IMMUNOCHEMICAL STUDIES OF TYPE II COLLAGEN DEGRADATION

IN BOVINE AND HUMAN ARTICULAR CARTILAGE

BY

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Abstract

A major limitation in analyzing collagen degradation in articular cartilage in arthritis has been the lack of a technique for identifying collagen breakdown in situ. A new methodology is described which utilizes immunochemical and immunohistochemical protocols and a well characterized antiserum that permits the detection and analysis of type II collagen degradation in human and bovine articular cartilages. The antiserum R181 specifically reacts with unwound alpha chains and the CNBr-derived peptides α1(II)CB11 and α1(II)CB8 of human and bovine type II collagens and not with helical collagens. Increased degradation of type II collagen was detected in bovine cartilage cultured with IL-1. Increased extractable α chains and smaller collagen fragments were detected immunochemically. Immunohistochemically, an increase in type II collagen degradation was detected in human cartilages cultured with interleukin-1. This could be inhibited by addition of a metalloproteinase inhibitor. All human arthritic cartilages showed more staining for type II collagen degradation than was seen in normal cartilages. Using light and electron microscopic analyses, distinct patterns of collagen degradation were seen in rheumatoid arthritic compared with osteoarthritic cartilages. The results suggest that type II collagen degradation is a significant and early event in cartilage pathology.

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Abbreviations used in this thesis

A	Absorbance
APMA	4-aminophenylmercuric acetate
BES	(N, N-bis [2-Hydroxyethyl-2- aminoethanesulfonic acid)
BSA	Bovine serum albumin
CNBr	Cyanogen bromide
DMEM	Dulbecco's modified Eagle's medium
DMEM-B	DMEM containing BSA 0.1 mg/ml
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetracetic acid
ELISA	Enzyme linked immunosorbent assay
GAG	glycosaminoglycan
HEPES	(N-[2-Hydroxyethyl] piperazine-N -[2-ethanesulfonic acid])
HFBA	Heptafluorobutyric acid
HPLC	High performance liquid chromatography
IL-1	Interleukin-1
NRS	Normal rabbit serum
PBS	Phosphate buffered saline
PVDF	Polyvinylidine difluoride membrane
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLS	Segment long spacing
TES	(N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid; 2-([2-Hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)ethanesulfonic acid)
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol

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Specific Aim

Most arthritic diseases result in the destruction of articular cartilage with a loss of function. The destruction is due, in part, to the degradation of the extracellular matrix, which is composed of primarily fibrillar collagen and aggregating proteoglycans. In articular cartilage, type II collagen fibrils are responsible for the tensile strength wheareas the proteoglycans provide the compressive stiffness necessary for normal articulation and function. The precise mechanisms by which these connective tissue components are degraded and the regulation of the degradation and synthesis is not fully understood. One major limitation to its clarification has been the lack of the ability to detect and analyze collagen degradation in health and disease. A technique for identifying collagen breakdown in situ did not exsist. This thesis describes immunochemical and immunohistochemical protocols which permit one to detect type II collagen degradation in articular cartilage in both humans and experimentally in bovine cartilages. The concept and design for these studies was based on the premise that when fibrillar collagens are cleaved and subsequent unwinding and fragmentation occurs, there is the exposure of hidden epitopes that are not exposed in the native collagen molecule. It was theorized that these determinants are usually sequestered in the triple helix and are not detectable until the helix unwinds and is fragmented and that experimentally, these antigenic epitopes could be used to generate a specific antiserum for degraded type II collagen.

This thesis describes the generation and characterization of an antibody (R181) that specifically reacts with fragmented and denatured human and bovine type II collagen alpha chains. The antiserum R181 does not react with helical type II collagen or any non-collagenous or collagenous protein in

articular cartilage, including the minor collagen types V, VI, IX and XI. This well characterized antiserum was then utilized as a tool to study the degradative changes in human normal and arthritic cartilages as well as bovine articular cartilage experimentally treated with IL-1. An antiserum with unique specificity as mentioned here provides a powerful tool to study normal cartilage remodeling, cartilage pathology and cartilage destruction via cytokines such as IL-1.

CHAPTER I

INTRODUCTION

I. PREFACE

This thesis will address various aspects of connective tissue collagen degradation in articular cartilage by describing experiments where the degradation of type II collagen was studied both immunochemically and immunohistochemically. As background, it is necessary to understand the anatomical features of the articulating joints and the biochemistry and immunology of the macromolecules which comprise articular cartilage. The resident cells of cartilage called chondrocytes will be discussed since their role in cartilage synthesis and turnover is paramount to these studies. The characteristics of cartilage addressed in detail in this thesis will be primarily those of the major fibrillar collagen found in articular cartilage, type II collagen. The degradation of articular cartilage collagen or the imbalance of cartilage matrix degradation and synthesis can result in several clinical conditions. Two such arthritides of clinical relevance, rheumatoid arthritis and osteoarthritis, will be described and were studied from the point of view of collagen degradation. The pathology and etiology of these clinical conditions and studies involving pathological human tissues will be discussed.

This "Introduction" does not presume to cover all the aspects of joint pathology relevant to articular cartilage destruction and loss of function, but rather presents a review of pertinent information required to understand and interpret the results presented in subsequent chapters.

II. JOINT ANATOMY

Diarthodial joints are classified according to the type of union at the point of contact of two or more bones. The proper movement of joint surfaces while weight bearing depends on the integrity of the components and the precise alignment of the proximal and distal skeletal structures. The type of joint relevant to this thesis is called a synovial joint and includes the majority of important articulations in the body. These joints are characterized by the free movement between two bones covered in hyaline cartilage surrounded by a synovial membrane, such as the knee, the joint between the tibia and femur. For the purposes of review, the synovial joint consists of four major components: a fibrous capsule, a synovial membrane, the synovial fluid and articular cartilage (see Figure 1). In certain joints, in addition to the cartilage lining the surface of the bones there are fibrocartilageous structures referred to as discs or menisci which separate the bones involved in articulation. The predominant collagen of the meniscus has been shown to be mostly type I collagen (1). Although these accessory structures found within synovial joints have all been shown to be important to the function and stability of the joint, the collagenous connective tissue studied in this thesis is exclusively articular cartilage.

A. Fibrous capsule

The fibrous capsule of the synovial joint is the peripheral fibrous membrane composed primarily of collagen fibers, which encases the joint cavity. This fibrous envelope is continuous with the perichondrium and the periostium of the adjacent bone and provides, in part, the joint stability, yet permits restricted flexibility. The stabilizing ligaments of the joint are found

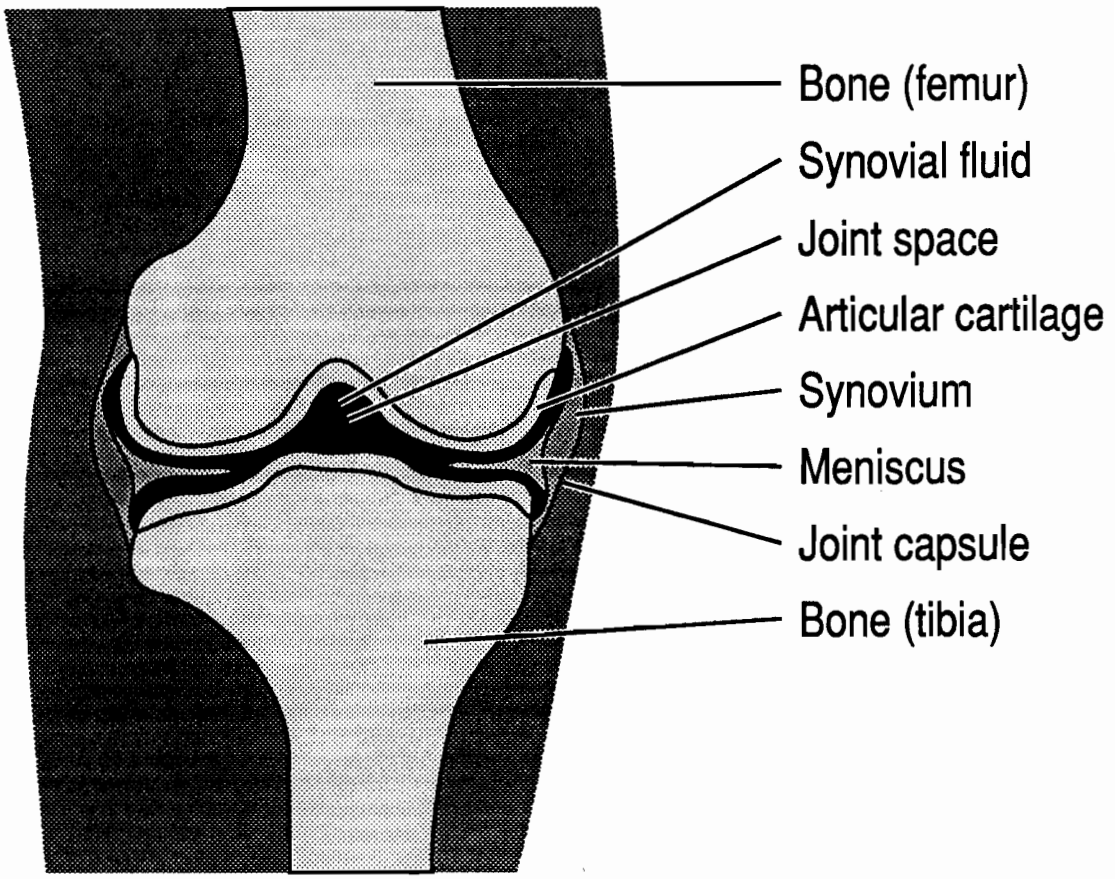


Figure 1 : Diagrammatic Representation of the Anatomy of the Synovial Joint of the Human Knee

combining with this fibrous capsule and together play a crucial role in stabilizing the joint. Ligaments under normal conditions function to facilitate joint motion about the most favorable axis. The ligaments are composed primarily of dense bundles of collagen fibers and elastin. Joints such as the knee are stabilized by both peripheral and internal fibrous structures referred to as collateral and cruciate ligaments, respectively. When the ligament is within the joint cavity, such as the anterior cruciate in the knee, the ligament itself is covered by the synovial membrane. It has been demonstrated that disruption of these ligaments and the hypermobility which results, lead to severe joint and cartilage degeneration (2).

The fibrous capsule is highly vascularized, contains a nerve network and is supplied by the lymphatics, all in contrast to that of articular cartilage which contains none of these structures. The sensitivity to pain has been shown to reside primarily in this capsule and to some degree in the synovial membrane which lines the inner surface of the fibrous capsule.

B. Synovial membrane

The synovial membrane is composed of an intimal layer of synovial cells overlying a layer of connective tissue, referred to as the subintimal layer. Normally this membrane covers all the intra-articular surfaces except the articular cartilage and the central portions of the menisci. Unlike most closed tissue spaces the joint is not lined by endothelium, but rather is formed from modified mesenchymal cells (3). Since there is no basement membrane, there is no structural barrier between the synovial membrane's vasculature and the synovial fluid. This continuous morphology permits an intimate involvement of the synovium and the joint capsule.

The synovial lining or intima is composed of two major, functionally distinct, cell types: 1) type A cells, with a more phagocytic macrophage-like function, 2) type B cells, with a synthetic fibroblast-like function (4). Normally the synovial lining is rarely more than one to four cell layers thick, according to location. In inflammatory diseases such as rheumatoid arthritis, the synovium undergoes proliferation and is characterized by a mononuclear cell infiltrate. The functions of the synovium are to both synthesize the components, such as hyaluronic acid, which are responsible for joint lubrication and to maintain a debris-free and germ-free environment by phagocytosis and pinocytosis. There is some evidence that both cell types and intermediate cells, can function in both the synthetic and phagocytic manner and that their morphology is representative of function (5-8).

Beneath the layer of synovial cells lies the subintimal layer composed of few cells among collagen fibrils, adipose tissue and a well developed vasculature. The extracellular matrix of the synovium is primarily made of collagen types I and III (9), although associated with the vascular endothelium and smooth muscle cells, collagen types IV and V can be found (10).

C. Synovial fluid

Joint tissues are bathed in a viscous friction-reducing fluid termed synovial fluid. Nutrition and lubrication are provided by the synovial fluid. Normal synovial fluid is a clear yellow plasma infiltrate to which hyaluronic acid has been added by the synthetic cells of the synovium. The unique morphology of the intimal layer and subintimal blood supply allows for fluid transport across the synovial membrane. It plays an important role in the maintainance of the intra-articular pressure, important in load bearing. The

dynamics of normal joint fluid production and resorption are maintained by the fluid flow of the microcirculation and lymphatic drainage.

In addition to the synovial cell product, hyaluronic acid, other glycoproteins and plasma components, such as α_2 macroglobulin, are typically present in small quantities (11,12). Since under normal conditions, there is very little synovial fluid present, more information is known about fluid found in abnormal joints. The concentration of small molecules in synovial fluid, such as electrolytes and glucose, is similar to that found in plasma. In rheumatoid arthritis, for example, the synovial fluid has been shown to contain larger proteins than normal, suggesting an altered selective exclusion, large numbers of inflammatory cells such as polymorphonuclear leukocytes and monocytes capable for matrix degradation, chemotactic factors including complement components, and proteinases. Specific proteinases important to these studies, such as collagenase, will be discussed in greater detail in subsequent sections.

Diffusion of molecules from the synovial fluid for both nutritional purposes and for stimulatory and inhibitory effects on chondrocyte function is well established. Earlier studies by Mankin (13) and Maroudas (14) using radiolabelled nucleotides, dyes etc. showed that solutes transfer well into cartilage from the synovial fluid surface. In addition, important to this thesis is the principle that molecules can diffuse in via the subchondral route since proteinases, such as collagenase, and molecules like IL-1 derived from this site are likely candidates for being involved in the matrix degradation observed in arthritic cartilages.

D. Articular cartilage

Articular cartilage is the smooth, resilient surface or covering of bone which permits normal joint motion. It is an avascular connective tissue composed of a unique combination of cells called chondrocytes and extracellular matrix. The extracellular matrix is composed of collagens and proteoglycans each of which will be discussed in a subsequent section. The major collagen of articular cartilage, as originally described by Miller (15) is type II collagen, the collagen type with which this thesis is primarily concerned. The tensile strength of cartilage is attributed to fibrils of type II collagen (16).

Articular cartilage of the synovial joint is referred to as hyaline cartilage. It appears macroscopically as milky in color, and is opaque with a smooth and glistening surface. This specialized connective tissue covers the articulating surfaces of bones: it has no perichondrium, the fibrous material that covers other hyaline cartilages. Articular cartilage peripherally fuses with the joint capsule and periosteum. It has been shown that the thickness of articular cartilage not only varies between individuals and from joint to joint, it varies within different regions of a particular joint. Studies of human and animal cartilages have shown that cartilage thickness is asymmetrical over the surface of weight bearing joints and that it is allometrically related to body weight and in certain joints, such as the knee, to the actual area of the tibial plateau (17,18). It has been observed that with increasing age cartilage thickness increases, perhaps as a mechanism for reducing the compressive forces between the surfaces. An increase in congruency between the surfaces results in a greater distribution of these stresses generated by weight bearing and motion (17,19). Specific age-related changes as they pertain to this work

will be discussed in a subsequent section.

1. Cartilage organization

Articular cartilage is organized into four zones commonly referred to as the following: superficial or tangential zone; mid or transitional zone; deep or radial zone; calcified zone (20-22) (See Figure 2). Cartilage is not a homogeneous tissue in regard to cellularity and is equally heterogeneous in matrix composition. Even though the cells of the cartilage, or chondrocytes, occupy less than 0.1% of cartilage volume (23,24) they are responsible for the synthesis and degradation of the extracellular matrix. Histologically, the matrix around the chondrocytes can be characterized as pericellular, that which is immediately surrounding the cell; territorial, representing that area adjacent to the chondrocyte; and interterritorial, the remainder of the matrix. Chondrocytes are in direct contact with the pericellular matrix, and not in a lacunae which is the cavity visualized in histological sections -an artefact of tissue preparation. Many ultrastructural studies have shown that these lacunae are in fact composed of a very thin network of collagen fibrils as well as proteoglycans (25,26). In addition, in the electron microscopic studies presented in this thesis the existence of a fine network of fibils can be observed in the pericellular sites with evidence of type II collagen degradation in the adjacent extracellular space (See Figure 38).

a. Superficial zone

The superficial zone is characterized by narrow collagen fibers of 30 nm in diameter or less, which are arranged in parallel or tangentially to the joint surface (27). Smaller and fewer proteoglycans are present in this area as has been measured biochemically and detected immunohistochemically (20,28).

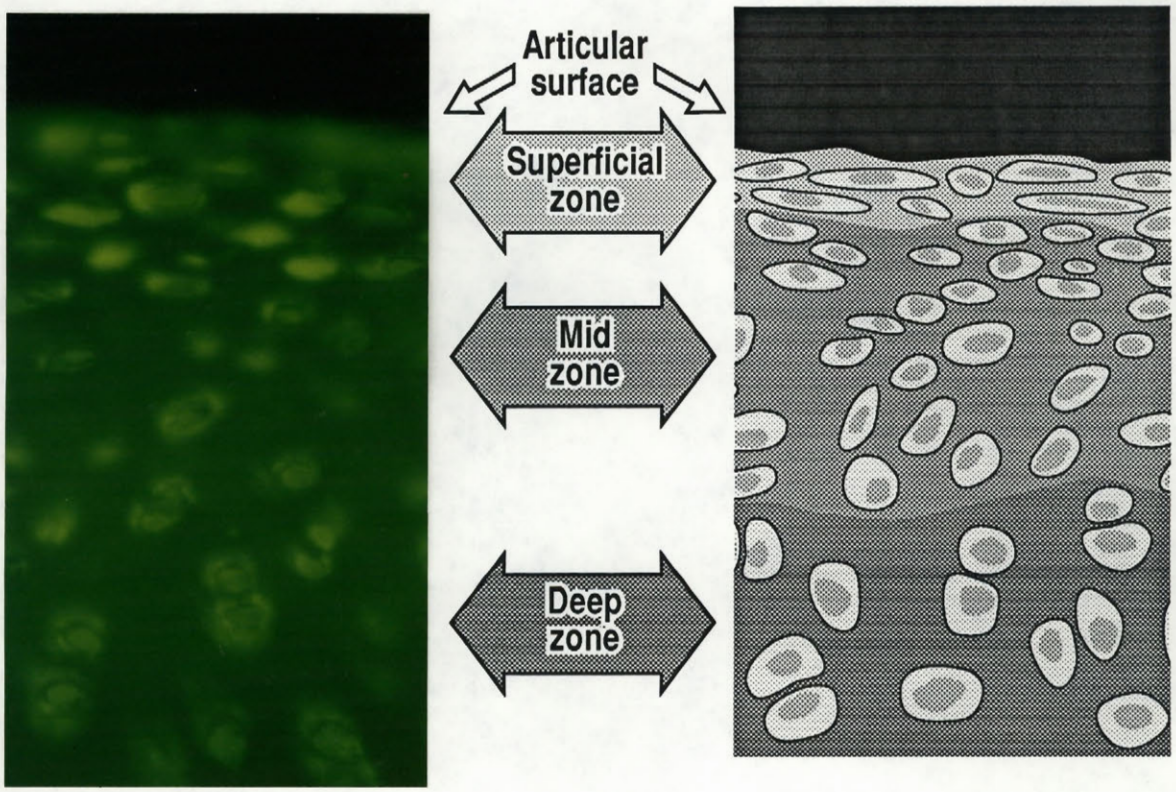


Figure 2 : Organization of Adult Articular Cartilage

Zonal organization of adult bovine articular cartilage is demonstrated in a micrograph (left panel) of full thickness cartilage section stained with R110 $F(ab')_2$ and fluoresceinated streptavidin as described in methods section. The right panel is a diagrammatic representation of the cellularity of cartilage observed in the micrograph. The tide mark which delineates the interface between articular cartilage and the calcified zone, the calcified zone itself and subchondral bone are not shown.

The relative sparsity of proteoglycans in the superficial zone is compensated for by the densely packed network of collagen fibers. This arrangement extends into the cartilage approximately 5 to 10 percent of the total cartilage thickness. Immunoreactive proteoglycans that are present in this superficial zone are not extractable in 4 M guanidine-hydrochloride as are most proteoglycans found elsewhere (29). These proteoglycans have been shown to be structurally unrelated to those that form large aggregates with hyaluronic acid and have been classified as dermatan sulfate proteoglycans (30,31).

The cellularity in this region is characterized by flattened chondrocytes lying parallel to surface, much like the arrangement of the collagen fibers. Even though the chondrocytes appear morphologically distinct from the chondrocytes found elsewhere in the cartilage, they contain all the usual organelles (i.e. multilobed nucleus, Golgi, mitochondria and rough endoplasmic reticulum) (32,33). Metabolic pathways of articular cartilage chondrocytes have not been extensively studied from a comparative point of view. Nonetheless, in general, articular-cartilage chondrocytes are equipped with enzymes such as those of the glycolytic pathway, lactic dehydrogenase and triosephosphate dehydrogenase, which have been detected in bovine articular cartilage (34). Lipids, such as triglycerides, cholesterol, and phospholipids, are present in articular cartilage both intra- and extracellularly. Based on wet weight, human articular cartilage has a total lipid content of approximately 0.32%-3.48% (35,36). Superficial zone chondrocytes are more closely associated with each other as can be observed in histological sections of cartilage cut perpendicular to the articular surface demonstrated in Figure 2. Whereas, as one looks deeper in the cartilage

section, away from the cartilage surface, they appear to increase in size, and take on an ovoidal shape and are more widely scattered in the matrix. The discoidal shape of superficial chondrocytes is consistent with what would be required of cells residing within an area of relatively little matrix and subjected to high shear forces and direct loading stresses.

The surface of cartilage is generally smooth, with few surface irregularities, except in cases of joint degenerative diseases such as osteoarthritis and rheumatoid arthritis. The pathological features of these diseases will be discussed in forthcoming sections. There have been a variety of studies that show irregularities and surface undulations on the surface of normal cartilages (37-39) although it has been suggested that these findings are artefactual as a results of tissue preparation, perhaps as a result of cutting and releasing tensions of the specialized matrix at the surface. Other studies have shown evidence of functional depressions or openings in the surface cartilage (40-42). These 'pores' are important in nutrient exchange and lubrication. Most studies of this type indicate that the surface is indeed not an inert and static covering of articulating surfaces, nor is it microscopically smooth and without subtle irregularities. Moreover, it may be that surface irregularities, in adult cartilage in particular, may relate more to the state of the cartilage and the manner in which it handles the shear forces and load stresses of weight-bearing and motion.

b. Mid zone

The mid zone is characterized by a randomly-organized network of larger collagen fibrils and an increase in large proteoglycans in comparison to the proteoglycan composition of the superficial zone. This transitional zone occupies approximately 40-45% of the articular cartilage. Because

proteoglycans are highly charged glycoproteins, they have been identified histologically using cationic dyes such as safranin o (43). Using these dyes the proteoglycans are shown to be abundant in this zone. Collagen fibrils in pericellular regions in the mid zone are usually smaller, oriented in a circular fashion and in association with proteoglycans, form a protective support for the chondrocyte (44). Away from the cells or in the territorial space the collagen fibrils are larger, ranging in width from 30-80 nm and are in general, more vertically arranged at right angles with the articular surface. The proteoglycans in the territorial zone have been shown to contain large aggregates of chondroitin sulfate and keratan sulfate proteoglycans (20).

Mid-zone chondrocytes are round in appearance and have all the usual intercellular organelles with, in particular, a well-developed rough endoplasmic reticulum characteristic of a cell engaged in active synthesis (45). These chondrocytes are more widely spaced than the cells of the superficial zone and are often seen in clusters.

Away from the specialized properties of the articular surface, the structure and function of articular cartilage as a weight-bearing network can be recognized. The interaction and orientation of both major components, fibrillar collagen which forms a restrictive scaffold and the greatly hyprophilic proteoglycans which create a resistive pressure due to their ability to imbibe water, exemplify the viscoelastic capabilities of load-bearing cartilage.

c. Deep zone

These special characteristics or features are carried into the remaining lower portion of articular cartilage which is referred to as the deep zone or by others, as the radial zone. This cartilage occupies the lower half (40-45%)

of the tissue and lies above the calcified cartilage and subchondral bone. The area is a continuation of the mid zone in regard to characteristics such as collagen fibrils and proteoglycan content. The collagen fibrils are largest in size of all zones (up to 100 nm in diameter) and are primarily oriented perpendicular to the articular surface. Some fibrils appear to be anchored in the calcified matrix below. These characteristics are indicative of the high degree of compressive stiffness of articular cartilage. Cartilage may lose some of its capacity to resist stress and become susceptible to degeneration and pathology. Studies have shown a loss of collagen and stabilized proteoglycans with increasing age at all levels (46,47). The largest proteoglycans are found in the interterritorial regions of the deep zone aggregating with hyaluronic acid (20,29). It is the interaction of these large proteoglycans spanning the spaces between the large collagen fibrils that could suggest that resistance to compression is at its greatest in the deep zone.

The chondrocytes of the deep zone are fewer in number, large and round and often are arranged in clusters or stacks of 2-3 cells. They are separated by more matrix. They contain an abundance of rough endoplasmic reticulum and a well developed Golgi. Chondrocytes in the deep and mid zones are characterized as having a more irregular plasma membrane than cells in the superficial zone. Chondrocytes have been observed with long extensions or processes, some of which can be very thin and branched (48).

d. Calcified zone

Lying beneath the deep zone of cartilage is the zone of calcified cartilage and subchondral bone. The junction between the non-calcified matrix and the adjacent calcified matrix is bounded by the tidemark, a

basophilic line seen on histological sections (49) (not included in Fig. 2). This structure is composed of collagen fibrils arranged in a variety of patterns, some passing through both calcified and non-calcified regions. It has been suggested that this distinct region is withstanding great stress and is responsible for maintaining the continuity between the two areas.

III. GENERAL PROPERTIES OF ARTICULAR CARTILAGE

Articular cartilage is a specialized tissue composed of a unique combination of cells and matrix, characteristics which were discussed in a preceeding section. One of the primary functions of articular cartilage is to perform in the joint as a shock absorber, to withstand the highly variable stress and compression of movement and weight-bearing. Another major function is to provide a smooth and resilient surface for motion between two bones to occur, maintaining an environment of low friction under conditions of high pressure and/or high velocity. These properties of articular cartilage are due to its principal components, collagen and proteoglycan. In a simplified but elegant way the macromolecular structure of cartilage can be thought of as a collagen fibril scaffold that forms a network of compartments which are filled with the highly polyanionic proteoglycans. The uniqueness of this viscoelastic tissue is in its ability to respond to selective and momentary compression and completely return to its original state. Biomechanical experiments have shown that even if a force is applied for hours, extruding water from the matrix, once stopped normal cartilage can return to its original configuration if it can freely imbibe water from its environment (46). In addition to playing a critical role in the biomechanics of articulation and cartilage resiliency, water is the most abundant constituent of normal cartilage. Water comprises 65-80% of the total wet weight of normal adult articular cartilage with little zonal variation (50). It has been shown that it is slightly higher in the superficial zone than in the rest of the cartilage (51). The swelling pressure of cartilage can be attributed to the highly charged water-binding glycosaminoglycans of the proteoglycans and the tensile strength of the collagen network determine its ability to resist deformation.

