EXPRESSION OF THE DISCOIDIN DOMAIN RECEPTORS, DDR-1 AND DDR-2 IN HUMAN NORMAL AND OSTEOARTHRITIC ARTICULAR CARTILAGES

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APRIL 2002

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Your file Votre référence
ISBN: 0-612-85819-7
Our file Notre référence
ISBN: 0-612-85819-7

Abstract

Osteoarthritis (OA) is a degenerative, age related disease of diarthrodial joints. In OA, there is increased cleavage of type II collagen by collagenases in articular cartilage. This excessive cleavage can be caused by the altered feedback regulation of gene expression in chondrocytes by collagen type II or its degradation products which may serve as regulators of collagenase synthesis and activity through a cell surface receptor-mediated mechanism. The aim of the present study was to investigate the expression and production of collagen receptors, DDR-1 and DDR-2 in human adult normal and OA articular chondrocytes and to determine whether this correlates with the expression of disease related genes such as type X collagen, a specific marker for chondrocyte hypertrophy. Immunofluorescence histochemistry using anti-peptide antibodies for the discoidin receptors was performed on fixed frozen sections of human articular cartilage. To investigate the expression of DDR genes, RNA extracted from isolated human adult normal and OA chondrocytes was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). Immunofluorescent localization results revealed the presence of DDR-2 but not DDR-1 on both normal and OA cartilages which suggests a role for DDR-2 as a cell surface regulator of cell-matrix interactions in both health and disease. DDR-1 mRNA was weakly expressed only in the normal adult cartilages which could explain the absence of detectable protein on the surface of the cells. As expected, DDR-2 mRNA was strongly expressed in all normal cartilage samples. In OA samples, the expression pattern varied in relationship to type X collagen expression. This could imply that at least in some cases a sequence of events independent of chondrocyte hypertrophy may lead to cartilage damage seen in OA. Taken together, the data indicate for the first

time the selective presence of DDR-2 on the surface of adult human articular chondrocytes and its variable expression in OA articular cartilage. Further studies could elucidate the exact nature of discoidin receptors interactions with collagen and its downstream signaling molecules to affect matrix turnover.

Résumé

L'arthrose est une maladie rhumatismale liée à l'âge caractérisée par la dégénéressence des cartilages articulaires. Dans l'arthrose, on constate un clivage accru du collagène de type II par les collagénases dans les cartilages articulaires. Ce clivage excessif peut être causé par l'altération du mécanisme de rétroaction de l'expression génétique dans les chondrocytes par le collagène de type II ou ses produits de dégradation qui peuvent réguler la synthèse et l'activité collagénique en vertu d'un mécanisme dont la médiation est assurée par les récepteurs à la surface des cellules. Le but de la présente étude est d'étudier l'expression et la production des récepteurs collagéniques, le DDR-1 et le DDR-2, dans les chondrocytes articulaires d'êtres humains adultes normaux et atteints d'arthrose, et de déterminer s'il y a une corrélation entre ce phénomène et l'expression des gènes liés à la maladie comme le collagène de type X, marqueur spécifique de l'hypertrophie des chondrocytes. L'histochimie par immunofluorescence au moyen d'anticorps antipeptidiques des récepteurs discoidiniques a été réalisée sur des coupes congelées fixes de cartilage articulaire humain.

Pour étudier l'expression des gènes DDR, l'ARN extrait des chondrocytes isolés d'êtres humains adultes normaux et atteints d'arthrose a été analysée par la technique de transcription inverse suivie de réaction en chaîne de la polymérase (RT-PCR). Les résultats de la localisation par immunofluorescence ont révélé la présence de DDR-2 mais pas de DDR-1 sur les cartilages de sujets normaux et souffrant d'arthrose, ce qui incite à croire que le DDR-2 pourrait être un régulateur à la surface des cellules des interactions cellule-matrice à la fois à l'état normal et à l'état pathologique. L'ARNm du DDR-1 n'était faiblement exprimé que dans les cartilages d'adultes à l'état normal, ce qui pourrait

expliquer l'absence de protéines décelables à la surface des cellules. Comme on s'y attendait, l'ARNm du DDR-2 était fortement exprimé dans tous les échantillons de cartilage normal. Dans les échantillons de cartilage arthrosique, le mode d'expression variait en fonction de l'expression du collagène de type X. Cela pourrait signifier qu'au moins dans certains cas, une séquence d'événements indépendants de l'hypertrophie des chondrocytes pourrait entraîner l'altération des cartilages que l'on constate dans l'arthrose. Prises ensemble, les données révèlent pour la première fois la présence sélective de DDR-2 à la surface des chondrocytes articulaires de l'être humain adulte et son expression variable dans les cartilages articulaires de sujets atteints d'arthrose. Des études plus fouillées permettront de mieux comprendre la nature exacte des interactions entre les récepteurs discoidiniques et le collagène et ses molécules de signalisation en aval qui affectent la vitesse de renouvellement de la matrice.

ACKNOWLEDGMENTS

I would like to extend my thanks and gratitude to all the individuals who helped me in various ways in the completion of this work. First and foremost, to my supervisor, Dr. A. Robin Poole, for providing a stimulating learning experience, the opportunity to pursue this degree, financial support, and for his continued oversight, wisdom and encouragement. To Dr. Fackson Mwale for supplying the anti-peptide antibodies and for his excellent advice and support. To Dr. Elena Tchetina for providing the primers necessary for the polymerase chain reaction and her skilled and patient teaching of the laboratory techniques. To Dr. Ginette Webb for her help and advice with the techniques necessary for tissue culture. To Mrs. Isabelle Pidoux for her excellent technical advice and her skilled teaching of Immunofluorescent histochemistry. To Ms. Guylaine Bedard for her help in preparing the figures. I would also like to acknowledge, with enormous gratitude, Drs M Tanzer and D Zukor for supplying the osteoarthritic cartilages.

I owe thanks to all my colleagues in the Joint Diseases Laboratory. It has been a privilege to work with them.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

1.1 Articular Cartilage

Articular cartilage is the thin layer of deformable, load-bearing material which lines the bony ends of all diarthrodial joints. The primary functions of articular cartilages are to distribute forces generated during joint loading, to stabilize and guide joint motion and to ensure with joint lubrication an almost frictionless articulation (1-3).

It consists of a composite extracellular matrix which is saturated with water. The water phase of cartilage constitutes from 65 to 85% of the total tissue weight and is important in controlling many physical properties (4-8). This extracellular matrix is synthesized and maintained by a sparse population of specialized cells, the chondrocytes. In adult human cartilage these cells may occupy as little as 2% of the total volume (104). The dominant structural components of the matrix are the collagen molecules (~ 75% by dry tissue weight) and the negatively-charged proteoglycans (~ 20% by dry tissue weight) (105). Collagen molecules, principally type II, assemble to form small fibrils and larger fibers with an orientation and dimension that vary throughout the depth of the cartilage layer (9-12). Type II collagen is the single most abundant protein in normal cartilage, comprising approximately 85-95% of the collagens (3). In mature cartilage, types V, VI, and IX each contribute 1-2% (9-10,106) and type XI adds another 2-3% (107).

Type II collagen: Type II collagen is synthesized as a high-molecular–weight precursor. The amino (N) and carboxy (C) propeptides are required for the correct

alignment of procollagen molecules during fibril assembly. They are removed by specific N and C-proteinases. The C-propeptide remains transiently within cartilage matrix after cleavage and binds Ca^{2+} (13). It was originally named chondrocalcin, because it was found concentrated at sites of mineral formation in calcifying cartilages during endochondral ossification in the growth plate. Since it binds calcium and hydroxyapatite, it might influence mineral growth (13). The collagen fibrils contain 300-nm-long type II tropocollagen molecules each containing a triple helix of three identical α chains with the nonhelical N and C terminal telopeptide domains. These are arranged in a quarter stagger as the fibril forms. In the adult, these collagen molecules are covalently joined by hydroxypyridinium cross-links. The presence of these cross-links serves to strengthen the collagen and may render it more resistant to proteolysis (13). In the adult cartilage the collagen fibrils are usually of larger diameter (~ 75 nm) in the middle and deep zones than in the superficial zone (30 nm). The turnover of type II collagen in this cartilage is probably very limited except in pericellular sites where there is evidence for limited type II cleavage and synthesis (14).

The primary proteoglycan of cartilage matrix is a very large molecule that aggregates with hyaluronic acid and is known as aggrecan (15-16).

Aggrecan: A single aggrecan molecule consists of a protein core and numerous glycosaminoglycan side chains. The glycosaminoglycans of articular cartilage are primarily chondroitin sulfate(CS) and keratan sulfate (KS). The core protein of aggrecan consists of three globular domains, one of which, the G1 domain at the amino terminus, binds to hyaluronan and therby retains these molecules within the

extracellular matrix. Close to the G1 domain is a second globular domain G2. Situated in a region of the core protein close to G2 is a keratan sulfate rich region in which up to 30 of these glycosaminoglycan chains are located. The keratan sulfate-rich region can bind to type II collagen thereby explaining the direct association of aggercan with collagen fibrils. Located between G2 and the G3 globular domain at the carboxy terminus is a chondroitin sulfate rich region containing up to 100 chains (16). Since its glycosaminoglycan chains can bind water, aggrecan creates a highly hydrated matrix, but hydration and swelling is limited by the collagen fibrillar network. Thus, aggrecan is only partially hydrated and exhibits a swelling pressure (13). Aggrecan is often concentrated in pericellular regions. This would theoretically provide further protection for the chondrocyte against compressive forces (13). The large size (Mw 50-100 x 10^6 Da) and complex structure of the proteoglycan aggrecan function to immobilize and restrain it within the collagen network, thus forming the solid matrix of articular cartilage (4,15,110).

The matrix of articular cartilage has a highly specific ultrastructure which may be divided into successive zones from the articular surface to the subchondral bone. Collagen fibers in the superficial zone of cartilage are densely packed and oriented parallel to the articular surface (9-12,17). The cells in this layer are flattened and have different matrix biosynthetic capacity than other chondrocytes. This surface zone is also characterized by a relatively low proteoglycan content and a low permeability to fluid flow (5,18,19). In the middle or transitional zone, the collagen fibers are reported to be either random (20,21) or radially oriented (12) and the proteoglycan content is at a maximum (5,18,22). This zone consists of rounded cells surrounded by

an extensive extracellular matrix. In the deep zone, adjacent to the zone of calcified cartilage and subchondral bone, the collagen fibers are larger and form bundles which are oriented perpendicular to the bone. In this zone cell volume is at its lowest and the cells are often grouped in clusters and resemble the hypertrophic chondrocytes of the growth plate and the proteoglycan content is again low (5,11,21,22). Cell density is at its highest at the articular surface and is progressively reduced in the mid and deep zones to about one-half to one-third that of the superficial zone. This cell density in the cartilage decreases with increasing age. Tensile properties are maximal at the articular surface. In osteoarthritis (OA) there is a loss of tensile properties and swelling pressure as collagen and proteoglycans are degraded and this loss results in mechanical decompensation (19).

Adjacent to the deep zone is the calcified zone. The junction is defined by a boundary called the tidemark. The calcified layer provides an interface with the subchondral bone with mechanical properties intermediate between those of cartilage and bone (13).

Physical Properties of the Extracellular Matrix

The structural organization of articular cartilage matrix gives this tissue special physical properties that enable it to absorb and disperse loads and in the presence of synovial fluid, to provide an almost frictionless articulating surface. Its rigidity provides shape and substance to structures such as embryonic limbs, the nose and pinnae of the ears and auditory bones, as well as the trachea and bronchioles and articular cartilages (13).

The tensile properties of cartilage are primarily determined by the collagenous fibrillar network. In adult articular cartilage, tensile properties are greatest in the

superficial zone where fibrils are aligned parallel to each other and to the articular surface. Cleavage of collagen by a collagenase leads to a loss of tensile stiffness and strength. Removal of proteoglycan does not affect the intrinsic tensile stiffness and strength of cartilage. However, the proteoglycan component, mainly aggrecan, likely retards the rate of stretch and alignment of collagen when a tensile load is suddenly applied. Cartilage in high-weight-bearing areas has an elevated content of proteoglycan but is not as stiff as cartilage in low-weight-bearing areas, which contain less proteoglycan (28).

