure containing the ligand binding site (such as I domain) and a cation binding site (Irie et al 1997; Mould et al 1998).

The β subunit, in the N-terminal region, has also been hypothesised to have an I-domain-like structure with cystine-rich repeats, containing sequences critical for ligand binding (Hynes 1992; Takada et al 1997). Integrin cytoplasmic domains provide one pathway linking integrin engagement with the cell's signal transduction apparatus (Jockusch et al 1995; Laflamme et al 1997).

Most integrins bind ligands that are components of ECM, e.g. fibronectin, collagen, and vitronectin (Humphries and Newham 1998); certain integrins can bind to soluble ligands (such as fibrinogen) or to counter receptors (such as intercellular adhesion molecules) on adjacent cells, leading to homo- or heterotypic aggregation (Kuhn and Eble 1994; Gahmberg et al 1998). Some of the integrin recognition sites in the ligands and counter receptors have been defined (Komoriya et al 1991; Newham et al 1997). The first binding site to be defined was the Arg-Gly-Asp (RGD) containing sequence present in fibronectin, vitronectin, and a variety of other adhesive proteins (Pierschbacher and Ruoslahti 1984; Hynes 1992)

Fig. 1.2 Structural features of integrin receptors (Hynes 1992).

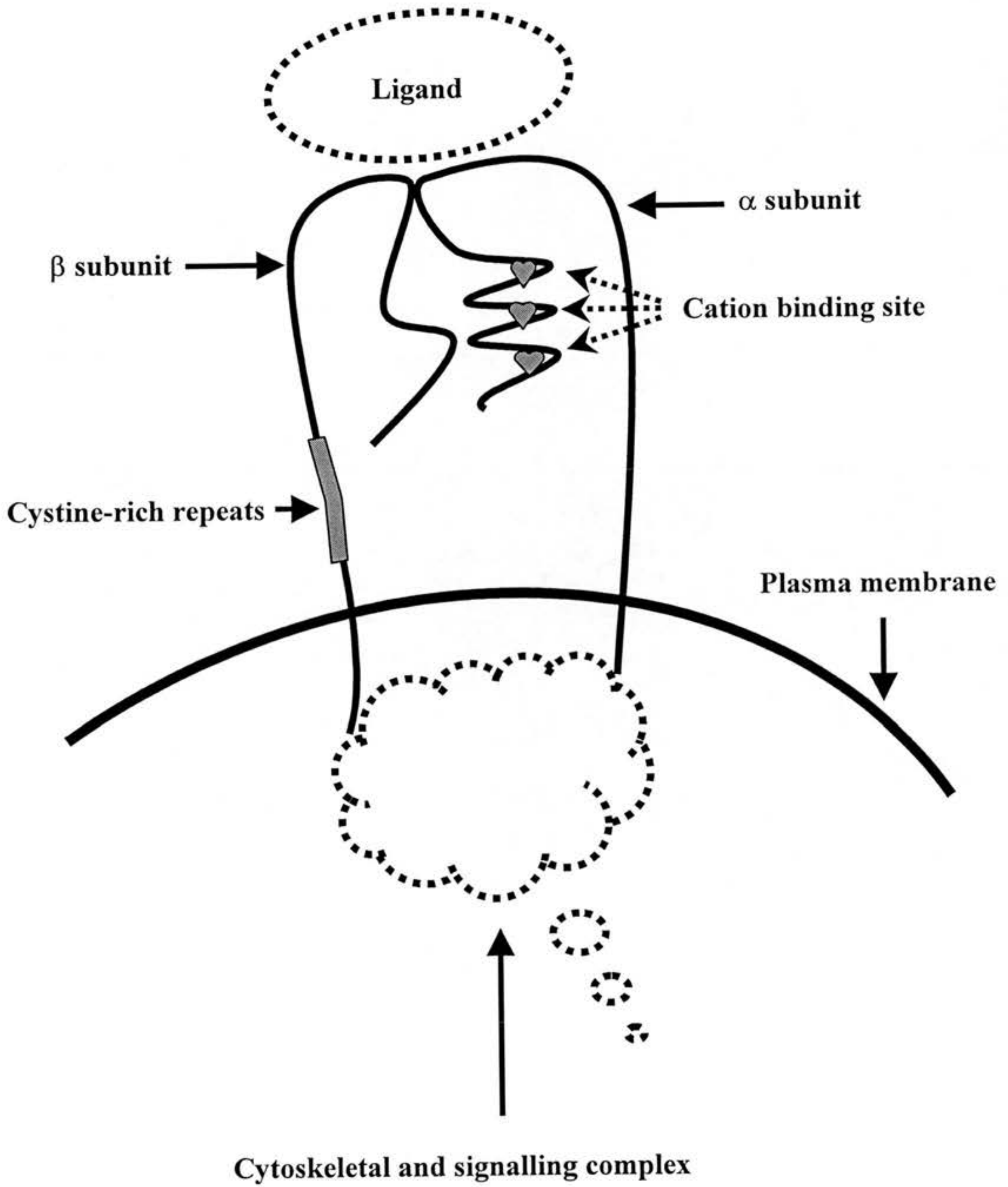


Table 1.3 Integrin subunits and combinations

	β1 (CD29)	β2 (CD18)	β3 (CD61)	β4 (CD104)	β5	β6	β7	β8
α1 (CD49a)	α1β1							
α2 (CD49b)	α2β1							
α3 (CD49c)	α3β1							
α4 (CD49d)	α4β1						α4β7	
α5 (CD49e)	α5β1							
α6 (CD49f)	α6β1			α6β4				
α7	α7β1							
α8	α8β1							
α9	α9β1							
α10	α10β1							
αV (CD51)	αVβ1		αVβ3		αVβ5	αVβ6		αVβ8
αIIb (CD41)			αIIbβ3					
αL (CD11a)		αLβ2						
αM (CD11b)		αMβ2						
αX (CD11c)		αXβ2						
αD (CD11d)		αDβ2						
αE (CD103)							αEβ7	

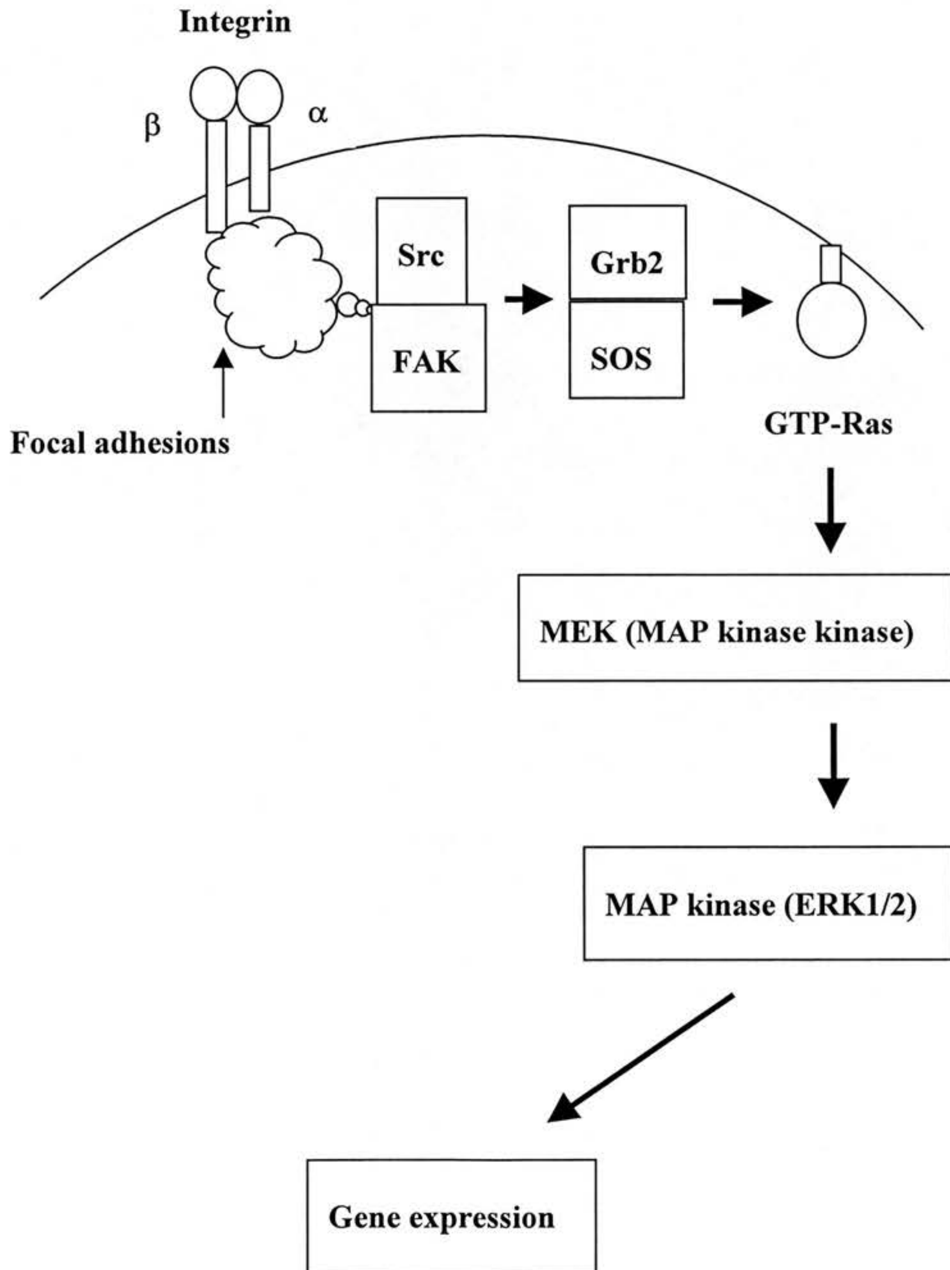
1.4.2. Integrin Function and Signalling

In cell types other than chondrocytes, integrins have been shown to play a role in the regulation of many cell functions, including embryonic development, maintenance of tissue architecture, differentiation, proliferation, programmed cell death, tumour metastasis, angiogenesis, haemostasis, and the response of cells to mechanical stress (Solursh 1989; Hynes 1992; Howe et al 1998; Frisch and Ruoslahti 1997; Giancotti 1997; Keely et al 1998; Clezardin 1998; Eliceiri and Cheresh 1999; Clemetson and Clemetson 1998; Ingber 1991; Shyy and Chien 1997). 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). Experiments using mutant or chimeric integrin structure have indicated that the α cytoplasmic domains regulate the specificity of the ligand-dependent interactions, whereas the β cytoplasmic domains are necessary and sufficient to target integrins to focal adhesions in a ligand-independent manner (Laflamme et al 1997). The assembly of focal contacts involves integrin engagement and interactions of focal adhesion proteins including α-actinin, talin, vinculin, tensin, paxillin, focal adhesion kinase (O'toole 1997; Guan 1997; Yamada 1997). The functional focal contacts play an important role in modulating cell adhesion and regulating changes in cell morphology and spreading. Focal contacts also serve as a framework for the association of signalling proteins to regulate cell behaviour (Clark and Brugge 1995).

Integrin-mediated signaling pathways can involve protein kinases, SH2-SH3 signaling molecules, small molecular weight GTPases, and phospholipid mediators (Kapron-Bras et al 1993; Schlaepfer et al 1994; Chen and Guan 1994; Chen et al 1994; Cohen et al 1995; Clark and Brugge 1995). Protein phosphorylation is amongst one of the earliest events occurring following integrin activation and involves protein tyrosine kinases or serine/threonine kinases (Schlaepfer and Hunter 1998). The tyrosine kinases which are activated include FAK, Src, Fgr, Csk, and Syk, these being activated by different integrins and are

cell type dependent (Kaplan et al 1994; Schaller and Parsons 1994; Sabe et al 1994; Lowell and Berton 1999; Tohyama et al 1994; Clark et al 1994). For example, FAK is a non-receptor protein tyrosine kinase that localizes to focal adhesions through a focal adhesion targeting domain and is activated by $\beta1$, $\beta2$, and $\beta3$ integrins (Hildebrand et al 1993; Lukashev et al 1994; Clark and Brugge 1995). The major serine/threonine kinases are PKC and MAP kinases (Vuori and Ruoslahti 1993; Chen et al 1994; Kolanus and Seed 1997; Howe et al 1998; Aplin et al 1998). PKC can also localize to focal adhesions (Jaken et al 1989; Woods and Couchman 1992) and is activated by phospholipid metabolites and calcium (Berridge 1987; Michell 1992). MAP kinase activation can be regulated by integrin (Chen et al 1994; Schlaepfer et al 1994). Crk and Grb2 are SH2-SH3 containing adapter proteins (Kapron-Bras et al 1993; Schlaepfer et al 1994) and PI-3 kinase and PLC are associated with second messenger activation (Chen and Guan 1994). Ras, Rho and mSOS are the representatives of small molecular weight GTPases that hydrolyze GTP into GDP (Keely et al 1998). Phospholipid mediators which can be activated include PIP-5 kinase, phospholipase A2 and 5-lipoxygenase (Clark and Brugge 1995). Other consequences of integrin activation include changes in intracellular calcium concentration, cytoskeletal organization and gene expression (Clark and Brugge 1995; Hering 1999; Schlaepfer and Hunter 1998). The integrin-mediated signalling pathways, in reality, probably involve numerous signalling molecules that converge on and diverge from the different pathways (Clark and Brugge 1995; Giancotti and Ruoslahti 1999). A single linear pathway of MAP kinase activation, however, is summarised to elucidate the signalling road taken (**Fig. 1.3**) (Clark and Brugge 1995; Robinson and Cobb 1997; Giancotti and Ruoslahti 1999).

Fig. 1.3 Integrin-dependent activation of the MAP kinase pathway (Clark and Brugge 1995; Robinson and Cobb 1997; Giancotti and Ruoslahti 1999).



Integrins can also interact with a variety of other transmembrane molecules including integrin-associated protein (IAP or CD47), transmembrane-4 superfamily (TM4 proteins), growth-factor receptors, glycosylphosphatidylinositol (GPI)-linked receptors, and caveolin (Yamada 1997; Porter and Hogg 1998). These complexes of integrins and partner receptors can be formed either in response to or independent of integrin activation and ligation. For example, IAP has been shown to bind to $\alpha V\beta 3$, $\alpha IIb\beta 3$, and $\alpha 2\beta 1$ (Gao et al 1996; Linderberg et al 1996; Chung et al 1997; Wang and Frazier 1998). IAP is a 50 kD single-chain protein composed of an extracellular immunoglobulin superfamily domain, five membrane-spanning sequences and a short cytoplasmic tail (Brown et al 1990; Rosales et al 1992; Linderberg et al 1993; Mawby et al 1994). Leukocyte CD31 engages IAP-$\alpha V\beta 3$ on the vascular endothelium and causes a Ca^{2+} influx, which then leads to endothelial retraction and loosening of the tight junctions to allow leukocyte migration (Cooper et al 1995; Imhof et al 1997; Schwartz et al 1993). Integrin-membrane protein complexes may therefore be the functional units to regulate or mediate integrin functions.

1.4.3. Integrin Expression by Human Chondrocytes

Integrin expression by human chondrocytes has been investigated utilising several techniques including immunohistochemistry, flow cytometry, immunoprecipitation, and northern blotting (Salter et al 1992; Woods et al 1994; Camper et al 1998). Cells from normal, osteoarthritic, tumour, fetal and adult tissue have been studied (Salter et al 1992; Woods et al 1994; Lapadula et al 1997; Tuckwell et al 1994; Durr et al 1993; Salter et al 1995). Most of the results obtained have given consistent findings, but some investigations have yielded conflicting results especially in the case of α chain integrins ($\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 6$) (Enomoto et al 1993; Durr et al 1993; Salter et al 1992, 1995). This has been attributed to differences in the methods used, i.e. immunohistochemical staining vs flow cytometry analysis (tissue sections vs isolated cells) (Lapadula et al 1997). The different observation of integrin expression by isolated chondrocytes or tissue

sections could be interpreted either as (1) an insufficient demasking of detected antigens in intact cartilage or (2) the absence of any integrin-mediated ECM interaction in native articular cartilage, for example, expression of the α2 integrin in the isolated chondrocytes but not in the native articular cartilage (Woods et al 1994; Lapadula et al 1997).

1.4.3.1. Integrin Expression by Normal Adult Human Chondrocytes

Salter et al (1992), using immunohistochemical staining of frozen sections of normal adult human articular cartilage, demonstrated that this tissue showed strong staining of α5β1 integrin. α1 and α3 integrin subunit expression was weak and variable. Chondrocytes did not express α2, α4, β2, and β3 integrin subunits. Using in situ immunohistochemical techniques, indirect immunofluorescence, flow cytometry and immunoprecipitation/SDS-PAGE methods, Woods et al (1994) undertook a detailed characterization of integrin expression by normal human articular chondrocytes including cryosectioned cartilage and collagenase-released chondrocytes. Monoclonal antibodies used included α1-α6, αV, β1, β3, β5 integrin subunits and heterodimeric αVβ3 and αVβ5 integrins. The results obtained using frozen sections and immunohistochemistry showed strong staining for β1, α5, αV, αVβ5, αVβ3, but negligible staining for α1, α2, α3, α4, α6, β2, and β3. The results obtained using isolated chondrocytes which were incubated overnight before being examined by flow cytometry, showed staining for β1, α5, αV, αVβ5, and α1 (the presence of the α1 subunit had not been detected in the frozen section), but no staining for α2, α4, α6, β2, β3, β5, and αVβ3 (αVβ3 was detected in the frozen section). The strength of staining for the α3 subunit varied between experiments. The results obtained using the immunoprecipitation/SDS-PAGE technique demonstrated the same types of integrin subunits observed using the flow cytometry technique. These findings established that normal articular chondrocytes strongly express the α1β1, α5β1, and αVβ5 integrin heterodimers,

and that lower levels of the $\alpha3\beta1$ and $\alpha V\beta3$ heterodimers are also present (Woods et al 1994).

Loeser et al (2000), using flow cytometry, demonstrated that primary chondrocytes (7 days culture) expressed $\alpha1$, $\alpha3$, $\alpha5$, and $\beta1$ integrin subunits, but no expression of $\alpha2$ and $\alpha4$ subunits. Yonezawa et al (1996), also using flow cytometry, showed that primary chondrocytes (overnight culture) expressed a high level of $\alpha5$ and $\beta1$ integrin subunits, low levels of $\alpha1$, $\alpha2$, and $\alpha3$, but no $\alpha4$, $\alpha6$, and $\beta3$ subunits. Expression of $\alpha2$ integrin was inconsistent. Laminin (the receptor for $\alpha6\beta1$ integrin) showed positive staining in sections of adult articular cartilage (Durr et al 1996). Antibodies against both laminin and $\alpha6\beta1$ integrin also revealed a strong pericellular reaction in sections of fetal or newborn epiphyseal cartilage (Durr et al 1996; Salter et al 1995). Using northern blot, immunoprecipitation and immunohistochemical methods human chondrocytes have also been shown to express $\alpha10\beta1$ integrin (Camper et al 1998).

In summary, normal adult human articular chondrocytes have been shown to express $\alpha1\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha V\beta5$, $\alpha V\beta3$, $\alpha6\beta1$, $\alpha10\beta1$, and $\alpha2\beta1$, in which the $\alpha1\beta1$ (receptor for collagen), $\alpha5\beta1$ (receptor for fibronectin) and the $\alpha V\beta5$ (receptor for vitronectin) heterodimers are consistently expressed.

1.4.3.2. Integrin Expression by Normal Fetal Human Chondrocytes

Durr et al (1993), using flow cytometry, demonstrated that fetal chondrocytes express the integrin subunits $\beta1$, αV, $\alpha5$, $\alpha6$, $\alpha2$, and also a low level of the $\alpha1$ subunit. The chondrocytes were isolated from fetal or newborn epiphyseal cartilage (autopsy material) with overnight culture (Durr et al 1993). Salter et al (1995) , using the technique of immunohistochemistry showed variation of integrin expression at different sites (articular, epiphyseal, growth plate, and meniscal cartilage) in human fetal knees. The results (**Table 1.4**) showed that

articular chondrocytes differed from epiphyseal, growth plate, and meniscal cells by staining intensity and pattern (Salter et al 1995). Cells from the epiphyseal area showed findings consistent with those obtained by Durr et al (1993). There was no expression of β3, β4, β6, or the α3 subunit by chondrocytes (Durr et al 1993; Salter et al 1995). Salter et al (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.

Table 1.4 Integrin expression by fetal knee joint chondrocytes

	β1	αVβ5	α1	α2	α5	α6	αV
articular cells	+	+	+	±	+	+weak*	+
epiphyseal cells	+	+	±	±	+	+	+
growth plate cells	+	+	–	–	+	+	+
meniscal cells	+	+	+	+strong*	+	+	+

+, >50% of cells stained positive; ±, <50% of cells stained positive; –, no cells staining.

*The terms strong and weak refer to the intensity of staining of the cells.

(Salter et al 1995)

1.4.3.3. Integrin Expression by Osteoarthritic Human Chondrocytes

Using the technique of flow cytometry, Lapadula et al (1997) showed that chondrocytes isolated from osteoarthritic articular cartilage expressed all the α chains (α1-6 and αV) and also β1 integrin. The α subunit most frequently expressed was α1, followed by α3, α5, α2, and αV, with lesser amounts of α4 and α6. It was found that expression of the subunits α2, α3, α5, and αV gradually decreased from minimally to the maximally damaged zones, suggesting that integrins may be involved in the pathogenesis of OA (Lapadula et al 1997).

Ostergaard et al (1998), using immunohistochemical techniques performed on cryostat sections of human articular cartilage from 12 topographically distinct sites from macroscopically normal and osteoarthritic femoral heads, showed that chondrocytes in normal and osteoarthritic cartilage expressed the integrin subunits α1, α5, αV, β1, β4, and β5. Chondrocytes in osteoarthritic cartilage, in addition, expressed the α2, α4, and β2 subunits. Superficial chondrocytes expressed more αV subunit than the deep zone chondrocytes. None of the subunits displayed topographical variation in expression in either normal or osteoarthritic cartilage (Ostergaard et al 1998). The results are summarised in **Table 1.5.**

Table 1.5 Integrin expression by normal and osteoarthritic chondrocytes

	α1	α2	α3	α4	α5	α6	αV	β1	β2	β3	β4	β5	β6
Normal cells	+	–	–	–	+	–	+	+	–	–	+	+	–
OA cells	+	+	–	±	+	–	+	+	±	–	+	+	–

+, positive staining. ±, occasional staining. –, negative staining. (Ostergaard et al 1998)

1.4.3.4. Integrin Expression by Human Chondrosarcoma Cells

The integrin profile for the human chondrosarcoma cell line HCS-2/8 has been examined using immunoprecipitation and fluorescence-activated cell sorter (FACS) analysis (Tuckwell et al 1994). Results using the immunoprecipitation technique showed that chondrosarcoma cells express the integrin $\alpha2\beta1$, $\alpha3\beta1$, $\alpha V\beta1$, and $\alpha V\beta3$, but either no or low levels of $\alpha1\beta1$ integrin. FACS data also identified $\alpha2$, αV, and $\beta1$ integrins and, in addition, showed the $\alpha5$ subunit to be present. No $\alpha4$ subunit was detected (Tuckwell et al 1994). Another chondrosarcoma cell line (105KC) was shown to express (using the immunoprecipitation technique) the integrin subunits $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, αV, and $\beta1$ (Holmvall et al 1995).

1.4.3.5. The Roles of Integrins in Chondrocytes

In cartilage, integrin-ECM interactions are thought to be important in many aspects, physiological and pathological, of chondrocyte function, including adhesion, spreading, proliferation, signal transduction, biomechanical regulation, chondrogenesis, and gene expression (Hering 1999). Chondrocyte $\beta1$ integrin-ECM interactions are required for chondrocyte survival, matrix deposition and differentiation in models of chondrocyte development (Hirsch et al 1997). Integrins mediate chondrocyte adhesion to many ECM proteins including type II collagen, type VI collagen, fibronectin, laminin, osteopontin, etc (Durr et al 1993; Enomoto et al 1993; Enomoto-Iwamota et al 1995; Tavella et al 1997; Camper et al 1997). Chondrocyte spreading and migration on type II collagen, type VI collagen, or fibronectin substrates in vitro is mediated by interactions with $\beta1$ integrins (Loeser 1993; Loeser et al 2000; Enomoto-Iwamoto et al 1997). The interaction of the $\alpha5\beta1$ integrin with fibronectin is necessary for adhesion, spreading, and proliferation of both chicken and rabbit chondrocytes (Enomoto-Iwamoto et al 1997; Tavella et al 1997). *In vitro* chondrogenesis (the differentiation of blastemal cells to chondroblasts and the formation of cartilage matrix) is inhibited by the function blocking anti-$\beta1$ integrin antibodies (Shakibaei

1998). Laminin-$\alpha3\beta1/\alpha6\beta1$ interactions are regulated by the ligand trend (depletion/reconstitution or competition experiments) during early chondrocyte differentiation (Tavella et al 1997). Other integrin-mediated attachment of chondrocytes to ECM proteins include $\beta1$-matrix Gla protein, $\beta3$-bone sialoprotein II, $\beta3$-osteopontin, and $\alpha2\beta1$-chondroadherin interactions (Loeser 1993; Camper et al 1997).

Integrins are involved in the regulation of cartilage matrix synthesis and integrity. Inhibition of integrin function inhibits type II collagen synthesis by chondrocytes in culture (Beekman et al 1997). However, integrins are also involved in cartilage breakdown. Fibronectin fragments stimulate chondrolysis and decrease proteoglycan synthesis in cartilage explants, which involve fibronectin-integrin interaction (Homandberg and Hui 1994). Ligation of $\alpha5\beta1$ integrin with fibronectin in cultured chondrocytes results in the formation of focal adhesion complexes comprising actin, FAK and the G protein Rho (Clancy et al 1997). Nitric oxide, a potential mediator of events occurring in osteoarthritis, can inhibit the assembly of the intracellular activation complex and the subsequent upregulation of proteoglycan synthesis that occurs following ligation of $\alpha5\beta1$ integrin to fibronectin (Clancy et al 1997).

Integrins have been shown to regulate the production of cytokines and inflammatory mediators by chondrocytes *in vitro.* Fibronectin has been shown to stimulate IL-6 production, which is mediated by interactions with $\alpha5\beta1$ integrins at the chondrocyte cell surface (Yonezawa et al 1996). Ligation of $\alpha5\beta1$ using activating monoclonal antibody JBS5 (which acts as agonist in a similar manner to fibronectin N-terminal fragment) has been shown to upregulate IL-6, IL-8, nitric oxide, and PGE2 production (Attur et al 2000). In contrast, the $\alpha V\beta3$ complex-specific function-blocking monoclonal antibody LM609 (which acts as an agonist similar to osteopontin) attenuated the production of IL-1β, nitric oxide, and PGE2 (Attur et al 2000). Treatment of cultured chondrocytes with TGF-β results in increased expression of $\alpha5\beta1$ integrin (Loeser et al 1995).

Signal transduction through integrins is known to upregulate metalloproteinase expression, which is mediated through IL-1 binding to its cell surface receptor (Arner and Tortorella 1995). Treatment of high density chondrocyte cultures with pronase and collagenase results in a 9-10 fold increase in aggrecan and link protein mRNA. These observed changes in mRNA expression were likely related to interactions between chondrocyte integrins and ECM (Hering et al 1994).

1.4.4. Integrins as Mechanotransduction Agents

Integrins can act as mechanoreceptors and transmit mechanical signals from the extracellular environment to the cytoskeleton (Ingber 1991; Davies 1995; Shyy and Chien 1997). Evidence shows that integrins can provide a gating function for signal transduction, by either supporting or prohibiting force transmission between ECM and the cytoskeleton (Ingber 1991). Wang et al (1993), using a magnetic twisting device applied mechanical forces directly to cell surface receptors. They showed that the integrin subunit β1 induced focal adhesion formation and supported a force-dependent stiffening response, whereas nonadhesion receptors did not (Wang et al 1993). Maniotis et al (1997) reported that living cells and nuclei are hard-wired. When integrins were stimulated by micromanipulating bound microbeads or micropipettes, cytoskeletal filaments reoriented, nuclei distorted, and nucleoli redistributed along the axis of the applied tension field. These effects were specific for integrins, independent of cortical membrane distortion, and were mediated by direct linkages between the cytoskeleton and nucleus (Maniotis et al 1997). Using a similar magnetic drag force device, intracellular Ca^{2+} concentration was shown to increase when the $\alpha2$ or the $\beta1$ integrin subunits were stressed, whereas mechanical loading of the transferrin receptor produced a significantly reduced effect (Pommerenke et al 1996). An increase in tyrosine phosphorylation was observed as a reaction to mechanical stress on the β1-subunits of the integrin family, whilst stress to the transferrin or low density lipoprotein receptors which have no connection to the cytoskeleton did

not produce this reaction (Bierbaum and Notbohm 1998; Schmidt et al 1998). These studies indicate that integrins are indeed able to transmit mechanical signals to the cell interior.

1.5. Mechanotransduction

Mechanoresponsiveness is a fundamental feature of all living cells (Ingber 1991, 1997; Chicurel et al 1998). Studies with cultured cells confirm that mechanical stresses can directly alter many cellular processes, including signal transduction, gene expression, growth, differentiation, and survival (Chen and Ingber 1999). For example in neonatal rat cardiac myocytes, cell stretch rapidly activates a plethora of second messenger pathways, including tyrosine kinases, $p21^{ras}$, mitogen-activated protein (MAP) kinases, S6 kinases ($pp90^{RSK}$), protein kinase C, phospholipase C, phospholipase D, phospholipase A_2 and cytochrome P450 pathways (Sadoshima and Izumo 1993). Evidence suggests that force is transmitted from the ECM via the plasma membrane into the cell interior and that mechanoreceptors are present at the cell surface (Wang et al 1993; Davies 1995; Maniotis et al 1997; Chen and Ingber 1999; Haidekker et al 2000), including those at the chondrocyte cell membrane (Wright et al 1996, 1997; Martina et al 1997; Grandolfo et al 1998; Guilak et al 1999). Mechanotransduction in endothelial cells and chondrocytes will now be discussed.

1.5.1 Mechanotransducers in Endothelial Cells

In endothelial cells, the transfer of blood flow shear stress forces into the cell occurs first at the luminal cell surface (Davies 1995). Plasma membrane molecules are therefore candidate mechanotransducers that generate intracellular biochemical signals. They include mechanosensitive ion channels (Adams et al 1989; Himmel et al 1993; Lansman et al 1987; Olesen and Bundgaard 1992, 1993), integrins (Ando et al 1988; Davies et al 1993, 1994; Davies and Tripathi 1993; Girard and Nerem 1993; Robotewskyj et al 1991) and G protein-linked receptors (Goligorsky 1988; Hudspeth 1989; Shepherd 1991; Robishaw and Foster 1989; Komaru et al 1994).

Shear stress-activated potassium channels and stretch-activated calcium ion channels are present in cultured endothelial cells (Olesen et al 1988; Lansman et al

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1987). Mechanically induced activity of an inward rectifying potassium current results in hyperpolarisation of the endothelial cells (Olesen et al 1988). A principal consequence of the activation of stretch-activated ion channels in vascular endothelial cells is the influx of calcium, resulting in their depolarisation of the vascular endothelial cells (Lansman et al 1987). The activities of mechanosensitive ion channels will influence downstream signalling pathways (Davies 1995).

Integrins with associated focal adhesion proteins locate at regions of the abluminal endothelial cell surface where membrane components contact the ECM and link to cytoskeleton (Burridge et al 1988; Madri et al 1988; Romer et al 1994; Yost and Hermann 1990). The most relevant integrins in endothelial cells include the fibronectin receptor $\alpha5\beta1$ and $\alpha v\beta1$, the laminin receptor $\alpha6\beta1$, the vitronectin receptor $\alpha v\beta3$ and $\alpha v\beta5$, and the basement membrane receptor $\alpha6\beta4$ (Charo et al 1990; Klein et al 1993). Shear stress applied to the luminal surface of cultured bovine endothelial cells or human umbilical vein endothelial cells (HUVECs) results in directional remodelling of the abluminal focal adhesion sites. This was observed directly in living cells using tandem scanning confocal microscopy (Davies et al 1993). Similar directional redistribution induced by shear stress also involves the focal adhesion proteins including focal adhesion kinase (pp125FAK), tensin, paxillin, and vinculin (Girard and Nerem 1993). Shear stress leading to activation of integrin associated pathways, for example FAK and MAP kinase signal-transduction pathways, has also been reported (Ishida et al 1996; Chen et al 1999). Available evidence thus suggests that integrins could act as mechanotransducers in endothelial cells.

G protein-linked mechanoreceptors have also been suggested to be involved in the response to shear stress (Shepherd 1991). A number of endothelial receptors, including those activated by ATP, thrombin, and bradykinin, are G protein linked (Himmel et al 1993). Activated G proteins regulate downstream signal transduction including activation of phospholipase C and protein kinase C, which

is common to several flow-initiated endothelial responses (Davies 1995). G protein-linked receptors also activate a MAP kinase cascade that transmits growth and differentiation signals from the cell surface to the nucleus (Cook et al 1993; Cook and McCormick 1994; Chiu et al 1999; Bao et al 2000). Other receptors including thrombomodulin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) have also been reported to be regulated by shear stress (Malek et al 1994; Nagel et al 1994; Ohtsuka et al 1993).

1.5.2. Mechanotransducers in articular chondrocytes

In articular chondrocytes, mechanosensitive ion channels and integrins have been suggested to be candidate mechanotransducers. Wright et al (1996) have shown that the hyperpolarisation response associated with cyclic strain of human articular chondrocytes follows activation of stretch-activated ion channels. Guilak et al (1999) showed findings consistent with these results. Mechanically induced calcium waves in bovine articular chondrocytes were inhibited by gadolinium and amiloride, agents known to block mechanosensitive ion channels (Yang and Sachs 1989; Hamill et al 1992). Stretch-activated potassium channels in pig articular chondrocytes have been identified using patch-clamp techniques (Martina et al 1997). Intercellular calcium waves induced by mechanical stimulation have been investigated using digital fluorescence video imaging, in primary cultures of rabbit articular chondrocytes. Intercellular Ca^{2+} spread is inhibited by 18alpha-glycyrrhetinic acid, indicating the involvement of gap junctions in signal propagation (Grandolfo et al 1998). By use of electrophysiological techniques, primary monolayer cultures of human articular chondrocytes have been shown to hyperpolarise following cyclical mechanical strain (Wright et al 1996). The transduction pathways involved in this hyperpolarisation response to cyclical strain involves $\alpha5\beta1$ integrin (Wright et al 1997). The downstream intracellular signalling pathways are inhibited by RGD-containing peptides or anti-integrin $\alpha5$ and $\beta1$ antibodies. These results suggest that $\alpha5\beta1$ integrin is an important

chondrocyte mechanoreceptor and a potential regulator of chondrocyte function (Wright et al 1997; Millward-Sadler et al 1999).

Integrins are also modulated by mechanical stress (Holmvall et al 1995). When chondrosarcoma cells were exposed to mechanical stimulation, mRNA expression of the $\alpha5$ integrin subunit was found to increase whilst expression of the $\beta1$, $\alpha2$, and αV did not increase significantly (Holmvall et al 1995). The effect of mechanical stress on integrin subunit expression has also been investigated in cells cultured on type II collagen-coated dishes with a flexible base. Mechanical stress increased mRNA expression of the $\alpha2$ integrin subunit whilst the levels of mRNA for integrin subunits $\beta1$, $\alpha1$, $\alpha5$, and αV showed no or only small changes, indicating that matrix proteins may modulate the expression of integrins during mechanical stress (Holmvall et al 1995).

1.5.3. Protein Phosphorylation Induced by Mechanical Stimulation

Protein tyrosine and serine/threonine kinases induce phosphorylation cascades (Ullrich and Schlessinger 1990; Fantl et al 1992; Hunter 1995). They transfer phosphate groups to target proteins following which phosphorylation cascades are activated. Protein tyrosine phosphorylation following mechanical stimulation has now been clearly identified in a number of cell systems, as an important occurrence both early in the mechanotransduction pathway and in downstream signalling events that result in regulation of gene transcription (Berk et al 1995; Davies 1995; Liu et al 1999). Mitogen-activated protein (MAP) kinases, including ERK1/2 and JNK, have been shown to be tyrosine phosphorylated after shear stress and mechanical stretching of a variety of cell types including bovine aortic endothelial cells (Tseng et al 1995), human umbilical vein endothelial cells (Ishida et al 1996; Pearce et al 1996), rat mesengial cells (Ishida et al 1999), rat cardiac myocytes (Yamazaki et al 1993), and human periodontal ligament cells (Matsuda et al 1998).

Human cell types studied with respect to the protein phosphorylation response to mechanical stimulation include platelets (Bromberg et al 1985; Kroll et al 1993; Nakai et al 1997), umbilical vein endothelial cells (Sokabe et al 1997; Okuda et al 1999; Li et al 1996; Ishida et al 1996; Dimmeler et al 1998; Pearce et al 1996; Chiu et al 1999; Bao et al 2000; Fisslthaler et al 2000; Akimoto et al 2000), gingival fibroblasts (Glogauer et al 1997), osteoblastic (or like) cells (Gebken et al 1999; Bierbaum and Notbohm 1998), intestinal epithelial cells (Han et al 1998a, 1998b), osteoblastic periodontal ligament cells (Matsuda et al 1998), and trabecular meshwork cells (Tumminia et al 1998). In platelets, the identified phosphorylated proteins are the myosin light chain (Bromberg et al 1985; Nakai et al 1997) and pleckstrin (Kroll et al 1993). In umbilical vein endothelial cells, the phosphorylated proteins include:

(1) the signalling molecules paxillin (Sokabe et al 1997), Crk-associated substrate (Okuda et al 1999), heat shock protein (Li et al 1996),
(2) kinases including focal adhesion kinase (Ishida et al 1996; Sokabe et al 1997), Akt kinase (Dimmeler et al 1998), ERK1/2 (Pearce et al 1996; Chiu et al 1999; Bao et al 2000), and endothelial nitric oxide synthase (Fisslthaler et al 2000).

A retinoblastoma protein (pRb) is dephosphorylated by inhibition of the activity of cyclin-dependent kinases (cdk) following 12 hours steady laminar shear stress in umbilical vein endothelial cells (Akimoto et al 2000). The level of cdk inhibitor p21 increased as a result of shear stress. These findings have been suggested that long term shear stress induces cell cycle arrest by upregulating p21 (Akimoto et al 2000).

Tyrosine phosphorylation of paxillin has been shown to occur in gingival fibroblasts after 1 or 5 min of mechanical stimulation, but is reduced after 10 min of the same stimulation regime (Glogauer et al 1997). A similar finding has been found in trabecular meshwork cells in which paxillin is tyrosine phosphorylated after 10 sec to 1 min of mechanical stimulation, but phosphorylation is reduced

after 2 min of stimulation (Tumminia et al 1998). ERK1/2 phosphorylation has also been shown to occur in osteoblastic like cells as a result of hypergravity stress (Gebken et al 1999). Mechanical stress caused the phosphorylation of c-Jun N-terminal kinase in osteoblastic periodontal ligament cells (Matsuda et al 1998). Some other proteins have also been found to be phosphorylated under mechanical stimulation and require to be further investigated (Razdan et al 1994; Oda et al 1995; Han et al 1998a, 1998b).

Phosphorylation events in response to mechanical stress can be immediate, transient, or long term effects. A summary of proteins phosphorylated in different human cell types following mechanical stimulation is shown in **Table 1.6**.

In human articular chondrocytes, protein phosphorylation following mechanical stimulation has been suggested (Wright et al 1997; Millward-Sadler et al 1999). However, the proteins which are phosphorylated have yet to be identified. Basdra et al (1994) applied a continuous compressive force of approximately 100 g/cm^2 for 90 min to cultured rat mandibular condylar chondrocytes. Using high resolution electrophoretic analysis of ^{32}P-labelled proteins, these workers showed specific dephosphorylation (and/or loss) of an acidic, 35-36 kD protein(s) and also of proteins overlapping with members of the ras superfamily of small GTP-binding proteins. This study provided the first evidence that mechanical pressure induces specific dephosphorylation events in condylar cartilage cells. The authors suggested that the events may indicate specific dephosphorylation due to a pressure-activated phosphatase, degradation of these phosphoprotein(s) triggered by mechanical stimulation, or dephosphorylation-targeted proteolysis (Basdra et al 1994).

Table 1.6 Proteins which are induced to be phosphorylated by applied mechanical stress in human cells (arranged in order of year of publication)

Cell type	Applied stress	Time	Protein phosphorylated*	Reference
Platelets	Platelet strip tension, 20-50 mg	10 min	Myosin light chain (20kD)	Bromberg et al 1985
Platelets	Shear stress, 90 dynes/cm^2	2 min	Pleckstrin (47 kD)	Kroll et al 1993
Platelets	Shear stress, 90 dynes/cm^2	2, 8, 15, 30, 60, 120 min	P-Y**: 29, 31, 36, 50, 58, 64, 76, 85, 105 kD	Razdan et al 1994
Platelets	Shear stress, 108 dynes/cm^2	30 sec, 1, 2 min	P-Y: 40, 58, 64, 70, 74, 85, 100, 130 kD	Oda et al 1995
HUVEC#	Shear stress, 12 dynes/cm^2	30, 60, 120 min	FAK (125 kD) tyrosine phosphorylation	Ishida et al 1996
HUVEC	Shear stress, 16 dynes/cm^2	30 min – 20 h	Heat shock protein (HSP27) serine phosphorylation	Li et al 1996
HUVEC	Shear stress, 2.92 dynes/cm^2	10 min	ERK2 (42kD)	Pearce et al 1996
HUVEC	Shear stress, 12 dynes/cm^2 (after adhesion to fibronectin for 10 min)	5 min	70 kD protein tyrosine phosphorylation (not paxillin)	Takahashi and Berk 1996
Gingival Fibroblasts	2 N/m^2	1, 5 min	Paxillin (68kD) tyrosine phosphorylation (reduction in 10 min)	Glogauer et al 1997

Table 1.6 Proteins which are induced to be phosphorylated by applied mechanical stress in human cells (arranged in order of year of publication) (continued)

Cell type	Applied stress	Time	Protein phosphorylated*	Reference
Platelets	Shear stress, 108 dynes/cm^2	45 sec	Myosin light chain	Nakai et al 1997
HUVEC	Uniaxial cyclic stretch (20% in length, 1 Hz)	15 min-1h	FAK & paxillin tyrosine phosphorylation	Sokabe et al 1997
Osteoblastic cells	Magnetic twist	10-30 min	40 kD proteins tyrosine phosphorylation	Bierbaum, Notbohm 1998
HUVEC	Shear stress, 15 dynes/cm^2	30, 60 min – 6 h	Akt kinase (protein kinase B)	Dimmeler et al 1998
Intestinal epithelial cells, Caco-2	10% stretch, 10 cpm	30 min	P-Y: 50, 60, 70, 120 kD	Han et al 1998a
Intestinal epithelial cells, Caco-2	10% stretch, 10 cpm	6 sec, 30 sec, 1, 5, 10, 30 min	P-Y: 50, 60, 70, 125 kD	Han et al 1998b
Osteoblastic periodontal ligament cells	9% stretch, 6 cycles/min	30, 60 min	c-Jun N-terminal kinase (JNK) phosphorylation	Matsuda et al 1998
HUVEC	20% stretch	15, 30, 60 min	FAK & paxillin tyrosine phosphorylation	Naruse et al 1998a,b

Table 1.6 Proteins which are induced to be phosphorylated by applied mechanical stress in human cells (arranged in order of year of publication) (continued)

Cell type	**Applied stress**	**Time**	**Protein phosphorylated***	**Reference**
Trabecular meshwork cells	10% uniaxial strain	10 sec, 30 sec, 1 min	Paxillin tyrosine phosphorylation (reduction in 2 min)	Tumminia et al 1998
HUVEC	Shear stress, 20 dynes/cm^2	10 min	ERK1/2 (p44/p42)	Chiu et al 1999
Osteoblast-like cells	Hypergravity stress 13 *g*	5, 10, 20, 30 min	ERK1/2 tyrosine phosphorylation	Gebken et al 1999
HUVEC	Shear stress, 12 dynes/cm^2	1, 5, 20 min	Crk-associated substrate p130cas tyrosine phosphorylation	Okuda et al 1999
HUVEC	Shear stress, 30 dynes/cm^2	12 h	Inhibit retinoblastoma protein (pRb) phosphorylation	Akimoto et al 2000
HUVEC	Impulse shear stress (abruptly applied for 3 sec), 16 dynes/cm^2	10 min	ERK1/2	Bao et al 2000
HUVEC	12 dynes/cm^2	1, 5 min	Endothelial nitric oxide synthase (eNOS) serine phosphorylation	Fisslthaler et al 2000

*Protein phosphorylation determined by assays or western blots using anti-phosphorylated antibodies.

**P-Y: phosphotyrosine.

#HUVEC: Human Umbilical Vein Endothelial Cells.

