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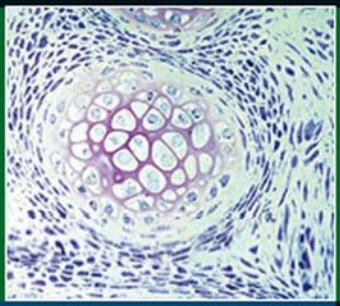
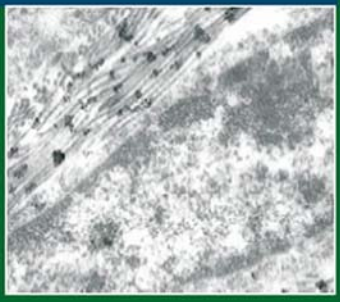


Diverse Roles of Integrin Receptors in Articular Cartilage



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With 36 Figures

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Abstract

Integrins are heterodimeric integral membrane proteins made up of α and β subunits. At least eighteen α and eight β subunit genes have been described in mammals. Integrin family members are plasma membrane receptors involved in cell adhesion and active as intra- and extracellular signalling molecules in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metastatic spread of tumour cells. Integrin beta 1 (β1-integrin), the protein encoded by the ITGB1 gene (also known as CD29 and VLAB), is a multi-functional protein involved in cell-matrix adhesion, cell signalling, cellular defense, cell adhesion, protein binding, protein heterodimerisation and receptor-mediated activity. It is highly expressed in the human body (17.4 times higher than the average gene in the last updated revision of the human genome). The extracellular matrix (ECM) of articular cartilage is a unique environment. Interactions between chondrocytes and the ECM regulate many biological processes important to homeostasis and repair of articular cartilage, including cell attachment, growth, differentiation and survival. The β1-integrin family of cell surface receptors appears to play a major role in mediating cell–matrix interactions that are important in regulating these fundamental processes. Chondrocyte mechanoreceptors have been proposed to incorporate β1-integrins and mechanosensitive ion channels which link with key ECM, cytoskeletal and signalling proteins to maintain the chondrocyte phenotype, prevent chondrocyte apoptosis and regulate chondrocyte-specific gene expression. This review focuses on the expression and function of β1-integrins in articular chondrocytes, its role in the unique biology of these cells and its distribution in cartilage.

1 Introduction

Articular cartilage is a specialised connective tissue with unique biological and mechanical properties which depend on the structural design of the tissue and the interactions between its unique resident cells, the chondrocytes, and the extracellular matrix (ECM) that makes up the bulk of the tissue (Buckwalter and Mankin 1998). Chondrocytes (Fig. 1) are the architects of the ECM (Muir 1995), building the macromolecular framework of the ECM from three distinct classes of macromolecules: collagens, proteoglycans, and noncollagenous proteins. Of the collagens present in articular cartilage, collagens type II, IX, and XI form a fibrillar meshwork that gives cartilage tensile stiffness and strength (Eyre 2004; Buckwalter and Mankin 1998; Kuettner et al. 1991), whereas collagen type VI forms part of the matrix immediately surrounding the chondrocytes, enabling them to attach to the macromolecular framework of the ECM and acting as a transducer of biomechanical and biochemical signals in the articular cartilage (Guilak et al. 2006; Roughley and Lee 1994). Embedded in the collagen mesh are large aggregating proteoglycans (aggrecan), which give cartilage its stiffness to compression, its resilience and contribute to its long-term durability (Dudhia 2005; Kiani et al. 2002; Luo et al. 2000; Roughley and Lee 1994). The extracellular matrix proteins in cartilage are of great significance for the regulation of the cell behaviour, proliferation, differentiation and morphogenesis (Kosher et al. 1973; Kosher and Church 1975; von der Mark et al. 1977; Hewitt et al. 1982; Sommarin et al. 1989; Ramachandrula et al. 1992; Ruoslahti and Reed 1994; Enomoto-Iwamoto et al. 1997; Gonzalez et al. 1993). Further, embedded in the meshwork are small proteoglycans, including decorin, biglycan and fibromodulin. Decorin and fibromodulin both interact with the type II collagen fibrils in the matrix and play a role in fibrillogenesis and interfibril interactions. Biglycan is mainly found in the immediate surrounding of the chondrocytes, where it may interact with collagen type VI (Buckwalter and Mankin 1998; Roughley and Lee 1994). Modulation of the ECM proteins is regulated by an interaction of a diversity of growth factors with the chondrocytes (Jenniskens et al. 2006; Trippel et al. 1989; Isgaard 1992; Hunziker et al. 1994; Sah et al. 1994). In fact, it has been reported recently that IGF-I and TGF-β stimulate the membrane expression of integrins, and that this event is accompanied by increasing adhesion of chondrocytes to matrix proteins (Loeser 1997). Other noncollagenous proteins in articular cartilage such as cartilage oligomeric matrix protein (COMP) are less well studied and may have value as a marker of cartilage turnover and degeneration (Di Cesare et al. 1996), while tenascin and fibronectin influence interactions between the chondrocytes and the ECM (Buckwalter and Mankin 1998; Burton-Wurster et al. 1997). The major cellular and molecular components of articular cartilage are shown in Fig. 2.

The ECM surrounds chondrocytes and protects them from biomechanical stress arising during normal joint motion, determines the types and concentrations of molecules that reach the cells and helps to maintain the chondrocyte phenotype. Throughout life, cartilage undergoes continuous internal remodeling as chondrocytes replace

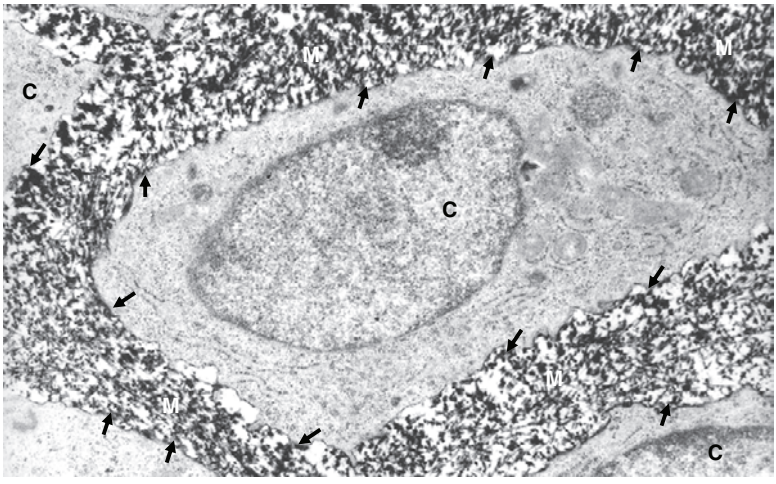

Fig. 1 Electron microscopic demonstration of chondrocytes. Typical chondrocyte (*C*) with smooth surface and numerous cavities of rough endoplasmic reticulum, mitochondria, other cell organelles, vacuoles and granules. Chondrocytes are embedded in a network of extra cellular matrix of thin irregularly running fibrils (*M*) closely attached to the cell surface (*arrows*)

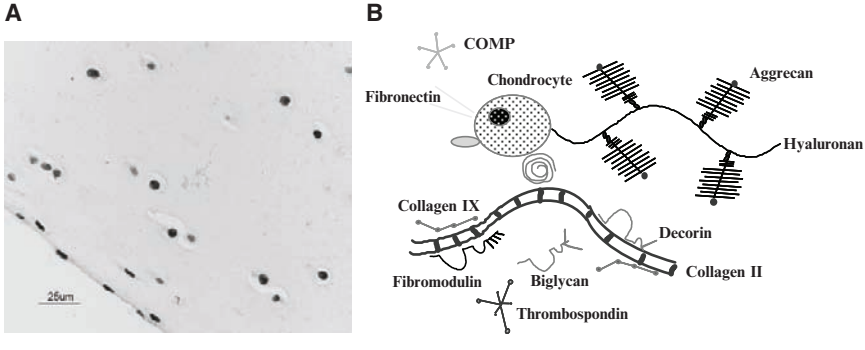

Fig. 2A, B Light microscopic appearance of equine articular cartilage and the major cellular and molecular components of articular cartilage. **A** Histological morphology of normal equine articular cartilage showing a relatively homogeneous tissue structure consisting of a single cell type, the chondrocyte. **B** Chondrocytes synthesise and maintain a macromolecular framework consisting of three distinct classes of macromolecules: collagens, proteoglycans, and noncollagenous proteins. Collagens (type II, IX, and XI) form the fibrillar meshwork of the ECM, whereas collagen type VI forms part of the peri-cellular matrix. Embedded in the collagen mesh are large aggregating proteoglycans (aggrecan). Also embedded in the matrix are small proteoglycans, including decorin, biglycan, fibromodulin and other noncollagenous proteins such as cartilage oligomeric matrix protein (COMP) and fibronectin

matrix macromolecules lost through degradation. Evidence indicates that ECM turnover depends on the ability of chondrocytes to detect alterations in the macromolecular composition and organisation of the matrix, including the presence of

degraded macromolecules, and to respond by synthesising appropriate types and amounts of new ECM components. It is known that mechanical loading of cartilage creates mechanical, electrical, and physicochemical signals that help to direct the synthesising and degrading activity of chondrocytes (Fig. 3) (Mobasheri et al. 2002a). In addition, the ECM acts as a signal transducer for the chondrocytes (Millward-Sadler and Salter 2004). A prolonged and severe decrease in the joint loading and usage leads to alterations in the composition of the ECM and eventually to loss of tissue structure and its specific biomechanical properties, whereas normal physical load on the joint stimulates the biosynthetic activity of chondrocytes and possibly the internal

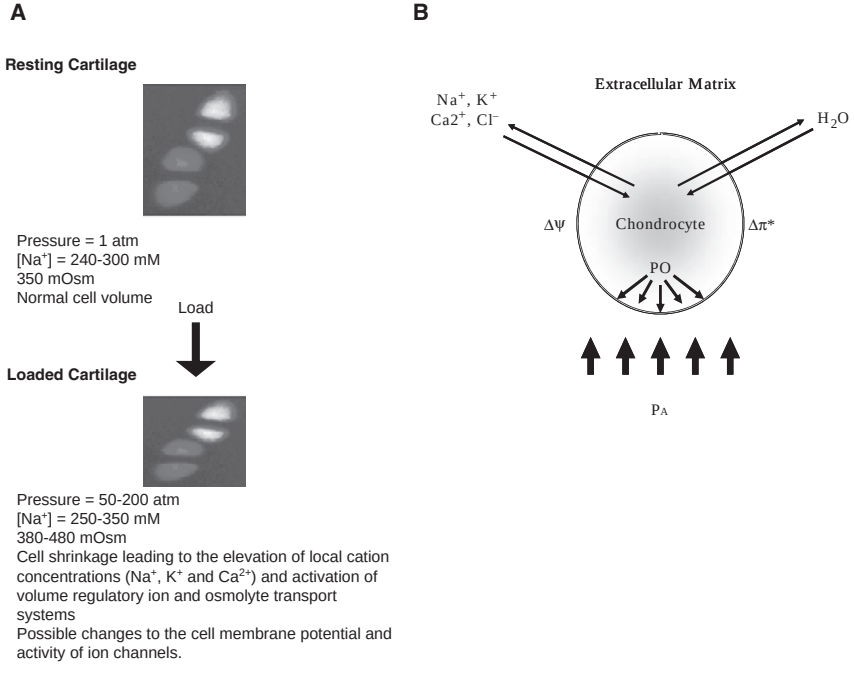

Fig. 3A, B The effects of mechanical load and the extracellular ionic and osmotic milieu on the physical environment of human articular chondrocytes. **A** Chondrocytes are excellent sensors of mechanical signals and respond to these signals in coordination with other environmental, hormonal and genetic factors to regulate metabolic activity. Resting articular cartilage experiences normal atmospheric pressure equivalent to 1 atmosphere (*atm*). Mechanically loaded articular cartilage is exposed to pressures as high as 50–200 atm. Mechanical loading of cartilage results in cell shrinkage, which in turn leads to the elevation of local cation concentrations (Na^+, K^+ and Ca^{2+}) and the activation of volume regulatory ion and osmolyte transport systems. In addition, there are changes to the cell membrane potential and activity of ion channels. **B** Chondrocytes are also excellent sensors of ionic and osmotic signals. This schematic illustrates the mechano-electrochemical responses in chondrocytes under mechanical load and the interaction between the extracellular matrix and chondrocytes. $\Delta\pi^*$ represents changes in osmotic pressure and $\Delta\psi$ symbolises the electrical membrane potential difference across the chondrocyte plasma membrane. (Adapted from Mobasheri et al. 2002)

tissue remodeling (Buckwalter and Lane 1997; Maffulli and King 1992). Ballistic, high-impact sports may result in long-term disturbances in the articular cartilage structure and function, since although articular cartilage can tolerate a tremendous amount of intensive and repetitive physical stress, it manifests a striking inability to heal even the most minor injury (Buckwalter and Martin 2004; Buckwalter 2003; Newman 1998; Buckwalter and Lane 1997). Furthermore, aging leads to alterations in the ECM composition and alters the biological functions of chondrocytes, including their ability to respond to a variety of stimuli such as growth factors (Hudelmaier et al. 2001; Eckstein et al. 2001; Ralphs and Benjamin 1994). All these alterations increase the probability of cartilage degeneration (Sarzi-Puttini et al. 2005; Buckwalter 2003; Poole 1999; Setton et al. 1999) and emphasise the importance of interaction of the chondrocytes with their surrounding ECM since this interaction regulates growth, differentiation and survival of the chondrocytes in normal and pathophysiological conditions.

An important facet of the biology and maintenance of articular cartilage is mediated by integrins, a family of heterodimeric transmembrane receptors that are abundantly expressed in articular cartilage. Integrins are intimately involved in chondrocyte adhesion to the ECM and signalling in a variety of processes, including chondrogenesis (Shakibaei et al. 1995), tissue repair and inflammation (Pulai et al. 2005; Zemmyo et al. 2003; Attur et al. 2000). Integrin beta 1 (β1-integrin), the protein encoded by the ITGB1 gene, is the primary focus of this review. β1-integrins play a key role in regulating a number of fundamental processes in chondrocytes; these include mechanotransduction, maintenance of the unique chondrocyte phenotype, regulation of chondrocyte-specific gene expression and inhibition of apoptotic cell death. This review will focus on the expression and function of β1-integrins in articular chondrocytes. We will discuss the research carried out over the last 20 years in our own laboratories and in the laboratories of others involved in studying chondrocyte ECM adhesion and signal transduction.

1.1 Integrins: Multifunctional Adhesion Molecules

Integrins (also known as very late antigen-integrins or VLA-integrins) are representative of a set of evolutionarily conserved, biologically important adhesion molecules (Buck and Horwitz 1987b). In fact, the sequences and genes of *Drosophila* integrins are quite closely related to vertebrate integrins, making it clear that integrins arose at a very early stage of evolution (Hynes 1992). Since the identification of the integrin receptor family approximately 20 years ago (Tamkun et al. 1986; DeSimone et al. 1987; Hynes et al. 1987), integrins and their ligands have been shown to play key roles in development, inflammatory responses, leukocyte traffic, hemostasis and cancer metastasis (Buck and Horwitz 1987a, b; Horton 1995; Van Waes 1995; Lohi et al. 1998; Menko et al. 1998).

They are heterodimeric glycoproteins consisting of one α and one β chain (Clark 1990). Each integrin subunit crosses the membrane once, with a long polypeptide tail in the extracellular space, and has two short cytoplasmic domains. Integrins are

the primary receptors for cell adhesion to the extracellular matrix proteins and in vertebrates they play important roles especially in maintaining cell–cell adhesion (Yong and Khwaja 1990; Clark 1990). Integrins convey transmembrane connections to the cytoskeleton and activate many intracellular signalling pathways (Buck and Horwitz 1987a). Ligand specificity depends on both α and β subunits where they recognise an RGD (Arg-Gly-Asp) sequence on the diverse ligands to which they bind (Buck and Horwitz 1987a). Integrins containing β1 or β3 chains are ECM receptors, whereas those containing β2 chains are leukocyte cell–cell receptors (Clark 1990). All β1-integrins bind to extracellular matrix molecules; in articular cartilage these include fibronectin, laminin and collagens (Buck and Horwitz 1987a; Cassiman 1989; Hemler et al. 1990). However, after extracellular ligand binding to an integrin, integrins cannot directly interact with the actin cytoskeleton but require associate molecules as mediators, and thus far more than ten molecules are known that bind to the cytoplasmic domain of the β1 subunit (Brakebusch and Fassler 2005). In addition to mediating cell adhesion, members of the β1 subfamily of integrins contribute to the organisation of the cytoskeleton and activate numerous intracellular signal transduction pathways (Juliano 1993; Akiyama 1996), thus controlling the behaviour of the cells including cell growth (Zhu and Watt 1996; Fang et al. 1996), differentiation (Riikonen et al. 1995; Streuli et al. 1995), cell morphology and migration (Chan et al. 1992; Bauer et al. 1993). Furthermore, it is known that different types of extracellular matrix components can selectively activate the expression of specific genes in cultured cells (Caron 1990; Streuli and Bissell 1990), and that integrin-mediated tyrosine phosphorylation is involved in immediate-early gene induction in monocytes (Lin et al. 1994).

Signal transduction through the VLA-5 fibronectin receptor has been shown to induce collagenase and stromelysin gene expression (Werb et al. 1989). Since increased amounts of fibronectin are present in the superficial layers of osteoarthritic cartilage (Jones et al. 1987), interactions between elevated fibronectin and the VLA-5 receptor will have profound effects on the expression and regulation of matrix metalloproteinases in that locality.

The interaction between chondrocytes and matrix proteins is mediated largely by the β1 subfamily of integrin receptors (Durr et al. 1993; Enomoto et al. 1993; Loeser 1993; Woods et al. 1994; Shakibaei 1995; Shakibaei et al. 1997; Enomoto-Iwamoto et al. 1997). Integrin-mediated adhesion to extracellular proteins (i.e. collagen II, fibronectin, laminin, vitronectin) can activate several cytoskeletal associated proteins, such as paxillin (Burridge et al. 1992), tensin (Bockholt and Burridge 1993) and intracellular signalling proteins such as FAK (focal adhesion kinase) (Schaller et al. 1993). In addition to their function as cell adhesion molecules in chondrocytes integrins are involved in cell–matrix interactions, cartilage remodelling and chondrogenesis (Shakibaei 1995; 1998; Shakibaei et al. 1993a), integrins also play an important role as signalling receptors in chondrocytes (Shakibaei et al. 1999). Co-localisation of β1 integrins (effectively chondrocyte-specific VLA-1, VLA-3 and VLA-5) with the insulin-like growth factor-I receptor (IGF-IR) suggests that matrix binding integrins and IGF-IR collaborate to regulate focal adhesion components in

focal adhesion sites and their downstream functions, including interactions with the cytoskeleton and mitogen-activated protein kinase signalling pathways (Shakibaei et al. 1999). Integrins also appear to play a key role in sustaining the chondrocyte phenotype by maintaining a constant link with the extracellular matrix, thus providing a critical survival signal.