The proteoglycans of the articular cartilage are negatively charged due to the presence of carboxyl and sulfate groups of the glycosaminoglycans, and so confer a net negative charge to the cartilage extracellular matrix. As a result, cartilage is highly hydrophilic, with a tendency to swell, in order to maintain mechanochemical equilibrium. This property significantly contributes to the mechanical function of articular cartilage by generating a large swelling pressure which facilitates load support and tissue recovery from deformations (16,23).

There are numerous other molecular species such as types IV and IX collagen, biglycan and decorin which contribute to the specialized function of articular cartilage.

1.2 Osteoarthritis (OA)

In a healthy joint, articular cartilage may withstand the large forces associated with weightbearing and joint motion over the lifetime of an individual. Osteoarthritis, represents a clinical collection of conditions involving a progressive pathological

alteration of joint structure which involves the degeneration of articular cartilage, a remodeling of sub-chondral bone and limited synovitis which lead to impaired joint motion, pain and disability (24,29). OA may be considered part of a process of an age-related change or a disease. It is twice as prevalent in women than men and increases in incidence with age. By radiographic examination, the disease has universal prevalence by age 80 (25) and most individuals have had some symptom, however mild, by that age. Changes that lead to the development of OA are slow. Osteoarthritis has been classified into primary (idiopathic) and secondary OA. In idiopathic OA, clinical presentation may result from change over a period of up to 30 years. The disease can involve one or two large joints or may be generalized and involve multiple joints, as in post-menopausal OA. Secondary forms of OA fall into two categories. One where there are risk factors affecting distribution and severity of loading on specific joints and another where there is a recongnizable gene defect (e.g. mutation in the cartilage collagen gene, COL 2A I) (1).

Joint injury with subsequent joint instability resulting from loss of ligamental or meniscal function are significant risk factors for OA (26). Injury resulting from under or over exercise has been reported as a risk for OA (27,38). Degeneration may also be accelerated by synovitis. Familial OA results from a genetic defect causing changes in cartilage matrix and physiology leading to the manifestation of joint degeneration following natural cessation of growth.

In brief, cartilage damage due to trauma, impact injuries, abnormal joint loading, excessive wear or as part of an ageing process can lead to changes in the composition, structure and material properties of the tissue. These alterations can compromise the

ability of cartilage to function and survive in the strenuous mechanical environment normally found in weight-bearing joints.

The Articular Cartilage in OA

The classic loss of articular cartilage seen in OA may be initiated as a focal process as is observed in experimental animal models of OA (30). The evidence for such a process in humans has been reported by Webb *et al* (G.R. Webb, A.R. Poole *et al.* manuscript in preparation). These focal lesions, which are commonly observed in ageing populations, may progressively enlarge and produce changes in loading. Degeneration is first observed at the articular surface where fibrillation occurs. This superficial fibrillation is associated with increased denaturation and loss of type II collagen as collagen fibrils are degraded (31,108). The cleavage is always more pronounced around chondrocytes from which the proteinases are released (32). This leads to a loss of tensile properties, particularly in OA susceptible joints such as the hip and knee (28). This damage to the fibrils leads also to a loss of the small proteoglycans decorin and biglycan and is accompanied by a loss of the large proteoglycan aggrecan (33).

Matrix metalloproteinases (MMPs) are generally considered to play the principal roles in the cleavage of macromolecules such as type II collagen and aggrecan. The MMPs exhibit considerable structural homology, are secreted as latent proenzymes and are activated extracellularly. They are named metalloproteinases because they contain an atom of zinc in their active site. Those MMPs that can cleave collagens are called collagenases. They cleave collagen type II at a specific site approximately three-quarters from the amino terminus. This results in unwinding or denaturation of the α chains which are then susceptible to secondary cleavage by collagenases and

other metalloproteinases such as stromelysin-1 and the gelatinases A and B (13). Of the many collagenases present in articular cartilage, collagenase-3 or MMP-13 is the most efficient at cleaving type II collagen (34,109). The collagenases 1, 2 and 3 like most other MMPs except stromelysin-3, are secreted as latent proenzymes. Stromelysin-1 can activate collagenase-1, gelatinase-A can activate collagenases-3 as can the membrane type 1-MMP (collagenase-4). Like other MMPs these activators may themselves be activated by plasmin (from plasminogen by urokinase type activator produced by chondrocytes) or by cysteine proteinases such as cathepsin B (33,35).

Activities of these MMPs are regulated not only at the levels of transcriptional activation, translation and extracellular proenzyme activation but also at the level of inhibition by tissue inhibitors of metalloproteinases, commonly known as TIMPs. There are four TIMPs, namely TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (36). In OA there is a deficiency of TIMPs (37). This clearly favors the excessive proteolysis that is observed in the diseased articular cartilage.

Mature articular chondrocytes do not ordinarily express type X collagen which is a small protein (59 kD), roughly half the size of type II collagen. Collagen type X is normally synthesized by hypertrophic chondrocytes in the growth plate in association with cartilage matrix resorption that precedes mineralization. But in OA its expression and synthesis is activated (39). In degenerate OA cartilage collagen type X is found in association with increased expression of the cell surface type II collagen receptor annexin V. These molecules are normally highly expressed by early hypertrophic chondrocytes. There is also increased expression of type II collagen (40-

42) and MMP-13 (30,34,43) which also occurs in the physis of the growth plate (44). These changes may represent a chondrocyte response to a damaged extracellular matrix with the reversion to a more fetal phenotype. The 'hypertrophic' changes are not however, usually extensive and are generally confined to more superficial cells remote from the advancing front of type II collagen cleavage and denaturation (1).

Mechanisms of Matrix Degradation

The degradation of matrix molecules is an integral feature of remodeling in growth and development and matrix turnover in the adult. It is not just a feature of pathology. It is believed to be carefully controlled by cytokines, growth factors and hormones that regulate the synthesis of proteinases, their inhibitors, and structural matrix molecules. Excessive proteolysis can occur as a result of an imbalance between the relative concentrations of a proteinase and its inhibitor. This can be physiologically regulated, as in the growth plate or it can be a pathologic event, such as in arthritis. Ordinarily, the synthesis and degradation of matrix molecules are finely balanced, particularly in cartilages that will survive, such as articular cartilages. In contrast, growth plate cartilages are transient and their matrix is short-lived and this is reflected by the structure of the matrix.

The reason for the increased degradation by MMPs in OA may lay in an ailing feedback mechanism in the cartilage. Collagen turnover, which is extremely slow for type II collagen in mature cartilage with an estimated half-life in the range of 100 years or more (45), increases substantially in osteoarthritis (46,47,109). This excessive cleavage may be caused by the altered feedback regulation of gene expression in chondroctyes by collagen or its degradation products. Evidence for such a mechanism comes from the observations that cultures of human OA articular

cartilage exhibited cyclic release of the collagenase-generated neoepitope of type II collagen (48). Collagen type II or its degradation products may feedback and regulate collagenase synthesis and activity in a cyclic fashion, through a cell surface receptor-mediated mechanism. The original fibroblast studies by Werb and Damsky (49) in 1989 revealed that a fragment of fibronectin containing an RGD (arg-gly-asp) sequence induced the expression of collagenase and stromelysin genes *in vitro*. This induction was shown to be a direct consequence of interaction with an integrin receptor. Subsequently, Homandberg et al. (50) showed that fibronectin fragments can enhance the release of active proteases from cartilage in explant cultures and suggested that fibronectin fragments may contribute to protease-mediated damage to cartilage. Studies done by T. Yasuda, M. Kobayashi, E. Tchetina, and A.R. Poole (Joint Diseases Laboratory, Shriners Hospital for Children, McGill University, Montreal, QC, 1999 ORS Proceedings) have shown that peptides of type II collagen prepared by cleavage of α-chains by cyanogen bromide (CNBr, cleaves at methionine residues) can stimulate an increase in collagenase mediated cleavage of type II collagen in pellet and explant cultures of bovine and human mature articular chondrocytes. Therefore, the interaction of type II collagen with chondrocytes may regulate collagenase activity and that this may be mediated by specific cell suface receptors that signal to the nucleus to alter gene expression.

1.3 Collagen Receptors

These are known to include annexin V (previously known as anchorin CII), the α2β1 and α5β1 integrin receptors, and the two discoidin receptors. Annexin V is a specific receptor for type II collagen in chondrocytes. It binds to the telopeptide region of type II collagen (51,52). It exhibits extensive homology with the calcium-binding proteins calpactin, lipocortin, and protein II. It also binds to type X collagen, as well as to the C-propeptide of type II procollagen. It is released from hypertrophic chondrocytes (51) following upregulation of MMP-13 expression (53). These are potent producers of the collagenase MMP-13 (54) which they use to rapidly resorb type II collagen. Annexin V might function as a mechanoreceptor on the cell surface, sensing strain in collagen fibers. Osteoarthritic cartilage is characterized by a significant upregulation of annexin V message and protein throughout the entire depth of the articular cartilage (51).

Integrins are transmembrane glycoproteins that are important in the adhesion of cells to extracellular matrix components, and may also play a role in cell-cell adhesion (55-59). Integrins are expressed in all cell types examined to date, and consist of α and β heterodimers, of which 15 α subunits and eight β subunits have been identified and cloned. Individual β subunits can heterodimerize with different α subunits and vice versa (although the later case is less frequent), forming a large number of different integrin family members. The β1 family of integrins represent the major class of cell substrate receptors with specificities primarily for collagens, laminins, and fibronectins. Further diversity is achieved by the fact that individual integrins can often bind more than one extracellular matrix ligand, and individual

ligands may be recognized by more than one integrin. The ligands that an integrin can bind will also vary between cell types, making it important to consider integrin function within the context of cell phenotype (57). Integrins may vary in cartilage according to its type, development, and disease. The α_v subunit is expressed more in the superficial zone. In osteoarthritic cartilage the $\alpha 2$, $\alpha 4$ and $\beta 2$ subunits are also expressed. The $\beta 1$ integrin is required for type X collagen deposition in matrix and cartilage growth (60). $\beta 1$ integrin can mediate attachment to type II collagen and fibronectin. Whereas the integrin-recognition sequence RGD inhibits attachment to fibronectin, it has no effect on type II collagen attachment. The major collagen binding integrins are $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 5\beta 1$ (61-66).

Recently two related tyrosine kinase receptors, DDR-1 and DDR-2, were shown to bind helical type II collagen. They may be involved in chondrocyte-collagen II interactions in human articular cartilage in health and disease. Since these two receptors were the focus of this study, a brief review is presented bellow.

1.4 Discoidin Domain Receptors (DDRs)

Discoidin receptors 1 and 2 (DDR-1 and DDR-2) are two closely related tyrosine kinase receptors. Receptor tyrosine kinases (RTKs) are a large family of transmembrane proteins composed of an extracellular ligand binding domain and a cytoplasmic catalytic domain that allow for specific decoding of extracellular signals and initiation of intracellular biochemical effects that regulate fundamentally important processes such as cell division, differentiation, movement, metabolism, and survival (67). The RTK-mediated signal transduction process involves receptor

dimerization, intermolecular autophosphorylation, and the binding of cellular substrates through the interaction of specific receptor phosphotyrosines with src homology 2 (SH2) domain sequences (68). The activated signaling pathways in concert with molecular effector and negative control systems ultimately define the specific response of the cell. Sequence analysis has indicated that vertebrate genomes encode a sizeable number of RTKs, which are commonly classified into subfamilies according to the arrangement of motifs in their extracellular domain (69).

The two recently identified DDRs are distinguished by a domain in their extracellular portions that has not been found in other receptor tyrosine kinases but was first noted in the discoidin I protein of the slime mold Dictyostelium discoideum (70) and thus termed the discoidin I domain. This sequence is found in several other extracellular proteins, notably in the coagulation factors V and VIII. In the slime mold, discoidins are expressed and secreted during the formation of the slug and the fruiting body, and they function as lectins by binding to N-acetyl-galactosamine and galactose (71).