The size of the proteoglycans and the degree of hydration, in part, are limited by the collagen network which entraps them. This varies throughout the cartilage zones, as discussed in preceeding sections and demonstrates the heterogeneous nature of articular cartilage. A variety of studies have demonstrated this heterogeneity of proteoglycans within the different regions of cartilage (20,52,53) suggesting that the swelling pressure and compressive stiffness also vary within the cartilage.

Several distinct collagen types have been identified within hyaline cartilage (54) with the major collagen type being type II (15). Type II collagen comprises 10-20% of the total weight of normal adult articular cartilage (50). Each of the collagenous components of cartilage will be addressed in detail in a forthcoming section. Changes in water content and macromolecular composition occur during degenerative diseases such as osteoarthritis and rheumatoid arthritis and will be discussed elsewhere in this thesis.

IV. CARTILAGE MACROMOLECULES

Most all connective tissue are constructed along similar principles, each adapted for a specific physiological function. As mentioned, this cartilage is composed of a tissue-specific collagen network or scaffold in which other components such as glycoproteins (proteoglycans), are entrapped or attached. The biomechanics of articulation require articular cartilage to be constructed in such a manner as to withstand extreme pressure, absorb shock and be resilient.

Various types of cartilage exist. One such group of cartilage is referred to a hyaline cartilage, and includes the cartilage discussed in this thesis, articular cartilage. In this section specific features of relevant collagens and proteoglycans will be discussed as they relate to this thesis. The major collagen of cartilage, type II collagen, is the collagen with which this thesis is primarily concerned and will be covered in detail. Although collagen is the most abundant protein in the body, much is still unknown particularly about the interaction of the various collagen types. The other collagens found in non-calcifying matrices, types V, VI, IX & XI (54,55) will be discussed more briefly and in the context of these experiments. Proteoglycans are critical to the function and integrity of articular cartilage, therefore some discussion of these large glycoproteins is essential.

A. Collagen

Collagens are proteins which structurally contain triple helical domain(s) composed of a repeating GLY-X-Y unit where X and Y can be any amino acid but are frequently proline and hydroxyproline, respectively. Functionally collagens participate in the extracellular construction and play a

role as primary supporting elements (56). To date, 12 genetically and structurally distinct collagen types have been identified and characterized to various degrees (57,58). Referred to as classical fibril-forming collagens, sharing some similar characteristics, are collagens type I, II and III. Based on sequence data of the primary structure of the pro-form of these collagens, they share 45-65% sequence homology (59). Nonetheless, these collagens, like all collagen, have a specific tissue distribution. The most abundant interstitial collagen is type I (the first to be identified and extensively studied) found in most tissues including bone, dentin and dermis. Fibrils of type III collagen form a fine reticular network and are found in tissues with type I, such as the dermis and blood vessel walls. Type II is the main collagenous protein of articular cartilage and in addition, is found in the vitreous body of the eye, and other hyaline cartilages. Various collagens have been studied for some 30 years. Even though this diverse family of collagen (including these interstitual collagen types I,II,III) are still the subject of many investigations. Fundamental questions, such as those addressing the interaction of collagens with each other and other extracellular matrix components, remain unanswered.

1. Collagen Immunology

Most proteins possess regions generally located on the outside or surface of the molecule which are referred to as antigenic determinants. These regions are usually composed of up to 15 different amino acid residues and the number of antigenic determinants is determined by the size and complexity of the protein (60,61). Antigenic determinants are a protein's unique sites which give it the ability to induce an antibody response when the protein comes in contact with cells of the immune system. This fundamental

concept is the basis for the foreigness of proteins between species and individuals. The degree of antigenicity of a particular protein is not only due to the linear amino acid sequence but rather a combination of the unique sequence and its secondary molecular structure. Amino acid sequence determinants or those polypeptides which still retain their antigenicity after extensive degradation and denaturation are referred to as sequential antigenic determinants. Earlier studies have indicated that linear arrays of amino acids within a protein with antigenic properties, can be as small as pentapeptides (62). Antigenic determinants which depend on the sequence secondary, tertiary or quarternary structure are referred to as conformational antigenic determinants (63).

In general, antibodies raised against a particular protein usually fail to react with other proteins except when the other protein possesses similar determinants, such as in the case of identical proteins from two different species. This cross-reactivity, when present, permits the preparation of antibodies for experimental purposes for most all proteins and is a concept when has critical relevance to the experiments described in this thesis. Conversely, if antibodies were raised against certain antigenic determinants which are species or age-specific, the characterization of unique molecules or structures which are different between species is possible.

Due to collagen's unique structural features, collagen can be thought of as a model protein for the study of protein antigenicity. The antigenic structure of collagen has been studied and the high degree of antigenic and structural diversity have been demonstrated in many studies over the past two decades and has been extensively reviewed by Timpl (63-65). Collectively these studies have identified three main groups of antigenic

determinants within the fibrillar collagen types I, II and III. These are: (1) helical or central determinants which are localized in the triple helix and are destroyed by denaturing or unwinding the helical alpha chain; (2) nonhelical central determinants which are only exposed after denaturing the triple helix; (3) terminal determinants which are those located in the nonhelical telopeptide extensions of the alpha chains which when present are not dependant on wound or unfolded alpha chains.

Antibody responses to helical determinants are most frequent and strong. When pepsin solubilized collagen is used as the immunogen, where the nonhelical terminal ends are removed the antibodies raised are specific for the helical or central determinants (66,67). Antibody responses are less common to central nonhelical determinants, although, it has been demonstrated in earlier studies and in the present study described in this thesis, that denatured or fragmented collagen can be antigenic and specific peptide antibodies be raised (68-71). Historically, the structural determinants of many of the collagens have been elucidated by localizing antibody reactions to distinct CNBr-derived collagen peptides (70,72,73).

Earlier studies have identified immunogenic determinants and antibody-reactive epitopes on unwound collagen alpha chains (63,67,74-76). Since these determinants are usually sequestered in the triple helix and not detectable until unwinding or fragmentation occurs, antibodies to these nonhelical central determinants provide experimentally unique tools to study collagen degradation. The understanding of the immunology of collagen as we know it, provided the basis for the development of the antiserum used and in the design of the studies described in this thesis. Experimentally, antibodies that only recognize epitopes which are found on unwound or

fragmented type II collagen (i.e. nonhelical central determinants) provide the opportunity to understand collagen degradation in a specialized tissue like cartilage both as a normal function of cartilage remodelling and in arthritic pathology.

2. Type II Collagen

a. Physicochemical properties

Characteristics of the type II collagen shared by other fibril-forming collagens are that each collagen molecule is composed of three polypeptide chains each made up of approximately 1000 amino acids with a molecular weight of approximately 95 kilodaltons. Each polypeptide chain is referred to as an α chain. Three α-chains together form what is called tropocollagen, which has a mass of approximately 285 kilodaltons. The molecular weight of collagen is unlike that of globular proteins, for example, due to its high concentration of low mass amino acids, such a glycine (33%) and proline (10%). This results in a mean residue weight of 91.6 daltons whereas the molecular properties of globular proteins such as BSA and phosphorylase, result in a mean residue weight closer to 115. This may explain why collagenous proteins appear to have a higher molecular weight (as much as 40% higher) on SDS PAGE than their actual size (77,78).

Type II collagen was first discovered in hyaline cartilage as a unique collagen composed of three identical α-chains, unlike that of type I which had been identified as having two different α-chains in a ratio of 2:1 (15,79). The nomenclature for collagen is indicative of the molecular form, that is type I collagen is designated as $[\alpha 1(I)]_2 \alpha 2$ and type II as $[\alpha 1(II)]_3$.

A unique feature of each α-chain is the repeating amino acid sequence where every third amino acid is a glycine often followed by either proline or

hydroxyproline. Another amino acid found in high concentrations in collagen in addition to hydroxyproline is hydroxylysine. Both are found in very few other proteins. Table 1 shows the amino acid composition per 1000 residues. It demonstrates the large proportion of hydroxyproline, the presence of 10 methionines, no cysteine, and a high carbohydrate content. The third position in the glycine-X-Y triplet is often a hydroxylated lysine residue in type II collagen and is a site for glycosylation (15).

Since type II collagen contains a high number of hydroxylysine residues it can contain a relatively large amount of carbohydrate. Hydroxylysine is most commonly glycosylated to form either galactosyl-hydroxylysine or glucosyl-galactosyl-hydroxylysine. Each α-chain is composed of a helical region (a left-handed helix) also referred to as the central region which accounts for 95% of the length of the molecule. Short non-helical regions or telopeptides are referred to as the NH_2-terminal region and the COOH-terminal region, respectively. Both non-helical regions are remnants of larger non-helical extensions which were enzymatically removed during collagen processing. These terminal regions in the final processed molecule are important sites for intermolecular crosslinks and will be discussed later.

Each α-chain is coiled together to form a right-handed helix with an approximate repeat of 100 Å. This conformation is strictly dependent on the unique feature of a glycine repeating every three amino acids along with the other amino acids mentioned in high concentrations. This arrangement around a common axis, forming a triple helix, is stabilized by hydrogen bonds between α-chains perpendicular to the helical axis. The arrangement of collagen molecules to form native fibrils is reflected in the image obtained after negative staining (with phosphotungstic acid) and electron microscopy.

The characteristic light and dark banding pattern with a repeat of 67 nm seen in negatively stained fibrils is indicative of the densely and loosely packed regions, respectively. These types of electron microscopic studies, usually using artificially aggregated fibrils called segment long spacing (SLS) crystallites, revealed the exact arrangement of collagen molecules in the native fibril, even without a precise knowledge of its primary structure (80). It was determined that the native fibril, of the fibrillar collagens type I, II, III were 4.4 D units in length, D being the length of one cross-striation period of 67 nm, and an overlap of approximately 30 nm (81). These determinations were confirmed years later using three dimensional analyses of the amino acid sequence and further interpretations of x-ray diffraction patterns (82-84).

The initial characterization of collagen was performed on material purified from connective tissues such as skin, bone and tendon. This was later to be referred to as type I collagen, whereas the major collagenous component of hyaline cartilages was identified later as distinct and referred to as type II collagen. The precise identities of these two, now well studied collagens, were based on the peptide maps obtained after cleavage with CNBr (85). The SLS crystallites of type II collagen were characterized by a similar banding pattern to that of type I collagen (86) and later to type III collagen (87) indicating the structural homology of these three classical fibril-forming collagens. The amino acid analyses show that these three genetically distinct collagens are similar although characterized by differences in sequence structure and specific amino acid content (Table 1).

Cleavage of collagens with CNBr has proven to be an invaluable tool in the characterization of the various collagen types. CNBr splits polypeptide

AMINO ACID	Residues/1000 Total Residues	
	HUMAN α1(II)[a]	HUMAN α1(I)[b]
3-Hydroxyproline	2	1
4-Hydroxyproline	97	108
Aspartic acid	43	42
Threonine	23	16
Serine	25	34
Glutamic acid	89	73
Proline	120	124
Glycine	333	333
Alanine	103	115
Half-cystine	0	0
Valine	18	21
Methionine	10	7
Isoleucine	9	6
Leucine	26	19
Tyrosine	2	1
Phenylalanine	13	12
Hydroxylysine	20	9
Lysine	15	26
Histidine	2	3
Arginine	50	50
Total	1000	1000
Gal-hydroxylysine	4	1
Glc-Gal-hydroxylysine	12	1

Data from Miller and Gay, 57, 88

[a] Purified from newborn human epiphysial cartilage.

[b] Purified from human placental tissue.

Table 1 : Amino Acid Composition of Human α1(II) Collagen Chains As Compared to Human Collagen α1(I) Chains

chains specifically at methionine residues (89) and when applied to collagen, results in relatively few fragments ranging in size from a few to close to 400 amino acids. CNBr-derived collagen fragments and subsequent sequence analyses, has permitted identification of each genetically distinct collagen species and continues to be a useful tool for the elucidation of the identities of new collagens. The generation and identification of CNBr-derived peptides of collagen type II are an important part of the studies described in this thesis.

b. **Biosynthesis**

As for all the fibril-forming collagens (I,II,III), the characteristics of the individual α chains and molecules derived thereof are well suited for their biological function, although they are considerably modified by post translational events. Collagens, like many proteins, are initially synthesized as larger precursor molecules modified by specific enzymic reactions to produce the functional collagen molecule. Again, the early investigations were performed mostly on type I collagen, but due to the high degree of homology between the fibril-forming collagens, the biosynthetic characteristics and processing are representative of each. First observed in tissue culture of human fibroblasts (90) and in cultures of rat calvaria (91) was the existence of a collagen precursor, referred to as procollagen. These newly synthesized collagenous polypeptide chains have an apparent molecular weight of 120 kilodaltons for α1(I) and 140 kilodaltons for α2(I) as compared to the resulting 95 kilodaltons for α chains of both species. Procollagen α1(II) (referred to as pro α1(II) is, in most structural aspects, quite similar to the pro α1(I), including propeptides, telopeptides and helical domain. It has been shown that α1(II) more closely resembles α1(I) than α2(I). For example,

the nucleotide sequence of cDNA to chick pro α1(II) mRNA has shown that the C-terminal end (or C-propeptide) of pro α1(II) contains the identical number of residues as the homologous sequence in pro α1(I). Further, the two sequences are identical in 71% of 246 amino acid residues (92). This is a somewhat higher degree of homology than is seen in the helical region.

The amino-and carboxy-terminal propeptides are cleaved by specific peptidases (called procollagen amino- and carboxy proteinases) before or during fibrillogenesis. These propeptides appear to be involved in the control of post-translational events and perhaps in the control of biosynthesis of collagen itself (93). Both propeptides are largely globular proteins and are thought to function in part to prevent intracellular fibril formation. In situations where the amino propeptide is not removed because of a defective enzyme, (i.e. dermatosporactic calves) the collagen molecules do not form intact fibrils. This results in the formation of a compromised biomechanical weak dermis. Other possible involvements of collagen propeptides have been proposed including that derived from the observation that the C-propeptide of α1(II) is associated with the calcification of cartilage (94,95).

c. Cross-linking

Fibrillar collagen's unique characteristics of strength and elasticity are in part contributed by the intramolecular cross-links which stabilize the molecule. Along with fibril diameter, orientation and density of fibrils, the degree of cross-linking combines to provide collagen with its structural function. The study of collagen cross-linking began with the observation by Gross et al that treating animals with the lathyrogen, β-aminoproprionitrile there was a dramatic increase in the amount of soluble collagen (96). More detailed amino acid sequence analysis of such collagen showed that the lysine

or hydroxylysine residue in the amino-terminal telopeptide was not oxidized to allysine as it was in collagen from normal rats (animals not treated with β-aminoproprionitrile) (97). This later was shown to be the situation with the oxidizable lysine or hydroxylysine in the carboxy-terminal telopeptide (98). These findings suggested that β-aminoproprionitrile, itself a compound isolated from the sweet pea, is inhibiting the formation of cross-links by interfering with the formation of aldehydes from the lysyl residues.

Cross-linking, an important posttranslational event, has been extensively studied and in part is well characterized. The initial events in the formation of cross-links are dependent on the enzyme, lysyl oxidase. This enzyme mediates the oxidative deamination of lysine and hydroxylysine residues yielding the respective semialdehydes, allysine and hydroxyallysine (97-99). This process occurs outside the cell and requires collagen aggregates or fibrils. It has been shown that lysyl oxidase has no effect on solubilized collagen molecules (100). The enzyme is tightly bound to collagen and is copper dependent. Although it has a particular affinity for the lysyl residues of the nonhelical peptides of the collagen molecule, it can also catalyze the oxidation of lysyl residues in the helical region at a much slower rate (101).

Intramolecular cross-links occur between the lysine and hydroxylysine-drived aldehydes within a collagen molecule, whereas, intermolecular cross-links occur between aldehydes and amino groups of lysyl residues located in different collagen alpha chains. Inhibitors of aldehyde formation, such as β-aminoproprionitrile, have revealed that aldehydic groups are essential for the formation of these cross-links.

Alterations in cross-linking occurring in a variety of connective tissue disorders, in particular those with a genetic basis. Among those conditions

are Marfans syndrome, osteogenesis imperfecta, and all types of Ehlers-Danlos syndrome, each with striking clinical features (102-104).

Aging has profound effects on the chemical and physical nature of collagen and many of these changes are related to the status of cross-links. For example, with age there is a progressive decrease in the amount of soluble collagen which can be extracted from any tissue such as skin. The physical property of tensile strength increases with advancing age suggesting that there are more nonreducible cross-links present (105). There is no direct evidence on the number of cross-links present in aging tissues although it has been indirectly shown that there is increased crosslinking with age (106). Reducible cross-links are altered or modified into nonreducible cross-links during the aging of collagen and can be demonstrated by the fact that the yield of acid extractable collagen is greater from fetal or young tissues as compared with mature adult tissues (107).

There have been studies addressing the question of these age-related changes in collagen cross-links and the possibility of in vivo reduction of lysyl residues has been ruled out (108) and others have shown that there is a possibility of oxidative modifications occurring with result in a peptide bond (109). Age-related changes and the importance of cross-links in predisposing collagen to degradation and or abnormal repair, and overall fibril stability, remain to be answered by further studies.

The stability of collagen molecules is dependant on an additional number of weaker forces that these covalent cross-links discussed here and include such things as hydrophobic bonds, ionic interactions, Van der Waals forces, and interactions with neighboring proteoglycans.

3. Minor collagens

It is well known that the most abundant protein in hyaline cartilage is type II collagen and its characteristics as a classical collagen have been discussed in the preceeding section. Even though the major collagenous component has ben identified for years and exhaustively characterized, recently several other collagens have been identified in hyaline cartilage or produced by chondrocytes. These collagens are part of the ever growing family of collagens which now include as many as 13 distinct gene products. These collagens discussed here which are found in articular cartilage are collagens types V, VI, IX, XI. Each is unique in its molecular form and chain organization. Although these collagens are present in lesser amounts than type II collagen, there role in cartilage appears to be anything but "minor". As these and other newly identified collagens are characterized as distinct molecules, their interactions and functions in the tissues in which they are found, continue to be the subject of many investigations.

a. Type V collagen

Type V collagen refers to a molecular species of collagen which is composed of three unique chains, $\alpha 1(V)$, $\alpha 2(V)$ and $\alpha 3(V)$, each with a molecular weight between 115kd and 125kd (110). Pepsin digestion removes non-collagenous peptide regions and results into a molecule which is commonly referred to as type V. Currently, three molecular forms of type V have been identified utilizing homotrimeric molecules of $\alpha 1(V)$ chains (111) and two different heterotrimeric forms $[\alpha 1(V)]_2\ \alpha 2(V)$ and $[\alpha 1(V)\ \alpha 2(V)\ \alpha 3(V)]$ where the former organization is most prevalent (112,113). Type V collagen (formerly referred to as AB collagen) comprises a small fraction of the total collagen pool and has been found in a variety of tissues including

cartilage (54) and cornea (116). The wide variety of tissue distribution implicates several cell types in its synthesis. Its identification in a number of tissues (by immunofluorescence and light and electron microscopy) has shown type V in close association with basement membranes and in fibrous stroma in tissues including tendon and cornea. Some controversy exists as to its presence and function, particularly in basement membranes and further studies have shown it to be present between collagen bundles probably functioning as a binding or connecting molecule (115). Perhaps one of the most important features to note is the fact that types I and V collagen are intimately associated and in fact have been shown to co-polymerize within the same fibrils network (116). Type V collagen can be found in most tissues where there is type I, whereas, type XI, another collagen with much similarity with type V molecules, is found in tissues in association with type II. The fact that there are a number of trimeric combinations and related molecules, suggests that they may have different functions in vivo with tissue-specific adaptations. Although, type V has been shown to be extractable in small amounts (approximately 1%) from cartilage (54), it was examined in these studies when characterizing the antiserum, R181, for possible cross-reactions with related and unrelated collagens.

b. Type VI collagen

Type VI collagen was first isolated from the wall of the aorta and has properties distinctly different from previously identified collagen (112). It has been characterized further to be a unique entity of the collagen group. It has a structure which does not allow the lateral aggregation which is characteristic of the interstitual collagens, types I, II and III. Previously, type VI was commonly referred to as intima collagen because it had

characteristics unlike the earlier described fibrillar collagens. The special characteristics of type VI are that it is composed of three chains, α1(VI), α2(VI), and α3(VI), which in their processed form have an approximate molecular weight of 140 kilodalton (114). The most prevalent molecular form is [α1(VI), α2(VI), α3(VI)]. The heterotrimer forms a dumbbell shaped molecule. Only a third of it is helical collagen and there are extensive disulfide cross-links (117,118). Pepsin digestion and reduction results in products of a molecular size between 40 and 70 kilodaltons (112,119). The short triple helical domains are terminated with globular domains at both ends which permit an end to end arrangement to form filaments or fibrils with a 100 nm repeat (120).

Type VI has been isolated from a variety of tissues including large arteries and small blood vessels, fetal membranes, skin, tendon and perichondrium (121). Although some controversy exists over its presence in hyaline cartilage it has been identified in articular cartilage by some authors (55) and in the intervertebral disc (122). For these reasons type VI was included in the characterization of the anti type II fragment antiserum (R181) used in study.

c. Type IX collagen

Closely associated with the predominant collagen type II fibrils is type IX collagen. Type IX collagen also contains a glycosaminoglycan side chain and thus can also be considered a proteoglycan (123). It has been shown that type IX collagen is arranged in a D-periodic fashion along type II fibrils (124). The intimate assocation of types II and IX collagens has further been supported by the studies showing the presence of aldehyde derived cross-links

between the two collagens (59,125).

Type IX collagen is composed of three different collagen chains designated α1 (IX), α2(IX), and α3(IX) each with a similar basic structure of an interupted helix. There are three helical domains separated by non-collagenous (non-helical) domains with non-helical domains at both ends. These chains are stabilized by interchain disulfide bridges. A chondroitin sulfate chain is attached to the amino terminal non-helical portion of α2(IX) (126). Previously, this collagen was identified as two fragments isolated from chick sternal cartilage and was referred to as HMW and LMW, for their high and low molecular size (127). Later studies showed that in fact HMW and LMW could be fractionated into three distinct chains and were found in an equal proportion when isolated from tissue (128). The nomenclature now used for type IX chain is as described above and the collagenous domains of each chain referred to as COL1, COL2 and COL3, and the four non-collagenous refered to as NC1, NC2, NC3 and NC4. In addition to the glycosaminoglycan attached to the amino terminal non-collagenous region of α2(IX), there is a long non-helical portion extending from the α1(IX) chain, resulting in a molecular weight of intact type IX molecules estimated ranging from 250-300 kilodaltons. This variation may be due to the glycosaminoglycan chains attached. From studies where type IX collagen was digested with chondroitinase ABC it was shown that the α2(IX) chain was reduced in size from approximately 115 kilodaltons to approximately 68 kilodaltons. Chondroitinase ABC treatment of α1(IX) and α3(IX) resulted in no change in migration in SDS-PAGE (129).

Functionally, type IX collagen appears to be intimately involved in the organization and the stabilization of type II fibrils in cartilage, although its

precise role is the subject of continued investigation. It has been shown that its synthesis is coordinately regulated with type II, (130) and a variety of studies have demonstrated the presence of aldehyde-derived cross-links between types II and IX collagen in hyaline cartilage (59,125). Immunolocalization studies have shown that type IX collagen is predominately present in the pericellular matrix and the intensity of staining increased when articular cartilage was pretreated with hyaluronidase. It has been suggested that type IX (and type XI) could be critical components in the fine fibrils which make up the matrix around chondrocytes. It could be involved in stabilizing the pericellular capsule. Some studies suggest type IX constitutes between 1-5% of the total collagen from hyaline cartilage (articular and sternal) (125,131) although other studies have suggested it could represent as much as 20% of the amount of synthesized type II (24).

d. Type XI

Type XI collagen was first identified in pepsin digests of mammalian hyaline cartilage as distinct chains by Burgeson and Hollister (132). On SDS-PAGE, α chains of type XI migrate slightly larger than those of type II and in fact, have a similar electrophoretic mobility to α1(V) and α2(V) chains. Initially this collagen was named 1α, 2α, and 3α and was also referred to as type K collagen by others in a review as recently as 1985 (133). Nevertheless, with the large body of data characterizing this as a unique heterotrimeric collagenous molecule, it has been proposed and accepted to be hence referred to as type XI and its constituent α chains as α1(XI), α2(XI) and α3(XI) (134). The CNBr-peptide map of the α1(XI) and α2(XI) chain is unique and differ from both type II and type V collagens. Interestingly, the α3 chain has a nearly identical CNBr-derived peptide map with the α1(II)

chain, with the α3(IX) peptides migrating more slowly than α1(II) peptides (132,135). Since they appear to have a nearly identical amino acid sequence with few variations the differences in α3(XI) and α1(II) are concluded to be post transitional modifications such as the degree of glycosolation. A heterogenous mixture of chain compositions appear to exist including chain combinations such as $[\alpha 1(XI)]_3$; $[\alpha 2(XI)]_2$, α1(XI), α1(XI); α2(XI), α3(XI). Adult cartilage contains α1 and α2 chains in a ratio greater than 1:1 and just less than 2:1 whereas, in fetal cartilages the ratio is more consistently 1:1. Due to the identification of these various collagens in articular cartilage and the apparent similarity of certain α chains, the collagen types V, VI, IX and XI were included in the antibody characterization experiments. Similarities exist between α1(V) and α1(XI), α2(XI) and α2(V), α3(XI) and α1(II).

Type XI collagen has been identified in a variety of cartilages from human and animal tissues including articular, growth plate and costochondral cartilages and in the intervertebral disc, a fibrocartilage. As a minor component, type XI represents approximately 3-5% by weight of the total collagen in a ratio of 1:30 to type II collagen. This coincides with the ratio of type V to type I in connective tissue other than cartilages (132). The role of type XI has not been elucidated. Certain functions which have been suggested by a variety of studies include, the regulation of type II fibril diameter; the mediation of the interaction of collagen and proteoglycans. This role of mediating physical interactions between connective tissue components has been supported by findings which show that, in vitro, type XI collagen can in solution or as fibrils bind to cartilage proteoglycans and further that this binding could be competed with by free sulfated glycosaminoglycans chains (136). This suggests a role for type XI fibrils in

providing a physical interaction with the cartilage proteoglycans and the type II fibrils with which they themselves are co-distributed. Under similar conditions type II fibrils themselves do not bind the various sulphated polysaccharides. Recently, using immunoelectron microscopy and immunofluorescence type XI was shown to be distributed throughout the type II fibril but was only detectable when the fibril structure was partially disrupted. In contrast the same authors showed that type II and type IX were detectable as part of intact fibrils in cartilage. This suggests that the tightly packed arrangement of type II collagen fibrils results in the inacessibility of the type XI collagen antibodies. These studies also indicated that along with types II, XI and IX are distributed throughout cartilage matrix (55).