1.6. Receptors for Activated C Kinase (RACKs)

1.6.1. Identification of RACKs

Protein kinase C (PKC), a serine/threonine kinase, has important actions in transmembrane signal transduction pathways and has been reported to regulate cell proliferation, differentiation, cell-to-cell interaction, secretion, cytoskeletal functions, gene transcription, apoptosis, and drug resistance (Nishizuka 1984; Young et al 1988; Chen and Yu 1994; Dekker and Parker 1994; Newton 1995; Cornford et al 1999). PKC isoenzymes are conventionally subdivided into classic, novel, and atypical type according to cofactor requirements (Kiley et al 1991; Nishizuka 1988, 1995). Classic PKCs (α, $\beta 1$, $\beta 2$, and γ) and novel PKCs (δ, ε, η, and θ) can be activated in vivo by the second messenger diacylglycerol generated by receptor-ligand binding and in vitro by phorbol esters (Mosior and Newton 1995). Classic type is also calcium-dependent (Nishizuka 1984, 1988, 1995; Newton 1995). In contrast, atypical PKCs (ζ, λ, μ, and ι) are calcium-independent and are not activated by phorbol esters (Naor et al 1988; Dekker and Parker 1994). PKC is translocated and activated in response to cellular stimulation (Disatnik et al 1994; Haller et al 1998). PKC may be activated by integrin-mediated signalling (Vuori and Ruoslahti 1993; Chun et al 1996). Lewis et al (1996) have shown that PKC activation by phorbol ester in $\alpha V\beta 5$-integrin expressing cells, induces spreading, increases colocalization of α-actinin, tensin, vinculin, and actin and triggers tyrosine phosphorylation of FAK. Haller et al (1998) demonstrate that integrin-induced PKCα and PKCε translocation to sites of focal adhesions, mediates vascular smooth muscle cell spreading. Wrenn and Herman (1995) have shown that PKCα is translocated from a cytosolic to a membrane fraction after integrin activation in pancreatic acinar cells. Ng et al (1999) showed that PKCα regulates $\beta 1$ integrin-dependent cell motility through association and control of integrin traffic.

PKC translocates from the cytosol to the particulate fraction on activation (Mochly-Rosen 1995). Immunological and biochemical data suggest that PKC may bind to proteins in the cytoskeletal elements in the particulate fraction and also in the nucleus. Mochly-Rosen et al (1991a) were the first to provide evidence for the presence of intracellular receptor proteins that bind activated PKC. Several proteins from the detergent-insoluble material of the particulate fraction bind PKC in the presence of phosphatidylserine and calcium; binding is further increased with the addition of diacylglycerol. Binding of PKC to two of these proteins is concentration-dependent, saturable, and specific. These binding proteins are termed Receptors for Activated C-Kinase, RACKs (Mochly-Rosen et al 1991a). PKC binds to RACKs via a site on PKC distinct from the substrate binding site. A sequence KGDYEKILVALCGGN that resembles the PKC binding site on these RACKs has been identified (Mochly-Rosen et al 1991b). The C2 region in the regulatory domain of PKC isozymes (α, β, and γ) is involved in binding of PKC to RACKs (Mochly-Rosen et al 1992; Ron et al 1995). The synaptic vesicle-specific p65 fragments, homologus to the C2 region of PKC, also bind to RACKs (Mochly-Rosen et al 1992).

Ron and co-workers isolated a cDNA clone encoding a 36 kD protein (RACK1) from a rat brain cDNA expression library (Ron et al 1994). RACK1 is a homolog of the β subunit of G proteins, containing seven WD40 repeat elements, and has a pseudo-RACK1 binding site on PKC (Ron et al 1994; Ron and Mochly-Rosen 1995). Genes encoding RACK1-like proteins have been isolated from a wide range of eukaryotic organisms (Vani et al 1997; Lardans et al 1998; Chou et al 1999; Taladriz et al 1999). The coatomer protein β′-COP is also a RACK (Csukai et al 1997). Similar to RACK1, β′-COP contains seven repeats of the WD40 motif and fulfils the criteria for RACKs. Activated PKCε colocalizes with β′-COP in rat cardiac myocytes and binds to Golgi membranes in a β′-COP-dependent manner (Csukai et al 1997).

1.6.2. The Role of RACK1

Ron et al (1995) have shown that the phorbol ester-induced translocation of the C2 region-containing PKC isozymes in cardiac myocytes is inhibited by C2 region-derived peptides which mimic the RACK1-binding sequence on PKCβ. Insulin-induced PKCβ translocation and function in Xenopus oocytes is also inhibited by C2 region-derived peptides (Ron et al 1995). This indicates that the RACK1 binding site on PKC is within the C2 region of the regulatory domain, providing a direct protein-protein interaction. Both RACK1 and activated PKCβII move together from one intracellular site to another (Ron et al 1999). RACK1 can also bind PKCα (Rotenberg and Sun 1998). DECA (an anti-tumor agent and PKC inhibitor) (Weiss et al 1987; Chen 1988; Rotenberg et al 1990) prevents PKCα translocation by inhibiting the formation of the PKCα-RACK1 complex (Rotenberg and Sun 1998). RACK1 levels, paralleled by PKC expression, increase in postnatal rat brain development (Battaini et al 1997). Battaini et al (1997) have suggested that RACK1 could be involved in synaptogenesis and myelination. Parallel modulation of RACK1 and PKCα, β isoforms is also found in the brains of morphine-treated rats (Escriba and Garcia-Sevilla 1999). RACK1 levels decrease in the ageing rat brain (Pascale et al 1996; Battaini et al 1997). Age-associated changes in the release of TNF-α from lipopolysaccharide (LPS)-stimulated rat alveolar macrophages, are correlated with a decrease in the level of RACK1. The observed decrease with age is associated with a defective PKC translocation, due to a reduction in the expression of RACK1, whereas no differences are detected in the expression of the LPS receptor (CD14) or total PKCα and βII in young and old rats (Corsini et al 1999). Battaini et al (1999) also report the presence of RACK1 immunoreactivity in human brain frontal cortex, where an impaired translocation of PKC has been demonstrated in Alzheimer's disease (Wang et al 1994a). A decrease in RACK1 content of the cytosol and membrane extracts in the brains of Alzheimer's disease patients has also been shown when compared with non-Alzheimer's controls (Battaini et al 1999). By comparison, the levels of the RACK1-related PKCβII were not altered in the same membrane extracts (Battaini et al 1999).

RACK1 can coimmunoprecipitate with β1 integrin in 293T cells (a derivative of human kidney embryonal fibroblasts containing the SV40 T antigen) and also with β2 integrin in JY lymphoblastoid cells (an Epstein-Barr virus-transformed human lymphoid cell line) (Liliental and Chang 1998). The association of RACK1 with integrins in vivo requires treatment with phorbol esters (Liliental and Chang 1998). RACK1 coordinates the binding of activated PKC and select pleckstrin homology domains in vitro (Rodriguez et al 1999) and also associates with PKCβ and the common beta-chain of the IL-5/IL-3/GM-CSF receptor (Geijsen et al 1999). RACK1, PKCβ, PKCθ, and spectrin can cotranslocate to the uropod in lymphocytes (Wang et al 1999). Padanilam and Hammerman (1997) have shown that enhanced expression of RACK1 following induction of acute ischemia in rat kidneys may regulate the process of regeneration of the proximal renal tubules following ischemic renal injury. Chang et al (1998) have identified RACK1 as a Src-binding protein. RACK1 inhibited the activity of Src tyrosine kinases and growth of NIH 3T3 cells, but did not inhibit the activities of serine/threonine kinases including PKC (Chang et al 1998).

Most studies have shown that RACK1-PKC binding is PKC activation dependent (Mochly-Rosen et al 1991a, 1991b; Ron et al 1994, 1995). However, RACK1 may interact with other molecules independent of PKC activation (Yarwood et al 1999; Brandon et al 1999; Luria et al 2000). RACK1 interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform in a yeast two-hybrid screen. This interaction is confirmed by coimmunoprecipitation of native RACK1 and PDE4D5 from human and mouse cell lines. The interaction is unaffected by stimulation of the cells with phorbol ester (Yarwood et al 1999). RACK1 has been shown to bind the intracellular domain of the GABA type A receptor β1 subunit, but PKC binding appears to be independent of this reaction (Brandon et al 1999). Luria et al (2000) have demonstrated that stimulation of mouse oocyte with phorbol ester results in a rapid translocation of cytosolic PKCα, but not PKCβI, PKCβII, or RACK1, to the

mouse oocyte plasma membrane. The distribution of PKCβII and RACK1 show no change (Luria et al 2000).

RACK1 has also been shown to interacted with the influenza A virus M1 protein (Reinhardt and Wolff 2000) and Epstein-Barr virus proteins (Smith et al 2000; Baumann et al 2000). RACK1 has therefore been suggested to play a role in the infectious cycle of some viruses.

1.7. Chondrocyte Mechanotransduction Pathway Studied Using Electrophysiological Techniques

1.7.1. Response of Plasma Membrane to Applied Hydrostatic Pressure in Articular Chondrocytes

Wright et al (1992) investigated the effects of applied hydrostatic pressure on the transmembrane potentials of articular chondrocytes. The hydrostatic pressure was entered on the cell culture by placing culture dishes in a sealed perspex pressure chamber with a gas inlet and outlet. Nitrogen gas was used to pressurise the cultures. Hyperpolarisation of the chondrocyte plasma membrane was induced by cyclic pressurization (0.33 Hz, 120 mmHg for 20 min) and depolarization was induced by continuous pressure (120 mmHg, 20 min). For the frequencies tested, the maximum values for chondrocyte hyperpolarization occurred at approximately 0.3-0.4 Hz. The mechanical stimulation regime (0.33 Hz, 120 mmHg, 20 min), similar to that used by Veldhuijzen et al (1979), was used to study the transduction pathway of the electrophysiological response of chondrocytes to mechanical stimulation.

By the use of pharmacological inhibitors, it was shown that the hyperpolarisation response in cultured human chondrocytes induced by cyclic pressurization involved Ca^{2+} activated K^+ channels and L-type calcium channels. Hyperpolarisation was also produced by addition of the calcium ionophore A23187 to the culture medium showing that a rise in intracellular Ca^{2+} concentration within the cell could induce the response. Plasma membrane histamine H1 and H2 receptors, and β-adrenoreceptors did not appear to be involved in the hyperpolarisation response. The studies also showed that the actin cytoskeleton, but not microtubules, was involved in the chondrocyte hyperpolarisation response (Wright et al 1992).

1.7.2. Effects of Intermittent Pressure-Induced Strain on the Electrophysiology of Cultured Chondrocytes: Evidence for the Presence of Stretch-Activated Ion Channels

The hyperpolarisation response of chondrocytes induced by cyclic hydrostatic pressure was further investigated. Cyclical pressure applied to the pressure chamber caused deformation of the base of the culture dish to which the chondrocytes were attached and therefore deformation (strain) on the chondrocytes (Wright et al 1996). This strain was measured by strain gauges attached to the base of the culture dish. It was found that the amplitude of the hyperpolarization response was proportional to the microstrain to which cells were subjected. The hyperpolarisation response did not occur when chondrocytes were subjected to cyclical pressurization in rigid glass culture dishes or when the plastic dishes were positioned in the pressurization chamber so as to avoid deformation of the base of the culture dish (Wright et al 1996).

Previous studies (Wright et al 1992) showed that the hyperpolarisation response involved Ca^{2+} activated K^{+} channels. Further experiments demonstrated that these channels were apamin-sensitive, charybdotoxin- and iberiotoxin-resistant, low-conductance ion channels (Blatz and Magleby 1986; Galvez et al 1990; Miller et al 1985; Hermann and Erxleben 1987). The involvement of L-type calcium channels was again confirmed by use of agents (somatostatin and cadmium chloride) known to block these channels (Tsunoo et al 1986; Narahashi et al 1987). EGTA in the culture medium (chelator of the extracellular Ca^{2+}) (Bers 1982) reduced the hyperpolarisation response to 48% of control values. Thapsigargin which raises intracellular Ca^{2+} by inhibition of Ca^{2+}-ATPase in endoplasmic reticulum (Thastrup 1990; Thastrup et al 1990; Lytton et al 1991) caused hyperpolarisation independent of mechanical strain but further hyperpolarisation of the cells occurred after cyclical pressurization. These findings suggest that chondrocyte hyperpolarization is dependent on intracellular free Ca^{2+} levels (Wright et al 1996).

1.7.3. Evidence of a Role for α5β1 Integrin as a Chondrocyte Mechanoreceptor

Integrins are a family of extracellular matrix receptors and could act as mechanochemical transducers (Ingber 1991). A series of experiments were undertaken to investigate a possible role for integrin molecules and integrin-associated signalling pathways in mediating the hyperpolarization response in cultured chondrocytes that follows cyclic strain (Wright et al 1997).

The standard regimen to induce cyclic strain was the same as for the previous studies (Wright et al 1996). Anti-integrin antibodies including those against α1, α3, α5, αV, αVβ3, and β1 were used to test for a possible role for integrins in the transduction process. The results showed that only α5 and β1 integrins appeared to be involved the chondrocyte hyperpolarization response induced by 0.33 Hz cyclic pressure-induced strain, whereas α1, α3, αV, and αVβ3 had no effects. The exposure of chondrocytes to GRGDSP peptide, which blocks integrin signalling, also resulted in inhibition of the hyperpolarization response, whereas the control peptide GRADSP had no effect on the response.

Using inhibitors of the intracellular signalling molecules, the hyperpolarisation response induced by cyclic pressure-induced strain was investigated (Wright et al 1997). It was found that the response was inhibited by cytochalasin D (which disrupts the actin cytoskeleton) (Casella et al 1981; Wang et al 1994b), genistein (tyrosine kinase inhibitor) (Akiyama et al 1987; O'Dell et al 1991), neomycin (phospholipase C inhibitor) (Liscovitch et al 1991), flunarizine (inhibitor of inositol triphosphate-mediated release of Ca^{2+} from the endoplasmic reticulum) (Maroto et al 1994), W7 (a calmodulin inhibitor) (Hidaka et al 1981) and H7, staurosporine, and calphostin C which are PKC inhibitors (Hidaka et al 1984; Tamakori et al 1986; Matsumoto and Sasaki 1989; Kobayashi et al 1989). However, indomethacin (a cyclo-oxygenase inhibitor) (Laneuville et al 1994), caffeic acid (lipoxygenase inhibitor) (Cho et al 1991; Sudina et al 1993), and ketoconazole (cytochrome P450 monoxygenase inhibitor) (Hosea 1998; Eagling et al 1998) had no effect on the

hyperpolarisation response. This suggested that the mechanotransduction pathway involves actin cytoskeleton, tyrosine kinase, PLC and PKC (Wright et al 1997).

1.7.4. Interleukin-4 in Chondrocyte Mechanotransduction

Studies in osteoblasts and endothelial cells have demonstrated the production of soluble factors, such as prostaglandins and nitric oxide, in response to mechanical stimulation (Somjen et al 1980; Ayajiki et al 1996). Further studies were undertaken to investigate whether soluble mediators were involved in the hyperpolarization response of articular chondrocytes to mechanical stimulation. Conditioned medium from mechanically stimulated chondrocytes, when added to unstimulated cells, caused membrane hyperpolarization of these cells of similar magnitude to that of the directly mechanically stimulated cells, demonstrating the presence of a soluble, transferable factor secreted by the mechanically stimulated chondrocytes. Conditioned medium experiments were carried out in the presence of anti-integrin antibodies. Medium from cells mechanically stimulated in the presence of anti-β1-integrin antibody did not significantly alter the membrane potential of these cells. In contrast, resting cells were still hyperpolarised by medium transferred from cells mechanically stimulated in the presence of anti-αVβ5-integrin antibody. This finding suggests that the secretion of soluble, transferable factor is β1 integrin-mediated (Millward-Sadler et al 1999).

A panel of recombinant human cytokines (IL-1β, IFN-γ, TGF-β, and IL-4, known to be involved in the regulation of chondrocyte metabolism and which potentially could function as autocrine/paracrine signalling molecules) (Yeh et al 1995; Shingu et al 1995; Martel-Pelletier et al 1999; Goldring 2000) were added to monolayer cultures of articular chondrocytes. Addition of IL-4 resulted in hyperpolarization response, whereas the other cytokines induced chondrocyte depolarization. Expression of IL-4 and IL-4 receptor in chondrocytes was then confirmed by the techniques of immunostaining and RT-PCR (Salter et al 1996b; Millward-Sadler et al 1999). Neutralizing antibodies to IL-4 and specific antibodies to IL-4R abolished

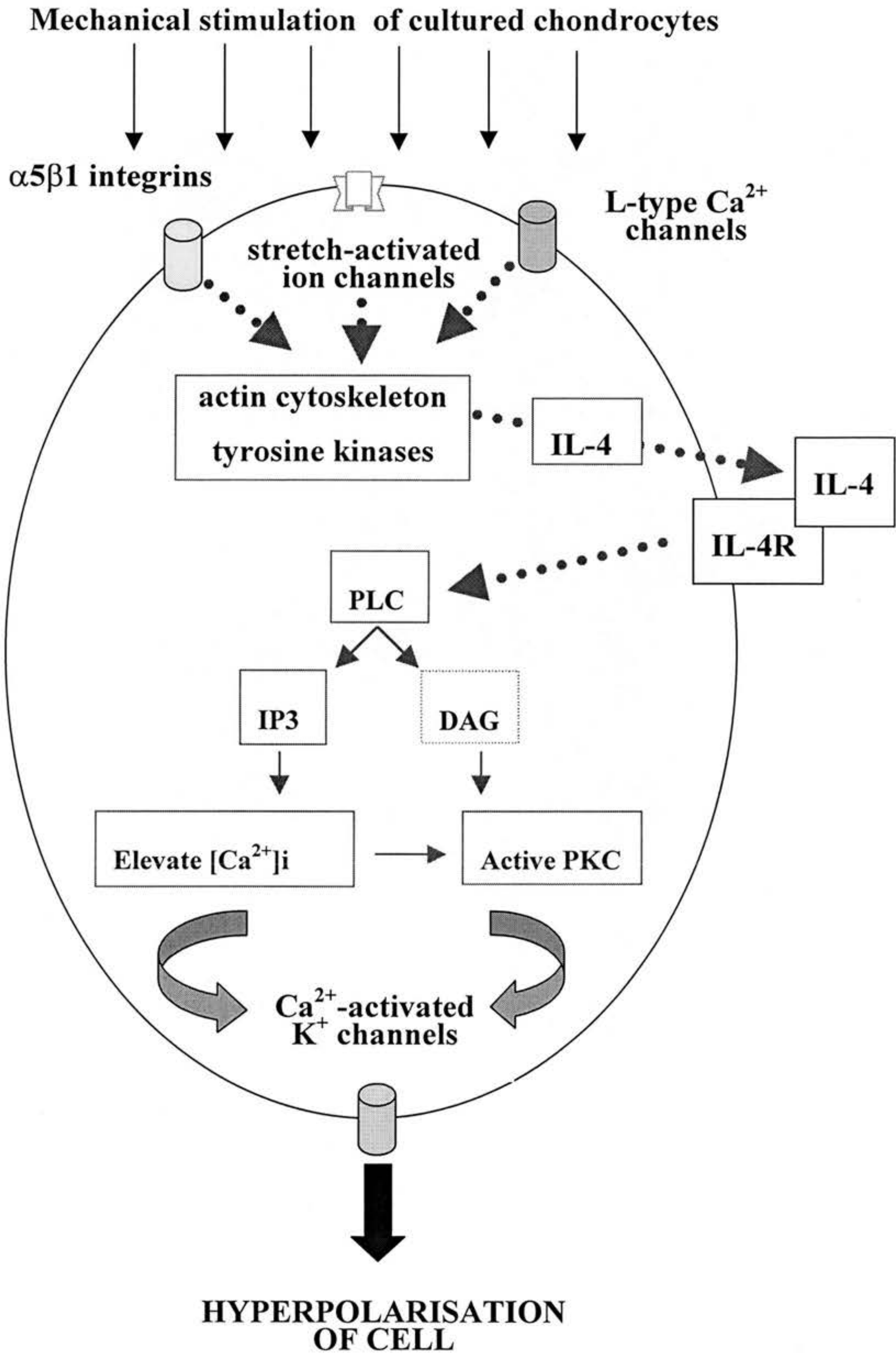
the hyperpolarization response to cyclic strain, whereas antibodies to IL-1β and its receptor had no effect. The results demonstrate that IL-4 is necessary for the hyperpolarization response to mechanical stimulation (Millward-Sadler et al 1999). The hyperpolarisation response of human articular chondrocytes to recombinant human IL-4 was unaffected by anti-β1 integrin antibody, gadolinium (a blocker of mechanosensitive ion channels) and genistein (a tyrosine kinase inhibitor), agents which have previously been shown to inhibit the hyperpolarisation response of human articular chondrocytes to mechanical stimulation (Wright et al 1996, 1997). These results indicated that mechanically induced secretion of IL-4 in the transduction pathways is downstream of activation of integrins, stretch-activated ion channels and tyrosine kinases. However, neomycin (an inhibitor of PLC), flunarizine (an inhibitor of IP3-mediated release of Ca^{2+} from the endoplasmic reticulum) and apamin (an SK channel blocker) each inhibited the chondrocyte hyperpolarisation response to IL-4, indicating IL-4 secretion is upstream of activation of PLC and IP3-mediated Ca^{2+} release which leads to the opening of SK channels and membrane hyperpolarisation (Millward-Sadler et al 1999).

1.7.5. Summary

Cultured articular chondrocytes subjected to cyclical strain at 0.33 Hz, 120 mmHg for 20 min undergo hyperpolarization of the plasma membrane by activation of Ca^{2+}-dependent K^+ channels. The mechanotransduction pathways involve the membrane receptor $\alpha5\beta1$ integrins and ion channels including stretch-activated ion channels and L-type Ca^{2+} channels. Intracellular signalling molecules include the actin cytoskeleton and tyrosine kinases with subsequent release of the chondroprotective cytokine interleukin 4. IL-4 downstream signalling involves PLC and IP3 pathways.

From the results obtained in above studies, the proposed mechanotransduction pathway of human articular chondrocytes to 0.33 Hz pressure-induced strain is summarised in **Fig. 1.4.**

Fig. 1.4 Proposed mechanotransduction pathway of human articular chondrocytes to 0.33 Hz pressure-induced strain.



1.8. Aims of the Work of the Thesis

Articular chondrocyte function is regulated partly by mechanical stimulation. The signal cascade activated in normal human articular chondrocytes by 0.33 Hz cyclical mechanical strain which results in membrane hyperpolarisation has been partially elucidated. The mechanotransduction pathway in human articular chondrocytes is integrin-mediated and involves protein tyrosine kinases. Integrin-mediated signalling pathway in other systems has been shown to activate tyrosine kinases and induce protein tyrosine phosphorylation (Davies 1995; Liu et al 1999). Accordingly part of the work of the thesis has been to investigate in more detail the tyrosine phosphorylation events that occur early in the transduction of the pressure-induced strain to human articular chondrocytes.

Pressure-induced strain of human articular chondrocytes at 0.33 Hz has been shown to induce activation of PKC (Wright et al 1997). As RACK1 has been shown to bind activated PKC and β1 integrin, experiments have also been performed to examine RACK1-β1-integrin association in response to 0.33 Hz pressure-induced strain.

In an attempt to understand the pathological processes occurring in diseased chondrocytes, experiments have also been undertaken to investigate whether chondrocytes from normal and osteoarthritic cartilage recognize and respond to cyclical strain in a similar manner. The electrophysiological response to pressure-induced strain has again been used as a marker of the effect.

CHAPTER 2. MATERIALS AND METHODS

All manipulation of human knee joint tissue and cells was carried out in a class I tissue culture hood. Gloves, laboratory coat and oversleeves were worn when handling biological tissue. If a given tissue was found to be infected, the joint was incinerated and the hood fumigated.

Antibodies and solutions used in this study are listed in appendices I and II.

2.1. Isolation and Culture of Chondrocytes

Human knee joints were obtained, 1-3 days after death, at hospital autopsy, with relatives' consent and ethical permission, opened under aseptic conditions and assessed and graded macroscopically for the presence or absence of osteoarthritis using the Collins/McElligott system (Collins and McElligott 1960; Midwood and Salter 1998). Representative sections were taken for histological confirmation of normal cartilage or osteoarthritic changes. Donors had died from a variety of diseases unrelated to the locomotor system and were undergoing routine hospital autopsy. Articular cartilage from different anatomical regions of the knee joint including the patella, tibial plateau, and femoral condyle were pooled. Normal and osteoarthritic cartilage were kept separately. Cartilage was cut into small pieces with a scalpel and incubated in antimicrobial solution (see appendix II) for 1 hour at room temperature (20°C). Cartilage fragments were washed twice with sterile phosphate buffered saline (PBS) and sequentially digested by enzymes at 37 °C in 95 per cent air / 5 per cent CO_2 with 0.25 % trypsin (Gibco, Paisley, UK) for 30 min and 3 mg/ml collagenase (Sigma, Dorset, England) for up to 48 hours.

The sample was checked for digestion by examination under the microscope. The cell suspension was collected using a sterile pastette and strained through a sterile strainer to remove undigested cartilage fragments. The cell suspension was centrifuged at 1000 rpm for 10 min, the supernatant discarded and the pellet re-suspended in PBS. This was repeated twice more to give three PBS washes. The cells were re-suspended in 10 ml Iscove's modified Dulbecco's medium (Gibco)

supplemented with 10% FCS (Sigma), 100 I.U./ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) and then filtered through a 70 micron cell sieve (Falcon, Becton Dickinson UK) to remove large cell clumps and pieces of debris. Cells were counted using a haemocytometer and viability checked using trypan blue dye. Cells were seeded in complete medium at a density of 5×10^4 (for electrophysiology) or 5×10^5 (for protein extraction) cells /ml in 55 mm petri dishes 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 unless otherwise stated. The day (16-20 hours) before mechanical stimulation was to be carried out, the culture medium containing serum was replaced by serum-free medium. Morphologically, the cells studied were typically flattened with a polygonal cell shape and did not show the fibroblastic appearance of dedifferentiated chondrocytes. Immunological staining confirmed production of similar ECM molecules (type II and type VI collagen and keratan sulphate) and expression of $\alpha5\beta1$ integrins, to that of human articular chondrocytes in vivo (Salter et al 1992; Loeser et al 1995) and after initial cell extraction (Jobanputra et al 1996).

2.2. Cell Culture of Immortalised Human Chondrocytes C-20/A4

The immortalised human chondrocyte cell line C-20/A4 was provided by Dr. Goldring (Beth Israel Deaconess Medical Center, New England Baptist Bone & Joint Institute, and Harvard Medical School, Boston, Massachusetts). C-20/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 et al 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 collagens II, IX, and XI and proteoglycans when cultured in an insulin-containing serum substitute (1%, v/v, Nutridoma-SP,

Boehringer Mannheim Biochemicals, Germany). C-20/A4 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma)/Ham's F12 (Sigma) (1:1, v/v) with 10% FCS (Sigma), 100 I.U./ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). The cell culture conditions were same as for the primary chondrocytes.

2.3. Assessment of Cartilage for Osteoarthritis

Joints were assessed for the presence or absence of osteoarthritis macroscopically and graded according to Collins/McElligott system (Collins 1960; Gardner 1992; Midwood 1998) (**TABLE 2.1.**). Representative sections were also taken for histological assessment.

TABLE 2.1. Collins macroscopic grading of osteoarthritis

GRADE	DESCRIPTION
I	Patches of fibrillation or softening in central area of articular cartilage. No area of whole thickness cartilage loss, no recognisable marginal osteophytosis and no detectable synovial inflammation.
II	Larger areas of fibrillation without denuding bone. Early marginal chondro-osteophytosis.
III	At least one area of whole thickness cartilage loss with bone exposure. More generalised 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.

2.4. Induction of Cyclical Mechanical Strain

The pressurisation apparatus (**Fig. 2.1**) used to induce cyclical mechanical strain has been previously described (Wright et al 1992, 1996). Flexible, plastic 55-mm tissue culture dishes (Nunc, Life technologies, UK) were placed in a sealed chamber with inlet and outlet ports. The culture dish was supported on six horizontal pins inserted into a brass cylinder attached to the base of the pressure vessel (**Fig. 2.2 and 2.3**). 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. A space of volume 8-ml (chamber B) was present between the base of the culture dish and the base of the pressure vessel. Gas entered this space from the main chamber (chamber A, volume 89 ml) via 18 holes each 2.2 mm in diameter in the brass cylinder. The chamber was pressurized with nitrogen gas from a cylinder, the frequency being dictated by an electronic timer controlling the inlet and outlet valves. Cyclical pressurization of this system induces deformation and strain on the base of the plastic tissue culture dish and its adherent cells due to a differential rate of rise of pressure above and below the culture dish. Previous electrophysiological studies in which cells have been subjected to 16 kPa above atmospheric pressure without associated deformation of the dishes (Pyrex glass petri dishes) to which they are adherent, have shown conclusively that this degree of pressure itself has no effect on membrane potential and that the electrophysiological response is wholly dependent on deformation of the base of the dish and adherent cells (Wright et al 1996).

In the present studies, a pressure of 1 Bar above atmospheric pressure which results in 3200 microstrain 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). Each experiment was carried out with chondrocytes from at least 3 different donors.

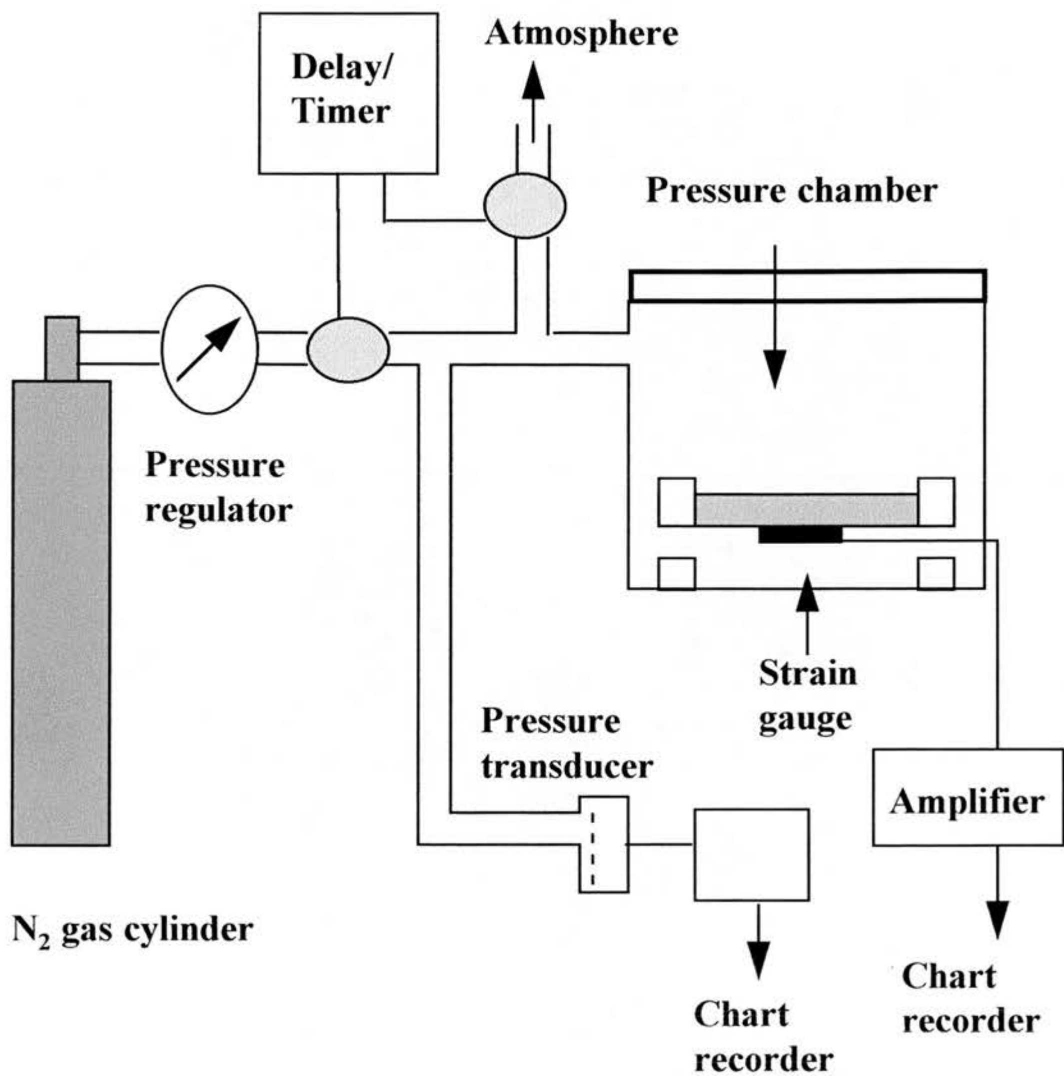


Fig. 2.1 Apparatus for application of intermittent pressure and recording microstrain

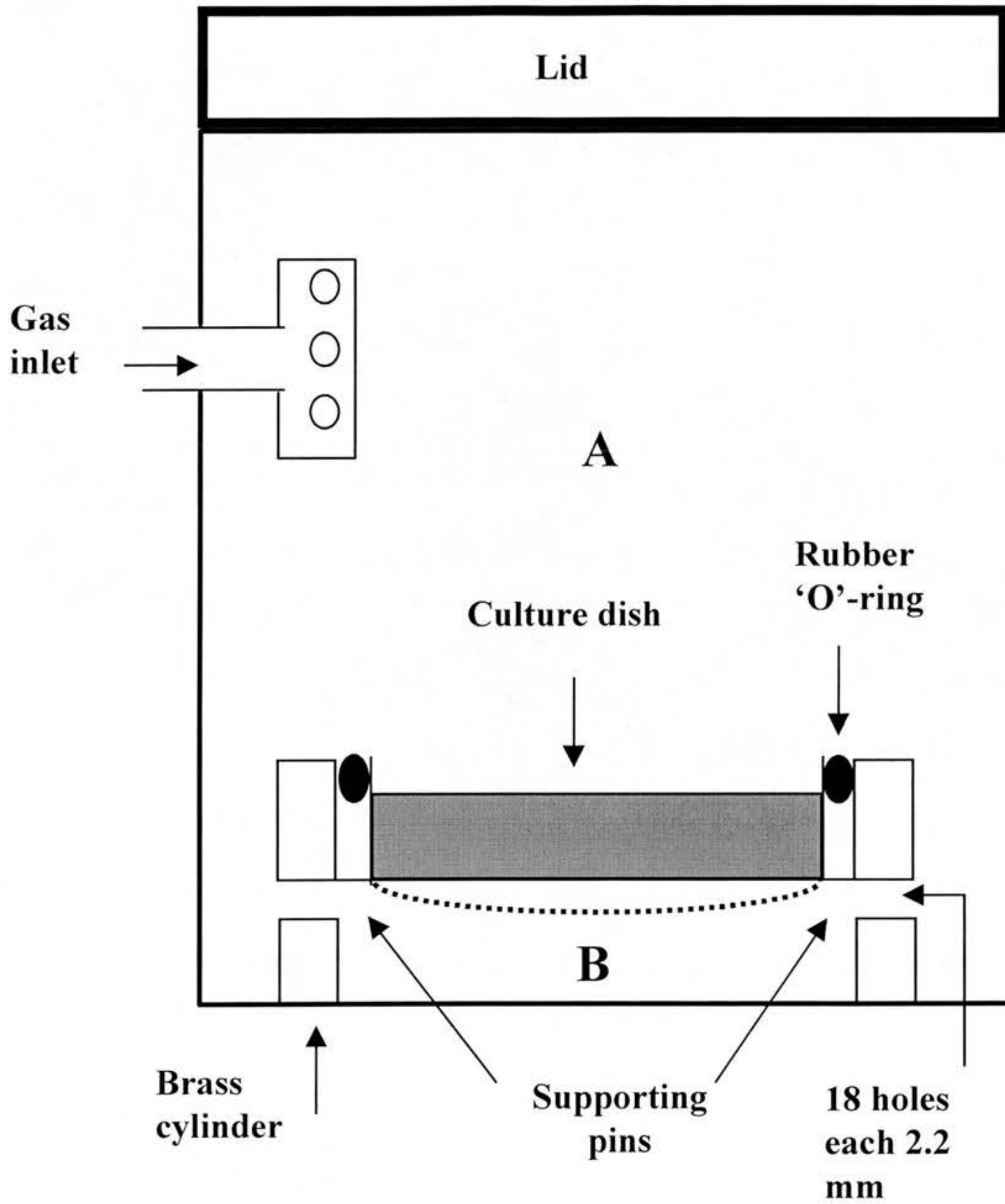


Fig. 2.2 Details of pressurisation chamber.

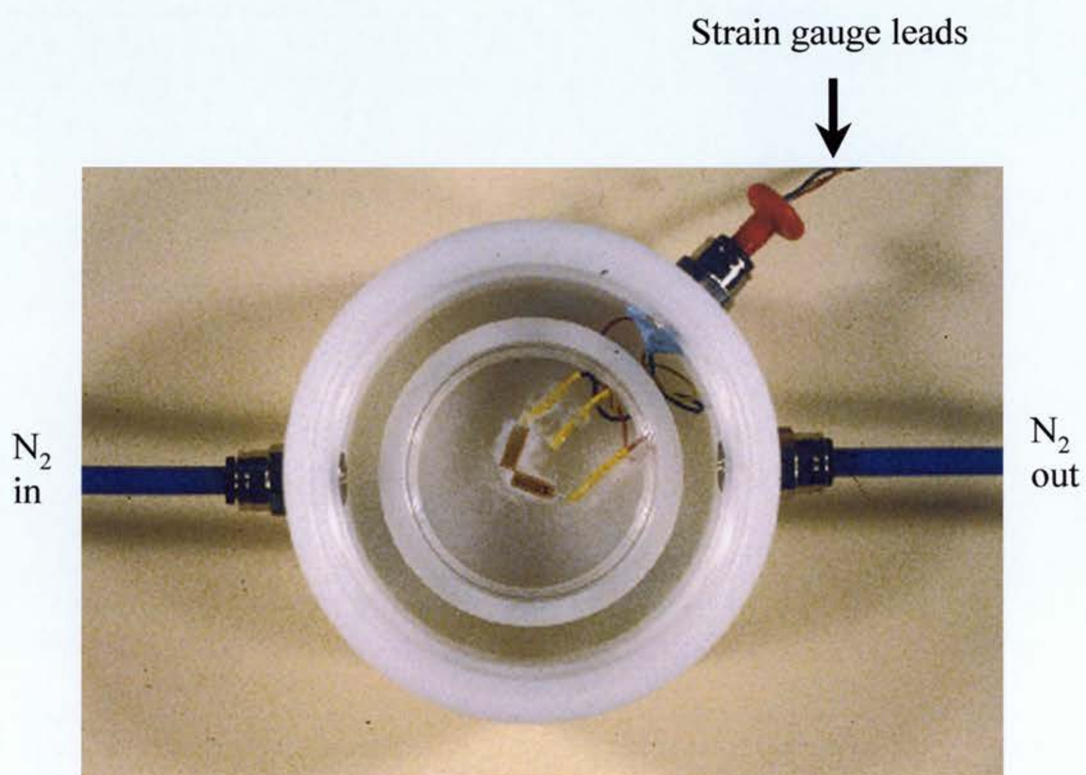


Fig.2.3 Pressurisation chamber with strain gauge.

2.5. Experimental Protocol for Tyrosine Phosphorylation Studies

Chondrocytes were subjected to pressure pulses of 1 atmosphere above atmospheric pressure, at a frequency of 0.33 Hz for either 1 or 5 minutes. To assess the role of integrins and stretch-activated ion channels in the induction of protein tyrosine phosphorylation, in separate experiments chondrocytes were incubated with 100 μg/ml of the synthetic hexapeptides GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) or GRADSP (Gly-Arg-Ala-Asp-Ser-Pro) (Novabiochem, Darmstadt, Germany) for 30 minutes or 10 μM gadolinium (Sigma Chemical Co.) for 10 minutes prior to mechanical stimulation. In separate experiments, the effects of IL-4 on chondrocyte tyrosine phosphorylation was examined by incubation of chondrocytes with recombinant IL-4 (10 ng/ml) (R&D Systems, Oxon, UK) for 1 min without mechanical stimulation.

2.6. Protein Extraction

Following mechanical stimulation, the dishes containing the cells were immediately washed with 10 ml ice-cold PBS containing 100 μM Na_3VO_4 (Sigma) and lysed in situ with 500 μl ice-cold lysis buffer at 4 $^{\circ}$C for 15 min. Lysis buffer contained 1% Igepal (Sigma), 100 μM Na_3VO_4, and the protease inhibitor cocktail tablet (Boehringer Mannheim, Germany). Lysates were collected by scraping the base of the culture dish with a cell scraper in the cold room at 4°C. Supernatants were collected after centrifugation at 13,000 rpm for 15 min. The pellet containing nuclear debris was discarded. The whole cell lysates were stored at -70 $^{\circ}$C for future use, including the separate storage of a 20 μl aliquot of the lysate for protein determination. The protein concentration in the lysates was determined using the Folin-Lowry assay method (Lowry et al 1951).

2.7. Lowry Determination of Protein Concentration (Lowry et al 1951)

Bovine serum albumin (BSA) (Sigma) standards increasing in concentration from 0 to 200 μg/ml (0, 10, 25, 50, 75, 100, 150, 200 μg/ml) were set up in triplicate and vortexed. Sample tubes were set up in triplicate by adding 5 μl sample to 195 μl 0.1 N 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 min. To each tube 100 μl Folin's reagent (Sigma) was added, the tubes were vortexed and allowed to stand at room temperature for 30 min. An aliquot of 200 μl of each sample was transferred to a 96 well tissue culture plate (Nunc, Life Technologies UK) and the absorbance read on a Dynatech MR 500 microplate reader at 570 nm. The average concentration for each triplicate sample was calculated by the software BioLinx 2.20 using a standard curve of BSA protein concentrations.

2.8. Immunoprecipitation

One ml aliquot of protein at a concentration of 500 μg/ml was used for immunoprecipitation.

For immunoprecipitation of phosphotyrosine, monoclonal anti-phosphotyrosine agarose beads (Sigma) were incubated with whole cell lysates for 4 hours at 4°C. The beads were sedimented by centrifugation at 13,000 rpm for 15 min, washed twice with ice-cold lysis buffer and once with ice-cold PBS containing 1 mM Na_3VO_4. SDS-sample buffer was added and the beads were then boiled for 5 min to dissociate the proteins.

For immunoprecipitation of pp125FAK (Santa Cruz Biotechnology, Inc., California, USA), paxillin (Chemicon International, Inc., California, USA) and β-

catenin (Transduction Laboratories, California, USA), whole cell lysates were incubated with primary antibodies for 1 hour and then protein A-Sepharose (Pharmacia LKB, Uppsala, Sweden) for 1 hour. Protein A was first incubated with goat anti-mouse antibody for immunoprecipitation with the monoclonal anti-β-catenin mouse IgG1 isotype antibody. For coimmunoprecipitation of β1 integrin and RACK1/PKCα, β1 integrin was immunoprecipitated with protein A-Sepharose (for mouse monoclonal IgG2a isotype) or protein G-Sepharose (for mouse monoclonal IgG1 isotype) (Pharmacia LKB). The immune complexes were then pelleted at 13,000 rpm, washed three times with ice-cold lysis buffer and released from protein A-Sepharose or protein G-Sepharose by the addition of a mixture of 25 μl of 10% SDS and 25 μl of sample buffer containing bromophenol blue dye. All of above procedures were carried out at 4°C. Equivalent amounts of immunoprecipitated proteins were separated using 7.5% or 10% SDS-PAGE under reducing conditions.

2.9. Western Blot

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

Proteins were analysed by SDS-PAGE according to the method of Laemmli (1970), using Bio-Rad equipment. Glass gel plates (12 cm x 10 cm and 12 cm x 9 cm), spacers, and grey silicone gaskets were cleaned with industrial methyl alcohol. Glass plate sandwiches were assembled into the clamp assembly and the unit transferred to the casting stand. A comb was placed in between the plates and a mark made 0.5 cm below the edge of the teeth. A separating gel solution was prepared of appropriate acrylamide (National diagnostics, UK) percentage (7.5% or 10%) for the resolution required. The solution was mixed and poured between the plates up to the mark using a 5 ml pasteur pipette. The solution was overlaid with distilled water and allowed to polymerise for 45 min. The overlaid distilled

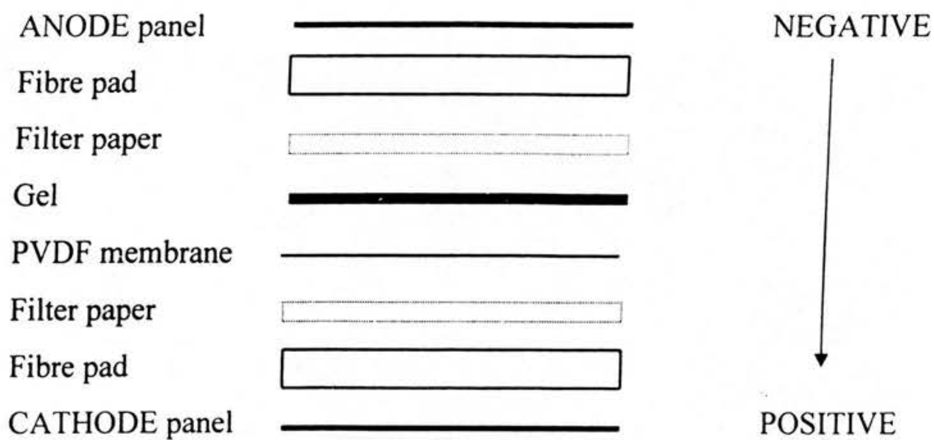
water was drained off and the surface of the gel dried with filter paper. The stacking gel solution was prepared (4%) and poured over the separating gel to the top of the plates. A 5 or 10 well comb was placed in the sandwich and the gel allowed to polymerise for 30 min. When the gel had polymerised, the comb was removed, the clamp assemblies removed from the casting stand and attached to the inner cooling core. The electrode buffer was prepared from a five times stock solution by diluting 80 ml of the stock solution with 320 ml distilled H_2O. Electrode buffer was added to fill the upper buffer chamber and the remainder placed into the lower buffer chamber. The protein samples were loaded onto the gel after 5 min boiling of the protein aliquot. The samples were electrophoresed through the gel at a constant voltage of 200 V and stopped when the line of bromophenol blue dye had reached the bottom of the resolving gel.