The DDRs have been cloned by several groups and given different names. DDr-1 was previously termed DDR (72), MCK-10 (73), EDDR1 (74), NEP (75), Cak (76), trkE (77), Ptk-3 (78), NTRK4 (79), and RTK6 (80). DDR-2 was previously termed Tyro10 (81), TKT (82), and CCK-2 (78). DDRs have been shown to be present on tumor cells (78,83,84). Using probes for both genes and in situ hybridization on adjacent sections of human ovary or lung carcinomas Alves et al. (78) have shown that DDR-1 is expressed in the tumor cells themselves, whereas DDR-2 is detected in the stormal cells surrounding the tumor.

In 1997, Vogel *et al.* (85) and Shrivastava *et al.* (70) independently identified collagens as ligands that bind and activate the DDRs. This provides an example of ligands shared by integrins and receptor tyrosine kinases and improves our understanding of the mechanisms by which cells perceive and respond to the extracellular matrix in their microenvironment. Vogel reported that the activation of overexpressed DDR-1 was triggered by collagen types I, II, III, IV, and V whereas DDR-2 was only activated by collagen types I, II, III, and V. It was further shown by Vogel *et al.* (85) that receptor activation required the native triple-helical structure of collagen and occurred over an extended period of time (2-18hrs). Collagen activation of DDR-1 was shown to induce phosphorylation of a docking site for the Shc phosphotyrosine binding domain. Activation of DDR-2 by collagen resulted in the up-regulation of matrix metalloproteinase-1 (MMP-1) expression. Therefore, it was suggested that since DDRs are triggered by collagen, and because activated DDR-2 promotes MMP-1 expression, a potential role for these two receptors would be in tumor cell activation and subsequent degradation of the matrix by metalloproteinases. Shrivastava *et al.* (70) proposed that a primary role for DDRs is to sense the quantity and configuration of collagens in the microenvironment and then to regulate the cellular response in terms of adhesion, migration, differentiation, survival, proliferation and matrix production.

1.5 Aim of the Study

Discoidin receptors are thought to play a role in the interactions of chondrocytes with the extracellular matrix of articular cartilage and the regulation of gene expression by these cells in health and disease. Since this interaction may change in osteoarthritis, the aim of this work was to determine whether human adult normal and osteoarthritic articular chondrocytes express and produce DDR-1 and DDR-2 collagen receptors and whether this correlates with the expression of disease related genes such as type X collagen.

CHAPTER 2: MATERIALS and METHODS

2.1 Source of Tissue

Human normal femoral condylar cartilage (7 patients), was obtained at autopsy at the Montreal General Hospital. The samples were taken 10 to 20 hours post mortem from patients with no history of joint disease or cytotoxic medications such as chemotherapy. The cartilage was normal upon macroscopic examination. Human femoral condylar cartilage (8 patients), was obtained from knee replacement surgery for OA at the Montreal General Hospital and the Jewish General Hospital, Montreal, Quebec, Canada (Courtesy of Dr. M Tanzer and Dr. D Zukor, respectively).

2.2 Antibodies (Abs)

A polyclonal rabbit antiserum was generated against a 13 amino acid peptide (Cys, Pro, Ala, Gly, Ser, Val, Phe, Pro, Lys, Glu, Glu, Glu, Tyr) mapping at the Discoidin-1-like domain of the human DDR-1 receptor (N-terminal, Fig. 1). Similarly, a polyclonal rabbit antiserum was made against a 16 amino acid peptide (Cys, Pro, Glu, Ile, Pro, Val, Gln, Pro, Asp, Asp, Leu, Lys, Glu, Phe, Leu, Gln) mapping at the Discoidin-1-like extracellular domain of the human DDR-2 receptor (Fig. 2). These Abs (1mg/ml) were prepared by Dr Fackson Mwale, Joint Diseases Laboratory, Shriners Hospitals for Children, Montreal, Quebec, Canada. F(ab′)2 preparation of Abs is presented below. Non-immune rabbit serum was used as negative control. Commercial polyconal Abs against human, rat and mouse DDR-1 (rabbit, 200 μg/ml), and DDR-2 (goat, 200 μg/ml) were from Santa Cruz Biotechnology (Santa Cruz, CA). Epitopes corresponded to amino acid sequences mapping at the carboxy terminus of the human receptors.

To control for the specificity of binding, both sets of Abs were pre-absorbed with their respective immunizing peptide epitopes (from Santa Cruz Biotechnology, in case of the commercial Abs). The peptides were used in five times the concentration of Ab (e.g. at 1/40 dilution or 5 μg/ml of the commercial Ab, 25 μg/ml of the peptide was added). Then the peptide-Ab mixture was incubated at 37°C for 1 hr and then at 4°C overnight.

Preparation of polyclonal antibodies. For anti-peptide antiserum production, female New Zealand White rabbits weighing 2.5-3.0 kg (Ferme des Chenes Bleues Inc. Montreal, Quebec, Canada) were initially immunized intramuscularly (thigh muscle) with 0.5 mg of peptide epitope conjugate with ovalbumin (OVA) in 0.25 ml phosphate buffer saline (PBS) [8.5g NaCl (BDH), 1.6g Na_2HPO_4 (BDH), 0.5g KH_2PO_4 (Fisher Scientific), 0.5g NaAzide (BDH), pH = 7.2)] and emulsified with 0.25 ml of complete Freund's adjuvant (Difco Laboratories. Inc., Detroit, MI). Booster injections of similar quantities of peptide-OVA emulsified with incomplete Freund's adjuvant (IFA; Difco Inc.) were given intramuscularly every 2-3 weeks. After the second booster, test bleeds were performed and antibody titers were determined by ELISA, as described below. In all cases, good titers were obtained after the two boosters. Animals were exsanguinated by cardiac puncture under anaesthesia and ~ 50 ml of serum was obtained.

Preparation of F(ab')$_2$. For immunoassays, F(ab')$_2$ Abs were obtained by pepsin digestion of each antiserum, as previously described (86). Briefly, the partially purified IgG was digested with pepsin at pH 4.0 for 8 hr. F(ab')$_2$ was concentrated and separated from low molecular weight digestion products by precipitation with 50% saturated ammonium sulfate. The Fc portion and undigested IgG were removed by AH-Sepharose-Protein A chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden).

ELISAs for determining antibody titer and specificity. The immunizing peptides (DDR-1 & DDR-2) conjugated to bovine serum albumin (BSA), were diluted to 20 μg/ml in 0.1 M carbonate buffer, pH 9.2 and 50 μl was added to each well (1 μg/well) of Immulon-2 flat-bottom tissue culture microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). After 24 hr at 4°C, the plates were washed three times with PBS containing 0.1% vol/vol Tween 20 (PBS-Tween; Sigma Chemical Co.). Non-coated binding sites were blocked by 150 μl/well of 1% wt/vol bovine serum albumin (BSA) in PBS (PBS-1% BSA) for 30 minutes at room temperature. The plates were washed once with PBS-Tween and 50 μl of serial doubling dilutions of the appropriate polyclonal antiserum preparation were added to individual wells. After 90 min at 37°C, the plates were washed three times with PBS-Tween. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1: 30,000 in PBS-1% BSA-Tween was added at 50 μl/well. After 1 hr at 37°C, the plates were washed three times with PBS-Tween and once with distilled water. Finally, 50 μl of freshly prepared alkaline phosphatase substrate, disodium *p*-nitrophenyl phosphate (Sigma) at 0.5mg/ml in 8.9 mM diethanolamine, 0.25mM $MgCl_2$, pH 9.8 was added to each well for 20-30 min at 37°C. The absorbances were measured at 405 nm on a plate reader (model Multiskan-MkII; ICN/ Flow, Mississauga, Ontario, Canada).

2.3 Immunofluorescent localization

Fresh human normal and osteoarthritic cartilage was embedded in the O.C.T. compound (polyvinyl alcohol, ethylene glycol, Tissue-Tek, Sakura, Torrance, CA). It was then cut into 6 μm full depth sections (from the superficial to the deep zone of cartilage) using a cryostat and attached to microscope slides pretreated with 3-aminopropyltriethoxysilane

(87) to ensure attachment. Briefly, a 2% solution of aminoalkylsilane in dry acetone was prepared by adding 5 ml 3- aminopropyltriethoxysilane [Sigma A-3648, stored at 4°C in a tightly closed container of anhydrous calcium sulfate (Drierite)] to 250 ml of dry acetone (99.5% pure).The 2% working solution was freshly prepared prior to use. The slides were dipped in the 2% AAS solution (250 ml) for 10 sec, rinsed 3 times in deionized water (5 dips each) and air dried overnight. The experimental slides with sections were stored at -20°C.

The tissue sections were fixed in 4% formaldehyde [freshly prepared from paraformaldehyde (BDH) in PBS, pH 7.6] for 5 min at room temperature, and then washed three times with PBS. The endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol (99.8%) for 10 min at room temperature. The sections were then washed in PBS and digested with chondroitinase ABC (ICN, 5u/ml) for 90 min at 37°C to remove the chondroitin sulfate chains of proteoglycans. After a wash in 0.1% BSA in PBS, the non-specific binding of the secondary Ab was blocked with 5% normal blocking serum (in PBS) of the same species for 30 min at room temperature. The slides were then incubated at room temperature with different dilutions (e.g. 1/40, 1/80, etc.) of Abs against the discoidin 1 and 2 receptors (either commercial or Dr Mwale's, see section 2.2) for 30 min. After three washes in 0.1% BSA in PBS, the slides were incubated for 45 minutes with the Alexa-bound secondary Ab (1/30 dilution in PBS of 60 µg/ml stock, 488 Ab, Molecular Probes, Eugene, OR). They were washed 3 times in PBS. The nuclei of the cells were counterstained in 1/100 dilution of ethidium bromide (EtBr, Sigma) solution (25 µg/ml ethidium bromide in 5% w/v EDTA in deionized water with 0.05% w/v NaAzide) for 1 min. The slides were rinsed in deionized water and wet mounted in

0.2M Tris (pH, 8.6) and glycerol (1: 9) and viewed within 24 hr with a Zeiss fluorescent photo microscope III.

2.4 Isolation of Chondrocytes from the Cartilage Matrix

Slices of both human normal and OA cartilages were removed aseptically using a scapel. The cartilage was washed twice in tissue culture medium (CM, see section 2.6) containing 1% Fungizone (Gibco) and weighed. The cartilage was finely chopped into cubes (2-3mm^3), and the chondrocytes released by sequential enzymatic digestion on a shaker at 37° C (88). Briefly the cartilage was incubated with 20ml of protease (Type I, from bovine pancreas, Sigma) at a concentration of 4mg/ml in CM, for an hour. The protease was removed and the cartilage chips washed in two 15ml volumes of CM. Twenty milliliters of Type IA collagenase (Sigma) at a concentration of 3 mg/ml in CM was added and incubated until all the cartilage had been digested. The cells, tissue and media were periodically removed from the container, passed through a cell strainer (40 μm, Falcon) to remove any cell debris and centrifuged (300g, 10min) to remove newly released chondrocytes. The undigested tissue and CM were returned to the digestion mixture. The released chondrocytes were washed twice in CM (300g, 7 min). The cells were counted on a haemocytometer. The cells were placed ($5x10^5$ cells/ml) into 25cm^2 tissue culture flasks (Falcon) coated with 2% agarose (Type VII, Sigma) and cultured for 24 hrs (37° C, 5% CO_2) in CM to allow for restoration of cell surface receptors.

2.5 Human Culture Medium (pH=7.4)

Hams F12 (Gibco) was supplemented with 2 mM L-Glutamine (Gibco) with 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 25 mM HEPES (Gibco), 1 mg/ml BSA (Sigma) and 10 µl/ml ITS [from stock solution containing 25mg insulin, 25mg transferrin, and 25µg sodium selenite (Boehringer Mannheim) in 50ml dH2O].