Several observations suggest that integrins collaborate with growth factors and that this collaboration is important for cell functions, such as adhesion, differentiation, growth, survival and other biological processes in different cell types (Vuori and Ruoslahti 1994; Arner and Tortorella 1995; Kinashi et al. 1995; Miyamoto et al. 1996; Mainiero et al. 1996; Schneller et al. 1997). IGF-I (insulin like growth factor-I) has been shown to stimulate the differentiation of chondrocytes and matrix synthesis (incorporation of sulphate into proteoglycans) in vivo and in vitro in numerous studies (Vetter et al. 1986; Trippel et al. 1989; Joosten et al. 1991; Hunziker et al. 1994; Sah et al. 1994). Miyamoto et al. have shown that the synergistic interaction between integrins and different growth factor receptors phosphorylate MAP kinases, which control a variety of cell functions, and that this collaboration is only possible when the integrins are aggregated and occupied by a ligand (Miyamoto et al. 1996).

Therefore integrins are at the centre of numerous normal physiological events and pathophysiological processes in human diseases: genetic, autoimmune, viral and bacterial. They are the target of effective therapeutic drugs against thrombosis and inflammation. Because of their diverse biological functions, integrins have become the most intensely studied and best-understood mammalian cell-adhesion receptors.

1.2 Integrin Structure–Function Relationships

Integrins are the major metazoan receptors for cell adhesion to extracellular matrix proteins and, in vertebrates, they play important roles in cell–cell adhesions, transmembrane connections to the cytoskeleton and the ECM and activation of many intracellular signalling pathways (Fig. 4) (Tamkun et al. 1986; DeSimone et al. 1987; Hynes et al. 1987). According to the Interpro[1] database, integrins have also been found in other living organisms including cyanobacteria, probably due to horizontal gene transfer (May and Ponting 1999).

The chondrocyte plasma membrane has long been identified as the site where mechanoreceptor complexes form and function, enabling chondrocytes to sense and respond to their mechanical environment (Wright et al. 1992, 1996; Stockwell 1991). Despite this realisation, an important question still remains unanswered: What are the molecular components of mechanoreceptor complexes in chondrocytes and how do they function? Accordingly, based on published information from a number of related and unrelated cell models we have formulated the following

[1] InterPro is a database of protein families, domains and functional sites in which identifiable features found in known proteins can be explored and applied to unknown protein sequences. Further information on InterPro can be found at: http://www.ebi.ac.uk/interpro/index.

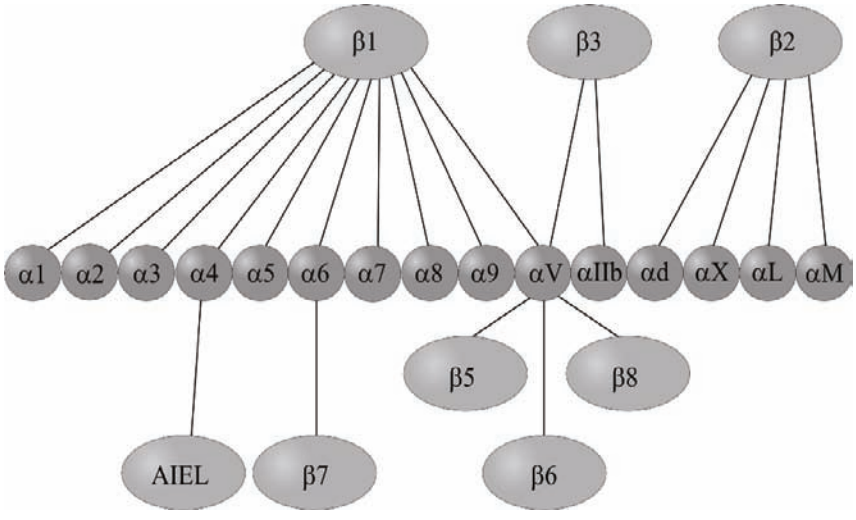

Fig. 4 Integrin receptors. Integrins are the major cell receptors for adhesion to extracellular matrix proteins and play important roles in cell–cell interactions by making transmembrane connections to the cytoskeleton and activating many intracellular signalling pathways. Integrins are heterodimeric proteins made up of alpha and beta subunits. At least 18 alpha and eight beta subunits have been described in the human genome. Integrin family members are membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, haemostasis, tissue repair, immune response and metastatic diffusion of tumour cells

hypothesis: We propose that chondrocyte mechanoreceptor complexes consist of plasma membrane resident integrin molecules associated with stretch-activated ion channels, key pericellular matrix macromolecules (i.e. collagen), serving as extracellular ligands for the receptor complex, and signalling and cytoskeleton complexes responsible for intracellular communication.

1.3 Structure of Integrins

Integrins are α-β heterodimers (Fig. 5); each subunit crosses the membrane once, with most of the polypeptide being in the extracellular space, and two short cytoplasmic domains. Most integrins recognise relatively short peptide motifs, and in general require an acidic amino acid to be present. Ligand specificity depends on both α and β subunits. Many integrins are expressed on cell surfaces in an inactive state in which they do not bind their ligands and do not convey signals (Hynes 1987, 1992; Ruoslahti and Pierschbacher 1987; Ruoslahti 1991). Integrins are known to frequently intercommunicate and the engagement of one may lead to the activation or inhibition of another.

Integrin cytoplasmic domains are normally less than 50 amino acids in length, with the β subunit sequences exhibiting greater homology to each other than the

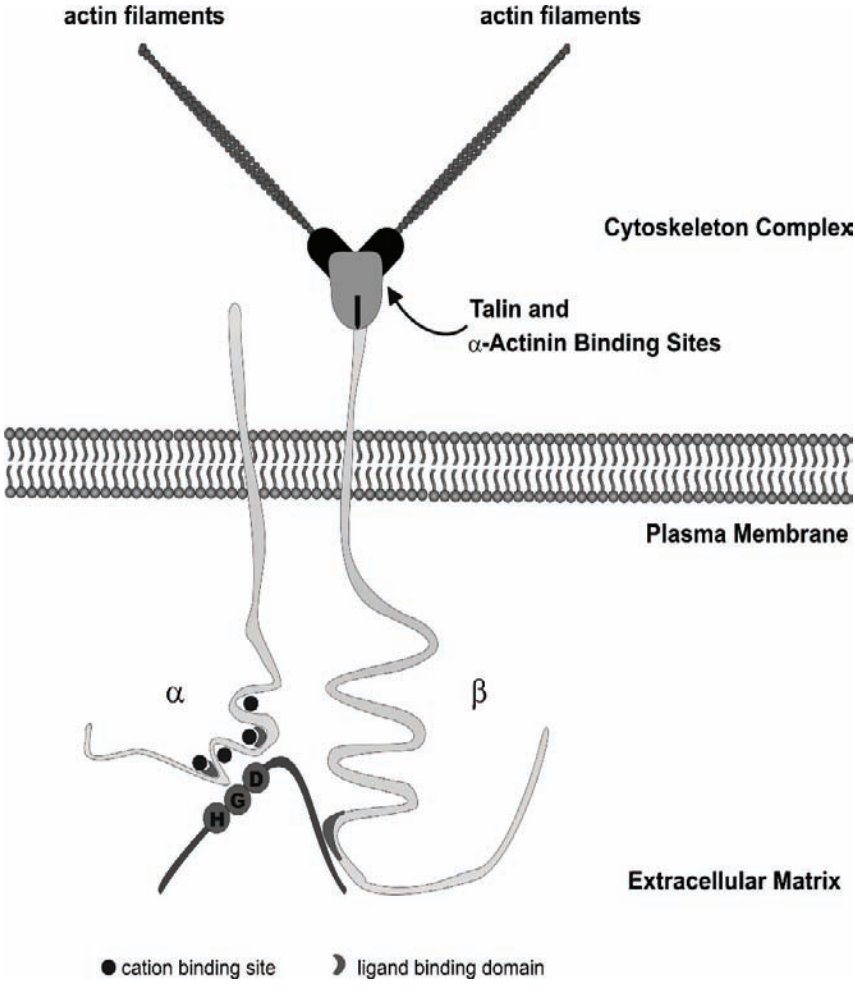

Fig. 5 Integrins are α-β heterodimers. Each integrin subunit crosses the membrane once, with most of the polypeptide in the extracellular space, and has two short cytoplasmic domains

α subunit sequences (Humphries et al. 2003). This is consistent with current evidence that the β subunit is the principal site for binding cytoskeletal and extracellular signalling molecules, whereas the α subunit has a regulatory role. Some integrin α subunits are cleaved post-translationally to produce a heavy and a light chain linked by a disulphide bond (Hynes 1987; Albelda and Buck 1990). Integrin α chains share a conserved sequence which is found at the beginning of the cytoplasmic domain, just after the end of the transmembrane region. The first ten residues of the α subunits' cytoplasmic domain appear to form an α helix that is terminated by a proline residue. The remainder of the domain is highly acidic in nature and loops back to contact

the membrane-proximal lysine anchor residue. Within the N-terminal domain of α subunits, seven sequence repeats, each of approximately 60 amino acids, have been found (Corbi et al. 1987). It has been predicted that these repeats assume the β propeller fold. The domains contain seven four-stranded beta-sheets arranged in a torus around a pseudosymmetric axis (Springer 1997). Integrin ligands and a putative Mg^{2+} ion are predicted to bind to the upper face of the propeller, in a manner analogous to the way in which the trimeric G-protein β subunit (G beta) (which also has a β propeller fold) binds the G protein α subunit (Springer 1997).

The C-terminal portion of the β subunits extracellular domain contains an internal disulphide-bonded cysteine-rich region, while the intracellular tail contains putative sites of interaction with a variety of intracellular signalling and cytoskeletal proteins such as focal adhesion kinase and alpha-actinin (Fig. 6). The first 20 amino acids of the β subunits' cytoplasmic domain are also alpha helical, but the final 25 residues are disordered and, apart from a turn that follows a conserved NPxY motif, appear to lack a defined structure, suggesting that a defined structure

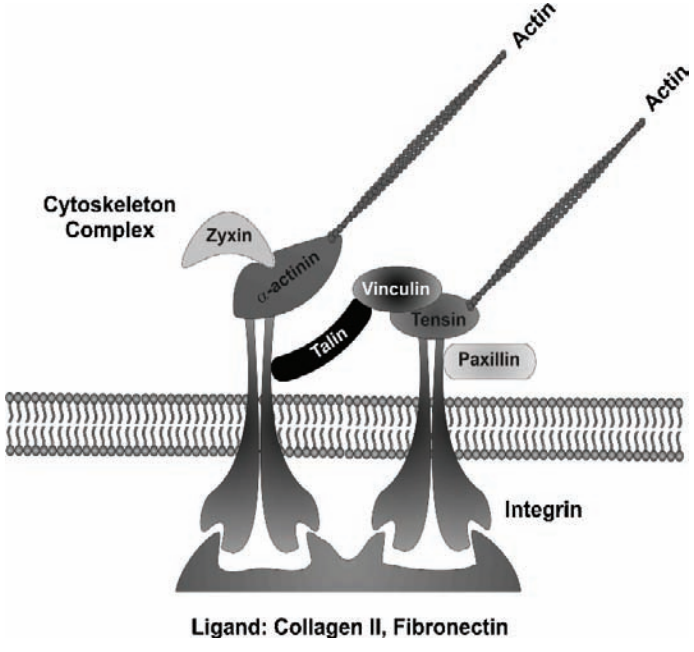

Fig. 6 Model of integrins attached to extracellular ligand and the cytoskeleton complex. The extracellular ligand binding site of integrin heterodimers is at the alpha–beta interface and binds to extracellular ligands (i.e. collagen II, fibronectin, laminin, vitronectin). The cytosolic domain of alpha-beta heterodimers binds cytoskeletal proteins. The integrin model illustrated in this figure shows two heterodimers attached to an extracellular ligand and to cytoskeletal associated proteins including paxillin, tensin, vinculin, talin, zyxin and α-actinin, which bind to actin filaments. This complex also has the ability to activate subcellular signalling proteins, such as FAK (focal adhesion kinase)

is only adopted on effector binding. Further, the two membrane-proximal helices contained in the β subunit mediate the link between the α and β subunits via a series of hydrophobic and electrostatic contacts.

1.4
EGF Domains in β1-Integrins

A sequence of approximately 30–40 amino-acid residues found in the sequence of epidermal growth factor (EGF) has been shown to be present, in a more or less conserved form, in the primary structure of integrins. The EGF-like domain motif is found in at least seven isoforms in the ITGB1 gene which encodes β1-integrin. The EGF motif is also present in a large number of β1-integrin ligands, mostly ECM-related proteins. The list of proteins currently known to contain one or more copies of an EGF-like pattern is large and highly varied[2]. An important common feature is that these repeats are found in the extracellular domain of membrane-bound proteins or in proteins known to be secreted (with certain exceptions). The EGF domain includes six cysteine residues which have been shown to be involved in the formation of disulphide bonds. The main structure is a two-stranded β sheet followed by a loop to a C-terminal short two-stranded sheet and subdomains of varying length between the conserved cysteines.

2
Integrins in Articular Cartilage

In the following sections, we primarily review our own work on the expression, tissue distribution and function of integrins in selected in vitro models of articular chondrocytes and limb bud mesenchymal cells.

2.1
Monolayer Cultures

2.1.1
Expression Pattern and Changes of Integrins on Chondrocytes in Monolayer Culture

The expression pattern of α1-, α3-, αv- and α5β1-integrins and their specific ligand binding were investigated in monolayer cultures of chondrocytes from 17-day-old mouse embryos using morphological and immunomorphological methods. After a 3-h culture period of chondrocytes from 17-day-old mouse embryos, numerous cells had already adhered in the form of a monolayer. After a 1-day culture period αv-, α3- and α5β1-integrins were observed on the chondrocytes. During the first 4 days, the number of the cells had increased, a matrix became perceptible. After

[2] See the Pfam protein domain database for more details: http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF07974

a 5-day culture period, the flat fibroblast-like cells, often of bipolar shape, increased in number at the expense of the chondrocytes. However, some fibroblast-like cells could always be demonstrated from the beginning of cultivation onwards. Using in vitro methods, we studied the expression of α1-, α3-, αv- and α5β1-integrins and their specific ligands in monolayer cultures of chondrocytes from 17-day-old mouse embryos (Shakibaei 1995) (Fig. 7). After 1-day in culture α3- (Fig. 7B), αv- and α5β1-integrins (not shown) were observed on limb bud-derived cells. Immuno-electron microscopy revealed integrin localisation to be predominantly in the contact areas of the cell surface with extracellular structures. α1-integrin was rarely detected (Fig. 7A). After a 5-day culture period, α3-integrin expression decreased (Fig. 7D) and was hardly recognisable on these cells by immunoelectron microscopy. On the other hand, the number of fibroblast-like cells with α1- (Fig. 7C), αv- and α5β1-integrins increased (not shown). Further, collagen type I and fibronectin could be demonstrated, as displayed ligands on the cell surface and the number of chondrocytes with collagen type II immunoreactivity continuously decreased (Fig. 8A–D). The α3-integrin combination is likely to be responsible for the binding of collagen type II, and α1-integrin may be involved in binding of collagen type I. This was the first study to demonstrate that dedifferentiation of chondrocytes to fibroblast-like cells in prolonged monolayer culture is accompanied by changes in integrin gene expression (Shakibaei 1995). Different mechanisms and reasons have been discussed for the dedifferentiation of chondrocytes in monolayer culture: (1) loosening of the interaction between chondrocytes and matrix compounds leading to dedifferentiation of

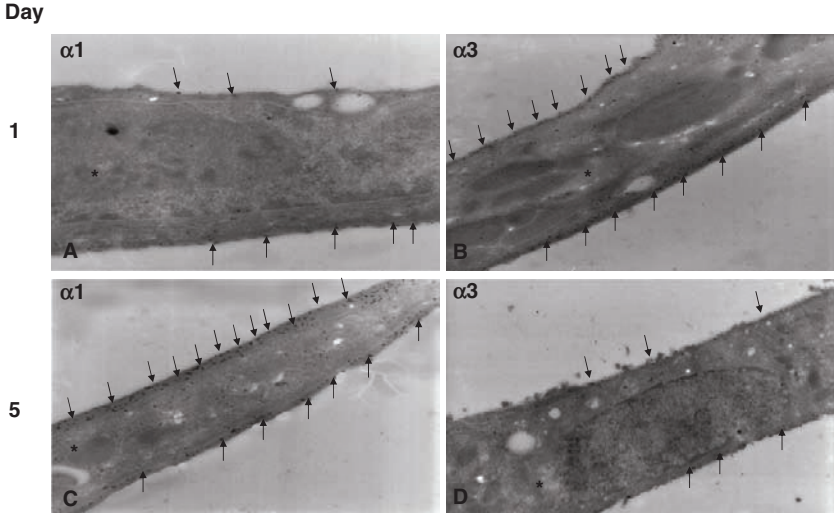

Fig. 7A–D Immuno-detection of integrins in chondrocytes in monolayer culture. **A, B** After 1–3 days in monolayer culture the chondrocytes (*) can be immuno-labelled with anti-α3-integrin (**B**), but not with anti-α1-integrin (**A**). **C, D** After 5 days in monolayer culture, only fibroblast-like cells are observed, which can be immuno-labelled with anti-α1-integrin (**C**), but not with anti-α3-integrin (**D**). ×15,000

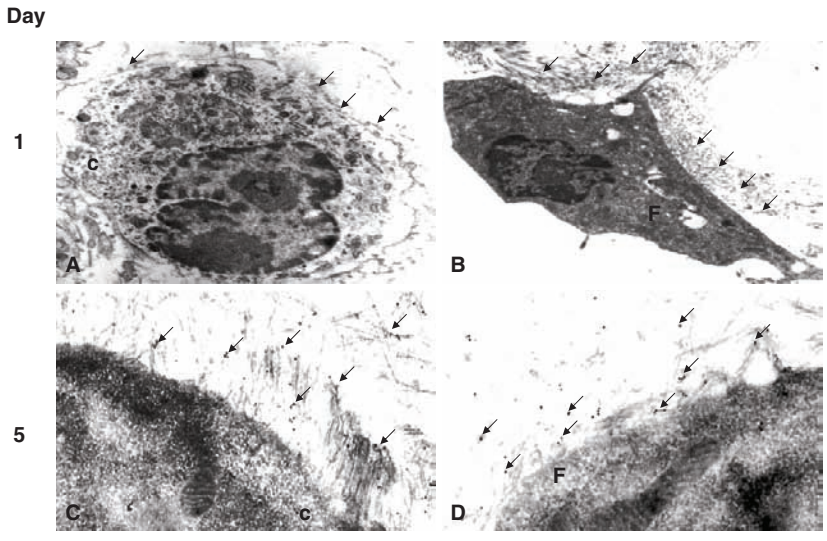

Fig. 8A–D Electron- and immuno-electron microscopic investigations of chondrocytes after 2 and 5 days in monolayer culture. After 2 days in monolayer culture the chondrocytes (*c*) exhibit a round to oval shape (**A**). A matrix can be observed on their surface (*arrows*) (**C**). Immuno-labelling against collagen type II (*arrows*) in the matrix shows that these are chondrocytes (*c*). After a 5-day culture period, increased fibroblast-like cells (*F*) with bipolar shape and long pseudopodia can be observed (**B**). They form a matrix with new collagenous fibrils, which is positively labelled with anti-collagen type I (*arrows*) (**D**). **A, B** ×10,000; **C, D** ×15,000

cells; (2) cell maturation; (3) ageing of cells and (4) contamination and overgrowth by fibroblasts in the culture. The behaviour and function of chondrocytes in long-term monolayer culture can thus be compared with the changes of chondrocytes in organoid culture in vitro (Shakibaei et al. 1993a). These methods also served to show which ligands preferentially bind to which integrins during this dedifferentiation in monolayer culture.