2.9.2. Electrophoretic Transfer of Proteins to Polyvinylidene Fluoride (PVDF) Membranes

The gel assembly was removed from the gel apparatus, the glass plates separated and the gel removed. For each gel a piece of PVDF membrane (Sigma, Millipore Immobilon-P) and two 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 100% methanol for 15 seconds and then transferred to a container of Milli-Q water for 2 min. The PVDF membrane, filter papers and fiber pads were equilibrated in transfer buffer for 15 min. The plastic holder for the gel membrane sandwich was opened, a fibre pad placed onto it followed in sequence by a piece of filter paper, the gel, the PVDF membrane, the second piece 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 lay between the cathode and the gel (**Fig. 2.4**). The tank was filled with transfer buffer sufficient to cover the gel/membrane sandwich without touching the electrodes and transfer was carried out at 100V for 1 hour, using a pre-frozen cooling unit.

Fig. 2.4

Assembly of tank transfer apparatus



2.9.3. 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 transfer apparatus and opened on a flat surface. The PVDF membrane was removed and marked for orientation by cutting off the top left hand corner. The PVDF membrane was then soaked in 20 ml 100% methanol for 10 seconds to drive out the water. The blot was then placed on a piece of filter paper for 15 min to evaporate the methanol. The blot was incubated with 10 ml 2% BSA (Sigma) in TBST overnight at 4°C for non-specific blocking. Next morning, the blocking solution was poured off and the membrane was washed 3 times with 20 ml TBST with rotation on an orbital shaker. The membrane was incubated with primary antibody diluted optimally in 5 or 10 ml TBST, for 1 hour with rotation at room temperature or overnight at 4°C and then washed 3 times, each for 5 min. The membrane was incubated with HRP-secondary antibody diluted 1:2000 in 10 ml TBST for 1 hour with rotation at room temperature, then washed as above. The proteins were then visualised by ECL or ECL Plus detection (Amersham, Buchinghamshire, UK). When the ECL detection system was used, a mixture of 1 ml of solution A and 1 ml of solution B were poured onto one membrane for 1 min. A mixture of solution A: solution B (40:1) was applied for 5 min when ECL Plus was utilised. The fluid was drained off and the membrane wrapped in Saran wrap. Any air pockets were smoothed out. The blot was placed in an autoradiography cassette and exposed to ECL hyperfilm (Amersham) for 1 min. The film was developed by hyperprocessor (Amersham) and the blot re-exposed as required depending on the strength of the signal.

Where indicated, immunoblots were stripped with a solution containing 62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 55 °C before reprobing.

2.9.4. Estimation of Protein Size

The molecular weight of proteins analysed by SDS-PAGE was determined by comparison with proteins of known size (multi-colored standard, Novex, UK) which were electrophoresed on the same gel.

2.10. Immunofluorescence

2.10.1 Single Immunofluorescent Labelling of Cultured Chondrocytes

Chondrocytes from normal adult articular cartilage were cultured at a concentration of $5x10^4$/ml as a monolayer for 1-2 weeks in 55 mm tissue culture Petri dishes (Nunc). The cells were washed with 10 ml TBS and then fixed using 2 ml of a 1:1 methanol/acetone mixture per dish for 5 min at -20 °C. The cells were permeabilised using 2 ml 0.2 % (v/v) Triton X-100 (Sigma) in TBS for 20 min. The cells were washed with 5 ml TBS with rotation three times, each for 5 min. Non-specific background was blocked by incubation with 2 ml 1% (w/v) BSA in TBS for 1 hour. The BSA was poured off and the cells incubated overnight at 4 °C with primary antibody diluted 1: 100 in 2 ml TBS. In the negative controls, the primary antibody was omitted. The cells were washed with 5 ml TBS three times for 5 min, with rotation, prior to being incubated with FITC or TRITC labelled secondary antibody diluted 1:100 in 2 ml TBS for 1 hour in the dark at room temperature. The cells were then washed with 5 ml TBS three times for 5 min with rotation and then mounted using immunofluorescent mountant (DAKO), the edges of the coverslip (previously cleaned with ethanol and allowed to air dry) secured by painting with clear nail varnish. The cells were visualised using a Zeiss axiophot fluorescence microscope.

2.10.2 Double Immunofluorescent Labelling of Cultured Chondrocytes for Confocal Microscopy

Human articular chondrocytes in 55 mm petri dishes were fixed in 5 ml 3.5% formaldehyde containing 1 mM $CaCl_2$ for 20 min at room temperature. After washing with 10 ml TBS, the cells were permeabilized with 5 ml 0.2% (v/v) Triton X-100 (Sigma) in TBS for 20 min, blocked by incubation with 2 ml 1% (w/v) BSA in TBS for 1 hour at room temperature and then incubated with following order of antibodies in 2 ml TBS, anti-pp125FAK or anti-α5-integrin for 6 hours at 4°C, FITC-conjugated secondary antibody for 1 hour at room temperature in the dark, second primary antibody (β-catenin) overnight at 4°C, and finally TRITC-conjugated secondary antibody for 1 hour at room temperature.

For colocalization of β1-integrin and RACK1, the staining order was anti-RACK1mouse monoclonal antibody for 2 hours at 4°C, goat anti-mouse TRITC-conjugated secondary antibody for 1 hour at room temperature, and finally anti-β1-integrin-FITC directly conjugated monoclonal antibody for 2 hours at 4°C. Cells were washed three times with 5 ml TBS after each antibody incubation. In the negative controls, primary antibodies were omitted. Cells were viewed by confocal microscopy and processed with confocal system software (Leica TCS NT, Leica Microsystems).

2.11. Electrophysiology

2.11.1. Preparation of Microelectrodes

Microelectrodes were made freshly for each experiment. The borosilicate glass capillaries GC150F-10, 1.5 mm outer diameter x 0.86 mm inner diameter standard wall with inner filament (Clark Electromedical Instruments, Reading, England) were used. One 10 cm glass capillary was pulled to yield two microelectrodes using a Narishige microelectrode puller (Narishige, Japan). After pulling, microelectrodes were cooled and stored in a clean container with tips uppermost.

2.11.2. Filling of Microelectrodes

A clean glass beaker with plasticine around its edge was filled with 3M KCl (223.5 g/l, filtered before use). The tips of the microelectrodes were placed in the 3M KCl and the shafts of the microelectrodes held by plasticine. The shaft of the microelectrode was filled with 3M KCl using a syringe and spinal needle. The tips of the microelectrodes filled by capillarity along the inner filament (**Fig. 2.5**).

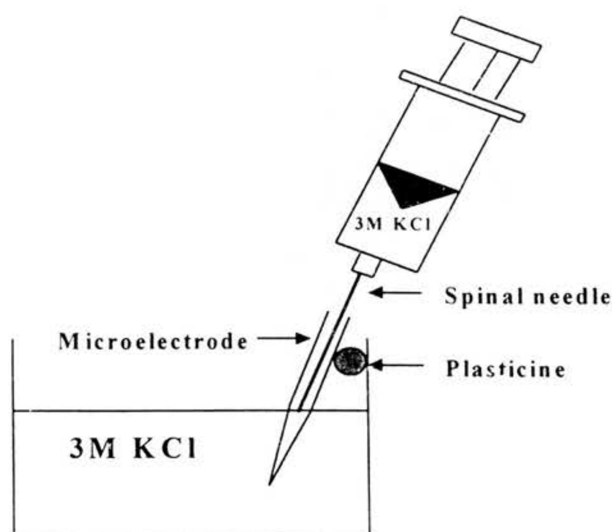


Fig. 2.5 Filling 3M KCl solution into microelectrode

Microelectrodes were not used for experimentation if air bubbles were visible within the microelectrode tip.

2.11.3. Electrophysiological Recording

These investigations were carried out at room temperature (20°C). Dishes of chondrocytes were placed on the stage of a Wild M40 inverted microscope (**Fig. 2.6**). Cells were observed under x200 magnification. A silver/silver chloride (Ag/AgCl) earth wire (non-polarisable for more stable recording) was placed in the periphery of the culture dish being examined. The central end of this wire was connected to the ground terminal of the input headstage. A microelectrode holder was used to connect the microelectrode to the headstage. A filled microelectrode was connected to microelectrode holder so that the Ag/AgCl wire of holder went down the shaft of microelectrode and made contact with 3M KCl in shaft of microelectrode. The microelectrode was passed over the Ag/AgCl wire until the blunt end of the microelectrode contacted the recess and pipette seat of the microelectrode holder (**Fig. 2.7**). The pipette cap was then turned to firmly hold the microelectrode in place in the holder. The central end of microelectrode holder was thus connected to the input of the headstage (**Fig. 2.8**). Using a Zeiss micromanipulator, the microelectrode connected to the headstage was lowered until its tip was beneath the level of the culture medium covering the chondrocytes. The tip of the microelectrode was then located using the x200 magnification.

Power to Axoclamp-2B (Microelectrode Clamp, Axon Instruments) was switched on (**Fig. 2.9**). Any voltage recorded at this time was offset to the zero base line on the oscilloscope (Gould Advance type OS 4000/4001), using the input offset potentiometer of the Axoclamp-2B. A Wheatstone bridge circuit incorporated into the circulating of the Axoclamp-2B allowed the determination of microelectrode resistance, whilst the experiment was in progress.

A command current of 5 nA was set with the step command thumbwheel switch. A voltage pulse was seen (**Fig. 2.10a**). The bridge dial was advanced until the voltage step was eliminated (**Fig. 2.10b**). The bridge was then in balance. The microelectrode tip resistance was then read from the bridge dial and was usually in the range of 30-50 MΩ which was for experimentation accepted. Lower tip resistance indicated tip damage and much higher resistances usually indicated air bubbles in the tip of the microelectrode.

A suitable cell was selected for impalement. The voltage-dependent oscillator was then switched on. This produced a note proportional to voltage. As the voltage changed on cell impalement, the note changed. This allows the experimenter to observe the microelectrode on cell impalement and not have to keep observing the oscilloscope monitor, as it is known when the microelectrode enters cell, from the change of note. The microelectrode was lowered into the cell using Zeiss micromanipulator. On cell impalement note changed as oscilloscope beam moved-down the oscilloscope face. The digital read out of membrane potential on Axoclamp-2B was then recorded.

The results from a cell were accepted if, on impalement, there was a rapid change in voltage to the level of the membrane potential of the cell and this voltage remained constant for at least 60 seconds. Membrane potentials of 5 or 10 isolated chondrocytes were recorded before and after the addition of a chemical reagent and again following the standard regimen of 20 min of cyclical mechanical strain.

A microelectrode was changed if i) the electrode resistance was lower than 20 MΩ (tip damage) or ii) electrode resistance increased dramatically, as this usually indicated electrode tip blockage with cellular material.

Fig. 2.6 Photograph of Wild M40 inverted microscope and micromanipulation system.

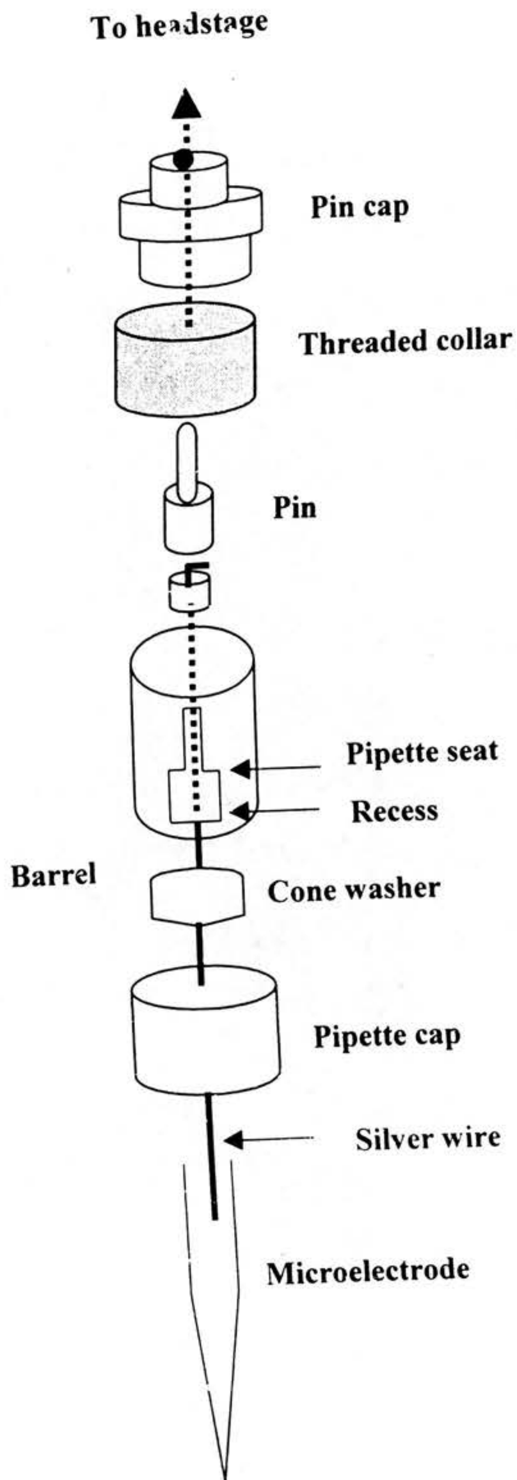


Fig. 2.7 Details of the microelectrode holder.

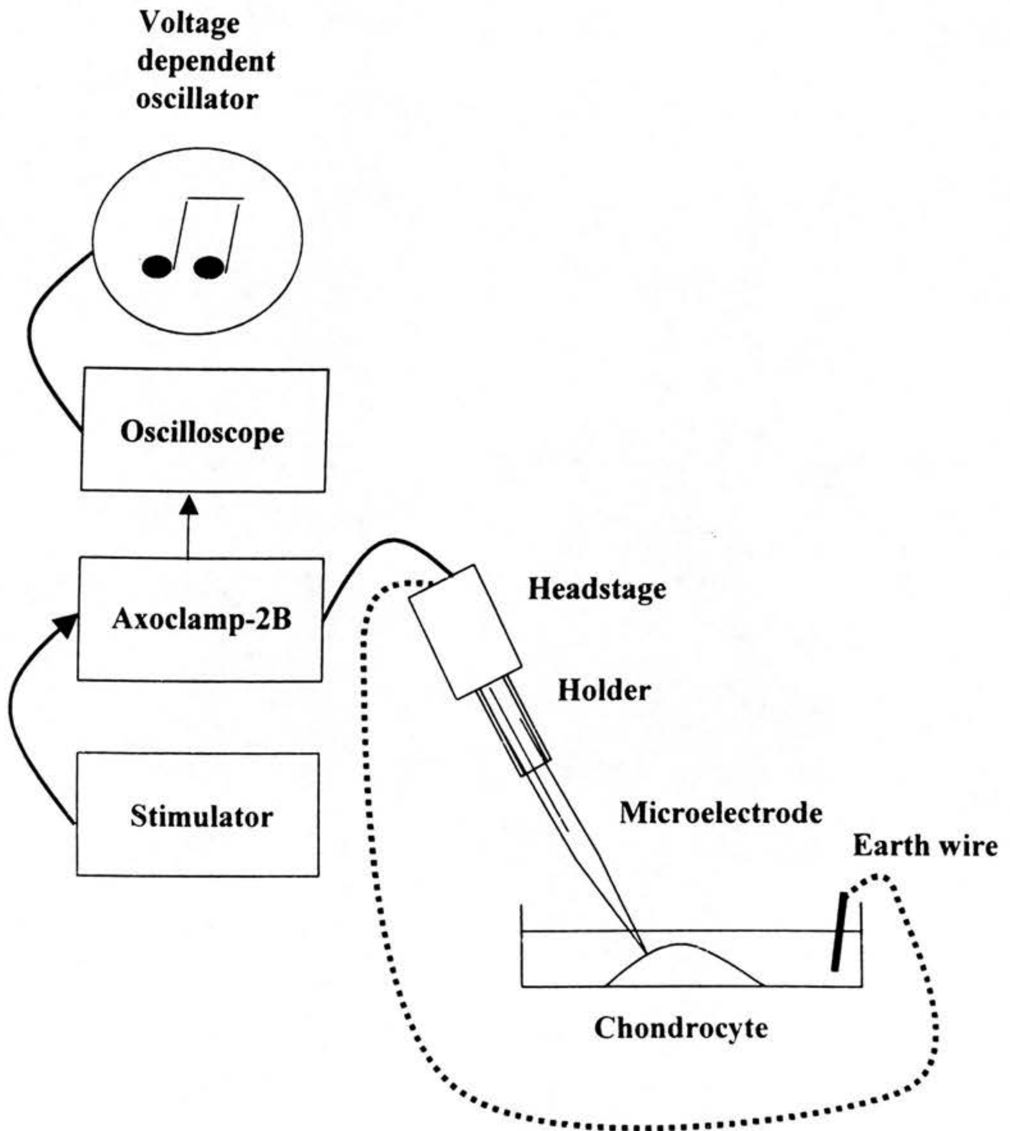


Fig. 2.8 Block diagram of Axoclamp-2B system.

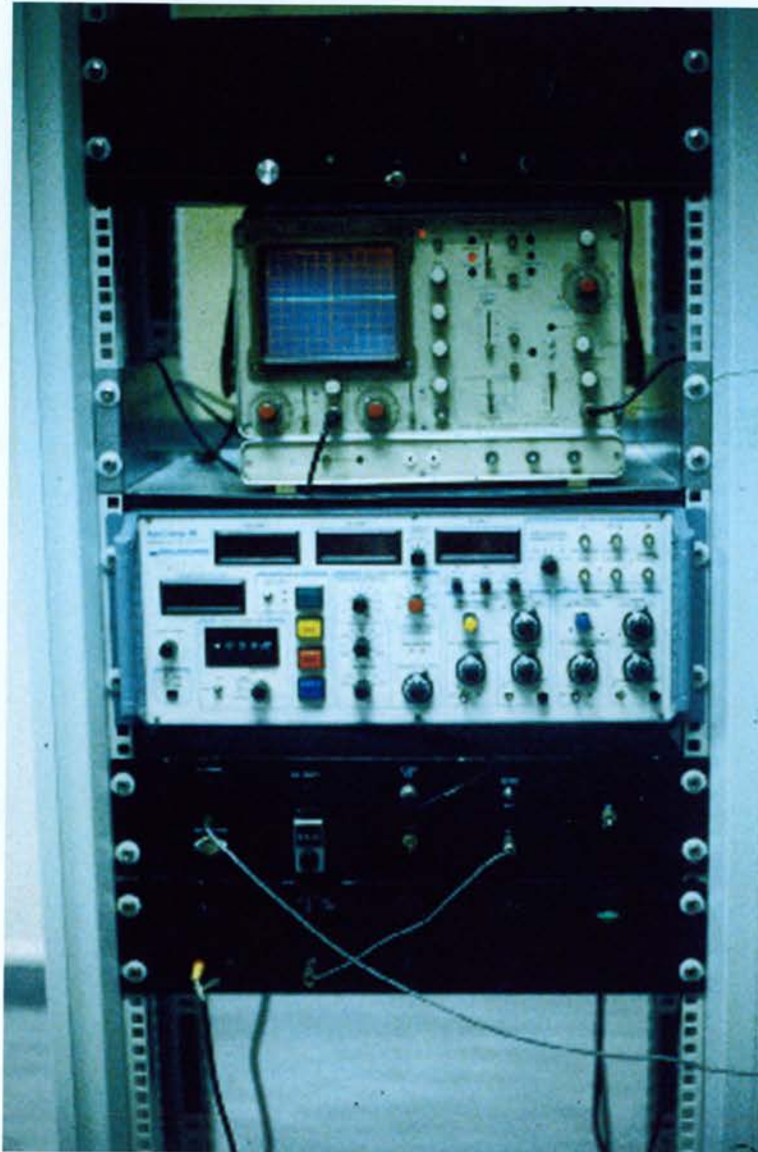
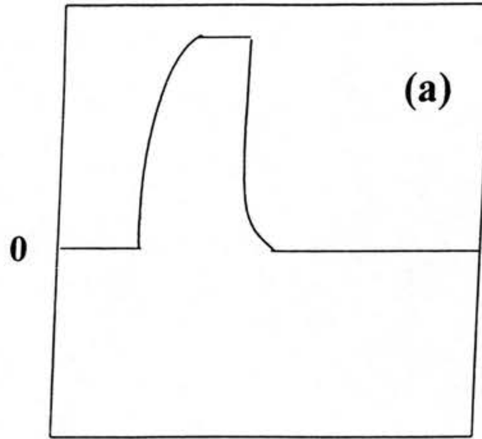


Fig. 2.9 Electrophysiological apparatus.



50 mV

1 ms

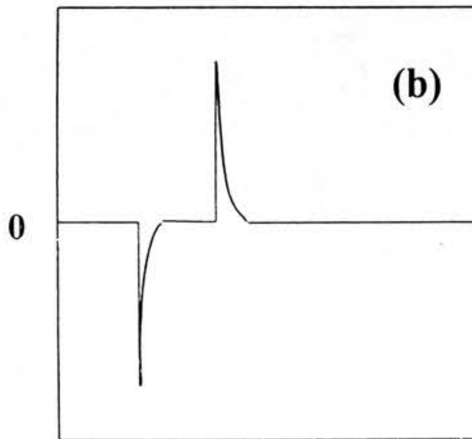


Fig. 2.10 Bridge balancing procedure.

2.12. Chemical Reagents

To investigate the mechanotransduction pathway induced by pressure-induced strain in cultured chondrocytes, a variety of reagents including inhibitors of intracellular mediators, antibodies, and cytokines were added separately to cell cultures. Membrane potentials of chondrocytes were measured before and either 10 or 30 min after the addition of a particular reagent. Chemical reagents were still contact with chondrocytes during the standardised pressurisation procedure and also when the post-strain membrane potentials of chondrocytes were being measured. The chemical reagents with their targets and concentrations utilised are shown in **Table 2.2**.

Table 2.2. Chemical Reagents

Reagent	Effect	Concentration Utilised*	Reference
Apamin	Blocker of Ca^{++}-activated K^{+} channels	4.9 μM	Blatz and Magleby 1986
Gadolinium	Blocker of stretch-activated ion channels	10 μM	Yang and Sachs 1989
Genistein	Tyrosine kinase inhibitor	40 μM	Akiyama et al 1987
Calphostin C	PKC inhibitor	1 μM	Kobayashi et al 1989
Staurosporine	PKC inhibitor	1 μM	Tamaoki et al 1986
Chelerythrine	PKC inhibitor	10 μM	Herbert et al 1990
Wortmannin	PI 3-kinase inhibitor	0.1 μM	Reinhold et al 1990
LY294002	PI 3-kinase inhibitor	30 μM	Vlahos et al 1994
Neomycin	PLC inhibitor	5 mM	Liscovitch et al 1991
Cytochalasin D	Disruption of actin cytoskeleton	2 μM	Wang et al 1994
Flunarizine	Inhibitor of inositol triphosphate-mediated release of Ca^{++} from the endoplasmic reticulum	50 μM	Maroto et al 1994

Table 2.2. Chemical Reagents (continued)

Reagent	Effect	Concentration utilised	Source
P4C10	Anti-β1 integrin Ab**	1 μg/ml	Gibco
TS2/16	Anti-β1 integrin Ab	1 μg/ml	Sanchez-Madrid
CD29	Anti-β1 integrin Ab	1 μg/ml	Serotec
P1F6	Anti-αVβ5 integrin Ab	1 μg/ml	Gibco
CD49e	Anti-α5 integrin Ab	1 μg/ml	Serotec
Anti-IL-4	Anti-IL-4 Ab	1μg/ml	R&D
Anti-IL-1β	Anti-IL-1β Ab	1 μg/ml	R&D
Anti-IL-1RI	Anti-IL-1 receptor type I Ab	10 μg/ml	R&D
Anti-IL-1RII	Anti-IL-1 receptor type II Ab	10 μg/ml	R&D
Anti-IL-2Rγ	Anti-IL-2 receptor gamma chain Ab	10 μg/ml	R&D
IL-4	Recombinant cytokine	10 ng/ml 10 pg/ml	R&D
IL-1β	Recombinant cytokine	10 ng/ml 10 pg/ml	R&D
Bric-126	Anti-CD47 Ab	1 μg/ml	IGBRL***

*Incubation period of recombinant cytokines and inhibitors of intracellular mediators: 10 min. Incubation period of antibodies: 30 min. ** Ab: Antibody.
*** IGBRL: International Blood Group Reference Laboratory (Bristol, UK)

2.13. Statistical Analysis

A Student t-test was used for statistical comparison between sample means, provided that the variance ratio of the two samples did not reach significance ($p<0.05$). Where this occurred, samples were compared using the non parametric Mann-Whitney U-test.

Significant results correspond to p values less than 0.05.

CHAPTER 3.

TYROSINE PHOSPHORYLATION IN NORMAL ARTICULAR CHONDROCYTES IN RESPONSE TO CYCLICAL STRAIN

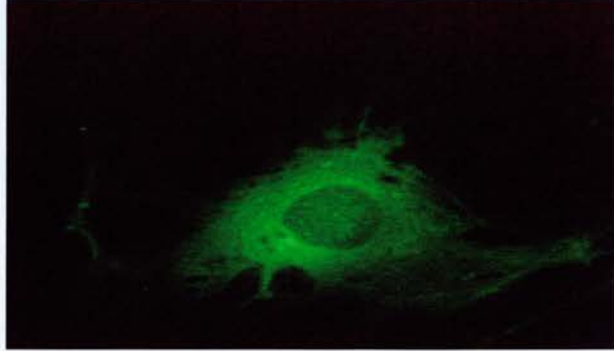
RESULTS

3.1. The Phenotype of Cultured Articular Chondrocytes

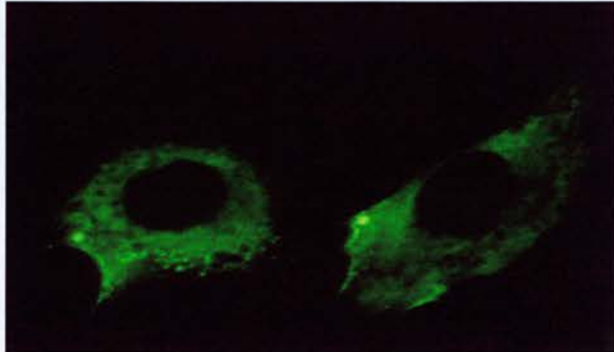
Chondrocytes were isolated from normal articular cartilage obtained from the femoral condyles of 4 females (mean age 71 years, range 63-83) and 6 males (mean age 66 years, range 45-88). Morphologically, the cells studied in 1-2 week cultures showed typically flattened and polygonal shape without fibroblastic appearance of dedifferentiated chondrocytes (Wright et al 1997; Midwood 1998). To identify whether the cells in vitro expressed similar ECM proteins and main integrin receptors identical to those of human articular chondrocytes in vivo (Salter et al 1992; Loeser et al 1995) and after initial cell extraction (Jopanbutra et al 1996), the cultured articular chondrocytes were subjected to immunofluorescent staining using monoclonal antibodies against type II collagen, type VI collagen, keratan sulphate and the integrin subunits $\alpha5$ and $\beta1$. The results demonstrated that in vitro, chondrocytes produced the type II and VI collagens and keratan sulphate (**Fig. 3.1**), and also the integrin subunits $\alpha5$ and $\beta1$ (**Fig. 3.2**).

Negative control

Collagen type II



Collagen type VI



Keratan sulphate

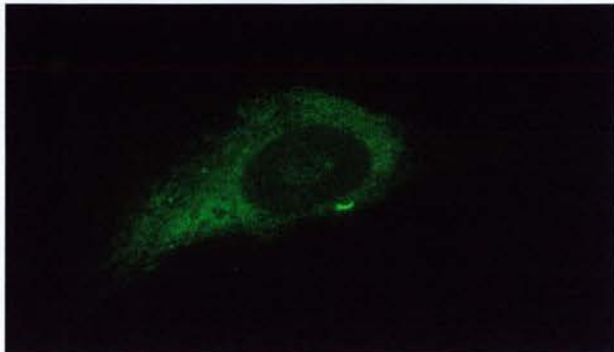
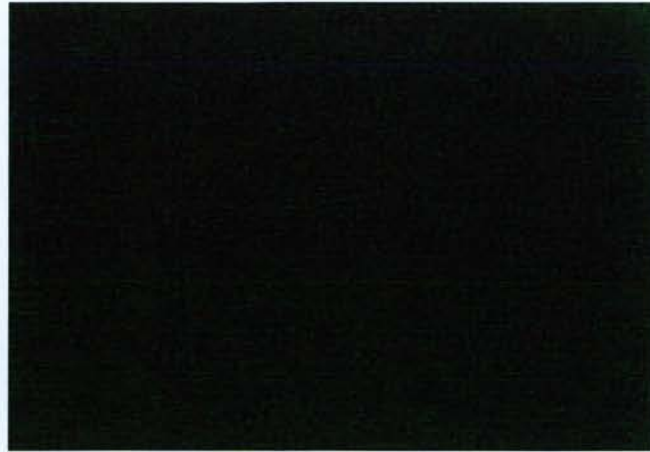
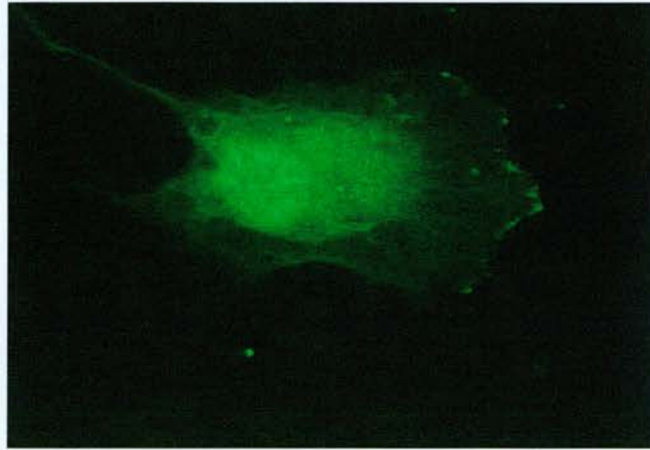


Fig. 3.1 Immunofluorescent staining of cultured chondrocyte: expression of type II, VI collagen and keratan sulphate (magnification x1800)

Negative control



$\alpha 5$ integrin



$\beta 1$ integrin

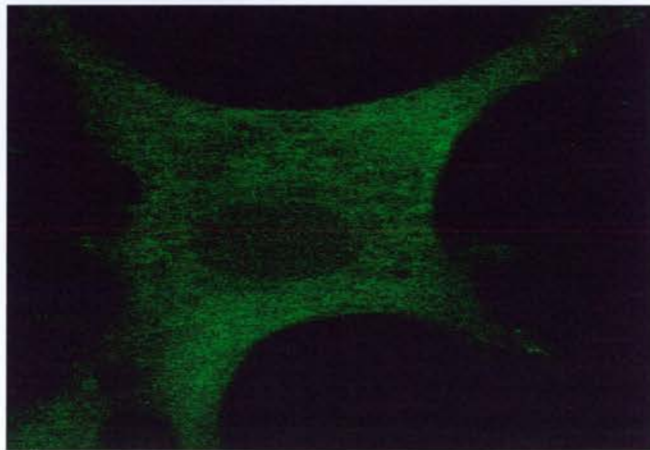


Fig. 3.2 Immunofluorescent staining of cultured chondrocyte: expression of $\alpha 5$, $\beta 1$ integrins (magnification x1890)

3.2. Protein Tyrosine Phosphorylation Induced by Cyclical Strain

Total cases used in this experiment included 16 males (mean age 67 years, range 45-86) and 12 females (mean age 72 years, range 53-93).

Chondrocytes were subjected to cyclical strain at 0.33 Hz for either 1 or 5 min following which the protein was extracted, quantified, tyrosine phosphorylated protein immunoprecipitated and finally detected by western blotting and immunoblotting. The results, shown in **Fig. 3.3,** are representative of experiments on chondrocytes from 6 different donors. Resting chondrocytes showed little intrinsic tyrosine phosphorylated proteins. After 1min mechanical stimulation at 0.33 Hz, three predominant bands of approximately 70, 90, and 125 kD were seen to be tyrosine phosphorylated. However, following mechanical stimulation for 5 min or longer time periods, tyrosine phosphorylation had decreased to baseline levels.

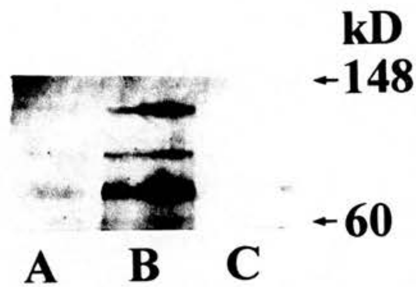


Fig.3.3 Mechanical stimulation induced tyrosine phosphorylation in human articular chondrocytes.

Tyrosine phosphorylated proteins in unstimulated chondrocytes and chondrocytes stimulated by 0.33 Hz cyclical strain were identified by immunoprecipitation and immunoblotting with anti-phosphotyrosine antibody. Tyrosine phosphorylation of three predominant proteins of approximately 70, 90 and 125 kD was increased after 1 min of 0.33 Hz cyclical strain (lane B), but had returned to unstimulated levels (lane A) after 5 min stimulation (lane C). Arrows in the right margin indicate positions of molecular mass markers.

3.3. Mechanical-Induced Tyrosine Phosphorylation Is Integrin and Stretch-Activated Ion Channel Dependent

Previous studies had indicated a signal cascade involving integrins and stretch-activated ion channels in the hyperpolarisation response of normal human articular chondrocytes to 0.33 Hz cyclical mechanical stimulation (Wright et al 1996, 1997).

In order to investigate whether these molecules and channels are involved in the events associated with tyrosine phosphorylation, chondrocytes were stimulated for 1 min in the presence of RGD containing peptides or gadolinium (**Fig. 3.4 & 3.5**). No increased protein tyrosine phosphorylation was observed in chondrocytes which had been treated with GRGDSP for 30 min prior to 1 min mechanical stimulation, whereas cells treated with GRADSP showed tyrosine phosphorylation of 3 proteins of identical molecular weight to cells mechanically stimulated in serum free media alone (**Fig. 3.4**).

Chondrocytes treated with gadolinium (10μM) for 10 min prior to 0.33 Hz mechanical stimulation, showed no induced tyrosine phosphorylation (**Fig. 3.5**).

Treatment of chondrocytes with GRGDSP and GRADSP for 30 min and gadolinium for 10 min, had no effect on protein tyrosine phosphorylation in the absence of mechanical stimulation.

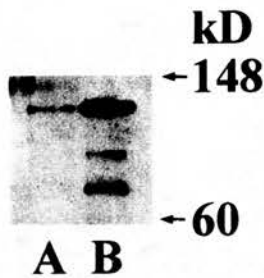


Fig. 3.4 Effect of GRGDSP peptide on mechanically-induced tyrosine phosphorylation.

Treatment of chondrocytes with 100µg/ml GRGDSP for 30 min prior to mechanical stimulation, abolished the tyrosine phosphorylation induced by 1 min 0.33 Hz pressure-induced strain (lane A), whereas treatment with the control peptide GRADSP (100µg/ml) had no effect (lane B). Tyrosine phosphorylated proteins in chondrocytes stimulated by 1 min 0.33 Hz cyclical strain were identified by immunoprecipitation and immunoblotting with anti-phosphotyrosine antibody. Results shown are from a single experiment and are representative of experiments on cells from 5 different donors.

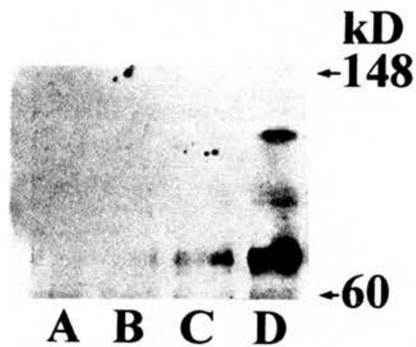


Fig. 3.5 Mechanical stimulation-induced tyrosine phosphorylation in chondrocytes is inhibited by gadolinium, an agent known to block stretch-activated ion channels. Tyrosine phosphorylated proteins were identified by immunoprecipitation of whole cell lysates and immunoblotting with anti-phosphotyrosine antibody. Lane A. resting cells; Lane B. 10μM gadolinium treatment for 10 min without 0.33 Hz cyclical strain; Lane C. 10μM gadolinium treatment for 10 min followed by 1 min 0.33 Hz cyclical strain; Lane D. 1 min 0.33 Hz PIS. There was no increase of tyrosine phosphorylation in cells which were treated with gadolinium with or without 0.33 Hz cyclical strain. Results shown are from a single experiment and are representative of experiments on cells from 3 different donors.

3.4. Tyrosine Phosphorylation of Focal Adhesion Proteins

To identify the specific proteins which were tyrosine phosphorylated in chondrocytes as a result of mechanical stimulation, antibodies against candidate molecules of appropriate molecular weight were used for immunoprecipitation and immunoblotting. Previous work has demonstrated that focal adhesion proteins associated with integrin signaling are phosphorylated on tyrosine residues following mechanical stimulation (Yano et al 1996; Seko et al 1999; Smith et al 1998) and so these proteins were initially investigated. Following immunoprecipitation with anti-phosphotyrosine antibodies and western blotting with specific antibodies against candidate molecules, the protein of approximately 70 kD was identified as paxillin and the 125 kD protein identified as pp125FAK (**Fig. 3.6**). This result was confirmed with the reverse experiment where immunoprecipitation with FAK or paxillin was first performed, followed by immunoblotting with anti-phosphotyrosine. The result showed increased tyrosine phosphorylation of FAK and paxillin following 1 min mechanical strain (**Fig. 3.7i & ii**). In an effort to identify the ~90 kD band, antibodies against α-actinin, vinculin, Stat 6, protein kinase C, and phosphatidylinositol 3-kinase p85, were tested and although these molecules were shown to be expressed by chondrocytes, they were not tyrosine phosphorylated following 1 min of mechanical stimulation. **Fig. 3.8** shows a representative blotting with anti-α-actinin which is expressed in the whole cell lysate, but is not present in the tyrosine phosphorylated immunoprecipitates from resting and mechanically stimulated cells.

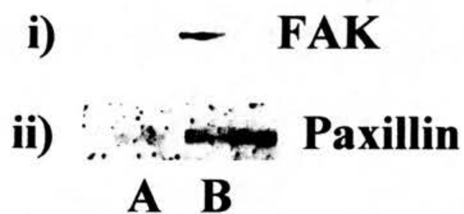


Fig. 3.6 Tyrosine phosphorylation of pp125FAK and paxillin as a result of 1 min of 0.33 Hz mechanical strain.

Results shown are from a single experiment and are representative of experiments on cells from 4 different donors.

(i) Immunoprecipitates of tyrosine phosphorylated proteins from chondrocytes were immunoblotted with pp125FAK antibody. Lane A. resting cells. Lane B. cells stimulated for 1 min by 0.33 Hz cyclical strain.

(ii) Immunoprecipitates of tyrosine phosphorylated proteins from chondrocytes were immunoblotted with anti-paxillin antibody. Lane A. resting cells. Lane B. cells stimulated for 1 min by 0.33 Hz cyclical strain.

(i)

(ii)

Fig. 3.7 FAK (i) and paxillin (ii) tyrosine phosphorylation induced by cyclical strain.

Immunoprecipitation with FAK or paxillin was first performed, followed by immunoblotting with anti-phosphotyrosine monoclonal antibody.

(i) Lane A. resting cells. Lane B. cells stimulated for 1 min by 0.33 Hz cyclical strain.

(ii) Lane A. resting cells. Lane B. cells stimulated for 1 min by 0.33 Hz cyclical strain. Lane C. cells stimulated for 5 min. Lane D. cells stimulated for 10 min. Lane E. cells stimulated for 20 min.

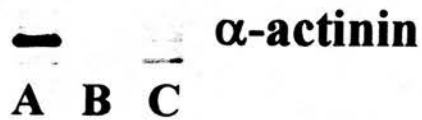


Fig.3.8 Tyrosine phosphorylation of α-actinin did not occur in response to 1 min of cyclical strain.

Lane A. whole cell lysate from resting cells. Lane B. tyrosine phosphorylated immunoprecipitates from resting cells. Lane C. tyrosine phosphorylated immunoprecipitates from cells which had been subjected to 1 min of mechanical stimulation by 0.33 Hz cyclical strain.

3.5. Identification of β-catenin in Human Articular Chondrocytes

The observation that integrin-linked kinase (ILK) is associated with integrin mediated β-catenin signaling (Novak et al 1998) and that adenomatous polyposis coli protein (APC), a molecule associated with β-catenin signaling (Polakis 1997) was expressed by human chondrocytes (Monaghan et al 1999), prompted the question as to whether the 90 kD protein could be β-catenin (McCrea and Gumbiner 1991), although to our knowledge expression of cadherin and β-catenin by articular chondrocyte had not previously been reported.

To investigate expression of these molecules, whole cell lysates of normal and osteoarthritic human adult articular chondrocytes were immunoblotted with antibodies against αE-catenin, β-catenin and γ-catenin. Lysates of HeLa cells, A549 lung epithelial cells, and chondrosarcoma cells were used as controls. Chondrocytes extracted from both normal and osteoarthritic cartilage expressed all three catenins whilst the chondrosarcoma cells expressed only αE-catenin and β-catenin (**Fig. 3.9i**). Probing with a pan cadherin antibody (Geiger et al 1990) showed bands in the lysates from HeLa cells, A549 lung epithelial cells and the chondrosarcoma cells but not in whole cell lysates of chondrocytes derived from normal or osteoarthritic articular cartilage (**Fig. 3.9ii**). Subsequent immunostaining of chondrocytes in culture and cryostat sections of fresh frozen normal and osteoarthritic human articular cartilage demonstrated expression of β-catenin by chondrocytes in vivo. **Fig. 3.10** showed positive immunostaining of β-catenin in chondrocytes in vitro.

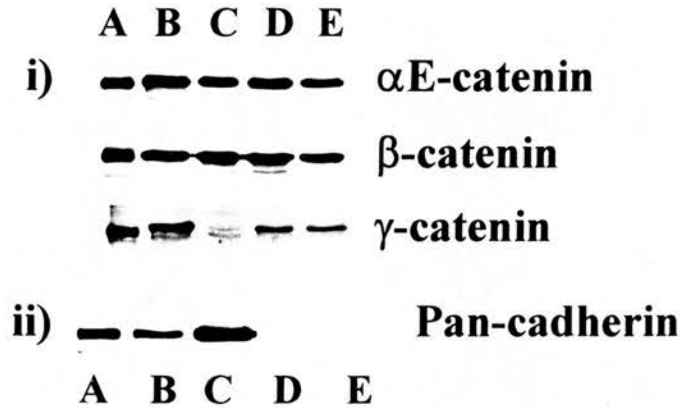
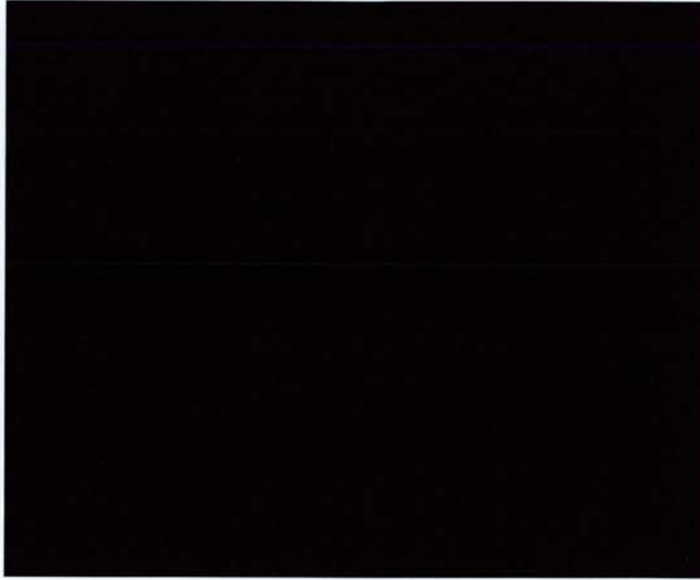


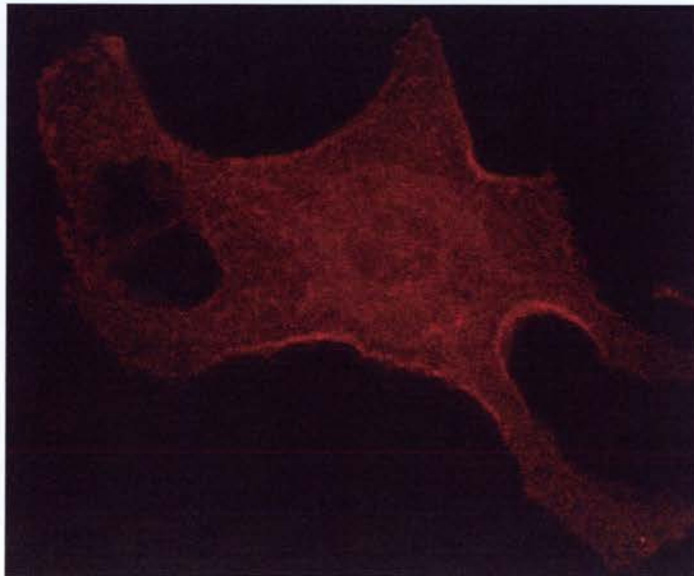
Fig. 3.9 Human articular chondrocytes express αE-catenin, β-catenin, and γ-catenin, but not cadherin. The results shown are from a single experiment and are representative of experiments on cells from 3 different donors.

i) Expression of catenin family. Equal amounts of whole cell lysates were immunoblotted with αE-catenin, β-catenin, and γ-catenin. Lane A. HeLa cells; Lane B. A549 lung epithelial cells; Lane C. chondrosarcoma cells; Lane D. normal human articular chondrocytes; Lane E. chondrocytes from osteoarthritic human cartilage. HeLa cells and A549 cells were used as positive controls. αE-catenin, β-catenin, and γ-catenin are expressed by normal and osteoarthritic chondrocytes whereas the chondrosarcoma cells expressed αE-catenin and β-catenin only.

ii) Cadherin expression. Following gel electrophoresis, whole cell lysates were immunoblotted with a pan-cadherin monoclonal antibody. Cadherin were not identified in lysates of normal and osteoarthritic chondrocytes (lanes D and E) whereas a single band of approximately 135 kD was identified in chondrosarcoma cells.