2.6 Total RNA Extraction and Isolation

About 10^7 isolated chondrocytes were used for each RNA isolation using a method modified from Chomczynski *et al.* (89). Briefly, the cells were solubilized in 6ml solution D [4M guanidine isothiocynate (Gibco), 25 mM sodium citrate (BDH), pH 7.0, 0.5% sarcosyl (Sigma), 100 mM 2-mercaptoethanol (Sigma)]. The cell mixture was homogenized by vortexing for 30 minutes. After centrifugation (500g, 10 min), the proteins and nucleic acids were precipitated by addition of 6 ml isopropanol (Fisher Scientific) overnight at -20° C. To pellet proteins and nucleic acids, the cell mixture was centrifuged for 20 min at 10,000g. The pellet was digested with 600µl Digestion buffer [10mM Tris, 5mM ethylene diamine tetra acetic acid (EDTA), 1% sodium dodecyl sulphate (SDS), pH 8.0] and 200 µl molecular biology grade proteinase K (14mg/ml, Roche) in a water bath at 50° C for 2hrs. The mixture was then extracted with 1 vol phenol (Gibco), 0.2 vol chloroform (Roche)-isoamylalcohol (Fisher Scientific) (48:1), and 0.1 vol 2 M sodium acetate, pH 4.0 and centrifuged at 10,000g (20 min, 4°C).

RNA was present in the aqueous phase which was removed and precipitated with 2 vol of 100% ethanol and 0.1 vol 3M sodium acetate overnight at -20°C. The RNA was recovered by centrifugation (16,000g, 30 min). The pellet was washed with 70% ethanol

to remove any excess salt and air-dried. The total RNA pellet was resuspended in diethyl pyrocarbonate (Sigma) treated (DEPC) water and the optical density (O.D) at 260 and 280 nm were recorded in a 1cm path length cuvette to quantitate the RNA content. (1 O.D. 260nm = 40μg RNA/ml) and assess the purity of the preparation (O.D 260/280nm > 1.8).

2.7 Reverse Transcription of RNA

Five micrograms of total RNA (from human normal or OA chondrocytes) were mixed with 1 μl oligo-dT (0.5 mg/ml, Pharmacia Biotech, Uppsala, Sweden) in sterile, distilled water to 11 μl, and heated to 70° C for 10 min in a thermal cycler (MJ Research, Inc. Watertown, MA). Then the mixture was quick chilled on ice and briefly centrifuged. Next, 4 μl of 5x First Strand Buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μl 0.1M 1.4-dithiothreitol (DTT) (both Gibco) and 1 μl 10mM 2′-deoxynucleoside 5′- triphosphate (dNTP) Mix (10 mM each dATP, dCTP, dGTP, dTTP at neutral pH, Pharmacia Biotech) were added and the mixture was incubated at 42 ° C for 2 min. Then 1μl (200 units) of SUPER SCRIPT II (RNase H Reverse Transcriptase, Gibco) was added and the mixture was incubated for 50 min at 42 ° C. The reaction was inactivated by heating at 70 ° C for 15 min.

2.8 Polymerase Chain Reaction (PCR)

One microliter of the cDNA prepared above was incubated with 2.5 μl 10x Buffer (Roche, 15 mM $MgCl_2$) 16 μl sterile, distilled water, 1μl dNTP Mix (Pharmacia Biotech), and 2 μl each of upstream and downstream oligonucleotide primers in a 100 μl

reaction tube. Following a cycle of denaturation at 95°C (10 min), 0.5 µl of Taq polymerase (Roche, 5 u/ml) was added to the mixture. Generally, the PCR protocol was 30 cycles at 94 °C for 1min, 50 °C for 1min and 72 °C for 5min. PCR product sizes were verified by electrophoresis (70 min at 100 volts, E-C apparatus corporations power supply) in 1% agarose gels in TAE (TAE = 40 mM Tris, 20 mM acetic acid/ 1 mM EDTA) containing 5 µl of a 1 mg/ml solution of EtBr in water. A 100 base pair DNA ladder was ran alongside the samples (Amersham). The primers (both from the coding region), GTGCTGATGCTCTGTAG (forward, 2731-2747) and GCCTATATGAGCTGATGCTTC (reverse, 2861-2881) were used in PCR to amplify the nucleotide sequence corresponding to the human DDR-1 receptor. For the human DDR-2 the primers (from the coding region) GAGAAGAGATACGAAGAACC (forward, 2849-2868) and CTGAGAGACAGAGGCTTGTT (reverse, 3021-3042) were used to amplify the sequence. To amplify human collagen type X, primers from the coding region, ACAGGAATGCCTGTGTCTGCTTTTACT (forward, 1653-1680) and GACCAGGTGTGGCTCCAGCTTCCCAATGC (reverse, 1956-1985) were used. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplifyed using the primers (from the coding region) GCTCTCCAGAACATCATCCCTGCC (forward, 605-628) and AGCTCATTTCCTGGTATGACAACG (reverse, 927-950) (primers courtesy of Dr Elena Tchetina, Joint Diseases Laboratory, Shriners Hospitals for Children, Montreal, Quebec, Canada). The PCR products were sequenced (Sheldon Biotechnology center, McGill University, Montreal, Quebec) to confirm their identity.

CHAPTER 3: **RESULTS**

Using the techniques described, femoral condyles of normal and osteoarthritic cartilages were examined for the presence of the DDR-1 and DDR-2 proteins and for the expression of the discoidin genes. Immunofluorescence histochemistry was used to examine fixed frozen sections of human articular cartilage while the gene expression studies were performed on isolated chondrocytes.

3.1 Localization Results

Human normal and OA femoral condylar cartilages were stained with the anti-peptide Abs (prepared by Dr F. Mwale) for discoidin-1 and discoidin-2. Figures shown are the results of 1/40 dilutions (25 μg/ml of Ab). To control for the specificity of staining, the Abs were pre-absorbed with the peptide epitope (125 μg/ml of peptide). As a negative control for staining, the sections were incubated with normal rabbit Fab fragments.

In 35 year old female normal cartilage, the section incubated with the DDR-1 Ab (Fig 3A) did not show staining when compared to the negative control (Fig 3B). The section stained with DDR-2 Ab was positive with pronounced green fluorescent cellular staining from the articular surface to the deep zone of the cartilage (Fig 3C & 3D). The nuclei of the cells fluoresced red/orange when stained with EtBr. These were clearly visible suggesting staining in the cytoplasm as well as the cell membrane.

The sections in figures 4A and 4B stained with DDR-2 show cellular staining. In the sections stained with Ab pre-absorbed with the peptide epitope (Fig 4C & 4D), the intensity of staining was visibly reduced. This observation confirmed that the cellular

staining observed was specific. Figure 5 shows other sections from the same normal tissue positively stained with DDR-2 Ab. Some of the staining is clearly focal and appears granular.

Cartilage from a normal 89 year old female, localized with DDR-1 Ab (Fig 6A) was also unstained as compared to a section incubated with normal rabbit Fab (Fig 6B). Another section from the same sample localized with DDR-2 Ab, again showed pronounced cellular staining throughout the section (Fig 6C).

Sections of OA cartilage from a 67 year old female did not show staining with DDR-1 Ab (Fig 7A). Sections from the same sample incubated with DDR-2 Ab showed positive cellular staining (Figures 7B, 8A & 8B) which could be clearly blocked with the peptide-absorbed Ab (Figures 7C & 8C).

Since fluorescence was weak in the sections stained with the commercial Abs against the discoidin receptors, a clear image could not be produced.

3.2 Analysis of Gene Expression

To investigate the expression of DDR genes, RNA extracted from isolated human adult normal and OA chondrocytes was analyzed by separate RT and PCR reactions. The expression pattern of DDR-1, DDR-2 and collagen type X were studied. Collagen type X, a short chain collagen first isolated from chick hypertrophic chondrocytes, is a specific marker for hypertrophic chondrocytes of the growth plate (90). It has been shown to be upregulated in human OA articular cartilage (39). It was used in the gene expression analysis to identify chondrocyte hypertrophy.

Chondrocytes from seven human normal and eight OA femoral condylar cartilages were examined. GAPDH was used as a house keeping gene to demonstrate equivalent cDNA loading in the gene expression experiments. Figure 9A shows the GAPDH results for the seven normal samples. The same results were observed for the eight OA samples (Fig. 9B).

The DDR-1 mRNA was found to be weakly expressed in all normal samples (Fig. 10A). No expression of DDR-1 was found in any of the OA samples (Fig. 10B). The DDR-2 mRNA was strongly expressed in all normal samples (Fig. 11A). DDR-2 expression pattern was reduced in two OA samples (Fig. 11B, 57M and 64M) while one of the samples did not express the gene (Fig. 11B, 81F). The only normal sample showing the expression of type X collagen was from a 58 year old male (Fig. 12A, 58M). Although macroscopically normal, this sample might have contained pre-osteoarthritic lesions. Early degenerative lesions observed at autopsy have been found to have OA-like features (G.R. Webb, A.R. Poole *et al.* manuscript in preparation). Type X collagen was expressed in six out of eight OA samples (Fig. 12B). Such expression pattern was expected in this group. The human gene expression results are summarized in Table 1.

1 mgpealssll llllvasgda dmkghfdpak cryalgmqdr tipdsdisas sswsdsta
61 hsrlessdgd gaw**cpagsvf pkeeeyl**qvd lqrlhlvalv gtqgrhaggl gkefsrsyrl
121 rysrdgrrwm gwkdrwgqev isgnedpegv vlkdlgppmv arlvrfypra drvmsvclrv
181 elygclwrdg llsytapvgq tmylseavyl ndstydghtv gglqygglgq ladgvvgldd
241 frksqelrvw pgydyvgwsn hsfssgyvem efefdrlraf qamqvhcnnm htlgarlpgg
301 vecrfrrgpa mawegepmrh nlggnlgdpr aravsvplgg rvarflqcrf lfagpwllfs
361 eisfisdvvn nsspalggtf ppapwwppgp pptnfsslel eprgqqpvak aegsptaili
421 gclvaiilll lliialmlwr lhwrrllsae rrvleeeltv hlsvpgdtil innrpgprep
481 ppyqeprprg npphsapcvp ngsaysgdym epekpgapll ppppqnsvph yaeadivtlq
541 gvtggntyav palppgavgd gpprvdfprs rlrfkeklge gqfgevhlce vdspqdlvsl
601 dfplnvrkgh pllvavkilr pdatknarnd flkevkimsr lkdpniirll gvcvqddplc
661 mitdymengd lnqflsahql edkaaegapg dgqaaqggpti sypmllhvaa qiasgmryla
721 tlnfvhrdla trnclvgenf tikiadfgms rnlyagdyyr vqgravlpir wmawecilmg
781 kfttasdvwa fgvtlwevlm lcraqpfgql tdeqvienag effrdqgrqv ylsrppacpq
841 glyelmlrcw sreseqrppf sqlhrflaed alntv

Figure 1. Discoidin domain receptor 1 protein sequence. The amino acids in bold were used to produce the antibody.

1 mipiprmplv lllllllilgs akaqvnpaic ryplgmsggh ipdeditass qwsestaaky
61 grldseegdg aw**cpeipvqp ddlkeflq**id lrtlhfitlv gtqgrhaggh giefapmyki
121 nysrdgsrwi swrnrhgkqv ldgnsnpydv flkdleppiv arfvrlipvt dhsmnvcmrv
181 elygcvwldg lvsynapagq qfvlpggsii ylndsvydga vgysmteglg qltdgvsgld
241 dftqtheyhv wpgydyvgwr nesatngfie imfefdrirn fttmkvhcnn mfakgvkifk
301 evqcyfrsea seweptavyf plvlddvnps arfvtvplhh rmasaikcqy hfadtwmmfs
361 eitfqsdaam ynnsgalpts pmapttydpm lkvddsntri ligclvaiif illaiiviil
421 wrqfwqkmle kasrrmldde mtvslslpse ssmfnnnrss spseqesnst ydrifplrpd
481 yqepsrlirk lpefapgeee sgcsgvvkpa qpngpegvph yaeadivnlq gvtggntycv
541 pavtmdllsg kdvaveefpr kllafkeklg egqfgevhlc evegmekfkd kdfaldvsan
601 qpvlvavkml radanknarn dflkeikims rlkdpniirl lavcitedpl cmiteymeng
661 dlnqflsrhe plsscssdat vsyanlkfma tqiasgmkyl sslnfvhrdl atrnclvgkn
721 ytikiadfgm srnlysgdyy riqgravlpi rwmswesill gkfttasdvw afgvtlwetf
781 tfcqeqpysq lsdeqvient geffrdqgrq iylpqpalcp dsvyklmlsc wrretkhrps
841 fqeihllllq qgae

Figure 2- Discoidin domain receptor 2 protein sequence. The amino acids in bold were used to produce the antibody.