B. Proteoglycans

The proteoglycan content of cartilage varies inversely to the collagen content (137). Proteoglycan content is lowest in the superficial zone, where collagen accounts for, in some cases, almost 90-100% of the dry weight (51). The proportion of collagen to proteoglycan in cartilage matrix varies not only within the different zones of articular cartilage but varies from individual to individual and with age. At various points in the Introduction, proteoglycans were mentioned where appropriate for this thesis. This section is added to overview the salient features unique to cartilage proteoglycans.

Proteoglycans are glycoproteins which consist of a core protein to which one or more glycosaminogycan chains is covalently bound. Previously these molecules were referred to as mucopolysaccharides or chondromucoproteins. There is a variety of proteoglycan types, classified in part by there GAG side chains and unique core proteins. There are four

general classes of GAG side chains defined by the repeating disaccharide backbones: chondroitin sulfate; dermatan sulfate; keratan sulfate; and heparan sulfate. The cartilage proteoglycan core protein is typically 220 kilodaltons and it contains two globular N-terminal domains (G1 and G2) one of which (G1) contains the region which interacts with link protein and hyaluronic acid to form aggregates (138,139). There is a third additional globular domains (G3) at the C-terminal end. A number of N-and O-linked oligosaccharides, are scattered throughout the molecule. Between G2 and G3 the keratan and chondroitin sulfate chains are located (140). Dermatan sulfate proteoglycans have been detected in articular cartilage (30,31). Two dermatan sulfate proteoglycans were identified and characterized by a much smaller molecular weight than the other species in cartilage. One species (designated DS-PGI) has a molecular weight ranging from between 165 and 285 kilodaltons with a core protein of approximately 45 kilodaltons. The other (DS-PGII) is smaller with a molecular weight between 87 and 120 kilodaltons. Following chondroitinase AC and ABC digestion, the core protein was determined to be approximately 45 kilodaltons (30). Chondroitin sulfate and keratan sulfate containing proteoglycans are characteristically the aggregating type and form large complexes. Since there can be approximately 100 chondroitin sulfate chains each with a molecular weight of 18-20 kilodaltons, these molecules have the potential of much larger molecular size than do the dermatan sulfate proteoglycans which do not bind to hyaluronic acid although the DS-PGI proteoglycans have been shown to self-associate (30).

The proteoglycans of hyaline cartilage are among the most complex of the proteoglycan molecules. The GAG chains found in cartilage are primarily

chondroitin sulfate, dermatan sulfate and keratan sulfate. These GAG chains vary greatly in chain length and do not, with the exception of hyaluronic acid, exist as free units in tissues but rather are bound as component parts of the proteoglycan molecules (141). An understanding of the structure of proteoglycan in cartilage comes from early electron microscopic studies where both monomers and aggregates were identified (133,142). These studies present an image of a proteoglycan molecule as a single protein core with GAG chains attached (subunit or monomers) or in a complex form where individual proteoglycan subunits are organized along a filament of hyaluronic acid (aggregate) (158). The appearance of the proteoglycan subunit takes on the classic appearance of a bottle brush. Aggregate stability depends on an additional protein called link protein (140). This interaction involves a distinct region of the core protein (G1 of the hyaluronic acid binding region) and the binding or specific interaction of the link protein with hyaluronic acid (143,144). Link protein, itself a glycoprotein, is approximately 45 kilodalton molecular weight. Several studies have implicated the degradation of link protein as a mechanism of destabilizing the aggregate form in vivo, by both proteinases and oxygen-free radicals (145). Link protein has been immunohistochemically shown to be present in articular cartilage and has been suggested as a pivotal component of the aggregate form (29,171) and may be deficient or degraded in aging or diseased cartilage.

In cartilage, a typical proteoglycan subunit consists of a core protein with as many as 100 chondroitin sulfate chains and 50 keratan sulfate chains attached (146). These subunits, each having a molecular weight of several million daltons, can complex in the aggregate form resulting in some of the largest molecular structures known. This specialized molecule and its large

hydrodyamic size are responsible for many of the unique properties of articular cartilage mentioned in Section III. Proteoglycan content in cartilage can occupy as much as 10% of the wet weight. Cartilage proteoglycans in solution have been shown to have the ability to imbibe 50ml of solvent per gram of dry weight (146). Both aggregating and non-aggregating proteoglycans make up the diverse populations of cartilage proteoglycans. The proteoglycans aggregating with hyaluronic acid form large molecular weight complexes, represent approximately 50-85% of the total proteoglycans in cartilage. There is a significant quantity of large non aggregating proteoglycan molecules also found in cartilage which do not bind hyaluronic acid. These occupy approximately 10 to 40% of the total proteoglycans in cartilage on a wet weight basis (147). In the specialized matrix of articular cartilage, these proteoglycans exert a swelling pressure on the fibrillar collagen network, endowing cartilage with its unique resilience to weight bearing and motion stresses.

V. COLLAGEN DEGRADATION

A. Proteinases

1. Collagenase

The changing composition of cartilage matrix during development, growth, and repair depends on a critical balance between the synthesis and degradation of cartilage components, collagen and proteoglycans. The degradation of articular cartilage is primarily mediated by proteinases and in particular by the class of proteinases referred to as metalloproteinases. Collagenase, the only known mammalian proteinase which can cleave helical collagen is operative at neutral pH and is widely distributed in mammalian tissues. Collagenase is collagen type specific and has been found in a variety of tissues including articular cartilage and is produced by a number of cell types including macrophages, synovial cells and fibroblasts (148-150). One basic tenet of mammalian collagenase is that it is responsible for the majority of collagen degradation in the extracellular matrix as an extracellular event. An enzyme which plays a critical role in cartilage integrity is collagenase which has been identified in the normal and arthritic human cartilage (150) and in the cartilage and pannus (151). Even though phagocytosis and lysosomal digestion of collagen may be also involved, a partial cleavage with collagenase is likely required for this to occur. The precise mechanisms by which the cartilage components, such as collagens and proteoglycans, are degraded under normal conditions (remodeled) and in disease are not fully understood. Collagenase plays a criticial role in the degradation of collagen by initially destabilizing the collagen helix by making a site specific cleavage of helical (mature) collagen (152). Incapable of maintaining a helical structure at physiological temperatures collagenase-

cleaved collagens unwind and become susceptible to further degradation by other proteinase present in the extracellular space. In this regard collagenase can be considered as the rate-limiting enzyme involved in collagen degradation.

The term collagenase has been used to refer to a particular protease which can cleave a peptide bond in the triple helical region of native collagen molecules. A simplistic way of classifying the heterogeneous groups of mammalian collagenases is by establishing 2 groups. One, referred to as the classical or interstitual collagenases cleave fibrillar collagen types I, II, and III. The other type specific collagenases are those which cleave one or more of the other collagen types such as type IV or V. A variety of molecular weight sizes have been estimated for vertebrate collagenases ranging from approximately 50 to 75 kilodaltons. Electrophoresis of enzyme secreted by human fibroblasts reveals characterisically 2 bands of 50 and 45 kilodaltons, respectively, where the proenzyme is slightly higher in molecular weight, approximately 60 and 55 kilodaltons, respectively (153). A large number of studies have characterized collagenases from a variety of sources, in both latent (proenzyme) and active form and all reflect molecular size characteristics within this range (reviewed by Stricklin and Hubbs) (154). The characteristic band of higher molecular weight has been shown to be a result of posttranslational glycosylation (155). Recent studies have characterized the gene for rabbit synovial cell collagenase as a 9.1 kilobase gene with 10 exons and 9 introns and has a high degree of similarity with other members of the metalloproteinase gene family (156).

Mammalian collagenase has a pH optimum of between pH 7 and 8 and is dependent on Ca^{2+} and Zn^{2+} for activity and stability. Divalent Zn^{2+} has

been determined to be at the active site of collagenases (157). A number of distinct genes and gene products have been identified for collagenase and comprise a collagenase gene family with at least 4 members (156,159). Classical collagenase cleaves helical collagens types I, II and III at a single locus involving either a Gly-Leu or Gly-Ile bond, at amino acids 775 and 776 (160,161). This results in two products referred to as TC^A and TC^B, the 3/4 part of the molecule and the 1/4 part, respectively. The specificities of the various tissue collagenases have not been fully clarified with regard to all the known collagen types. This is of particular interest since by definition, all collagens contain a helical portion although because of the presence of non-helical and globular domains they may not require cleavage with collagenase to permit susceptibility to proteolytic degradation. Other factors may also play a role in collagenase susceptibility, such as degree of cross-linking. Recent studies have shown that purified human rheumatoid synovial collagenase did not degrade types IX and XI collagen at low temperatures (25°C) and high temperatures (35°C) while cleavage of types II and X collagens occurred at the same temperatures (162). These authors showed that even though types IX and XI collagens were not degraded by purified collagenase in vitro, these cartilage collagens were susceptible to degradation by conditioned medium from IL-1 stimulated human chondrocytes, thus indicating the chondroctyes have the ability to synthesize different collagen-degrading enzymes. The precise molecules elucidated by chondrocytes and the regulation of their synthesis have not been fully characterized.

Central to maintaining an intact connective tissue matrix, is the regulation of collagenase. In part, this can depend on the activation of latent

proenzyme (163). Physiologically, a number of proteolytic enzymes are capable of activating procollagenase including plasmin produced by plasminogen-activator (164), plasma kallikrein (163) and stromelysin (166,167). A natural inhibitor (or group of inhibitors) of collagenase has been identified in cartilage and other tissues with an approximate molecular size of 28 kilodaltons. It is referred to as TIMP, for tissue inhibitor(s) of metalloproteinases (168-170). Mediators of proteinase secretion and activation and inhibition are covered in more detail Section VI. C.

2. Cathepsins and Elastase

A number of other enzymes are capable of degrading collagens in the non-helical regions and in collagen-cleaved collagens. The importance of proteinase which cleave the non-helical regions (eg. telepeptides) is that this cleavage can disrupt the stability of the fibril, since these regions in tropocollagen contain interchain cross-links. Cathepsin B and cathepsin G, cysteine and serine proteinases, respectively, are examples of enzymes which have been shown to cleave non-helical collagen (171). As these destabilized molecules denature, further degradation can occur by gelatinase, and presumably a myriad of peptidases and nonspecific proteinases, to further degrade the collagen molecule. These mechanisms have not been fully investigated. In addition to finding in cartilage inhibitiors of metalloproteinases, such as TIMP, there are inhibitors of serine proteinases and cysteine proteinases. The major inhibitors of these classes of proteinase are α 1-proteinase inhibitor for serine proteinases (172) and the cystatins for cysteine proteinases (173). An additional small molecular weight (13 kilodaltons) inhibitor of cysteine proteinases also has been identified in cartilage (170). Although the mediators and inhibitors of proteinase activity

have been characterized in vitro their ability to difuse into cartilage is related to there molecular size and the state of the collagen and proteoglycan matrix. Most certainly, molecules as large as α2 macroglobulin, a general proteinase inhibitor found in serum with a molecular size of 718 kilodaltons (174), would be unable to freely diffuse in cartilage without significant matrix degradation and loss (50).

The environment is important for these processes, since pH significantly affects these enzymes. Even though cathepsin B can degrade collagen types I, II and III (171,175) it is inactivated (denatured) at neutral pH, the pH of cartilage, and is only active for a short period of time in the extracellular environment around chondrocytes (176). The proteolytic enzymes cathepsin G and elastase originate from polymorphonuclear leucocytes which are mainly concentrated in the synovial cavity in rheumatoid arthritis. A recent study has shown that polymorph elastase has no effect on native collagen types II, IX and XI but did degrade denatured collagen types IX and XI (162). These studies confirm the resistance of native helical type II collagen to proteolytic activity by known proteases. Further studies on the specific enzymes (possible "cross-linkases") involved in the degradation of this impressively stable molecule are needed.

3. Stromelysin

Metalloendopeptidases or metalloproteinases are names that refer to the group of connective tissue degrading enzymes which include collagenase, and stromelysin. Previously, stromelysin was referred to as proteoglycanase and now it is accepted that proteoglycanase and collagenase activator proteinase are all terms referring to the same enzyme (177). Using the nomenclature MMP for matrix metalloproteinase, stromelysin would be

referred to as MMP-3. Within the past decade the biochemistry, functional properties, and genetic structure have begun to be elucidated. The metalloproteinase, which we now know as stromelysin, was identified in a variety of cell types including: rabbit fibroblasts (178,179) human synovial cells (180) rabbit bone cells (181), human fibroblasts (182).

Stromelysin, like collagenase is secreted in a pro-form and requires activation, which can occur in vitro by trypsin or APMA. Prostromelysin isolated from rabbit synovial fibroblasts has an apparent molecular weight on SDS-PAGE of 51 kilodaltons (183). The size varied after activation as shown by these authors and was converted to an active species ranging in molecular size from 41 to 25 kilodaltons. The molecular mass for human stromelysin has been shown to be very similar to that of other species. Human prostromelysin appears on SDS-PAGE as two species approximately 57 and 60 kilodaltons and an active form of approximately 45 kilodaltons (184-186). The pH optimum for stromelysin isolated from human cartilage on proteoglycan monomer has been shown to be pH 5.5 (185) and other have shown rabbit fibroblast stromelysin to be active at neutral pH (183).

Purified human and rabbit stromelysin have been shown to degrade proteoglycan, casein, fibronectin, laminin, and type IV collagen. Neither degraded type I collagen (183,186). Stromelysin and collagenase are induced and secreted coordinately by such agents a phorbol 12-myristate 13-acetate and 12-tetradecanoylphorbol 13-acetate (167,177,186). Stromelysin is inactivated by the metal chelator, EDTA and TIMP and resembles the other metalloproteinases.

Perhaps one of the most relevant aspects of the recent data on stromelysin which is pertinent to this thesis is its function as an activator of

procollagenase (166). Since collagenase is secreted as a proenzyme and requires activation once in the extracellular space, the activator (or activators) of latent collagenase are of paramount importance to collagen degradation. Recently, it has been shown using natural and recombinant collagenase and stromelysin that not only are the two enzymes coordinately regulated but stromelysin can act as the activator of procollagenase, in vitro (166). Use of recombinant enzymes confirmed the observations earlier identifying stromelysin as an activator of collagenase (187,188). The activator of stromelysin in vivo has been suggested as plasmin, although this remains to be clearly established (189,190). Fini et al identified a 1.9 kilobase cDNA clone which codes for a rabbit metalloproteinase of 53.9 kilodalton protein and has high homology with rabbit stromelysin (191). This proteinase can also activate collagenase (human synovial cell) and is coordinately suppressed and enhanced by dexamethasone or phorbol myristate acetate, respectively. Mediators such as IL-1 have also been studied for there ability to regulate stromelysin. It has been shown that IL-1β regulates human stromelysin at the transcriptional level (192).

Clearly, the role of stromelysin in the regulation of collagen degradation and in connective tissue breakdown itself, such as its ability to degrade proteoglycan, is a complex one and requires further study. Molecular cloning of the various members of the metalloproteinase family of enzymes will clearly define this group and identify their roles in the maintenance of connective tissue matrix integrity.

VI. MEDIATORS AND STIMULATORS OF PROTEINASE SECRETION

A. Interleukin 1

Interleukin 1 has been the subject of much research over the past decade. During such time, its identity and actions have been both elucidated and widely debated. A ubiquitous molecule first identified as a heat-labile protein found in acute leukocytic exudate which produced fever when injected into animals, this activity was called endogenous pyrogen (193). It was later shown to augment T lymphocyte responses and thus play a critical role in the immune response (194,195). Prior to the application of recombinant DNA technology and the cloning of IL-1, similar molecules having a variety of activities, were described. This resulted in a confusing list of names, including those of interest to this report such as, mononuclear cell factor, (196) and catabolin, (197). It is now clear that these cytokines are the same molecule which has been called interleukin 1.

IL-1 has two forms (α and β). Even though these are distinct gene products, they recognize the same receptor and share similar, if not identical, biological properties (review by Dinarello) (198,199). As a biologically active polypeptide, IL-1 has a molecular weight in humans of approximately 17 kilodaltons. Both α and β forms are synthesized as a 31 kilodalton precursor. The entire genes for human IL-1 α and β have been cloned (200). The amino acid sequence for α and β forms is highly conserved within a species (e.g. 75-78% for IL-1β) (199). Although IL-1 has biological similarities and some sequence homology with other cytokines, such as tumor necrosis factor (TNF) and both acidic and basic fibroblast growth factors, the use of recombinant IL-1 α and β has permitted the study of Il-1, independent of other cytokines. In the studies presented in this thesis, the effect of

exogenously added IL-1 β has been assessed on bovine and human cartilage explants utilizing recombinant IL-1 β.

IL-1 has been shown to be produced by a variety of lymphoid and non-leukocytic cells (including chondrocytes and fibroblasts) and has been associated with diverse natural biological processes. It has been shown to participate in various pathological processes (196,201,202). Its effect on proteinase secretion has been widely studied either using purified or, recombinant IL-1, or medium containing IL-1 (159). Many early studies with cartilage were carried out using conditioned culture medium from monocyte/mononuclear cell cultures (203,204). Some studies have further linked the effects of IL-1 and cartilage pathology with studies showing that collagen itself can act to stimulate its production (205) and that prostaglandin production can also be increased by IL-1. Prostaglandins are also potent molecules in a variety of effector pathways, both immune and non-immune (206,207). Of particular interest to the work in this thesis is the evidence of a direct involvement of IL-1 in articular cartilage destruction. In addition to in vitro studies of the effects of IL-1, a variety of authors have identified IL-1 in joint fluids of patients with a variety of arthritic conditions including rheumatoid arthritis and osteoarthritis (208,209).

IL-1 has been shown to be a potent inducer of collagenase and stromelysin production (proteoglycanase) from cells including chondrocytes (197,201,210,211). Besides stimulating the release of prostaglandins and metalloproteinases, IL-1 has been shown to stimulate the release of plasminogen activator from chondrocytes (212). Both plasminogen activator and stromelysin are capable of activating collagenase (166). This sequence of events, schematically represented in Figure 3, is suggested as one possible

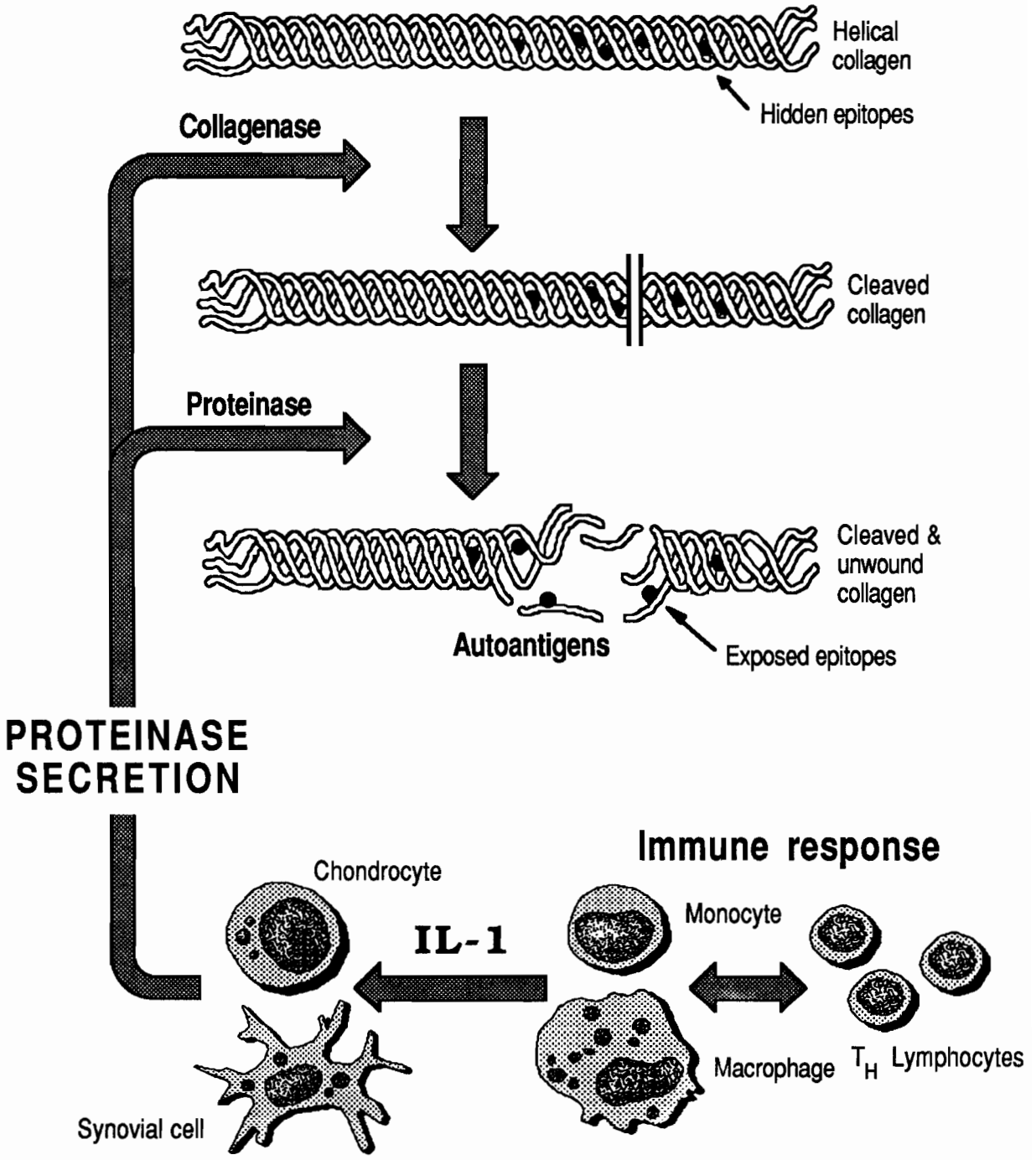


Figure 3 : Proteolytic Destruction of Cartilage Collagen

cascade for the degradation of collagen and ultimate destruction of articular cartilage. Other lines of investigation linking IL-1 with the erosive processes in chronic inflammation include studies where intra-articular injection of IL-1 has been shown capable of inducing inflammation and cartilage degradation in rabbit knee joints (203,213). While certain problems exist with these types of animal studies, they provide circumstantial evidence that IL-1 acts in vivo to mediate cartilage breakdown.

Inhibition of IL-1 can be achieved from a variety of points, including, receptor antagonists and molecules that limit transcription of IL-1 and the IL-1 receptor (reviewed by J.W. Larrick) (214). An important area which needs further study, is the identification and characterization of specific inhibitors of IL-1. Certain studies have elucidated inhibitors of IL-1, presumably derived from macrophage/monocyte populations and identified in the urine and serum of patients with diseases such as chronic juvenile arthritis (215). Other studies have shown that a molecule with an approximate molecular weight of 22 kilodaltons, purified from the urine of patients with monocytic leukemia, blocked the stimulation by IL-1 of both collagenase and prostaglandin E_2 in human synovial cells and fibroblasts (216). A molecule of similar size from human monocytes is produced upon stimulation with immune complexes and bears IL-1 inhibitory activity when tested in the IL-1 dependent mitogenesis assay using thymocytes (217). Since determination of inhibitory activity in vitro with partially purified factors does not guarantee that they function in vivo, further studies with recombinant molecules will elaborate the precise mechanisms of the regulation of IL-1. None the less, the importance of IL-1 in connective tissue remodelling and disease processes is undisputed.

B. Autocrine factors

Recently, Brinckerhoff and colleagues reported the identification of two autocrine proteins which induce the synthesis of collagenase from rabbit fibroblasts cultures (218). These factors were shown to be produced by rabbit synovial fibroblasts in culture upon stimulation with either phorbol myristate acetate or crystals of mono-sodium urate monohydrate. The proteins of 12 and 14 kilodaltons were purified from serum-free conditioned medium and amino terminal sequences were determined. The proteins induced in this model systems were shown by SDS-PAGE to be of approximately 57,14, and 12 kilodaltons, the largest corresponding to collagenase and/or stromelysin. Within the amino acid sequences determined there was a high degree (60-80%) of similarity betwen human serum amyloid A and the 14 kilodalton protein and with human β2-microglobulin and the 12 kilodalton protein. Although the mechanism by which these autocrine molecules act to stimulate collagenase synthesis is not known, it is clear that their effect is not limited to their autocrine function. It has been shown that purified human-serum amyloid A, (once thought to be produced by hepatocytes) can stimulate cultures of rabbit fibroblasts to synthesize collagenase (219. Endogenously, these molecules could be produced by fibroblasts of the joint tissues. This production could be influenced by products of macrophages such as IL-1 or other cells of the inflammatory response, and play an important role in the normal maintenance of and remodelling of the extracellular matrix. Over-production or an imbalance in the regulation of these recently described molecules may represent a mechanism which could play a pivotal role in the destructive process seen in the arthritic diseases.

C. Proteinase inhibitors

Just as important to the balance of extracellular matrix synthesis and degradation as the catabolic proteinases, are the inhibitors of these enzymes. Any imbalance between enzyme and inhibitor could result in an uncontrolled degradation of the substrate, collagen, as in the situation described in Figure 3. Further, action of collagenase, on fibrillar collagen results in an unwinding of the triple helical structure at physiological temperature and increased susceptibility to other proteinases. Molecules involved in inhibiting in vivo collagenase activity are referred to as TIMP or tissue inhibitor(s) of metalloproteinases and have a molecular weight of approximately 28 kilodaltons, depending on the tissue or source (168-170). As discussed in preceding sections, normal adult articular cartilage is permeable to molecules up to approximately 40 kilodaltons (50). For example, the small inhibitors of cysteine proteinases of approximately 13 kilodaltons (174) could readily diffuse into intact cartilage, wherease another important broad spectrum proteinase inhibitor, α2-macroglobulin with a very large molecular size of approximately 718 kilodaltons, could be excluded from affecting much of intact cartilage (174). After extensive degradation, cartilage could be more permeable to both inhibitors, such as α_2-macroglobulin and the proteinases themselves, each derived from serum and/or cells of the synovium and inflammatory response. Presumably, under normal conditions, the articular surface is more directly affected by mediators and inhibitors of proteinase activity, which originate from the synovium and blood supply, than are cells and matrix remote from the surface. An important point to make which is discussed again in this thesis, is that mid/deep zone cartilage and cartilage cells could be affected by factors coming from an inflammatory

response in the subchondral bone marrow. Some studies have demonstrated subchondral inflammation in the rheumatoid joint (220,221). Moreover, it can be suggested by the data presented here in this thesis that deep zone chondrocytes are very active in both osteoarthritis and rheumatoid arthritis, presumably stimulated by mediators like IL-1 to produce matrix degrading enzymes.