Negative control



β-catenin

Fig. 3.10 Immunofluorescent staining of cultured chondrocyte: expression of β-catenin (magnification x2025)

3.6. Tyrosine Phosphorylation of β-catenin Induced by Mechanical Stimulation

Following identification of β-catenin in adult human chondrocytes, experiments were undertaken to establish whether β-catenin was indeed the 90 kD protein tyrosine phosphorylated following 1 min of mechanical stimulation. Normal human articular chondrocytes were subjected to 1 min cyclical strain, the extracted proteins immunoprecipitated with anti-phosphotyrosine, and immunoblotted with β-catenin monoclonal antibody. The results show that β-catenin is tyrosine phosphorylated in human chondrocytes following 1 min of 0.33Hz mechanical stimulation (**Fig. 3.11i**). Experiments in which immunoprecipitation with anti-β-catenin was undertaken before immunoblotting with the phosphotyrosine monoclonal antibody showed similar results, with a single band of approximately 90 kD being identified. Incubation of chondrocytes with GRGDSP, but not GRADSP for 30 min, inhibited the mechanically-induced tyrosine phosphorylation of β-catenin (**Fig. 3.11ii**).

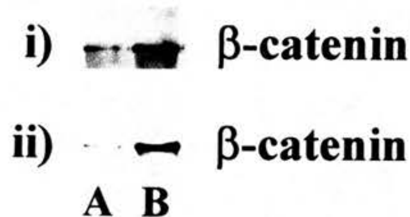


Fig. 3.11 Tyrosine phosphorylation of β-catenin following mechanical stimulation of human articular chondrocytes. Results shown are from a single experiment and are representative of experiments on cells from 4 different donors.

i) Immunoprecipitates of tyrosine phosphorylated proteins from resting chondrocytes (lane A) and from chondrocytes following 1 min of 0.33 Hz cyclical strain (lane B) were immunoblotted with β-catenin monoclonal antibody. Tyrosine phosphorylation of β-catenin was increased following 1 min 0.33 Hz cyclical strain.

ii) Immunoprecipitates of tyrosine phosphorylated proteins from chondrocytes stimulated by 0.33 Hz cyclical strain for 1 min following treatment with GRGDSP (lane A) or GRADSP (lane B) were immunoblotted with β-catenin mAb. GRGDSP inhibited mechanical-induced tyrosine phosphorylation of β-catenin.

3.7. Coimmunoprecipitation of β-Catenin with pp125FAK and Colocalization with pp125FAK and α5-Integrin

Whole cell lysates of mechanically stimulated and unstimulated human articular chondrocytes were immunoprecipitated with anti-β-catenin monoclonal antibody and following gel electrophoresis the precipitate was immunoblotted with antibodies against a number of focal adhesion proteins including vinculin, pp125FAK and paxillin, and also α5 and β1 integrins. The results showed coimmunoprecipitation of pp125FAK with β-catenin in both unstimulated cells and also in cells which had been subjected to 1 min mechanical stimulation (**Fig. 3.12**), whereas no coimmunoprecipitation of the other molecules investigated was observed. Coimmunoprecipitation of pp125FAK and β-catenin was unchanged following 30 min incubation of chondrocytes with GRGDSP or GRADSP prior to mechanical stimulation (**Fig. 3.13**). Double staining immunofluorescence showed colocalization of β-catenin with pp125FAK and α5 integrin at the chondrocyte cell membrane (Fig. **3.14**). The colocalization at the cell periphery was confirmed by computer analysis (TCS NT version 1.0, Leica Lasertechnik GmbH Heidelberg) showing yellow pixels after merging the green and red images and the same location of the peak of the fluorescent intensity curve. In **Fig. 3.15** is shown the colocalization of FAK (green curve) and β-catenin (red curve) and in **Fig. 3.16** the colocalization of α5 integrin (green curve) and β-catenin (red curve).

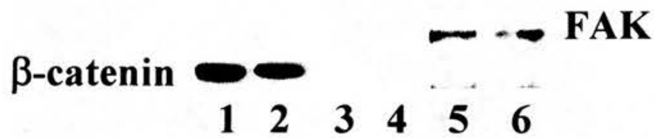


Fig. 3.12 β-catenin coimmunoprecipitates with pp125FAK in human articular chondrocytes.

Results shown are from a single experiment and are representative of experiments on cells from 5 different donors. Protein extracts from chondrocytes, either resting (lanes 1, 3 and 5) or following 1 min 0.33 Hz cyclical strain (lanes 2, 4 and 6) were immunoprecipitated with β-catenin and immunoblotted with antibodies to: β-catenin (lanes 1, 2); secondary antibody alone (lanes 3, 4); and pp125FAK (lanes 5, 6).

Fig. 3.13 Coimmunoprecipitation of β-catenin and FAK, the association of which was unchanged following 30 min incubation of chondrocytes with GRGDSP or GRADSP. Lane A. cells treated with GRGDSP followed by 1 min cyclical strain. Lane B. cells treated with GRADSP followed by 1 min cyclical strain. Lane C. cells treated with GRGDSP without mechanical stimulation. Lane D. cells treated with GRADSP without mechanical stimulation.

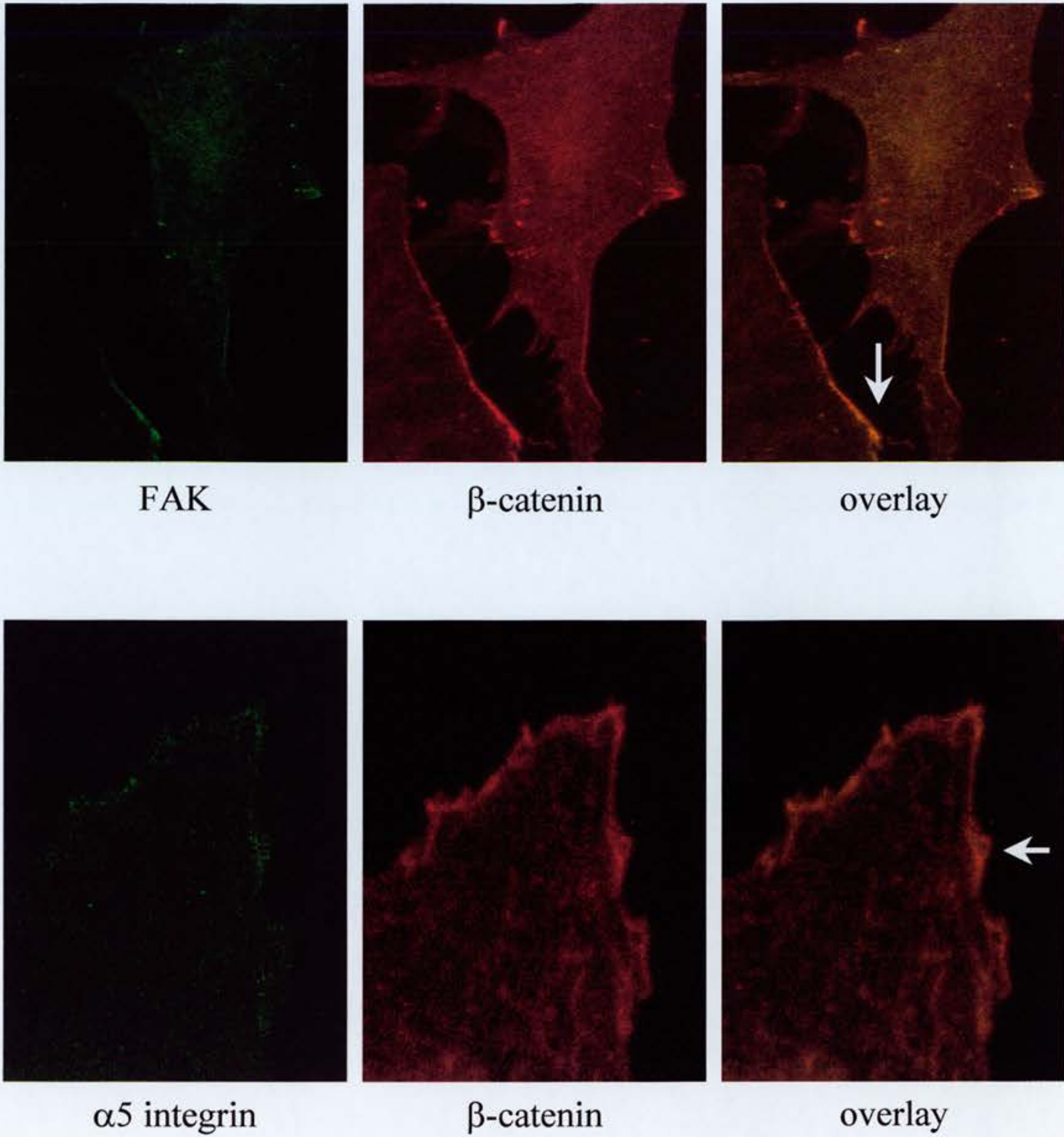
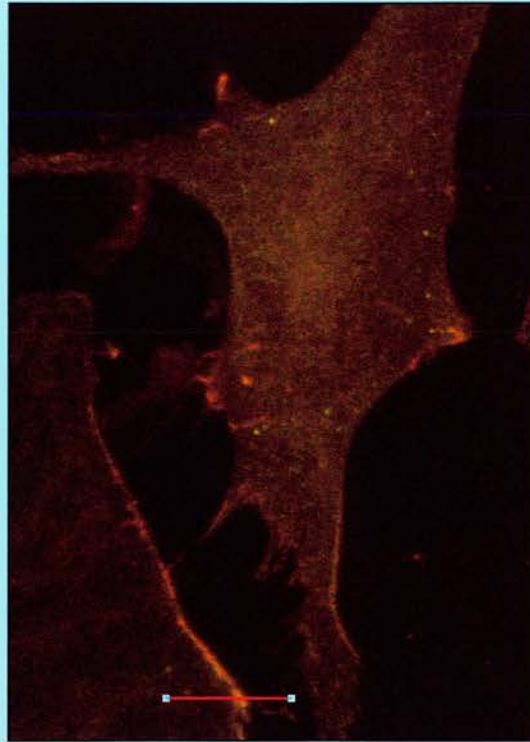
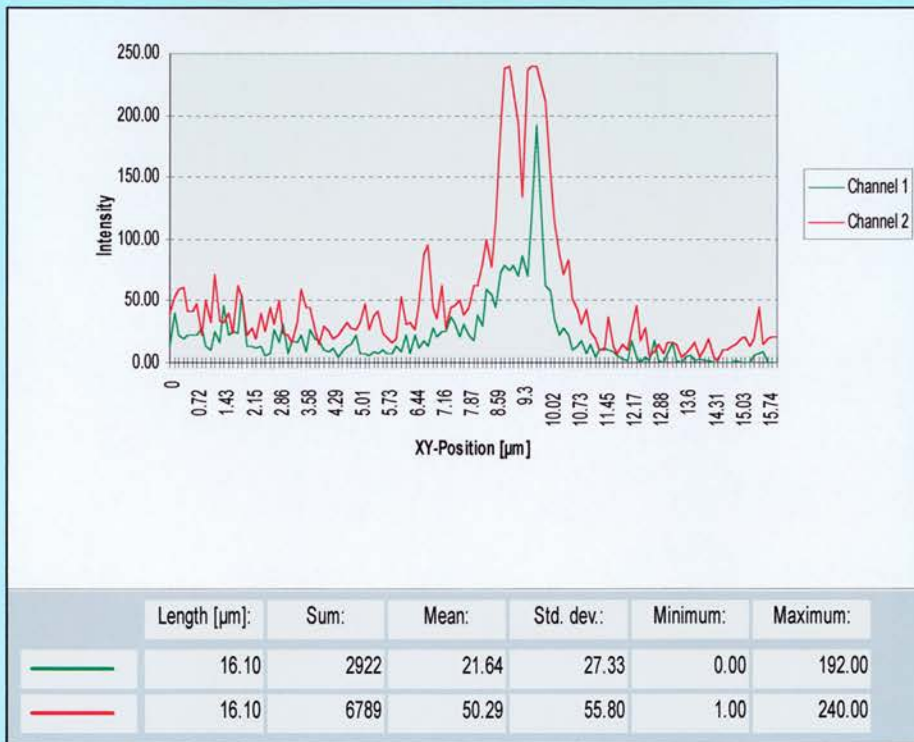


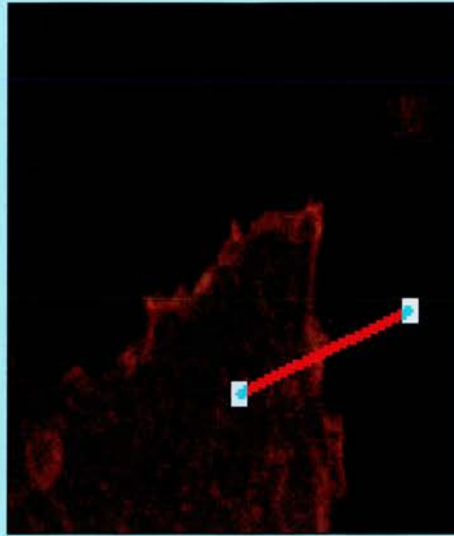
Fig. 3.14 β-catenin colocalizes with pp125FAK and α5 integrin in human articular chondrocytes. Results shown are from a single experiment and are representative of experiments on cells from 3 different donors. Unstimulated human articular chondrocytes were fixed and subjected to double immunofluorescence staining for: Detection of pp125FAK (green) and β-catenin (red) (upper row) and detection of α5 integrin (green) and β-catenin (red) (lower row). Colocalization sites (arrows) appear as yellow pixels after merging the images. (magnification: upper row x1510, lower row x4530)

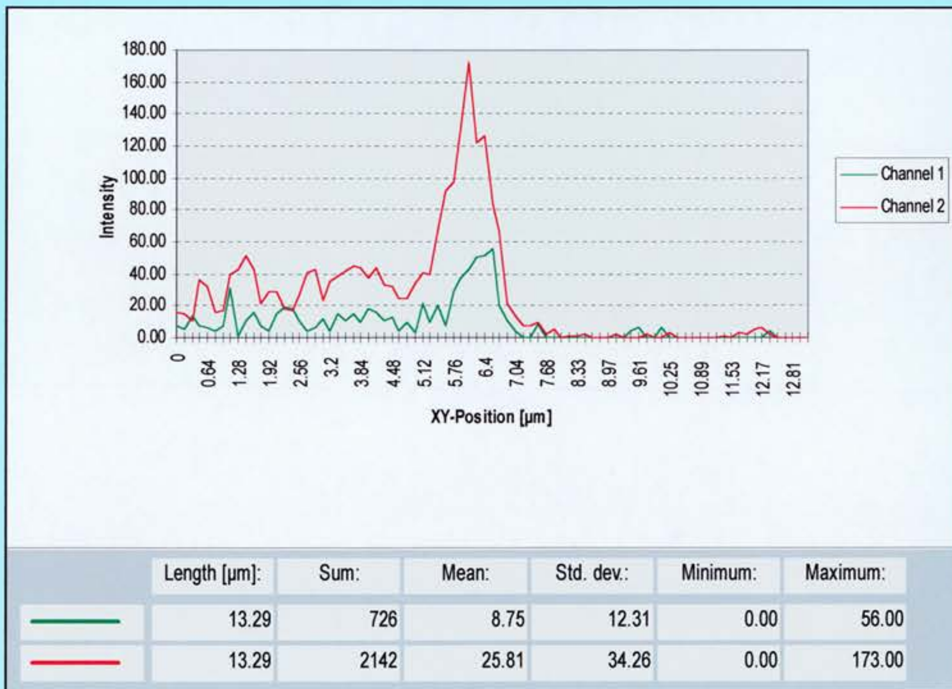




	Length [µm]:	Sum:	Mean:	Std. dev.:	Minimum:	Maximum:
(green line)	16.10	2922	21.64	27.33	0.00	192.00
(red line)	16.10	6789	50.29	55.80	1.00	240.00

Fig. 3.15 Colocalization of FAK and β-catenin. Unstimulated human articular chondrocytes were fixed and subjected to double immunofluorescence staining for detection of pp125FAK (green curve) and β-catenin (red curve) examined by confocal microscopy. The peak of both fluorescent intensity curves at the same position indicates colocalization sites.





	Length [µm]:	Sum:	Mean:	Std. dev.:	Minimum:	Maximum:
green	13.29	726	8.75	12.31	0.00	56.00
red	13.29	2142	25.81	34.26	0.00	173.00

Fig. 3.16 Colocalization of α5 integrin and β-catenin. Unstimulated human articular chondrocytes were fixed and subjected to double immunofluorescence staining for α5 integrin (green curve) and β-catenin (red curve) examined by confocal microscopy. The peak of both fluorescent intensity curves at the same position indicates colocalization sites.

DISCUSSION

This study has demonstrated that 0.33 Hz mechanical stimulation of normal human articular chondrocytes results in a rapid tyrosine phosphorylation of pp125FAK, paxillin and β-catenin, the process being dependent on integrins and stretch-activated ion channels. Immunoprecipitation of β-catenin with pp125FAK and double staining immunofluorescence, was also demonstrated the colocalization of β-catenin with α5 integrin and pp125FAK. This supports a role for integrin-β-catenin signaling in the chondrocyte response to mechanical stimulation at 0.33 Hz.

3.8. Mechanically-induced Protein Tyrosine Phosphorylation Is Integrin and Mechanosensitive Ion Channel Dependent

It has been shown that cyclical mechanical stimulation, at 0.33 Hz, results in membrane hyperpolarisation of human articular chondrocytes as a result of activation of SK channels (Wright et al 1992). The use of pharmacological inhibitors of intracellular signalling molecules, stretch-activated ion channels and specific antibodies against integrins, cytokines and cytokine receptors has enabled the signalling cascade activated in human articular chondrocytes by 0.33 Hz pressure-induced strain which results in membrane hyperpolarisation to be partially elucidated (Wright et al 1996, 1997; Millward-Sadler et al 1999). These studies have shown that membrane hyperpolarisation is the result of mechanically-induced secretion of IL-4. Secreted IL-4 binds to type II IL-4 receptors and stimulates a signal cascade involving phospholipase C and PKC, leading to activation of SK channels. Specific blockade of α5β1 integrins, stretch-activated ion channels and tyrosine kinases inhibits mechanically-induced membrane hyperpolarisation, but has no effect on the hyperpolarisation produced by exogenously added IL-4, which is consistent with a role for these molecules upstream of IL-4 release, in the mechanotransduction pathway. As both RGD peptides and gadolinium inhibit

mechanically-induced tyrosine phosphorylation of pp125FAK, paxillin and β catenin, it appears that integrins and stretch-activated ion channels are involved in events upstream of tyrosine phosphorylation, in the mechanotransduction cascade.

Mechanical stresses applied to a system such as chondrocytes attached to a tissue culture dish are transferred to adherent cells through their adhesive contacts with the surrounding extracellular matrix. RGD containing peptides will compete with RGD containing matrix proteins for integrin binding and will decrease cell binding with matrix proteins. It has been shown previously that recognition of the 0.33 Hz mechanical stimulus by human articular chondrocytes (and human bone cells) is via α5β1 integrin (Wright et al 1997; Salter et al 1997). This integrin is the classical fibronectin receptor and binds to fibronectin via an RGD motif (Hynes 1992; Pierschbacher and Ruoslahti 1984). In the presence of RGD peptides, cell attachment to fibronectin via α5β1 integrin is inhibited and therefore mechanical stresses which would normally be transferred to the cell via α5β1-fibronectin associations and which result in activation of intracellular signaling cascades, will be abrogated.

A wide range of cells express mechanosensitive ion channels which permit the flow of ions across the cell membrane in response to shear and stretch (Lansman et al 1987; Adams et al 1989; Sachs 1991; Olesen and Bundgaard 1992, 1993; Himmel et al 1993). These mechanosensitive ion channels are blocked by gadolinium (Yang and Sachs 1989) which has previously been shown to inhibit the hyperpolarisation response of bone cells and chondrocytes to 0.33 Hz mechanical stimulation (Wright et al 1997; Salter et al 1997). The present study has shown that both integrin function and SAC activity are required for tyrosine phosphorylation of pp125FAK, paxillin and β-catenin in the response of the chondrocyte to cyclical mechanical stimulation. A transient increase in intracellular Ca^{2+} concentration as a result of its entry from the extracellular fluid through gadolinium sensitive ion channels appears to be one of the earliest events involved in the response of chondrocytes, endothelial cells and lung cells to mechanical stress (Guilak et al 1999; Goligorsky

1988; Liu et al 1994) and may be necessary for integrin-dependent tyrosine phosphorylation of focal adhesion associated molecules (Alessandro et al 1998). Gadolinium sensitive ion channels have also been shown to be involved in the activation of c-src and in the tyrosine phosphorylation of FAK and paxillin, as induced by cyclical mechanical stretch, in human umbilical endothelial cells (Naruse et al 1998a, 1998b). In contrast, the stretch-induced expression of early response genes and increased protein synthesis of cardiac myocytes is not inhibited by either gadolinium or RGD containing peptides (Sadoshima et al 1992). Mechanical stimulation-induced activation of ERK1/2 in endothelial cells (Ikeda et al 1998, 1999), MAP kinase activation (Yamazaki et al 1998) and STAT1 and STAT3 phosphorylation (Pan et al 1999) in cardiomyocytes are also not inhibited by gadolinium. The response of cells, including chondrocytes and bone cells, to mechanical stimulation is in part dependent on the nature of mechanical stimulus (Salter et al 1997; Sah et al 1989). A variety of different mechanotransduction pathways, with participation of different signaling molecules, in the recognition and cellular responses to mechanical stimuli, are likely to exist (Salter et al 1997).

3.9. Tyrosine Phosphorylation of Focal Adhesion Proteins

Increased tyrosine phosphorylation of paxillin and pp125FAK following mechanical stimulation, has been shown by other workers using different cell types which have been stimulated by a variety of *in vitro* techniques (Ishida et al 1996; Yano et al 1996; Smith et al 1998; Li et al 1997). This suggests their widespread involvement in the mechanotransduction processes, although the time course of the tyrosine phosphorylation events varies between different cell types. Yano et al. (1996), using bovine aortic endothelial cells, showed that the level of phosphotyrosine in pp125FAK was increased between 30 min and 4 hours of cyclic strain, whereas tyrosine phosphorylation of paxillin was only significantly increased after 4 hours of mechanical stimulation. In contrast, Smith et al. (1998) demonstrated that smooth muscle cells subjected to 30 min of mechanical strain had increased levels of tyrosine phosphorylation of both pp125FAK and paxillin.

Tyrosine phosphorylation of paxillin was sustained for at least 4 hours after the discontinuance of the strain. Tyrosine activation of paxillin was of particular interest in smooth muscle cell morphology because paxillin was concentrated in focal adhesions, sites of contractile filament termination, and dense bodies. The transient tyrosine phosphorylation events described in the present study have also been shown to be present in other systems. Rat cardiac myocytes show a transient increase in FAK tyrosine phosphorylation, maximal at 5 min following mechanical stimulation (Seko et al 1999). Sadoshima and Izuma (1993) have shown a transient increase in tyrosine phosphorylation of 120 kD and 60 kD proteins, most likely FAK and paxillin, in cardiac myocytes, which is maximal at 1 min and returns to resting levels by 5-10 minutes. The tyrosine phosphorylation and dephosphorylation events may be associated with the activation of tyrosine phosphatase. The recognised importance of paxillin and pp125FAK tyrosine phosphorylation in integrin adhesion-activated signaling cascades (Burridge et al 1992) would be consistent with similar roles for these molecules in integrin-activated mechanotransduction.

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 organised 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-autophoshorylation 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 subunits (Giancotti and Ruoslahti 1999). These could be possible mechanisms by which integrin stimulation resulting from mechanical stress leads to tyrosine phosphorylation.

Protein tyrosine phosphorylation can regulate protein function by promoting protein-protein interactions with conserved motifs, such as SH2 domains or PTB (phosphotyrosine binding) domains, which bind phosphorylated tyrosine residues within specific amino acid motifs (Songyang et al 1993; Kavanaugh et al 1995). These interactions then provide important links to downstream signalling pathways (Laflamme et al 1997).

3.10. Tyrosine Phosphorylation of β-catenin Induced by Mechanical Stimulation

The identification of the tyrosine phosphorylation of β-catenin in adult human articular chondrocytes following mechanical stimulation was an unsuspected finding. Expression of β-catenin does not appear to be an in vitro artefact, as immunohistochemical studies of both normal and osteoarthritic human articular cartilage show in vivo expression of β-catenin (Salter et al unpublished observation). A role for β-catenin in the signal transduction pathways of a number of different cell types is now recognised (Gumbiner 1995; Daniel and Reynolds 1997). β-catenin is known to interact with adhesion proteins, transcription factors, tyrosine kinases, phosphatases as well as the tumor suppressor adenomatous polyposis coli (APC) protein (Ilyas and Tomlinson 1997; Korinek et al 1997; Morin et al 1997; Rubinfeld et al 1997; Daniel and Reynolds 1997; Ben-Ze'ev and Geiger 1998). β-catenin is a component of the adherans junction and acts as a physical linker of the cytoskeleton to cadherin (Aberle et al 1994; Jou et al 1995). Tyrosine phosphorylation of cadherin-catenin complexes has been shown to modulate cell adhesiveness (Daniel and Reynolds 1997). N-cadherin has been shown to be involved in chick embryonic limb chondrogenesis (Oberlender and Tuan 1994a) although its expression is lost in mature cartilage (Oberlender and Tuan 1994b). Consistent with these observations we were unable to identify cadherin expression in human articular chondrocytes obtained from adult individuals. Adherans junctions support calcium dependent cell-cell adhesion, but in vivo, articular

chondrocytes are separated from neighbouring cells by a pericellular and a territorial matrix and do not normally make cell-cell contact (Young and Heath 2000). Similarly, in our in vitro culture system, chondrocytes are grown in sparse monolayer culture and cell contact is minimal. These cells do however express αE-catenin and γ-catenin as well as β-catenin, suggesting roles for these molecules in non-cadherin associated signaling. Interestingly, cultured chondrosarcoma cells showed upregulation of cadherin expression and loss of γ-catenin. The reason for this differential expression is not clear, but it may be associated with neoplastic transformation. For example, it has been shown that overexpression of γ-catenin in SV40-transformed 3T3 cells can suppress the tumorigenicity of the cells in mice and also that transfection of γ-catenin into a human renal carcinoma cell line resulted in a dose-dependent suppression of tumor formation by these cells in nude mice (Simcha et al 1996).

Inhibition of mechanically induced tyrosine phosphorylation of β-catenin by RGD containing peptides, colocalization of β-catenin with both pp125FAK and α5 integrin in chondrocytes, as well as coimmunoprecipitation of pp125FAK with β-catenin would suggest a previously unrecognized role for β-catenin in integrin-mediated mechanotransduction. Evidence is accumulating for the association of β-catenin with integrin signaling in other systems via a serine–threonine protein kinase (integrin-linked kinase (ILK)) (Novak et al 1998). ILK interacts with the cytoplasmic domains of the integrin β1, β2, and β3 subunits and has been shown to induce translocation of β-catenin to the nucleus, where it is involved in enhancement of LEF-1 transcriptional activity (Novak et al 1998). ILK activity is also stimulated following cell adhesion to fibronectin as a result of the action of PI3-kinase (Delcommenne et al 1998; Wu et al 1998).

The kinase involved in tyrosine phosphorylation of β-catenin following mechanical stimulation remains to be identified. Tyrosine phosphorylation of β-catenin has been shown to be mediated by both receptor tyrosine kinases and cytoplasmic protein kinases of the Src family (Daniel and Reynolds 1997). Thus, β-catenin

binds to, and is phosphorylated by, the epidermal growth factor receptor (Hoschuetzky et al 1994) and β-catenin is heavily tyrosine-phosphorylated in Src-transformed cells (Behrens et al 1993). Co-immunoprecipitation of pp125FAK with β-catenin in the present study implies a direct functional relationship. pp125FAK may either induce tyrosine phosphorylation of β-catenin directly, or in association with Src family kinases, which have been shown to be involved in fibronectin / α5β1 integrin mediated signaling (Parsons and Parsons 1997; Hanks and Polte 1997) on which the chondrocyte responses to mechanical stimulation at 0.33 Hz are dependent (Wright et al 1997).

In summary, this study has shown that 0.33 Hz mechanical stimulation of human adult articular chondrocytes results in transient tyrosine phosphorylation of the protein kinase pp125FAK, paxillin, a focal adhesion complex adaptor protein and the multifunctional signaling molecule, β-catenin. Tyrosine phosphorylation of all three molecules in response to mechanical stimulation is integrin-dependent and requires the participation of gadolinium sensitive SAC. Colocalization of α5 integrin with β-catenin and coimmunoprecipitation of pp125FAK with β-catenin, support the existence of a novel integrin-β-catenin signaling pathway in normal chondrocytes which is activated by cyclical mechanical strain. The roles of β-catenin in downstream events such as IL-4 release (Millward-Sadler et al 1999) and changes in proteoglycan synthesis (Bavington et al 1996) which occur as a result of mechanical stimulation of human articular chondrocytes in our in vitro system, remain to be defined.

CHAPTER 4.

INVOLVEMENT OF RACK1 IN THE RESPONSE OF CHONDROCYTES TO MECHANICAL STIMULATION

RESULTS

Total cases used in this experiment included 7 males (mean age 71 years, range 45-79), 10 females (mean age 64 years, range 51-83) and the immortalized human chondrocyte cell line C-20/A4.

4.1. Response of the Immortalized Human Chondrocyte Cell Line C-20/A4 to Cyclical Mechanical Stimulation

To overcome the difficulties in obtaining sufficient cartilage from autopsy cases, the immortalized human chondrocyte cell line C-20/A4 (Goldring et al 1994) was introduced to the chondrocyte mechanotransduction study.

In a preliminary series of experiments the electrophysiological response of the cell line to pressure-induced strain was examined to see if the cells behaved in a similar manner to primary human chondrocytes. C-20/A4 cells at a density of 5×10^4 were subjected to 0.33 Hz cyclical mechanical strain for 20 min. The membrane potentials of ten cells were examined before and after cyclical mechanical strain. A hyperpolarisation response, similar to that of primary normal human articular chondrocytes, was induced by mechanical stimulation. **Table 4.1** shows the results from two representative experiments. Assessment of tyrosine phosphorylation in C-20/A4 cells following mechanical stimulation was performed. Cells were subjected to 0.33 Hz cyclical strain for 1, 5, and 10 min, conditions identical to those studied with primary chondrocytes. The results showed (**Fig. 4.1**) that protein tyrosine phosphorylation was upregulated following 1 and 5 min stimulation, however had

declined the level after 10 min stimulation. More bands (approximately 8 bands) were found to be present than in primary normal chondrocytes. Paxillin was also identified as a tyrosine phosphorylated protein in C-20/A4 cells (**Fig. 4.2**).

Table 4.1 C-20/A4 cell hyperpolarisation response to cyclical mechanical strain

	n	Membrane potential (-mV) (mean±SEM)			
		Resting	Post strain	%change post strain	P value
Dish 1	10	13.7±0.6	19.1±0.4	+39	<0.0001
Dish 2	10	14.5±0.7	20.0±0.4	+38	<0.0001

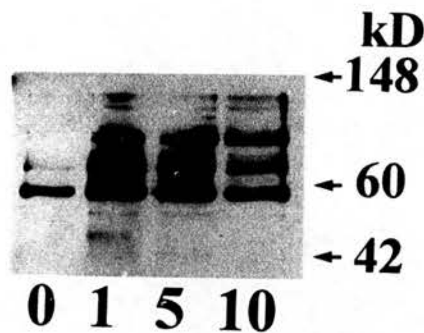


Fig. 4.1 Mechanical stimulation induced tyrosine phosphorylation in the immortalized human articular chondrocyte cell line C-20/A4. Tyrosine phosphorylated proteins in unstimulated chondrocytes and chondrocytes stimulated by 0.33 Hz cyclical strain were identified by immunoprecipitation and immunoblotting with anti-phosphotyrosine monoclonal antibody. Protein tyrosine phosphorylation was upregulated after 1 min (lane 1) and 5 min (lane 5) of mechanical stimulation compared with resting cells (lane 0). However, the level had declined after 10 min (lane 10) of mechanical stimulation. Arrows in the right margin indicate positions of molecular mass markers.

IP: Phosphotyrosine
IB: Paxillin

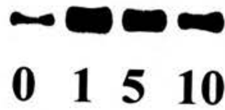


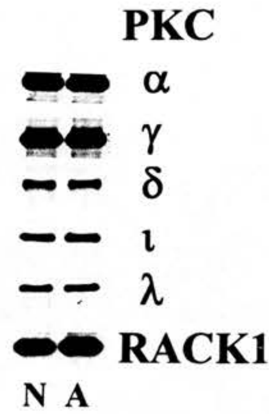
Fig. 4.2 Paxillin tyrosine phosphorylation in C-20/A4 cells in response to cyclical mechanical strain. Lane 0: resting cells. Lane 1: cells after 1 min cyclical strain. Lane 5: cells after 5 min cyclical strain. Lane 10: cells after 10 min cyclical strain. The peak tyrosine phosphorylation of paxillin was induced by 1 min of mechanical stimulation.

4.2. Expression of PKC Isozymes and RACK1 in Primary Chondrocytes and C-20/A4 Cells

To investigate the expression of PKC isozymes in cultured chondrocytes, whole cell lysates of primary cultured chondrocytes and C-20/A4 cells were subjected to western blotting with a panel of antibodies against the PKC isozymes including subtype α (82 kD), β (80 kD), γ (80 kD), δ (78 kD), ε (90 kD), η (82 kD), θ (79 kD), ι (74 kD), λ (74 kD), and RACK1 (36 kD). The results showed that PKC α, γ, δ, ι, λ, and RACK1 were expressed in normal and osteoarthritic cultured chondrocytes (**Fig. 4.3i**). C-20/A4 cells expressed isozymes α, ι, λ, and RACK1, similar to primary cultured chondrocytes, but no expression of isozymes γ and δ. PKCε was expressed in low amounts by C-20/A4 cells, but not by primary cultures of human chondrocytes (**Fig. 4.3ii**).

To assess whether RACK1 was associated with PKC, protein lysates from primary cultures of normal chondrocytes (three cases) were immunoprecipitated with anti-PKC monoclonal antibody (Sigma) and then immunoblotted with anti-RACK1 monoclonal antibody. The anti-PKC monoclonal antibody (clone MC5) used binds to the α, $\beta 1$ and $\beta 2$ PKC isoforms, but not the γ isoform. Coimmunoprecipitation of RACK1 with PKC was shown to occur (**Fig.4.4**).

(i)



(ii)

Fig. 4.3 Identification of PKC isozymes in primary cultured chondrocytes and C-20/A4 cells.

(i) Normal (lane labelled "N") and osteoarthritic (lane labelled "A") cultured chondrocytes expressed PKC α, γ, δ, ι, λ, and RACK1.

(ii) The immortalized human articular chondrocyte cell line C-20/A4 expressed PKC isozymes α, ι, λ, and very low levels of ε subtype**,** and RACK1.

IP: PKC

Fig. 4.4 Coimmunoprecipitation of RACK1 with PKC. Cell lysates from three cultures of normal chondrocytes were immunoprecipitated with PKC and then immunoblotted with RACK1. (The PKC monoclonal antibody used recognized PKC isozymes α and β, but not the γ isoform. Since PKCβ was not present in the articular chondrocytes, it indicated that RACK1 here bound to PKCα.)

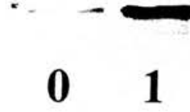
4.3. RACK1 and β1-Integrin Association in Cultured Chondrocytes

Primary articular chondrocytes were subjected to 1 min of 0.33 Hz mechanical stimulation. The cells, after cyclical strain, along with unstimulated control cells, were washed and extracted. The proteins (500 μg/ml), were immunoprecipitated with monoclonal anti-β1-integrin antibody, and then immunoblotted with monoclonal anti-RACK1 antibody. The results showed upregulation of β1-integrin-RACK1 interaction following 1 min mechanical stimulation in normal articular chondrocytes (**Fig. 4.5i**). The same experiments were carried out with OA chondrocytes. However, the results showed no upregulation of β1-integrin-RACK1 interaction after 1 min mechanical stimulation in these cells (**Fig. 4.5ii**).

By immunofluorescence, in vitro chondrocytes were stained with anti-β1-integrin-FITC and anti-RACK1 monoclonal antibodies. The double stained immunofluorescence specimens were viewed using confocal microscopy. After merging of the images, β1-integrin and RACK1 were shown to colocalize together (**Fig. 4.6**).

(i)

IP: β1 integrin
IB: RACK1



(ii)

IP: β1 integrin
IB: RACK1

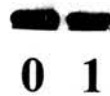
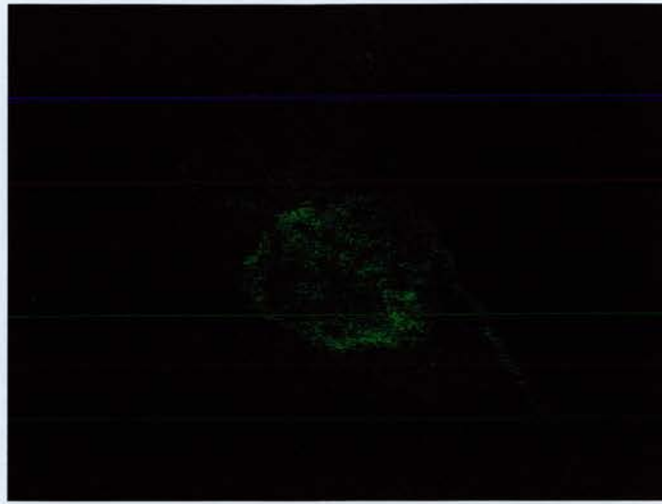


Fig. 4.5

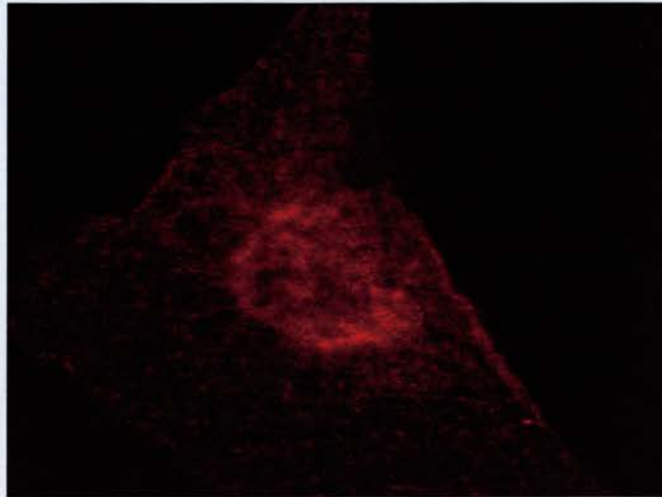
(i) Upregulation of β1-integrin-RACK1 interaction after 1 min cyclical strain in normal articular chondrocytes. Left lane (labelled "0") was unstimulated cells and right lane (labelled "1") was mechanically stimulated cells.

(ii) No upregulation of β1-integrin-RACK1 interaction after 1 min cyclical strain in OA chondrocytes. Left lane (labelled "0") was unstimulated cells and the right lane (labelled "1") was mechanically stimulated cells.

β1 integrin



RACK1



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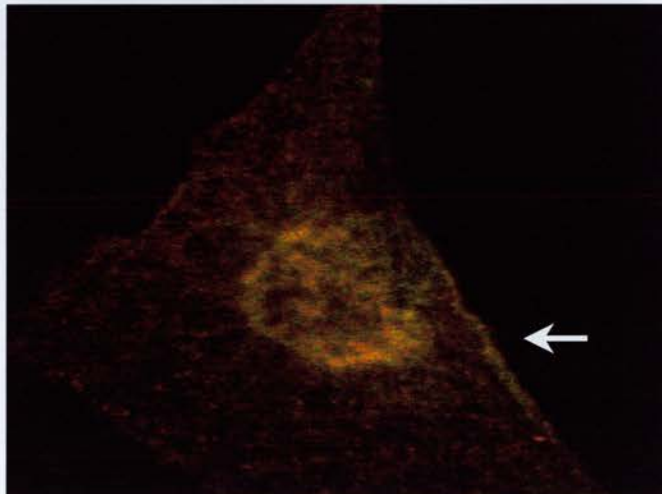


Fig. 4.6 Colocalization of β1 integrin and RACK1. Unstimulated human articular chondrocytes were subjected to double immunofluorescent staining for detection of β1 integrin (green) and RACK1(red). Colocalization sites (arrow) appear as yellow pixels after the images were merged. (magnification x2133)

4.4. Effects of the PKC Inhibitor Chelerythrine and RGD Peptides

The role of the PKC inhibitor chelerythrine on the β1 integrin and RACK1/PKCα association response to mechanical strain was investigated. Four groups of primary chondrocytes and C-20/A4 cells were taken:

Group 1	Resting cells.
Group 2	Cells receiving 1 min of mechanical stimulation.
Group 3	Cells + chelerythrine 10 μM, 30 min.
Group 4	Cells + chelerythrine 10 μM, 30 min + 1 min of cyclical mechanical strain.

Cell lysates were prepared and immunoprecipitated with β1 integrin and then immunoblotted with RACK1 or PKCα. The results showed that the upregulation of β1-integrin-RACK1 and β1-integrin-PKCα interaction induced by mechanical stimulation was inhibited by chelerythrine (**Figs. 4.7 & 4.8**).

In order to investigate whether integrin signalling pathways are involved in the upregulation of integrin-RACK1 interaction, primary chondrocytes and C-20/A4 cells were mechanically stimulated for 1 min in the presence of RGD containing peptides. The results showed that GRGDSP inhibited integrin-RACK1/PKCα interaction induced by mechanical stimulation, whereas the control peptide GRADSP had no effect (**Figs. 4.9 & 4.10**).

IP: β1 integrin
IB: RACK1

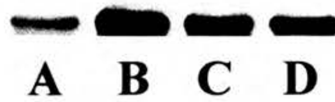


Fig. 4.7 PKC inhibitor inhibited the upregulation of β1-integrin-RACK1 interaction induced by mechanical stimulation in C-20/A4 cells. Lane A: resting cells. Lane B: cells stimulated for 1 min by 0.33 Hz cyclical strain. Lane C: 10 μM chelerythrine chloride treatment for 30 min without mechanical stimulation. Lane D: 10 μM chelerythrine chloride treatment for 30 min following 1 min cyclical mechanical strain.

IP: β1 integrin
IB: PKCα

Fig. 4.8 PKC inhibitor inhibited the upregulation of β1-integrin-PKCα interaction induced by mechanical stimulation in C-20/A4 cells. Lane A: resting cells. Lane B: cells stimulated for 1 min by 0.33 Hz cyclical strain. Lane C: 10 μM chelerythrine chloride treatment for 30 min without mechanical stimulation. Lane D: 10 μM chelerythrine chloride treatment for 30 min following 1 min cyclical mechanical strain.

IP: β1 integrin
IB: RACK1

Fig. 4.9 GRGDSP inhibited the upregulation of β1 integrin-RACK1 interaction induced by mechanical stimulation in primary chondrocytes.

Lane A: cells treated with GRGDSP followed by 1 min cyclic strain. Lane B: cells treated with GRADSP followed by 1 min cyclic strain. Lane C: cells treated with GRGDSP without mechanical stimulation. Lane D: cells treated with GRADSP without mechanical stimulation.

Fig. 4.10 GRGDSP inhibited the upregulation of β1 integrin-PKCα interaction induced by mechanical stimulation in primary chondrocytes.
Lane A: cells treated with GRGDSP followed by 1 min cyclic strain. Lane B: cells treated with GRADSP followed by 1 min cyclic strain.

DISCUSSION

The results of the present study have identified an association between RACK1 and β1 integrin. This association is increased following 0.33 Hz cyclical mechanical stimulation, both in normal articular chondrocytes and also in the immortalized chondrocyte cell line C-20/A4. OA chondrocytes do not show this increased association under the same conditions. RACK1 is a receptor for activated PKC. The PKC inhibitor chelerythrine abolishes the upregulation of the β1-integrin-RACK1/PKCα association response to cyclical mechanical stimulation. The association of β1 integrin-RACK1 and β1 integrin-PKCα following mechanical stimulation is mediated by an integrin dependent signalling pathway.

4.5. PKC Activation and β1 Integrin-RACK1 Association in Chondrocyte Mechanical Stimulation

Protein kinase C comprises a family of more than 10 distinct serine/threonine kinase isoenzymes that have important actions in transmembrane signal transduction pathways (Cornford et al 1999). Different tissues appear to have their own characteristic patterns of PKC isozyme expression and different isozymes appear to have different biological functions (Battaini et al 1997; Haller et al 1998; Cornford et al 1999). This study has shown that primary cultures of human articular chondrocytes express the PKC isozymes α, γ, δ, ι, and λ. The immortalized chondrocyte cell line C-20/A4 expresses a different pattern, i.e. α, ι, λ and low levels of ε subtype. Both primary chondrocytes and immortalized cells express significant levels of RACK1. RACK1 is coimmunoprecipitated with PKC (The anti-PKC antibody clone MC5 recognizes the PKC isoforms α and β, but not the γ isoform (Young et al 1988; van der Zee et al 1995)).