*** Both protein sequences obtained from:**
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein
Using search terms, DDR1 and DDR2

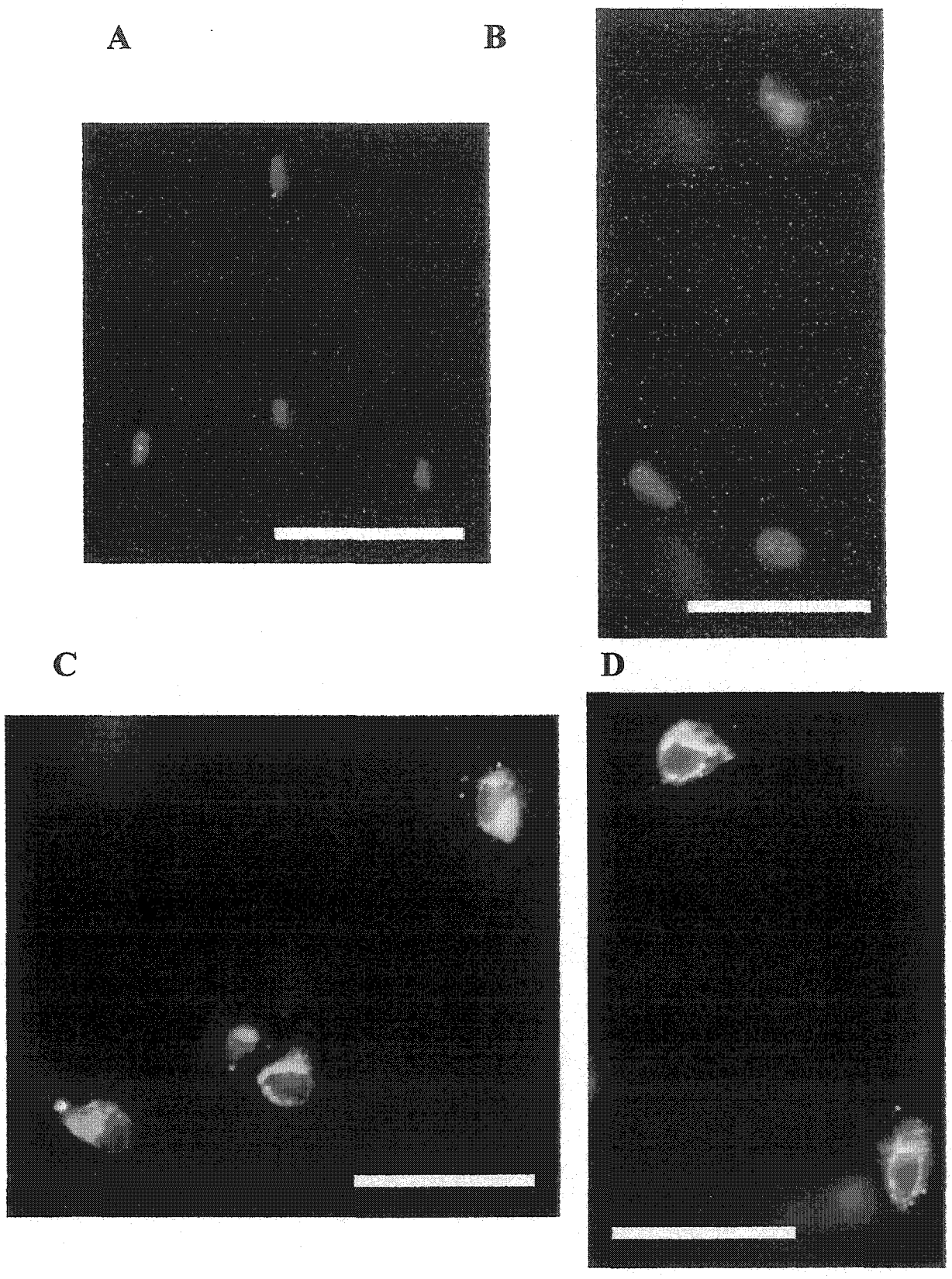


Figure 3 .Immunolocalization in human normal femoral condylar cartilage (35 year old female). (A) Section stained with DDR-1 Ab, bar = 50μm (B) Section stained with normal rabbit Fab. (C & D) Sections stained with DDR-2 Ab. B & C, bar = 30μm.

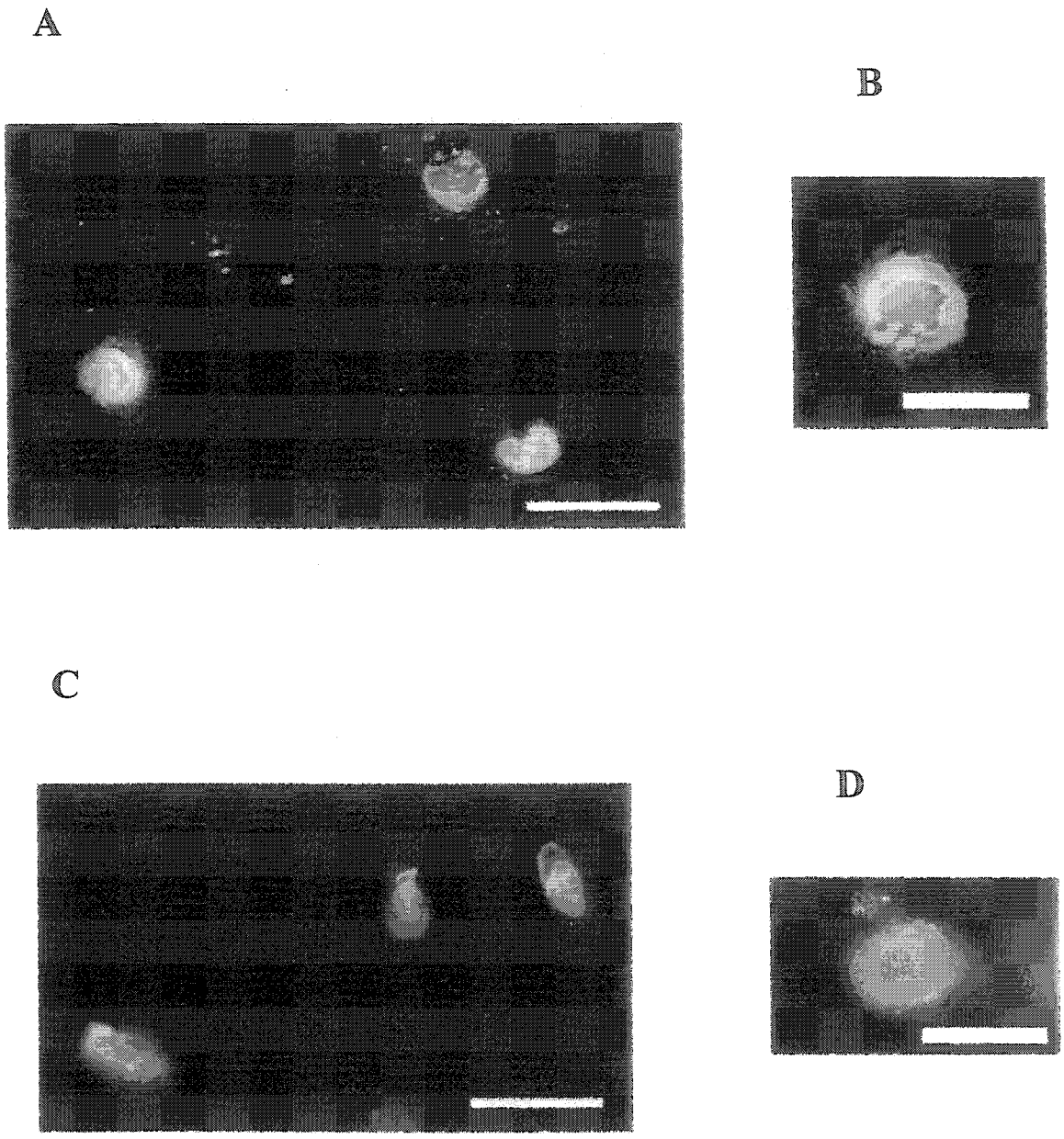


Figure 4. .Immunolocalization of DDR-2 receptor in human normal femoral condylar cartilage (35 year old female). (A,B) Sections stained with DDR-2 Ab. (C,D) Control sections stained with peptide absorbed DDR-2 Ab. A & C, bar = 30μm. B & D, bar = 10 μm

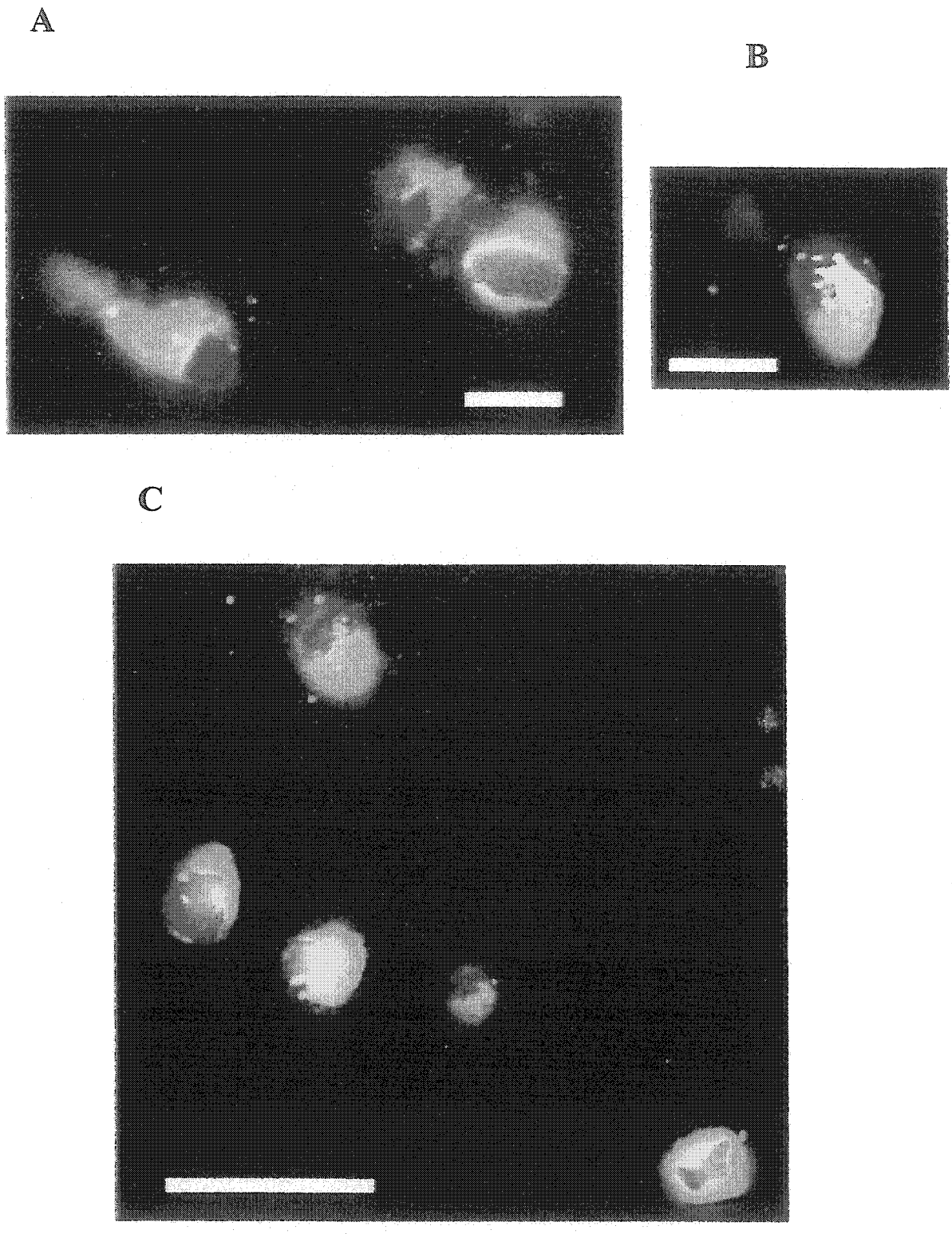


Figure 5. Immunolocalization of DDR-2 receptor in human normal femoral condylar cartilage(35 year old female). (A, B) Bar = 10μm (C) Bar = 30μm