2.1.2 Integrin Expression and Collagen Type II are Implicated in the Maintenance of Chondrocyte Morphology in Monolayer Culture

It is well established that chondrocytes grown in monolayer culture at low density and, with the addition of serum, dedifferentiate after several days, changing their cell shape to fibroblast-like cells and reducing synthesis of collagen type II and cartilage-specific proteoglycans (von der Mark et al. 1977). Cell–matrix interactions play an essential role in the cartilage tissue. Chondrocyte development, differentiation, function, phenotype and survival are determined by the properties of the extracellular matrix (Kosher and Church 1975; Lash and Vasan 1978; von der Mark 1980; von der Mark et al. 1977). We wanted to establish a system to optimise the cultivation of chondrocytes

in monolayer culture and to slow down their dedifferentiation and apparent overgrowth by fibroblast-like cells. For this purpose, freshly isolated chondrocytes from 17-day-old mouse embryos were grown either on plastic or on collagen type II-coated cell culture flasks. Using this model, chondrocytes grown on plastic cell culture flasks changed their phenotype to fibroblast-like cells after 5 days in culture. On the other hand, cells grown on collagen type II maintained their spherical phenotype for more than 2 weeks in monolayer culture (Fig. 9). After a culture period of 17 days, the remaining chondrocytes had maintained their round to oval shape. They were embedded in a network of newly formed collagenous fibrils. The surface was therefore recognisable only in certain areas or after collagenase treatment. At the end of the 2nd week of the culture period, more and more cells detached from the substrate, leaving a network of fibrillar material, partly in the form of a pericellular capsule. In experiments without collagen type II, a mixed culture consisting of fibroblast-like cells and round chondrocytes was observed from the beginning onwards. Chondrocytes from 17-day-old mouse fetuses undoubtedly consist of a heterogeneous cell population.

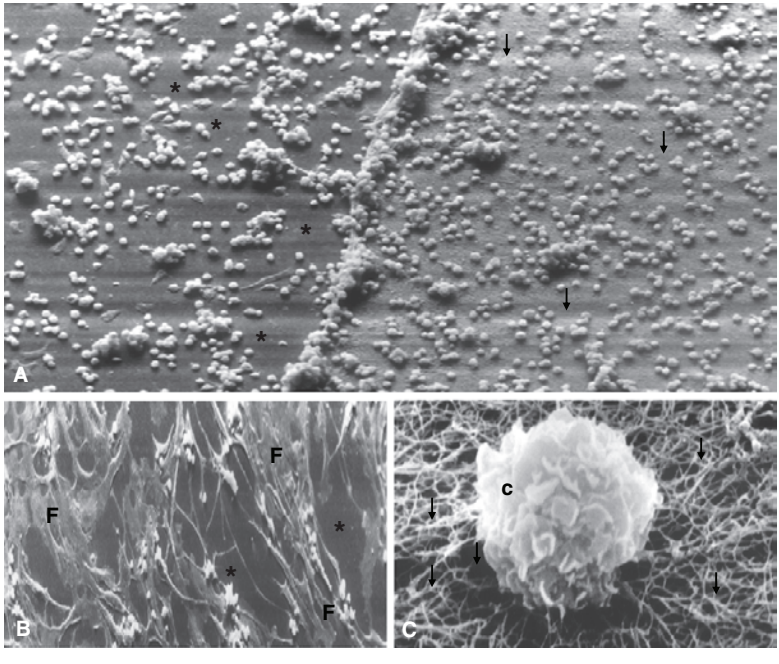

Fig. 9A–C Scanning electron micrographs of chondrocytes in monolayer culture. **A** Half of the glass plate was coated with collagen type II (*arrows*), the other half was uncoated (*). After a 1-day culture period on collagen type II, only round to oval chondrocytes could be seen. On the uncoated half, round chondrocytes as well as flat fibroblast-like cells adhered. ×200. **B** After 5 days in monolayer culture on the uncoated half, an overgrowth of fibroblast-like cells (*F*) was observed. ×400. **C** 5 days of culture on a collagen type II-coated substrate (*arrows*): the chondrocyte phenotype was maintained for the entire culture period. ×600

Fibroblast-like cells attached to the outside of the cartilage and perichondral cells are also certainly released following enzymatic treatment. The fibroblast-like cells, which increase in number in the absence of collagen coating, derive from this source. Hence, some of the fibroblast-like cells might have been formed in this way. It has to be considered that due to fibroblast-like cells other collagen types, proteoglycans, factors and matrix-degrading enzymes (e.g. metalloproteinases) get into the medium and additionally influence the behaviour or dedifferentiation of the chondrocytes. Hence, the absence of fibroblast-like cells on collagen coating after 10 days in vitro largely depends on the missing overgrowth and its possible consequences. Conversely, a positive effect of collagen type II on the stabilisation of chondrocyte phenotype and differentiation cannot be excluded, considering the better adhesion and higher proliferation rate.

Immunomorphological studies showed that chondrocytes grown on collagen type II, produced collagen type II, fibronectin and expressed β1-integrins on the plasma membrane from day 1 until the end of the culture period (Fig. 10). Further, treatment of these cultures with β1-integrin antibodies reduced chondrocyte adhesion on the collagen type II-coated surface by about 70% compared with control rabbit IgG, which indicates the involvement of β1-integrins in the adhesion of chondrocytes to these matrix components (Fig. 11). The data from this study suggest that collagen type II present in the surrounding of the chondrocytes influences their phenotype and that this interaction depends on the complement of β1-intrgins (Shakibaei et al. 1997). This is strong evidence that indicates a participation of integrins of the β1 group and their potential role in this adhesion process. It is most likely that the influence of collagen type II on cellular behaviour (differentiation, cell shape, secretion product) depends on the integrins to participate in intracellular signalling.

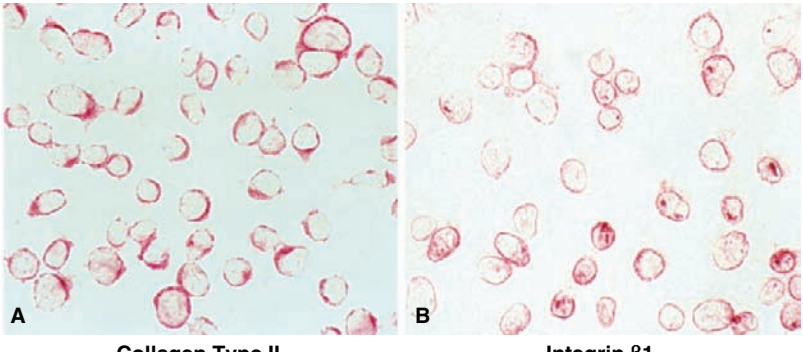

Fig. 10A, B Immuno-labelling of collagen type II and β1-integrin by the APAAP method. **A** The round to oval chondrocytes produced collagen type II, which was seen as a matrix around the cells for the entire culture period. **B** Immuno-labelling against β1-integrins: the integrins where observed on round to oval chondrocytes during the entire culture period. ×360

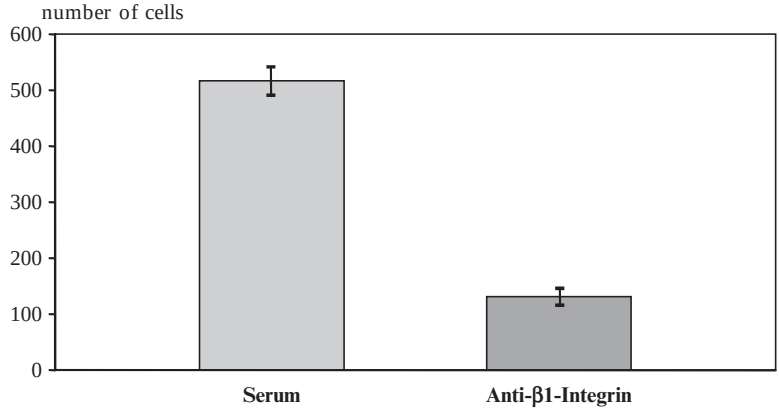

Fig. 11 Attachment inhibition assay of chondrocytes by integrin antibodies; control rabbit IgG. After a short treatment with collagenase (3 min), the cells were preincubated for 15 min with 100 µl/ml of rabbit IgG (control NS) and anti-β1-integrin prior to their addition to glass plates coated with collagen type II. The frequency of the number of adhered cells was evaluated by scoring 500 cells from 30 different microscopic fields in each situation. The graph highlights the number of adhered cells; mean values are shown with standard deviations (n=3 independent experiments)

The preferential adhesion of chondrocytes on collagen type II-coated substrates and the missing adhesion of fibroblast-like cells indicate a specific interaction. Some integrins of the β1 group are available on the surface of chondrocytes for a specific binding to extracellular matrix. This integrin pattern occurs in cartilage in vivo and in vitro. Consequently, any blockage of integrins of this group should fundamentally influence the adhesion behaviour. These findings speak for a participation of integrins of the β1 group in this adhesion process.

2.1.3 Signal Transduction by β1-Integrin Receptors in Chondrocytes In Vitro: Collaboration with IGF-IR

After the adhesion of chondrocytes in monolayer culture on dishes coated with collagen type I or on plastic, they dedifferentiate to fibroblast-like cells. They lose their polygonal phenotype and become a spindle-like shape that resembles that of fibroblasts. Under these conditions, they produce collagen type I instead of type II and fewer proteoglycans (Benya et al. 1978; Shakibaei 1995). Chondrocytes grown on collagen type II survive for a long period of time without changing their morphology or their secretion products (Shakibaei et al. 1997). The extracellular matrix proteins, such as collagens and vitronectin, are of great significance for the regulation of cell proliferation, differentiation, morphogenesis and function (Enomoto-Iwamoto et al. 1997; Gonzalez et al. 1993; Kosher and Church 1975; Ruoslahti and Reed 1994; von der Mark et al. 1977). The interaction between

chondrocytes and matrix proteins is mediated largely by the β1 subfamily of integrin receptors (Durr et al. 1993; Enomoto et al. 1993; Enomoto-Iwamoto et al. 1997; Loeser 1993; Shakibaei et al. 1997; Shakibaei et al. 1995; Woods et al. 1994). This interaction plays a crucial role in regulating several biological phenomena, including cell morphology, gene expression and cell survival, inducing signal transduction pathways that change cell behaviour (Giancotti and Mainiero 1994; Hungerford et al. 1996; Varner et al. 1995; Wary et al. 1996). The interaction between extracellular matrix components and integrins leading to the specific activation of intracellular signalling proteins has been studied in many cell types, such as fibroblasts and platelets, but not yet in chondrocytes. The goal of this study was to investigate the hypothesis that the integrin-dependent intracellular response modulated by IGF-I in chondrocytes requires the ligation of integrins to specific cartilage matrix proteins (collagen type II).

Integrin expression can be regulated by growth factors, including IGF-I and TGF-β (Loeser 2000). Tissue engineering studies have shown that growth factors (e.g. IGF-I, TGF-β) entrapped in synthetic ECM scaffolds and released under specific conditions affect chondrocyte behaviour and phenotype (van der Kraan et al. 2002). The presence of growth factors such as IGF-1, TGF-β and BMP-7 induce various signalling pathways that aid in transducing phenotypically genuine gene expression by chondrocytes (Yoon and Fisher 2006). In our own studies, we examined the mechanisms by which collagen-binding integrins co-operate with insulin-like growth factor-I (IGF-I) receptors (IGF-IR) to regulate the chondrocyte phenotype and control differentiation (Shakibaei et al. 1999). Adhesion of chondrocytes to anti-β1-integrin antibodies or collagen type II leads to the phosphorylation of proteins with apparent molecular weights of 200 kDa, 190 kDa, 125 kDa, 100 kDa and 80–40 kDa (Fig. 12). Furthermore, adhesion of IGF-I-treated chondrocytes to collagen type II or anti-β1-integrin antibodies revealed in tyrosine phosphorylation of almost the same set of proteins, but was significantly higher than of those cultivated on collagen type II not treated with IGF-I. No significant tyrosine phosphorylation of proteins could be shown in chondrocytes plated on collagen type I or on poly-L-lysine or coated with anti-major histocompatibility complex (MHC) with or without exposure to IGF-I, suggesting that the phosphorylation of signalling proteins was induced by binding to specific matrix components (Fig. 12). After immunoprecipitation with anti-β1-integrin antibodies, the samples were probed by immunoblotting with anti-FAK. The results indicate that β1-integrins interact with FAK, in chondrocytes adhering to collagen type II (Fig. 13). The results showed a marked time-dependent increase in expression of FAK in chondrocytes cultured on collagen type II and treated with IGF-I. Adhesion of chondrocytes to collagen type II leads to binding of β1-integrin and cytoskeletal and signalling proteins localised at focal adhesions; these proteins include α-actinin, vinculin, paxillin and focal adhesion kinase (FAK). These then stimulate the recruitment of docking proteins such as Shc (Src-homology collagen). Exposure of collagen type II-cultured chondrocytes to IGF-I leads to interaction of Shc with the IGF-IR and with β1 (Fig. 14), α1- and α5-integrins, but not with α3-integrin, as we could show with a series of co-immunoprecipitation experiments. Shc then

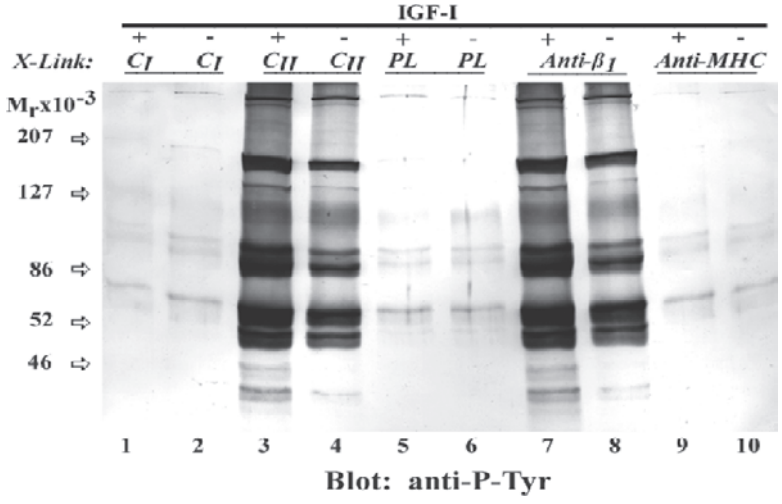

Fig. 12 Tyrosine phosphorylation induced by interaction with extracellular matrix components and integrins. Serum-starved human chondrocytes were plated on dishes coated with collagen types I (CI), II (CII), poly-L-lysine (*PL*), anti-β1-integrin and anti-MHC antibodies, then either treated with IGF-I (100 ng/ml) or left untreated in serum-free medium for 1 h. Equal amounts of total proteins were separated by 7.5% SDS-PAGE and analysed by Western blotting with anti-P-tyrosine (*anti-P-Tyr*) antibody

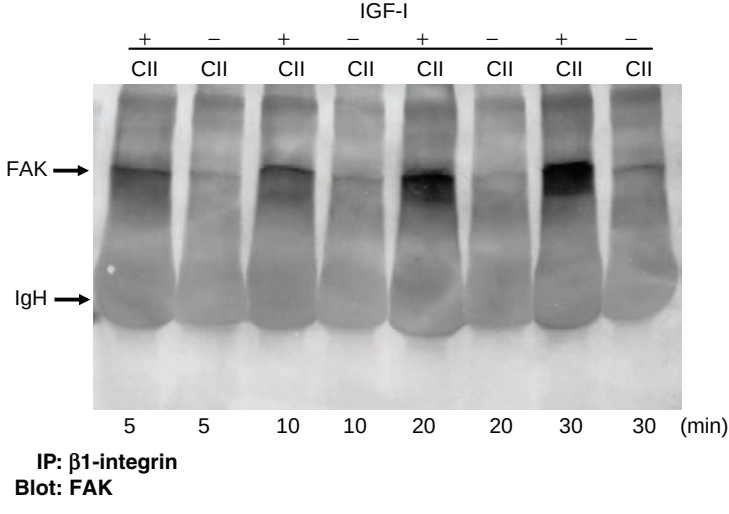

Fig. 13 The cytoplasmic domain of β1-integrin interacts with intracellular signalling proteins. Serum-starved human chondrocytes were plated on dishes coated with collagen type II (CII) then either treated with IGF-I (100 ng/ml) or left untreated for 5, 10, 20 and 30 min. Bound chondrocytes were lysed and immunoprecipitated with anti-β1 cytoplasmic domain (β1) antibody and for the control with normal IgG-serum. Immunoprecipitates were separated by 7.5% SDS-PAGE and analysed by immunoblotting with anti-FAK (focal adhesion kinase). *IgH*, immunoglobulin heavy chain

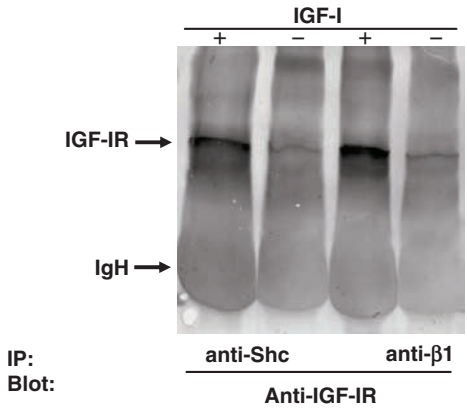

Fig. 14 The IGF-IR binds to the adaptor protein Shc as well as to integrin receptors in chondrocytes. Serum-starved human chondrocytes were plated onto dishes coated with collagen type II and then treated with IGF-I (100 ng/ml) or left untreated for 1 h. Equal amounts of total proteins were lysed and immunoprecipitated with anti-Shc and anti-β1-integrin antibodies. Immunoprecipitates were separated by 7.5% SDS-PAGE and analysed by Western blotting with anti-IGF-IR antibodies. *IgH*, immunoglobulin heavy chain

associates with growth factor receptor-bound protein 2 (Grb2), an adaptor protein and extracellular signal-regulated kinase. The expression of the docking protein Shc occurs only when chondrocytes are bound to collagen type II or integrin antibodies and increases when IGF-I is added, suggesting a collaboration between integrins and growth factors in a common or shared biochemical signalling pathway. Furthermore, these results indicate that focal adhesion assembly may facilitate signalling via Shc, a potential common target for signal integration between integrin and growth-factor signalling regulatory pathways. Thus, the collagen-binding integrins and the IGF-IR co-operate to regulate focal adhesion components and these signalling pathways have common targets (Shc-Grb2 complex) in subcellular compartments, thereby linking to the Ras-mitogen-activated protein kinase signalling pathway (Fig. 15). Indeed, the synergistic interaction between integrins and growth factor receptors phosphorylates MAP kinases, which control a variety of cell functions, and this collaboration is only possible if the integrins are already aggregated and occupied by a ligand (Fig. 16) (Shakibaei et al. 1999). It is tempting to hypothesise that the integrins mediate collagen type II-induced differentiation and adhesion of chondrocytes by interacting with the growth factor that stimulates chondrocyte adhesion and activates IGF-IR, leading to the intracellular signalling required for the stabilisation the chondrocyte phenotype. Furthermore, all these observations support the hypothesis that focal adhesion assembly may facilitate signalling via Shc, a common point for signal integration and potentiation between integrin- and growth factor signalling pathways in chondrocytes. The collaborative effect of integrins on IGF-IR signalling pathway in chondrocytes may be one of the

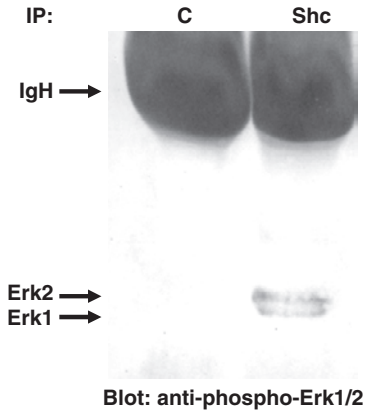

Fig. 15 Association of Shc proteins with activated Erk1/2 in chondrocytes. Serum-starved human chondrocytes were plated onto dishes coated with collagen type II and then treated with IGF-I (100 ng/ml) or left untreated for 1 h. Equal amounts of total proteins were lysed and immunoprecipitated using anti-Shc antibody and for the control normal IgG-serum (*C*). Immunoprecipitates were separated by 10% SDS-PAGE and analysed by immunoblotting with anti-activated Erk1/2 antibodies. *IgH*, immunoglobulin heavy chain

major molecular mechanisms controlling cytoskeletal organisation, cell differentiation and cell survival.