On activation, PKCα translocates in association with its receptor molecule RACK (Rotenberg and Sun 1998). It has been shown that PKC is rapidly activated (within

one minute) following mechanical stretch, in cardiac myocytes (Sadoshima and Izumo 1993). The results of the present study show that a rapid increase in β1 integrin-RACK1 association occurs in primary chondrocytes and in the immortalised chondrocyte cell line (C-20/A4), following mechanical stimulation. This increased interaction is inhibited by the PKC inhibitor chelerythrine, which interacts with the catalytic domain of PKC (Herbert et al 1990). Since:

(i) calcium ions are involved in the chondrocyte mechanotransduction pathway (Wright et al 1992, 1996),

(ii) PKCα binds to RACK1 (Vani et al 1997),

(iii) PKCα is expressed by both primary chondrocytes and C-20/A4 cells but PKCγ is only expressed by primary chondrocytes,

the PKCα isozyme was chosen for study in the experiments investigating PKCα-β1 integrin association. These experiments have shown PKCα-β1 integrin association in primary chondrocytes and immortalized cells and that this interaction is increased following mechanical stimulation; the interaction is inhibited by the PKC inhibitor chelerythrine. The baseline level of β1 integrin/RACK1 or β1 integrin/PKCα association is different between resting cells and resting cells with chelerythrine treatment alone. The mechanism is unclear. One possible explanation is that the PKC inhibitor chelerythrine interacts with the catalytic domain (Herbert et al 1990) and RACK1 interacts with the regulatory domain (Ron and Mochly-Rosen 1995). RACK1/PKC regulatory domain/β1 integrin association therefore increases by unknown mechanisms. However, these results suggest that PKCα is activated following mechanical stimulation, which is consistent with previous studies (Wright et al 1997; Millward-Sadler et al 1999) and that PKCα activation is necessary for the β1 integrin-RACK1 association. In contrast, PKC does not appear to be involved in the depolarisation response to mechanical stimulation in OA chondrocytes (Millward-Sadler et al 2000a), nor does upregulation occur of β1 integrin-RACK1 association in OA cells following mechanical stimulation. β1 integrin-RACK1/PKCα association, or its lack of association, may therefore play a role in the regulation of chondrocyte function in normal and diseased cartilage.

Increased association of β1 integrin and RACK1/PKCα which is induced by cyclical mechanical strain, is inhibited by incubation with RGD containing peptides. These findings suggest that mechanotransduction via integrins is necessary for association between RACK1/PKCα and β1 integrin following 0.33 Hz mechanical stimulation. The transduction and conversion of cyclical mechanical stimuli into biochemical responses are complex processes. Many molecules as for example in focal adhesion- integrin, CD47, FAK, paxillin, β-catenin and RACK1/PKCα are involved and these molecules are thought to interact in a complicated dynamic manner (Clark and Brugge 1995; Giancotti and Ruoslahti 1999). Their roles in interleukin-4 secretion induced by cyclical mechanical stimulation or in the aberrant pathways activated in OA chondrocytes by mechanical stimulation, remain to be elucidated.

4.6. Response of the C-20/A4 Cells to Cyclical Mechanical Stimulation

The immortalized cell line C-20/A4 has been established from human articular cartilage (Goldring et al 1994). C-20/A4 cells synthesize and secrete extracellular matrix molecules including type II collagen, proteoglycan, and chondroitin-4- and chondroitin-6- sulfate (Goldring et al 1994). It has been shown that in these cells, exposure to IL-1β decreases the levels of type II collagen mRNA and increases the levels of mRNA for collagenase, stromelysin and the gene products of the immediate early response genes (egr-1, c-fos, c-jun, and jun-B). As a consequence of these results, it has been suggested that the C-20/A4 cell line can be used as a model for the study of normal and pathological repair mechanisms in cartilage (Goldring et al 1994).

The present study has shown that in C-20/A4 cells a hyperpolarisation response, similar to primary normal chondrocytes, is induced by 0.33 Hz cyclical mechanical strain. On pressure-induced strain, proteins tyrosine phosphorylated include paxillin, a focal adhesion complex adaptor protein and an increased association of

$\beta1$ integrin-RACK1/PKCα is also induced by mechanical stimulation. These findings suggest that the immortalized human chondrocyte cell line C-20/A4 could be used as a model to examine chondrocyte mechanotransduction events.

However, not all characteristics are the same between primary chondrocytes and immortalized cells (Goldring et al 1994; Loeser et al 2000). For example, chondrocyte integrin expression by C-20/A4 cells, including $\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, and $\beta1$ subunits have been analysed using the technique of flow cytometry. The specific relative fluorescence intensity from high to low, was integrin subunits $\alpha3$, $\alpha5$, $\beta1$, $\alpha2$, $\alpha1$, but no $\alpha4$ subunit was detected. C-20/A4 cells expressed higher levels of the integrin subunits $\alpha2$, $\alpha3$, $\alpha5$ and $\beta1$, but lower levels of the $\alpha1$ integrin subunit, compared to primary cells. Differences in integrin subunit expression may therefore alter the adhesive manner in which chondrocytes interact with type II collagen in the ECM (Loeser et al 2000). Dedifferentiation with time in culture and passage number should be checked regularly in the use of cell line model and primary cells.

CHAPTER 5.

ELECTROPHYSIOLOGY OF ATICULAR CHONDROCYTES FROM OA CARTILAGE

RESULTS

The full information from which the values given in the Tables are derived can be found in Appendix III. The total cases used in this section of the experimental work include 6 females (mean age 71 years, range 63-81) and 9 males (mean age 69 years, range 45-81). Results shown are from a single representative experiment. Experiments were repeated on chondrocytes from more than one individual and were reproducible between donors. In some instances replicate experiments were carried out by other members of the research group (MO Wright, B Groenendijk) with consistent results.

5.1. Electrophysiological Response of OA Chondrocytes to Cyclical Strain

Chondrocytes from normal and diseased areas of OA joints were subjected to mechanical stimulation at 0.33 Hz for 20 min. The pressure pulses of 1 atmosphere above atmospheric pressure were used and resulted in 3200 microstrain on the base of the dish (Millward-Sadler et al 2000b). The electrophysiological response of OA chondrocytes to this cyclical strain was found to be membrane depolarisation (**Table 5.1a**), which differed from the response obtained in normal articular chondrocytes i.e. membrane hyperpolarisation (**Table 5.1b**). The baseline resting membrane potential and degree of response varied from experiment to experiment in both normal and OA chondrocytes when different batches of cells were used. Cell density and the time period of chondrocytes in vitro may contribute to the variation.

Table 5.1a Electrophysiological response of OA chondrocytes to cyclical strain

	Membrane potential (-mV) (mean±SEM)			
n*	Resting	Post strain	% change post strain	P value
5	30.0±0.8	16.4±1.0	-45	<0.0001
10	27.4±0.8	18.7±1.1	-32	<0.0001
5	32.6±0.7	21.0±0.3	-36	<0.0001

*n: cell number measured.

Table 5.1b Electrophysiological response of normal articular chondrocytes to cyclical strain

	Membrane potential (-mV) (mean±SEM)			
n	Resting	Post strain	% change post strain	P value
10	45.1±0.9	56.0±0.9	+24	<0.0001
5	29.4±0.5	42.0±0.7	+43	<0.0001
5	22.4±0.8	30.2±0.6	+35	<0.0001

5.2. Role of the Apamin-Sensitive Ion Channel in the OA Chondrocyte Depolarisation Response to Cyclical Strain

The hyperpolarisation response observed in normal articular chondrocytes subjected to cyclical strain follows activation of apamin-sensitive Ca^{2+}-dependent K^{+} ion channels (Wright et al 1996, 1997). The same experiment was performed to investigate whether there was any role for apamin-sensitive ion channels in the depolarisation response of OA chondrocytes to cyclical strain. Apamin at a concentration of 4.9 μM was incubated with OA cells for 10 min prior to cyclical mechanical strain. The membrane potentials of ten cells were recorded prior to and after the addition of apamin and also after the period of 0.33 Hz mechanical strain. The exposure of the cells to apamin alone produced no significant change in membrane potential. When the OA cells were subjected to cyclical strain in the presence of apamin, membrane depolarisation response was still observed (**Table 5.2**), suggesting that apamin-sensitive ion channels are not involved in the OA chondrocyte depolarisation response to cyclical strain.

Table 5.2 The effect of apamin on the depolarisation response of OA chondrocytes to cyclical strain

		Membrane potential (-mV) (mean±SEM)				
Reagent	n	Resting	Apamin alone	Post strain	% change post strain	P value
Control	10	29.9±0.9		22.9±0.9	-23	<0.0001
apamin	10	38.3±0.9	36.8±0.7	28.1±1.0	-24	<0.0001*

* Compared with reagent alone.

5.3. Effect of Anti-Integrin Antibodies on the Depolarisation Response of OA Chondrocytes to Cyclical Strain

The role of specific integrin molecules in the transduction process was examined using antibodies against integrin subunits known to be expressed by human articular chondrocytes. Antibodies present during strain and when post strain membrane potentials were being recorded. An investigation in the effect of anti-β1 integrin antibody (TS2/16) on normal chondrocytes was repeated. It was confirmed that the hyperpolarisation response induced by mechanical strain was blocked by this antibody (**Table 5.3a**).

The anti-integrin antibodies utilised (1 μg/ml for 30 min at 37°C) had no effect on the resting membrane potential in normal and OA chondrocytes. Both the anti-β1 integrin antibody (CD29) and the anti-α5 integrin antibody (CD49e) inhibited the depolarisation response of OA chondrocytes to 0.33 Hz mechanical strain. In contrast the anti-αVβ5 integrin antibody (P1F6) had no effect on the electrophysiological response of these cells to identical stimulation (**Table 5.3b**).

Table 5.3a Effect of anti-β1 integrin antibody (TS2/16) on the hyperpolarisation response of normal chondrocytes to cyclical strain

Reagent	n	Membrane potential (-mV) (mean±SEM)			% change post strain	P value
		Resting	Antibody Alone	Post strain		
Control	5	22.4±0.8		30.2±0.6	+35	<0.0001
Anti-β1	5	23.6±0.5	25.6±1.5	23.0±0.7	-10	NS*

*NS: not significant. Compared with reagent alone.

Table 5.3b The effect of anti-integrin antibodies on the depolarisation response of OA chondrocytes to cyclical strain

Reagent	n	Membrane potential (-mV) (mean±SEM)			% change post strain	P value
		Resting	Antibody alone	Post strain		
control	5	32.6±0.7		21.0±0.3	-36	<0.0001
anti-β1	5	32.4±0.8	32.0±0.9	32.0±1.5	0	NS*
anti-αVβ5	5	34.2±0.7	33.8±0.6	23.6±0.5	-30	<0.0001
control	10	32.6±1.0		24.4±0.7	-25	<0.0001
anti-α5	5	33.4±1.5	31.2±1.5	31.2±1.9	0	NS*

*NS: not significant. Compared with antibody alone.

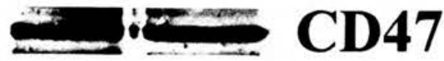
5.4. Effect of Anti-CD47 (Bric-126) on the Response of Cultured Chondrocytes to Cyclical Strain

Integrin-associated protein (IAP or CD47) is known to interact with integrins on the cell membrane to form distinct complexes that recruit signaling molecules activated in a number of integrin-mediated responses (Porter and Hogg 1998). Using the techniques of immunoblotting, immunoprecipitation and confocal microscopy, CD47 expression by normal and OA cultured chondrocytes was investigated. Both normal and OA cultured chondrocytes were shown to express CD47 (**Fig. 5.1i**), coimmunoprecipitation of CD47 and $\alpha 5$ integrin (**Fig. 5.1ii**) and colocalization with $\alpha 5$ integrin (**Fig. 5.2**). Results shown are from normal chondrocytes.

To investigate the role of CD47 in the hyperpolarisation response of normal chondrocytes to cyclical strain, the anti-CD47 monoclonal antibody (Bric-126) was incubated with normal articular chondrocytes at 37°C for 30 min. The antibody itself was found to have no effect on the membrane potential, but it blocked the hyperpolarisation response which is normally induced by cyclical strain (**Table 5.4a**).

Bric-126 was also found to inhibit the depolarisation response of OA chondrocytes to mechanical stimulation (**Table 5.4b**).

(i)



(ii)

IP: α5 integrin
IB: CD47

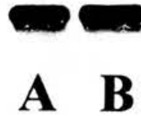
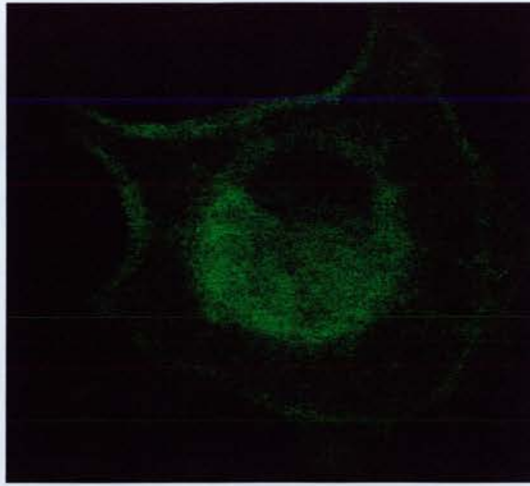


Fig. 5.1

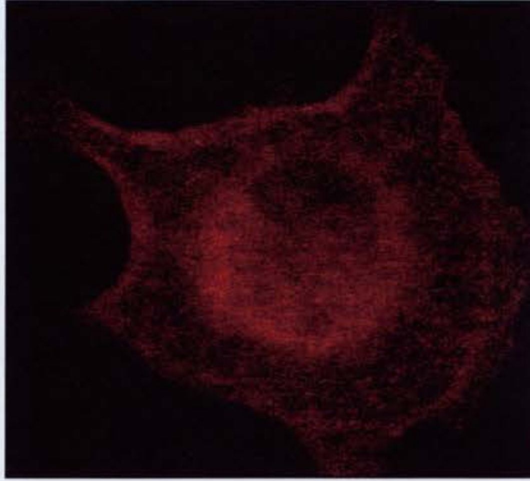
(i) Expression of CD47 by normal cultured chondrocytes (two cases).

(ii) Coimmunoprecipitation of CD47 with α5 integrin. Lane A: resting cells. Lane B: cells with 20 min mechanical stimulation at a frequency of 0.33 Hz.

α5 integrin



CD47



overlay

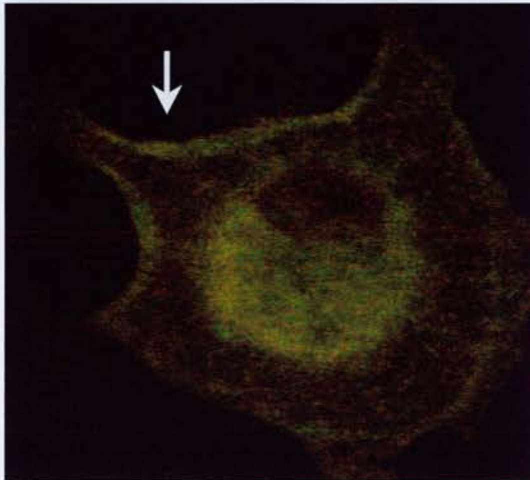


Fig. 5.2 Colocalization of CD47 and α5 integrin. Unstimulated normal human articular chondrocytes were subjected to double immunofluorescent staining for detection of α5 integrin (green) and CD47 (red). On confocal microscopy, colocalization sites (arrows) appear as yellow pixels, after merging the images. (magnification x2067)

Table 5.4a Effect of Bric-126 on the membrane hyperpolarisation response of normal articular chondrocytes to cyclical strain

		Membrane potential (-mV) (mean±SEM)				
Reagent	n	Resting	Bric-126 alone	Post strain	% change post strain	P value
Control	5	25.0±0.4		30.2±0.6	+21	<0.0001
Bric-126	5	26.2±0.8	27.2±0.4	27.6±1.2	+2	NS*

*NS: not significant. Compared with antibody alone.

Table 5.4b Effect of Bric-126 on the depolarisation response of OA chondrocytes to cyclical strain

		Membrane potential (-mV) (mean±SEM)				
Reagent	n	Resting	Bric-126 alone	Post strain	% change post strain	P value
control	10	37.1±1.5		31.8±1.3	-14	0.02
Bric-126	10	38.4±0.8	39.9±0.9	39.2±1.0	-2	NS*

*NS: not significant. Compared with antibody alone.

5.5. Effect of Inhibitors of Intracellular Signalling Molecules on the Depolarisation Response of OA Chondrocytes to Cyclical Strain

The effects of chemical agents known to interfere with integrin-associated intracellular signalling pathways, were tested for their effects on the depolarisation response to cyclical mechanical strain in OA chondrocytes. These inhibitors included cytochalasin D (a disruptor of the actin cytoskeleton), genistein (a tyrosine kinase inhibitor), staurosporine and calphostin C (protein kinase C inhibitors), and wortmannin and LY294002 (PI-3 kinase inhibitors).

Of the reagents tested, only cytochalasin D and calphostin C had a significant effect on the resting membrane potential, inducing a depolarisation of the cells.

The depolarisation response observed in OA chondrocytes following 0.33 Hz cyclical strain was inhibited by genistein, wortmannin and LY294002, but in contrast, disruption of actin cytoskeleton by cytochalasin D and inhibition of PKC by staurosporine and calphostin C had no effect on the depolarisation response (**Table 5.5a**).

Experiments using normal chondrocytes, showed that both genistein (a tyrosine kinase inhibitor) and chelerythrine chloride (PKC inhibitor) prevented the hyperpolarisation response to 0.33 Hz cyclical strain, consistent with previously reported results (**Table 5.5b**).

Table 5.5a Effects of inhibitors of intracellular signalling molecules on the depolarisation response of OA chondrocytes to cyclical strain

		Membrane potential (-mV) (mean±SEM)				
Reagent	n	Resting	Reagent alone	Post strain	% change post strain	P value*
control	10	45.5±2.0		37.6±0.7	-17	0.006
cytochalasin D	10	48.4±1.4	39.8±0.8#	31.5±0.7	-21	<0.0001
control	10	47.7±1.2		36.7±0.7	-23	<0.0001
wortmannin	10	48.1±1.4	44.2±1.0	45.9±1.0	+4	NS
LY294002	10	47.1±1.2	46.5±0.7	45.2±0.9	-3	NS
control	10	37.4±0.9		28.8±0.7	-23	<0.0001
genistein	10	38.1±0.8	36.2±1.0	36.6±0.9	+1	NS
control	5	35.0±2.0		27.4±0.8	-22	0.02
staurosporine	5	35.2±1.0	38.2±1.5	29.8±1.7	-22	0.006
calphostin C		35.6±1.7 (n=10)	28.8±2.0^ (n=5)	21.2±2.2 (n=5)	-26	0.04

*Compared with reagent alone. NS: not significant.

# P<0.0001 versus Resting.

^ P=0.03 versus Resting.

Table 5.5b Effect of genistein and chelerythrine chloride on the hyperpolarisation response of normal chondrocytes to cyclical strain

		Membrane potential (-mV) (mean±SEM)				
Reagent	n	Resting	Reagent alone	post strain	% change post strain	P value
Control	5	29.4±0.5		42.0±0.7	+43	<0.0001
Genistein	5	32.8±0.6	34.4±0.6	34.2±0.7	-0.6	NS*
Chelerythrine	5	32.0±0.8	35.2±0.4	31.6±1.3	-10	NS*

*NS: not significant. Compared with reagent alone.

5.6. Effect of Cytokines on the Membrane Potential of Normal and OA Chondrocytes

In separate experiments the membrane potentials of normal and OA chondrocytes were measured before and 10 min after the addition of recombinant IL-1β or IL-4 to the culture medium at a concentration of either 10 ng/ml or 10 pg/ml. Two dose levels were used, since the effect of cytokines on the membrane potential of human chondrocytes was dose-dependent over a range between 100 fg/ml and 10 ng/ml. A maximal response was obtained at concentrations of 5-10 pg/ml (Millward-Sadler et al 1999).

Addition of IL-4 to mechanically unstimulated normal chondrocytes resulted in membrane hyperpolarisation, whereas the addition of IL-1β induced membrane depolarisation (**Table 5.6**).

Under similar conditions to those described above, both cytokines resulted in membrane depolarisation of OA chondrocytes (**Table 5.7a & 5.7b**).

Experiments were then performed in an attempt to elucidate the relationship between IL-4 and IL-1β. OA chondrocytes were treated with either (i) anti-IL-4 antibody (1 μg/ml) for 10 min prior to incubation with recombinant IL-1β (10 pg/ml) for 10 min, or (ii) anti-IL-1β antibody (1 μg/ml) for 10 min prior to incubation with recombinant IL-4 (10 pg/ml) for 10 min. The antibodies of anti-IL-4 and anti-IL-1β alone produced no effect on the membrane potential of OA chondrocytes. In the presence of anti-IL-1β antibody, the addition of IL-4 caused a membrane depolarisation of OA chondrocytes. However, in a separate experiment, in the presence of anti-IL-4 antibody, the addition of IL-1β caused no significant change in membrane potential of the cells (**Table 5.8**).

Table 5.6 Effect of IL-4 and IL-1β (10ng/ml) on the membrane potential of mechanically unstimulated normal chondrocytes

		Membrane potential (-mV) (mean±SEM)			
Reagent	n	Resting	With reagent	% change	P value
IL-4	5	24.6±0.5	32.2±1.2	+31	0.002
IL-1β	5	21.8±1.3	14.2±0.7	-35	0.002

Table 5.7a Effect of IL-4 and IL-1β (10 ng/ml) on the membrane potential of mechanically unstimulated OA chondrocytes

		Membrane potential (-mV) (mean±SEM)			
Reagent	n	Resting	With reagent	% change	P value
IL-4	10	42.5±1.0	36.9±0.9	-13	0.0004
IL-1β	10	33.1±1.2	28.0±1.2	-15	0.008

Table 5.7b Effect of IL-4 and IL-1β (10 pg/ml) on the membrane potential of mechanically unstimulated OA chondrocytes

		Membrane potential (-mV) (mean±SEM)			
Reagent	n	Resting	With reagent	% change	P value
IL-4	10	45.7±1.0	40.1±1.1	-12	0.001
IL-1β	10	46.3±1.1	40.2±1.0	-13	0.0009

Table 5.8 Effect of IL-4 + anti-IL-1β or IL-1β + anti-IL-4 on the membrane potential of OA chondrocytes

Membrane potential (-mV) (mean±SEM)					
n	Resting	Anti-IL-1β	Anti-IL-1β + IL-4 (10 pg/ml)	% change	P value
10	45.5±1.6	45.7±1.2	40.0±0.8	-13	0.0009
n	Resting	Anti-IL-4	Anti-IL-4 + IL-1β (10 pg/ml)	% change	P value
10	43.0±1.3	42.4±0.9	41.6±1.0	-2	NS*

*NS: not significant. Compared with antibody alone.

5.7. Effect of IL-1 Receptor and IL-2 Receptor γ Antibodies on the Depolarisation Response of OA Chondrocytes to Cyclical Strain

In order to examine the cytokine receptor types which may be involved in the depolarisation response of OA chondrocytes to pressure-induced strain, experiments were undertaken using anti-IL-1 receptor type I and type II antibodies. In addition, the IL-2 receptor γ chain is known to be shared by receptor complexes activated by the cytokines IL-2, IL-4, IL-7, IL-9 and IL-15 (Demoulin & Renauld 1998). Experiments were therefore also performed using the anti-IL-2 receptor γ chain antibody to investigate whether the IL-2 receptor γ chain is involved in the IL-4 signaling in the depolarisation response of OA chondrocytes to 0.33 Hz mechanical strain.

In separate experiments, cells were incubated with antibodies to the IL-1 receptor types I and II and also the IL-2 receptor γ chain at a concentration of 10 μg/ml for 10 min before being subjected to 0.33 Hz cyclical strain for 20 min. The results are shown in **Table 5.9**.

The antibodies by themselves had no effect on resting membrane potential. Anti-IL-1 receptor I antibody and anti-IL-2 receptor γ antibody inhibited the depolarisation response to cyclical strain, whereas anti-IL-1 receptor type II had no significant effect on the electrophysiological response to cyclical strain.

Table 5.9 Effect of anti-IL-1 RI, RII, and anti-IL-2 Rγ on the depolarisation response of OA chondrocytes to cyclical strain

		Membrane potential (-mV) (mean±SEM)				
Reagent	n	Resting	Antibody	Post strain	% change	P value*
control	10	29.4±1.5		21.7±0.7	-26	0.003
anti-IL-1RI	10	32.2±1.7	35.8±1.7	34.4±1.8	-4	NS
anti-IL-1RII	10	32.6±1.1	34.5±1.2	30.2±0.8	-13	0.008
control	10	43.1±1.1		36.7±0.8	-15	0.0002
anti-IL-2Rγ	10	43.9±0.7	42.9±1.3	42.2±1.1	-2	NS

*Compared with reagent alone. NS: not significant.

DISCUSSION

5.8. The Altered Electrophysiological Response of OA Chondrocytes to Cyclical Strain

These studies have demonstrated that chondrocytes derived from OA cartilage show a consistent and reproducible membrane depolarisation response to 0.33 Hz cyclical mechanical stimulation. The depolarisation response is associated with activation of a tetrodotoxin sensitive Na^+ channel (Millward-Sadler et al 2000a), but not the apamin-sensitive ion channel which is the channel involved in the hyperpolarisation response of normal articular chondrocytes (Wright et al 1996). Using RTPCR, it has been shown that both normal and OA chondrocytes express SK channels (Millward-Sadler et al 2000a). It is therefore likely that failure hyperpolarisation on pressure-induced strain is the result of activation of different signalling pathways, as suggested by the results observed using the chemical inhibitors of intracellular signalling molecules.

The electrophysiological response of both normal and OA chondrocytes to cyclical strain involves $\alpha5\beta1$ integrins, gadolinium sensitive ion channels and integrin-associated protein (CD47) in the plasma membrane. However, the involvement of intracellular signalling molecules is different. Neither the actin cytoskeleton nor protein kinase C, which are molecules involved in the hyperpolarisation response to pressure-induced strain of chondrocytes derived from normal cartilage, are necessary for the depolarisation response of chondrocytes from osteoarthritic cartilage. In addition, the electrophysiological response of normal and OA chondrocytes to recombinant IL-4 is also different (hyperpolarisation and depolarisation, respectively).

Chondrocytes are surrounded by the presence of the collagen and proteoglycan rich ECM in vivo and the degree of force which is directly transmitted to the chondrocyte itself has not been clearly defined but potentially is of a lesser degree

(Millward-Sadler et al 2000a). In the system used in the present project, a plastic Petri dish of cells is placed on the platform in the pressure chamber, producing two spaces above and below the dish. 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 due to differences in volumes of the spaces involved and the relative inaccessibility of the space below the dish for entering gas (Wright et al 1996). 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 the dish. It is believed that the response of the cells in this system is secondary to stretch rather than pressure for a number of reasons. When a nondeformable glass dish is set in the pressure chamber, no hyperpolarization response is elicited in normal chondrocytes to PIS. Similarly, when a deformable plastic dish is suspended in the center of the chamber (allowing free flow of gas without deformation of the base of the dish), there is no electrophysiological response of the cells to the increased pressure. Furthermore, a system developed recently shows that the base of the Petri dish is deformed as a result of increased gas pressure on the underside of the dish while the pressure in the upper side remains unchanged. In this new system, chondrocytes are subjected to stretch without an increase in pressure and the electrophysiological response is identical to that in the system used in this thesis (Millward-Sadler et al 2000a). During the cyclical stimulation, the microstrain on the base of the dish is 3200, which is equivalent to 0.32% deformation. Movement of fluid is slight on the mdeium surface. The cells will be subjected to fluid flow although the degree is not known. It is possible that fluid flow also contributes to the cell stimulus in the system. Additional studies are required to ascertain whether osteoarthritc chondrocytes to pressure in the system respond in a similar manner.

5.9. CD47 Is Involved in Chondrocyte Mechanotransduction

This study has shown that cultured chondrocytes express CD47 and that it associates with $\alpha 5$ integrin. Functionally, the normal chondrocyte hyperpolarisation response induced by cyclical mechanical strain at a frequency of 0.33 Hz for 20 min is inhibited by incubation with anti-CD47 antibody (Bric-126). The depolarisation response seen in OA chondrocytes induced by same regime is also abolished by Bric-126. The results indicate that CD47 is involved in the mechanotransduction pathway activated by 0.33 Hz cyclical mechanical strain in chondrocytes.

It has been shown that CD47 and integrin are physically and functionally associated (Lindberg et al 1993). It has been shown that $\alpha 5\beta 1$ integrin has a role as a chondrocyte mechanoreceptor (Wright et al 1997). In human bone cells, CD47 antibody inhibits the hyperpolarisation response induced by 0.33 Hz mechanical stimulation, but has no effect on the depolarisation response induced by 0.104 Hz mechanical stimulation (Salter et al 1997). It might therefore have a role in the sensing by the cell of different frequencies of mechanical strain. CD47 has been shown to associate with the integrins $\alpha V\beta 3$, $\alpha IIb\beta 3$, $\alpha 2\beta 1$, and $\alpha V\beta 5$ (Porter and Hogg 1998). In articular chondrocytes, using the techniques of confocal microscopy and coimmunoprecipitation, CD47 has been shown to be associated with $\alpha 5$ integrin. It is therefore possible that, in articular chondrocytes, both $\alpha 5\beta 1$ integrin and CD47 together sense the stretch and initiate the response to mechanical stimulation. It has been shown that CD47 exerts its effects on $\alpha V\beta 3$ function via a G-protein, acting upstream of a common cell-spreading pathway mediated by PI 3-kinase, PKC and tyrosine kinases (Gao et al 1996). CD47 has been shown to play a role in the tyrosine phosphorylation of FAK (Gao et al 1996; Chung et al 1997). These signalling molecules, including PI 3-kinase, PKC, tyrosine kinases and FAK, are also the components of integrin-mediated signalling pathways (Clark and Brugge 1995). Our data show that the chondrocyte mechanotransduction pathways activated by 0.33 Hz cyclical strain involve tyrosine kinases, FAK and PKC. These

consistent findings suggest that CD47 has an important role in chondrocyte mechanotransduction.

How CD47 transduces mechanical stimulation into a biochemical response, however, remains unclear. It has been shown that mechanical stimulation, at 0.33 Hz, resulting in chondrocyte membrane hyperpolarisation, requires activation of stretch-activated ion channels and L-type calcium channels (Wright et al 1992, 1996). It has been proposed that CD47 has a role as a membrane calcium channel (Schwartz et al 1993). As such, CD47 may play a role in intracellular calcium ion regulation in the mechanotransduction pathways activated in chondrocytes by mechanical stimulation. The transduction events associated with CD47, however, need to be further elucidated.

5.10. The Role of PKC and the Actin Cytoskeleton in the OA Chondrocyte Depolarisation Response to Cyclical Strain

The hyperpolarisation response in normal chondrocytes and also the depolarisation response in OA chondrocytes induced by 0.33 Hz cyclical mechanical stimulation initially involves $\alpha5\beta1$ integrins, stretch-activated ion channels and CD47, followed by the subsequent activation of tyrosine kinases and phospholipase C. However, staurosporine, calphostin C (PKC antagonists) (Tamaoki et al 1986; Kobayashi et al 1989) and cytochalasin D (a substance known to disrupt the actin cytoskeleton) (Casella et al 1981), which inhibit the hyperpolarisation response in normal chondrocytes, had no effect on the depolarisation response in OA chondrocytes, suggesting that activation of PKC and the actin cytoskeleton are not involved in the depolarisation response induced in OA chondrocytes by 0.33 Hz pressure-induced strain. However, the PI 3-kinase inhibitors wortmannin and LY294002 (Reinhold et al 1990; Vlahos et al 1994) abolish the depolarisation response of OA chondrocytes

suggesting involvement of PI 3-kinase in the particular signal transduction pathway activated in OA chondrocytes.

The difference in responses to cyclic mechanical stimulation is a further example of phenotypic difference between normal and OA chondrocytes. Other well recognised differences include altered production of ECM molecules, cytokines and proteases (Ostergaard and Salter 1998). These changes in OA cartilage can influence chondrocyte activity by modifying cell-matrix interactions and may therefore influence integrin-mediated signalling (Jopanbutra et al 1996; Ostergaard et al 1998; Loeser et al 1995; Lapadula et al 1998). Studies have shown that PKC might play a role in the pathogenesis of osteoarthritis (Hamanishi et al 1996; Satsuma et al 1996; Fukuda et al 1997; Tanaka et al 1998; Piperno et al 1998). Satsuma et al (1996) have shown that PKC isozymes are redistributed in normal and OA cartilage. Adhesion of cells derived from intact cartilage to fibronectin was decreased by the addition of a PKC inhibitor to the cell culture medium (Piperno et al 1998). Intra-articular administration of a PKC activator (e.g. 12-O-tetradecanoyl-phorbol-13-acetate (TPA)) prevented the development of experimental osteoarthritis (Hamanishi et al 1996). A low frequency and magnitude of cyclic tensile stretch increased proteoglycan synthesis, but high frequency and magnitude of stretch decreased proteoglycan synthesis and also reduced the activity of PKC (Fukuda et al 1997). Thus, it has been suggested that PKC is involved in the mechanical stress-mediated degradation of articular cartilage.

Protein kinase C does not appear to be involved in the depolarisation response of OA chondrocytes to cyclical mechanical strain, which is consistent with the findings from the RACK1 studies of the present work, which showed an increased association of RACK1 and β1 integrin in normal chondrocytes, but not in OA chondrocytes. For, since as RACK1 is a receptor for activated PKC and as PKC is not involved in the depolarisation response to pressure-induced strain in OA chondrocytes, it is very improbable that an increased association of RACK1 and β1 integrin would be seen.

The role of the actin cytoskeleton in osteoarthritis is still unclear. Frenkel et al (1996) have shown that exposure to nitric oxide, which plays a significant role in the pathogenesis of osteoarthritis (Studer et al 1999), inhibited attachment of chondrocytes to fibronectin and disrupted the assembly of actin filaments. It has also been shown that nitric oxide disrupts fibronectin-induced assembly of a subplasmalemmal actin/Rho A/focal adhesion kinase signalling complex, which is associated with $\alpha5\beta1$ integrin (Clancy et al 1997; Clancy 1999). These findings, plus the lack of inhibition by cytochalasin of the depolarisation response to pressure-induced strain, would suggest that the integrin mediated intracellular pathways activated in chondrocytes by 0.33 Hz cyclical mechanical strain are altered in osteoarthritis.

5.11. The Role of Cytokines in the OA Chondrocyte Depolarisation Response to Cyclical Strain

OA is characterized by progressive cartilage degradation, in which ECM integrity is no longer maintained and the homeostasis of catabolic cytokines (i.e. IL-1β, TNFα), anabolic cytokines (i.e. IGF, TGFβ), anti-inflammatory cytokines (i.e. IL-4, IL-10, IL-13) and the cytokine receptor antagonist IL-1Ra is disturbed (Martel-Pelletier et al 1999). The electrophysiological studies using normal articular chondrocytes have shown that IL-4 causes chondrocyte membrane hyperpolarisation, whereas IL-1β induces a depolarisation response in mechanically unstimulated cells. The membrane hyperpolarisation response of normal chondrocytes to 0.33 Hz mechanical stimulation did not involve IL-2Rγ (Millward-Sadler et al 1999).

The data from OA chondrocytes show an aberrant response. Both IL-1β and IL-4 cause membrane depolarisation. IL-4 induces membrane depolarisation in OA chondrocytes treated with IL-1β antibodies, whereas IL-1β does not cause membrane depolarisation when OA chondrocytes are first incubated with IL-4 antibody. This suggests that IL-4 secretion is a downstream event of IL-1β secretion

in OA chondrocytes in response to 0.33 Hz mechanical stimulation. Furthermore, cytokine receptor studies show that IL-1β signalling involves the IL-1 type I receptor (IL-1RI), but not the type II receptor (IL-1RII) and the IL-2 receptor gamma chain is involved in the cytokine signalling pathways which are activated.

Proinflammatory cytokines are believed to play a pivotal role in the initiation and development of the OA process, among which IL-1β and TNFα appear prominent (Martel-Pelletier et al 1999). IL-1 can induce chondrocytes and synovial cells to produce other cytokines such as IL-6, IL-8 and leukemia inhibitory factor (LIF) as well as its own production, and also to stimulate proteases and PGE_2 production (Goldring 2000). The biological activation of cells by IL-1 is mediated through association with type I and type II IL-1 receptor (Slack et al 1993). In cartilage cells, the IL-1RI, which has a higher affinity for IL-1β than for IL-1α, appears responsible for signal transduction (Martel-Pelletier et al 1992; Arend 1993; Sadouk et al 1995). The number of IL-1 type I receptors is significantly increased in OA chondrocytes (Martel-Pelletier et al 1992). This fact may explain the findings that IL-1RI, not IL-1RII, is involved in the depolarisation response to 0.33 Hz mechanical stimulation seen in OA chondrocytes.

The anti-inflammatory cytokine IL-4 has been shown to be produced by synovial membrane cells and chondrocytes of cartilage (Bendrups et al 1993; Salter et al 1996b), and is found in increased levels in the synovial fluid of OA patients (Bendrups et al 1993). IL-4 can suppress the synthesis of IL-1β and TNFα in OA synovial tissue (Bendrups et al 1993). IL-4 anti-inflammatory properties include decreased production of IL-1β, TNFα and MMP, upregulation of IL-1Ra and TIMP-1, and inhibition of PGE_2 release (Hart et al 1989; Essner et al 1989; Donnelly et al 1990; Vannier et al 1992; Hart et al 1995; Shingu et al 1995; Alaaeddine et al 1999; Millward-Sadler et al 2000b). Although a shift in the balance between pro- and anti-inflammatory cytokines is believed to contribute to the

destructive processes in osteoarthritis, their interactions or modulatory effects in the metabolism of joint articular tissue remain unclear.

This study has shown that the IL-2 receptor gamma chain (cγ) is involved in the depolarisation response of OA chondrocytes to 0.33 Hz cyclical mechanical strain, but not in the hyperpolarisation response of normal chondrocytes (Millward-Sadler et al 1999). This suggests that the altered electrophysiological response results from different IL-4 receptors being involved in cell signalling. IL-4 exerts its actions via type I receptors (IL-4Rα/cγ) in OA chondrocytes and via type II receptors (IL-4Rα/IL-13R) in normal chondrocytes (Millward-Sadler et al 1999). Significant differences in signaling cascades activated by IL-4 type I and type II receptors have been identified. It has been found that signal transduction through IL-4Rα/cγ results in tyrosine phosphorylation of JAK1 and JAK3 whereas signaling through IL-4Rα/IL-13R results in tyrosine phosphorylation of JAK1 and TYK2 (Murata and Puri 1997; Ryan et al 1996). IL-4 receptor can also activate STAT5 by a cγ and JAK3 dependent mechanism (Lischke et al 1998).

In summary, in cultured OA chondrocytes, 0.33 Hz cyclical mechanical stimulation induces a membrane depolarisation response via activation of tetrodotoxin sensitive sodium channels. The mechanotransduction pathway is mediated by integrins, stretch-activated ion channels and CD47 and involves the intracellular signalling molecules tyrosine kinases, PLC and PI 3-kinase, but not PKC and the actin cytoskeleton. The cytokines IL-1β and IL-4 are also involved in the signal transduction pathway.

CHAPTER 6. CONCLUSIONS

Cyclical mechanical stimulation at a frequency of 0.33 Hz results in a hyperpolarisation response of normal cultured chondrocytes via activation of Ca^{2+}-activated K^+ ion channels. The mechanotransduction pathway involves $\alpha5\beta1$ integrins, stretch-activated ion channels, the actin cytoskeleton and tyrosine kinases, with subsequent release of the chondroprotective cytokine interleukin 4. PLC, IP3 and PKC are involved in the downstream signalling events initiated by interleukin-4.

This study has identified that CD47, tyrosine phosphorylation of focal adhesion kinase, β-catenin and paxillin, and β1 integrin-RACK1/PKCα association are involved in the mechanotransduction pathway activated by 0.33 Hz cyclical mechanical strain in normal cultured chondrocytes. The proposed mechanotransduction pathway of the hyperpolarisation response in normal cultured chondrocytes is summarised in **Fig.6.1.**

The electrophysiological response of OA chondrocytes to 0.33 Hz cyclical mechanical strain involves integrins, CD47, stretch-activated ion channels, tyrosine kinases, the cytokines IL-1β and IL-4 and PLC and PI-3 kinase, leading to a membrane depolarisation via activation of tetrodotoxin sensitive Na^+ channels (**Fig.6.2**). However, the intracellular signalling molecules PKC and actin cytoskeleton are not involved in the mechanotransduction pathway.

It is unclear why chondrocytes from osteoarthritic cartilage should show differences in their response to 0.33 Hz mechanical stimulation. Possibilities may include changes in cell-extracellular matrix interactions. These may arise secondary to extracellular matrix degradation and production of new matrix molecules such as tenascin (Salter 1993). In addition altered integrin expression in osteoarthritic chondrocytes (Ostergaard et al 1998) may also lead to alternative cell-matrix

interactions and signalling. Additionally, changes in local cytokine production may influence integrin signalling and cell responses to mechanical stimuli. For example, in osteoarthritis there is an increased production of a variety of locally acting signalling cytokines including interleukin 1 (Towle et al 1997) and tumor necrosis factor (Shinmei et al 1991) which may regulate integrin mediated signalling pathways. It has also been shown that nitric oxide and nitric oxide-producing cytokines can exert profound effects on fibronectin-$\alpha5\beta1$ integrin signalling in chondrocytes (Clancy et al 1997). The intracellular signalling processes may therefore alter and then interfering with integrin signalling by intracellular cross talk and inside-out mechanisms or by transcriptional control of aggrecan, protease and cytokine receptor gene expression to produce a different electrophysiological response.

Abnormalities of mechanotransduction leading to aberrant chondrocyte activity in diseased articular cartilage may be important in the progression of osteoarthritis. It will be important to further investigate this possibility and to assess whether such changes, should they exist, are reversible and indicate novel means of treating cartilage degradation in osteoarthritis.

Fig. 6.1 Proposed mechanotransduction pathway in normal cultured chondrocytes to 0.33 Hz cyclical mechanical strain.

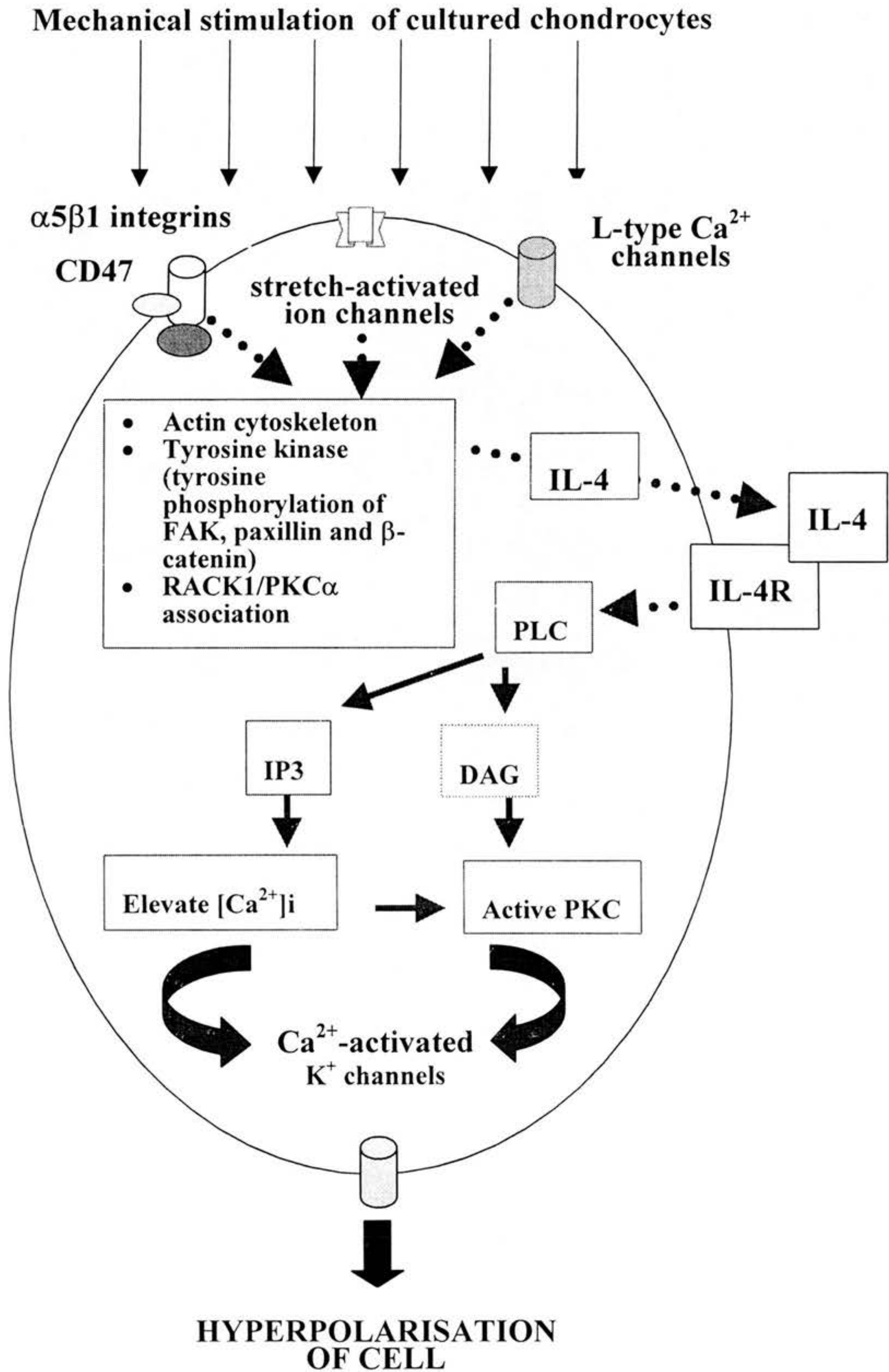
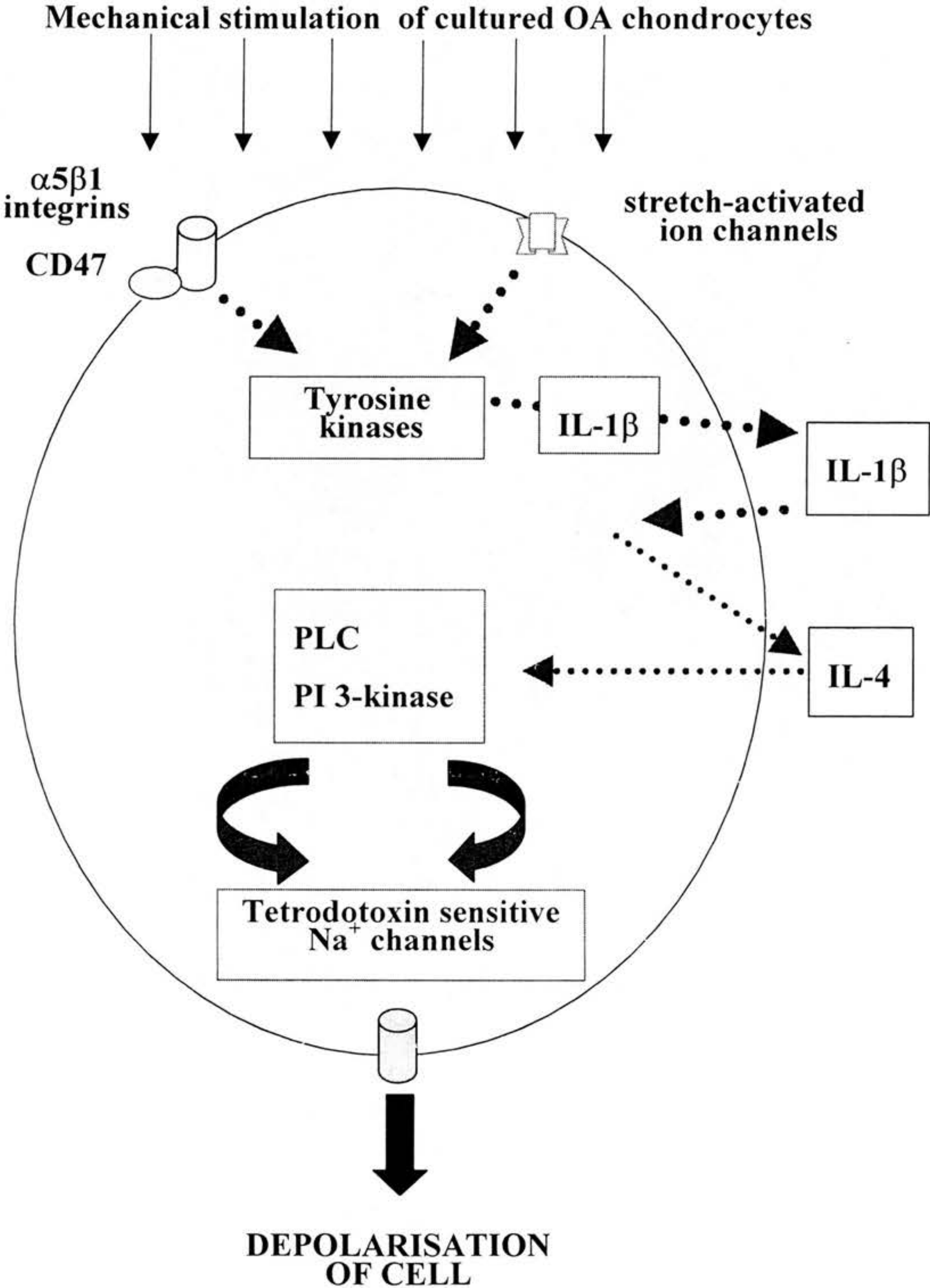


Fig. 6.2. Proposed mechanotransduction pathway in OA chondrocytes to 0.33 Hz cyclical mechanical strain.