A valid approach to the inhibition of proteinase activity in vivo, would be to decrease the production of molecules known to stimulate proteinase secretion, such as IL-1. Inhibitors of the naturally occuring activators of latent metalloproteinases such as plasmin and cathepsin β (222) could also affect the degradative processes. In the case of collagenase, a reduction in the availability of metal co-factors by metal chelators could also play an important role in the regulation of proteinase activity and the maintainance of matrix integrity.

D. Connective tissue autoimmunity

Many investigators have studied the humoral and cellular immune responses to connective tissue components in arthritic patients, including those with chronic inflammatory arthritis, and in a variety of animal models. Since cartilage matrix components, such as type II collagen and proteoglycans are under normal conditions sequestered from the immune system (due to the absence in cartilage of a direct vascular and lymphatic supply), their excessive release from articular cartilage during proteolysis and or inflammation could be important in developing autoimmune responses. As schematically represented in Figure 3, fragments of collagen or exposed antigenic epitopes on non-native collagen could act as autoantigens and

stimulate more proteinase secretion and further degradation of cartilage collagen (and proteoglycan) resulting in a perpetuation of the destructive processes (223). Cellular immune responses have been measured in response to native and denatured types I, II and III collagens in the peripherial blood of patients with connective tissue diseases, particularly those with a chronic inflammatory component (75,224-226). Other studies have shown that degraded cartilage components such as human type II collagen peptides can stimulate cultured human synovial fibroblasts and explants of human rheumatoid synovial tissue, to secrete collagenase (227).

Animal studies have produced a larged body of evidence linking connective tissue autoimmunity with arthritic diseases. Cellular immune responses to collagen, in particular to fragments of types I and III collagens (primary collagens of synovial tissue), have been identified in animal models of arthritis, including rabbits with Mycobacterial induced inflammatory arthritis (228,229). In addition, the development of a rodent model of arthritis which is induced by injection of helical, homogolous or heterologous type II collagen, has provided further evidence of the importance of connective tissue antigens in the development and persistence of arthritic diseases. The animal model has permitted a vast area of study concerned with the antigenic components of the type II collagen molecule and with the cells (lymphocytes) or antibodies which are responsible for disease expression (230-234).

Although it is difficult to clearly assess the precise autoimmune connective tissue components in human disease it is even more unlikely that reasearch in this area will assign a single epitope or molecule the exclusive role of a causative or responsible agent for the destruction of articular

cartilage in the arthritic diseases. There is, however, a large body of evidence including what has been aforementioned which links autoimmune responses to cartilage components to the chronicity and progression of many of the inflammatory arthritic diseases. Very recently, a study was reported extending others data linking antibody levels to various collagens with rheumatoid arthritis (313). This study concluded that anti-collagen antibodies may be produced to a wide variety of collagen epitopes and that the heterogeneity of antibodies may be a result of the different components being exposed and degraded during the course of the disease. These authors studied the levels of antibodies to native and denatured types I, II, IX and XI collagens from rheumatoid arthritic patients. Having already established that these patients had antibodies to native type II collagen they were interested in any association with the minor collagens of cartilage, types IX and XI. These findings along with similar studies dating back to pioneering studies in this area, such as those by Andriopoulos et al. (235) provide sound evidence that collagen autoimmunity exists but still leaves open the question of whether it is a primary or secondary phenomenon.

VII. ARTHRITIC DISEASES

A. Osteoarthritis

1. General clinical features and etiology

Osteoarthritis (or osteoarthrosis or degenerative joint disease) is the most prevalent form of arthritic disease and in some respects can be considered as an arthritis of aging or of "wear and tear". Osteoarthritis is characterized by a progressive degeneration and loss of articular cartilage with associated bony changes, both at the joint margins and in subchondral bone. In general terms, osteoarthritis can be classified as primary (idiopathic) where there is an absence of etiologic factors (as in the case with some age-related changes) or as secondary, where there is a clear local or systemic causative factor, such as joint trauma or skeletal abnormalities. Symptoms and clinical manifestations include joint stiffness, motion limitation and, progressively developing joint pain which can be a secondary event as a result of the degeneration of joint tissues (236). Synovitis is commonly associated with chronic (late stage) degenerative disease or acute episodes (237). The symptoms as they relatate to the classification of the arthritides are quite variable and at times overlap with other joint disease (167). The American College of Rheumatism sets classification criteria for osteoarthritis as for all rheumatoid diseases. These criteria allow scientists and practioners to classify these diseases by combining clinical, radiologic and laboratory findings and are constantly being reviewed and updated (238).

The clinical features, such as pain and motion abnormality, are the first indications of disease and further, often indicate appreciable and advanced pathology. Symptomatic pain arises from associated joint tissues since cartilage itself has no nerve supply. Affected joints can be isolated, bilateral

(eg. both knees) or systemic. Most commonly joints effected are: the knee, hip, the extremities (hands and feet) and spine, with the major weight bearing joints of the knee and hip being most effected.

2. Pathology

Pathological changes in osteoarthritic cartilage both reflect degradative and proliferative changes. Gross changes in the cartilage include surface erosions, fibrillation, and cleft formation. As the disease progresses, the erosion of cartilage can be progressive to the extent where there is a total loss of cartilage down to subchondral bone. Historically, the destructive processes were thought to be predominantly in the superficial zones of cartilage particularly in the earlier stages and caused by factors released into the synovial fluid including factors such as IL-1 and degradative proteinases. The progressive degeneration of cartilage is a result of the chondrocytes ability to ultimately destroy its own matrix. Data presented in this thesis provide direct evidence that these degradative changes are indeed occuring and, moreover, are occurring throughout the cartilage, including the predominant superficial zone changes. Proliferative changes, in contrast, include chondrocyte clustering and osteophyte formation observed predominately at the jont margins. Chondrocyte clustering is often seen in histological sections associated with fibrillation and clefts presumably as an attempt at repair.

Biochemical characteristics of osteoarthritic cartilage include a net loss of proteoglycan and collagen (239-241). As is presented and discussed in this thesis, there is appreciable damage to collagen fibrils which results in a loss of structural integrity and an inability of the fibrillar collagen to retain proteoglycan. Although the predominant collagen of adult articular cartilage

is type II, both type I and type III have been identified in human osteoarthritic cartilage, perhaps indicative of a repair process (242).

Proteoglycans in osteoarthritis have been extensively studied. Using metachromatic dyes and immunostaining, a loss of proteoglycans is associated with cartilage degradation in osteoarthritis (243). In experimental osteoarthritis in the dog (rupture of anterior cruciate ligament), it has been shown that not only is there an early loss of proteoglycan at the surface but there are changes in proteoglycan content indicating both a synthesis, degradation and reorganization (244). Changes in proteoglycans are in content and character. A review of a variety of studies has shown that in early disease there is an increase in chondroitin sulfate content in relationship to keratan sulfate whereas in late stage disease there is net loss of proteoglycan (see review by Poole) (223).

Clinically, osteoarthritis remains a major problem with little effective treatment short of joint replacement. Further understanding of the mechanisms of the destructive processes involved in the joint destruction and ultimate loss of function, will provide the basis for therapeutic intervention and early detection.

B. Rheumatoid arthritis

1. General clinical features and etiology

Rheumatoid arthritis describes an inflammatory condition which is chronic and systemic and can involve many joints of the body. The common feature which brings a variety of articular and extraarticular manifestations together in the term rheumatoid arthritis, is synovial inflammation. This articular inflammation contributes to the progressive joint destruction and

deformity which are paramount features of this diseases. Mononuclear cell infiltration, hyperplasia, and fibrosis are all common features of synovial inflammation. Increased production of synovial fluid occurs. This contains a myriad of immunue cells, cytokines and mediators of inflammation and proteolysis (see Figure 3). Polymorphonuclear leucocytes and monocytes are present and capable of producing degradative enzymes and may contribute (at least in the superficial zones) to the progressive degradation of articular cartilage (245,246).

Extraarticular features which are characteristic of rheumatoid arthritis include neuropathy, arteritis, scleritis, nodule formation, splenomegaly, and lymphadenopathy all of which were once thought to be sequelae of the primary disease and now accepted as integral components of systemic arthritis (247). Clinical symptoms of rheumatoid arthritis are not completely dissimilar from those described for osteoarthritis. In addition to joint pain, stiffness and limited movement, these are the classical signs of inflammation: edema, erythema, heat and tenderness. A combination of clinical features, discomfort and severe disability may occur in acute flare-up or as chronic episodes which last for an individuals entire life. Symptoms are most often gradual in developing and commonly occur symmetrically (eg. both knees or hands). Since there is major erosion of cartilage, bone, tendons and ligaments with pannus formation and proliferation, progression of the disease results in disabillity deformities and joint immobility.

Although the disease can occur in any individual regardless of age, race etc., there is a plethora of epidemiological information available. Its prevalence in the United States is estimated to be between 0.3% and 1.5% of the population. Women are affected 2 to 3 times as often as men and with

the peak of incidence in the 4th and 5th decades (248). From studies of HLA class II gene products in rheumatoid patients, individuals with haplotype DR-4 are several times more susceptible to developing rheumatoid arthritis (249,250). Since class II histocompatibility antigens are involed in immune responses, particularly antigen presentation, these associations are undoubtedly important and continue to be investigated.

Laboratory findings unique to rheumatoid arthritis are headed by antibodies which are specific for antigenic determinants on the Fc portion of IgG (i.e. rheumatoid factor antibodies) (251). Since the existence of rheumatoid factors occurs in 1-5% of non arthritic individuals there presence does not necessarily indicate disease, nor do their absence precude the possiblity of the development of disease. High levels of these antibodies are generally associated with active disease and severity and in addition usually indicate a high frequency of secondary complication and poor prognosis.

Many studies and as many thoughts exist on the etiology of rheumatoid arthritis. No conclusive agent or agents can be identified, viral, bacterial or otherwise. What is clear is that this disease is intimately linked with an immune response. Autoimmunity, particularly to connective tissue antigens, may be involved in the chronicity of arthritis (223). Evidence of autoimmunity to molecules such as type II collagen and proteoglycans exsists from a number of studies (75,228,252-253). There is some association with cellular and humoral immunity to collagen type II in patients with rheumatoid arthritis (227,254). Moreover, type II collagen can be injected into rodents and an erosive inflammatory joint disease ensues (255). Since cartilage specific molecules such as type II collagen and aggregating proteoglycan are sequestered from the immune system under normal conditions, the

degradation of such molecules and their exposure could lead to their action as autoantigens thereby stimulating the degradative process even further (as outlined in figure 3).

2. Pathology

Many of the early changes in the joints are related to synovial tissue, hyperplasia of both type A and B synovial cells, increased vascularity and mononuclear cell and neutrophil infiltration. Immune complexes of antibodies with a wide range of antigens have been identified, such as byproducts of inflammatory processes, mainly fibrin, collagen, digested IgG and DNA. These complexes trigger further responses and release of hydrolytic, proteolytic enzymes, and arachidonic acid metabolites from cells. All these mediators are themselves capable of furthering inflammatory and/or degradative processes. Collagenolytic activity is increased as a result of increased collagenase production by synovial cells in response to products of these processes such as IL-1 (256). Cartilage collagen has been thought to be degraded and lost from arthritic cartilage secondarily to the loss of proteoglycan. Studies have indicated collagenolytic activity in surface layers of cartilage and at the interface of synovium and cartilage (151,223,257,258) presumably as a result of the local environment (eg. synovial fluid). As will be discussed later, the studies presented here in this thesis draw attention to two important findings, in this regard, one being evidence of early degradation of fibrillar type II collagen and the significant amount of collagen breakdown occuring throughout the cartilage zones, and in particular, remote from the surface. Ultrastructurally, damaged collagen fibrils have been detected in pericellular and interterritorial regions of cartilage (259).

Proteoglycans have been less extensively studied in rheumatoid cartilage and these changes are still uncertain. The loss of proteoglycan has been identified particularly in the superficial zone and in vitro studies have shown that proteoglycans can be degraded by factors contributed by rheumatoid synovium (260). These degradation products are of a variety of types and do not aggregate with hyaluronic acid. Extrapolated to the in vivo situation, proteoglycans degraded by an inflammed synovium would be incapable of fulfilling their role in cartilage and would thus compromise the function of cartilage. The association of proteoglycan fragments with disease activity has been studied by a number of laboratories using RIA detecting fragments in synovial fluid (261). Studies of the biochemical and immunological parameters are difficult for a number of reasons, including the difficulty in obtaining normal tissue and synovial fluids, and although diseased joints are accessable at the time of total arthroplasty at this stage of degeneration there is a minimum of cartilage remaining. Inflammatory changes are also recognized in subchondral sites in rheumatoid arthritis and are associated with bone resorption and loss. These changes can result as well from the action of cytokines such as IL-1 and tumor necrosis factor (194,262). Cellular infiltration from subchondral bone into cartilage has also been observed (263-265) which could lead to degradative and biosynthetic effects seen in deep zone cartilage.

CHAPTER 2

MATERIALS AND METHODS

I. REAGENTS, CHEMICALS, MATERIALS AND EQUIPMENT

The following list of reagents and chemicals, with their sources, refers to specific materials used in the experimental aspects of this thesis. All other chemicals used in this work were high quality, reagent grade chemicals from general laboratory suppliers.

Pepsin	Cooper Biomedicals, Malvern PA
Cesium Chloride	Accurate Chemicals, Westbury, NY
Cyanogen Bromide	Aldrich Chemical Co., Milwaukee, WI
Sodium dodecyl sulfate	Schwarz/Mann Biotech, Cleveland, OH
Acrylamide	Bio-Rad, Richmond, CA
^{14}C-methylated molecular weight standards	" " "
Coomassie Blue R-250	" " "
Freunds complete adjuvant	Difco, Detroit, MI
Freunds incomplete adjuvant	" " "
AH-Sepharose-Protein A	Pharmacia, Uppsala, Sweden
Ketamine-HCl	Rogar/STP Inc., Montreal, Quebec
Xylazine	Hauer-Lockhard, Bayvet Div., Miles Laboratories, Rexdale, Ontario.
Sodium pyruvate	Gibco, Grand Island, NY
Non-essential amino acids	" " "
Dulbecco's modified Eagles med.	Flow Laboratories, McLean, VA
Fungizone	" " "

Normal donkey serum	SEROTEC/Daymar Laboratories, Toronto, Ont.
Chondroitinase ABC	Miles Laboratories, Napierville, IL
Keratanase	" " "
OCT embedding medium	" " "
Hyaluronidase (testicular, type VI-S)	Sigma, St. Louis, MO
4-aminophenylmercuric acetate	" " "
Disodium p-nitrophenylphosphate (Sigma-104)	
Gentamicin	" " "
Bovine serum albumin (RIA grade, crystallized)	" " "
Trypsin Type XIII	" " "
2-mercaptoethanol	" " "
Cyclohexylamino propane sulfonic acid	" " "
Pepstatin A	" " "
Phenylmethylsulfonyl fluoride	" " "
HEPES	" " "
BES	" " "
TES	" " "
Paraformaldehyde	BDH Chemicals, Montreal, Quebec
Gelatin	" " " "
Iodoacetamide	" " " "
Nonidet P40	" " " "
Gum arabic	" " " "
Silver lactate	ICN Canada, Montreal, Quebec
Uranyl acetate	Fisher Canada, Montreal, Quebec
Permount	" " " "
Hydroquinone	" " " "

Diethanolamine	Fisher Canada, Montreal, Quebec
Lead citrate	Polyscience, Warrington, PA
Normal ascites fluid	BRL - Bethesda Research Laboratory Bethesda, MD
IL-1β (human recombinant)	Ciston, Pine Brook, NJ
Biotinylated donkey anti-rabbit Ig	Amersham, Arlington Heights, IL
Fluorescein-labelled streptavidin	" "
Gold-labelled streptavidin	" "
^{125}I-Protein A	" "
Nitrocellulose Membrane (Transblot)	" "
Polyvinylidine difluoride membrane (Immobilon)	Millipore, Bedford, MA
X-Omat AR X-ray film	Kokak, Rochester, NY
Amicon membrane filter VM 30	Amicon Div., W.R. Grace & Co. Danvers, MA.

Equipment and instrumentation that was used as mentioned in this thesis and bears noting is listed below:

Tissue-TEK II Cryostat	Miles Laboratories, Napierville, IL
Mini-Protean Apparatus	Bio-Rad Laboratories, Richmond, CA
Eppendorf Microcentrifuge 5313	Fisher Canada, Montreal, Quebec
Cronex Lightning Plus Intensifying Screen	Dupont, Wilmington, DE
Applied Biosystems Microsequencer Model 470A	Applied Biosystems, Foster City, CA

Savant Speed Vac	Emmerston Instruments, Scarborough, Ont.
Microdialysis apparatus	(BRL) Bethesda Research Laboratories, Bethesda, MD
Thertek Microtiter Reader	Flow Laboratories, McLean, VA
96-well flat bottom tissue culture plate (No. 76-032-05)	" " " "
Linbro 12-well tissue culture plate (No. 76-053-05)	" " " "
Beckman HPLC System, Model 110A pumps, Model 421 controller, model 160 detector	Beckman Instruments, Palo Alto, CA
VYDAC C18 column TP201 (4.6 x 250 mm)	CSC Inc., Montreal, Quebec
VYDAC C18 column 218TPB (25 x 0.94 cm)	" " "
Amicon ultrafiltration cell, 25 ml	Amicon Div., W.R. Grance & Co. Danvers, MA.

II. TISSUE

A. Animal

Bovine condylar articular cartilage was obtained from animals (12-18 months of age) from a local abbatoir shortly after sacrifice. For tissue culture studies, condylar cartilage was aseptically removed from the metacarpal-phalangeal joint. Bovine nasal cartilage and bovine skin were obtained from similar animals for the isolation and purification of type II and I collagen, respectively. Bovine growth plate cartilage was removed from the femoral condyle of near term fetuses of animals 12-18 months of age.

B. Human

Human articular cartilages were obtained from the femoral condyles of adult knee joints with the following exceptions: radial head cartilage from patient KE (33 year old female, rheumatoid arthritic, rheumatoid factor negative); tibial plateau cartilage from patient CP (73 year old male, osteoarthritic). All arthritic cartilages were obtained at surgery from rheumatoid or osteoarthritic patients undergoing total joint arthroplasty or at the time of autopsy within 10 hours post mortem. Four osteoarthritic cartilages were examined from patients PS, 69 year old male; VM, 74 year old female; CP, 73 year old male; CR, 63 year old female. All patients were diagnosed as having classical non-inflammatory osteoarthritis. Histologically each cartilage had identifiable surface fibrillation. Rheumatoid cartilages were obtained from patients LM (65 year old female) and DK (63 year old female) and both were rheumatoid factor positive. Cartilage from two rheumatoid factor negative patients

AD (59 year old male) and KE (33 year old female), were also examined.

Non-arthritic (normal) cartilages were obtained from four individuals within 10 hours post mortem at the time of autopsy, each having no known history nor signs of arthritic/joint abnormalities. The following age-matched cartilages were examined: 73 year old male, 61 year old male, 56 year old male and 71 year old female. All tissues were handled identically and processed for immunohistochemistry, as described below.

For the purposes of preparing human cartilage explants, non-arthritic articular cartilage was obtained at autopsy within 10 hours post mortem, from the femoral condyle of individuals of ages 47, 59 and 63. All individuals were males and the causes of death were cerebral hemmorage, carcinoma, and cardiac disease, respectively. Cartilage which appeared macroscopically healthy was asceptically removed and cut into approximately 2 mm cubes. For studies where cartilage was examined immunohistochemically for type II collagen degradation, full thickness pieces of cartilage were used (approx. 3mm x 6mm x 2mm) to allow correct orientation and permit examination of events in the various cartilage zones. Culture experiments were performed as described in section VII.

III. COLLAGEN PREPARATION

A. Extraction and Purification

Type II collagen was extracted and purified from adult bovine nasal septum or adult human femoral condylar cartilage by differential salt precipitation as described by Miller (15). A schematic of the procedure is represented in Figure 4. Briefly, the cartilage was minced with a scalpel and twice extracted with 4 M guanidine-HCl in 0.15 mM potassium acetate, pH 6.3 to remove the proteoglycan. The resulting residue was rinsed in water and dissolved in 0.5 M acetic acid containing 1 mg/ml pepsin for 24 h at 4°C, with stirring. After centrifugation at 15,000 g_{av} for 20 min the supernatant was adjusted to 0.9 M NaCl by slow addition of crystalline salt. The resultant precipitate was recovered by centrifugation and redissolved in 50mM Tris-HCl, pH 7.5 containing 1 M NaCl and dialyzed extensively against 20mM Na_2HPO_4. The precipitate was redissolved in 0.5 M acetic acid and NaCl was added to a final concentration of 2.22 M NaCl. The precipitated type II collagen was collected by centrifugation and redissolved in 0.5 M acetic acid and exhaustively dialyzed against the same to remove residual NaCl. In most cases the selective salt precipitation was repeated on small quantities (25-100mg) two or three times to ensure purity and to remove contaminating proteins and smaller than α chain collagen fragments. The collagen was then lyophilized, checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described below, and stored at -20°C.

Bovine type I collagen was isolated from bovine skin by differential salt precipitation as previously described (266). Bovine collagen types V, VI, IX and XI were kindly provided by Dr. David R. Eyre (Department of

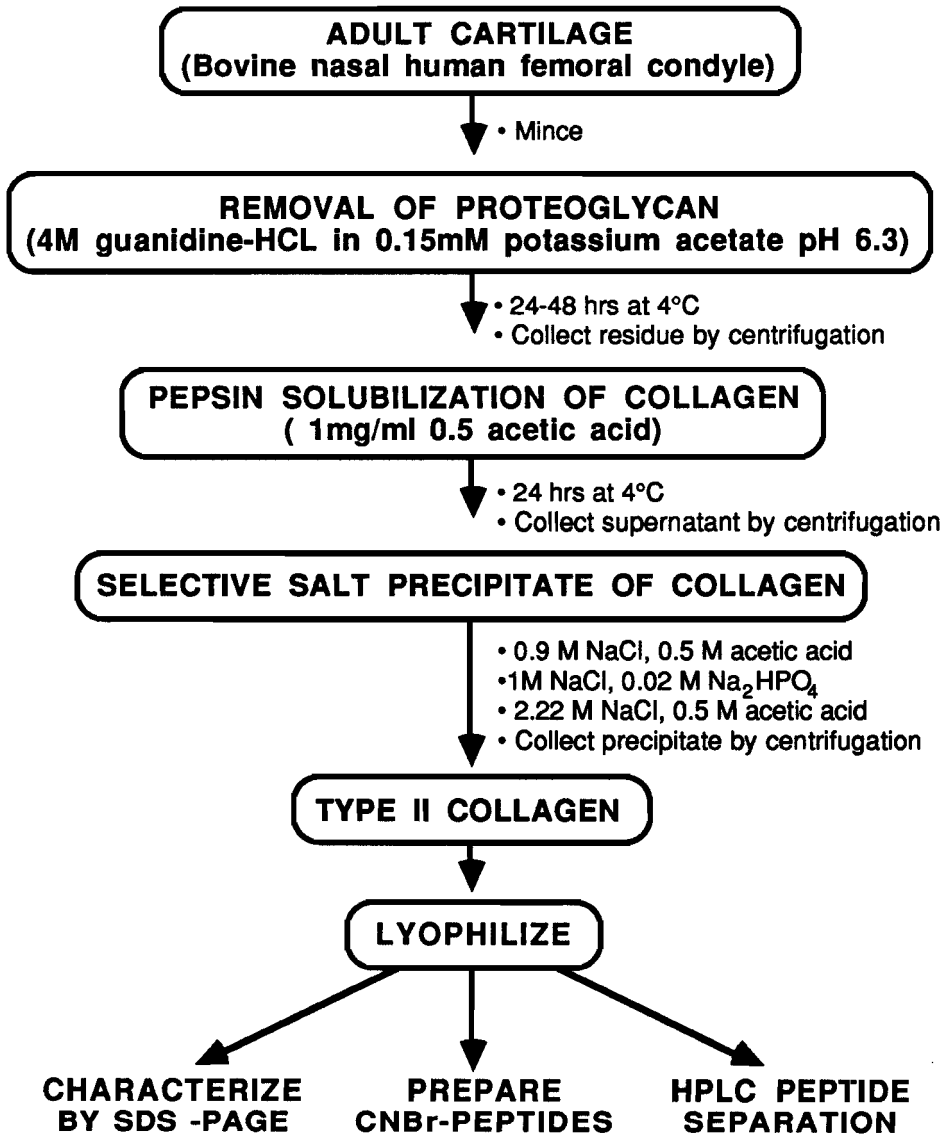


Figure 4 : Preparation of Type II Collagen

Orthopedics, University of Washington, Seattle, WA). Each was determined to be pure by SDS-PAGE analysis and used for ELISA analysis. Human type II collagen was a generous gift from Dr. Michel van der Rest (Genetics Unit, Shriners Hospital for Crippled Children, Montreal, Quebec).

B. Density gradient centrifugation

Bovine growth plate or articular cartilage and normal human cartilage were dissected into small pieces of 1 mm^3 and extracted with 4 M guanidine-HCl, 50mM EDTA, 0.15 M sodium acetate, pH 7.0, containing proteinase inhibitors as described for use in the immunohistochemical protocol (Section VI). The extraction and subsequent fractionation were performed as described elsewhere (267). The caesium chloride density gradient centrifugation was performed under associative conditions as described, resulting in fractions designated A1 through A6 (nomenclature as described by Heinegård) (268).

C. Cyanogen bromide cleavage of collagen

Collagens were cleaved at methionine residues with cyanogen bromide (CNBr) using a method based on others (269,270). Collagens were dissolved in 70% formic acid at a concentration of 5 mg/ml to which was added 12 mg/ml of CNBr. The tubes were flushed with nitrogen, sealed and kept at 26°C. The digestion was terminated by a ten-fold dilution with distilled water. After lyophilization the collagen peptides were stored at -20°C. Purity and characterization were determined by SDS-PAGE. Initially, the reaction was carried out for 5 hrs. with intermittent shaking, although this resulted in a considerable amount of collagen not completely

cleaved. Many bands on SDS-PAGE appear larger than the largest single CNBr-derived peptide, α1(II)CB10). To improve the yield of individual peptides several changes were made including: increasing the reaction time to overnight (18-20 h), performing the reaction under reducing conditions and using fetal bovine type II collagen. Reduction had no effect on the completeness of cleavage. This suggests that reducible cross-links are not involved in preventing cleavage at each methionine, nor are there reducible cross-links between α-chains which result in dimeric peptides. When CNBr-derived peptides of type II collagen purified from fetal and adult bovine cartilages were compared by SDS-PAGE, no significant difference could be observed in the cleavage products (Figure 8). One protein band was identified present in the fetal collagen peptides which is not present in the adult preparation. This presumably represents either an incomplete cleavage between two peptides or one or more peptides joined together by the presence of interchain cross-links.