The Ras-MAPK pathway regulates such essential cellular functions as growth and differentiation through the phosphorylation and consequent activation of a cascade of proteins. Ras is a membrane-anchored intracellular GTP binding protein that on binding of its transcellular receptor tyrosine kinase (RTK) activates the ser/thr kinase Raf. Raf in turn phosphorylates MEK, a mitogen-activated protein kinase kinase (MAPKK). The activated MAP kinases (also known as extracellular signal-related kinases [Erk]) migrate from the cytosol to the nucleus where they regulate the activity of transcription factors such as *jun*, c-*fos* and c-*myc* (Lenormand et al. 1993b). These transcription factors regulate the transcription of genes and thus orchestrate various cellular responses. One of the transcription factors phosphorylated as a result of Ras-MAPK activation is *jun*, which may play a role in the regulation of anoikis, since the *jun*-N-terminal kinase (JNK) pathway is activated in and promotes apoptosis in response to the loss of cell-matrix contact (Cardone et al. 1997).

2.1.4 Inhibition of MAPK Pathway Induces Chondrocyte Apoptosis

As stated above, the mitogen-activated protein kinase (MAPK) pathway is stimulated by adhesion of human chondrocytes to anti-β1-integrin antibodies or collagen type II in vitro. These mechanisms most likely prevent chondrocyte dedifferentiation

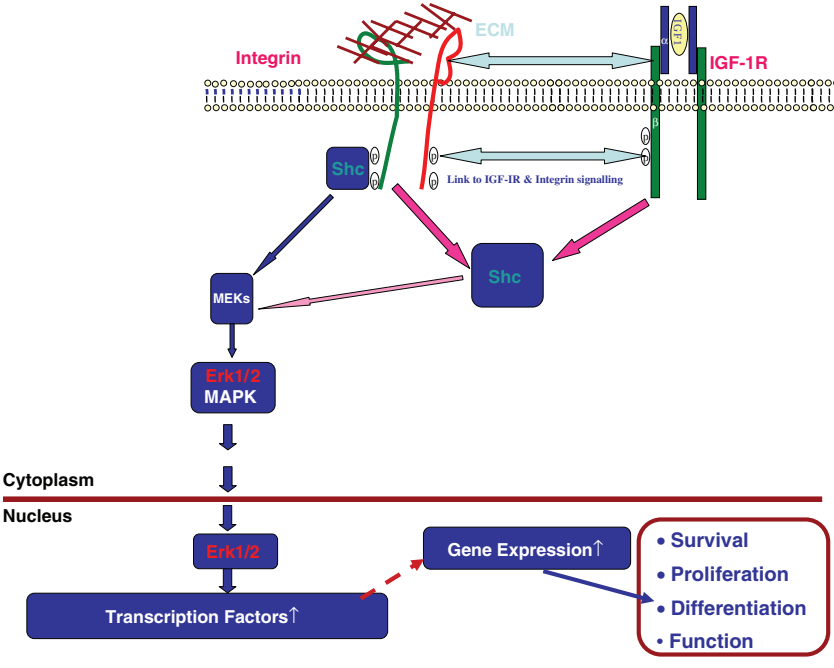

Fig. 16 Working model of IGF-I-induced stability of the chondrogenic potential in human articular chondrocytes in vitro. Binding of extracellular matrix (*ECM*) to its receptor (integrins) and binding of IGF-I to IGF-IR activates and binds the integrins to the IGF-IR. This complex associates with the docking protein Shc. The intensity of association is increased when IGF-I is added. Shc protein binds either to the IGF-IR or distinct integrin receptors, suggesting a collaboration between integrins and growth factors in a common/shared biochemical signalling pathway. This collaboration is only possible if the integrins are already aggregated and occupied by a ligand. Shc then associates with Grb2 and with phospho-Erk, leading to an activation of the Ras-mitogen-activated protein kinase signalling pathway, which controls a variety of cell functions

to fibroblast-like cells and chondrocyte death. Apoptosis, or programmed cell death, plays a key role in embryogenesis, immunological competence and tissue homeostasis for cell removal and can be distinguished biochemically and morphologically from cell necrosis, which is a passive, energy-independent form of cell death. Chondrocyte degradation and death occurs in enchondral ossification as well as in age-associated arthropathies such as osteoarthritis. Chondrocyte apoptosis, can be induced in vitro by a variety of agents, such as nitric oxide, oxygen radical scavengers, tumor necrosis factor and interleukin-1β.

As stated above, we showed, by adhesion of human chondrocytes to anti-β1-integrin antibodies or to collagen type II in vitro, that the mitogen-activated

protein kinase (MAPK) pathway is stimulated by β1-integrins (Shakibaei et al. 1999). This mechanism most likely prevents chondrocyte dedifferentiation to fibroblast-like cells and chondrocyte apoptosis. It is important to know the apoptosis pathways of chondrocytes because inhibition of chondrocyte apoptosis may be of therapeutic value after cartilage injury and in arthritis. Apoptosis is also important in physiological conditions such as chondrogenesis and during enchondral ossification. To investigate whether the mitogen-activated protein kinase (MAPK) pathway downstream of the β1-integrin receptor plays an essential role in differentiation and survival of the chondrocytes, we blocked the mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) (MEK), a kinase upstream of the kinase Erk by using the pharmacological drug U0126. Exposure of chondrocytes to U0126 caused a cellular morphology typical of apoptosis in chondrocytes, with nuclear changes including chromatin condensation into dense areas along the nuclear membrane and nuclear fragmentation. The cellular membrane was irregular (bleb formation) and cytoplasmic vacuoles (dilatation of mitochondria and endoplasmic reticulum) could be seen (Fig. 17). In addition, this exposure caused activation of caspase-3, a member of the aspartate-specific cysteine proteases and an enzyme that plays a key role in apoptosis, in a dose-dependent manner. Western blot analysis with an antibody specific for dually phosphorylated Erk showed that collagen type II induced phosphorylation of Erk1/2 was specifically blocked by

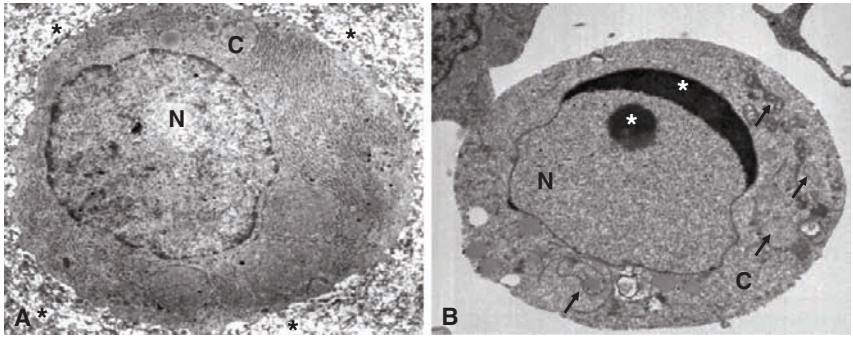

Fig. 17A, B Electron micrographs of chondrocytes. **A** Electron microscopic investigation of an untreated vital chondrocyte (*C*) exhibiting a smooth surface, a large nucleus (*N*) with much loosely packed and functionally active euchromatin and little dense, functionally inactive heterochromatin and numerous cavities of rough endoplasmic reticulum. The chondrocyte is surrounded by a pericellular matrix sheath closely attached to the cell membrane. ×10,000. **B** After treatment with the MAPK inhibitor U0126 for 1 h, a representative apoptotic chondrocyte has nuclear changes with peripheral segregation and aggregation of chromatin into dense areas (*) along the nuclear membrane, swellings and dilatations of cell organelles (mitochondria and endoplasmic reticulum) (*arrows*), and bleb formation at the cell membrane. ×10,000

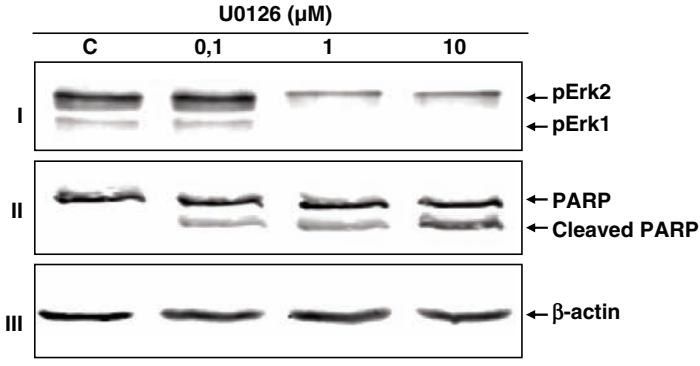

Fig. 18 Inhibition of Erk1/2 activity and cleavage of PARP after treatment with U0126. Serum-starved human chondrocytes were plated on dishes coated with collagen type II and then treated with various concentrations of U0126: 0 μM, 0.1 μM, 1 μM and 10 μM for 1 h. The cells were immuno-labelled with anti-phospho Erk1/2 (I) and anti-PARP (II). The results showed a distinctive and significant dose-dependent decrease of phosphorylated Erk1/2 and cleaved PARP. Expression of the housekeeping protein β-actin (III) was not affected by co-treatment with U0126. *C*, control

U0126 in a dose-dependent manner (Fig. 18). In U0126-treated chondrocytes, the cleavage of 116-kDa poly (ADP-ribose) polymerase (PARP), an enzyme assisting in the repair of DNA strands, resulted in the 85-kDa apoptosis-related cleavage fragment and was associated with caspase-3 activity (Fig. 18). Immunohistochemical analysis showed that U0126 treated chondrocytes were caspase-3 positive (not shown). These results indicate that the MEK/Erk signal transduction pathway is downstream of the β1-integrin receptor and that it is involved in the maintenance of chondrocyte differentiation and survival (Fig. 19) (Shakibaei et al. 2001). These observations led to further studies of the role of the mitogen-activated protein kinase pathways in chondrocytes.

2.1.5 Expression of the VEGF Receptor-3 in Osteoarthritic Chondrocytes and Association with β1-Integrins

Increased angiogenesis is known to occur in rheumatoid arthritis and osteoarthritis (Giatromanolaki et al. 2001; Fearon et al. 2003). The occurrence of pro-angiogenic growth factors such as VEGF (Pufe et al. 2001a, b, c), hepatocyte growth factor (Pfander et al. 1999)and fibroblast growth factor (Kusafuka et al. 2002) together with endocrine hormones such as leptin (Kume et al. 2002) may lead to the invasion of blood vessels from the subchondral bone or cartilage-covered pannus tissue into the cartilage (Afuwape et al. 2002). The expression

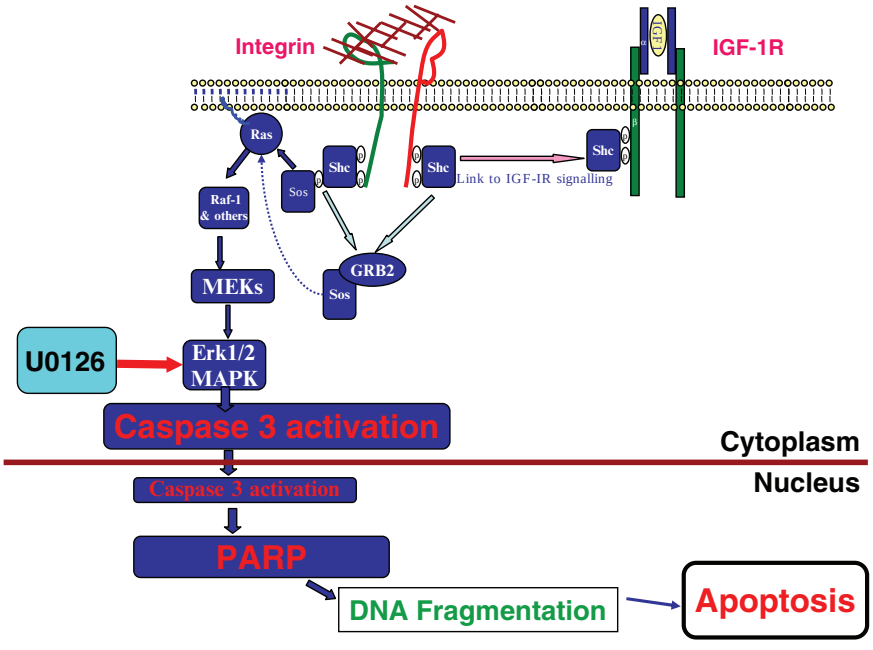

Fig. 19 Working model of how the inhibition of the mitogen-activated protein kinase kinase induces apoptosis of human chondrocytes. This model addresses the important role of the Ras-MAP kinase signalling pathway (MAPK pathway) in the stimulation of human chondrocyte differentiation. Induced signals by integrins or transmembrane G proteins lead to the activation of the Ras-MAP Kinase signalling pathway. Activation of the MAPK pathway may be a mechanism by which integrins regulate gene expression. In previous studies, we showed that activation of the MAPK pathway regulates the activity of a number of intracellular signalling proteins through phosphorylation. Erk1/Erk2, a downstream kinase of the MAPK pathway regulates the expression of various transcription factors. Specific inhibition of Erk1/Erk2 results in cleavage of PARP in human chondrocytes in vitro. Cleavage of caspase-3 and PARP are common features of apoptosis, therefore the specific inhibition of the Ras-mitogen activated kinase leads to apoptosis

of two VEGF receptors (VEGFR-1, VEGFR-2) has been shown recently in cartilage (Enomoto et al. 2003). The co-expression of the VEGF receptor VEGFR-2 and VEGF in chondrocytes indicates that VEGF activates chondrocytes in an autocrine or paracrine manner (Pufe et al. 2001a, b, c). On the other hand, ligands for VEGFR could penetrate from pannus tissue or synovial fluid into cartilage matrix and activate chondrocytes in conjunction with existing pro-inflammatory cytokines (i.e. by elevating glucose uptake and utilisation and stimulating catabolic activities; (Mobasheri et al. 2002b, 2002c; Richardson et al. 2003). In addition, it has been shown in endothelial cells, that the interaction of VEGFR-3 with VEGF modulates

the activity of VEGFR-2 (Matsumura et al. 2003). Whether a similar cross-talk modulates the sensitivity of osteoarthritic chondrocytes to VEGFR-2 requires further investigation. Thus far, little is known about the occurrence of VEGFR-3 and its real ligands, VEGF-C or -D, in cartilage. Since VEGF-C was detected in the synovial membrane of osteoarthritic joint samples (Paavonen et al. 2002; Wauke et al. 2002), one may speculate that VEGF-C reaches the synovial fluid and penetrates into the cartilage. In a recent study, we have demonstrated, for the first time, VEGFR-3, the receptor for VEGF-C in arthritic cartilage samples. Furthermore, we found that expression was stimulated in vitro by IL-1β, a pro-inflammatory cytokine that plays a key role in pathogenesis of arthritis.

Continuing with the theme of co-localisation, we set out to determine if enhanced expression of vascular endothelial growth factor and vascular endothelial growth factor receptor (VEGFR)-1 and -2 in chondrocytes of osteoarthritic cartilage are accompanied by changes in integrin expression. We initially demonstrated that VEGFR-3 (FLT-4) is expressed in chondrocytes by immunohistochemistry and immuno-electron microscopy. We then looked for a possible functional co-localisation of VEGFR-3 with β1-integrin; by using an established co-immunoprecipitation assay, we found a functional complex between the β1-integrin and VEGFR-3 in IL-1β-stimulated chondrocytes, indicating that activated VEGFR-3 may interact with β1-integrin and associated subcellular pathways in osteoarthritic chondrocytes (Fig. 20). Given that β1-integrins are also associated with other surface receptors and proteins in chondrocytes that mediate catabolic cartilage destruction in arthritis (i.e. the urokinase-type plasminogen activator receptor and matrix metalloproteinases) (Schulze-Tanzil

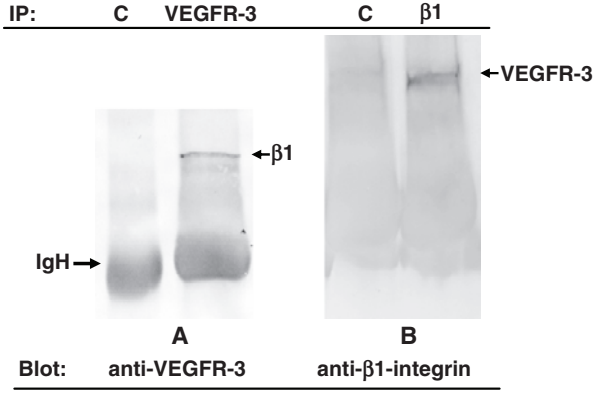

Fig. 20A, B Co-immunoprecipitation assays and detection of VEGFR-3 and β1-integrin. **A** Immunoprecipitation with anti-β1-integrin antibodies or normal IgG serum (*C*) followed by immunoblotting with VEGFR-3 antibodies. **B** Immunoprecipitation with anti-VEGFR-3 antibodies or normal IgG serum (*C*) followed by immunoblotting with anti-β1-integrin antibodies. *C*, samples precipitated with control antibodies; *IgH*, immunoglobulin heavy chain

et al. 2001; Schwab et al. 2004), we hypothesise that signal transduction by these receptor complexes via β1-integrins may play a crucial role in pathogenesis of osteoarticular disorders such as osteoarthritis (Shakibaei et al. 2003).