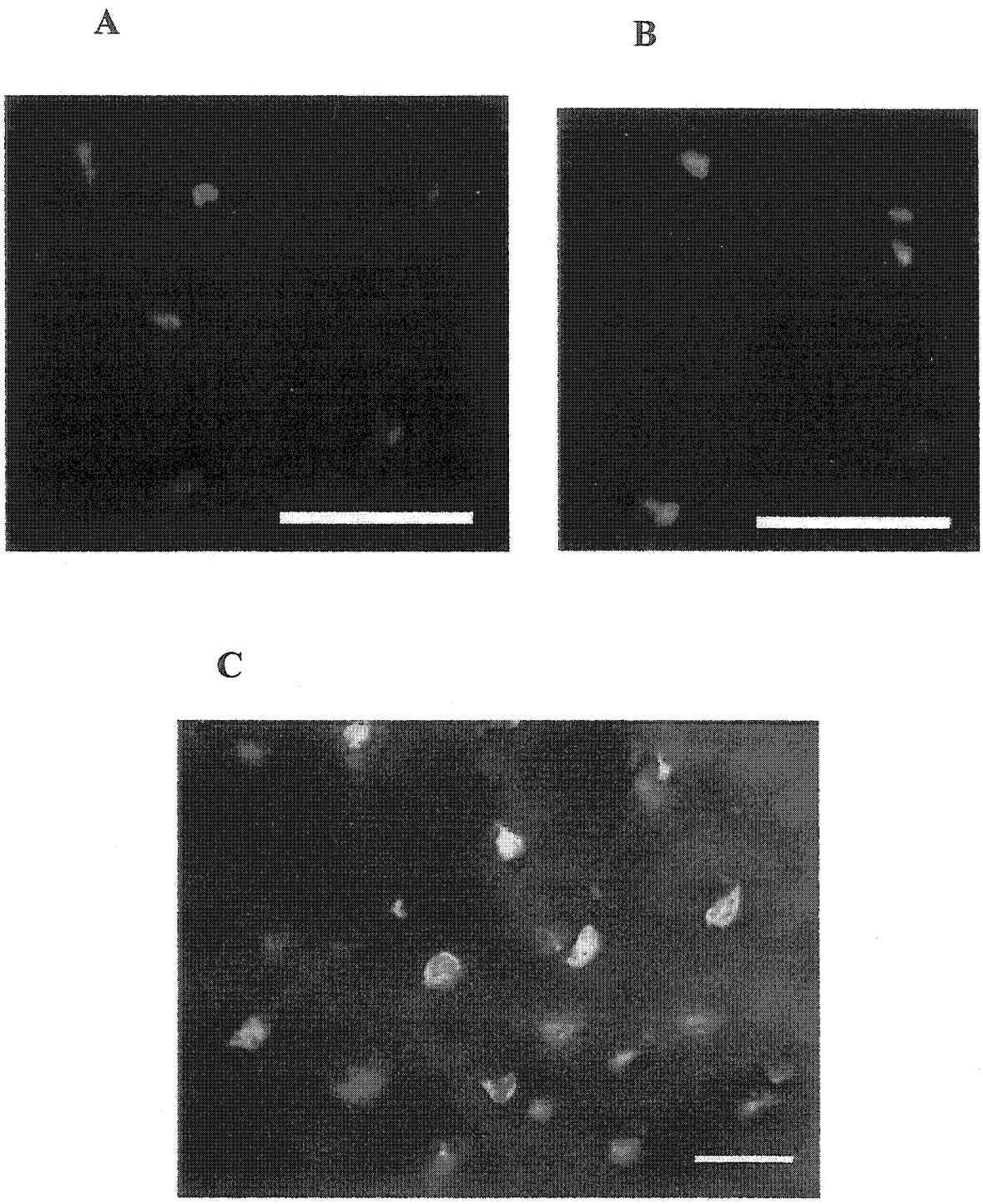


Figure 6. Immunolocalization in human OA femoral condylar cartilage (89 year old female).(A) Section stained with DDR-1Ab. (B) Section stained with normal rabbit Fab. (C) Section stained with DDR-2Ab. A & B, bar = 50μm. C, bar = 30μm.

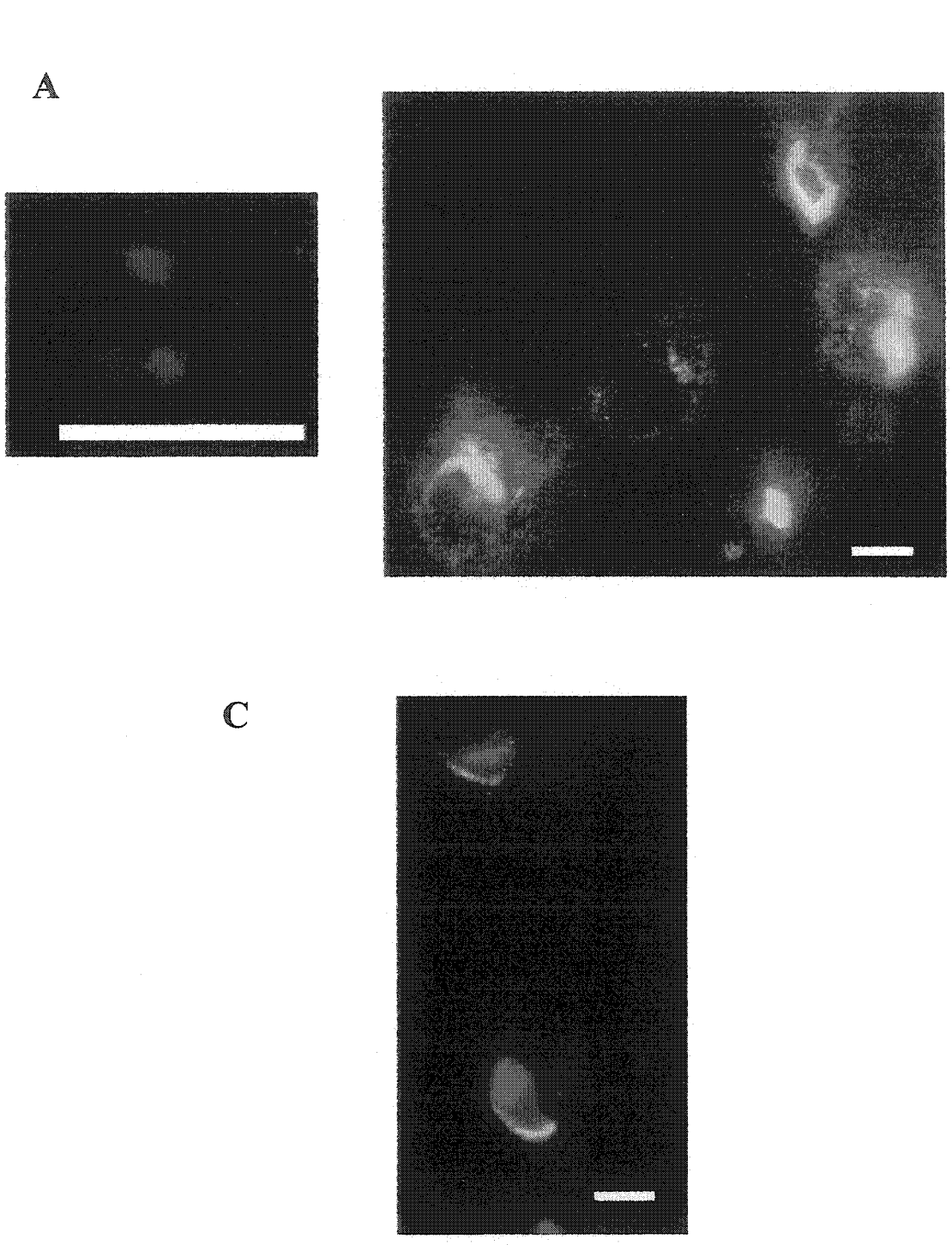


Figure 7. Immunolocalization in human OA femoral condylar cartilage (67 year old female). (A) Section stained with DDR-1 Ab, (B) Section stained with DDR-2 Ab,(C) Control section stained with peptide absorbed DDR-2 Ab. A, bar = 30μm. B & C, bar = 10μm.

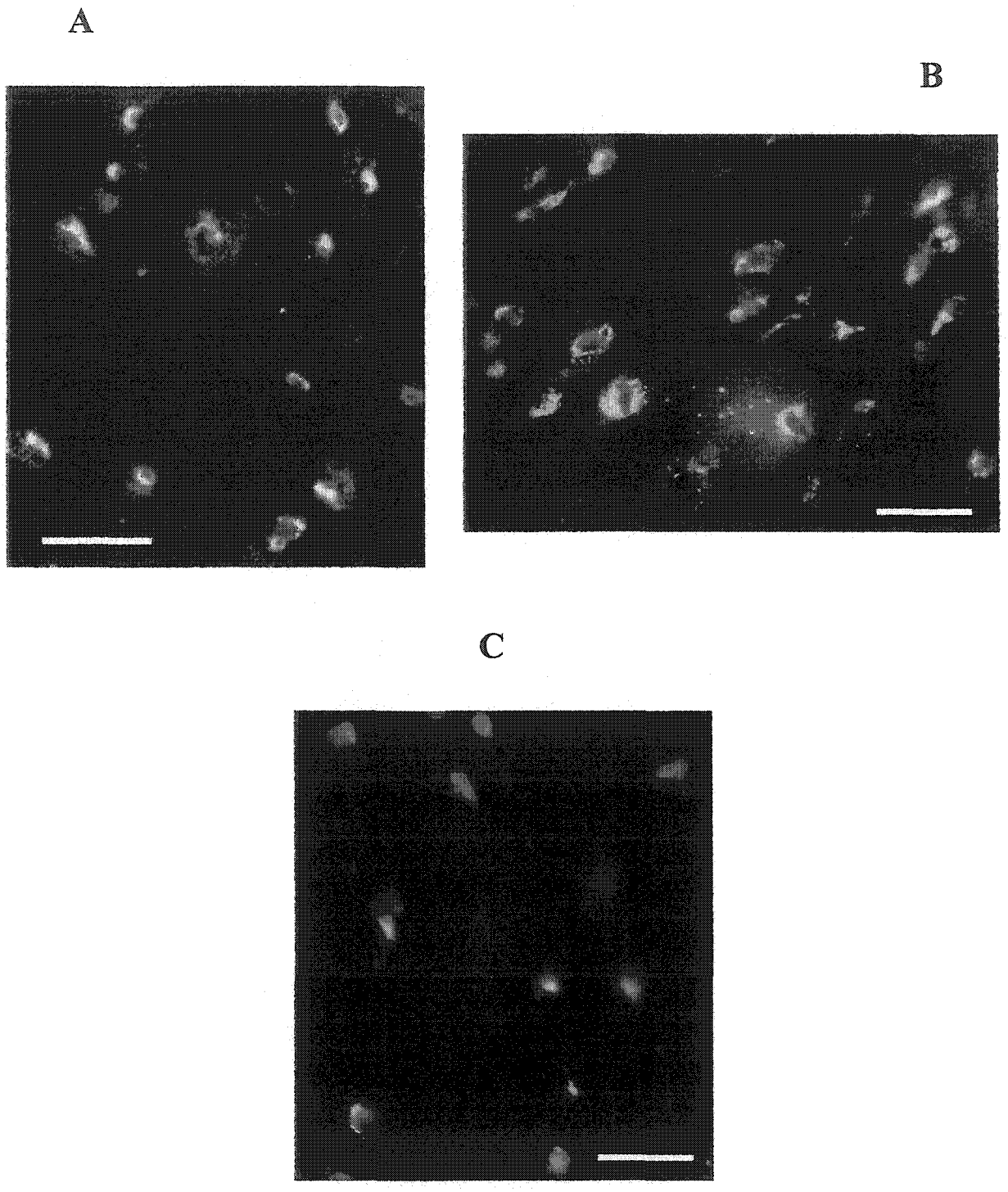


Figure 8. Immunolocalization in human OA femoral condylar cartilage (67 year old female).(A & B) Sections stained with DDR-2 Ab. (C) Control sections stained with peptide absorbed DDR-2 Ab. A, bar = 50μm. B & C, bar = 30 μm