D. Reversed-phase high performance liquid chromatography

Purified bovine or human type II collagen or CNBr-derived type II collagen peptides were characterized by reversed-phase HPLC using a method described by van der Rest et al. (271). Collagens were prepared as described elsewhere and were rehydrated from their freeze-dried state, in 0.01 M HFBA in 35% (v/v) acetonitrile in water (solution A of the mobile phase gradient). The acetonitrile used was HPLC grade and water was deionized and passed through a Sep-PAK C-18 column. Both mobile phase solvents were filtered through a 0.22 micron Millipore filter and degassed for 10 min. The HPLC system consisted of two pumps with a gradient

programmer. The optimal separation conditions were obtained when using 0.01 M HFBA as the ion-pairing mobile phase in a linear gradient of acetonitrile and water (35%-60%). The separation was performed during a 1 h linear gradient with a flow rate of 1 ml/min for the analytical column and 3 ml/min for the semi-preparative column. Column eluates were monitored for UV absorbance at 214 nm using an in-line Beckman Model 16D spectrophotometer. Because of the size and special characteristics of collagen/collagen peptides a separation column with a large pore size and phase was used. According to the amount of material to be chromatographed an analytical column (4.6 x 250 mm) or a semi-preparative column (25 x 0.94 cm) was used. The column fractions were collected, the peaks pooled, and dried in polyethylene tubes using a Savant Speed-Vac centrifuge evaporator. Fractions were examined by SDS-PAGE as described in Section V, B.

IV. ANTISERA

A. Antibody Production

Female New Zealand white rabbits weighing 2.5 kg (Ferme des Chenes Bleues Inc., Montreal, Quebec) were maintained on rabbit chow (Ralston Purina Inc., Montreal, Quebec). Prior to immunization, serum was obtained from a preimmune blood sample of 30 ml which was taken by cardiac puncture under anesthesia using a combination of ketamine-HCl, (25 mg/kg) with xylazine (3 mg/kg).

For the antiserum, R181, the primary immunization consisted of 5 mg bovine type II collagen CNBr-derived peptides in 0.5 ml 0.9% NaCl, 0.01% acetic acid emulsified with 0.5 ml complete Freund's adjuvant. Half total volume was injected intramuscularly into each hind leg. Booster injections of 1 mg bovine type II CNBr-peptides emulsified with incomplete Freund's adjuvant as before were injected either intramuscularly or subcutaneously or both, every 2-3 weeks. Each booster was followed 2 weeks later by a test bleed and antibody titer determination by ELISA.

After two booster injections the antiserum was of sufficient titer and 50 ml of blood were obtained by cardiac puncture. For immunohistochemical studies a $F(ab')_2$ preparation was prepared by pepsin digestion, as described in this section subsection B. In general, antisera were stored at -20°C except for a small aliquot of a working preparation. Repeated freezing and thawing was avoided.

Antisera R167 and R254 were prepared in this laboratory and used in these studies as a positive control for cartilage matrix components. For antiserum R167, the immunogen was fractions A4,5,6 of an associative cesium chloride density gradient centrifugation of bovine fetal growth

plate cartilage. To prepare antiserum R254, fractions A5 and A6 of a similar centrifugation gradient of bovine fetal growth plate were used to immunize the rabbit. Both antisera were prepared by a similar immunization protocol as described for R181, and were shown by ELISA to react with multiple proteins of whole-bovine cartilage extracts (unpublished observations, A.R. Poole). Antiserum R110 was prepared and characterized in this laboratory by a similar protocol as described for R181 with bovine proteoglycan, fractions D1,2,3 from a dissociative cesium chloride density gradient centrifugation.

The mouse monoclonal antibody AN9P1 was utilized to immunohistochemically identify the presence of proteoglycan in articular cartilage. The monoclonal was a gift from C. Webber (Joint Diseases Laboratory, Shriners Hospital, Montreal) and has been characterized and described by Witter et al (261). AN9P1 has been shown to react with keratan sulfate or keratan sulfate stubs when they are bound to cartilage proteoglycan core protein. The monoclonal was raised as an ascitic fluid, therefore normal mouse ascitic fluid was utilized as a control when AN9P1 was used. Both were used at a dilution of 1/32 in PBS containing 1% BSA and 0.25% v/v Nonidet P40, as was used for the other antibodies. When these reagents were used immunohistochemically, a second step antibody was needed in order to utilize the biotinylated donkey anti-rabbit antibody, used with the other antisera. A rabbit antiserum to mouse IgG, prepared in this laboratory, was used at a dilution of 1/10 in PBS as described above.

B. Preparation of $F(ab')_2$ for immunohistochemistry

To reduce nonspecific binding and facilitate the penetration of the antibodies throughout the tissues, $F(ab')_2$ was prepared. Each antiserum used in the immunohistochemical protocol was used as a $F(ab')_2$ preparation, including non-immune rabbit serum which was used as a control. The biotinylated second step antiserum (donkey anti-rabbit $F(ab')_2$) was commercially obtained as a $F(ab')_2$ preparation. For all other antisera the procedure used was as previously described by Poole et al. (29,178). Briefly, after sufficient time for clotting to occur (2-4 h, the blood was centrifuged at approximately 1200xg using a general laboratory centrifuge at room temperature for 20 minutes. The resulting serum was removed and the immunoglobulin fraction precipitated two times using 50% (v/v) saturated ammonium sulfate. This was achieved by the slow addition of an equal volume of saturated ammonium sulfate, while stirring. The final precipitate was dissolved in PBS and dialyzed against the same with several changes overnight at 4°C. The resulting partially pure whole IgG preparation was digested with pepsin at 1 mg pepsin per mg of IgG in PBS with an adjusted pH of 4.0. After digestion for 18 h at 4°C the Fc portion and any undigested IgG was removed by absorption to AH-Sepharose-Protein A (272). The Sepharose was removed by gentle centrifugation, washed two times with PBS and concentrated using an Amicon ultrafiltration cell with a YM30 filter. The A_{280} was monitored to ascertain effective washing. Each serum was handled in an identical manner. The protein concentration was determined on the final concentrate. The protein concentration was calculated based on the assumption that 1mg/ml of protein has an absorbance of 1.3 using a cell

with a 1cm path length (29).

Generally, small aliquots of each serum were stored at 4°C 1 mg/ml containing 1mg/ml protein and sodium azide at a final concentration of 0.05% (w/v), to prevent contamination.

V. ANTIBODY CHARACTERIZATION

A. Enzyme-linked immunosorbent assay

Purified collagens were dissolved in 0.1 M acetic acid at 2 mg/ml and diluted in 0.1 M carbonate buffer, pH 9.0 to 50 µg/ml. Fifty µl were added to each well of a 96-well flat bottom tissue culture microtiter plate. Where indicated, collagens were denatured after dilution by heating for 15 min at 60°C. Collagens were bound overnight at 4°C to ensure the integrity of native collagens. The assay was continued as previously described (273) with the following modifications. The labelled second step antibody was a porcine anti-rabbit $F(ab')_2$ (29) labelled with alkaline phosphatase (274) which was prepared in this laboratory. The substrate used was disodium p-nitrophenylphosphate (Sigma 104) at 0.5 mg/ml in diethanolamine buffer (9.6% diethanolamine, 0.2 mM $MgCl_2$, pH 9.8). Routinely, the plates were incubated with the substrate at 37°C for 1-2 h before reading the optical density at 405 nm using a Titertek microtiter plate reader Immune serum R181 was compared in all tests to the preimmune serum of R181.

B. Electrophoresis and immunoblot analyses

SDS-PAGE of collagens was performed according to Laemmli (275) in 1 mm thick 10% slab gels (16 x 18 cm format) with two exceptions where noted: 1) a 7.5 -15% gradient gel was used to characterize CNBr-derived peptides of type II collagen, and 2) the Mini protean apparatus (7.5% gel, 1 mm thick, 7 cm x 8 cm) was used for α1(II) chain analysis. Collagens in sample buffer containing 0.35 M 2-mercaptoethanol were heated to 60°C for 10 min and centrifuged for 2 min in a fixed speed Eppendorf centrifuge 5313 prior to loading to remove any undissolved material.

Molecular weight standards used were the following: [^{14}C]-methylated proteins which have approximate molecular weights (in kilodaltons) as indicated: myosin, 200; phosphorylase b, 92; BSA, 68; ovalbumin, 46; carbonic anhydrase, 30; lysozyme, 14. Gels were stained with Coomassie blue R-250 in 40% v/v methanol and 10% v/v acetic acid in water or transferred to nitrocellulose. Electrophoretic transfer to nitrocellulose was performed according to Towbin et al. (276) except that the transfer was carried out in methanol-free electrode buffer of 25 mM TRIS, 192 mM glycine, pH 8.3. This resulted in a more complete transfer of collagens, since some collagen peptides are not transferred efficiently with methanol present (G.R. Dodge, unpublished observations). The membrane was blocked for 16 h at 4°C with 3% BSA in 10 mM sodium potassium phosphate, pH 7.2, containing 0.145 M NaCl and 0.05% NaN_3 (PBS). After rinsing with PBS for 15 min at room temperature the membrane was incubated for 1.5 h at room temperature with the antiserum (R181) or a mixture of antisera R254 and R167, or preimmune serum, each at 10% concentration after dilution in PBS containing 1% BSA. After exhaustive washing with PBS containing 0.01% Tween 20, the immunoblot was incubated for 1.5 h at room temperature with ^{125}I Protein A (10^5 cpm/ml) in PBS containing 1% BSA. The membrane was then washed thoroughly in PBS, air dried and the radioactivity was visualized by radioautography using X-OMAT AR X-ray film. Routinely, the exposure time was overnight at -70°C with the use of a Cronex lightning plus intensifying screen.

C. Protein sequence analysis

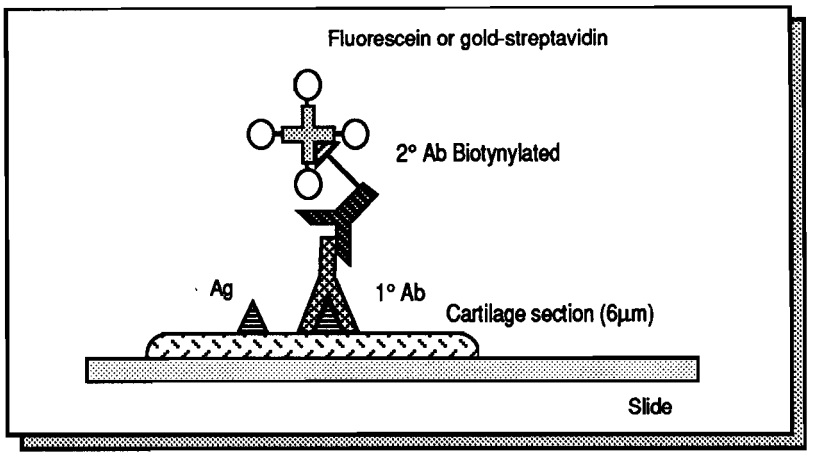
CNBr-derived peptides of bovine type II collagen were electrophoresed in 1 mm thick 10% slab gels as described above. Separated proteins were electrophoretically transferred onto a PVDF membrane as described by Matsudaira for sequencing small quantities of proteins (277). Briefly, the PVDF membrane is quickly stained with Coomassie Blue R250 after transfer and destained sufficiently to identify the protein bands. For the purposes of peptide identification, three major bands were cut out and their amino acid sequences determined by automated Edman degradation using an Applied Biosystems microsequencer, Model 470A. The derivatized amino acids were identified by on line high performance liquid chromatography using an Applied Biosystems analyzer, Model 120A.

VI. IMMUNOHISTOCHEMICAL METHOD

Bovine and human articular cartilages were mounted in O.C.T. embedding media, frozen and 6 μm thick sections were cut perpendicular to the articular surface at -20°C using a Tissue-Tek II cryostat. Sections were picked up on glass microscope slides, precoated with a solution of 0.5% (w/v) gelatin in H_2O containing 1 mM chromium potassium sulfate as described by Pappas (278) to enhance adherence of the tissue sections to the slide.

A flow chart of the general immunohistochemical protocol is presented in figure 5, the details are included in the following text or where specific applications are discussed.

Sections were either immediately frozen and stored at -20°C or used immediately. Sections were fixed for a specific time as described in each experiment, in 4% formaldehyde freshly prepared from paraformaldehyde in PBS, pH 7.4 for 5 min (29) washed in PBS for 15 min and then for 15 min in 0.1% normal donkey serum in PBS, to block unreactive aldehyde groups for 15 min. These and subsequent manipulations were at room temperature. To enhance permeability of the tissue, sections were treated with chondroitinase ABC at 0.0125 units/50 μl/section in Tris-acetate buffer, pH 7.6, for 90 min at 37°C in a humid environment (29). Keratanase was added to the enzyme digestion solution for human tissue at 0.025 units/50 μl/section and the digestion was conducted for 18 h at 37°C in a humidified chamber. After enzymatic pretreatment, all tissues were washed twice for 10 min with PBS containing 0.1% BSA. Proteinase inhibitors included during enzyme treatment were: pepstatin (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM), iodoacetamide (1 mM), and EDTA (1 mM).



FIXATION	• 4% Formaldehyde in PBS 4 min
WASH/BLOCK	• PBS 10 min • PBS with 0.1% Normal donkey serum 10 min
CHONDROITINASE ABC TREATMENT	• 90 min 37 °C
WASH	• PBS with 0.1% BSA
1° ANTIBODY F(ab')$_2$	• Diluted in PBS with 0.1% nonidet 1% BSA 60 min RT
WASH	
2° ANTIBODY F(ab')$_2$	• Biotinylated donkey anti-rabbit diluted in 1.0% BSA/PBS 30 min RT
WASH	

DETECTION SYSTEM	• Fluorescein-streptavidin diluted in 1.0% BSA/PBS 20 min RT
WASH	
COUNTERSTAIN	• Ethidium bromide 1 min
WASH	
MOUNT	• Tris-glycerol
EXAMINE IMMEDIATELY	

DETECTION SYSTEM	• Gold-streptavidin diluted in 1.0% BSA/PBS 20 min RT
WASH	
DRY	
SILVER ENHANCE	
DRY	
MOUNT	• Permount
EXAMINE	

Figure 5 : General Outline of Immunohistochemical Protocol Used for Bovine Articular Cartilage

Sections were incubated for 90 min at room temperature in a humidified chamber with 50 µl/section of R181 $F(ab')_2$ or normal rabbit serum (NRS) $F(ab')_2$ diluted in PBS containing 1% BSA including 0.25% v/v Nonidet P40 at a concentration of 0.1 mg/ml. Controls were prepared by absorbing R181 $F(ab')_2$ before use with 1 mg/ml of denatured type II collagen at 37° for 1 h followed by 18 h at 4°C and centrifugation before use to remove any precipitate. Sections were washed twice as above for 10 min after each antibody incubation. A biotin-streptavidin detection system was used consisting of a donkey $F(ab')_2$ anti-rabbit Ig labelled with biotin, and either fluorescein-labelled streptavidin or gold(5-15nm)-labelled streptavidin as the indicator. The biotinylated donkey anti-rabbit $F(ab')_2$ was used at a dilution of 1/100 in PBS with 1% BSA at 50 µl/section for 1 h. After washing twice, the sections were treated with 50 µl of the fluorescein-labelled streptavidin at a dilution of 1/50 in PBS with 1% BSA for 20 min at room temperature. After washing in PBS, the sections that were stained with fluorescein-labelled streptavidin were counter-stained with 0.001% (w/v) ethidium bromide in H_2O for 1 min. (to counterstain nuclei which fluoresce red) and mounted in approximately 25 µl of a mixture of TRIS-glycerol, pH 8.6 (279). Slides were examined for fluoresence immediately with incident illumination using a Zeiss photomicroscope III fitted with a 75 watt Xenon lamp.

In studies where gold-labelled streptavidin was used, the silver enhancement method of Danscher and Nörgaard (280), as modified by others (E. Lee & A.R. Poole, manuscript in preparation), was utilized. Thus after sections were incubated with the biotinylated donkey anti-rabbit $F(ab')_2$ and washed as described above, the sections were incubated with

gold (5-15 nm)-labelled streptavidin at a dilution of 1/20 in PBS containing 1% BSA for 30 min at room temperature. After washing twice, the slides were dried slowly and held at 4°C until next day, or when convenient, before proceeding. The slides were then dipped in a 0.5% gelatin in water and allowed to air dry. The physical developer was prepared by mixing a 50% solution of gum arabic in water with 6.67 ml of 2.0 M citric buffer, 10 ml of 50 mM hydroquinone and 10 ml of 40 freshly prepared silver lactate in the dark. The slides were dipped in this solution for 1 h at 30°C in the dark, after which the slides were flushed with tap water at approximately 40°C for 30 min before exposure to light. The slides were then air dried and the sections mounted in Permount. Using the light microscope the presence of a positive reaction could be visualized as a black to brownish, grain-like staining.

VII. EXPERIMENTAL MODEL OF COLLAGEN DEGRADATION

A. Bovine articular cartilage explant cultures

For culture of cartilage explants, serum-free Dulbecco's modified Eagle's medium (DMEM-B) was used with the following additions: BSA (crystallized), 0.1 mg/ml; gentamicin sulfate, 100 µg/ml; sodium pyruvate, 1 mM; non-essential amino acids, 0.1 mM; 2-mercaptoethanol, $5x10^{-5}$M. Bovine metacarpal cartilage was cut into small uniform pieces of approximately 3mm x 7mm x 1mm. Tissue was rinsed, first with BSA-free DMEM containing gentamicin at 200 µg/ml and fungizone at 5 µg/ml (Flow Laboratories, McLean VA) and then with regular BSA-free DMEM. Explants were randomly and equally distributed into 12-well, 2.4 cm diameter, Linbro tissue culture plates with each well containing 2 ml DMEM-B. Human recombinant interleukin-1 β (IL-1β) (5 half-maximal units/ml) was added where indicated. Explants were maintained for up to 14 days at 37°C in a humidified incubator containing 5% CO_2 in air. Medium was changed every two days, with fresh addition of rIL-1β to the stimulated cultures. Tissues were removed at intervals, rinsed with PBS and prepared for immunohistochemistry as described below. Culture media were stored at -20°C until assayed for collagenase activity.

In some experiments, an aliquot of each culture medium was examined for the presence of type II collagen (α-chain or fragments) by SDS-PAGE and immunoblotting with R181 used as the means of detecting collagen. The details of these experiments are described in this section.

B. Cartilage extraction

Freshly isolated bovine articular cartilage, and cartilage cultured with or without IL-1, were subjected to extraction with 4M guanidine HCl in 0.15 mM potassium acetate, pH 6.3 including the proteinase inhibitors as described for use in the immunohistochemical protocol. Equivalent quantities (approximately 25 mg) of cultured and noncultured cartilage were cut into small pieces of less than 2 mm^3 and extracted in 0.7 ml of the extraction solution, with rigorous shaking for 48 h at 4°C. The residual cartilage was removed by centrifugation for 15 min at 100g. The extracts were prepared for electrophoresis and immunoblotting by removing the guanidine HCl using microdialysis against 0.5 M acetic acid. Each dialysate was collected and concentrated by freeze-drying using a Savant Speed Vac and rehydrated with 150 µl of electrophoresis sample buffer.

C. Amino acid analysis

To determine whether or not collagen or collagen fragments thereof were secreted into the medium and/or were extractible from bovine cartilage explants, amino acid analyses were performed. Medium removed from explant cultures every two days, was freeze-dried using a Savant Speed Vac. Each aliquot of spent medium was an original volume of 1.5 ml, and after dehydration, rehydrated in 10x less volume (150 µl) of 0.1 M acetic acid. Of this solution aliquots were taken for SDS-PAGE and amino acid analysis. For amino acid analysis, 25 µl was removed and dehydrated using the Savant Speed Vac Evaporator after which the material was dissolved in 6 M HCl, flushed with nitrogen and hydrolyzed at 110°C for 20 h. After drying under vacuum the samples were dissolved in 100 µl of 0.2 M citrate buffer, pH 2.2.

Aliquots were analyzed using a Durrum amino acid analyzer using a 0.2 M citrate buffer system of pH 3.25, pH 4.25 and pH 7.9. For the purposes of these investigations samples were evaluated for the presence of hydroxyproline. This was done by comparing peaks of each chromatogram with those of standards.

D. Immunochemical detection of collagen and collagen fragments

The presence of collagen and/or collagen degradation products in the media from explant cultures were immunochemically detected using SDS-PAGE and immunoblotting with R181. In these experiments, the aliquots (150 µl) of culture media were dried using a Savant Speed Vac in Eppendorf microfuge tubes. The resulting dry material was rehydrated in 2x SDS-PAGE sample buffer and prepared for electrophoresis by warming to 60°C for 10 min. Samples were electrophoresed under reducing conditions as described in Section V, B. Each sample was loaded at 25 µl, which represents one-third the total original volume.

E. Collagenase assay

To measure collagenase activity, acid soluble guinea pig skin collagen generously provided by Dr. Elaine Golds (Joint Diseases Laboratory, Shriners Hospital for Crippled Children, Montreal, Quebec) was acetylated with ^{14}C-acetic anhydride (10-30 mCi/mmol) and used in the standard fibril assay as described by Cawston and Barrett (281). The assay was routinely performed for 18 h at 35°C in the presence of 4-aminophenylmercuric acetate 1 mM (APMA) to activate the latent enzyme. Test media were thawed before assay and centrifuged for 5 min using a fixed speed Eppendorf centrifuge model

5313 to remove any cells or debris.

F. Immunohistochemistry

Tissue explants of bovine articular cartilage were removed from culture at various intervals, rinsed with PBS and embedded in OCT embedding media and frozen at -20°C. Using the cryostat as described above, 6 µm thick sections were cut applied to precoated microscope slides (see Section VI) and stored frozen until immunohistochemical experiments were performed. Sections of bovine cartilage were fixed in freshly prepared 4% formaldehyde prepared as described above, for 5 min. After rinsing with PBS and blocking with donkey normal serum (0.1% in PBS) these bovine cartilage sections were incubated with chondroitinase ABC at 0.0125 units/50µl section in TRIS acetate buffer pH 7.6 for 90 min. at 37°C to remove the chondroitin sulfate side chains of the proteoglycan. This is done to increase the accessibility of the antibodies. The sections were then handled as described in the general protocol for immunohistochemistry (Figure 5), Section VI.

To address the question of whether or not the chondroitinase treatment exposed epitopes of native collagen which reacted with the antiserum R181, the following experiment was performed. Full thickness pieces of normal bovine articular cartilage were incubated in DMEM with 0.125% (w/v) trypsin type XIII (Sigma No. T8642), 1 mM EDTA, for 3 h at 37°C. The enzyme activity was blocked by washing the tissue for 15 min with 1% normal donkey serum at room temperature. Frozen sections were prepared, fixed and immunolocalization performed as described.

VIII. STUDIES OF HUMAN ARTICULAR CARTILAGE DEGRADATION

A. Human articular explant cultures

Human articular cartilage explants were maintained in culture in DMEM-B supplemented with L-ascorbic acid, (0.1mM) and with HEPES, (15mM); BES, (10mM), TES (10mM); to maintain pH 7.5 in an atmospheric incubator at 37°C. Explants were randomly and equally distributed (approximately 200mg) into 12-well, 2.4. cm diameter, Linbro tissue culture plates with each well containing 2 ml of supplemented DMEM-B for 24 h before beginning the experiment. After this initial rest period, the cultures were stimulated with 1 unit/ml recombinant human IL-1β where indicated. In some experiments the proteinase inhibitor, U24522 (R-S)-N-[2-[-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl]-L-leucyl-L-phenylalaninylamide) (supplied by Dr. G. DiPasquale, Stuart-Pharmaceuticals, Div. of ICI Americas, Inc., Wilmington, DE) was used. It was handled in the following manner. A 20 mM stock solution of U24522 was prepared by dissolving the compound in DMSO (vehicle) and in cultures where required, a small aliquot (2µl) was added to each culture well for a final concentration of 0.01 mM. As control for the toxicity of DMSO in the culture system, 0.05% DMSO was added to IL-1 stimulated and unstimulated explant cultures. Tissues from these cultures were immunohistochemically evaluated, in parallel with those from cultures with the inhibitor. Viability was assessed in an experiment where cartilage was cultured with IL-I including vehicle control, the inhibitor at the highest concentration tested (0.05mM), or vehicle alone. Using similar tissue as that already described from day 14 explant cultures, explants were labelled with ^{3}H-leucine (25µCi/ml) in DMEM-B with the additives described above, for 24 h at 37°C in an atmospheric incubator. The explants were then

washed in 3 changes of PBS (20ml for 10 min) alone remove the incorporated ^{3}H-leucine. The incorporation of ^{3}H-leucine into newly synthesized proteins was determined by scintillation counting an aliquot of the tissue digests after papain treatment. Tissue digestion was performed on the entire explant culture using papain (Sigma) at a concentration of 0.4mg/ml in 0.2m TRIS pH 7.0, containing 5mM EDTA and 5mM DTT, for 2h at 60°C.

The effects of IL-1 on human articular cartilage and the effects of the proteinase inhibitor U24522, were studied by measuring the collagenase and proteoglycan activity (unreported work of Dr. J. Mort, Joint Diseases Laboratory, Shriners Hospital, Montreal, Quebec) in the media during the culture period of 14 days and the presence or absence of immunohistochemically-detectable degraded type II collagen. The collagenase activity was measured by the standard fibril assay as described in Section VII, E with the addition of a dialysis step. The media collected every two days were dialyzed against assay buffer using the Bethesda Research Laboratory microdialysis apparatus to remove the inhibitor.

Frozen sections were made of the tissue from these cultures as described in the general description of the immunohistochemical protocol and handled identically as described for other human cartilages (section VIII, B). Streptavidin-gold with silver enhancement was used for detection of staining at the light microscopic level (Section VI).

B. Immunohistochemistry

Human articular cartilage obtained as described, from normal (non-arthritic) and arthritic patients, was cut into pieces of approximately 2 mm x 7 mm x 2 mm maintaining the orientation of the articular surface. It was

embedded in OCT embedding medium at -20°C. Six micron thick sections were cut perpendicular to the articular surface and handled as described in the immunohistochemical protocol, except for these specific steps. Frozen sections were allowed to thaw and were fixed for 8 min in 4% formaldehyde/PBS. Prior to the tissue permeabilization step, the tissues were washed and blocked as described in Section VI. In addition to the chondroitinase ABC used to remove the chondroitin sulfate from the proteoglycans, keratanase was added to the enzyme digestion solution at a concentration of 0.025 units/50 µl/section.

In experiments where the murine monoclonal antibody, AN9P1 was used, the ascites fluid containing the antibody was diluted 1/32 in PBS containing 1% BSA and 1% v/v Nonidet P40, as used for other antibodies. Normal ascites fluid was used as a control diluted in the same manner. When AN9P1 was used a second step antibody was needed in order to utilize the biotinylated-donkey anti-rabbit antibody. A rabbit antiserum to mouse IgG, prepared in this laboratory, was used at a dilution of 1/10 in PBS as described above. Reaction was visualized by silver enhancement of the gold-labelled streptavidin as described in Section VI.