2.1.6 Effects of Curcumin on IL-1β-Induced Inhibition of Collagen Type II, β1-Integrin Synthesis and Activation of Caspase-3 in Human Chondrocytes In Vitro

The pathogenesis of osteoarthritis is dominated by a cascade of pro-inflammatory cytokines governed by the cytokines TNF-α and IL-1β (Blanco et al. 1999). Besides enhancing the production of catabolic enzymes such as matrix metalloproteinases (MMPs) that lead to cartilage matrix destruction and cartilage loss, these pro-inflammatory cytokines also effectively induce apoptosis in articular chondrocytes (Heraud et al. 2000; Aizawa et al. 2001). Apoptosis is known to play a significant role in the pathogenesis of osteoarthritis (Blanco et al. 1998; Kim et al. 2000; Aigner and Kim 2002).

In previous studies, we and others have shown that the dietary phytochemical curcumin, inhibited several pro-inflammatory and catabolic effects of IL-1β and other cytokines in articular chondrocytes. Curcumin inhibited MMP production, COX-2 secretion and also prevented inhibition of cartilage-specific matrix production by IL-1β (Li et al. 2001; Liacini et al. 2002; Schulze-Tanzil et al. 2004; Sylvester et al. 2004). Curcumin (diferuloylmethane) is a component of the rhizomes of *Curcuma domestica* which derives from *Curcuma longa*. Curcumin has been proposed as a naturally occurring chemotherapeutic agent for cancer therapy since it reduces tumour cell survival, tumour expansion, and secondary inflammation via inhibition of the ubiquitous transcription factor nuclear factor-κB (NF-κB) (Mukhopadhyay et al. 2001; Bharti et al. 2003).

Here we summarise some of the data from our studies on the effects of curcumin on human chondrocytes. Positive labelling of collagen type II was visible around chondrocytes in control cultures. Stimulation with 10 ng/ml IL-1β led to decreased collagen type II immunolabelling around chondrocytes in a time-dependent manner. Co-treatment with curcumin appeared to increase collagen type II production (Fig. 21). Transmission electron microscopic investigation of monolayer chondrocytes revealed a flattened shape with small cytoplasmic processes, a large mostly euchromatic nucleus with nucleoli and a well-structured cytoplasm. Treatment of chondrocytes with IL-1β resulted in degenerative changes such as swelling of rER and clustering of swollen mitochondria and other cellular organelles. After longer incubation periods (30 min), more severe features of cellular degeneration were observed in response to IL-1β treatment such as areas of condensed heterochromatin in the cell nuclei. The flattened monolayer chondrocytes became more and more rounded and lost their microvilli-like processes. Chondrocytes that were pretreated with IL-1β and then co-treated with IL-1β and 50 μM curcumin showed less severe cellular degeneration at the ultrastructural level as early as 5 min after co-treatment. In the presence of curcumin (for 15–30 min), the morphological degenerative features of chondrocytes almost disappeared (Fig. 22). Chondrocytes were

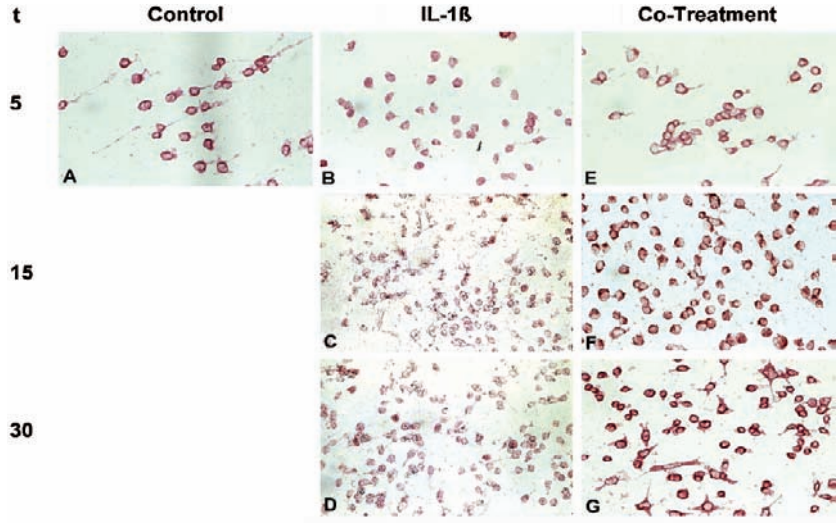

Fig. 21A–G Effect of curcumin on IL-1β-induced inhibition of collagen type II production as revealed by APAAP. Serum-starved human articular chondrocytes were either untreated (control, **A**) or exposed to 10 ng/ml IL-1β alone for 5, 15, 30 min or prestimulated for 30 min with 10 ng/ml IL-1β before being co-treated with IL-1β and 50 μM of curcumin for 5, 15, 30 min and labelled using the APAAP technique. In response to IL-1β, a decrease in collagen type II synthesis was observed (**B–D**), which was reversed in a time-dependent manner by co-treatment with curcumin (**E–G**). **A–G**: ×160. (Reproduced from Mobasheri et al. 2007 with copyright permission from Elsevier Science)

immunolabelled with anti-β1-integrin antibodies and gold coupled secondary antibodies for subsequent investigation by electron microscopy. IL-1β alone led to decrease in β1-integrin complement of chondrocyte cell membrane (Fig. 23B–D) compared to the untreated control cultures (Fig. 23A). In response to co-treatment with IL-1β and curcumin, an increase in cell surface labelling for β1-integrins was evident, suggestive of a modest stimulatory effect of curcumin on β1-integrin synthesis in chondrocytes (Fig. 23E–G). As early as 5 min after treatment with IL-1β, small distinct caspase-3-positive areas appeared at the margin of the chondrocyte nuclei. After 30 min, many areas positive for activated caspase-3 were present in the cell nuclei indicating the onset of early apoptosis. In chondrocytes prestimulated with IL-1β for 30 min, the labelling for activated caspase-3 decreased by curcumin co-treatment in a time-dependent manner. After 30 min of co-treatment with 50 μM curcumin and 10 ng/ml IL-1β, the IL-1β-induced labelling for activated caspase-3 almost disappeared, suggesting reversibility of caspase-3 activation at these stages of cytokine stimulation (Fig. 24A–G). The inhibition of collagen type II and β1-integrin synthesis by IL-1β was reversed by co-treatment with 50 μM curcumin and IL-1β (Fig. 25).

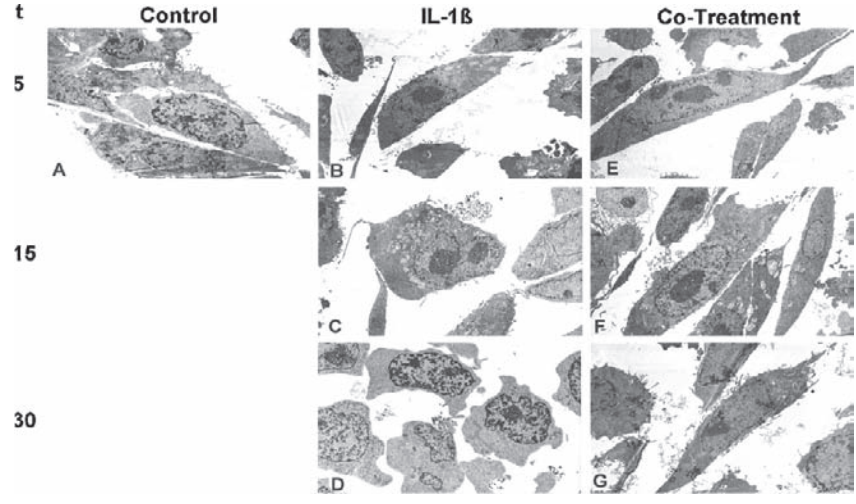

Fig. 22A–G Ultrastructural changes induced by IL-1β in chondrocytes were reversed by curcumin. Serum-starved human articular chondrocytes were either untreated or exposed to 10 ng/ml IL-1β alone for 5, 15, 30 min or prestimulated for 30 min with 10 ng/ml IL-1β before being co-treated with IL-1β and 50 μM of curcumin for 5, 15, 30 min and investigated using the transmission electron microscope technique. Chondrocytes treated with 10 ng/ml IL-1β exhibited characteristic features of degeneration such as annular chromatin condensation at the nuclear envelope of chondrocytes, swelling of mitochondria and rER in a time-dependent manner (**B–D**). Chondrocytes that were pretreated with IL-1β and then co-treated with IL-1β and 50 μM curcumin showed less severe cell degeneration at the ultrastructural level in a time-dependent manner (**E–G**). In control cultures, no ultrastructural changes were observed (**A**). **A–G**: ×5,000. (Reproduced from Mobasheri et al. 2007 with copyright permission from Elsevier Science)

Curcumin has been shown as a potent inhibitor of the NF-κB pathway (Bharti et al. 2003). Additionally, curcumin also antagonised the AP-1 and the c-Jun N-terminal kinase signalling pathways (Chen and Tan 1998).

Cytokines such as IL-1β and TNF-α induce nuclear translocation of NF-κB. NF-κB integrates several signalling pathways. Many pro-inflammatory effects of IL-1β and TNF-α in arthritis are regulated by activated NF-κB. NF-κB is a heterodimer consisting of two subunits. The most common subunits, which have also been demonstrated in chondrocytes, are the p65 and p50 subunits. These molecules are trapped in the cytoplasm as an inactive complex by association with an inhibiting IκBα subunit. In response to phosphorylation, IκBα dissociates from the complex and the p65 and p50 subunits are free to translocate to the cell nucleus activating gene expression by binding to DNA-NF-κB recognition sites in the promotors of various NF-κB-regulated genes. NF-κB is known to be involved in the regulation of a wide array of genes, among them those regulating cell cycle and survival (Kumar et al. 2004). NF-κB has also been shown in mitochondria, suggesting that it may be involved in regulation of apoptosis (Cogswell et al. 2003), since these cell organelles play a regulatory role in apoptosis (Green and Reed 1998; Loeffler and Kroemer 2000).

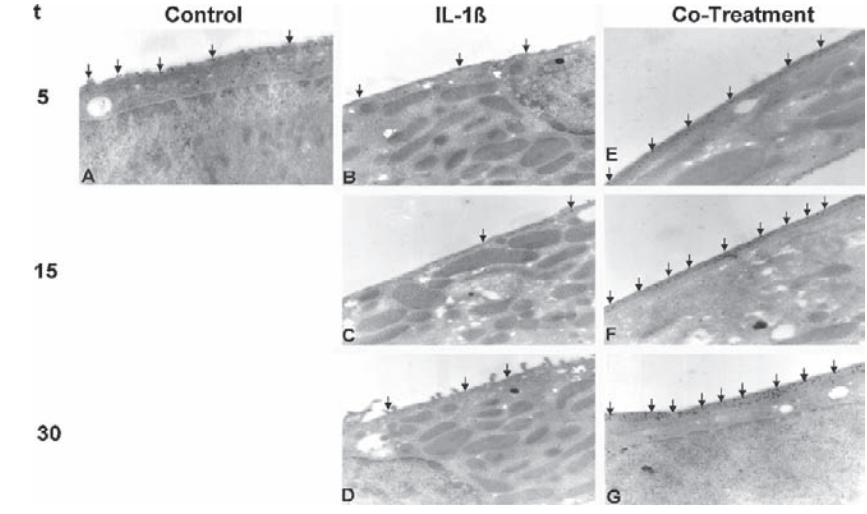

Fig. 23A–G Immunoelectron microscopic investigation of the effects of IL-1β and curcumin on β1-integrin expression in chondrocytes. Chondrocytes either treated with 10 ng/ml IL-1β or prestimulated with 10 ng/ml IL-1β before being co-treated with IL-1β and curcumin or not treated were immunolabelled with anti-β1-integrin antibodies and gold-coupled secondary antibodies. 10 ng/ml IL-1β alone resulted in decreased β1-integrin labelling (**B–D**). In response to curcumin co-treatment, an increase in cell surface labelling for β1-integrin was evident (**E–G**). **A–G**: ×10,000. (Reproduced from Mobasheri et al. 2007 with copyright permission from Elsevier Science)

The role of NF-κB in chondrocyte apoptosis induced by pro-inflammatory cytokines has not been investigated yet. In other cell types, NF-κB has been implicated to play a regulatory role in apoptosis (Barkett and Gilmore 1999). In immortalised cancer cells, curcumin has been shown to promote apoptosis, but in other cell types curcumin exhibited anti-apoptotic properties (Aggarwal 2000a, b). Maintenance of cartilage-specific matrix components is the prerequisite for chondrocyte survival (Yang et al. 1997). β1-integrins are transmembrane cell matrix signal transduction receptors on chondrocytes' surface, mediating the essential cell matrix interaction of them (Cao and Yang 1999). One important signal transduction pathway activated by these integrin receptors is the MAPKinase pathway (Schulze-Tanzil et al. 2001; Shakibaei et al. 1999). Particularly, β1-integrins regulate the interaction between chondrocytes and the pericellular collagen type II network (Cao and Yang 1999). Disruption of cell matrix communication by inhibition of the MAPKinase pathway has been shown to lead to chondrocyte apoptosis evident by caspase-3 activation and cleavage of the poly(ADP)ribose polymerase (Shakibaei et al. 2001). In most cell types, the apoptotic program is executed by a family of cysteine proteases called caspases. Activation of caspases such as caspase-3 plays a pivotal role in apoptosis. Caspases are expressed as pro-enzymes. After processing by proteolytic cleavage into the active form, caspase-3 forms heterotetramers (Cohen 1997; Thornberry and Lazebnik 1998).

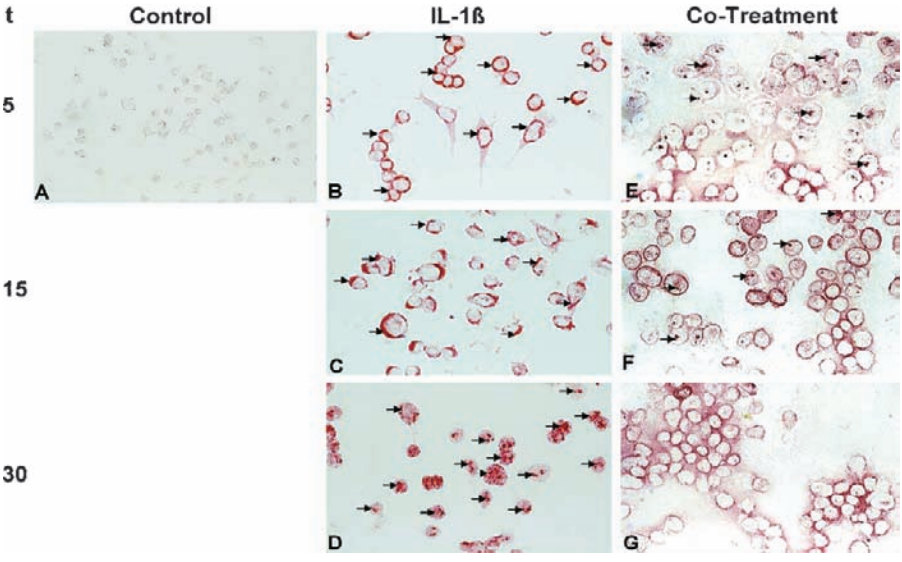

Fig. 24A–G Curcumin antagonised IL-1β-induced caspase-3 activation as revealed by APAAP. Serum-starved human articular chondrocytes were treated as described above. In response to IL-1β stimulation, activation of caspase-3 was evident in a time-dependent manner in chondrocyte nuclei (**B–D**). Co-treatment with curcumin led to decreased labelling for activated caspase-3 in a time-dependent manner (**E–G**). In control cultures, no caspase-3 activation was evident (**A**). **A–G**: ×160. (Reproduced from Mobasheri et al. 2007 with copyright permission from Elsevier Science)

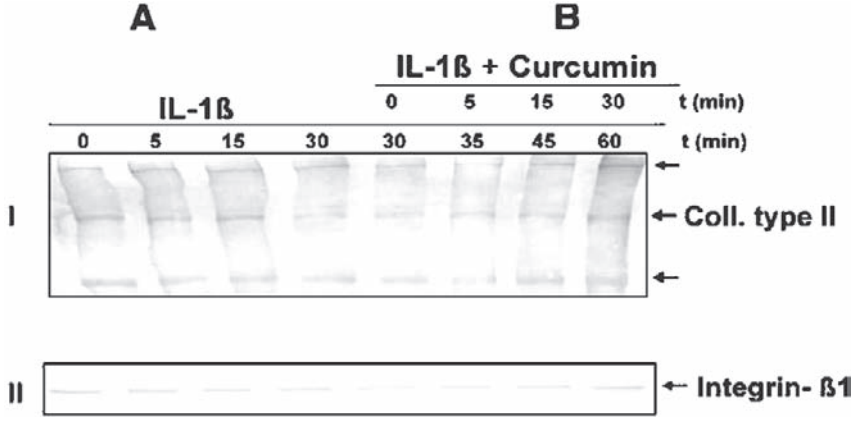

Fig. 25A, B Effect of curcumin on IL-1β-induced suppression of cartilage-specific collagen type II production and β1-integrin synthesis as revealed by Western blot analysis. To evaluate collagen type II and β1-integrin synthesis in response to IL-1β and curcumin, semi-quantitative Western blot analysis was performed. IL-1β led to inhibition of collagen type II and β1-integrin synthesis on the cell surface in a time-dependent manner, a process which was reversed by treatment with 50 μM curcumin. (Reproduced from Mobasheri et al. 2007 with copyright permission from Elsevier Science)

Curcumin inhibits IL-1β-induced caspase-3 activation and matrix-production in chondrocytes, suggesting that this naturally occurring chemotherapeutic and anti-inflammatory agent may reduce, block or reverse cartilage breakdown in degenerative joint diseases such as osteoarthritis. Furthermore, it is well known that curcumin has antioxidant properties (Aggarwal et al. 2003, 2007; Khanna et al. 2007). Proinflammatory cytokines increase reactive oxygen species (ROS) (Shakibaei et al. 2005b), which may then function as additional inflammatory signalling molecules. These interesting and important in vitro effects of curcumin potentially make it an ideal nutraceutical chondroprotective agent that may find future use as an adjunct to other anti-rheumatoid and inflammatory drugs (Shakibaei et al. 2005a).