REFERENCES

Aberle H, Butz S, Stappert J, Weissig H, Kemler R, Hoschuetzky H 1994 Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J Cell Sci* 107:3655-3663.

Adams DJ, Barakeh J, Laskey R, van Breemen C 1989 Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J* 3:2389-2400.

Akesson K 1999 Osteoarthritis and degenerative spine pathologies. In: Seibel MJ, Robins SP, Bilezekian JP ed. Dynamics of bone and cartilage metabolism. New York: Academic Press. P637-648.

Akimoto S, Mitsumata M, Sasaguri T, Yoshida Y 2000 Laminar shear stress inhibits vascular endothelial cell proliferation by inducing cyclin-dependent kinase inhibitor p21. *Circ Res* 86(2):185-190.

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y 1987 Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 262(12):5592-5595.

Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH 1986 Tensile properties of knee joint cartilage: I. Influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res* 4:379-392.

Alaaeddine N, Di Battista JA, Pelletier JP, Kiansa K, Cloutier JM, Martel-Pelletier J 1999 Inhibition of tumor necrosis factor alpha-induced prostaglandin E2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways. *Arthritis Rheum* 42:710-718.

Albelda SM, Buck CA 1990 Integrins and other cell adhesion molecules. *FASEB J* 4:2868-2880.

Alessandro R, Masiero L, Lapidos K, Spoonster J, Kohn EC 1998 Endothelial cell spreading on type IV collagen and spreading-induced FAK phosphorylation is regulated by Ca^{2+} influx. *Biochem Biophys Res Commun* 248:635-640.

Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenward R, Hochbert M, Howell D, Kaplan D, Koopman W, Longley S, Mankin H, McShane DJ, Medsger T, Meenan R, Mikkelsen W, Moskowitz R, Murphy W, Rothschild B, Segal M, Sokoloff L, Wolfe F 1986 Development of criteria for the classification and reporting of osteoarthritis-classification of osteoarthritis of the knee. *Arthritis Rheum* 29:1039-1049.

Altruda F, Cervella P, Tarone G, Botta C, Balzac F, Stefanuto G, Silengo L 1990 A human integrin β1 subunit with a unique cytoplasmic domain generated by alternative mRNA processing. *Gene* 95:261-266.

Andersen KL, Norton LA 1991 A device for the application of known stimulated orthodontic forces to human cells in vitro. *J Biomech* 24:649-654.

Ando J, Komatsuda T, Kamiya A 1988 Cytoplasmic calcium responses to fluid shear stress in cultured vascular endothelial cells. *In Vitro Cell Dev Biol* 24:871-877.

Aplin AE, Howe A, Alahari SK, Juliano RL 1998 Signal transduction and signal modulation by cell adhesion receptors: The role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev* 50:197-263.

Arend WP 1993 Interleukin-1 receptor antagonist. *Adv Immunol* 54:167-227.

Argraves WS, Suzuki S, Arai H, Thompson K, Pierschbacher MD, Ruoslahti E 1987 Amino acid sequence of the human fibronectin receptor. *J Cell Biol* 105:1183-1190.

Armstrong CG, Mow VC 1982 Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. *J Bone Joint Surg* 64A:88-94.

Armstrong CG, O'Connor P, Gardner DL 1992 Mechanical basis of connective tissue disease. In: Gardner DL ed. Pathological basis of the connective tissue diseases. London: Edward Arnold. P261-281.

Arnaout MA 1990 Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* 75:1037-1050.

Arner EC, Tortorella MD 1995 Signal transduction through chondrocyte integrin receptors induces matrix metalloproteinase synthesis and synergizes with interleukin-1. *Arthritis Rheum.* 38:1304-1314.

Athnansiou KA, Agarwal A, Dzida FJ 1994 Comparative study of the intrinsic mechanical properties of the human acetabular and femoral head cartilage. *J Orthop Res* 12:340-349.

Attur MG, Dave MN, Clancy RM, Patel IR, Abramson SB, Amin AR 2000 Functional genomic analysis in arthritis-affected cartilage: yin-yang regulation of inflammatory mediators by alpha 5 beta 1 and alpha V beta 3 integrins. *J Immunol* 164(5):2684-2691.

Ayajiki K, Kindermann M, Hecker M, Fleming I, Busse R 1996 Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. *Circ Res* 78:750-758.

Banes AJ, Gilbert J, Taylor D, Monbureau O 1985 A new vacuum-operated stress-providing instrument that applies static or variable duration cyclic tension or compression to cells in vitro. *J Cell Sci* 75:35-42.

Bao X, Clark CB, Frangos JA 2000 Temporal gradient in shear-induced signaling pathway: involvement of MAP kinase, c-fos, and connexin 43. *Am J Physiol* 278(5):H1598-1605.

Barre PE, Redini F, Boumediene K, Vielpeau C, Pujol JP 2000 Semiquantitative reverse transcription-polymerase chain reaction analysis of syndecan-1 and –4 messages in cartilage and cultured chondrocytes from osteoarthritic joints. *Osteoarthritis Cartilage* 8:34-43.

Basdra EK, Huber LA, Komposch G, Papavassiliou AG 1994 Mechanical loading triggers specific biochemical responses in mandibular condylar chondrocytes. *Biochim Biophys Acta* 1222:315-322.

Battaini F, Pascale A, Lucchi L, Pasinetti GM, Govoni S 1999 Protein kinase C anchoring deficit in postmortem brains of Alzheimer's disease patients. *Exp Neurol* 159(2):559-564.

Battaini F, Pascale A, Paoletti R, Govoni S 1997 The role of anchoring protein RACK1 in PKC activation in the ageing rat brain. *Trends Neurosci* 20(9):410-415.

Baumann M, Gires O, Kolch W, Mischak H, Zeidler R, Pich D, Hammerschmidt W 2000 The PKC targeting protein RACK1 interacts with the Epstein-Barr virus activator protein BZLF1. *Eur J Biochem* 267(12):3891-3901.

Bavington C, Wright MO, Jobanputra P, Lin H, Morrison PT, Brennan FR, Salter DM, Nuki G 1996 Accelerated proteoglycan synthesis in human chondrocytes following cyclical pressurisation is mediated by β1 integrin and stretch activated ion channels. *Br J Rheumatol* 35(Suppl A1):110.

Beekman B, Verzijl N, Bank RA, von der Mark K, Te Koppele JM 1997 Synthesis of collagen by bovine chondrocytes cultured in alginate; Post translational modifications and cell matrix interaction. *Exp Cell Res* 237: 135-141.

Behrens J, Vakaet L, Friis R, Winterhager E, Van Roy F, Mareel MM, Birchmeier W 1993 Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphoryiation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J Cell Biol* 120:757-766.

Belkin AM, Zhidkova NI, Balzac F, Altruda F, Tomatis D, Maier A, Tarone G, Koteliansky VE, Burridge K 1996 β_{1D} integrin displaces β_{1A} isoform in striated

muscles: localization at junctional structures and signaling potential in non-muscle cells. *J Cell Biol* 132:211-226.

Belloli DM, Williams JL, Park JY 1991 Calculated strain fields on cell stressing devices employing circular diaphragms as substrates. *Trans Orthop Res Soc* 16:399

Bendrups A, Hilton A, Meager A, Hamilton JA 1993 Reduction of tumor necrosis factor alpha and interleukin-1 beta levels in human synovial tissue by interleukin-4 and glucocorticoid. *Rheumatol Int* 12:217-220.

Benya PD, Shaffer JD 1982 Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* 30:215-224.

Ben-Ze'ev A, Geiger B 1998 Differential molecular interactions of β-catenin and plakoglobin in adhesion, signaling and cancer. *Curr Opin Cell Biol* 10:629-639.

Berk BC, Corson MA, Peterson TE, Tseng H 1995 Protein kinases as mediators of fluid shear stress stimulated signal transduction in endothelial cells: a hypothesis for calcium-dependent and calcium-independent events activated by flow. *J Biomech* 28:1439-1450.

Berridge MJ 1987 Inositol triphosphate and diacylglycerol: two interacting second messengers. *Annu Rev Biochem* 56:159-193.

Bers DM 1982 A simple method for the accurate determination of free [Ca] in Ca-EGTA solutions. *Am J Physiol* 242:C404-408.

Bierbaum S, Notbohm H 1998 Tyrosine phosphorylation of 40 kDa proteins in osteoblastic cells after mechanical stimulation of beta1-integrins. *Eur J Cell Biol* 77:60-67.

Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Wart HV, Poole AR 1997 Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99:1534-1545.

Bjelle AO 1975 Content and composition of glycosaminoglycans in human knee joint cartilage. Variation with site and age in adults. *Conn Tiss Res* 3:141-147.

Blanco FJ 1999 Catabolic events in osteoarthritic cartilage. *Osteoarthritis Cartilage* 7:308-309.

Blatz AL , Magleby KL 1986 Single apamin-blocked Ca-activated K+ channels of small conductance in cultured rat skeletal muscle. *Nature (London)* 323:718-720.

Brandon NJ, Uren JM, Kittler JT, Wang H, Olsen R, Parker PJ, Moss SJ 1999 Subunit-specific association of protein kinase C and the receptor for activated C kinase with GABA type A receptors. *J Neurosci* 19(21):9228-9234.

Brighton CT, Strafford B, Gross SB, Leatherwood DF, Williams JL, Pollack SR 1991 The proliferative and synthetic response of isolated calvarial bone cells of rats to cyclic biaxial mechanical strain. *J Bone Joint Surg [Am]* 73:320-331.

Brocklehurst R, Bayliss MT, Maroudas A Coysh HL, Freeman MAR, Revell PA, Ali SY 1984 The composition of normal and osteoarthritic articular cartilage from human knee joints. *J Bone Joint Surg* 66:95-106.

Bromberg ME, Sevy RW, Daniel JL, Salganicoff L 1985 Role of myosin phosphorylation in contractility of a platelet aggregate. *Am J Physiol* 249 (3 Pt 1):C297-303.

Brown E, Hooper L, Ho T, Gresham H 1990 Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *J Cell Biol* 111(6 Pt 1):2785-2794.

Bullough PG 1992 The pathology of osteoarthritis. In: In: Moskowitz RW, Howell DS, Goldberg VM, Mankin HJ ed. Osteoarthritis: Diagnosis and medical/surgical management. Philadelphia: WB Saunders. P39-69.

Burridge K, Fath K, Kelly T, Nuckolls G, Turner C 1988 Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* 4:487-525.

Burridge K, Turner CE, Romer LH 1992 Tyrosine phosphorylation of paxillin and $pp125^{FAK}$ accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J Cell Biol* 119:893-903.

Burton-Wurster N, Butler M, Harter S, Colombo C, Quintavalla J, Swartzendurber D, Arsenis C, Lust G 1986 Presence of fibronectin in articular cartilage in two animal models of osteoarthritis. *J Rheumatol* 13:175-182.

Burton-Wurster N, Horn VJ, Lust G 1988 Immunohistochemical localization of fibronectin and chondronectin in canine articular cartilage. *J Histochem Cytochem* 36:581-588.

Burton-Wurster N, Todhunter RJ, Lust G 1993 Animal models of osteoarthritis. In: Woessner JF, Howell DS ed. Joint cartilage degradation: basic and clinical aspects. New York: Marcel Dekker. P347-384.

Byers PD, Contepomi CA, Farkas TA 1970 A post mortem study of the hip joint including the prevalence of the features of the right side. *Ann Rheum Dis* 29:15-31.

Camper L, Heinegard D, Lundgren-Akerlund E 1997 Integrin alpha2beta1 is a receptor for the cartilage matrix protein chondroadherin. *J Cell Biol* 138(5):1159-1167.

Camper L, Hellman U, Lundgren-Akerlund E 1998 Isolation, cloning, and sequence analysis of the integrin subunit alpha10, a beta1-associated collagen binding integrin expressed on chondrocytes. *J Biol Chem* 273:20383-20389.

Casella JF, Flanagan MD, Lin S 1981 Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. *Nature* 293(5830):302-305.

Caterson B, Lowther DA 1978 Changes in the metabolism of the proteoglycans from sheep articular cartilage in response to mechanical stress. *Biochim Biophys Acta* 540:412-422.

Centrella M, McCarthy TL, Canalis E 1988 Skeletal tissue and transforming growth factor β. *FASEB J* 2:3066-3073.

Chang BY, Conroy KB, Machleder EM, Cartwright CA 1998 RACK1, a receptor for activated C kinase and a homolog of the β subunit of G proteins, inhibits activity of Src tyrosine kinases and growth of NIH 3T3 cells. *Mol Cell Biol* 18(6):3245-3256.

Chano T, Tanaka M, Hukuda S, Saeki Y 2000 Mechanical stress induces the expression of high molecular mass heat shock protein in human chondrocytic cell line CS-OKB. *Osteoarthritis Cartilage* 8(2):115-119.

Charo IF, Nannizzi L, Smith JW, Cheresh DA 1990 The vitronectin receptor alpha v beta 3 binds fibronectin and acts in concert with alpha 5 beta 1 in promoting cellular attachment and spreading on fibronectin. *J Cell Biol* 111:2795-2800.

Chen BM, Grinnell AD 1995 Integrins and modulation of transmitter release from motor nerve terminals by stretch. *Science* 269:1578-1580.

Chen CS, Ingber DE 1999 Tensegrity and mechanoregulation: from skeleton to cytoskeleton. *Osteoarthritis Cartilage* 7:81-94.

Chen HC, Guan JL 1994 Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc Natl Acad Sci USA* 91:10148-10152.

Chen KD, Li YS, Kim M, Li S, Yuan S, Chien S, Shyy JY 1999 Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. *J Biol Chem* 274(26):18393-18400.

Chen LB 1988 Mitochondrial membrane potential in living cells. *Annu Rev Cell Biol* 4:155-181.

Chen Q, Kinch MS, Lin TH, Burridge K, Juliano RL 1994 Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J Biol Chem* 269:26602-26605.

Chen Y, Yu L 1994 Differential regulation by cAMP-dependent protein kinase and protein kinase C of the μ opioid receptor coupling to a G protein activated K^+ channel. *J Biol Chem* 269:7839-7842.

Chicurel ME, Chen CS, Ingber DE 1998 Cellular control lies in the balance of forces. *Curr Opin Cell Biol* 10:232-239.

Chiu JJ, Wung BS, Hsieh HJ, Lo LW, Wang DL 1999 Nitric oxide regulates shear stress-induced early growth response-1. Expression via the extracellular signal-regulated kinase pathway in endothelial cells. *Circ Res* 85(3):238-246.

Cho H, Ueda M, Tamaoka M, Hamaguchi M, Aisaka K, Kiso Y, Inoue T, Ogino R, Tatsuoka T, Ishihara T 1991 Novel caffeic acid derivatives: extremely potent inhibitors of 12-lipoxygenase. *J Med Chem* 34:1503-1505.

Chou YC, Chou CC, Chen YK, Tsai S, Hsieh FMJ, Liu HJ, Hseu TH 1999 Structure and genomic organization of porcine RACK1 gene. *Biochim Biophys Acta* 1489:315-322.

Chung J, Gao AG, Frazier WA 1997 Thrombspondin acts via integrin-associated protein to activate the platelet integrin αIIbβ3. *J Biol Chem* 272:14740-14746.

Chun JS, Ha MJ, Jacobson BS 1996 Differential translocation of protein kinase C epsilon during HeLa cell adhesion to a gelatin substratum. *J Biol Chem* 271:13008-13012.

Clancy R 1999 Nitric oxide alters chondrocyte function by disrupting cytoskeletal signaling complexes. *Osteoarthritis Cartilage* 7:399-400.

Clancy RM, Rediske J, Tang XY, Nijher N, Frenkel S, Philips M, Abramson SB 1997 Outside-in signaling in the chondrocyte. Nitric oxide disrupts fibronectin-induced assembly of a subplasmalemmal actin rho A focal adhesion kinase signaling complex. *J Clin Invest* 100:1789-1796.

Clark EA, Brugge JS 1995 Integrins and signal transduction pathways: the road taken. *Science* 268: 233-239.

Clark EA, Shattil SJ, Ginsberg MH, Bolen J, Brugge JS 1994 Regulation of the protein tyrosine kinase pp72syk by platelet agonists and the integrin alpha IIb beta 3. *J Biol Chem* 269:28859-28864.

Clemetson KJ, Clemetson JM 1998 Integrins and cardiovascular disease. *Cell Mol Life Sci* 54:502-513.

Clezardin P 1998 Recent insights into the role of integrins in cancer metastasis. *Cell Mol Life Sci* 54:541-548.

Cohen GB, Ren R, Baltimore D 1995 Modular binding domains in signal transduction proteins. *Cell* 80:237-248.

Collins DH 1949 Pathology of articular and spinal diseases. London: Edward Arnold. P74-115.

Collins DH, McElligott TF 1960 Sulphate uptake by chondrocytes in relation to histological changes in osteoarthritic human articular cartilage. *Ann Rheum Dis* 19:318-330.

Cook SJ, McCormick F 1994 Ras blooms on sterile ground. *Nature (London)* 369:361-362.

Cook SJ, Rubinfeld B, Albert I, McCormick F 1993 RapV12 antagonizers Ras-dependent activation of Erk-1 and Erk-2 by LPA and EGF in rat-1 fibroblasts. *EMBO J* 12:3475-3485.

Cooper D, Lindberg FP, Gamble JR, Brown EJ, Vadas MA 1995 Transendothelial migration of neutrophils involves integrin-associated protein (CD47). *Proc Natl Acad Sci USA* 92:3978-3982.

Cornford P, Evans J, Dodson A, Parsons K, Woolfenden A, Neoptolemos J, Foster CS 1999 Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer. *Am J Pathol* 154:137-144.

Corsini E, Battaini F, Lucchi L, Marinovich M, Racchi M, Govoni S, Galli CL 1999 A defective protein kinase C anchoring system underlying age-associated impairment in TNF-α production in rat macrophages. *J Immunol* 163:3468-3473.

Csukai M, Chen CH, De Matteis MA, Mochly-Rosen D 1997 The coatomer protein β′-COP, a selective binding protein (RACK) for protein kinase Cε. *J Biol Chem* 272(46):29200-29206.

Curley GP, Blum H, Humphries MJ 1999 Integrin antagonists. *Cell Mol Life Sci* 56:427-441.

Daniel JM, Reynolds AB 1997 Tyrosine phosphorylation and cadherin/catenin function. *Bioessays* 19:883-891.

Davies PF 1995 Flow-mediated endothelial mechanotransduction. *Physiol Rev* 75(3):519-560.

Davies ME, Dingle JT, Pigott R, Power C, Sharma H 1991 Expression of intercellular adhesion molecule 1 (ICAM-1) on human articular cartilage chondrocytes. *Connect Tissue Res* 26:207-216.

Davies PF, Robotewskyj A, Griem ML 1993 Endothelial cell adhesion in real-time. Measurements in vitro by tandem scanning confocal image analysis. *J Clin Invest* 91:2640-2652.

Davies PF, Robotewskyj A, Griem ML 1994 Quantitative studies of endothelial cell adhesion: directional remodeling of focal adhesion sites in response to flow forces. *J Clin Invest* 93:2031-2038.

Davies PF, Tripathi SC 1993 Mechanical stress mechanisms and the cell. An endothelial paradigm. *Circ Res* 72:239-245.

Dekker LV, Parker PJ 1994 Protein kinase C: a question of specificity. *Trends Biochem Sci* 19:73-77.

Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S 1998 Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc Natl Acad Sci USA* 95:11211-11216.

Demoulin JB, Renauld JC 1998 Signalling by cytokines interacting with the interleukin-2 receptor gamma chain. *Cytokines Cell Mol Ther* 4:243-256.

DeWitt MT, Handley CJ, Oakes BW, Lowther DA 1984 In vitro response of chondrocytes to mechanical loading. The effect of short term mechanical tension. *Connect Tissue Res* 12:97-109.

Dimmeler S, Assmus B, Hermann C, Haendeler J, Zeiher AM 1998 Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. *Circ Res* 83(3):334-341.

Disatnik MH, Buraggi G, Mochly-Rosen D 1994 Localization of protein kinase C isozymes in cardiac myocytes. *Exp Cell Res* 210:287-297

Doege KJ, Sasaki M, Horigan E, Hassell JR, Yamada Y 1987 Complete primary structure of the rat cartilage proteoglycan core protein deduced from cDNA clones. *J Biol Chem* 262:17757-17767.

Doege KJ, Sasaki M, Kimura T, Yamada Y 1991 Complete coding sequence and deduced primary culture of the human cartilage large aggregating proteoglycan aggrecan. Human-specific repeats and additional alternatively spliced forms. *J Biol Chem* 266:894-902.

Donnelly RP, Fenton MJ, Finbloom DS, Gerrard TL 1990 Differential regulation of IL-1 production in human monocytes by IFN-gamma and IL-4. *J Immunol* 145:569-575.

Durr J, Goodman S, Potocnik A, von der Mark H, von der Mark K 1993 Localization of β1 integrins in human cartilage and their role in chondrocyte adhesion to collagen and fibronectin. *Exp Cell Res* 207:235-244.

Durr J, Lammi P, Goodman SL, Aigner T, von der Mark K 1996 Identification and immunolocalization of laminin in cartilage. *Exp Cell Res* 222:225-233.

Eagling VA, Tjia JF, Back DJ 1998 Differential selectivity of cytochrome P450 inhibitors against probe substrates in human and rat liver microsomes. *Br J Clin Pharmacol* 45:107-114.

Eggli PS, Hunziker EB, Schenk RK 1988 Quantitation of structural features characterizing weight- and less-weight-bearing regions in articular cartilage: a steriological analysis of medial femoral condyles in young rabbits. *Anatom Rec* 222:217-227.

Eliceiri BP, Cheresh DA 1999 The role of αv integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *J Clin Invest* 103:1227-1230.

Enomoto-Iwamoto M, Iwamoto M, Nakashima K, Mukudai Y, Boettiger D, Pacifici M, Kurisu K, Suzuki F 1997 Involvement of alpha 5 beta 1 integrin in matrix interactions and proliferation of chondrocytes. *J Bone Miner Res* 12:1124-1132.

Enomoto-Iwamoto M, Iwamoto M, Pacifici M, Boettiger D, Kurisu K, Suzuki F 1995 The interaction of alpha 5 beta 1 integrin with fibronectin is required for proliferation of chondrocytes. *Trans Orthop Res Soc* 20:396.

Enomoto M, LeBoy PS, Menko AS, Boettinger D 1993 Beta 1 integrins mediate chondrocyte interaction with type I collagen, type II collagen and fibronectin. *Exp Cell Res* 205:276-285.

Escriba PV, Garcia-Sevilla JA 1999 Parallel modulation of receptor for activated C kinase 1 and protein kinase C-α and β isoforms in brains of morphine-treated rats. *Br J Pharmacol* 127:343-348.

Essner R, Rhoades K, McBride WH, Morton DL, Economou JS 1989 IL-4 down-regulates IL-1 and INF gene expression in human monocytes. *J Immunol* 142:3857-3861.

Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M, McCormick F, Williams LT 1992 Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* 69:413-423.

Fisslthaler B, Dimmeler S, Hermann C, Busse R, Fleming I 2000 Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. *Acta Physiol Scand* 168(1):81-88.

Fornaro M, Languino LR 1997 Alternatively spliced variants: a new view of the integrin cytoplasmic domain. *Matrix Biol* 16:185-193.

Fosang AJ, Hardingham TE 1989 Isolation of the N-terminal globular protein domains from cartilage proteoglycans. Identification of G2 domain and its lack of interaction with hyaluronate and link protein. *Biochem J* 261:801-809.

Frenkel SR, Clancy RM, Ricci JL, DrCesare PE, Rediske JJ, Abramson SB 1996 Effects of nitric oxide on chondrocyte migration, adhesion, and cytoskeletal assembly. *Arthritis Rheum* 39:1905-1912.

Frisch SM, Ruoslahti E 1997 Integrins and anoikis. *Curr Opin Cell Biol* 9:701-706.

Fujisawa T, Hattori T, Takahashi K, Kuboki T, Yamashita A, Takigawa M 1999 Cyclic mechanical stress induces extracellular matrix degradation in cultures chondrocytes via gene expression of matrix metalloproteinases and interleukin-1. *J Biochem* 125(5):966-975.

Fukuda K, Asada S, Kumano F, Saitoh M, Otani K, Tanaka S 1997 Cyclic tensile stretch on bovine articular chondrocytes inhibits protein kinase C activity. *J Lab Clin Med* 130:209-215.

Gahmberg CG, Valmu L, Fagerholm S, Kotovuori P, Ihanus E, Tian L, Pessa-Morikawa T 1998 Leukocyte integrins and inflammation. *Cell Mol Life Sci* 54:549-555.

Galvez A, Gimenez-Gailego G, Reuben JP, Roy-Contancin L, Feigenbaum P, Kaczorowski GJ, Garcia ML 1990 Purification and characterization of a unique, potent peptidyl probe for the high conductance calcium-activated potassium channel from the venom of the scorpion Buthus tamulus. *J Biol Chem* 265:11083-11090.

Gao AG, Lindberg FP, Dimity JM, Brown EJ, Frazier WA 1996 Thrombospondin modulates $\alpha V\beta 3$ function through integrin-associated protein. *J Cell Biol* 135:533-544.

Gardner DL 1992 Osteoarthritis and allied diseases. In: Gardner DL ed. Pathological basis of the connective tissue diseases. London: Edward Arnold. P842-923.

Gebken J, Luders B, Notbohm H, Klein HH, Brinckmann J, Muller PK, Batge B 1999 Hypergravity stimulates collagen synthesis in human osteoblast-like cells: evidence for the involvement of p44/42 MAP-kinase (ERK1/2). *J Biochem* 126(4):676-682.

Geiger B, Volberg T, Ginsberg D, Bitzur S, Sabanay I, Hynes RO 1990 Broad spectrum pan-cadherin antibodies, reactive with the C-terminal 24 amino acid residues of N-cadherin. *J Cell Sci* 97:607-614.

Geijsen N, Spaargaren M, Raaijmakers JA, Lammers JW, Koenderman L, Coffer PJ 1999 Association of RACK1 and PKCbeta with the common beta-chain of the IL-5/IL-3/GM-CSF receptor. *Oncogene* 18(36):5126-5130.

Giancotti FG 1997 Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr Opin Cell Biol* 9:691-700.

Giancotti FG, Ruoslahti E 1999 Integrin signaling. *Science* 285:1028-1032.

Gilbert JA, Banes AJ, Link GW 1989 Characterization of the surface strain applied to cyclically stretched cells in vitro. *Trans Orthop Res Soc* 14:249.

Girard PR, Nerem RM 1993 Endothelial cell signaling and cytoskeletal changes in response to shear stress. *Front Med Biol Eng* 5:31-36.

Glogauer M, Arora P, Yao G, Sokholov I, Ferrier J, McCulloch CA 1997 Calcium ions and tyrosine phosphorylation interact coordinately with actin to regulate cytoprotective responses to stretching. *J Cell Sci* 110(Pt 1):11-21.

Goldring MB 2000 The role of the chondrocyte in osteoarthritis. Arthritis Rheum 43:1916-1926.

Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, Arbiser JL, Apperley JF 1994 Interleukin-1β-modulated gene expression in immortalized human chondrocytes. *J Clin Invest* 94:2307-2316.

Goligorsky MS 1988 Mechanical stimulation induces Ca_i^{2+} transients and membrane depolarization in cultured endothelial cells. Effects on Ca_i^{2+} in co-perfused smooth muscle cells. *FEBS Lett* 240:59-64.

Gordon T 1968 Osteoarthrosis in US adults. In: Bennett PH, Wood PHN ed. Population studies of the rheumatic diseases. New York: Excerpta Medica Fundation. P391-397.

Grandolfo M, Calabrese A, D'andrea P 1998 Mechanism of mechanically induced intercellular calcium waves in rabbit articular chondrocytes and in HIG-82 synovial cells. *J Bone Miner Res* 13:443-453.

Guan JL 1997 Role of focal adhesion kinase in integrin signaling. *Int J Biochem Cell Biol* 29:1085-1096.

Guilak F, Bachrach NM 1993 Compression-induced changes in chondrocyte shape and volume determined in situ using confocal microscopy. *Trans Am Orthop Res Soc* 18:619.

Guilak F, Zell RA, Erickson GR, Grande DA, Rubin CT, McLeod KJ, Donahue HJ 1999 Mechanically induced calcium waves in articular chondrocytes are inhibited by gadolinium and amiloride. *J Orthop Res* 17(3):421-429.

Gumbiner BM 1995 Signal transduction of beta-catenin. *Curr Opin Cell Biol* 7:634-640.

Haidekker MA, L'Heureux N, Frangos JA 2000 Fluid shear stress increases membrane fluidity in endothelial cells: a study with DCVJ fluorescence. *Am J Physiol* 278(4):H1401-1406.

Hakomori S, Fukuda M, Sekiguchi K, Carter WG 1984 Fibronectin, laminin and other extracellular glycoproteins. In: Piez KA, Reddi AH ed. Extracellular Matrix Biochemistry. New York: Elsevier P229-275.

Haller H, Lindschau C, Maasch C, Olthoff H, Kurscheid D, Luft FC 1998 Integrin-induced protein kinase Cα and Cε translocation to focal adhesions mediates vascular smooth muscle cell spreading. *Circ Res* 82:157-165.

Hamanishi C, Hashima M, Satsuma H, Tanaka S 1996 Protein kinase C activator inhibits progression of osteoarthritis induced in rabbit knee joints. *J Lab Clin Med* 127:540-544.

Hamill OP, Lane JW, McBride DW 1992 Amiloride: a molecular probe for mechano-sensitive channels. *Trends Pharmacol Sci* 13:373-376.

Han O, Li GD, Sumpio BE, Basson MD 1998a Strain induces Caco-2 intestinal epithelial proliferation and differentiation via PKC and tyrosine kinase signals. *Am J Physiol* 275(3 Pt 1):G534-541.

Han O, Sumpio BE, Basson MD 1998b Mechanical strain rapidly redistributes tyrosine phosphorylated proteins in human intestinal Caco-2 cells. *Biochem Biophys Res Commun* 250(3):668-673.

Hanks SK, Polte TR 1997 Signaling through focal adhesion kinase. *Bioessays* 19:137-145.

Hardingham TE, Beardmore-Gray M, Dunham DG, Ratcliffe A 1986 Cartilage proteoglycans. Functions of proteoglycans. *Ciba Found Symp* 124:30-46.

Hardingham TE, Fosang AJ 1992 Proteoglycans: many forms and many functions. *FASEB J* 6:861-870.

Hart PH, Ahern MJ, Smith MD, Finlay-Jones JJ 1995 Comparison of the suppressive effects of interleukin-10 and interleukin-4 on synovial fluid macrophages and blood monocytes from patients with inflammatory arthritis. *Immunology* 84:536-542.

Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA 1989 Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc Natl Acad Sci USA* 86:3803-3807.

Hasegawa S, Sato S, Saito S, Suzuki Y, Brunette DM 1985 Mechanical stretching increases the number of cultured bone cells synthesizing DNA and alters their pattern of protein synthesis. *Calcif Tissue Int* 37:431-436.

Heinegard D, Hascall VC 1974 Aggregation of cartilage proteoglycans. III. Characteristics of the proteins isolated from trypsin digests of aggregates. *J Biol Chem* 249:4250-4256.

Heinegard D, Saxne T, Lorenzo P 1999 Noncollagenous proteins: glycoproteins and related molecules. In: Seibel MJ, Robins SP, Bilezekian JP ed. Dynamics of bone and cartilage metabolism. New York: Academic Press. P59-69.

Helminen HJ, Jurvelin J, Kiviranta I, Paukkonen K, Saamanen AM, Tammi M 1987 Joint loading effects on articular cartilage: a historical review. In Helminen HJ, Kiviranta I, Tammi M, Sasmanen AM, Paukkonen K, and Jurvelin J: Joint Loading: biology and health of articular structures. P1-46.

Hemler ME 1990 VLA proteins in the integrin family: structures, functions and their role on leukocytes. *Annu Rev Immunol* 8:365-400.

Herbert JM, Augereau JM, Gleye J, Maffrand JP 1990 Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* 172:993-999.

Hering TM, Kollar J, Huynh TD, Varelas JB, Sandell LJ 1994 Modulation of extracellular matrix gene expression in bovine high density chondrocyte cultures by ascorbic acid and enzymatic resuspension. *Arch Biochem Biophys* 314:90-98.

Hering TM 1999 Regulation of chondrocyte gene expression. *Front Biosci* 4:D743-761.

Hermann A, Erxleben C 1987 Charybdotoxin selectively blocks small Ca-activated K channels in aplysia neurons. *J Gen Physiol* 90:27-47.

Hidaka H, Inagaki M, Kawamotos S, Sasaki Y 1984 Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* 23:5036-5041.

Hidaka H, Sasaki Y, Tanaka T, Endo T, Ohno S, Fujii Y, Nagata T 1981 N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc Natl Acad Sci USA* 78:4354-4357.

Hildebrand JD, Schaller MD, Parsons JT 1993 Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *J Cell Biol* 123:993-1005.

Himmel HM, Whorton AR, Strauss HC 1993 Intracellular calcium, current, and stimulus-response coupling in endothelial cells. *Hypertension Dallas* 21:112-127.

Hirsch MS, Lunsford LE, Trinkhaus V, Svoboda KKH 1997 Chondrocyte survival and differentiation *in situ* are integrin mediated. *Developmental Dynamics* 210: 249-263.

Hoch DH, Grodzinsky AJ, Koob TJ, Albert ML, Eyre DR 1983 Early changes in material properties of rabbit articular cartilage after menisectomy. *J Orthop Res* 1:4-12.

Hodge WA, Fijan RS, Carlson KL, Burgess RG, Harris WH, Mann RW 1986 Contact pressures in the human hip joint measured in vivo. *Proc Natl Acad Sci USA* 83:2879-2883.

Holmvall K, Camper L, Johansson S, Kimura JH, Lundgren Akerlund E 1995 Chondrocyte and chondrosarcoma cell integrins with affinity for collagen type II and their response to mechanical stress. *Exp Cell Res* 221:496-503.

Homandberg GA, Hui F 1994 Arg-Gly-Asp-Ser peptide analogs supress cartilage chondrolytic activities integrin binding and non-binding fibronectin fragments. *Arch Biochem Biophys* 310:40-48.

Hoschuetzky H, Aberle H, Kemler R 1994 Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J Cell Biol* 127:1375-1380.

Hosea NA 1998 Oxidation of nonionic detergents by cytochrome P450 enzymes. *Arch Biochem Biophys* 353:365-373.

Hotchin NA, Hall A 1995 The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. *J Cell Biol* 131:1857-1865.

Hounsell EF, Feeney J, Scudder P, Tang PW, Feiz T 1986 H-NMR studies at 500 MHz of a neutral disaccharide and sulphated di-g-tetra-, hexa- and larger oligosaccharides obtained by endo-β-galactosidase treatment of keratan sulphate. *Eur J Biochem* 157:375-384.

Howe A, Aplin AE, Alahari SK, Juliano RL 1998 Integrin signaling and cell growth control. *Curr Opin Cell Biol* 10:220-231.

Howell DS, Treadwell BV, Trippel SB 1992 Etiopathogenesis of osteoarthritis. In: Moskowitz RW, Howell DS, Goldberg VM, Mankin HJ ed. Osteoarthritis: Diagnosis and medical/surgical management. Philadelphia: WB Saunders. P233-252.

Hudspeth AJ 1989 How the ear's works work. *Nature Lond* 341:397-404.

Hughes PE, Pfaff M 1998 Integrin affinity modulation. *Trends Cell Biol* 8:359-364.

Humphries MJ, Newham P 1998 The structure of cell-adhesion molecules. *Trends Cell Biol* 8:78-83.

Hung CT, Williams JL 1994 A method for inducing equi-biaxial and uniform strains in elastomeric membranes used as cell substrates. *J Biomech* 27:227-232.

Hunter T 1995 Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. *Cell* 80:237-248.

Hynes RO 1992 Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.

Ikeda M, Takei T, Mills I, Kito H, Sumpio BE 1999 Extracellular signal-regulated kinases 1 and 2 activation in endothelial cells exposed to cyclic strain. *Am J Physiol* 276 (2 Pt 2):H614-622.

Ikeda M, Takei T, Mills I, Sumpio BE 1998 Calcium-independent activation of extracellular signal-regulated kinases 1 and 2 by cyclic strain. *Biochem Biophys Res Commun* 247:462-465.

Ilyas M, Tomlinson IPM 1997 The interactions of APC, E-cadherin and β-catenin in tumor development and progression. *J Pathol* 182:128-137.

Imhof BA, Weeransinghe D, Brown EJ, Lindberg FP, Hammel P, Piali L, Dessing M, Gisler R 1997 Cross talk between alpha(v)beta3 and alpha4beta1 integrins regulates lymphocyte migration on vascular cell adhesion molecule 1. *Eur J Immunol* 27:3242-3252.

Ingber DE 1991 Integrins as mechanochemical transducers. *Curr Opin Cell Biol* 3:841-848.

Ingber DE 1997 Tensegrity: the architectural basis of cellular mechanotransduction. *Ann Rev Physiol* 59:575-599.

Irie A, Kamata T, Takada Y 1997 Multiple loop structures critical for ligand binding of the integrin α4 subunit in the upper face of the β-propeller mode 1. *Proc Natl Acad Sci USA* 94:7198-7203.

Ishida T, Haneda M, Maeda S, Koya D, Kikkawa R 1999 Stretch-induced overproduction of fibronectin in mesangial cells is mediated by the activation of mitogen-activated protein kinase. *Diabetes* 48:595-602.

Ishida T, Peterson TE, Kovach NL, Berk BC 1996 MAP kinase activation by flow in endothelial cells. Role of beta 1 integrins and tyrosine kinases. *Circ Res* 79(2):310-316.

Jaken S, Leach K, Klauck T 1989 Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. *J Cell Biol* 109:697-704.

Jobanputra P, Lin H, Jenkins K, Bavington C, Brennan FR, Nuki G, Salter DM, Godolphin JL 1996 Modulation of human chondrocyte integrins by inflammatory synovial fluid. *Arthritis Rheum* 39:1430-1432.

Jockusch BM, Bubeck P, Giehl K, Kroemker M, Moschner J, Rothkegel M, Rudiger M, Schluter K, Stanke G, Winkler J 1995 The molecular architecture of focal adhesions. *Annu Rev Cell Dev Biol* 11:379-416.

Jorgensen F, Ohmori H 1988 Amiloride blocks the mechano-electrical transduction channel of hair cells in the chick. *J Physiol* 403:577-588.

Jou TS, Stewart DB, Stappert J, Nelson WJ, Marrs JA 1995 Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. *Proc Natl Acad Sci USA* 92:5067-5071.

Jurvelin J, Helminen H, Lauritsalo S 1985 Influences of joint immobilization and running exercise on articular cartilage surfaces of young rabbits. *Acta Anat* 122:62-68.

Kaplan KB, Bibbins KB, Swedlow JR, Aranud M, Morgan DO, Varmus HE 1994 Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. *EMBO J* 13:4745-4756.

Kapron-Bras C, Fitz-Gibbon L, Jeevaratnam P, Wilkins J, Dedhar S 1993 Stimulation of tyrosine phosphorylation and accumulation of GTP-bound p21ras upon antibody-mediated alpha 2 beta 1 integrin activation in T-lymphoblastic cells. *J Biol Chem* 268:20701-20704.

Kavanaugh WM, Turck CW, Williams LT 1995 PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science* 268:1177-1179.

Keely P, Parise L, Juliano R 1998 Integrins and GTPase in tumour cell growth, motility and invasion. *Trends Cell Biol* 8:101-106.

Kempson GE, Muir H, Pollard C, Tuke M 1973 The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297:465-472.

Kiley SC, Parker PJ, Fabbro D, Jaken S 1991 Differential regulation of protein kinase C isozymes by thyrotropin-releasing hormone in GH4C1 cells. *J Biol Chem* 266:23761-23768.

Kiviranta I, Tammi M, Jurvelin J, Arokoski J, Saamanen AM, Helminen H 1988 Moderate running exercise augments glycosaminoglycans and thickness of articular cartilage in the knee joint of young beagle dogs. *J Orthop Res* 6:188-195.

Klein SF, Giancotti F, Presta M, Arbelda SM, Buck CA, Rifkin DB 1993 Basic FGF modulates integrin expression in microvascular endothelial cells. *Mol Biol Cell* 4:873-892.

Kobayashi E, Nakano H, Morimota M, Tamaoki T 1989 Calphostic C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kianse C. *Bichem Biophys Res Commun* 159(2):548-553.

Kolanus W, Seed B 1997 Integrins and inside-out signal transduction: converging signals from PKC and PIP3. *Curr Opin Cell Biol* 9:725-731.

Komaru T, Wang Y, Akai K, Sato K, Sekiguchi N, Sugimura A, Kumagai T, Kanatsuke H, Shirato K 1994 Pertussis toxin-sensitive G protein mediates coronary microvascular control during autoregulation and ischemia in canine heart. *Circ Res* 75:556-566.

Komoriya A, Green LJ, Mervic M, Yamada SS, Yamada KM, Humphries MJ 1991 The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *J Biol Chem* 266:15075-15079.

Korinek V, Barker N, Morin PJ, Wichen DV, Weger RD, Kinzler KW, Vogelstein B, Clevers H 1997 Constitutive transcriptional activation by a β-catenin-Tcf complex in APC-/- colon carcinoma. *Science* 275:1784-1787.

Kroll MH, Hellums JD, Guo Z, Durante W, Razdan K, Hrbolich JK, Schafer AI 1993 Protein kinase C is activated in platelets subjected to pathological shear stress. *J Biol Chem* 268(5):3520-3524.

Kuhn K, Eble J 1994 The structural bases of integrin-ligand interactions. *Trends Cell Biol* 4:256-260.

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Laflamme SE, Homan SM, Bodeau AL, Mastrangelo AM 1997 Integrin cytoplasmic domains as connectors to the cell's signal transduction apparatus. *Matrix Biol* 16:153-163.

Laneuville O, Breuer DK, DeWitt DL, Hla T, Funk CD, Smith WL 1994 Differential inhibition of human prostaglandin endoperoxide H synthases-1 and –2 by nonsteroidal anti-inflammatory drugs. *J Pharmacol Exp Ther* 271:927-934.

Languino LR, Ruoslahti E 1992 An alternative form of the integrin β1 subunit with a variant cytoplasmic domain. *J Biol Chem* 267:7116-7120.