C. Electron microscopy

Sections of human articular cartilage were prepared as described for light microscopy in Section VI. Each 6 µm thick section was fixed for 20 min in 4% formaldehyde in PBS pH 7.4 at 4°C. Tissues were kept at 4°C and washed in ice cold PBS for 10 min. To block the unreactive aldehyde groups, 5 sections were blocked with 5% normal donkey serum for 30 min at room temperature. After a rinse in PBS for 10 min the tissue was permeabilized

with 0.2% hyaluronidase in PBS with proteinase inhibitors, as described in the immunohistochemical protocol, for 1 h at 37°C. Reaction with either R181 or NRS $F(ab')_2$ was performed in regard to dilutions and washes as for light microscopy except that 100 µl of the appropriate primary antibody was incubated with the tissue overnight at 4°C instead of 90 min. at room temperature. The second step antiserum, the biotinylated donkey anti-rabbit, was incubated for 3 h at room temperature. The streptavidin-gold (15 nm) was also incubated overnight at 4°c.

After a wash in PBS containing 0.15% BSA for 2 h the tissues were dehydrated with sequential 10 min intervals in 50%, 70%, 95% and 100% ethanol. The tissues were then covered with a 1:1 mixture of ethanol and Spurr for 1 h, then a 1:2 mixture for an additional hour prior to embedding in 100% Spurr resin which was placed in a gelatin capsule and positioned on top of the tissue section as it remained on the glass slide. This was allowed to cure overnight in a desiccator. After detachment from the microscope slide the embedded tissue was sectioned using a glass knife and a Reichert-Jung microtome. Sections were placed on grids and stained with lead nitrate and uranyl acetate. Tissue was examined with a Phillips 400 electron microscope.

CHAPTER 3

RESULTS

I. COLLAGEN PURIFICATION AND CHARACTERIZATION

A. Preface

The purity of any protein is paramount to generating a useful antiserum. The interpretation and conclusions drawn from the studies described in this thesis critically depend on the antibody specificity and an understanding of the immunological and biochemical properties of collagen, primarily type II collagen. Presented in this section are the results from experiments involving isolation and biochemical characterisation of the immunogen used to generate the antiserum designated R181. The specificity of this antiserum has been assessed using purified type II collagen and cyanogen bromide derived peptides of type II collagen, both prepared as described in this thesis. Other bovine collagens, namely type V, VI, IX & XI were purified in either Dr. David Eyre's laboratories or Dr. Michel van der Rest's as described in Materials and Methods. Cyanogen bromide derived peptides of human type II collagen were prepared in an identical manner as were bovine type II peptides.

B. Type II collagen

Based on the special properties of fibrillar collagens, type II collagen was purified from the adult bovine nasal septum. Following the method described in Chapter 2 and outlined in Figure 4 (15) type II collagen was obtained. Final purification was selective salt precipitation and in most cases was repeated on small quantities (25-100 mg) two or three times to increase

purity and remove small fragments or contaminating proteins. In figure 6, purified bovine type II collagen was heat denatured at 60°C for 3 min or boiled for 3 min and separated by a gradient (7.5%-15%). Lanes B and D contain two separate collagen preparations which were heated to 60°C for 3 min. These demonstrate the homogeneous α-chain composition of type II collagen (15). There is no evidence for the presence of an α2 chain, which migrates slightly faster than the α1 chain, nor are there any other contaminating proteins present. In comparison, lane C contains 100 μg of the identical collagen preparation seen in lane B except it has boiled for 3 min prior to loading. This resulted in a reduction in high molecular weight (γ and β) components and an increase in small fragments, clearly seen as smaller than the intact α-chain. With this observation, all subsequent samples prepared for SDS-PAGE were heated to 60°C rather than boiling. In some the presence of small molecular size-collagenous fragments were observed in certain preparations. These fragments were seen by both using the general protein stain, Coomassie blue, and immunoblotting analysis with R181.

Using purified bovine type II collagen, a mixture of peptides was generated using cyanogen bromide which results in cleavage at methionine residues, of which there are 10 per α chain (88,282). After taking several approaches to assure complete digestion as described in Chapter 2, the optimal cleavage was obtained after extending the reaction time from 4 h to 16-18 h. In Figure 7, adult bovine intact collagen α chains are shown in lane A and type II collagen CNBr-derived peptides prepared in this manner from the same collagen are seen in lane A are shown in lane B. Precise peptide identification was obtained by sequence analysis (Chapter 3, section II). There were no intact α chains remaining (as was the case from reaction mixtures at

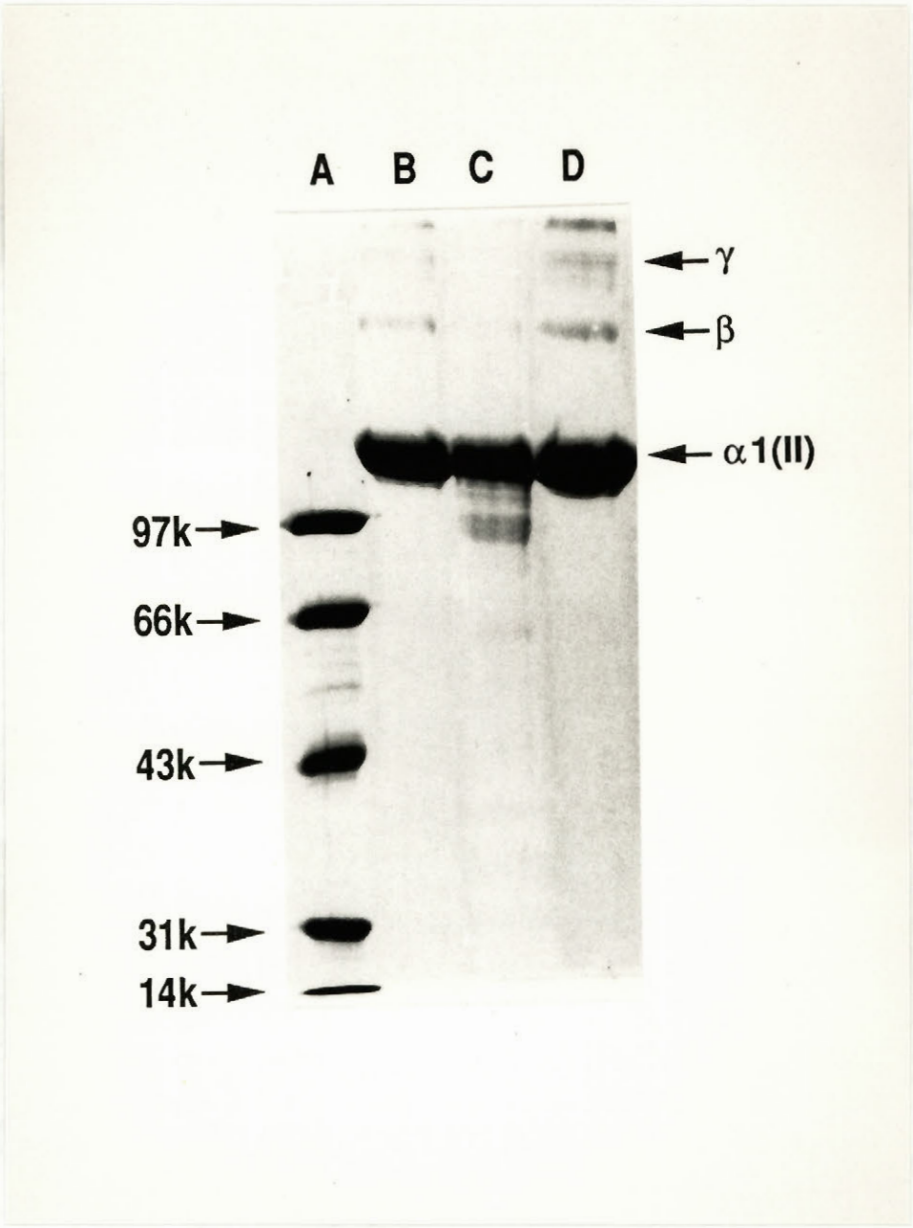


Figure 6 : SDS-PAGE (7.5%) of Purified Bovine Type II Collagens Demonstrating Fragmentation of Collagen After Boiling.

In lane A are molecular weight standards where k=1000. Lanes B and D contain 100μg of bovine type II collagen which was heated to 60° for 3 min prior to loading. The dimers (β) and trimers (γ) of α chains are indicated. Lane C is 100μg of the identical collagen preparaton as in lane B, but was boiled for 3 min prior to loading. Proteins have been stained with Coomassie blue.

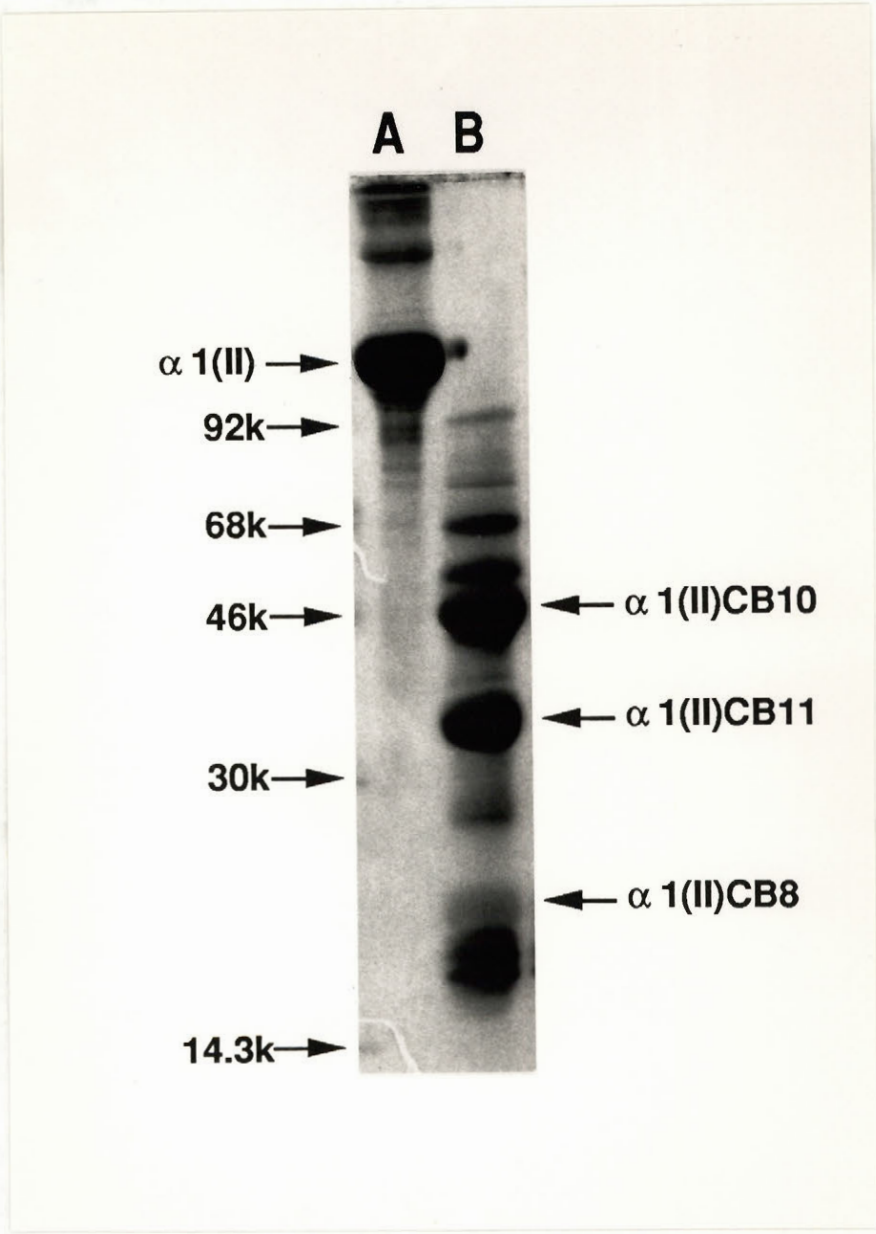


Figure 7 : SDS-PAGE of Purified Bovine Type II Collagen α Chains and CNBr-Derived Peptides

Purified bovine type II collagen α chains and CNBr-derived peptides of bovine type II collagen were separated by SDS-PAGE. (7.5%-15% gradient) Lane A represents 15 μg/lane of purified α chains and lane B, 60 μg/lane of CNBr-derived peptides after staining with Coomassie blue. Molecular weight markers were used as reference points and are indicated.

4 h). Nonetheless, there were several bands which represent incomplete digestion products or peptides with crosslinks to other peptides. Based on the known position of methionine residues and the relative size of each peptide, any bands larger than α1(II)CB10 represents a di- or tri-peptide.

One of the parameters examined to determine whether or not non-reducible crosslinks were playing a role in the profile obtained for CNBr-derived peptides, was to compare the peptides obtained from adult and fetal bovine tissues. In Figure 8, the similarities between these tissues are observed. Here it is demonstrated by the reaction with the antiserum R181 after SDS-PAGE and immunoblotting. Lanes A and C show the major reactive band as the type II α-chain in both fetal and adult derived collagen preparations. Each show a preponderance of small molecular weight, collagenous fragments in addition to the large molecular weight γ and β components. The existence of small collagenous fragments in these collagen preparations is largely due to two factors: the fact that both are overloaded and that the immunoblotting is much more sensitive than Coomassie blue staining. In addition, this can be seen by comparing the reaction seen in Figure 8, lanes A or C, with 15μg per lane with that observed in Figure 13, where only 5 μg of type II collagen is stained with Coomassie blue in lane A and after immunoblotting with R181 in lane B.

Although little significant difference is observed in the native type II collagen preparations of adult and fetal bovine cartilages, some differences in the peptide profiles can be seen. There are several large molecular weight reactive protein bands present in lane B in the adult collagen which are absent the fetal preparation in lane D. This provides further evidence that covalent crosslinks are more prevalent in aged tissue and that most probably

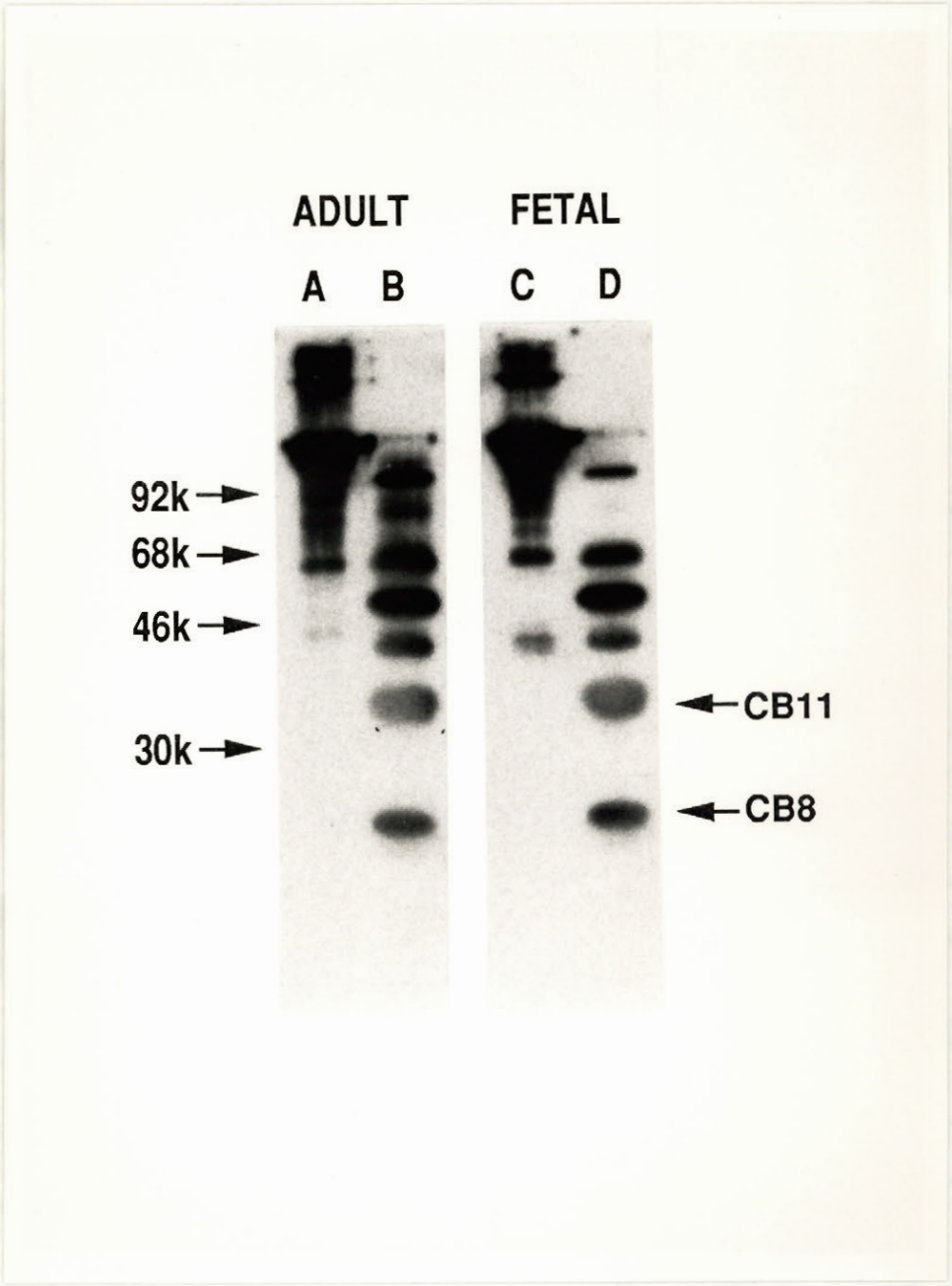


Figure 8 : Demonstration of Similarity Between Adult and Fetal Bovine Type II Collagen CNBr-Derived Peptides by Minigel SDS-PAGE (8%) and Immunoblotting with R181.

Lanes A and C contain 15 µg each of type II collagen; lanes B and D contain 35µg each of type II collagen CNBr-derived peptides. Lanes A and B were derived from adult cartilage whereas lanes C and D were from fetal cartilage. Reaction of antiserum R181 has been detected by ^{125}I-protein A. Molecular weight standards are indicated.

the peptides that are larger than the largest single peptide (α1(II)CB10) are due to the presence of crosslinks rather than incomplete cleavage. Routinely, therefore, collagen peptides were prepared as described using the extended cleavage time; it was deduced to be the most completely cleaved collagen obtainable by this technique. Consistently, the peptide profiles from different collagen preparations were identical. CNBr-derived peptides from human type II collagen were also prepared and gave a similar pattern. The R181 reaction profile for human type II peptides is compared to that of bovine in Chapter 3, section II, B.

C. HPLC analysis of bovine type II collagen

HPLC permits the rapid and automated analysis of minute amounts of materials. Van der Rest et al, have demonstrated the utility of reversed phase HPLC techniques in the analysis of small quantities of the fibrillar collagens and peptides thereof (271,283). According to the column used, these techniques can be utilized as a preparative step in purification or can be purely analytical. For the purposes of this study and analyzing native type II collagen, an analytical column (Vydac 218TPB) was used although in other studies collagen peptides were isolated and retrieved from a semi-preparative column (Vydac) with a diameter of 19cm. A mobile phase containing 0.01 M HFBA in acetonitrile and H_2O was used for its volatile properties, important in retrieving and analyzing fractions.

Represented in Figure 9 is a chromatogram of a typical analysis of purified bovine type II collagen. A 1h linear gradient of acetonitrile and H_2O containing 0.01 M HFBA was applied. Fractions representing peaks labelled A and B were pooled, dried using the Savant Speed Vac and weighed. The

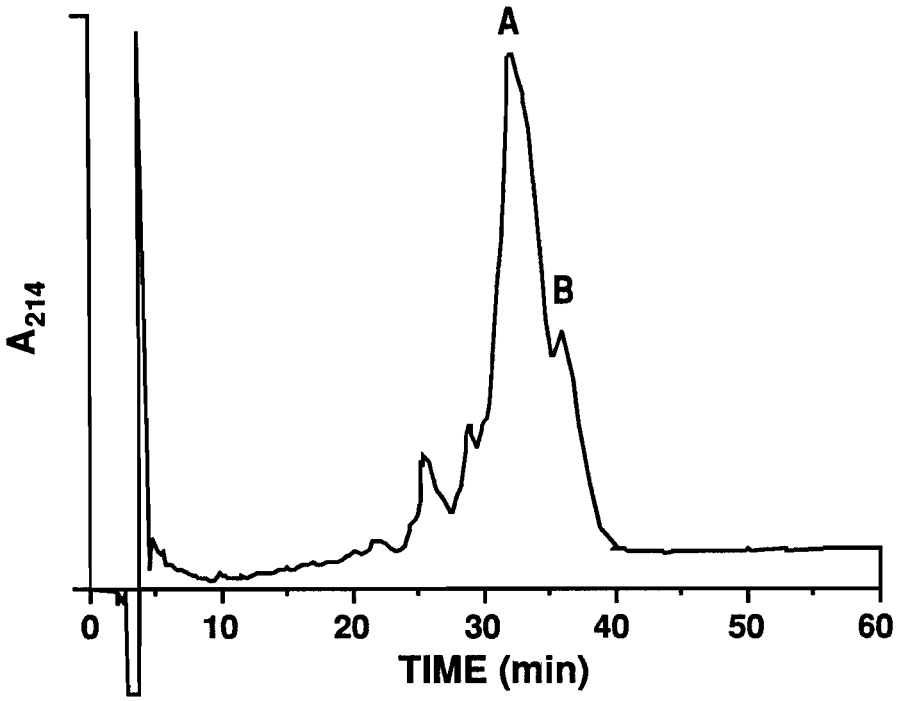


Figure 9 : Reversed-phase HPLC Analysis of Bovine Type II Collagen.

Using a VYDAC 218TPB column as described in the Methods section, 0.5 mg of purified bovine type II collagen was chromatographed over a 1 hr linear gradient of acetonitrile and H_2O (35%-60%) with 0.01M HFBA. Peaks A and B were collected, freeze-dried and analyzed by SDS-PAGE.

average recovery obtained for 15 chromatographic runs was approximately 75%. The relatively insoluble nature of collagen contributes to the loss which mostly occurs in the loading of the sample. Peaks A and B were thought to represent the α-chains and high molecular weight dimers and trimers, respectively. Each peak was loaded and electrophoresed in an 8% polyacrylamide gel. (Figure 10). Based on dry weight approximately 50 μg of collagen was added to each lane. As can be seen, peak A represents the bulk of the purified α-chain component, whereas peak B contains α chains (monomers, β components (dimers) and γ components (trimers). These data is evidence of the purity in regard to collagen type of the bovine type II collagen preparation.

D. Summary

The following summarizes the data presented on collagen purification and characterization.

1. Purified type II collagen was prepared from bovine and human nasal cartilage.
2. Purity of type II collagen preparation was assessed by SDS-PAGE and resulted in identification of a single band indicative of the three identical α chains which comprise type II collagen.
3. Fragments of type II collagen were generated as artifacts of the pre-loading boiling step most commonly used in SDS-PAGE. Warming to 60°C for as little as 3 min was sufficient to denature the collagen molecule in reducing sample buffer.
4. Small quantities of fragments of type II collagen could be also detected in collagen preparations by immunoblotting with R181 which could not

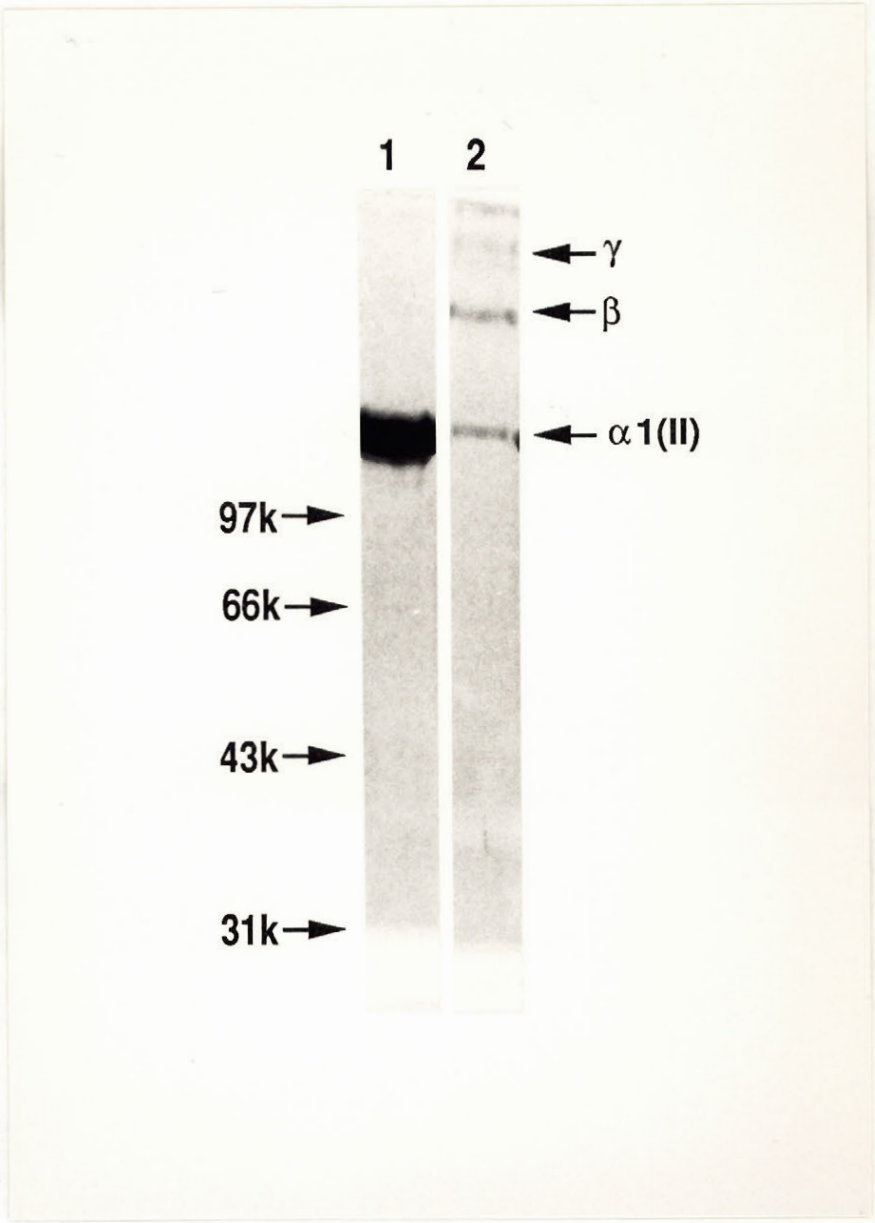


Figure 10: SDS-PAGE of Peaks A and B From Reversed -Phase HPLC Analysis of Bovine Type II Collagen.