GAPDH Expression

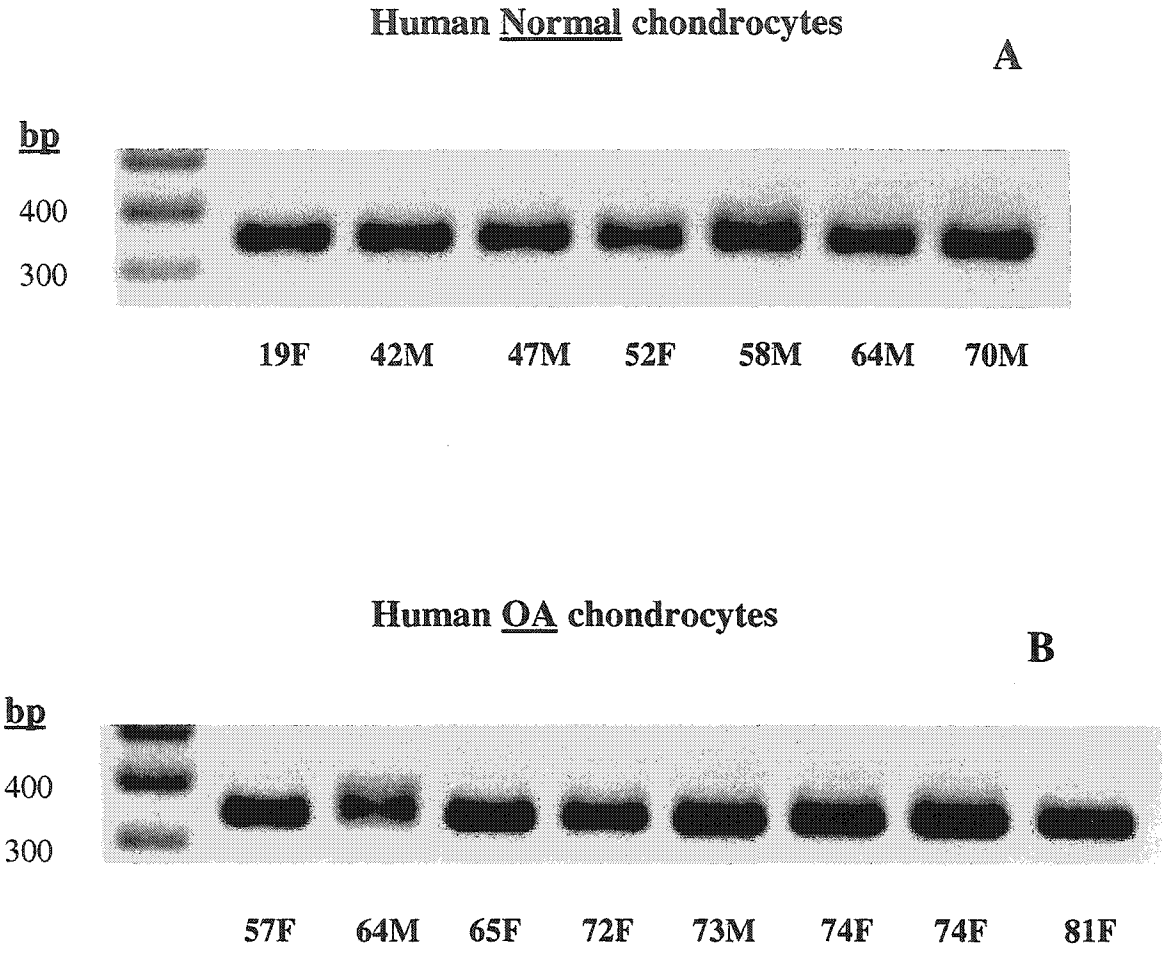
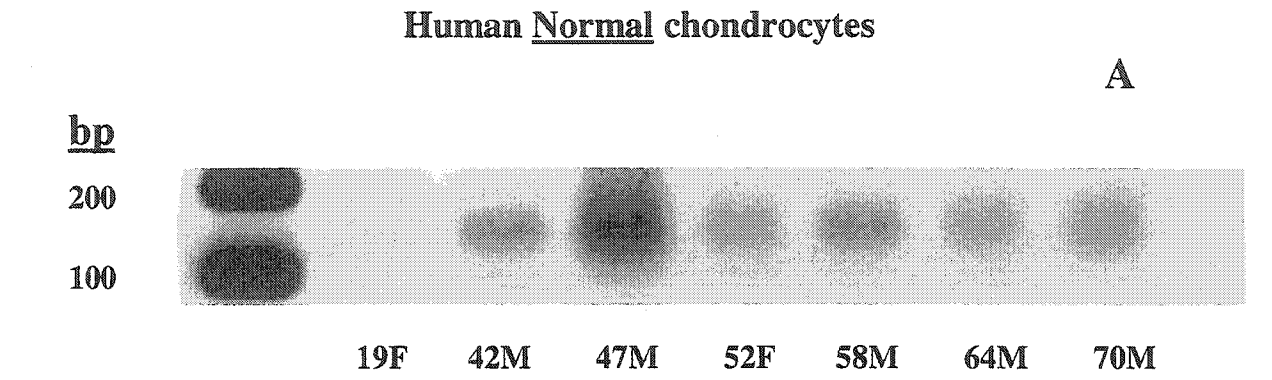


Figure 9. (A) Normal articular chondrocytes, n = 7.
(B) OA articular chondrocytes, n = 8.
GAPDH gene fragment = 350 bp

DDR-1 Expression



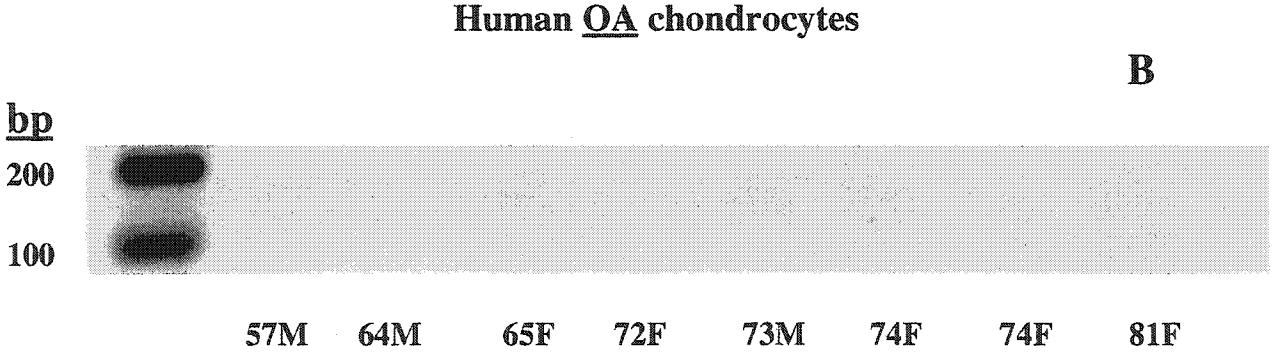


Figure 10. Discoidin receptor-1 gene expression. (A) Normal articular chondrocytes, n = 7. (B) OA articular chondrocytes, n = 8.
DDR-1 gene fragment = 150 bp

DDR-2 Expression

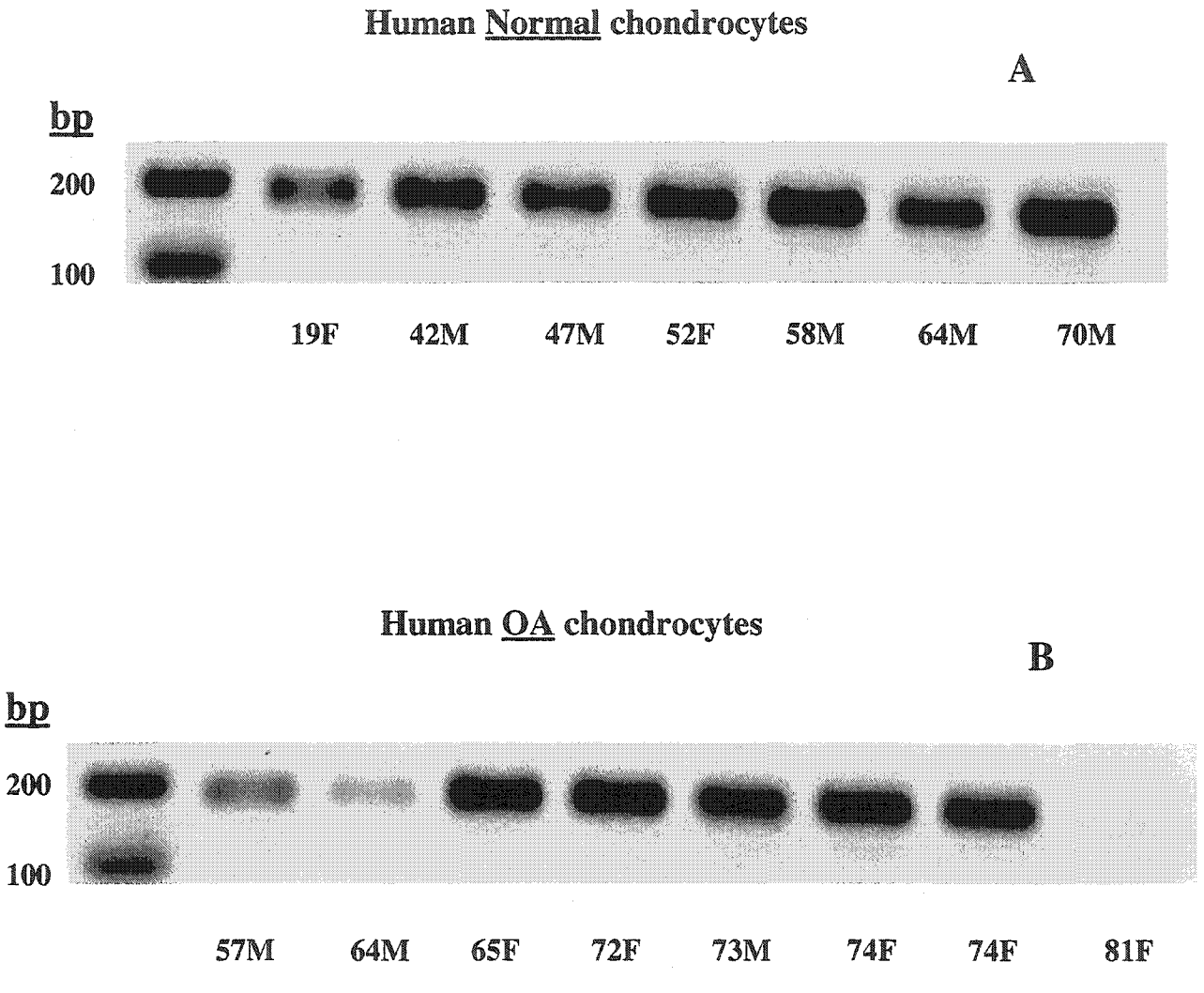


Figure 11. Discoidin receptor-2 gene expression. (A) Normal articular chondrocytes, n = 7. (B) OA articular chondrocytes, n = 8. DDR-2 gene fragment = 192 bp