Lansman JB, Hallam TJ, Rink TJ 1987 Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? *Nature (London)* 325:811-812.

Lapadula G, Iannone F, Zuccaro C, Grattagliano V, Covelli M, Patella V, Lo Bianco G, Pipitone V 1998 Chondrocyte phenotyping in human osteoarthritis. *Clin Rheumatol* 17:99-104.

Lapadula G, Iannone F, Zuccaro C, Grattagliano V, Covelli M, Patella V, Lo Bianco G, Pipitone V 1997 Integrin expression on chondrocytes: correlations with the degree of cartilage damage in human osteoarthritis. *Clin Exp Rheumatol* 15:247-254.

Lardans V, Serra E, Capron A, Dissous C 1998 Characterization of an intracellular receptor for activated protein kinase C (RACK) from the mollusc Biomphalaria glabrata, the intermediate host for Schistosoma mansoni. *Exp Parasitol* 88(3):194-199.

Lark MW, Bayne EK, Flanagan J, Harper CF, Hoerrner LA, Hutchinson NI, Singer II, Donatelli SA, Weidner JR, Williams HR, Mumford RA, Lohmander LS 1997 Aggrecan degradation in human cartilage: evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. *J Clin Invest* 100:93-106.

Lawrence JS, Bremner JM, Bier F 1966 Osteoarthrosis. Prevalence in the population and relationship between symptoms and x-ray changes. *Ann Rheum Dis* 25:1-24.

Lee GM, Johnstone B, Jacobson K, Caterson B 1993 The dynamic structure of the pericellular matrix on living cells. *J Cell Biol* 123:1899-1907.

Lee RC, Rich JB, Kelley KM, Weiman DS, Mathews MB 1982 A comparison of in vitro cellular responses to mechanical and electrical stimulation. *Am Surg* 48:567-574.

Lewis JM, Cheresh DA, Schwartz MA 1996 Protein kinase C regulates alpha V beta 5-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. *J Cell Biol* 134:1323-1332.

Li S, Kim M, Hu YL, Jalali S, Schlaepfer DD, Hunter T, Chien S, Shyy JY 1997 Fluid shear stress activation of focal adhesion kinase. Linking to mitogen-activated protein kinases. *J Biol Chem* 272:30455-30462.

Li S, Piotrowicz RS, Levin EG, Shyy YJ, Chien S 1996 Fluid shear stress induces the phosphorylation of small heat shock proteins in vascular endothelial cells. *Am J Physiol* 271(3 Pt 1):C994-1000.

Liliental J, Chang DD 1998 Rack1, a receptor for activated protein kinase C, interacts with integrin β subunit. *J Biol Chem* 273(4):2379-2383.

Lindberg FP, Gresham HD, Reinhold MI, Brown EJ 1996 Integrin-associated protein immunoglobulin domain is necessary for efficient vitronectin bead binding. *J Cell Biol* 134:1313-1322.

Lindberg FP, Gresham HD, Schwartz E, Brown EJ 1993 Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in αVβ3-dependent ligand binding. *J Cell Biol* 123:485-496.

Lischke A, Moriggl R, Brandlein S, Berchtold S, Kammer W, Sebald W, Groner B, Liu X, Hennighausen L, Friedrich K 1998 The interleukin-4 receptor activates STAT5 by a mechanism that relies upon common gamma-chain. *J Biol Chem* 273:31222-31229.

Liscovitch M, Chalifa V, Danin M, Eli Y 1991 Inhibition of neural phospholipase D activity by aminoglycoside antibiotics. *Biochemical J* 279 (pt 1): 319-321.

Liu M, Tanswell AK, Post M 1999 Mechanical force-induced signal transduction in lung cells. *Am J Physiol* 277(4):L667-L683.

Liu M, Xu J, Tanswell AK, Post M 1994 Inhibition of strain-induced fetal rat lung cell proliferation by gadolinium, a stretch-activated channel blocker. *J Cell Physiol* 161:501-507.

Loeser RF 1993 Integrin-mediated attachment of articular chondrocytes to extracellular matrix proteins. *Arthritis Rheum* 36:1103-1109.

Loeser RF, Carlson CS, McGee MP 1995 Expression of beta-1 integrins by cultured articular chondrocytes and in osteoarthritic cartilage. *Exp Cell Res* 217:248-257.

Loeser RF, Sadiev S, Tan L, Goldring MB 2000 Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins in mediating chondrocyte adhesion to type II and VI collagen. *Osteoarthritis Cartilage* 8:96-105.

Lohmander LS, Ionescu M, Jugessur H, Poole AR 1999 Changes in joint cartilage aggrecan after knee injury and in osteoarthritis. *Arthritis Rheum* 42:534-544.

Lotz M, Blanco FJ, von Kempis J, Dudler J, Maier R, Villiger PM, Geng Y 1994 Cytokine regulation of chondrocytes functions. *J Rheumatol* 22(suppl.43):104-108.

Lowell CA, Berton G 1999 Integrin signal transduction in myeloid leukocytes. *J Leukoc Biol* 65:313-320.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275.

Lukashev ME, Sheppard D, Pytela R 1994 Disruption of integrin function and induction of tyrosine phosphorylation by the autonomously expressed beta 1 integrin cytoplasmic domain. *J Biol Chem* 269:18311-18314.

Luria A, Tennenbaum T, Sun QY, Rubinstein S, Breitbart H 2000 Differential localization of conventional protein kinase C isoforms during mouse oocyte development. *Biol Reprod* 62(6):1564-1570.

Lytton J, Westlin M, Hanley MR 1991 Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J Biol Chem* 266:17067-17071.

Madri JA, Pratt BM, Yannariello-Brown J 1988 Matrix driven cell size change modulates aortic endothelial cell proliferation and sheet migration. *Am J Pathol* 132:18-27.

Malek AM, Jackman R, Rosenberg RD, Izumo S 1994 Endothelial expression of thrombomodulin is reversibly regulated by fluid shear stress. *Circ Res* 74:852-860.

Maniotis AJ, Chen CS, Ingber DE 1997 Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc Natl Acad Sci USA* 94:849-854.

Mankin HJ, Dorfman H, Lipiello L, Zarins A 1971 Biochemical and Metabolic Abnormalities in Articular Cartilage from Osteoarthritic Human Hips II. Correlation of Morphology with Biochemical and Metabolic Data. *J Bone Joint Surg* 53-A:523-537.

Mankin HJ, Lippiello L 1970 Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. *J Bone Joint Surg* 52:424-434.

Maroto R, De la Fuente MT, Artalejo AR, Abad F, Lopez MG, Garcia-Sancho J, Garcia AG 1994 Effects of Ca^{2+} channel antagonists on chromaffin cell death and cytosolic Ca^{2+} oscillations induced by veratridine. *Eur J Pharmacol* 270(4):331-339.

Maroudas A 1980 Physical chemistry of articular cartilage and the intervertebral disc. In: Sokoloff L ed. The joints and synovial fluid. New York: Academic Press. P239-291.

Martel-Pelletier J, Alaaeddine N, Pelletier JP 1999 Cytokines and their role in the pathophysiology of osteoarthritis. *Front Biosci* 4:D694-703.

Martel-Pelletier J, McCollum R, Di Battista JA, Faure MP, Chin JA, Fournier S, Sarfati M, Pelletier JP 1992 The interleukin-1 receptor in normal and osteoarthritic human articular chondrocytes. Identification as the type I receptor and analysis of binding kinetics and biologic function. *Arthritis Rheum* 35:530-540.

Martina M, Mozrzymas JW, Vittur F 1997 Membrane stretch activates a potassium channel in pig articular chondrocytes. *Biochim Biophys Acta* 1329:205-210.

Matsuda N, Morita N, Matsuda K, Watanabe M 1998 Proliferation and differentiation of human osteoblastic cells associated with differential activation of MAP kinases in response to epidermal growth factor, hypoxia, and mechanical stress in vitro. *Biochem Biophys Res Commun* 249(2):350-354.

Matsumoto H, Sasaki Y 1989 Staurosporine, a protein kinase C inhibitor interferes with proliferation of arterial smooth muscle cells. *Biochem Biophys Res Commun* 158:105-109.

Mawby WJ, Holmes CH, Anstee DJ, Spring FA, Tanner MJ 1994 Isolation and characterization of CD47 glycoprotein: a multispanning membrane protein which is the same as integrin-associated protein (IAP) and the ovarian tumor marker OA3. *Biochem J* 304(Pt 2):525-530.

Mayne R, Brewton RG 1993 Extracellular matrix of cartilage: collagen. In: Woessner JF Jr, Howell DS ed. Joint cartilage degradation: basic and clinical aspects. New York: Marcel Mekker. P81-108.

McCrea PD, Gumbiner BM 1991 Purification of a 92-kDa cytoplasmic protein tightly associated with the cell-cell adhesion molecule E-cadherin (Uvomorulin). *J Biol Chem* 266:4514-4520.

Michell RH 1992 Inositol lipids in cellular signalling mechanisms. *Trans Biochem Sci* 17:274-276.

Midwood KS 1998 Expression and function of NG2/HMPG in human cartilage. The University of Edinburgh: PhD Thesis.

Midwood KS, Salter DM 1998 Expression of NG2 proteoglycan in human articular chondrocytes. *Osteoarthritis cartilage* 6(5):297-305.

Miller C, Moczydlowski E, Latoire R, Philips M 1985 Charybdotoxin, a protein inhibitor of single Ca^{2+} -activated K^{+} channels from mammalian skeletal muscle. *Nature* (London) 313:316-318.

Millward-Sadler SJ, Wright MO, Davies LW, Nuki G, Salter DM 2000b Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes. *Arthritis Rheum* 43:2091-2099.

Millward-Sadler SJ, Wright MO, Lee HS, Caldwell H, Nuki G, Salter DM 2000a Altered electrophysiological responses to mechanical stimulation and abnormal signalling through $\alpha5\beta1$ integrin in chondrocytes from osteoarthritic cartilage. *Osteoarthritis Cartilage* 8:272-278.

Millward-Sadler SJ, Wright MO, Lee HS, Nishida K, Caldwell H, Nuki G, Salter DM 1999 Integrin-regulated secretion of interleukin 4: a novel pathway of mechanotransduction in human articular chondrocytes. *J Cell Biol* 145:183-189.

Mitrovic D, Quintero M, Stankovic A, Ryckewaert A 1983 Cell density of adult human femoral condylar articular cartilage. Joints with normal and fibrillated surfaces. *Lab Invest* 49:309-316.

Mochly-Rosen D, Khaner H, Lopez J 1991a Identification of intracellular receptor proteins for activated protein kinase C. *Proc Natl Acad Sci USA* 88:3997-4000.

Mochly-Rosen D, Khaner H, Lopez J, Smith BL 1991b Intracellular receptors for activated protein kinase C. *J Biol Chem* 266(23):14866-14868.

Mochly-Rosen D 1995 Localization of protein kinases by anchoring proteins: A theme in signal transduction. *Science* 268:247-251.

Mochly-Rosen D, Miller KG, Scheller RH, Khaner H, Lopez J, Smith BL 1992 p65 fragments, homologus to the C2 region of protein kinase C, bind to the intracellular receptors for protein kinase C. *Biochemistry* 31:8120-8124.

Monaghan H, Kongkanuntn R, Bubb VJ, Wyllie AH, Salter DM 1999 Expression of APC in human bone and cartilage. *J Pathol* 187:38A.

Morgelin M, Paulsson M, Hardingham TE, Heinegard D, Engel J 1988 Cartilage proteoglycans. Assembly with hyaluronate and link protein as studied by electron microscopy. *Biochem J* 253:175-185.

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW 1997 Activation of β-catenin-Tcf signaling in colon cancer by mutation in β-catenin or APC. *Science* 275:1787-1790.

Moses MA, Sudhalter J, Langer R 1990 Identification of an inhibitor of neovascularization from cartilage. *Science* 248:1408-1410.

Mosior M, Newton AC 1995 Mechanism of interaction of protein kinase C with phorbol esters. Reversibility and nature of membrane association. *J Biol Chem* 270:25526-25533.

Moskowitz RW 1992 Introduction. In Moskowitz RW, Howell DS, Goldberg VM, Mankin HJ ed. Osteoarthritis: Diagnosis and medical/surgical management. Philadelphia: WB Saunders. P1-7.

Mould AP, Burrows L, Humphries MJ 1998 Identification of amino acid residues that form part of the ligand-binding pocket of integrin α5β1. *J Biol Chem* 273:25664-25672.

Mow VC, Wang CC, Hung CT 1999 The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage. *Osteoarthritis cartilage* 7:41-58.

Muir H 1977 Molecular approach to the understanding of osteoarthrosis. *Ann Rheum Dis* 36:199-208.

Muir H, Carney SL 1987 Pathological and biochemical changes in cartilage and other tissues of the canine knee resulting from induced joint instability. . In Helminen HJ, Kiviranta I, Tammi M, Sasmanen AM, Paukkonen K, and Jurvelin J: Joint Loading: biology and health of articular structures. P47-63.

Muir H, Hardingham TE 1975 Structure of proteoglycan. In: Whelan WJ ed. MTP international review of science. Biochemistry series one. Vol.5: Biochemistry of Carbohydrates. Baltimore: University Park Press. P153-222.

Muller JM, Chilian WM, Davis MJ 1997 Integrin signaling transduces shear-stress dependent vasodilation of coronary arterioles. *Circ Res* 80:320-326.

Murata T, Puri RK 1997 Comparison of IL-13- and IL-4-induced signaling in EBV-immortalized human B cells. *Cell Immunol* 175:33-40.

Murrell GA, Jang D, Williams RJ 1995 Nitric oxide activates metalloprotease enzymes in articular cartilage. *Biochem Biophys Res Commun* 206:15-21.

Nagel T, Resnick N, Atkinson WJ, Dewey CF Jr., Gimbrone MA Jr. 1994 Shear stress selectively upregulates ICAM-1 expression in cultured human vascular endothelial cells. *J Clin Invest* 94:885-891.

Nakai K, Hayashi T, Nagaya S, Toyoda H, Yamamoto M, Shiku H, Ikeda Y, Nishikawa M 1997 Shear stress-induced myosin association with cytoskeleton and phosphorylation in human platelets. *Life Sci* 60(11):PL181-191.

Naor Z, Shearman MS, Kishimoto A, Nishizuka Y 1988 Calcium-independent activation of hypothalamic type I protein kinase C by unsaturated fatty acids. *Mol Endocrinol* 2(11):1043-1048.

Narahashi T, Tsunoo A, Yoshii M 1987 Characterization of two types of calcium channels in mouse neuroblastoma cells. *J Physiol* (London) 383:231-249.

Naruse K, Sai X, Yokoyama N, Sokabe M 1998a Uni-axial cyclic stretch induces c-src activation and translocation in human endothelial cells via SA channel activation. *FEBS Lett* 441(1):111-115.

Naruse K, Yamada T, Sai XR, Hamaguchi M, Sokabe M 1998b Pp125FAK is required for stretch dependent morphological response of endothelial cells. *Oncogene* 17:455-463.

Nemoto O, Yamada H, Kikuchi T, Shinmei M, Obata K, Sato H, Seiki M 1997 Suppression of matrix metalloproteinase-3 synthesis by interleukin-4 in human articular chondrocytes. *J Rheumatol* 24:1774-1779.

Newham P, Craig SE, Seddon GN, Schofield NR, Rees A, Edwards MR 1997 α4 integrin binding interfaces on VCAM-1 and MAdCAM-1. *J Biol Chem* 272:19429-19440.

Newton AC 1995 Protein kinase C: structure, function, and regulation. *J Biol Chem* 270:28495-28498.

Ng T, Shima D, Squire A, Bastiaens PIH, Gschmeissner S, Humphries MJ, Parker PJ 1999 PKCα regulates β1 integrin-dependent cell motility through association and control of integrin traffic. *EMBO J* 18:3909-3923.

Nishizuka Y 1984 The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* 308:693-698.

Nishizuka Y 1988 The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 334(6184):661-665.

Nishizuka Y 1995 Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 9:484-496.

Novak A, Hsu SC, Leung-Hagesteijn C, Radeva G, Papkoff J, Montesano R, Roskelley C, Grosschedl R, Dedhar S 1998 Cell adhesion and the integrin-linked kinase regulate the LEF-1 and β-catenin signaling pathways. *Proc Natl Acad Sci USA* 95:4374-4379.

Oberlender SA, Tuan RS 1994a Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. *Development* 120:177-187.

Oberlender SA, Tuan RS 1994b Spatiotemporal profile of N-cadherin expression in the developing limb mesenchyme. *Cell Adhes Commun* 2:521-537.

Oda A, Yokoyama K, Murata M, Tokuhira M, Nakamura K, Handa M, Watanabe K, Ikeda Y 1995 Protein tyrosine phosphorylation in human platelets during shear stress-induced platelet aggregation (SIPA) is regulated by glycoprotein (GP) Ib/IX as well as GP IIb/IIIa and requires intact cytoskeleton and endogenous ADP. *Thromb Haemost* 74(2):736-742.

O'Dell TJ, Kandel ER, Grant SG 1991 Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature* 353(6344):558-560.

Ohtsuka A, Ando J, Korenaga R, Kamiya A, Toyama-Sorimaci N, Miyasaka M 1993 The effect of flow on the expression of vascular adhesion molecule-1 by cultured mouse endothelial cells. *Biochem Biophys Res Commun* 193:303-310.

Okuda M, Takahashi M, Suero J, Murry CE, Traub O, Kawakatsu H, Berk BC 1999 Shear stress stimulation of p130(cas) tyrosine phosphorylation requires calcium-dependent c-Src activation. *J Biol Chem* 274(38):26803-26809.

Olesen SP, Bundgaard M 1993 ATP-dependent closure and reactivation of inward rectifier K^+ channels in endothelial cells. *Circ Res* 73:492-495.

Olesen SP, Bundgaard M 1992 Chloride-sensitive channels of large conductance in bovine aortic endothelial cells. *Acta Physiol Scand* 144:191-198.

Olesen SP, Clapham DE, Davies PF 1988 Hemodynamic shear stress activates a K^+ current in vascular endothelial cells. *Nature (London)* 331:168-170.

Osborne KD, Trippel SB, Mankin HJ 1989 Growth factor stimulation of adult articular cartilage. *J Orthop Res* 7:35-42.

Ostergaard K, Petersen J, Andersen CB, Bendtzen K, Salter DM 1997a Histologic/histochemical grading system for osteoarthritic articular cartilage. Reproducibility and validity. *Arthritis Rheum* 40:1766-1771.

Ostergaard K, Salter DM, Andersen CB, Petersen J, Bendtzen K 1997b CD44 expression is up-regulated in the deep zone of osteoarthritic cartilage isolated from human femoral heads. *Histopathology* 31: 451-459.

Ostergaard K, Salter DM 1998 Immunohistochemistry in the study of normal and osteoarthritic human articular cartilage. *Prog Histochem Cytochem* 33:93-165.

Ostergaard K, Salter DM, Petersen J, Bendtzen K, Hvolris J, Andersen CB 1998 Expression of alpha and beta subunits of the integrin superfamily in articular cartilage from macroscopically normal and osteoarthritic human femoral heads. *Ann Rheum Dis* 57: 303-308.

O'Toole TE 1997 Integrin signaling: Building connections beyond the focal contact? *Matrix Biol* 16:165-171.

Padanilam BJ, Hammerman MR 1997 Ischemia-induced receptor for activated C kinase (RACK1) expression in rat kidneys. *Am J Physiol* 272(Renal Physiol. 41):F160-166.

Palmoski MJ, Brandt KD 1981 Running inhibits the reversal of atrophic changes in canine knee cartilage after removal of a leg cast. *Arthritis Rheum* 24:1329-1337.

Palmoski MJ, Brandt KD 1984 Effects of static and cyclic compressive loading on articular cartilage plugs in vitro. *Arthritis Rheum* 27:675-681.

Palmoski MJ, Perricone E, Brandt KD 1979 Development and reversal of a proteoglycan aggregation defect in normal canine knee cartilage after immobilization. *Arthritis Rheum* 22:508-517.

Pan J, Fukuda K, Saito M, Matsuzaki J, Kodama H, Sano M, Takahashi T, Kato T, Ogawa S 1999 Mechanical stretch activates the JAK/STAT pathway in rat cardiomyocytes. *Circ Res* 84:1127-1136.

Parsons JT, Parsons SJ 1997 Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. *Curr Opin Cell Biol* 9:187-192.

Pascale A, Fortino I, Govoni S, Trabucchi M, Wetsel WC, Battaini F 1996 Functional impairment in protein kinase C by RACK1 (receptor for activated C kinase 1) deficiency in aged rat brain cortex. *J Neurochem* 67(6):2471-2477.

Paul JP 1980 Joint kinetics. In: Sokoloff L ed. The joints and synovial fluid. Vol.2. NewYork: Academic press. P139-176.

Paulsson M, Morgelin M, Wiedemann H, Beardmore-Gray M, Dunham D, Hardingham T, Heinegard D, Timpl R, Engel J 1987 Extended and globular protein domains in cartilage proteoglycans. *Biochem J* 245:763-772.

Pearce MJ, McIntyre TM, Prescott SM, Zimmerman GA, Whatley RE 1996 Shear stress activates cytosolic phospholipase A2 (cPLA2) and MAP kinase in human endothelial cells. *Biochem Biophys Res Commun* 218:500-504.

Pelletier JP, Martel-Pelletier J, Howell DS, Ghandhur-Mnaymneh L, Enis JE, Woessner JF 1983 Collagenase and collagenolytic activity in human osteoarthritic cartilage. *Arthritis Rheuma* 26:63-68.

Peracchia F, Ferrari G, Poggi A, Rotilio D 1991 IL-1β-induced expression of PGDF-AA isoform in rabbit articular chondrocytes is modulated by TGF-β1. *Exp Cell Res* 193:208-212.

Phillips FM, Pottenger LA, Hay RV 1990 In situ cross-linking of cartilage proteoglycans. *J Orthop Res* 8:189-198.

Pierschbacher MD, Ruoslahti E 1984 Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 309:30-33.

Piperno M, Reboul P, Hellio le Graverand MP, Peschard M, Annefeld M, Richard M, Vignon E 1998 Osteoarthritc cartilage fibrillation is associated with a decrease in chondrocyte adhesion to fibronectin. *Osteoarthritis Cartilage* 6:393-399.

Polakis P 1997 The adenomatous polyposis coli (APC) tumor suppressor. *Biochim Biophys Acta* 1332:F127-147.

Pommerenke H, Schreiber E, Durr F, Nebe B, Hahnel C, Moller W, Rychly J 1996 Stimulation of integrin receptors using a magnetic drag force device induces an intracellular free calcium response. *Eur J Cell Biol* 70:157-164.
Poole AR 1986 Proteoglycan in health and disease: structures and functions. *Biochem J* 236:1-14.

Poole AR 1997 Cartilage in health and disease. In: Koopman WJ ed. Arthritis and allied conditions: a textbook of rheumatology. Baltimore: Williams and Wilkins. P255-308.

Poole AR, Pidoux I, Reiner A, Rosenberg L 1982 An immunoelectron microscope study of the organization of proteoglycan monomer, link protein and collagen in the matrix of articular cartilage. *J Cell Biol* 93:921-937.

Poole AR, Reiner A, Tang LH, Rosenberg L 1980 Proteoglycans from bovine nasal cartilage. Immunochemical studies of link protein. *J Biol Chem* 255:9295-9305.

Poole AR, Rizkalla G, Ionescu M 1991 Increased content of the C-propeptide of type II collagen in osteoarthritic human articular cartilage. *Trans Orthop Res Soc* 16:343.

Porter JC, Hogg N 1998 Integrins take partners: cross-talk between integrins and other membrane receptors. *Trends Cell Biol* 8:390-396.

Radin EL, Martin RB, Burr DB, Caterson B, Boyd RD, Goodwin C 1984 Effects of mechanical loading on the tissues of the rabbit knee. *J Orthop Res* 2:221-234.

Razdan K, Hellums JD, Kroll MH 1994 Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s). *Biochem J* 302(Pt3):681-686.

Reinhardt J, Wolff T 2000 The influenza A virus M1 protein interacts with the cellular receptor of activated C kinase (RACK) 1 and can be phosphorylated by protein kinase C. *Vet Microbiol* 74(1-2):87-100.

Reinhold SL, Prescott SM, Zimmerman GA, McIntyre TM 1990 Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB J* 4(2):208-214.

Roberts S, Weightman B, Urban JPG, Chapell D 1986 Mechanical and biochemical properties of human articular cartilage in osteoarthritic femoral heads and in autopsy specimens. *J Bone Joint Surg [Br]* 68:278-288.

Robinson MJ, Cobb MH 1997 Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 9:180-186.

Robishaw JD, Foster KA 1989 Role of G proteins in the regulation of the cardiovascular system. *Annu Rev Physiol* 51:229-244.

Robotewskyj A, McKibben S, Duli RO, Griem ML, Davies PF 1991 Dynamics of focal adhesion site remodeling in living endothelial cells in response to shear stress forces using confocal image analysis. *FASEB J* 5:A527.

Rodan GA, Bourret LA, Harvey A, Mensi T 1975b Cyclic AMP and cyclic GMP. Mediators of the mechanical effects on bone remodelling. *Science* 189:467-469.

Rodan GA, Mensi T, Harvey A 1975a A quantitative method for the application of compressive forces to bone in tissue culture. *Calcif Tissue Res* 18:125-131.

Rodriguez MM, Ron D, Touhara K, Chen CH, Mochly-Rosen D 1999 RACK1, a protein kinase C anchoring protein, coordinates the binding of activated protein kinase C and select pleckstrin homology domains in vitro. *Biochemistry* 38(42):13787-13794.

Romer LH, McLean N, Turner CE, Burridge K 1994 Tyrosine kinase activity, cytoskeletal organization, and motility in human vascular endothelial cells. *Mol Biol Cell* 5:349-361.

Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D 1994 Cloning of an intracellular receptor for protein kinase C: A homolog of the β subunit of G proteins. *Proc Natl Acad Sci USA* 91:839-843.

Ron D, Jiang A, Yao L, Vagts A, Diamond I, Gordon A 1999 Coordinated movement of RACK1 with activated βIIPKC. *J Biol Chem* 274(38):27039-27046.

Ron D, Luo J, Mochly-Rosen D 1995 C2 region-derived peptides inhibit translocation and function of β protein kinase C in vivo. *J Biol Chem* 270(41):24180-24187.

Ron D, Mochly-Rosen D 1995 An autoregulatory region in protein kinase C: The pseudoanchoring site. *Proc Natl Acad Sci USA* 92:492-496.

Rosales C, Gresham HD, Brown EJ 1992 Expression of the 50-kDa integrin-associated protein on myeloid cells and erythrocytes. *J Immunol* 149:2759-2764.

Ross MH, Romrell LJ, Kaye GI 1995 Cartilage. In Histology: a text and atlas, third edition. P132-149.

Rotenberg SA, Smiley S, Ueffing M, Krauss RS, Chen LB, Weinstein IB 1990 Inhibition of rodent protein kinase C by the anticarcinoma agent dequalinium. *Cancer Res* 50:677-685.

Rotenberg SA, Sun XG 1998 Photoinduced inactivation of protein kinase C by dequalinium identifies the RACK-1-binding domain as a recognition site. *J Biol Chem* 273(4):2390-2395.

Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P 1997 Stabilization of β-catenin by genetic defects in melanoma cell lines. *Science* 275:1790-1792.

Ruoslahti E 1991 Integrins. *J Clin Invest* 87:1-5.

Ryan JJ, McReynolds LJ, Keegan A, Wang LH, Garfein E, Rothman P, Nelms K, Paul WE 1996 Growth and gene expression are predominantly controlled by distinct regions of the human IL-4 receptor. *Immunity* 4:123-132.

Sabe H, Hata A, Okada M, Nakagawa H, Hanafusa H 1994 Analysis of the binding of the Src homology 2 domain of Csk to tyrosine-phosphorylated proteins in the suppression and mitotic activation of c-Src. *Proc Natl Acad Sci USA* 91:3984-3988.

Sachs F 1991 Mechanical transduction by membrane ion channels: a mini review. *Mol Cell Biochem* 104:57-60.

Sadoshima J, Izumo S 1993 Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J* 12:1681-1692.

Sadoshima J, Takahashi T, Jahn L, Izumo S 1992 Roles of mechano-sensitive ion channels, cytoskeleton, and contractile activity in stretch-induced immediate-early gene expression and hypertrophy of cardiac myocytes. *Proc Natl Acad Sci USA* 89:9905-9909.

Sadouk M, Pelletier JP, Tardif G, Kiansa K, Cloutier JM, Martel-Pelletier J 1995 Human synovial fibroblasts coexpress interleukin-1 receptor type I and type II mRNA: The increased level of the interleukin-1 receptor in osteoarthritic cells is related to an increased level of the type I receptor. *Lab Invest* 73:347-355.

Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD 1989 Biosynthetic response of cartilage explants to dynamic compression. *J Orthop Res* 7:619-636.

Salter DM, Godolphin JL, Gourlay MS 1995 Chondrocyte heterogeneity: immunohistologically defined variation of integrin expression at different sites in human fetal knees. *J Histochem Cytochem* 43:447-457.

Salter DM, Godolphin JL, Gourlay MS, Lawson MF, Hughes DE, Dunne E 1996a Analysis of human articular chondrocyte CD44 isoform expression and function in health and disease. *J Pathol* 179:396-402.

Salter DM, Hughes DE, Simpson R, Gardner DL 1992 Integrin expression by human articular chondrocytes. *Br J Rheumatol* 31:231-234.

Salter DM, Nuki G, Wright MO 1996b Interleukin-4 expression in normal and osteoarthritic human articular cartilage. *J Rheumatol* 23:1314-1315.

Salter DM, Robb JE, Wright MO 1997 Electrophysiological responses of human bone cells to mechanical stimulation: Evidence for specific integrin function in mechanotransduction. *J Bone Miner Res* 12:1133-1141.

Salter DM 1993 Tenascin is increased in cartilage and synovium from arthritic knees. *Br J Rheumatol* 32:780-786.

Sandy JP, Adams ME, Billingham MEJ, Plaas A, Muir H 1984 In vivo and in vitro stimulation of chondrocyte biosynthetic activity in early experimental osteoarthritis. *Arthritis Rheum* 27:388-397.

Satsuma H, Saito N, Hamanishi C, Hashima M, Tanaka S 1996 Alpha and epsilon isozymes of protein kinase C in the chondrocytes in normal and early osteoarthritic articular cartilage. *Calcif Tissue Int* 58:192-194.

Schaffer JL, Newman NC, Gray ML, Gerstenfeld LC 1993 Osteoblast phenotypic expression after mechanical perturbation with a dynamic and isotropic and biaxially uniform strain. *J Bone Miner Res* 8:S367.

Schaffer JL, Rizen M, L'Italien GJ, Benbrahim A, Megerman J, Gerstenfeld LC, Gray ML 1994 Device for the application of a dynamic biaxially uniform and isotropic strain to a flexible cell culture membrane. *J Orthop Res* 12:709-719.

Schaller MD, Parsons JT 1994 Focal adhesion kinase and associated proteins. *Curr Opin Cell Biol* 6:705-710.

Schlaepfer DD, Hanks SK, Hunter T, van der Geer P 1994 Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* 372:786-791.

Schlaepfer DD, Hunter T 1998 Integrin signalling and tyrosine phosphorylation: just the FAKs? *Trends Cell Biol* 8:151-157.

Schmidt C, Pommerenke H, Durr F, Nebe B, Rychly J 1998 Mechanical stressing of integrin receptors induces enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. *J Biol Chem* 273(9):5081-5085.

Schwartz E, Leveille C, Stevens J, Oh W 1981 Proteoglycan structure and metabolism in normal and osteoarthritic cartilage of guinea pigs. *Arthritis Rheum* 24:1528-1529.

Schwartz MA, Brown EJ, Fazeli B 1993 A 50-kDa integrin-associated protein is required for integrin-regulated calcium entry in endothelial cells. *J Biol Chem* 268:19931-19934.

Seireg A and Arvikar RJ 1975 The prediction of muscular load sharing and joint forces in the lower extremities during walking. *J Biomech* 8:89-102.

Seko Y, Takahashi N, Tobe K, Kadowaki T, Yazaki Y 1999 Pulsatile stretch activates mitogen-activated protein kinase (MAPK) family members and focal

adhesion kinase (p125(FAK)) in cultured rat cardiac myocytes. *Biochem Biophys Res Commun* 259:8-14.

Setton LA, Elliott DM, Mow VC 1999 Altered mechanics of cartilage with osteoarthritis: human osteoarthritis and an experimental model of joint degeneration. *Osteoarthritis Cartilage* 7:2-14.

Setton LA, Mow VC, Howell DS 1995 Changes in the shear properties of canine knee cartilage resulting from anterior cruciate transection. *J Orthop Res* 13:473-482.

Setton LA, Zimmerman JR, Mow VC, Muller FJ, Pita JC, Howell DS 1990 Effects of disuse on the tensile properties and composition of canine knee joint cartilage. *Trans Orthop Res Soc* 15:155.

Shakibaei M 1998 Inhibition of chondrogenesis by integrin antibody *in vitro*. *Exp Cell Res* 240: 95-106.

Shepherd GM 1991 Sensory transduction: entering the mainstream of membrane signalling. *Cell* 67:845-851.

Shingu M, Miyauchi S, Nagai Y, Yasutake C, Horie K 1995 The role of IL4 and IL6 in IL1-dependent cartilage matrix degradation. *Br J Rheumatol* 34:101-106.

Shinmei M, Masuda K, Kikuchi T, Shimomura Y, Okada Y 1991 Production of cytokines by chondrocytes and its role in proteoglycan degradation. *J Rheumatol* 27(Suppl):89-91.

Shyy JYJ, Chien S 1997 Role of integrins in cellular responses to mechanical stress and adhesion. *Curr Opin Cell Biol* 9:707-713.

Simcha I, Geiger B, Yehuda-Levenberg S, Salomon D, Ben-Ze'ev A 1996 Suppression of tumorigenicity by plakoglobin: an augmenting effect of N-Cadherin. *J Cell Biol* 133:199-209.

Simon WH, Mak AF, Spirt A 1989 The effect of shear fatigue on bovine articular cartilage. *J Orthop Res* 8:86-93.

Sinclair J ed. Collins Cobuild English Dictionary. London: HarperCollins Publisher. P661.

Slack J, McMahan CJ, Waugh S, Schooley K, Spriggs MK, Sims JE, Dower SK 1993 Independent binding of interleukin-1 alpha and interleukin-1 beta to type I and type II interleukin-1 receptors. *J Biol Chem* 268:2513-2524.

Slowman SD, Brandt KD 1986 Composition and glycosaminoglycan metabolism of articular cartilage from habitually loaded and habitually unloaded sites. *Arthritis Rheum* 29:88-94.

Smith PG, Garcia R, Kogerman L 1998 Mechanical strain increases protein tyrosine phosphorylation in airway smooth muscle cells. *Exp Cell Res* 239:353-360.

Smith PR, de Jesus O, Turner D, Hollyoake M, Karstegl CE, Griffin BE, Karran L, Wang Y, Hayward SD, Farrell PJ 2000 Structure and coding content of CST (BART) family RNAs of Epstein-Barr virus. *J Virol* 74(7):3082-3092.

Smith RL, Donlon BS, Gupta MK, Mohtai M, Das P, Carter DR, Cooke J, Gibbons G, Hutchinson N, Schurman DJ 1995 Effects of fluid-induced shear on articular chondrocyte morphology and metabolism in vitro. *J Orthop Res* 13(6):824-831.

Sokabe M, Naruse K, Sai S, Yamada T, Kawakami K, Inoue M, Murase K, Miyazu M 1997 Mechanotransduction and intracellular signaling mechanisms of stretch-induced remodeling in endothelial cells. *Heart Vessels Suppl* 12:191-193.

Solursh M 1989 Differentiation of cartilage and bone. *Curr Opin Cell Biol* 1: 989-994.

Somjen D, Binderman I, Berger E, Harell A 1980 Bone remodelling induced by physical stress is prostaglandin E2 mediated. *Biochim Biophys Acta* 627:91-100.

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BJ, Birge RB, Fajardo EJ, Chou MM, Hanafusa H, Schaffhausen B, Cantley LC 1993 SH2 domains recognize specific phosphopeptide sequences. *Cell* 72:767-778.

Spirt AA, Mak AF, Wassell RP 1989 Nonlinear viscoelastic properties of articular cartilage in shear. *J Orthop Res* 7:43-49.

Springer TA 1990a Adhesion receptors of the immune system. *Nature* 346:425-434.

Springer TA 1990b The sensation and regulation of interactions with the extracellular environment: the cell biology of lymphocyte adhesion receptors. *Annu Rev Cell Biol* 6:359-402.

Springer TA 1997 Folding of the N-terminal, ligand-binding region of integrin alpha-subunits into a beta-propeller domain. *Proc Natl Acad Sci USA* 94:65-72.

Studer R, Jaffurs D, Stefanovic-Racic M, Robbins PD, Evans CH 1999 Nitric oxide in osteoarthritis. *Osteoarthritis Cartilage* 7:377-379.

Stupack DG, Shen C, Wilkins JA 1994 Control of lymphocyte integrin function: evidence for multiple contributing factors. *Cell Immunol* 155(1):237-245.

Sudina GF, Mirzoeva OK, Pushkareva MA, Korshunova GA, Sumbatyan NV, Varfolomeev SD 1993 Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. *FEBS Lett* 329:21-24.

Suzuki S, Naitoh Y 1990 Amino acid sequence of a novel integrin beta-4 subunit and primary expression of the messenger RNA in epithelial cells. *EMBO J* 9:757-763.

Swoboda B, Pullig O, Kirsch T, Kladny B, Steinhuaser B, Weseloh G 1998 Increased content of type VI collagen epitopes in human osteoarthritic cartilage: Quantitation by inhibition ELISA. *J Orthop Res* 16:96-99.

Takada Y, Kamata T, Irie A, Puzon-McLaughin W, Zhang XP 1997 Structural basis of integrin-mediated signal transduction. *Matrix Biol* 16:143-151.

Takahashi M, Berk BC 1996 Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. *J Clin Invest* 98(11):2623-2631.

Taladriz S, Gonzalez-Aseguinolaza G, Marquet A, Larraga V 1999 Cloning, molecular analysis and differential cell localisation of the p36 RACK analogue antigen from the parasite protozoon Crithidia fasciculata. *FEBS Lett* 443:375-380.

Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F 1986 Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem Biophys Res Commun* 135:397-402.

Tamkun JW, DeSimone DW, Fonda D, Patel RS, Buck C, Horwitz AF, Hynes RO 1986 Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell* 46(2):271-282.

Tanaka S, Hamanishi C, Kikuchi H, Fukuda K 1998 Factors related to degradation of articular cartilage in osteoarthritis: a review. *Semin Arthritis Rheum* 27:392-399.

Tavella S, Bellese G, Castagnola P, Martin I, Piccini D, Doliana R, Colombatti A, Cancedda R, Tacchetti C 1997 Regulated expression of fibronectin, laminin and related integrin receptors during the early chondrocyte differentiation. *J Cell Sci* 110 (Pt 18):2261-2270.

Teshima R, Treadwell B, Trahan C, Mankin H 1983 Comparative rates of proteoglycan synthesis and size of proteoglycans in normal and osteoarthritic chondrocytes. *Arthritis Rheum* 76:1225-1230.

Thastrup O, Cailen PJ, Drobuii BK, Hanley MR, Dawson AP 1990 Thapsigargin, a tumour promotor, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum. *Proc Natl Acad Sci USA* 87:2466-2470.

Thastrup O 1990 Role of Ca^{2+}-ATPase in regulation of cellular Ca^{2+} signaling, as studied with the selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. *Agents Actions* 29:8-15.

Thompson R, Oegema T 1979 Metabolic activity of articular cartilage in osteoarthritis. *J Bone Joint Surg* 61:407-416.

Tohyama Y, Yanagi S, Sada K, Yamamura H 1994 Translocation of p72syk to the cytoskeleton in thrombin-stimulated platelets. *J Biol Chem* 269:32796-32799.

Towle C, Hung H, Bonassar L, Treadwell B, Mangham D 1997 Detection of interleukin-1 in the cartilage of patients with osteoarthritis: possible autocrine/paracrine role in pathogenesis. *Osteoarthritis Cart* 5:293-300.

Tseng H, Peterson TE, Berk BC 1995 Fluid shear stress stimulates mitogen-activated protein kinase in endothelial cells. *Circ Res* 77:869-878.

Tsunoo A, Yoshii M, Narahashi T 1986 Block of calcium channels by enkephalin and somatostatin in neuroblastoma-glioma hybrid NG108-15 cells. *Proc Natl Acad Sci USA* 83:9832-9836.

Tuckwell DS, Ayad S, Grant ME, Takigawa M, Humphries MJ 1994 Conformation dependence of integrin-type II collagen binding. Inability of collagen peptides to support alpha 2 beta 1 binding, and mediation of adhesion to denatured collagen by a novel alpha 5 beta 1-fibronectin bridge. *J Cell Sci* 107 (pt4):993-1005.

Tumminia SJ, Mitton KP, Arora J, Zelenka P, Epstein DL, Russell P 1998 Mechanical stretch alters the actin cytoskeletal network and signal transduction in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci* 39(8):1361-1371.

Urban JP 1994 The chondrocyte: a cell under pressure. *Br J Rheumatol* 33:901-908.

Ullrich A, Schlessinger J 1990 Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203-212.

Vandenburgh HH 1988 A computerized mechanical cell stimulation for tissue culture: effects on skeletal muscle organogensis. *In Vitro Cell Dev Biol* 24:609-619.

Van der Flier A, Kuikman I, Baudoin C, van der Neut R, Sonnenberg A 1995 A novel $\beta 1$ integrin isoform produced by alternative splicing: unique expression in cardiac and skeletal muscle. *FEBS Lett* 369:340-344.

Van der Zee EA, Bolhuis JJ, Solomonia RO, Horn G, Luiten PG 1995 Differential distribution of protein kinase C (PKC alpha beta and PKC gamma) isoenzyme immunoreactivity in the chick brain. *Brain Res* 676:41-52.

van Kampen GPJ, van de Stadt RJ 1987 Cartilage and chondrocyte responses to mechanical loading in vitro. In Helminen HJ, Kiviranta I, Tammi M, Sasmanen AM, Paukkonen K, and Jurvelin J: Joint Loading: biology and health of articular structures. P112-125.

Vani K, Yang G, Mohler J 1997 Isolation and cloning of a Drosophila homolog to the mammalian RACK1 gene, implicated in PKC-mediated signalling. *Biochim Biophys Acta* 1358:67-71.

Vannier E, Miller LC, Dinarello CA 1992 Coordinated antiinflammatory effects of interleukin 4: interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. *Proc Natl Acad Sci USA* 89:4076-4080.

Veldhuijzen JP, Bourret LA, Rodan GA 1979 In vitro studies of the effect of intermittent compressive forces on cartilage cell proliferation. *J Cell Physiol* 98:299-306.

Villiger PM, Terkeltaub R, Lotz M 1992 Monocyte chemoattractant protein-1 (MCP-1) expression in human articular cartilage: Induction by peptide regulatory factors and differential effects of dexamethasone and retinoic acid. *J Clin Invest* 90:488-496.

Vlahos CJ, Matter WF, Hui KY, Brown RF 1994 A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002*).* *J Biol Chem* 269(7):5241-5248.

Vuori K, Ruoslahti E 1993 Activation of protein kinase C precedes alpha 5 beta 1 integrin-mediated cell spreading on fibronectin. *J Biol Chem* 268:21459-21462.

Walsh DA, Sledge CB, Blake DR 1997 Biology of the normal joint. In: Kelley WN, Harris Jr ED, Ruddy S, Sledge CB ed. Textbook of rheumatology. Philadelphia: W.B. Saunders Company. P1-22.

Wang HY, Pisano MR, Friedman E 1994a Attenuated protein kinase C activity and translocation in Alzheimer's disease brain. *Neurobiol Aging* 15:293-298.

Wang N, Butler JP, Ingber DE 1993 Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 260:1124-1127.

Wang WH, Cassola A, Giebisch G 1994b Involvement of actin cytoskeleton in modulation of apical K channel activity in rat collecting duct. *Am J Physiol* 267(4 pt 2):F592-598.

Wang XQ, Frazier WA 1998 The thrombospondin receptor CD47 (IAP) modulates and associates with alpha2 beta1 integrin in vascular smooth muscle cells. *Mol Biol Cell* 9:865-874.

Wang XY, Ostberg JR, Repasky EA 1999 Effect of fever-like whole-body hyperthermia on lymphocyte spectrin distribution, protein kinase C activity, and uropod formation. *J Immunol* 162(6):3378-3387.

Webb G, Westacott C, Elson C 1997 Chondrocytes tumor necrosis factor receptors and focal loss of cartilage in osteoarthritis. *Osteoarthritis Cart* 5:427-437.

Weiss MJ, Wong JR, Ha CS, Bleday R, Salem RR, Steele GD, Chen LB 1987 Dequalinium, a topical antimicrobial agent, displays anticarcinoma activity based on selective mitochondrial accumulation. *Proc Natl Acad Sci USA* 84:5444-5448.

Williams JL, Chen JH, Belloli DM 1992 Strain fields on cell stressing devices employing clamped circular elastic diaphragms as substrates. *J Biomech Eng* 114:377-384.

Woo SLY, Kwan MK, Coutts RD, Akeson WH 1992 Biomechanical considerations. In: In Moskowitz RW, Howell DS, Goldberg VM, Mankin HJ ed. Osteoarthritis: Diagnosis and medical/surgical management. Philadelphia: WB Saunders. P191-211.

Woods A, Couchman JR 1992 Protein kinase C involvement in focal adhesion formation. *J Cell Sci* 101:277-290.

Woods VL Jr, Schreck PJ, Gesink DS, Pacheco HO, Amiel D, Akeson WH, Lotz M 1994 Integrin expression by human articular chondrocytes. *Arthritis Rheum* 37:537-544.