Lane 1 is material from peak A, lane 2 is material from peak B collected and pooled from the chromatograph represented in Figure 9. Trimers, dimers, and monomeric α chains are indicated as γ, β, α, respectively. Electrophoresis was carried out in a 8% gel in the large format as described. Molecular weight standards are indicated.

be detected by the conventional protein stain, Coomassie blue. This indicates the high degree of sensitivity obtained with immunoblotting and detection with a specific antiserum.

5. Peptides were generated using cyanogen bromide with optimal cleavage obtained with an extended reaction time of 18 h.

6. Comparison of adult and fetal bovine type II collagen and CNBr-derived peptides was performed. There was a striking similarity between peptide profiles on immunoblots when using R181 as the antiserum. A few high molecular weight multi-peptides were absent in the fetal cartilage collagen preparation indicating the presence of non-reducible crosslinks in adult tissues.

7. CNBr derived peptides were first identified by relative molecular size based on the known sequence of type II collagen and the position of methionine residues. The precise identities of three major peptides, α1(II)CB8,10,11 were established by amino acid sequencing.

8. Reversed-phase HPLC analysis of bovine type II collagen revealed its purity by demonstration of a large major peak containing homogeneous α chains with a minor peak containing cross-linked dimers and trimers.

II. CHARACTERIZATION OF THE ANTIBODIES

A. Preface

Central to this thesis is the precise characterization of the primary antiserum which reacts with degraded or denatured type II collagen, designated R181. In this section, data will be presented which discerns the specificity of the antibodies of R181. Initial information was obtained using ELISA and bovine type II collagen purified as described, and purified collagens kindly provided by Dr. David Eyre (University of Washington, Seattle) in the case of bovine types V,VI,IX,XI and by Dr. Michel van der Rest (Genetic Unit, Shriners Hospital, Montreal, Quebec) for human type II collagen. As the study progressed, so did the sophistication of the analytical work. Subsequent characterization was performed by SDS-PAGE and immunoblot analyses along with microsequence analyses of isolated peptides. Except where mentioned, characterization was done using adult bovine type II collagen. Each analysis will be presented along with the appropriate data. A summary of the characterization of R181 will be included at the end of this section.

B. ELISA analyses of antiserum R181

1. Bovine collagens

The possible reactivity of antibodies in antiserum R181 with various cartilage collagenous components was investigated by ELISA analysis. Purified collagens associted with the non-calcifying cartilage matrix (284), types II, V, VI, IX and XI were tested as native collagens. In the same experiment the immunogen, bovine type II CNBr-derived peptides was tested and as seen in Figure 11 (upper left hand panel). This reaction had a titer of

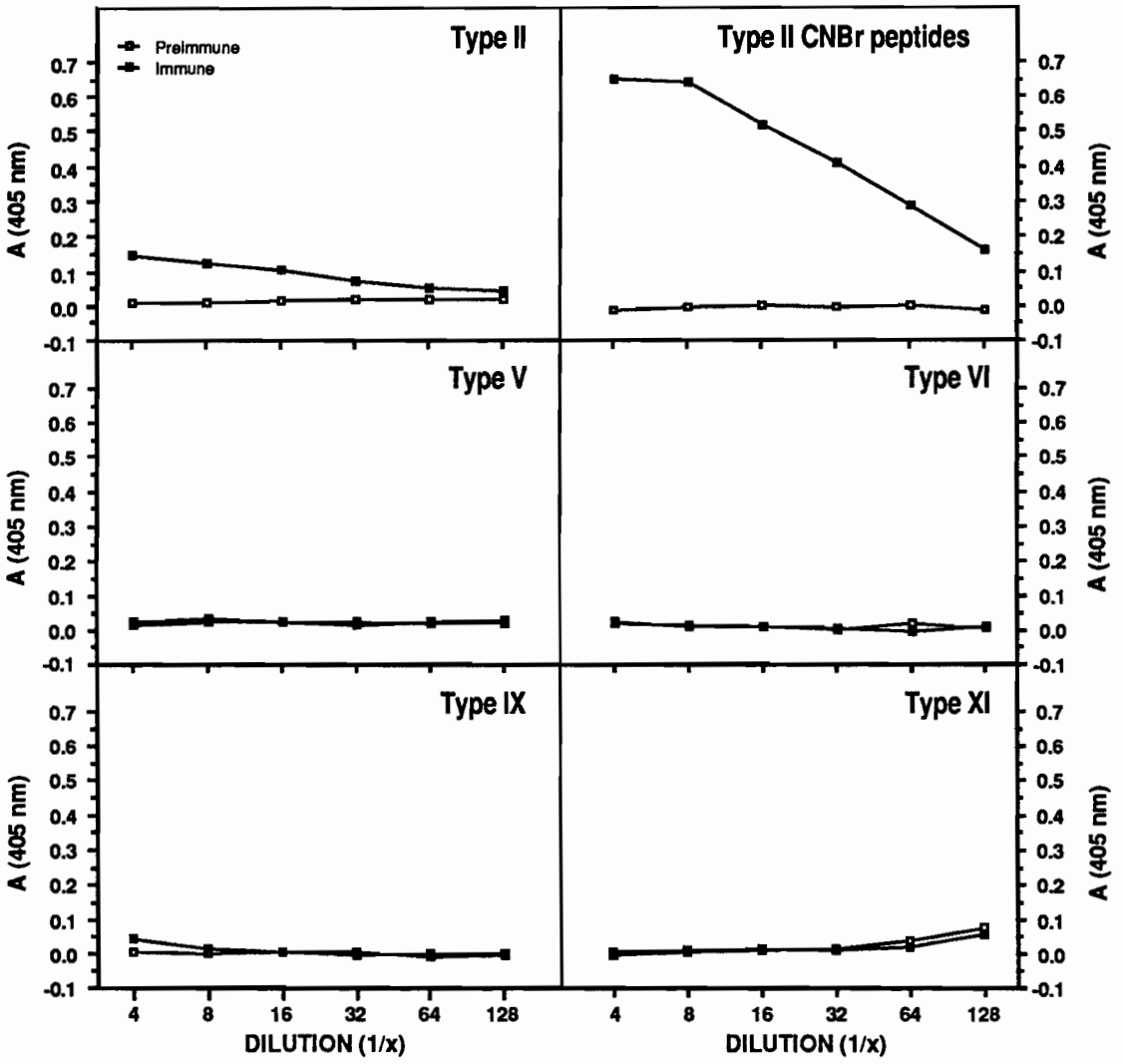


Figure 11 : ELISA Analyses of Antiserum (R181) with Purified Native Bovine Cartilage Collagens.

To determine specificity of antibodies the reaction was measured to native collagen types II, V, VI, IX, and XI and compared to the reaction determined to the immunogen, CNBr-derived peptides of a bovine type II collagen.

1/128. The reaction observed with the mixture of peptides was relatively low, throughout several experiments. Antibody titers were assessed to denatured types II, V, VI, IX and XI and compared to the reaction to CNBr-derived peptides of type II (Figure 12). The strongest reactions were detected to the immunogen (type II CNBr-derived peptides) and denatured type II collagen. No reaction was observed to any collagen tested, native or denature, in the preimmune serum.

A small reaction with native type II collagen (highest A_{405} = 0.140) observed at the highest concentrations of serum appears to be a result of trace amounts of denatured or fragmented collagen in the type II preparation as a result of purification and handling. This was demonstrated by analysis of type II collagen by SDS-PAGE followed by electrotransfer to nitrocellulose and immunostaining with R181. At low collagen concentrations, as shown in Figure 12, a small reaction is detected with material of a smaller molecular size than on intact α chains. This was not detected with conventional protein staining with Coomassie blue. This point was addressed in this chapter, section I, B of the collagen purification and characterization section (Figures 8 and 13) where data presented there further confirmed the existence of small collagen fragments detectable with antibody but not with conventional PAGE staining (except when overloaded).

2. Human collagens

The reactivity of antiserum R181 with human type II collagen was also determined by ELISA (Figure 14). Native, denatured, and CNBr-derived peptides of human type II collagen were examined and compared in their reactivity to bovine type II collagen CNBr-derived peptides. Equivalent reactions were seen to human and bovine type II CNBr-derived collagen

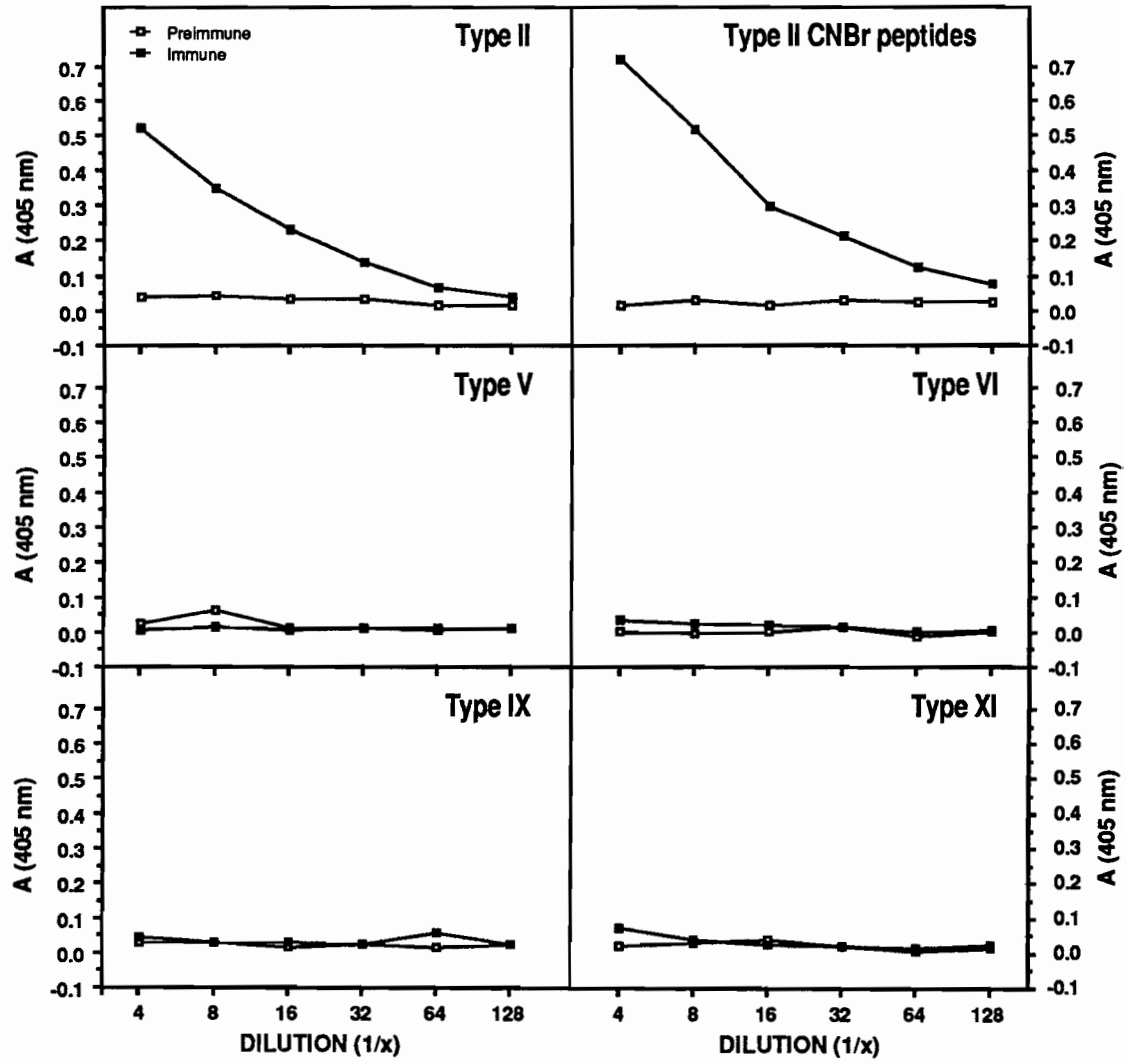


Figure 12 : ELISA Analyses of Antiserum (R181) with Purified Denatured Bovine Cartilage Collagens.

To determine specificity of antibodies the reaction was measured to heat-denatured collagen types II, V, VI, IX, and XI and compared to the reaction determined to the immunogen, CNBr-derived peptides of a bovine type II collagen.

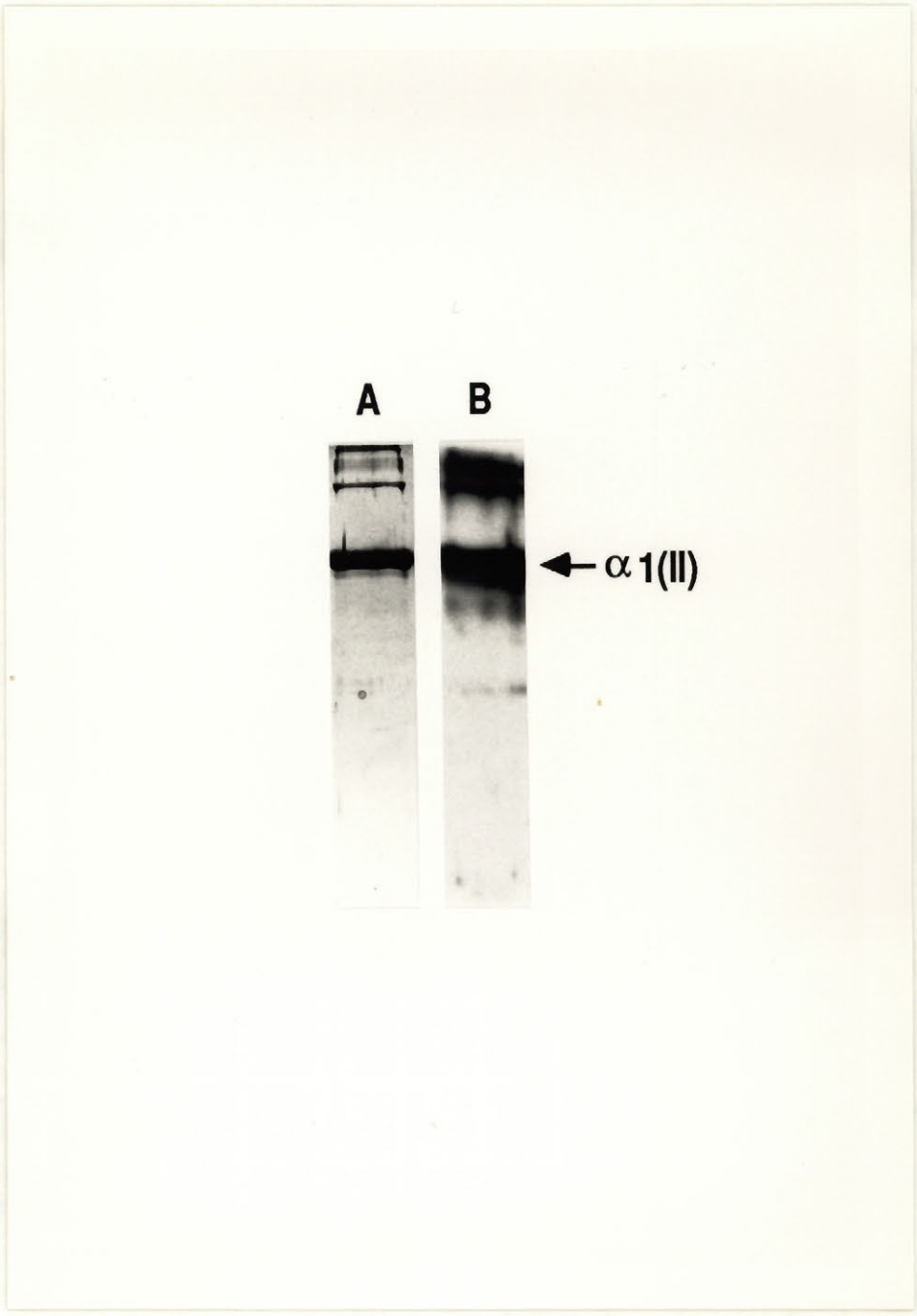


Figure 13 : Demonstration of Small Quantities of Immunoreactive Fragments of Type II Collagen in Purified Bovine Type II Collagen

Purified bovine type II collagen (5 μg) was characterized in SDS-PAGE (7.5%) using a Bio-Rad Mini protean apparatus. Lane A represents the protein stained with Coomassie Blue; lane B is an autoradiograph of an identical track after transfer onto nitrocelluose and incubation with R181, after detection with ^{125}I-Protein A. The immunoreactive β and γ components of higher molecular weight can also be seen.

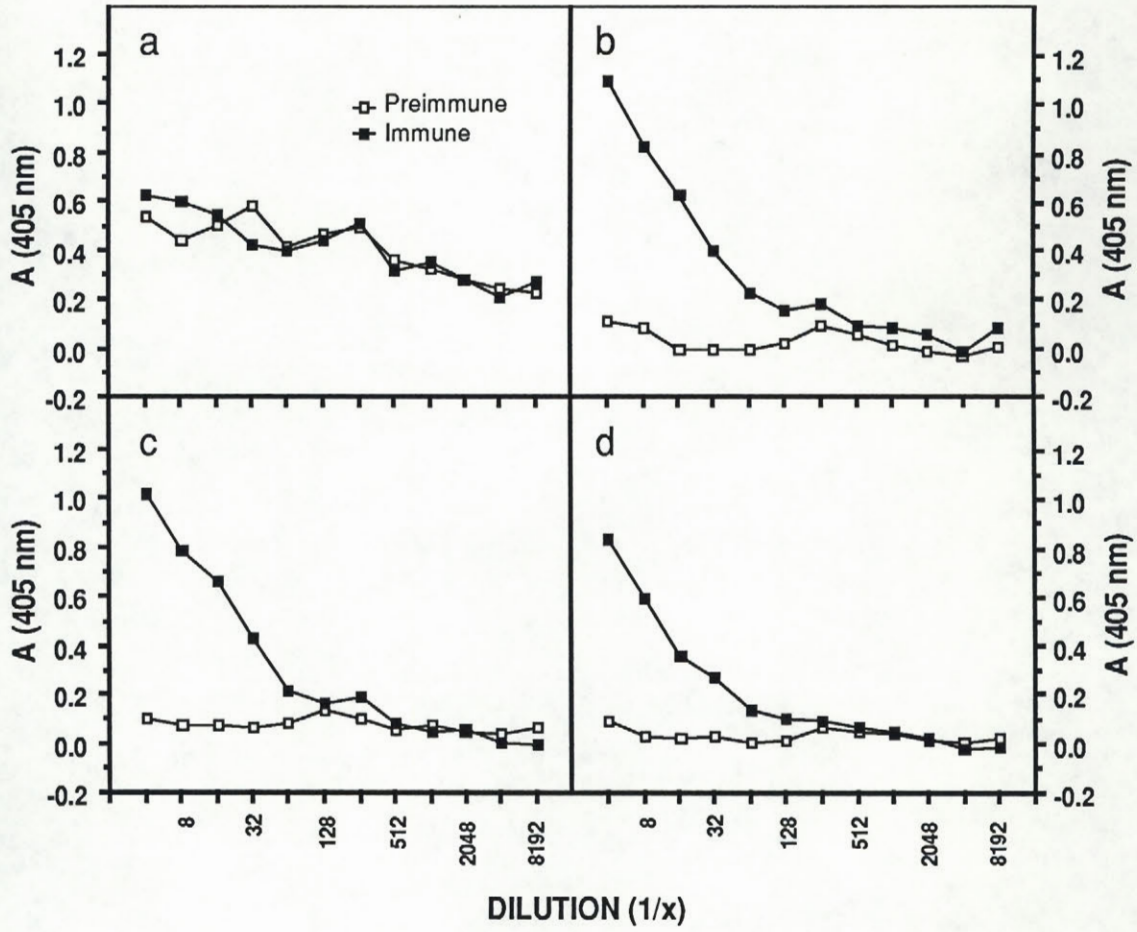


Figure 14 : ELISA Analyses of Antiserum (R181) to Demonstrate Cross-Reaction With Human Type II Collagen

Binding of preimmune and immune R181 to human type II collagens (a) native, (b) denatured and (d) CNBr-derived peptides are shown. For comparison, binding to the immunogen, bovine type II collagen CNBr-derived peptides (c) is shown.

peptides and denatured type II collagen, whereas in the case of human type II there was no reaction with native human type II collagen over and above that shown by preimmune serum. Preimmune R181 showed no reaction with any of the other collagens tested.

C. Biochemical characteristics

1. SDS-PAGE and immunoblot analyses

To determined which CNBr-derived peptides of bovine type II collagen were recognized by R181 antibodies, SDS-PAGE and immunoblotting techniques were employed. Initially, CNBr-derived peptides were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose. In Figure 15 two such electrophoretic profiles are presented, where lane A represents all peptides in the mixture CNBr-derived peptides of type II collagen stained with Coomassie and lane B represents only those peptides reactive with R181. The two largest peptides are identified as α1(II)CB10 and α1(II)CB11, composed of approximately 320 and 270 amino acids, respectively. The most revealing data from this comparison of peptides stained with Coomassie blue and those recognized by R181 and detected with ^{125}I-protein A, is the lack of antibody reaction with α1(II)CB10 but reactivity with α1(II)CB11. The other major reactive single peptide identified is a α1(II)CB8, approximately 170 amino acids. Although these peptides are artificially generated by a cleavage at each methionine residue, the antibody reaction, <u>in situ</u> can be related to post-collagenase cleavage events where unwinding of the α chains occurs with exposure of antigenic epitopes. Any reactive bands present above the peptide identified as α1(II)CB10 represent multiple peptides or crosslinked peptides based on the

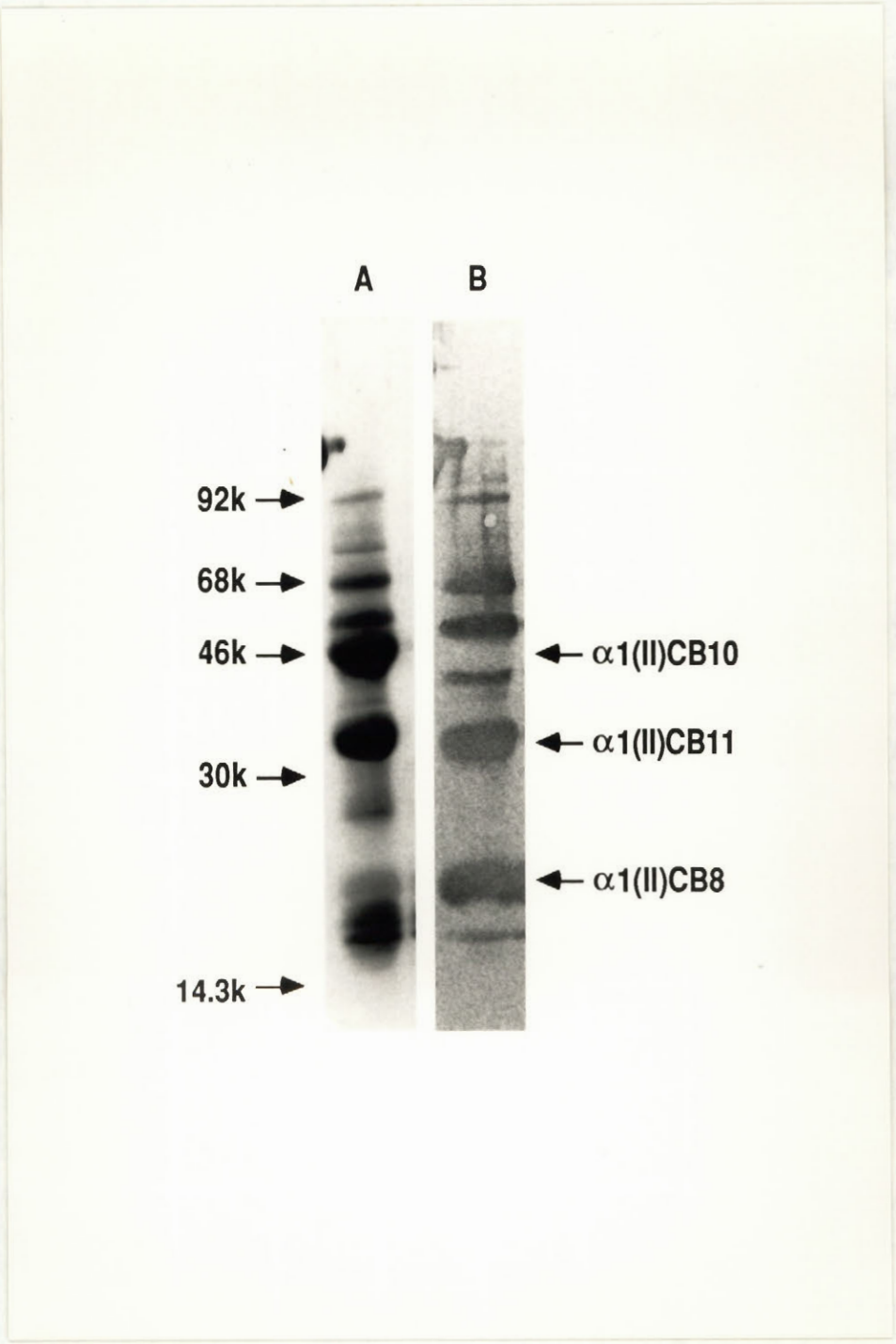


Figure 15 : Identification of CNBr-Derived Peptides of Bovine Type II Collagen.

CNBr-derived peptides (60 µg/lane) of bovine type II collagen were separated by SDS-PAGE (7.5%-15% gradient). Lane A represents the peptides after staining with Coomassie blue. An identical track was transfered to nitrocellulose and incubated with R181 and ^{125}I-Protein A (lane B). Peptides identified by microsequencing analysis are indicated. Radiolabeled molecular weight markers were used as reference points and are indicated.

molecular size and positions of methionine residues. The three peptides precisely labelled, were identified as such only after sequencing the first 10-20 amino acids from the amino terminal end and relating these sequences to the known primary structure of the bovine α chain (285).