2.2 High-Density Cultures

2.2.1 ECM Changes Following Long-Term Cultivation of Cartilage (Organoid/High-Density Cultures)

High-density (organoid or micromass) cultures of prechondrogenic mesenchymal cells derived from limb buds of 12-day-old mouse embryos give rise to typical cartilaginous tissue which develops after 7 days in vitro (Fig. 26A). We have undertaken immunomorphological studies to show that these cartilage-like entities contain the major components of the ECM, such as collagen type II (Fig. 26B) and cartilage-specific proteoglycans (Shakibaei et al. 1993b). After a 3-week cultivation period, hypertrophic cartilage cells are increasingly apparent in this culture system. Many of these cells (as well as normal chondrocyte-like cells) detach from the matrix from the second week in vitro onwards to take on a fibroblast-like appearance. During this time, thick (25–65 nm) collagen fibrils occur at the surface of these cells (Fig. 26C). We have used immunomorphology to show that these thick fibrils contain collagen type I (Fig. 26D) (Shakibaei et al. 1993b). Hence in older cartilage cultures, chondrocyte-like cells change their phenotype and biosynthesis programme. The high-density and organoid culture systems provided us with an experimental system for studying the dedifferentiation of cartilage and possibly also for the transformation of cartilage cells to osteoblast-like cells.

In a related study, we used limb buds from 12-day-old mouse embryos to develop an in vitro chondroblast/chondrocyte model and co-cultured them with mouse peritoneal macrophages in high-density or monolayer cultures to create an in vitro model of inflammation (Shakibaei and Mohamed-Ali 1994). The result of this experimental approach was an extensive breakdown of the cartilage matrix. Numerous chondrocyte-like cells detached from the ECM and adopted a fibroblast-like morphology. Immunomorphological methods showed that the expression of collagen type II and fibronectin was diminished and several integrins (β1, α3-, and α5 β1 types) disappeared from the surface of chondrocytes during this process (not shown). The degradative alterations observed could be due to pro-inflammatory and matrix degrading products of macrophages and activated

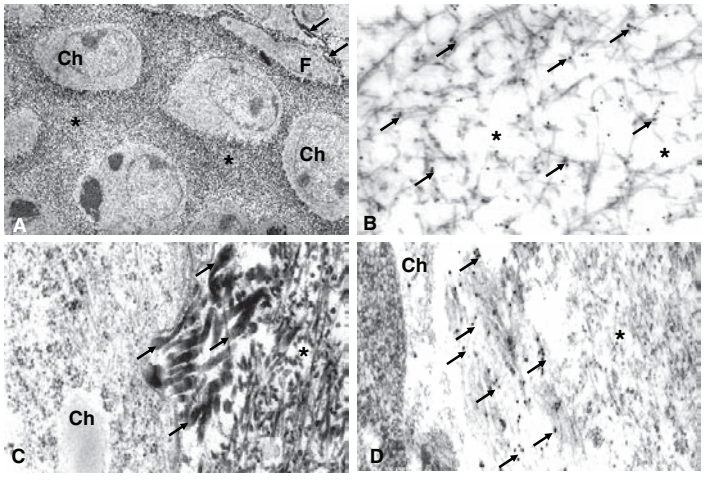

Fig. 26A–D Electron microscopic demonstration of cartilage developed in organoid culture after 7 and 21 days. **A** Electron microscopic demonstration of cartilage developed in organoid culture after 7 days: The flat fibroblast-like cells (*F*) are embedded in a dense mass of thick collagen fibrils (*arrows*). Typical chondrocytes (*Ch*) are embedded in the cartilage matrix (*). ×6,500. **B** Immuno-labelling with collagen type II (*arrows*) is only demonstrable in the cartilage matrix (*), ×32,000. **C** Electron microscopic demonstration of cartilage developed in organoid culture after 21 days: Occurrence of thick collagenous fibrils (*arrows*) at the surface of the chondrocytes (*Ch*); (*), typical cartilaginous matrix). ×32.000. **D** Immuno-labelling with collagen type I is only demonstrable in these thick fibrils but not in the cartilage matrix (*). ×60,000

chondrocytes (Shakibaei and Mohamed-Ali 1994). This co-culture model should be useful for studying the expression of ECM components and the turnover of ECM proteins and cell receptors in pathological and inflammatory conditions.

2.2.2 Expression of Integrins in Ageing Cartilage Tissue In Vitro

As we have demonstrated in our monolayer studies, chondrocyte–matrix interactions are important for chondrocyte function, and integrins play an essential role in these interactions as transmitters of extracellular signals. In one of our earliest studies on integrins, we used immunomorphological techniques (light and electron microscopy) and demonstrated the presence of integrin receptors for collagen type I (β1α1 and β1α2) on the surface of ageing chondrocytes (Shakibaei et al. 1993a). Cultures of chondrocytes in their prime (i.e. from juvenescent cartilage tissue) exhibited only β1α3- and β1α5-receptors and labelling of β1α1- and β1α2-integrins was not seen in this case. Our results demonstrated that after the occurrence of thick collagen fibrils, chondrocytes express new integrin receptors (β1α1 and β1α2) on the cell membrane. Thus, phenotypic changes in ageing

and dedifferentiating cartilage tissue in vitro involve changes in the biosynthesis programme of matrix components and alterations in the expression of integrins (Fig. 27A–C) (Shakibaei et al. 1993a). These initial observations served as a platform for further studies aimed at gaining a better understanding and interpretation of cartilage changes in vivo during ageing and under pathological conditions.

2.2.3 Changes in Integrin Expression During Chondrogenesis In Vitro

Pioneering studies by Daniels and Solursh demonstrated that the process of chondrogenesis can be modulated by the cytoskeleton and the extracellular matrix (Daniels and Solursh 1991). The use of cartilage organoid culture systems allows detailed investigation of chondrogenesis from early blastema condensation until cartilage maturation. This chondrogenic development is accompanied by an enlargement of cartilage nodules, which is not due to

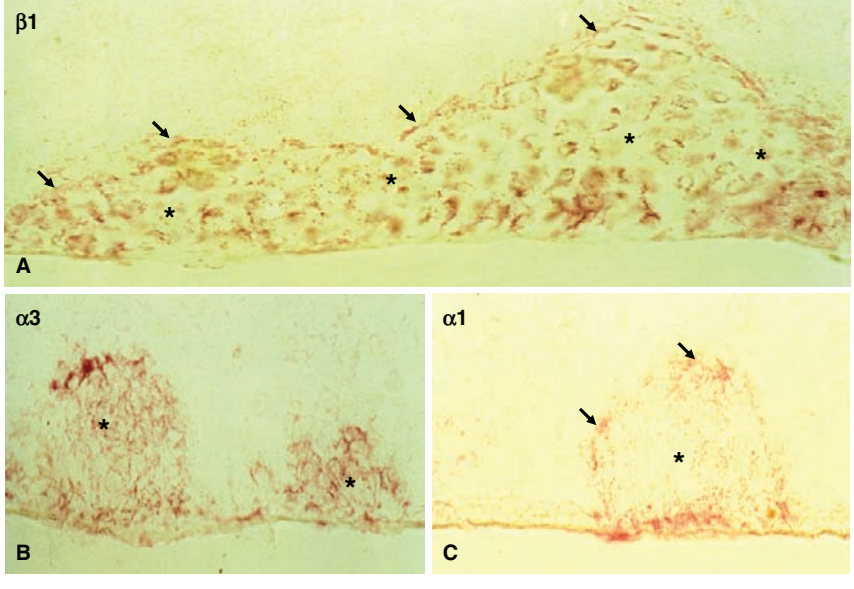

Fig. 27A–C Immuno-labelling of β1-, α1- and α3-integrins as revealed by APAAP in cartilage grown in organoid culture. **A** Demonstration of the β1-integrin chain after 14 days of culture. Positive labelling of the β1-chain was shown on the surface of the cells in the perichondrium (*arrows*) as well as in the cartilage nodules (*). **B** Demonstration of the α3-integrin chain after 14 days of culture. This integrin was found mainly in the cartilage nodules (*). **C** Demonstration of the α1-integrin chain after 14 days of culture. This integrin was found mainly in the perichondrium (*arrows*) and also around some cells in the cartilage nodules (*). **A** ×360; **B, C** ×240

cellular proliferation, but to appositional growth. This includes the transition of perichondral cells into chondrocytes. These conclusions are based on the virtual absence of mitotic figures in cartilage and perichondrium and on the continuous increase in nodule size during cultivation. We studied the distribution of α1- and α3-integrins as well as collagen types I and II by immunofluorescence and immuno-electron microscopy during chondrogenesis in vitro (Shakibaei et al. 1995). In our studies, we incubated mesenchymal cells derived from limb buds of 12-day-old mouse embryos in high-density organoid culture and studied the expression of the above-mentioned proteins after various time periods. Within the first 2 days of the culture period, only α1-integrin could be detected. Formation of cartilage-specific matrix with collagen type II production on day 3 was accompanied by the occurrence of α3-integrin. On day 7, α3 was present only in the newly formed cartilage nodules, whereas α1 was strongly expressed in the perichondrium and was further more or less homogeneously distributed in the surrounding mesenchyme. On day 14, α1-integrin was again detectable in the cartilage; this can be related to the observation

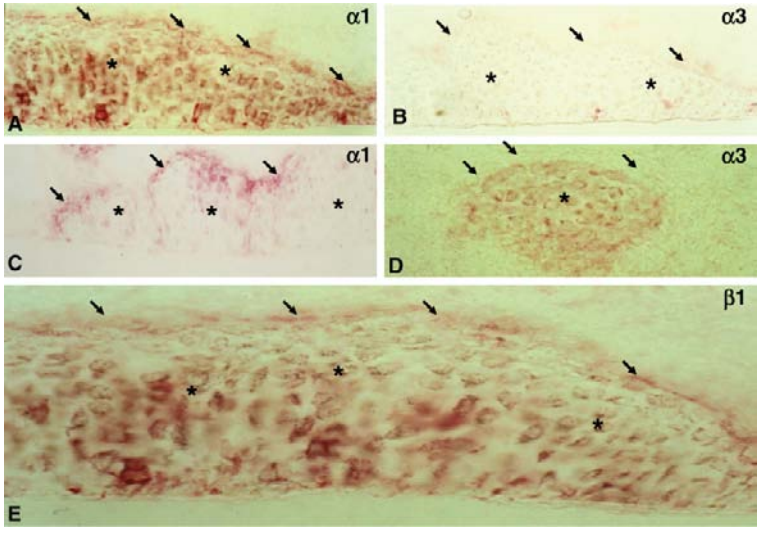

Fig. 28A–E Immuno-labelling of β1-, α1- and α3-integrins as revealed by APAAP during chondrogenesis of limb bud mesenchymal cells from 12-day-old mouse embryos grown in organoid cultures. **A, B** After a culture period of 2 days, α1-integrin is uniformly detectable within the mesenchymal cell mass (*) and in the perichondrium (*arrows*), whereas α3-integrin can not be found. **C, D** After a culture period of 7 days, α1-integrin is detectable mainly in the perichondrium and mesenchyme (*arrows*), whereas α3-integrin can be found mainly within the cartilage nodules (*), but not in the perichondrium (*arrows*). **E** After a culture period of 7 days, β1-integrin is detectable both in the perichondrium (*arrows*) and cartilage nodules (*). **A–D** ×240; **E** ×360

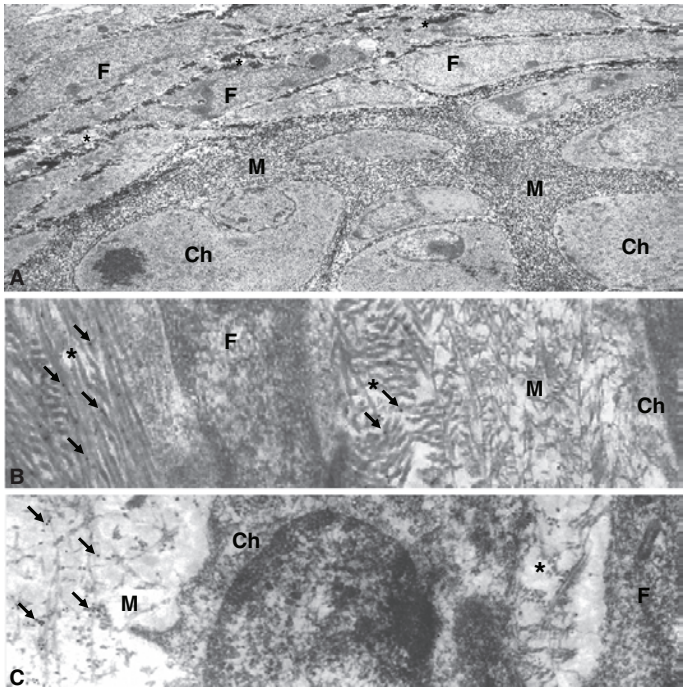

Fig. 29A–C Electron microscopic demonstration of cartilage developed in organoid culture after 7 days. **A** The zone of perichondrium/cartilage is shown. Flat fibroblast-like cells (*F*) are embedded in a dense mass of thick collagen fibrils (*). Typical chondrocytes (*Ch*) are embedded in the cartilage matrix (*M*). ×6,500. **B** Immuno-labelling with collagen type I. Gold particles (*arrows*) are detectable only in the perichondrium on collagenous fibrils (*). Gold particles (*arrows*) fail to occur in the cartilage matrix (*M*). ×40,000. **C** Immuno-labelling (*arrows*) with collagen type II is only demonstrable in the cartilage matrix (*M*), not in the perichondrium (*). ×32,000

that dedifferentiation of chondrocytes in ageing cartilage is also accompanied by the occurrence of collagen type I and α1-integrin (Figs. 28A–E and 29A–C). These results and the work of others (Loeser 2000, 2002; Knudson and Loeser 2002) suggest that a strict correlation exists between the collagen type synthesised by chondrocyte-progenitor cells and the appropriate collagen receptor presented on the surface of the cells. Co-localisation of α1-integrin and collagen type I, on the one hand, and α3-integrin and collagen type II and their changes during development and dedifferentiation indicate, on the other hand, a coupling in expression or activation of integrins with collagen synthesis. However, it may be suggested that changing the ligand (from collagen type I to type II, and vice versa) induces the corresponding expression or presentation of the receptor for the new ligand. It has been reported that the regulation of integrin receptor distribution on the surface of cells can be influenced by ligand occupancy.

2.2.4 Inhibition of Chondrogenesis by Incubating Chondrocyte Cultures with an Anti-integrin Antibody

In the first 24 h of the organoid culture period, cells sorted out and formed prechondrogenic areas, composed of densely packed cells with intercellular interactions (gap junctions), which were surrounded by a perichondrium of flat fibroblast-like cells, resembling the situation of the early-stage in vivo chondrogenesis. The extensive cell–cell interactions during the first step of chondrogenesis are crucial. The presence of Ca^{2+} and cell adhesion molecules for cell aggregation as well as the expression of adhesive molecules such as neural cell adhesion molecules (N-CAM), N-cadherin and hyaluronan during blastemal cell differentiation, suggest that cell aggregation is mediated by specific molecules and surface receptors. However, it is still unclear whether integrins play an important role during the early stages of differentiation of prechondrogenic cells to chondroblasts.

The ability to modulate chondrogenesis by altering the cytoskeleton and the extracellular matrix (Daniels and Solursh 1991) inspired us to further investigate the potential function inhibiting properties of anti-integrin antibodies. The functions of integrins during the process of chondrogenesis were investigated by using anti-integrin antibodies on mesenchymal cells from limb buds of 12-day-old mouse embryos. The cells were treated with anti-β1-, -α1-, and -α5-integrin antibodies (a) from day 1 to day 3 and (b) from day 3 to day 7 of cultivation. The total culture period in these studies was 7 days. The presence of exogenous anti-β1-, but not -α1- and -α5-integrin antibodies, from day 1 to 3 completely inhibited the differentiation of blastemal cells to chondroblasts and the formation of cartilage matrix (Fig. 30C). Conversely, the presence of exogenous anti-β1-, -α1-, and -α5-integrin antibodies from day 3 of cultivation onwards had no apparent effect. Immunoblotting (not shown) and immuno-morphological evaluation of the cultures treated with anti-beta 1 antibody from day 1 to day 3 revealed a unique pattern of integrin and collagen expression which consisted of β1-, α1-, α5β1-integrins (not shown) and collagen type I (Fig. 30D). The cartilage-specific chondroitin sulfate proteoglycan (CSPG) could not be demonstrated in these cultures. The cultures treated later (day 3 to day 7) revealed expression of β1-, α3-, α5β1-integrin, and αvβ3-integrins, collagen types I and II, and CSPG identical to that of the untreated controls (Fig. 30B). These results led to the conclusion that β1-integrins play a crucial role in early cartilage differentiation and point to possible cell–matrix interactions during the induction phase of chondrogenesis (Shakibaei 1998). Recent work on in vitro chondrogenesis of bone marrow-derived human mesenchymal stem cells in collagen type II scaffolds revealed the necessity of functional β1-integrin on the cell surface for appropriate cell-mediated assembly and collagen fibre reorganisation (Chang et al. 2007). The strong inhibitory effect on chondrogenesis in response to integrin-mediated adhesion shows that integrins, according to their function as cell adhesion- and signalling receptors during embryogenesis, play a central role in cell–initial matrix interaction in chondrogenesis. This provides additional evidence that integrins play a vital role in chondrogenesis and should be targeted in future tissue engineering approaches.