Wrenn RW, Herman LE 1995 Integrin-linked tyrosine phosphorylation increases membrane association of protein kinase C alpha in pancreatic acinar cells. *Biochem Biophys Res Commun* 208:978-984.

Wright MO, Jobanputra P, Bavington C, Salter DM, Nuki G 1996 Effects of intermittent pressure-induced strain on the electrophysiology of cultured human chondrocytes: evidence for the presence of stretch-activated membrane ion channels. *Clin Sci* 90:61-71.

Wright MO, Nishida K, Bavington C, Godolphin JL, Dunne E, Walmsley S, Jobanputra P, Nuki G, Salter DM 1997 Hyperpolarisation of cultured human chondrocytes following cyclical pressure-induced strain: evidence of a role for $\alpha5\beta1$ integrin as a chondrocyte mechanoreceptor. *J Orthop Res* 15:742-747.

Wright MO, Stockwell RA, Nuki G 1992 Response of plasma membrane to applied hydrostatic pressure in chondrocytes and fibroblasts. *Connect Tissue Res* 28:49-70.

Wu C, Keightley SY, Leung-Hagesteijn C, Radeva G, Coppolino M, Goicoechea S, McDonald JA, Dedhar S 1998 Integrin-linked protein kinase regulates fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. *J Biol Chem* 273:528-536.

Yamada KM 1997 Integrin signaling. *Matrix Biol* 16:137-141.

Yamazaki T, Komuro I, Kudoh S, Zou Y, Nagai R, Aikawa R, Uozumi H, Yazaki Y 1998 Role of ion channels and exchangers in mechanical stretch-induced cardiomyocyte hypertrophy. *Circ Res* 82:430-437.

Yamazaki T, Tobe K, Hoh E, Maemura K, Kaida T, Komuro I, Tamemoto H, Kadowaki T, Nagai R, Yazaki Y 1993 Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. *J Biol Chem* 268:12069-12076.

Yang XC, Sachs F 1989 Block of stretch-activated ion channels in Xenopus oocytes by gadolinium and calcium ions. *Science* (Washington DC) 243:1068-1071.

Yano Y, Geibel J, Sumpio BE 1996 Tyrosine phosphorylation of $pp125^{FAK}$ and paxillin in aortic endothelial cells induced by mechanical strain. *Am J Physiol* 271(2 Pt 1):C635-649.

Yarwood SJ, Steele MR, Sctoland G, Houslay MD, Bolger GB 1999 The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. *J Biol Chem* 274(21):14909-14917.

Yeh LA, Augustine AJ, Lee P, Riviere LR, Sheldon A 1995 Interleukin-4, an inhibitor of cartilage breakdown in bovine articular cartilage explants. J Rheumatol 22:1740-1746.

Yonezawa I, Kato K, Yagita H, Yamauchi Y, Okumura K 1996 VLA-5-mediated interaction with fibronectin induces cytokine production by human chondrocytes. *Biochem Biophys Res Comm* 219:261-265.

Yost JC, Hermann IM 1990 Substratum-induced stress fiber assembly in vascular endothelial cells during spreading in vitro. *J Cell Sci* 95:507-520.

Young B, Heath JW 2000 Skeletal tissue. In: Young B and Heath JW ed. Wheater's functional histology: a text and colour atlas. London: Churchill Livingstone. P172-173.

Young S, Rothbard J, Parker PJ 1988 A monoclonal antibody recognising the site of limited proteolysis of protein kinase C. Inhibitor of down regulation in vivo. *Eur J Biochem* 173:247-252.

Zhidkova NI, Belkin AM, Mayne R 1995 Novel isoform of β1 integrin expressed in skeletal and cardiac muscle. *Biochem Biophys Res Commun* 214:279-285.

Zhu WB, Mow VC, Koob TJ, Eyre DR 1993 Viscoelastic shear properties of articular cartilage and the effects of glycosidase treatment. *J Orthop Res* 11:771-781.

APPENDIX I

Monoclonal antibodies used in this study.

ANTIBODY	CLONE	TYPE	SOURCE
Anti-type II collagen	6B3	Mouse monoclonal IgG	Chemicon International Ltd. Harrow, UK
Anti-keratan sulphate	MAB 2022	Mouse monoclonal IgG2b	Chemicon International Ltd. Harrow, UK
Anti-β1 integrin	TS2/16	Mouse monoclonal	Francisco Sanchez-Madrid, Madrid
Anti-β1 integrin	P4C10	Mouse monoclonal IgG1	Gibco, Life technologies, UK
Anti-β1 integrin	JB1a	Mouse monoclonal IgG	Chemicon International Ltd. Harrow, UK
Anti-CD29	JB1B	Mouse monoclonal IgG2a	Serotec Ltd. Oxford, UK
Anti-CD29-FITC	3S3	Mouse monoclonal IgG1	Serotec Ltd. Oxford, UK
Anti-CD29	3S3	Mouse monoclonal IgG1	Serotec Ltd. Oxford, UK
Anti-αVβ5 integrin	P1F6	Mouse monoclonal IgG1	Chemicon International Ltd. Harrow, UK
Anti-CD49e	SAM-1	Mouse monoclonal IgG2b	Serotec Ltd. Oxford, UK
Anti-CD47	Bric-126	Mouse monoclonal IgG2b	International Blood Group Reference Laboratory, UK
Anti-phosphotyrosine agarose	PT-66	Mouse monoclonal IgG1	Sigma-Aldrich Com. Ltd. Dorset, UK
Anti-phosphotyrosine HRP conjugate	PY-20	Mouse monoclonal IgG2b	Amersham Life Science, Buckinghamshire, UK

Monoclonal antibodies used in this study (continued).

ANTIBODY	CLONE	TYPE	SOURCE
Anti-phosphoserine agarose	PSR-45	Mouse monoclonal IgG1	Sigma-Aldrich Com. Ltd. Dorset, UK
Anti-phosphoserine	PSR-45	Mouse monoclonal IgG1	Sigma-Aldrich Com. Ltd. Dorset, UK
Anti-α-actinin	BM-75.2	Mouse monoclonal IgM	Sigma-Aldrich Com. Ltd. Dorset, UK
Anti-vinculin	hVIN-1	Mouse monoclonal IgG1	Sigma-Aldrich Com. Ltd. Dorset, UK
Anti-protein kinase C	MC5	Mouse monoclonal IgG2a	Sigma-Aldrich Com. Ltd. Dorset, UK
Anti-Pan Cadherin	CH-19	Mouse monoclonal IgG1	Sigma-Aldrich Com. Ltd. Dorset, UK
Anti-Paxillin	MAB 3060	Mouse monoclonal IgG1	Chemicon International Ltd. Harrow, UK
Anti-IL-4	34019. 111	Mouse monoclonal IgG2b	R&D systems Europe Ltd. Oxon, UK
Anti-IL-1β	2805.31	Mouse monoclonal IgG1	R&D systems Europe Ltd. Oxon, UK
Anti-IL-1 RI	35730. 111	Mouse monoclonal IgG1	R&D systems Europe Ltd. Oxon, UK
Anti-IL-1 RII	32437. 111	Mouse monoclonal IgG2a	R&D systems Europe Ltd. Oxon, UK
Anti-IL-2 Rγ	38024. 111	Mouse monoclonal IgG1	R&D systems Europe Ltd. Oxon, UK

Monoclonal antibodies used in this study (continued).

ANTIBODY	CLONE	TYPE	SOURCE
Anti-PKCα	3	Mouse monoclonal IgG2b	Transduction Laboratories California, USA
Anti-PKCβ	36	Mouse monoclonal IgG2b	Transduction Laboratories California, USA
Anti-PKCγ	5	Mouse monoclonal IgG1	Transduction Laboratories California, USA
Anti-PKCδ	14	Mouse monoclonal IgG2b	Transduction Laboratories California, USA
Anti-PKCε	21	Mouse monoclonal IgG2a	Transduction Laboratories California, USA
Anti-PKCη	31	Mouse monoclonal IgG1	Transduction Laboratories California, USA
Anti-PKCθ	27	Mouse monoclonal IgG2a	Transduction Laboratories California, USA
Anti-PKCι	23	Mouse monoclonal IgG2b	Transduction Laboratories California, USA
Anti-PKCλ	41	Mouse monoclonal IgG1	Transduction Laboratories California, USA
Anti-RACK1	20	Mouse monoclonal IgM	Transduction Laboratories California, USA
Anti-β-catenin	14	Mouse monoclonal IgG1	Transduction Laboratories California, USA

Polyclonal antibodies used in this study.

ANTIBODY	TYPE	SOURCE
Anti-PI 3-kinase p85	rat polyclonal	Upstate Biotechnology, New York, USA
Anti-α5 integrin	rabbit polyclonal	Chemicon International Ltd. Harrow, UK
Anti-FAK	rabbit polyclonal	Santa Cruz, California, USA
Anti-PYK2	goat polyclonal	Santa Cruz, California, USA
Anti-αE catenin	goat polyclonal	Santa Cruz, California, USA
Anti-γ catenin	goat polyclonal	Santa Cruz, California, USA
Anti-STAT 6	rabbit polyclonal	Chemicon International Ltd. Harrow, UK
Anti-NK1R	rabbit polyclonal	Sigma, Paisley UK
Anti-type VI collagen	rabbit polyclonal	Southern Bionuclear Services, Reading UK
FITC/TRITC conjugated anti-mouse Ig	goat polyclonal	Harlan Sera-Lab, Loughborough UK
FITC/TRITC conjugated anti-rabbit Ig	goat polyclonal	Harlan Sera-Lab, Loughborough UK
Anti-mouse Ig HRP	rabbit polyclonal	DAKO, Cambridgeshire, UK
Anti-rabbit Ig HRP	swine polyclonal	DAKO, Cambridgeshire, UK
Anti-goat Ig HRP	rabbit polyclonal	DAKO, Cambridgeshire, UK
Anti-rabbit Ig HRP	goat polyclonal	Santa Cruz, California, USA
Anti-mouse Ig	goat polyclonal	Transduction Laboratories California, USA

APPENDIX II

Solutions used in this study.

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, C-8051): 30 mg per sample was made up in 10 ml of serum free Iscove's modified Dulbecco's medium (Gibco) and filter sterilised. One unit will hydrolyze 1.0 μmole of furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) per min at 25°C at pH 7.5 in the presence of calcium ions. Two batches were used: Lot 57H6832 (1.4 FALGPA hydrolysis units/mg solid and 47 caseinase units/mg soild) and 68H8628 (1.2 FALGPA hydrolysis units/mg solid and 35 caseinase units/mg solid).

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

Solutions used for Lowry determination of protein concentration

Alkaline carbonate solution

To 49 ml of reagent C, add 49 ml of reagent D. Then add 1 ml of reagent A, followed by 1 ml of reagent B. Prepare freshly.

(A) Copper sulphate solution (1%) 1 g $CuSO_4$ in 100 ml dH_2O

(B) Sodium potassium tartrate (2%) 2 g NaKtartrate in 100 ml dH_2O

(C) Sodium carbonate (4%) 20 g Na_2CO_3 in 500 ml dH_2O

(D) Sodium hydroxide (0.2N) 4 g NaOH in 500 ml 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

Final Acrylamide Conc. (%)	dH_2O (ml)	1.5 M Tris-HCl pH 8.8 (ml)	10% (w/v) SDS (μl)	Stock 30 % Acrylamide (ml)	10 % APS (μl)	TEMED (μl)
7.5	4.85	2.50	100	2.50	50	5
10	4.05	2.50	100	3.30	50	5

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

Stacking gel composition

Final Acrylamide Conc. (%)	dH_2O (ml)	0.5M Tris-HCl pH 6.8 (ml)	10% (w/v) SDS (μl)	Stock 30 % Acrylamide (ml)	10 % APS (μl)	TEMED (μl)
4	6.1	2.5	100	1.3	50	10

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

1.5M Tris-HCl pH 8.8

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

5X electrode buffer

7.5 g Tris

36 g Glycine

2.5 g SDS

made up to 500 ml with dH_2O and stored at 4°C

Sample buffer (for 8 ml)

3.6 ml dH_2O

1.0 ml 0.5 M Tris pH 6.8

1.6 ml 10 % SDS

0.8 ml 1 M dithiothreitol (DTT)

0.8 ml glycerol

0.2 ml 0.05 % (w/v) bromophenol blue

Solutions used for electrophoretic transfer to PVDF membrane

Transfer buffer

made up fresh each time.

3.03 g Tris

14.4 g Glycine

200 ml methanol

800 ml dH_2O

Solutions used for development of blot

10X TBS

To 500 ml of 0.5 M Tris was added approximately 200 ml 1 N HCl until the pH was 7.6, solution made up to 2000 ml with dH_2O and pH re-adjusted to pH 7.6.

1X TBS

10X TBS was diluted 1:10 with normal saline (42.5 g sodium chloride in 5 litre dH_2O).

TBST (Tween20, 0.1%)

500 μl Tween20 (Sigma) was dissolved in 500 ml TBS

Blocking solution

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

APPENDIX III

Original data from which the results given in the Tables of Sections 4.1 and Chapter 5 are calculated. The table number corresponds to the Results section.

Table 4.1 C-20/A4 cell hyperpolarisation response to cyclical mechanical strain

		Membrane potential (-mV)	
	n	Resting	Post strain
Dish 1	10	12,14,13,18,11	20,19,20,18,17
		15,15,14,13,12	18,21,19,18,21
Dish 2	10	13,12,11,18,16	22,21,20,19,20
		17,14,14,14,16	19,18,20,19,22

Table 5.1a Electrophysiological response of OA chondrocytes to cyclical strain

Donor	n	Membrane potential (-mV)	
		Resting	Post strain
81/M	5	30,31,27,31,31	15,14,16,20,17
74/M	10	25,28,30,24,28 27,25,30,31,26	16,16,20,25,24 16,20,18,15,17
69/F	5	31,34,33,34,31	21,22,21,20,21

Table 5.1b Electrophysiological response of normal articular chondrocytes to cyclical strain

Donor	n	Membrane potential (-mV)	
		Resting	Post strain
80/M	10	41,42,46,50,44 48,45,46,46,43	57,60,57,54,53 56,58,52,60,53
71/M	5	28,29,29,30,31	40,41,43,42,44
67/F	5	21,20,23,24,24	29,29,31,32,30

Table 5.2 The effect of apamin on the depolarisation response of OA chondrocytes to cyclical strain

Donor	Reagent	Membrane potential (-mV)		
		Resting	Apamin alone	Post strain
73/F	control	31,31,32,32		23,26,20,21,20
		25,26,30,27		26,19,24,23,27
		33,32		
	apamin	37,37,39,33	40,37,38,35,38	27,28,25,22,29
		39,38,42,42	34,34,37,40,35	30,30,26,31,33
		35,41		
74/M	control	37,39,38,44		40,35,38,34,37
		41,49,50,51		37,36,38,40,41
		53,53		
	apamin	53,43,47,46	52,47,48,52,42	36,37,39,40,39
		43,41,48,52	41,45,46,45,40	43,37,41,38,39
		44,45		

Table 5.3a Effect of anti-β1 integrin antibody (TS2/16) on the hyperpolarisation response of normal chondrocytes to cyclical strain

Donor	Reagent	Membrane potential (-mV)		
		Resting	antibody alone	Post strain
74/M	control	22,20,24,23,23		32,30,29,29,31
	anti-β1	24,22,24,25,23	25,29,29,22,23	22,21,25,24,23

Table 5.3b The effect of anti-integrin antibodies on the depolarisation response of OA chondrocytes to cyclical strain

Donor	Reagent	Membrane potential (-mV)		
		Resting	antibody alone	Post strain
69/F	control	31,34,33,34 31		21,22,21,20,21
	anti-β1	32,35,32,33 30	30,32,34,34,30	29,33,35,35,28
	anti-αVβ5	35,33,35,36 32	32,35,34,33,35	23,24,22,24,25
50/M	control	31,34,34,35 40,30,30,31 30,31		25,26,21,23,25 21,25,26,25,27
	anti-α5	38,30,35,30 34	36,30,29,28,33	30,34,37,29,26
74/M	control	43,46,38,39 45		32,31,31,30,30
	anti-α5	42,41,39,43 38	42,38,42,43,39	37,43,46,44,42

Table 5.4a Effect of Bric-126 on the membrane hyperpolarisation response of normal chondrocytes to cyclical strain

		Membrane potential (-mV)		
Donor	Reagent	Resting	Bric-126 alone	Post strain
67/F	control	25,26,24,24,26		29,29,31,32,30
	Bric-126	26,24,25,28,28	27,28,27,28,26	30,31,26,26,25

Table 5.4b The effect of Bric-126 on the depolarisation response of OA chondrocytes to cyclical strain

		Membrane potential (-mV)		
Donor	Reagent	Resting	Bric-126 alone	Post strain
45/M	control	33,33,35,30		28,28,33,27,36
		40,46,41,36		35,36,35,26,34
		40,37		
	Bric-126	40,37,35,37	41,39,41,45,36	42,42,36,40,35
		36,40,43,37	38,37,43,39,40	35,41,44,40,37
		40,39		

Table 5.5a Effects of inhibitors of intracellular signalling molecules on the depolarisation response of OA chondrocytes to cyclical strain

Donor	Reagent	Membrane potential (-mV)		
		Resting	Reagent alone	Post strain
73/F	control	31,31,32,32 25,26,30,27 33,32		23,26,20,21,26 19,24,23,20,27
	cytochalasin D	37,40,38,38 40,35,35,34 36,34	36,35,25,28,28 29,33,25,26,24	16,21,15,21,24 22,25,25,19,20
74/M	control	37,39,38,44 41,49,50,51 53,53		40,35,38,34,37 37,36,38,40,41
	cytochalasin D	52,51,50,40 44,48,55,46 48,50	37,43,42,37,41 38,37,40,40,43	35,30,34,34,28 30,31,31,29,33
	wortmannin	47,46,41,49 49,48,47,51 53,44	41,42,49,46,47 50,46,43,40,40	44,42,39,50,50 39,47,45,39,46
	LY294002	45,47,47,45 52,43,43,53 40,46	44,45,46,50,49 45,44,53,42,50	45,50,47,46,40 43,51,45,47,42

Table 5.5a Effects of inhibitors of intracellular signalling molecules on the depolarisation response of OA chondrocytes to cyclical strain (continued)

Donor	Reagent	Membrane potential (-mV)		
		Resting	Reagent alone	Post strain
63/F	control	42,43,50,45		39,36,40,40,35
		44,52,51,49		37,36,35,33,36
		51,50		
	wortmannin	49,42,54,49	43,42,41,51,46	46,42,46,49,47
		50,44,48,55	46,45,40,44,44	48,40,45,46,50
		42,48		
	LY294002	44,45,41,51	49,47,50,45,48	48,46,48,43,45
		46,46,54,45	44,46,47,44,45	43,45,40,50,44
		48,51		
50/M	control	41,38,38,36		26,29,26,31,31
		32,37,39,34		30,28,28,32,27
		40,39		
	genistein	38,42,38,40	41,34,35,35,32	42,36,33,39,34
		40,35,40,35	39,38,34,34,40	34,40,37,35,36
		37,36		
50/M	control	35,42,32,30		28,29,29,25,26
		36		
	staurosporine	36,33,38,36	40,40,37,33,41	25,30,34,33,27
		33		
	calphostin C	42,41,40,34	33,34,28,25,24	25,23,26,17,15
		28,30,30,39		
		32,40		

Table 5.5a Effects of inhibitors of intracellular signalling molecules on the depolarisation response of OA chondrocytes to cyclical strain (continued)

Donor	Reagent	Membrane potential (-mV)		
		Resting	Reagent alone	Post strain
74/M	control	43,46,38,39 45		32,31,31,30,30
	calphostin C	56,60,60,53 52,55,56,54 50,53	41,37,40,42,37 43,36,40,44,36	20,20,21,15,19 14,19,18,23,15

Table 5.5b Effect of genistein and chelerythrine chloride on the hyperpolarisation response of normal chondrocytes to cyclical strain

Donor	Reagent	Membrane potential (-mV)		
		Resting	Reagent alone	Post strain
71/M	control	28,29,29,30,31		40,41,43,42,44
	genistein	32,34,34,33,31	33,35,33,35,36	35,35,32,33,36
	chelerythrine	32,32,31,30,35	34,36,36,35,35	36,33,30,30,29

Table 5.6 Effect of IL-4 and IL-1β (10 ng/ml) on the membrane potential of mechanically unstimulated normal chondrocytes

Donor	Reagent	Membrane potential (-mV)	
		Resting	With reagent
74/M	IL-4	26,25,23,24,25	30,36,31,34,30
81/M	IL-1β	24,20,25,18,22	12,14,15,14,16

Table 5.7a Effect of IL-4 and IL-1β (10 ng/ml) on the membrane potential of mechanically unstimulated OA chondrocytes

Donor	Reagent	Membrane potential (-mV)	
		Resting	With reagent
73/M	IL-4	44,43,42,45,40	39,33,38,40,35
		39,46,37,43,46	35,41,38,34,36
	IL-1β	29,36,29,28,37	23,24,28,28,24
		34,30,35,35,38	30,33,25,32,33
74/M	IL-4	40,42,43,43,49	31,25,34,25,38
		50,41,42,46,42	26,28,27,31,32
	IL-1β	45,49,55,47,52	41,40,33,37,38
		45,43,47,44,42	42,39,37,37,34

Table 5.7b Effect of IL-4 and IL-1β (10 pg/ml) on the membrane potential of mechanically unstimulated OA chondrocytes

		Membrane potential (-mV)	
Donor	Reagent	Resting	With reagent
73/F	IL-4	36,37,42,44,35	35,35,34,36,34
		38,38,43,39,38	35,36,38,32,38
	IL-1β	38,37,41,45,44	38,36,35,35,39
		38,37,40,42,36	33,34,33,38,35
81/F	IL-4	44,48,50,44,48	42,46,42,39,36
		44,51,43,43,42	36,38,38,40,44
	IL-1β	51,48,51,44,47	38,40,44,35,38
		49,41,43,47,42	40,43,37,44,43

Table 5.8 Effect of IL-4 + anti-IL-1β or IL-1β + anti-IL-4 on the membrane potential of OA chondrocytes

	Membrane potential (-mV)		
Donor	Resting	Anti-IL-1β Ab. alone	Anti- IL-1β + IL-4 (10 pg/ml)
74/M	37,42,48,53,44 50,51,40,44,46	44,47,41,51,40 48,46,43,50,47	42,40,39,39,38 43,37,39,45,38
Donor	Resting	Anti-IL-4	Anti-IL-4 + IL-1β (10 pg/ml)
74/M	44,42,42,42,51 39,43,49,41,37	46,40,40,46,45 44,43,39,43,38	44,48,39,39,44 41,40,41,43,37

Table 5.9 Effect of anti-IL-1 RI, RII, and anti-IL-2 Rγ on the depolarisation response of OA chondrocytes to cyclical strain

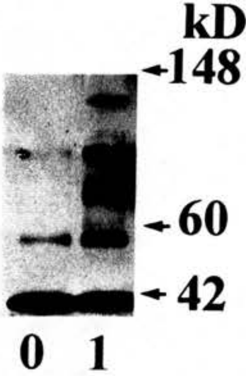
Donor	Reagent	Membrane potential (-mV)		
		Resting	Antibody alone	Post strain
74/M	control	33,32,30,26 26		21,21,22,23,24 19,25,24,20,18
	anti-IL-1RI	29,32,40,29 28,37,26,26 35,40	33,34,40,29,36 45,30,32,44,35	32,39,38,42,40 38,31,29,30,35
	anti-IL-1RII	30,35,29,38 29,36,29,34 36,30	35,33,30,40,34 38,40,30,32,33	27,28,30,31,31 35,30,33,28,29
45/M	control	35,34,30,30 36,34,31,30 36,31		24,25,25,26,30 22,22,31,28,27
	anti-IL-1RI	37,35,39,40 40	41,40,35,36,42	43,42,38,39,40
	anti-IL-1RII	40,36,35,35 47,48,41,33 33,41	43,41,40,42,35 47,34,38,46,47	33,36,38,39,35 34,40,35,34,41

Table 5.9 Effect of anti-IL-1 RI, RII, and anti-IL-2 Rγ on the depolarisation response of OA chondrocytes to cyclical strain (continued)

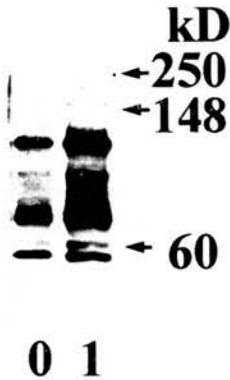
Donor	Reagent	Membrane potential (-mV)		
		Resting	Antibody alone	Post strain
75/F	control	33,56,46,50 43,48,46,48 42,38		43,35,35,29,30 28,26,40,34,32
	anti-IL-1RI	48,42,52,39 39,42,40,52 50,43	50,44,43,41,43 54,46,44,49,41	46,48,40,50,53 54,40,44,39,46
	anti-IL-1RII	40,39,40,46 40,38,39,45 44,41	45,47,42,42,45 48,50,46,45,50	35,38,40,41,50 34,44,36,47,43
63/F	control	40,46,43,40 41,51,42,44 41,43		38,33,35,35,38 37,36,42,34,39
	IL-2Rγ	42,45,41,45 42,46,44,43 48,43	45,40,40,48,39 47,39,42,49,40	40,46,40,41,41 46,39,49,42,38
74/M	control	37,39,38,44 41,49,50,51 53,53		40,35,38,34,37 37,36,38,40,41
	IL-2Rγ	43,42,44,51 55,50,43,48 50,46	52,49,46,43,55 40,43,47,48,41	46,52,40,41,45 52,50,47,40,49

APPENDIX IV

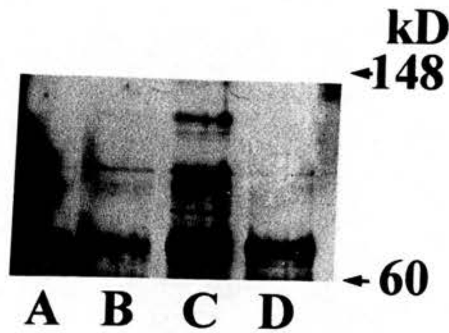
The results by western blot and confocal microscopy are associated with this thesis. The parenthesis shows the OA day-book number.



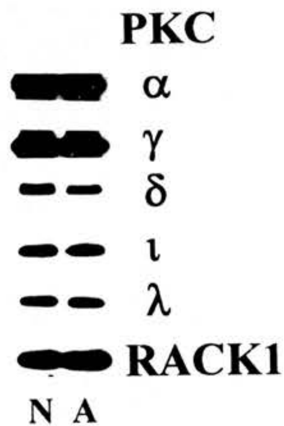
Mechanical stimulation induced tyrosine phosphorylation in human articular chondrocytes. 0: resting cells. 1: 1 min of 0.33 Hz cyclical strain. (No. 10250)



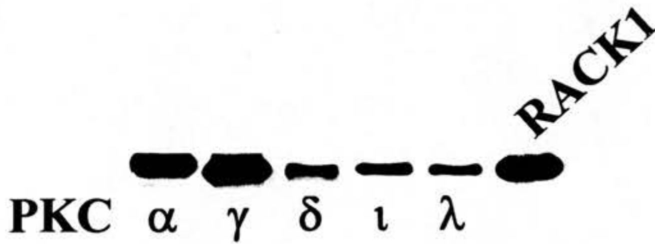
Mechanical stimulation induced tyrosine phosphorylation in human articular chondrocytes. 0: resting cells. 1: 1 min of 0.33 Hz cyclical strain. (No. 10258)



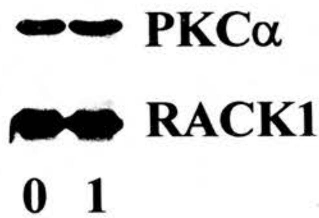
Effect of RGD peptide on mechanically-induced tyrosine phosphorylation. A: chondrocytes incubated with GRADSP only. B: cells incubated with GRGDSP only. C: cells treated with GRADSP and then subjected to 1 min of cyclical strain. D: cells treated with GRGDSP and then subjected to 1 min of cyclical strain. (No. 11370)



Identification of PKC isozymes in primary cultured chondrocytes. N: normal adult chondrocytes. A: chondrocytes from osteoarthritic cartilage. (No. 11051)



Identification of PKC isozymes in primary normal chondrocytes. (No. 11104)



PKCα and RACK1 in primary normal chondrocytes. 0: whole cell lysates from resting cells. 1: whole cells lysates from cells subjected to 1 min of 0.33 Hz cyclical strain. (No. 12457)

Upregulation of β1 integrin-RACK1 interaction after 1 min cyclical strain in C-20/A4 cells. Immunoprecipitated with β1 integrin and immunoblotted RACK1. A: unstimulated cells. B: mechanically stimulated cells.

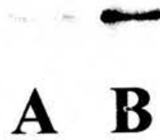
PKC inhibitor inhibited the upregulation of β1 integrin-RACK1 interaction induced by mechanical stimulation. Immunoprecipitated with β1 integrin and immunoblotted with RACK1. A: resting cells. B: cells stimulated with 1 min of cyclical strain. C: cells incubated with chelerythrine only. D: cells incubated with chelerythrine and then subjected to 1 min of cyclical strain. (No. 12187)

PKC inhibitor inhibited the upregulation of β1 integrin-PKCα interaction induced by mechanical stimulation. Immunoprecipitated with β1 integrin and immunoblotted with PKCα. A: resting cells. B: cells stimulated with 1 min of cyclical strain. C: cells incubated with chelerythrine only. D: cells incubated with chelerythrine and then subjected to 1 min of cyclical strain. (No. 12459)

IP: β1 integrin
IB: RACK1

GRGDSP inhibited the upregulation of β1 integrin-RACK1 interaction induced by mechanical stimulation in primary chondrocytes. A: cells treated with GRGDSP followed by 1 min cyclical strain. B: cells treated with GRADSP followed by 1 min strain. C: cells treated with GRGDSP only. D: cells treated with GRADSP only. (No. 12810)

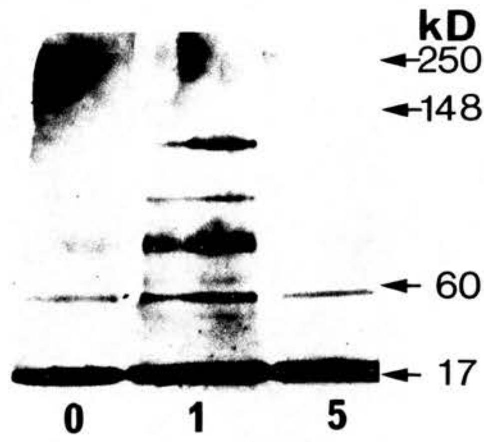
IP: β1 integrin
IB: PKCα



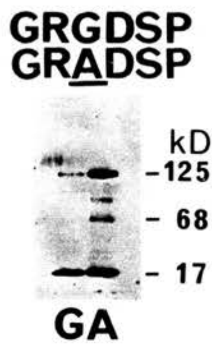
GRGDSP inhibited the upregulation of β1 integrin-PKCα interaction induced by mechanical stimulation in primary chondrocytes. A: cells treated with GRGDSP followed by 1 min cyclic strain. B: cells treated with GRADSP followed by 1 min cyclic strain. (No. 12835)

IP: α5 integrin
IB: CD47

Coimmunoprecipitation of α5 integrin and CD47 in primary articular chondrocytes. A: resting cells. B: cells subjected to 10 min cyclic strain. C: cells subjected to 20 min cyclic strain. (No. 12035)

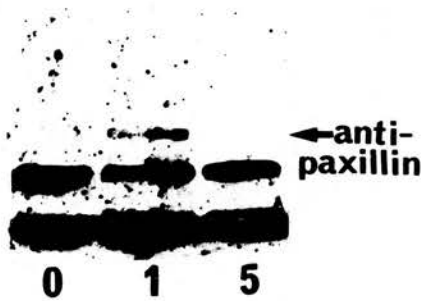


Whole gel corresponds to the Fig. 3.3 (Page 103).



Whole gel corresponds to the Fig. 3.4 (Page 105).

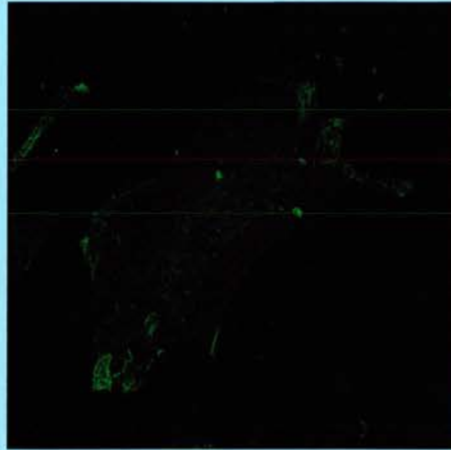
Whole gel corresponds to the Fig. 3.6i (Page 108).



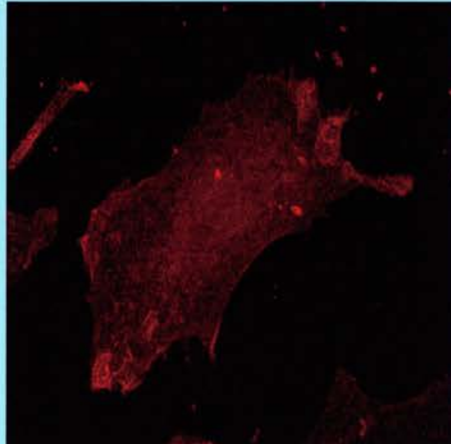
Whole gel corresponds to the Fig. 3.6ii (Page 108).

Colocalization of FAK and β-catenin

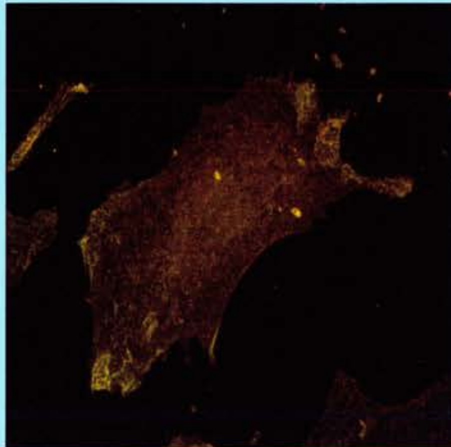
FAK



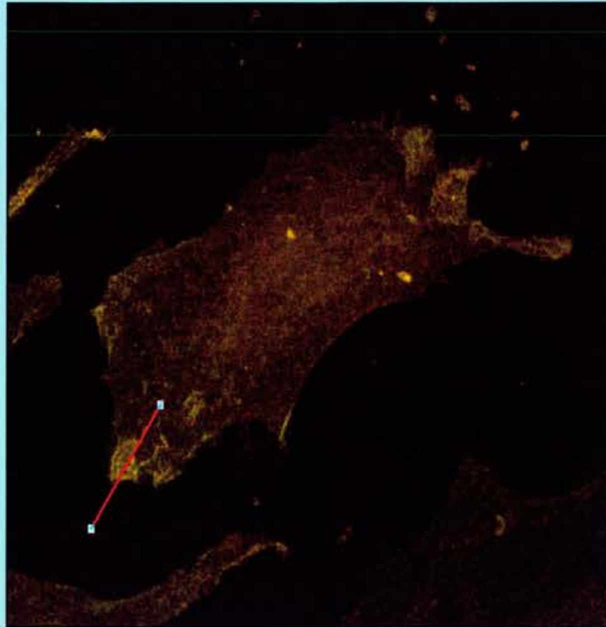
β-catenin

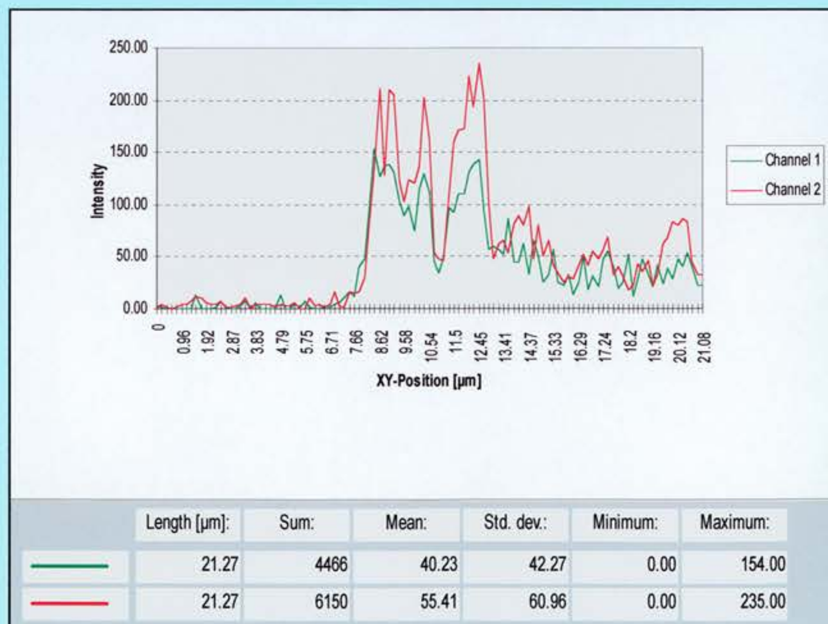


overlay



Colocalization of FAK and β-catenin



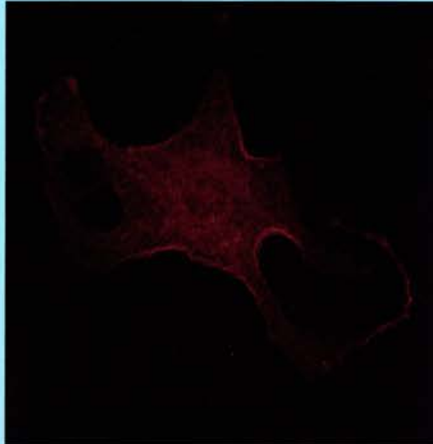


	Length [µm]:	Sum:	Mean:	Std. dev.:	Minimum:	Maximum:
(green line)	21.27	4466	40.23	42.27	0.00	154.00
(red line)	21.27	6150	55.41	60.96	0.00	235.00

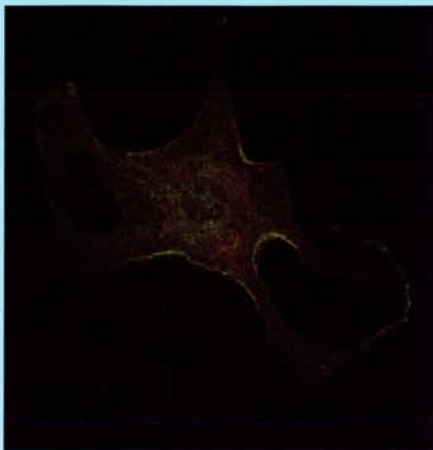
Colocalization of α5 integrin and β-catenin

α5-integrin

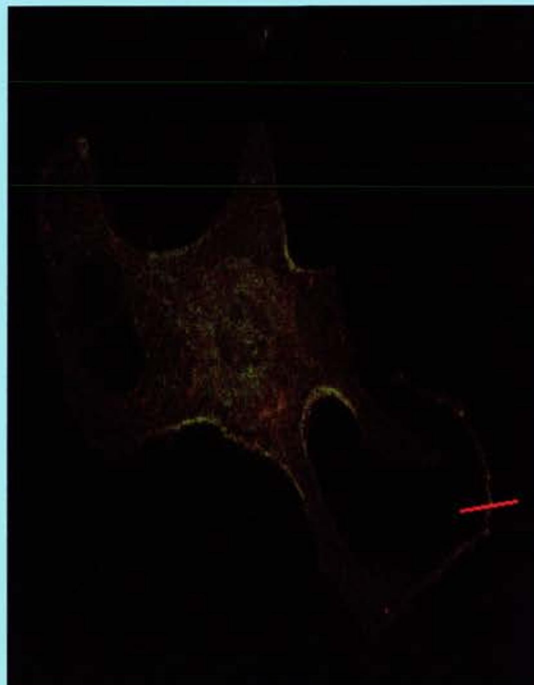
β-catenin

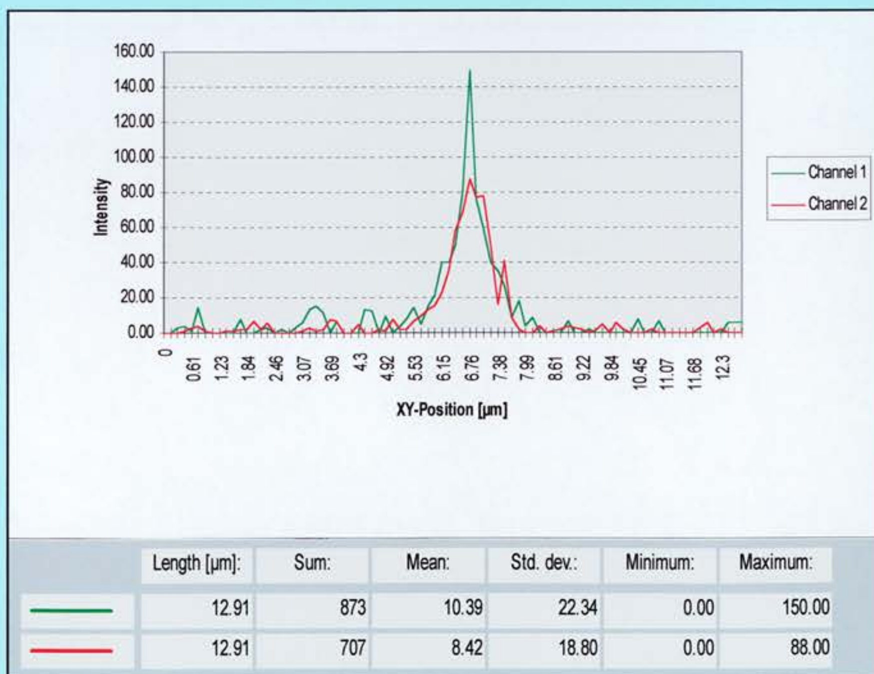


overlay



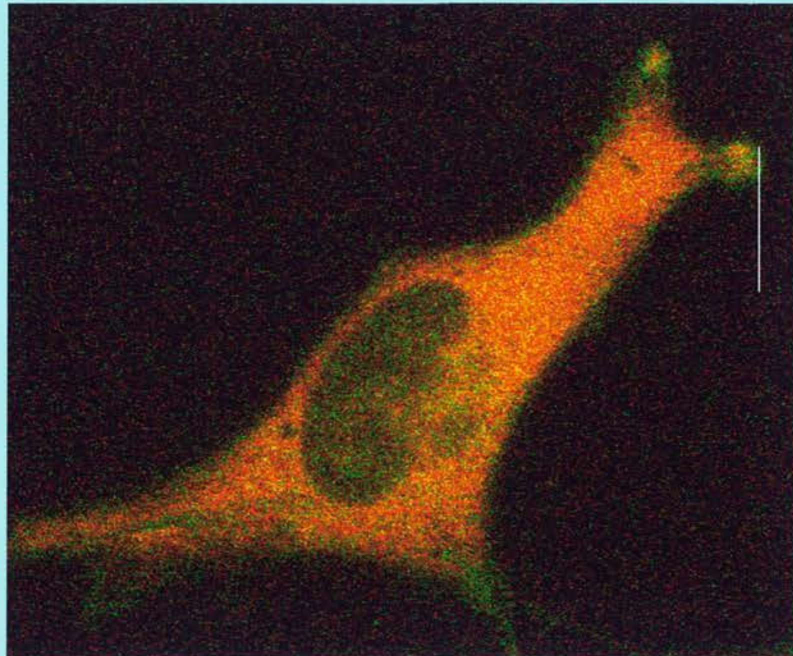
Colocalization of α5 integrin and β-catenin

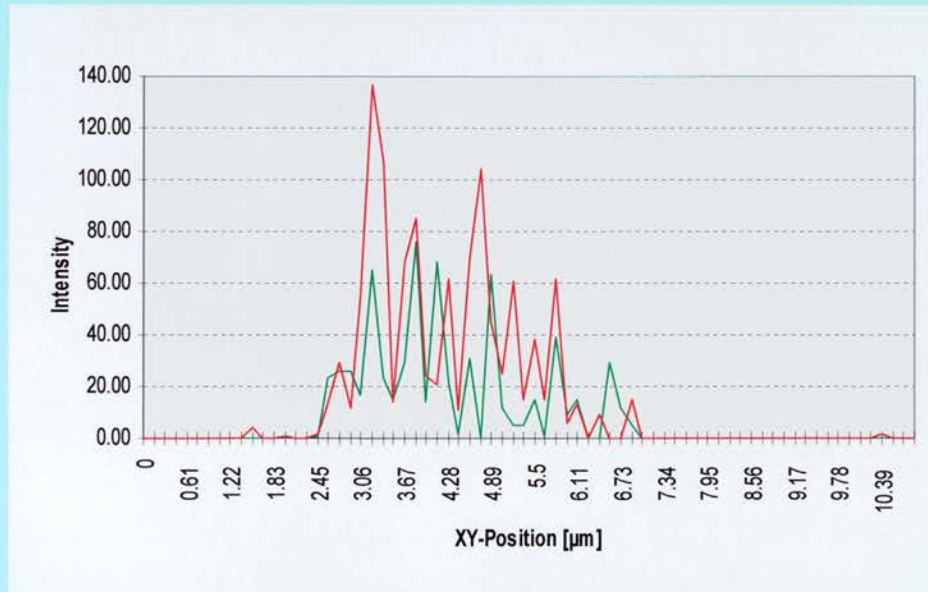




	Length [µm]:	Sum:	Mean:	Std. dev.:	Minimum:	Maximum:
(green line)	12.91	873	10.39	22.34	0.00	150.00
(red line)	12.91	707	8.42	18.80	0.00	88.00

Colocalization of RACK1 and β1 integrin





TRITC: RACK1
FITC: β1 integrin