Type X Collagen Expression

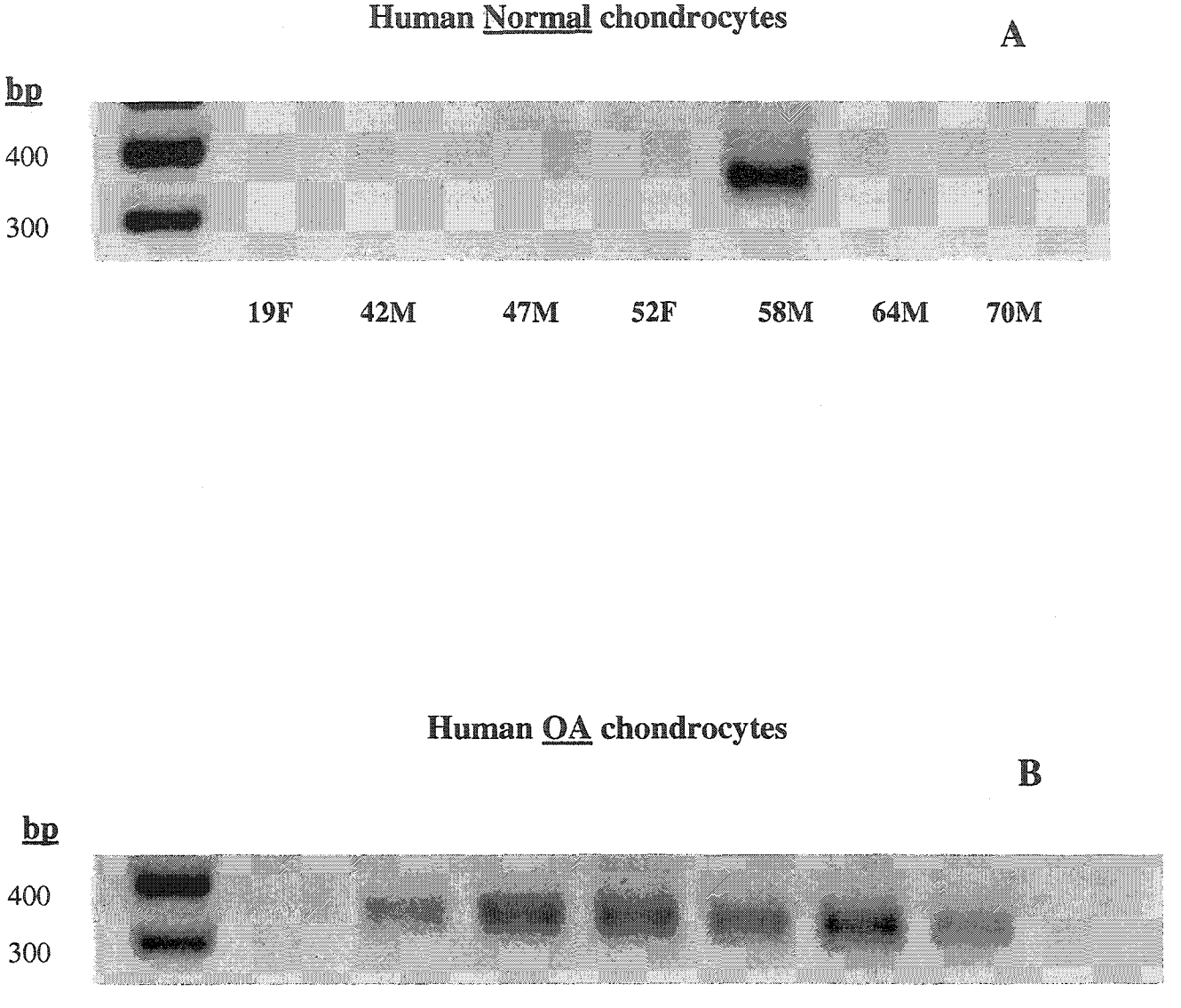


Figure 12 . Collagen type X gene expression. (A) Normal articular chondrocytes,n = 7. (B) OA articular chondrocytes, n = 8.
Type X collagen gene fragment = 360 bp

Table 1: RT- PCR Results of Human Adult Articular Chondrocytes

Cartilage	Age	Sex	DDR1	DDR2	Col X
Normal	19	F	+/-	++	-
	42	M	+	++	-
	47	M	+	++	-
	52	F	+	++	-
	58	M	+	++	+
	64	M	+	++	-
	70	M	+	++	-
OA	57	F	-	+	+/-
	64	M	-	+	+
	65	F	-	++	++
	72	F	-	++	++
	73	M	-	++	+
	74	F	-	++	++
	74	F	-	++	+
	81	F	-	-	-

PCR Band :

+/- : Detectable
+ : Clearly defined
++ : Strong

CHAPTER 4: **Discussion**

4.1 Overview

Osteoarthritis (OA) is a major worldwide health problem due to its debilitating and progressive nature. It results in high morbidity and a marked decrease in the quality of life. Improvements in understanding the fundamental pathways governing the health and disease of articular cartilage have lead to significant advances in the medical and surgical management of OA. The classic loss of articular cartilage seen in OA is associated at the molecular level with increased denaturation and loss of type II collagen by collagenases. The increased cleavage of type II collagen correlates with increased expression and content of various metalloproteinases including stromelysin-1 (MMP-3), gelatinases A (MMP-2), and B (MMP-9) and collagenase-1 (MMP-1), collagenase-2 (MMP-8), collagenase-3 (MMP-13) and membrane type 1-MMP (MT1-MMP)(1).

What causes the increase in synthesis and activation of these MMPs at the cellular level is still not clearly understood. There is evidence for an involvement of cytokines such as interleukin-1 and tumor necrosis factor α and cell matrix interactions (91). It is possible that some collagen fragments may stimulate chondrocyte-mediated cartilage resorption via cell surface receptor activation just as in fibroblasts, MMP-1 is upregulated through an RGD-integrin receptor activation (49).

In this study the presence of the DDR-1 and DDR-2 receptors and the expression of these genes were examined for the first time in human adult femoral condylar cartilages. These two receptors of the discoidin domain receptor tyrosine kinase subfamily bind and are activated by collagens, in particular the fibril-forming collagens (70, 85).

Little is known about the possible downstream events mediated by DDRs. DDR-1 is able to bind the adaptor protein Shc, however, the MAP kinase pathway is apparently not activated under these conditions (85). *In vitro* activation of DDR-2 has been reported to induce expression of the collagenase, MMP-1 (85), one of a group of enzymes that are involved in remodeling the ECM during morphogenesis, tissue repair (92), and in arthritis (14,104). It has been shown that metalloproteinases can control the availability of several growth factors to their receptors (93-96). This raises the interesting possibility that collagen-induced activation of DDR-2 results in degradation of the ECM and allows crosstalk with other signaling systems.

The physiological functions of the widely expressed DDRs are not well understood. Recently, it was shown that DDR-1 signaling is essential for cerebellar granule cell differentiation (97). Interestingly, DDR-1 is over expressed in fast-growing tumors (78), suggesting a possible role for DDRs as collagen sensors on the surface of tumor cells (85).

To analyze the *in vivo* role of DDR-1, Vogel et al. (98) introduced a deletion into the DDR-1 gene in the mouse germ line. Mice lacking DDR-1 were small, and mutant females showed multiple reproductive defects. The majority of females were unable to give birth because developing blastocysts did not implant. Successfully reproducing females were unable to nourish their litters because the mammary gland epithelium failed to secrete milk. Independently, Labrador et al. (99) generated DDR-2 deficient mice. Lack of DDR-2 signaling was compatible with prenatal development, yet it reduced chondrocyte proliferation and bone growth during postnatal development. Wounded skin of DDR-2 null mice contained fewer proliferating cells than skin of wild-type mice. They

concluded from these physiological studies, combined with *in vitro* proliferation assays that there is a requirement for DDR-2 in the proliferation of chondrocytes and fibroblasts.

It was therefore hypothesized that discoidin receptors may play a role in the interactions of chondrocytes with the ECM and the regulation of gene expression by these cells in osteoarthritic articular cartilage may change in relationship to the induction of pathology. In this study the expression and production of DDRs by human adult normal and OA articular chondrocytes and their correlation with expression of OA related genes, such as type X collagen, was investigated.

4.2 Immunolocalization

The immunofluorescent localization results revealed the presence of DDR-2 on both normal and osteoarthritic cartilages. The staining pattern was consistent with a cell surface protein. As mentioned earlier, DDR-2 has been shown to have a role in chondrocyte and fibroblast cell proliferation. It is then logical to believe that DDR-2 plays a fundamental role in the cartilage possibly as a cell surface regulator of cell-matrix interactions, because it directly binds the most abundant extracellular matrix component, collagen. The role of DDR-2 may be as a linker to collagen in the surrounding microenvironment, thereby influencing cell signaling and the cellular response in terms of differentiation, proliferation, and most importantly extracellular matrix remodeling. As expected, this fundamental function would then operate in pathological as well as the physiological state of the tissue, but because of changes in matrix collagen structure in OA, cell signaling may then change as part of altered interactions with the receptor.

Discoidin domain receptor-1 protein was not detected in the normal cartilage samples nor the diseased osteoarthritic cartilage. DDR-1 is widely expressed during mammalian embryonic development and strikingly high levels of DDR-1 are seen in fast growing invasive mammary, ovarian, and lung tumors (78), in keeping with the increased proliferative rates in these tumors. In addition, Vogel et al (98) showed that ablation of DDR-1 in the mouse resulted in a severe postnatal growth reduction. These findings may suggest a role for DDR-1 in cartilage growth and differentiation but not in matrix homeostasis in the adult nor in pathology.

4.3 Gene expression

The discoidin 1 RNA was weakly expressed and then only in the normal adult cartilages. This weak expression probably accounts for the lack of detectable protein on the surface of the cells. The mild expression of the DDR-1 gene in the normal state of the tissue might be disrupted in the pathological OA state. This could explain the negative localization results of the OA samples.

As expected from the localization results, the DDR-2 mRNA was strongly expressed in all normal samples. This again suggests a basic role for DDR-2 in chondrocyte interactions with the extracellular matrix. DDR-2 gene expression was less consistent in the OA samples. This could perhaps be more clearly explained by considering the collagen type X expression pattern. Collagen type X is a specific marker for chondrocyte hypertrophy in the growth plate and has been shown to be upregulated in human OA articular cartilage (39,90). In this analysis, the normal samples (except for one sample with possible pre-osteoarthritic lesions) did not express the type X gene. In the

OA samples, low or undetectable levels of DDR-2 expression could be correlated with weak or undetectable type X collagen gene expression. In the osteoarthritic samples with strong DDR-2 expression, collagen type X was also clearly expressed. It was observed by Meijers *et al.* that the appearance of type X collagen was not associated with the degree of degeneration or the severity of the disease in the OA cartilage (100). It has been reported by Lapadula *et al.* that integrin expression on chondrocyes correlates negatively with the severity of cartilage damage (101). Also, cell-matrix interactions play a pivotal role in the induction of the hypertrophic phenotype (102). Therefore, it is possible that cartilage damage was more pronounced in those cartilage samples with reduced DDR-2 expression. This could have then led to decreased cell-ECM interaction and reduction of the hypertrophic phenotype leading to lack or reduced expression of type X collagen. From this one could speculate that at least in some cases a sequence of events independent of chondrocyte hypertrophy may lead to cartilage damage seen in OA. However, the exact nature and condition for such interactions will require further investigation.

4.4 Concluding remarks

In summary, this study has shown for the first time the selective presence of discoidin domain receptor 2 on the surface of adult human articular chondrocytes and has identified changes in its expression in OA articular cartilage. How discoidin receptors interact with their ligand collagen and its downstream signaling molecules and affect matrix turnover remain to be studied.

In the future, experiments could be designed to study the role of DDR-1 in prenatal cartilage development in an experimental model such as mouse or rabbit. Also, the relationship of DDR-2 expression to cartilage damage needs to be further elucidated. It would be interesting to use the DDR-2 knockout mouse model (99) to study the conditions of chondroctye differentiation in articular cartilage and the extent of matrix degeneration in the absence of DDR-2 receptors. The presence of the hypertrophic phenotype and expression of type X collagen in this model would also be investigated.

In addition, using methods applied by Neuhold *et al.* (103), transgenic mice over-expressing the DDR-2 receptor could be made to study the effect on the integrity of articular cartilage and expression of such genes as collagenase-3 (MMP-13), type X collagen and other markers of matrix degradation. Briefly, a cartilage-specific promoter would be used to target a constitutively active (i.e. phosphorylated) DDR-2 receptor to the hyaline cartilages and joints of transgenic mice.

Hopefully, these studies will prove useful in providing important new insights into the physiology of articular cartilage, pathophysiology of OA and identification of new therapeutic targets for monitoring, intervention and improving the long term outcome of therapy in OA patients.

CHAPTER 5: **References**

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