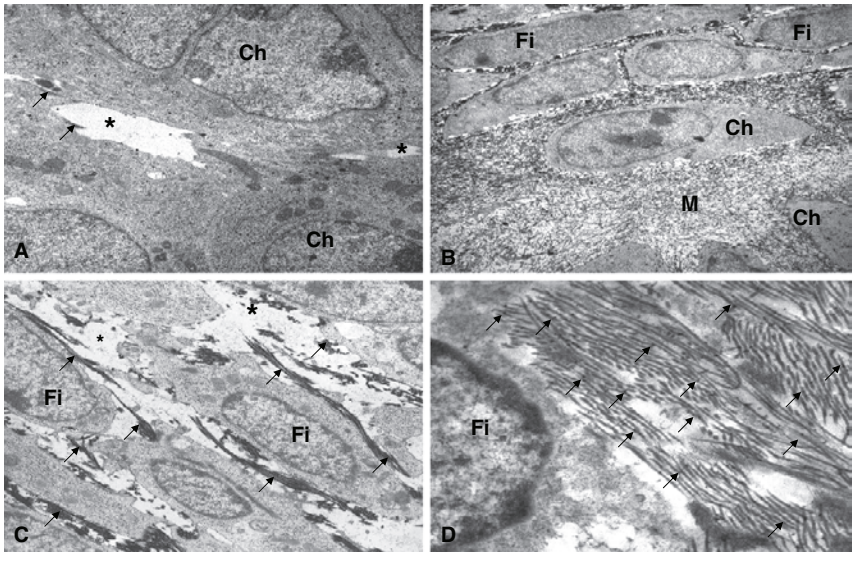

Fig. 30A–D Electron microscopy of limb bud mesenchymal cells grown at high density in organoid culture on days 2 and 7 of the culture period. **A** Electron microscopy of the organoid culture after 2 days: cells are separated from each other by an extracellular space (*) in which matrix material is present (*arrows*). They resemble typical chondroblasts. ×6,500. **B** Electron microscopy of the periphery of a cartilage nodule after 7 days in organoid culture. The flat fibroblast-like cells (*F*) of the perichondrium are embedded in dense bundles of thick collagenous fibrils. Typical chondrocytes (*Ch*) are embedded in the cartilage matrix (*M*). ×6,500. **C** Electron microscopy of mesenchymal cells from limb buds of mouse embryos (day 12) after 7 days *i*n vitro treated with anti-β1-integrins from the beginning of the cultivation. Inhibition of chondrogenesis and transformation to fibroblast-like cells (*F*) are shown. ×6,500. **D** The fibroblast-like cells (*F*) synthesised collagen type I (*arrows*). ×40,000

2.2.5 Integrin Expression During Differentiation of Mesenchymal Limb Bud Cells to Chondrocytes in Alginate Culture

The use of alginate beads has been increasingly adopted to allow investigators to stabilise the unique chondrocyte phenotype in vitro (Grandolfo et al. 1993; Guo et al. 1989). The aim of this study was to define the behaviour of mesenchymal cells (from 12-day-old mouse limb buds) in alginate cultures. For the optimisation of cartilage cell cultures, we employed the alginate system and encapsulated mesenchymal cells from 12-day-old mouse limb buds. They were grown in alginate beads for up to 4 weeks (Shakibaei and De Souza 1997). The mesenchyme of 12-day-old limb buds consists of different cell populations. Therefore, it must be considered that due to enzymatic treatment not only fibroblast-like or undifferentiated mesenchymal cells, but also prechondrocytes are released. Cultivation of these

different cell types mostly yields mixed cultures. In most culture systems (monolayer and mass cultures), these mixed populations do not allow the development of pure chondrocyte cultures. We found that a sub-population of these cells differentiated to chondrocytes and exhibited a stable phenotype right up to the very end of the culture period (Fig. 31A). After 3–4 days, a cartilage-specific matrix started to develop in these beads (Fig. 31B–D). Fibroblast-like cells present in the mixed cultures did not survive. The fibroblast-like or undifferentiated mesenchymal cells,

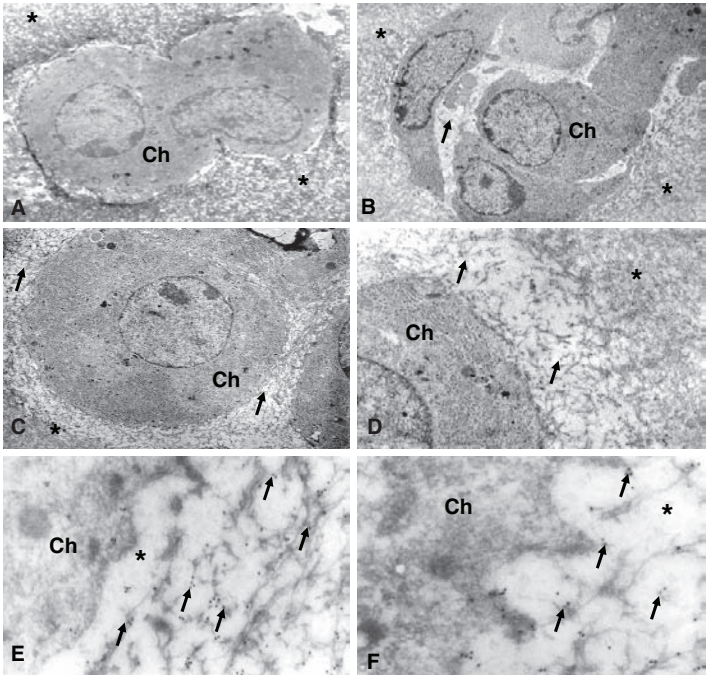

Fig. 31A–F Electron microscopic demonstration of limb bud cells from 12-day-old mouse embryos after a culture period of 1 day (**A**), 4 days (**B**) and 7 days (**C, D**) in alginate (*). After 1 day, alginate cultures of undifferentiated limb buds from 12-day-old mouse embryos showed cell aggregates (**A**). After 3–4 days, chondrocytes (*Ch*) developed from aggregated prechondrogenic mesenchymal cells (**B**). Typical cartilage cells were mainly round to oval, with a well-developed rough endoplasmic reticulum, a large Golgi apparatus and other organelles, such as mitochondria and small vacuoles. The chondrocytes (*Ch*) formed a matrix, thus moving apart from one another. **C** The matrix (*arrows*) was closely attached to the cell membrane. **D** The matrix in cartilage tissue consisted of thin collagenous fibrils (*arrows*) which ran singly and irregularly (**C, D**). The morphology of the chondrocytes and the cartilage matrix was almost unchanged after 14 and 21 days. Immuno-labelling using antibodies against collagen type II (**E**) and chondroitin sulfate-proteoglycan (**F**) at the electron microscope level showed gold particles (*arrows*) only over the typical cartilage matrix (*). Labelling with antibodies against collagen type I could not be demonstrated (not shown). **A, B** ×7,000; **C** ×10,000; **D** ×15,000; **E, F** ×40,000

the so-called anchorage-dependent cells, however, require adhesion or anchoring. Since this is not possible in agar and alginate, they perish. Moreover, the interaction between alginate (which carries a negative charge) and chondrocytes probably induces a stimulation of the differentiation of the determined prechondrogenic mesenchymal cells to chondrocytes and a stabilisation of the phenotype of the differentiated cells. In fact, chondrocytes also secrete growth/differentiation substances that promote their own differentiation. The alginate environment most likely prevents diffusion of these substances thereby permitting their progression. Hence, after a 7-day culture period, chondrocytes and other mesenchymal cells can be distinguished by necrosis and differentiation. After dissolution of alginate by chelating agents, a pure chondrocyte population can, therefore, be obtained.

Dissolution of alginate beads and centrifugation resulted in chondrocytes that did not lose their contact with the ECM (not shown). Cultivation of these chondrocytes in mass culture yielded pure chondrocyte populations. Immuno-electron microscopy revealed expression of collagen type II, fibronectin, decorin and chondroitin sulfate-proteoglycans in the alginate-cultured chondrocytes and in mass cultures (Figs. 31E–F, 32A, B) (Shakibaei and De Souza 1997). A number of publications have reported on the cultivation of differentiated chicken, bovine, human and pig chondrocytes in alginate culture. These cells synthesise collagen type II and cartilage-specific proteoglycans (Guo et al. 1989; Hauselmann et al. 1992; Grandolfo et al. 1993) (Petit et al. 1996). The question was whether the morphologically undifferentiated mesenchymal cells of limb buds can differentiate to chondrocytes in alginate culture before cartilage formation (day 12). We further addressed whether the cell–matrix relationship persists after the release of chondrocytes from alginate. The extracellular matrix in cartilage plays an essential role in the behaviour, differentiation and proliferation of chondrocytes. They can be grown further in mass culture. Under these conditions, chondrocytes produce a pure cartilage cell culture containing collagen type II, fibronectin and proteoglycans in the matrix.

2.2.6 β1-Integrins Exist in the Cartilage Matrix

As presented above, we demonstrated the presence and necessity of β1-integrins on chondrogenic progenitor cells for chondrogenesis in vitro (Shakibaei et al. 1995; Shakibaei and Mohamed-Ali 1994). We then further investigated the distribution of integrins in the cartilage matrix by using immuno-electron microscopy and by immunoprecipitation methods. Cartilage tissue of limb buds of 12-day-old mouse embryos was treated with collagenase and the cell-free and cellular protein-free supernatant was removed and used for immunoprecipitation experiments. Immunoprecipitation with antibodies against β1-, α1-, α3-, and α5β1-integrins and collagen type II, followed by immunoblotting with the same antibodies demonstrated the presence of these integrins and collagen type II in the supernatant (not shown). The integrins found in the cartilage matrix could have been either secreted or shed by the cells (Figs. 33A, B, 34A, B). These results

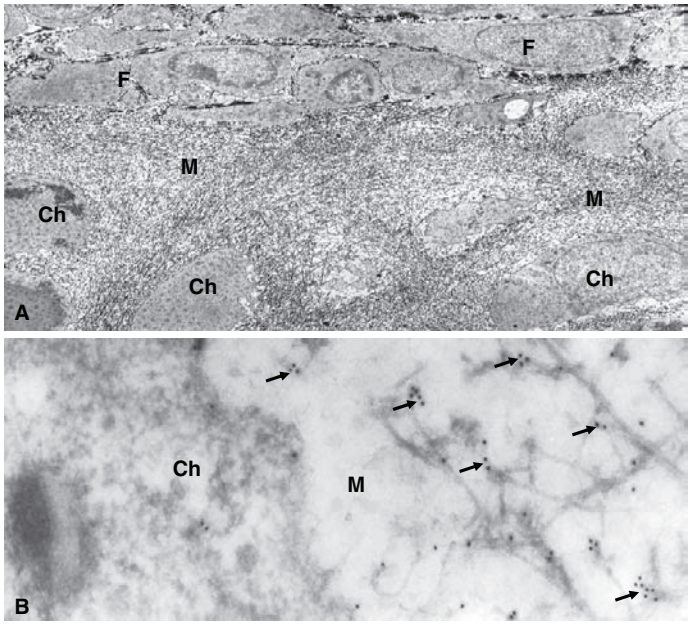

Fig. 32A, B After dissolution of alginate by Ca^{2+}-free medium, the chondrocytes (*Ch*) could be isolated and grown in organoid culture in vitro. During these processes, the cells did not lose contact with the extracellular matrix (not shown), and even maintained their matrix capsule. After cultivation of these cells in organoid culture for 7 days, a pure cartilage culture formed (**A**) showing the typical components of the cartilage matrix (*M*). The flat fibroblast-like cells (*F*) of the perichondrium are embedded in dense bundles of thick collagenous fibrils. Typical chondrocytes (*Ch*) are embedded in the cartilage matrix (*). ×6,500. Immuno-labelling using antibodies against collagen type II (**B**) at the electron microscope level showed gold particles (*arrows*) only over the typical cartilage matrix (*). ×50,000

suggest that integrins are also present in the ECM, away from the chondrocyte plasma membrane (Shakibaei and Merker 1999). Integrins are anchored in the cell membrane through a hydrophobic transmembrane segment. One possible explanation for their localisation in the matrix could be exocytotic secretion or shedding. A number of studies have shown that cell adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1) can be shed from endothelial cells in culture, from melanoma and ovarian carcinoma cells and that VCAM-1 (vascular cell adhesion molecule-1) can be shed from activated endothelial cells in culture. Moreover, it should be noted that not only adhesion molecules, but also other cell membrane receptors such as cytokines and growth-factor receptors and the transferrin receptor are released into the extracellular fluid and plasma. The question as to whether these integrin molecules have a function in the cartilage matrix, such as interlinking, matrix organisation or stabilisation of matrix components still remains to be elucidated.

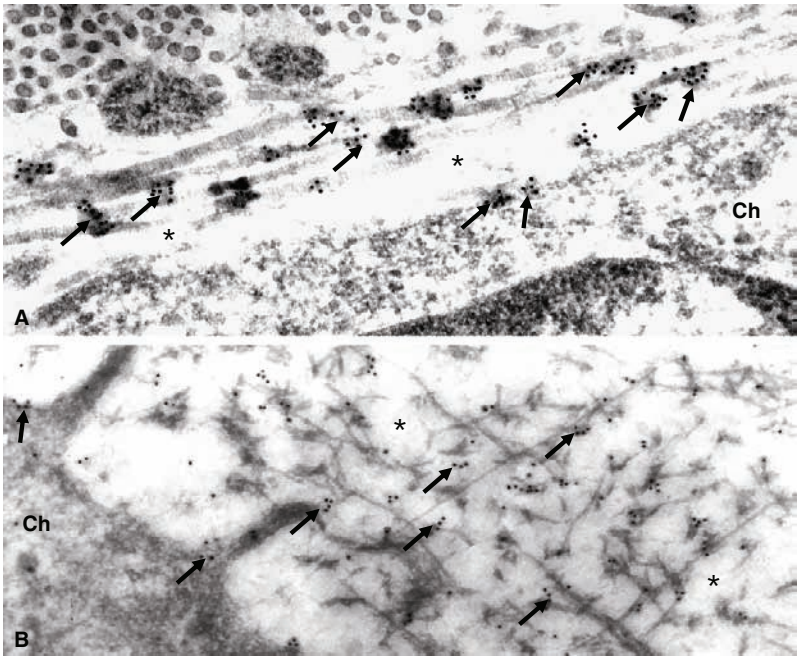

Fig. 33A, B Immuno-electron microscopic demonstration of α1- and α3-integrins in cartilage organoid culture after 7 days using immunogold labelling. **A** Gold labelling (*arrows*) at the plasma membrane of chondrocytes (*Ch*) and in the cartilage matrix (*) with antibodies against α1-integrin. ×50,000. **B** Gold labelling (*arrows*) at the plasma membrane of chondrocytes (*Ch*) and in the cartilage matrix (*) with antibodies against α3-integrin. ×50,000

2.2.7 Integrins and Matrix Metalloproteinases Co-localise in the Extracellular Matrix of Chondrocyte Cultures

Finding β1-integrins in the cartilage matrix suggested that these proteins may be implicated in the assembly of its three-dimensional architectural scaffold. The mechanism for this event is not yet established. Matrix metalloproteinases (MMPs) may be involved in an integrin-shedding mechanism and matrix β1-integrins may act to alter MMP activity. To address this question, we designed experiments to determine whether β1-integrins and MMPs co-localise in chondrocytes or in the ECM of cartilage. We studied high-density cultures of limb buds of 12-day-old mouse embryos by using double immunofluorescence, immuno-electron microscopy and by co-immunoprecipitation in order to examine the localisation of β1-integrins and matrix metalloproteinases (MMP-1, MMP-3 and MMP-9) in cartilage. We found that all the selected MMPs co-localised with β1-integrins in high-density cartilage cultures. Immunogold and immunofluorescence co-labelling of both β1-integrins and MMPs was observed not only at the surface of chondrocytes but

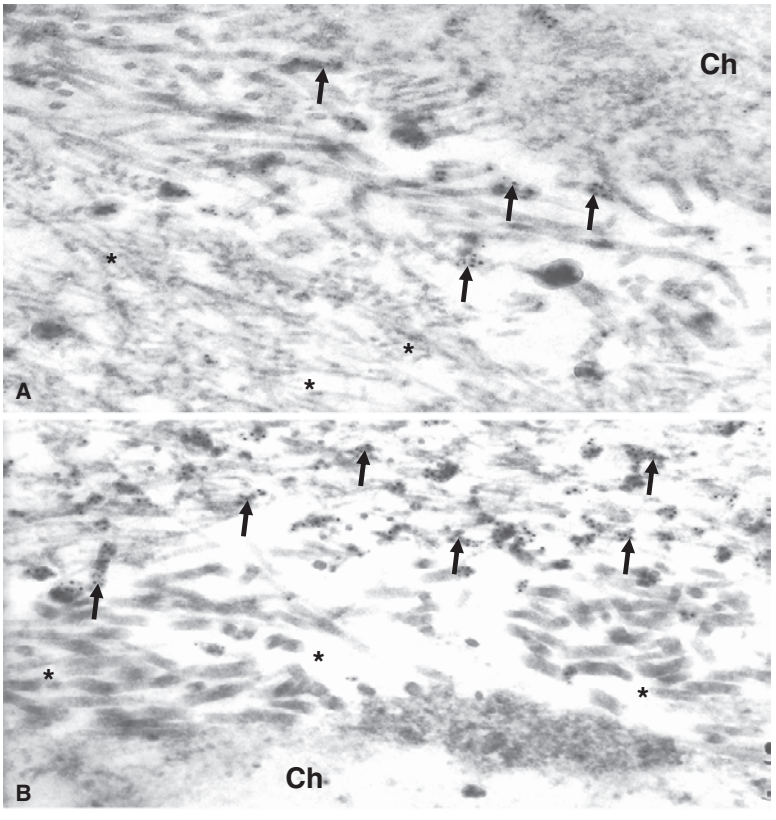

Fig. 34A, B Immuno-electron microscopic demonstration of α1- and α3-integrins in ageing cartilage organoid culture after 21 days using immunogold labelling. **A** Gold labelling with antibodies against α1-integrins (*arrows*) can be observed only over thick collagenous fibrils, but not in the typical cartilage matrix (*). **B** Gold labelling with antibodies against α3-integrins (*arrows*) can be observed only in the typical cartilage matrix, but not in the thick collagenous fibrils (*). ×40,000

also in pericellular spaces between collagen fibrils in the extracellular matrix (ECM) (Fig. 35A, B). Immunoprecipitation experiments suggest that a functional association exists between MMPs and β1-integrins in chondrocytes as already described in other cell types (Schulze-Tanzil et al. 2001). These observations set the scene for further co-localisation-type studies, which allowed us to test various hypotheses implicating integrin function in other important biological processes in chondrocytes (see later sections). Some indications for a functional association of MMPs and integrins in other cell types have been reported previously. It was found that MMP-2 and αvβ3 (vitronectin receptor) are specifically co-localised and functionally associated on angiogenic blood vessels and melanoma cells in vivo. The naturally occurring MMP-2 breakdown product (PEX) inhibits binding of MMP-2 to the vitronectin receptor.

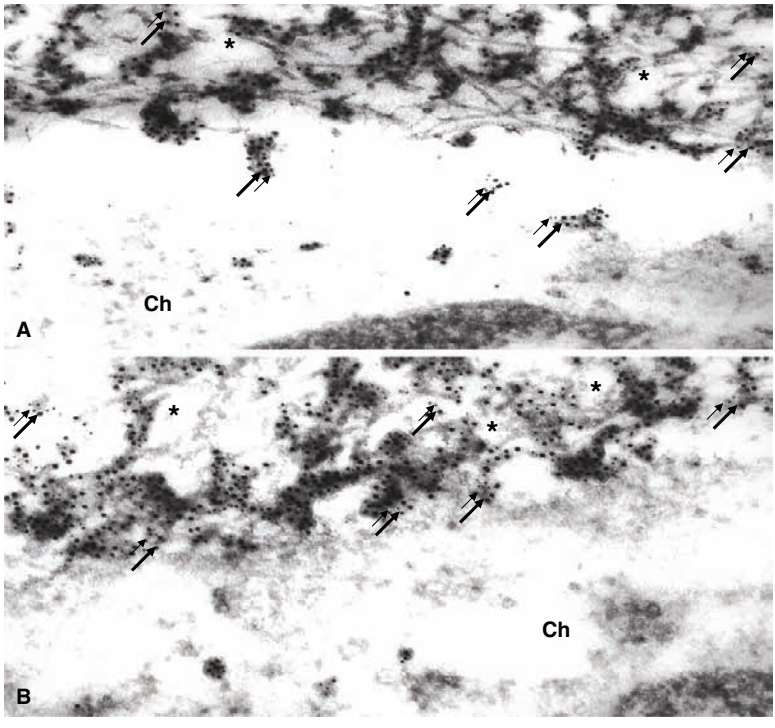

Fig. 35A, B Double immuno-electron microscopic demonstration with the anti-cytoplasmic domain of β1-integrin antibody and either anti-MMP1 (**A**) or anti-MMP3 (**B**) antibodies. Both gold-labelled antibodies (*arrows*) integrin (10-nm gold particles, *large arrows*) and MMP (5-nm gold particles, *small arrows*) are co-localised and concentrated at the cell membrane of the chondrocytes (*Ch*) as well as distributed in the ECM (*) between collagen fibrils. ×80,000

Integrin receptors are also involved in regulation of particular MMP gene expression. For example, the vitronectin receptor participates in regulation of MMP-2 at the transcriptional level in melanoma cells. The fibronectin receptors (α5β1 and α4β1) take part in regulation of MMP-1, MMP-3 and MMP-9 in fibroblasts, and integrin α2β1 is a positive regulator of collagenase MMP-1 and collagen α1 (I) gene expression.

2.2.8 Integrins and Stretch-Activated Cation Channels: Putative Components of Chondrocyte Mechanoreceptors

Articular cartilage is a unique connective tissue that experiences a variety of stresses, strains and loading pressures that result from normal activities of daily living and physical activity (Lane Smith et al. 2000). The ability to perceive mechanical signals and respond in a co-ordinated fashion to biomechanical stimuli is a fundamental

property of articular chondrocytes. Chondrocytes (Archer and Francis-West 2003) detect and respond to applied mechanical loads by altering their metabolic state through a process known as mechanotransduction (Urban 1994). Mechanical load is an important regulator of metabolic and biosynthetic activity in articular chondrocytes (Urban 1994). Mechanically induced cell membrane deformation is one of a number of possible mechanotransduction pathways by which chondrocytes sense and respond to changes in their mechanical environment (Knight et al. 1998; Guilak 1995; Guilak et al. 1995). Changes in ionic and osmotic pressure, ion transport, fluid flow and electrical current across the chondrocyte membrane are important mechano-electrochemical phenomena in loaded articular cartilage. In many cell types, mechanical stimulation induces increases of the cytosolic free Ca^{2+} concentration that propagates from cell to cell as an intercellular Ca^{2+} wave (Berridge and Dupont 1994; Amundson and Clapham 1993; Cornell-Bell and Finkbeiner 1991). The generation of intracellular calcium waves within chondrocytes and intercellular Ca^{2+} signals may provide mechanisms to co-ordinate tissue responses in cartilage physiology and biomechanical function (D'Andrea et al. 2000). Changes in amplitude or frequency of load have significant effects on the production of matrix macromolecules and of pro-inflammatory agents leading to cartilage breakdown (Urban 1994). With inappropriate mechanical loading of the joint, as occurs with traumatic injury, ligament instability, bony malalignment or excessive weight bearing, cartilage exhibits manifestations characteristic of osteoarthritis (OA). The composition of cartilage reflects the net response of the chondrocytes to the prevailing loading pattern, with cartilage proteoglycan content being highest in heavily loaded regions, and removal of load, leading to cartilage thinning and proteoglycan loss (Urban 1994). Breakdown of cartilage matrix in OA involves degradation of the extracellular matrix macromolecules and decreased expression of chondrocyte matrix proteins necessary for normal joint function. OA cartilage often exhibits increased amounts of type I collagen and synthesis of proteoglycans characteristic of immature cartilage.

Cellular mechanotransduction is defined as a series of dynamic processes that allow living cells to convert biomechanical stimuli into biochemical activity. Articular cartilage is loaded under normal gravity (static loading) and frequently loaded during physical activity (dynamic loading). Mechanotransduction in articular cartilage refers to the many cellular and extracellular matrix mechanisms by which chondrocytes quantitatively modulate the rates of matrix synthesis and degradation and alter the composition of the extracellular matrix which consists primarily of type II collagen and aggregating proteoglycans which give cartilage the ability to resist tensile stress and physical load, respectively (Benjamin et al. 1994).

Mechano-electrochemical responses of chondrocytes under mechanical load involve changes in osmotic pressure and the electrical membrane potential difference across the chondrocyte plasma membrane. Chondrocytes are therefore excellent sensors of biomechanical, ionic, osmotic and electrical signals and respond to these varied signals in coordination with other environmental, hormonal and genetic factors to regulate metabolic activity (Mobasheri et al. 2002a).

Despite this realisation, very little is known about the molecular details of mechanotransduction in chondrocytes.

The focus of work on chondrocyte mechanotransduction has shifted from the biochemical responses of the extracellular matrix to the chondrocyte and its plasma membrane. We need to gain a better understanding of the signalling and regulatory pathways activated during mechanical signal transduction in normal articular cartilage. This knowledge may be important for formulating therapeutic strategies for the rational design of pharmaceutical compounds capable of modulating the metabolic and biosynthetic activities of chondrocytes.

Immunohistochemical investigations of chondrocytes have shown the expression of aquaporin water channels (Mobasheri et al. 2004; Trujillo et al. 2004; Mobasheri and Marples 2004) as well as ENaC (Trujillo et al. 1999), NMDA (Orazizadeh et al. 2006; Salter et al. 2004; Shimazaki et al. 2006), calcium (Shakibaei and Mobasheri 2003; Wang et al. 2003; Yellowley et al. 2002; Guilak et al. 1999), chloride (Tsuga et al. 2002; Sugimoto et al. 1996) and sodium (Mobasheri et al. 2002a; Kizer et al. 1997) ion channels. The most widely reported ion channels of chondrocytes are, however, the potassium ion channels.

We have previously reviewed the role of mechanoreceptors, β1-integrins, stretch-activated cation channels and intracellular messengers (i.e. cAMP, intracellular Ca^{2+}) and their involvement in chondrocyte mechanotransduction (Mobasheri et al. 2002a). We have proposed a chondrocyte mechanoreceptor model incorporating key extracellular matrix macromolecules, β1-integrins, mechanosensitive ion channels, the cytoskeleton and subcellular signal transduction pathways that maintain the chondrocyte phenotype, prevent chondrocyte apoptosis and regulate chondrocyte-specific gene expression (Mobasheri et al. 2002a).

2.2.9 β1-Integrins Co-localise with Selected Ion Channels in Mechanoreceptor Complexes of Mouse Limb-Bud Chondrocytes

Having proposed this model, we set about to determine if β1-integrins associate with any ion channels or membrane transporters. Interactions between chondrocytes and their extracellular matrix are partly mediated by β1-integrin receptors and recent studies by our group have shown that β1-integrins co-localise with a variety of cytoskeletal complexes, signalling proteins and growth factor receptors. Further, interaction of mechanosensitive ion channels and integrins has been proposed to participate in chondrocyte mechanotransduction (Lee et al. 2000; Guilak et al. 1999; Mobasheri et al. 2002a). Accordingly, we investigated the possible co-localisation of β1-integrin with two candidate mechanosensitive ion channels and a P-type ATPase in mouse limb-bud chondrocytes. We found, using indirect two-fluorochrome immunofluorescence, that the α subunits of Na, K-ATPase, the epithelial sodium channel (ENaC) and the voltage-activated calcium channel (VACC) co-localise with β1-integrin in limb-bud chondrocytes (Shakibaei and Mobasheri 2003) (Fig. 36).

Further proof for this concept was provided by co-immunoprecipitation experiments which revealed co-localisation and association of β1-integrins with ENaC, VACC and Na, K-ATPase (Fig. 36). This observation initially surprised us, but a more detailed study of the existing literature revealed that cellular responses and signalling cascades initiated by the influx of calcium or sodium through putative mechanosensitive channels in mechanically responsive cells may be regulated more effectively if such channels were organised around integrins with receptors, kinases and cytoskeletal complexes strategically clustered about them. Furthermore, the proximity of ATPase ion pumps such as Na, K-ATPase to chondrocyte mechanoreceptor complexes could possibly facilitate rapid homeostatic responses to the ionic perturbations brought about by activation of mechanically gated cation channels and efficiently regulate the intracellular milieu of chondrocytes (Shakibaei and Mobasheri 2003).

The role of mitogen-activated protein kinases (MAPKs) in chondrocyte mechanotransduction has been investigated using a well-defined in vitro model employing a laminar flow chamber (Hung et al. 2000). The authors hypothesised that mitogen-activated protein kinase (MAPK) participates in fluid flow-induced chondrocyte mechanotransduction. The extracellular signal-regulated kinases 1 and 2 (Erk1/2) were activated 1.6- to threefold after 5–15 min of fluid flow exposure. Activation of Erk1/2 was also observed in the presence of both 10% FBS and 0.1% BSA, suggesting that the flow effects do not require serum agonists. Treatment with thapsigargin or EGTA had no significant effect on the ERK1/2 activation response to laminar-flow, suggesting that $Ca2^{+}$ mobilisation is not required for this particular response. These

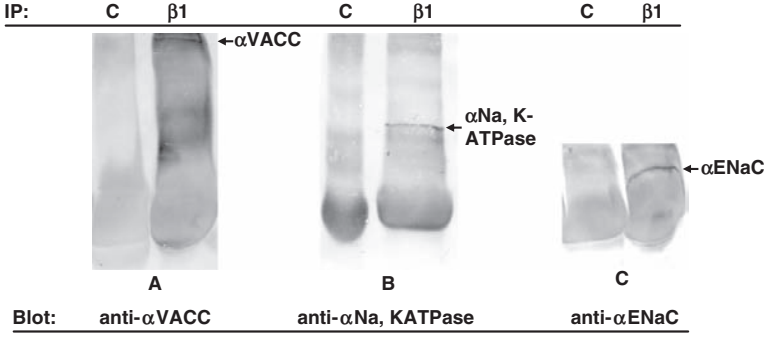

Fig. 36A–C Co-immunoprecipitation assays and immunoblotting of β1-integrin, αNa, K-ATPase, αENaC and αVACC. **A** Immunoprecipitation with anti β1-integrin antibody followed by immunoblotting with anti-αVACC antibody. B Immunoprecipitation with anti-β1-integrin antibody followed by immunoblotting with anti-αNa, K-ATPase antibody. **C** Immunoprecipitation with anti-β1-integrin antibody followed by immunoblotting with anti-αENaC antibody. In each panel, the left-hand Western blot lanes labelled *C* indicate control immunoprecipitates using nonimmune control anti-rabbit immunoglobulin antibody. Visualisation was performed through nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (p-toluidine salt)

investigators also assessed downstream effects of the activated MAPKs and found that 2 h after exposure of transfected chondrocytes to fluid laminar-flow aggrecan promoter activity was significantly decreased by 40%. The observed response was blocked by treatment of chondrocytes with the MEK-1 inhibitor PD98059. These findings demonstrate that fluid flow-induced mechanical signals activate the MEK-1/ERK signalling pathway in articular chondrocytes and result in downregulation of aggrecan gene expression. Thus, the MEK-1/ERK signalling pathway also appears to be involved in mechanotransduction and cell viability in chondrocytes.

2.2.10 Inhibition of Integrin Function Results in Chondrocyte Apoptosis

Chondrocytes bind to extracellular matrix proteins via α5β1-integrin whose binding to the extracellular matrix protein fibronectin leads to the assembly of a focal adhesion site or focal adhesion complex. These sites contain a number of cytoskeletal proteins, linker proteins and protein kinases, and they are responsible for relaying the adhesion-anchorage signals. Integrin signalling is further implicated in studies into the effects of extracellular matrix anchorage signals on cell survival (Frisch and Francis 1994). Disrupting interactions between cells and the extracellular matrix may induce apoptotic cell death. This phenomenon, termed anoikis, was first described in fibroblasts and has since been observed in chondrocytes. Chondrocyte apoptosis has been proposed to contribute to the pathogenesis of human and animal osteoarthritis (Mobasheri 2002).

Integrins function both as cell adhesion receptors and as intracellular signalling receptors. Currently more than ten molecules are known to bind to the cytoplasmic tail of β1-integrins (Brakebusch and Fassler 2005), important in chondrocytes. Among the downstream factors in these signalling pathways is the docking protein Shc, involved in transduction between receptor tyrosine kinase RTK and Ras proteins. In addition to activating the Ras-MAPK cascade, it is apparent that interactions exist between integrins and the IGF-IR, as discussed earlier (Shakibaei et al. 1999). We have shown that chondrocytes cultured on type II collagen and exposed to IGF-1 display a significantly greater density of focal adhesions when compared to cultures without IGF-I (Shakibaei et al. 1999). We observed increased phosphorylation of tyrosine residues in intracellular integrin-dependent signalling proteins when IGF-I is present and the docking transduction protein Shc forms a complex with growth factor receptor-bound protein 2 (Grb 2) inducing Ras activation.

The intracellular signalling of β1-integrin ligation by such extracellular matrix components as type II collagen involves the Ras-MAPK pathway (Shakibaei et al. 1999). This ubiquitous system regulates such essential cellular functions as growth and differentiation through the phosphorylation and consequent activation of a cascade of proteins. Ras is a membrane-anchored intracellular GTP-binding protein that on binding of its transcellular receptor tyrosine kinase (RTK) activates the ser/thr kinase Raf. Raf in turn phosphorylates MEK, a mitogen activated protein kinase kinase (MAPKK). The activated MAP kinases (also known as extracellular signal-

related kinases (Erk)) migrate from the cytosol to the nucleus where they regulate the activity of transcription factors such as jun, c-fos and c-myc (Lenormand et al. 1993a). These transcription factors regulate the transcription of genes and thus orchestrate various cellular responses. One of the transcription factors phosphorylated as a result of Ras-MAPK activation is jun, which may play a role in the regulation of anoikis, since the jun-N-terminal kinase (JNK) pathway is activated in and promotes apoptosis in response to the loss of cell–matrix contact (Cardone et al. 1997).

The importance of the Ras-MAPK pathway to chondrocyte survival has been clearly demonstrated by the induction of chondrocyte apoptosis following the inhibition of MEK (Shakibaei et al. 2001). Blocking MEK using a selective inhibitor interrupts the phosphorylation cascade and prevents activation of Erk1/Erk2. These kinases would normally translocate to the nucleus and regulate the phosphorylation status of transcription factors, hence regulating gene expression (Lenormand et al. 1993a). Interruption of the pathway produces apoptosis possibly as the result of increased expression of pro-apoptotic proteins or repression of anti-apoptotic proteins. Alternatively, the inhibition of Erk1/Erk2 may prevent inactivation of pro-apoptotic factors (Shakibaei et al. 2001). Other downstream effector molecules mediate integrin–cytoskeleton interaction. Integrin molecules lack an actin-binding domain and therefore cytoskeletal interactions are mediated by integrin-associated molecules such as α-actinin, talin, filamin and integrin-linked kinase (Ilk) (Brakebusch and Fassler 2005). It has been reported that integrin-mediated interaction with the chondrocyte cytoskeleton is mediated via the Integrin-linked-kinase (Ilk), which contains a multi-domain protein composed of four amino-terminal ankyrin (ANK) repeats, a pleckstrin homology-like domain and a carboxy-terminal serine/threonine kinase domain (Hannigan et al. 1996), which acts primarily as an adaptor protein between integrin adhesion sites and actin (Grashoff et al. 2003). Ilk can interact with α- and β-parvin, which are members of a family of actin-binding proteins (Nikolopoulos and Turner 2000; Olski et al. 2001) and deletion of the integrin-linked kinase gene leads to disruption of this interaction and delays assembly of focal-adhesion sites, stress fibre formation and cell spreading (Grashoff et al. 2003). Therefore, integrin-mediated adhesion to the extracellular matrix and the intracellular signalling pathways thus activated are likely to be important for chondrocyte survival and the physiological maintenance of the chondrocyte phenotype. The same interrelationships are likely to be equally important for chondrocyte mechanical signal transduction.

3 Concluding Remarks

Although this review has covered the diverse roles of integrin receptors in articular cartilage, we suspect that the research carried out to date has merely revealed the tip of the iceberg. Here, we summarise the main points concerning the involvement of integrins in cartilage function and pathophysiology:

During growth and development, the skeletal system, particularly the cartilage lining the articulating surfaces of bones, optimises its extracellular matrix architecture

by subtle adaptations to the prevailing mechanical loads. The mechanisms for adaptation involve a multi-step process of cellular mechanotransduction including mechanocoupling, conversion of mechanical forces into local mechanical signals, (i.e. shear stresses, that initiate a response by chondrocytes), biochemical coupling, transduction of a mechanical signal to a biochemical response involving pathways within the cell membrane and cytoskeleton; and finally modulation of gene expression and cell behaviour.

In normal cartilage, the integrin family of cell surface receptors plays a major role in mediating cell–matrix interactions. Chondrocyte integrins serve as receptors for fibronectin ($\alpha5\beta1$), collagen types II and VI ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$), laminin ($\alpha6\beta1$), vitronectin and osteopontin ($\alpha v\beta3$). Integrin-binding stimulates intracellular signalling which can affect gene expression and regulate chondrocyte function. Further, integrin expression can be regulated by growth factors including IGF-I and TGF-β (Loeser 2000; van der Kraan et al. 2002). In cartilage tissue engineering, the integrin family of cell surface receptors has been proposed as important sinks for trapping growth factors essential for maintaining the chondrocyte phenotype in three-dimensional scaffolds (van der Kraan et al. 2002). Thus, by providing a link between the ECM and the cytoskeleton, integrins may be important transducers of mechanical stimuli. Indeed $\alpha5\beta1$-integrin has been proposed as a mechanoreceptor (Salter et al. 2001) along with soluble mediators including substance P and interleukin 4 (Millward-Sadler and Salter 2004), but downstream signalling cascades and cell responses are largely unstudied (Salter et al. 2001).

Integrins are also important in the growth plate where chondrocytes proliferate and differentiate in order to bring about longitudinal bone growth. The interaction of cells with the extracellular matrix significantly influences differentiation and growth and these processes involve integrin as well as nonintegrin cell surface receptors such as annexin V and CD44 (Egerbacher and Haeusler 2003; Yoon and Fisher 2006).

In arthritis and specifically osteoarthritis, abnormal integrin expression alters cell/ECM signalling and modifies chondrocyte synthesis, followed by an imbalance of catabolic, pro-inflammatory cytokines over regulatory factors (Iannone and Lapadula 2003). For example, the pro-inflammatory mediator nitric oxide exerts its detrimental effects on chondrocytes by inhibiting collagen and proteoglycan synthesis, enhancing apoptosis, and diminishing β1-integrin-dependent adhesion to the ECM (Amin and Abramson 1998). Another example is fragments of fibronectin (Fn-fs) that bind β1-integrin and initiate catabolic events by enhancing levels of pro-inflammatory cytokines. Fn-fs also upregulate matrix metalloproteinase (MMP) expression, significantly enhance degradation and loss of proteoglycan (PG) from cartilage and temporarily suppress PG synthesis, all of which contribute to the pathogenesis of osteoarthritis (Homandberg 1999).

Although progress has been made on unravelling the role of integrins in articular cartilage, further studies are required to uncover thus far unresolved issues and identify potential new roles for integrins in cartilage physiology and pathophysiology.

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