Further characterization was performed in R181 in respect to an unrelated collagen (i.e. type I) and non-collagenous components of articular cartilage. This investigation was important in order to utilize the antibodies in cartilage sections where the complete complement of cartilage components exists. Purified type I and type II collagens, type II CNBr peptides and whole cartilage extract and fractions thereof separated by cesium chloride density gradient centrifugation, were electrophoresed in a 10% SDS-polyacrylamide gel under reducing conditions, and the separated proteins were then electrophoretically transferred onto nitrocellulose. The transblot was either incubated with preimmune, immune R181 serum or a mixture of R254 and R167, each at a dilution of 10%. As seen in figure 16a, preimmune R181 showed no reactivity to any of the proteins examined, whereas immune serum R181 (Figure 16b) reacted with purified, denatured bovine α1(II) chains, and peptides of bovine type II collagen. No reaction was detected with purified bovine type I collagen, nor with any proteins found in the bovine cartilage extract except for a weak band of reactivity detected in fraction number 6 of the cesium chloride density gradient, corresponding in size to the α1(II) chains. In order to demonstrate the presence of other cartilage proteins on the transblot which were unreactive with R181, the identical transblot was incubated with a mixture of two antisera designated R167 and R254. As can be seen in Figure 16c, these antisera react with a wide variety of proteins which are present on the nitrocellulose, none of

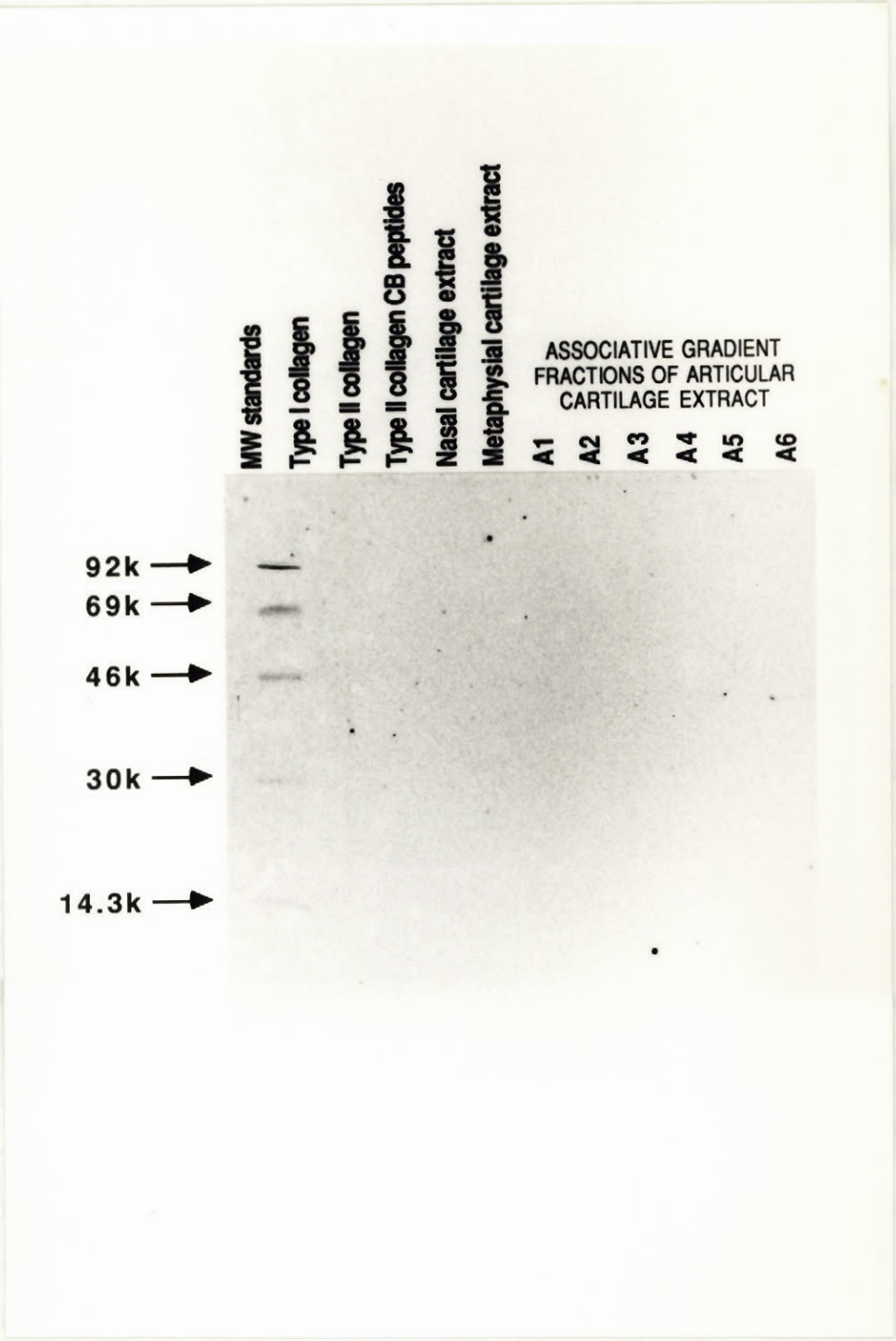


Figure 16a : Specifity of Antiserum R181 Is Shown By Immunoblotting.

Various bovine collagens (20 µg per lane of native types I and II: 60 µg per lane type II CNBr-derived peptides), fractionated and whole bovine cartilage extracts (approximately 10 µl of total extract or fraction) were separated by SDS-PAGE (10%) and electrophoretically transferred to nitrocellulose. A radioautograph is shown of the reaction with *preimmune R181*. Antibody reactions are detected by using ^{125}I-protein A. Reference molecular weight markers are indicated. (k=1000)

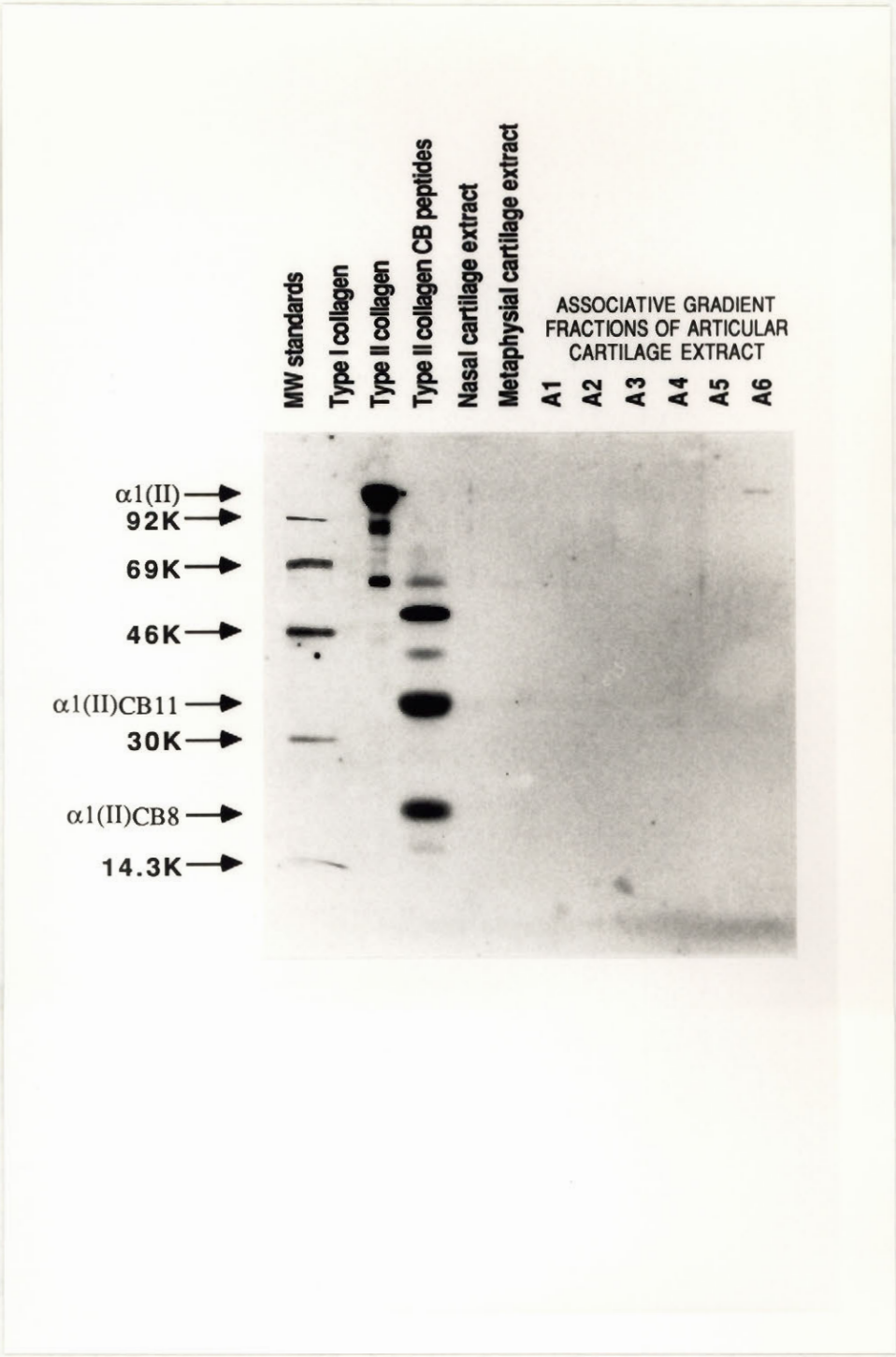


Figure 16b : Specifity of Antiserum R181 Is Shown By Immunoblotting.

Various bovine collagens (20 µg per lane of native types I and II: 60 µg type II CNBr-derived peptides per lane), fractionated and whole bovine cartilage extracts (approximately 10 µl of total extract or fraction) were separated by SDS-PAGE (10%) and electrophoretically transferred to nitrocellulose. A radioautograph is shown of the reaction with *immune R181*. Antibody reactions are detected by using ^{125}I-protein A. Reference molecular weight markers are indicated. (k=1000)

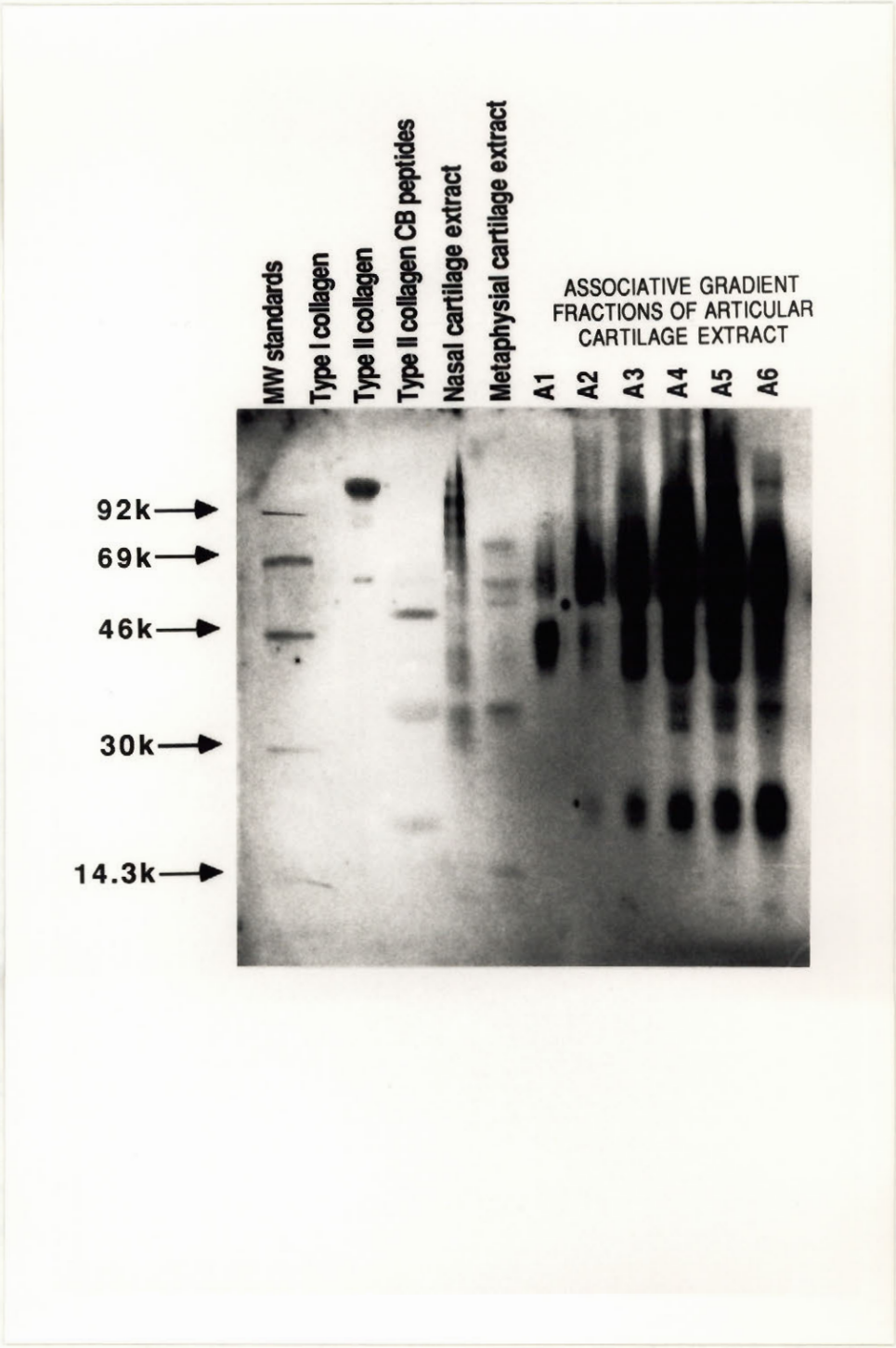


Figure 16c : Specifity of Antiserum R181 Is Shown By Immunoblotting.

The identical immunoblot which is represented in figure 16b was reacted with antisera R167 and R254. These antisera were generated to cartilage extract components as described in Methods and were used as a positve control for identifying matrix components. Antibody reactions are detected by using ^{125}I-protein A. Reference molecular weight markers are indicated. (k=1000)

which react with R181 except type II collagen α chains and type II CNBr-derived peptides. CNBr-derived peptides of type II collagen, which have epitopes reactive with R181 were identified by their relative molecular sizes using SDS-PAGE analyses and by microsequencing of these electrophoretically separated peptides. In Figure 14b the major antibody reactive peptides were identified as α1(II)CB8 and α1(II)CB11.

To determine whether or not R181 cross-reacted with human type II collagen peptides derived by cleavage with CNBr, immunoblot comparison analysis was performed between human and bovine type II collagen. Bovine and human type II collagen and CNBr-derived peptides thereof along with a whole cartilage extract of human articular cartilage, were electrophoresed by SDS-PAGE (10%) and electrophoretically transferred, to nitrocellulose. Immunoblots were either incubated with 10% (v/v) R181 or 10% (v/v) preimmune serum. Figure 17 illustrates the radioautograph of the immunoblot showing the specific reaction of R181 with both bovine and human type II collagen. The two species are strikingly similar in both peptides and in R181 reactivity. The two major reactive peptides are identified. No reaction was observed with an identical blot incubated with preimmune R181, just as previously demonstrated with bovine collagens. Since proteinacious components of human articular cartilage are represented in the whole cartilage extract (Figure 17, lane E) the lack of any reaction except for the band in the α-chain position provides the basis for extending the studies to human tissue.

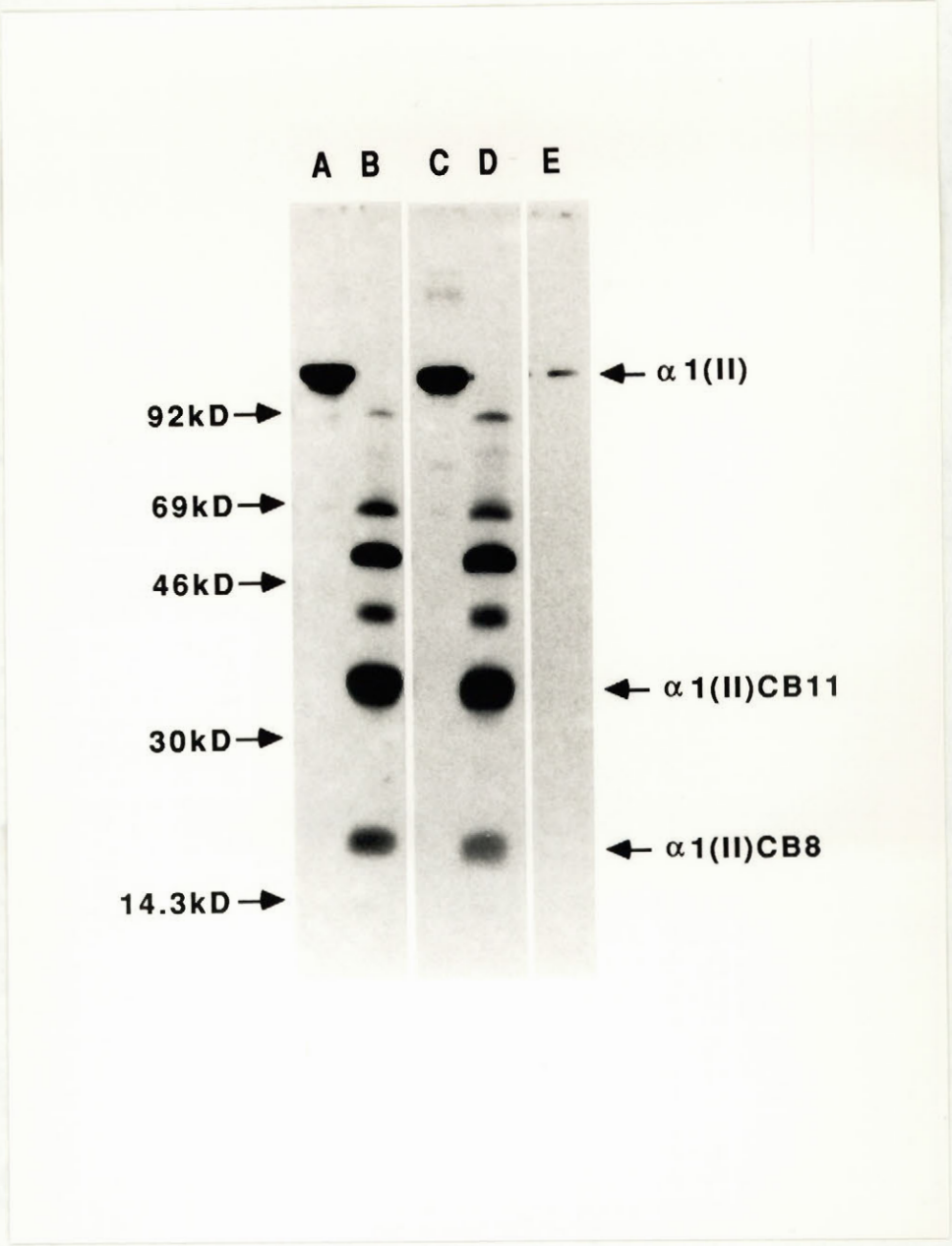


Figure 17 : Reaction Specifity of Antiserum R181 with Human Type II Collagen α Chains, CNBr-Derived Peptides and an Unfractionated Extract of Human Articular Cartilage.

After SDS-PAGE (10%) and electrophoretic transfer onto nitrocellulose, immunoblots were prepared. Shown are radioautographs of immunoblots incubated with immune R181. In lane A, 15 µg purified bovine type II collagen; lane B, 60 µg bovine type II collagen CNBr-derived peptides; lane C, 15 µg human type II collagen; lane D, 60 µg human type II collagen CNBr-derived peptides; lane E, 10µl unfractionated extract of human articular cartilage. Molecular weight markers are indicated where k=1000. Reaction of R181 has been detected by using ^{125}I-protein A. Positions of α1(II), α1(II)CB11 and α1(II)CB8 are indicated. No reactive bands were observed in an identical immunoblot incubated with preimmune R181 (not shown).

2. Sequence analysis of type II collagen peptides

Precise identification of CNBr-derived peptides of bovine type II collagen was made only after protein sequence analysis. The first 10-20 amino acids of selected peptides were sequenced and compared with the amino acid sequences which have been published (285) (Figure 18). As mentioned, initial identification was made by conventional means of comparing size and mobility in PAGE, for the sequence studies peptides from an identical SDS-PAGE as shown in Figure 15 was used. Therefore, the reactive bands clearly identified in Figure 15 could be sequenced and assigned their position in the collagen molecule. Two R181 reactive bands, α1(II)CB8 and α1(II)CB11, were identified by amino acid sequencing and a major band, as seen by Coomassie Blue staining, was identified by its amino acid sequence as α1(II)CB10. This peptide was clearly unreactive with the antiserum R181 (Figure 15). Based on previous studies of CNBr derived peptides of type II collagen (286), the largest peptide is α1(II)CB10. Therefore any peptide species larger than α1(II)CB10 results from incomplete cleavage at methionine residues or the presence of non-reducible crosslinks. Attempts were made at sequencing several other peptide species, although analyses revealed multiple peptides, perhaps a result of mobility similarity (overlapping) or size identity. No conclusions could be made on the identity of other bands sequenced except for those revealed as α1(II)CB8, 10 and 11.

D. Summary

The following summarizes the data presented in the section on specificity of the antiserum R181 as determined by ELISA analyses immunoblotting and microsequencing.

Residue α1(II)CBII		Residue α1(II)CB8		Residue α1(II)CB10	
124	GLY	403	GLY	552	HYP
125	PRO	404	PHE	553	GLY
126	ARG	405	HYP	554	GLU
127	GLY	406	GLY	555	ARG
128	LEU	407	PRO	556	GLY
129	HYP	408	HYL	557	ALA
130	GLY	409	GLY	558	ALA
131	GLU	410	ALA	559	GLY
132	-	411	ASN	560	ILE
133	GLY	412	GLY	561	ALA
		413	GLU	562	GLY
		414	HYP	563	PRO
		415	GLY	564	HYL
		416	LYS	565	GLY
		417	ALA	566	ASP
				567	ARG
				568	GLY
				569	ASP
				570	VAL
				571	GLY

Figure 18 : Amino Acid Sequence Analysis of CNBr-Derived Peptides of Bovine α1(II).

Individual peptide bands blotted onto PVDF membranes were cut out and sequenced from the NH_2-terminal by limited Edman degradation as described in Materials and Methods. Presented are the sequence analyses and their corresponding residue number as matched with data from a number of laboratories as compiled by D. Galloway (285).

1. R181 displays the strongest reaction to the immunogenic antigen, bovine type II CNBr-derived peptides.
2. An equivalent reaction was observed to denatured bovine type II collagen.
3. No reaction was detected to native or denatured collagen types V, VI, IX, and XI.
4. No significant reaction was observed to any collagen, in any form, by preimmune R181.
5. R181 reacts with human type II CNBr-derived peptides, and denatured type II, although shows reaction to native type II.
6. R181 was shown to react specifically with denatured type II collagen of both bovine and human origin.
7. R181 reacts specifically with two major peptides of bovine and human type II collagen, α1(II)CB8 and α1(II)CB11 and is unreactive with the largest CNBr-derived peptide α1(II)CB10.
8. Whole, or fractionated bovine articular cartilage extracts show no reactive component other than the α-chain of type II collagen. No reaction was detected to a non-cartilage collagen, type I collagen.
9. The lack of reaction with any proteins in the unfractionated human cartilage extract (except for the α-chain of type II collagen) confirms the similarity of the human cartilage reaction profile of antiserum R181 with that of the reaction profile with bovine cartilage.
10. Microsequencing was successfully applied to electrophoretically separated type II collagen peptides after transfer onto PVDF membranes.

11. Amino acid sequence determination of the first 10-20 amino terminal residues was sufficient to match the obtained sequences with those published and ascribe the correct identity to three major peptides, α1(II)CB8, 10 and 11.

12. **Combined conclusion:** R181 specifically reacts with bovine and human type II collagen peptides α1(II)CB8 and α1(II)CB10 along with other minor peptides. This reactivity can be detected in denatured α chains but not when the collagen is in a native conformation.

These data demonstrate the specificity of R181 for denatured bovine and human type II collagen and two CNBr-derived peptides of type II collagen. The two major reactive peptides are located well within the helical region of the alpha chain in the N-terminal half of the molecule. Figure 19 represents a CNBr-derived peptide map of bovine type II collagen based on a review by Fietzek and Kuhn (286), illustrating the relative positions of α1(II)CB8, α1(II)CB11, their relative size and the cleavage position of mammalian collagenase. This demonstrates that the epitopes which are recognized by this polyclonal antiserum are with collagen peptides which are located towards the N-terminus of the cleavage site of mammalian collagenase (61).

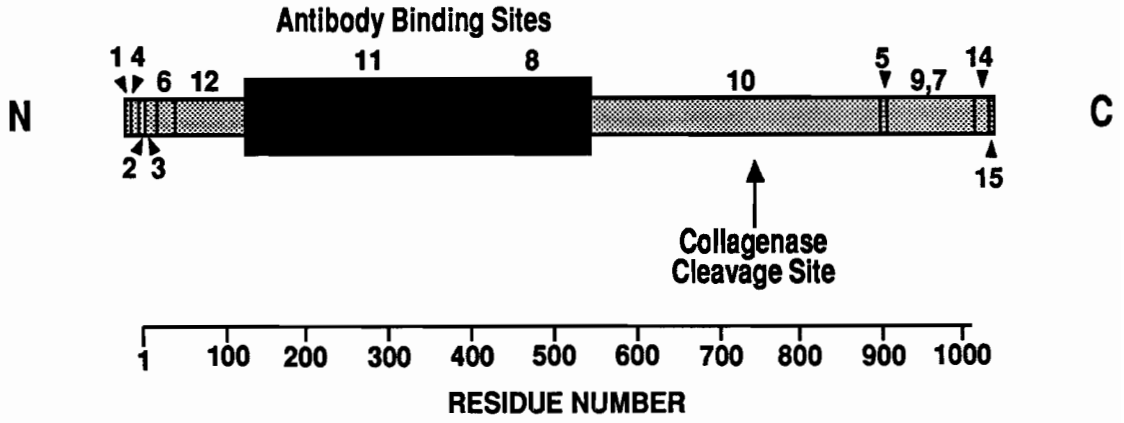


Figure 19 : Diagrammatic Representation of Position and Relative Sizes of CNBr-Derived Peptides of Bovine Type II Collagen.

R181-reactive peptides, the cleavage site for mammalian collagenase and the identities of CNBr-derived peptides are indicated.

III. ARTICULAR CARTILAGE DEGRADATION IN CULTURE

A. Preface

Cartilage explant cultures have been successfully utilized to investigate in vivo events since the first description of cartilage tissue culture (287). For the purposes of studying articular cartilage degradation and synthesis, explant cultures have been widely used by some workers studying cartilage metabolism and macromolecule biosynthesis (288) and by others investigating the breakdown of proteoglycan and collagen in response to various stimuli (289-291). Proteoglycan degradation is commonly monitored by detecting the release of glycosaminoglycans into the explant culture medium whereas collagen breakdown has historically been assessed by measuring the release of hydroxyproline, into the explant culture medium (288,289).

This section describes experiments using explants of bovine articular cartilage and immunochemical and immunohistochemical methods to detect type II collagen degradation and turnover. This explant model of articular cartilage degradation utilizing well defined serum-free medium allows for the investigation of the effects of molecules such as IL-1. Medium changes every two days enables enzyme levels to be determined over a period of time. In the experiments where IL-1 was added to explant cultures, it was added back at each change of culture media. Therefore the available concentration was kept at a constant level throughout the experiment. In these studies the effects of IL-1 on collagenase production/secretion was determined by measuring collagenase activity in the culture medium collected at periodic intervals. The biological effects of IL-1 on enzyme production and subsequent action was investigated immunohistochemically and immunochemically by examining the tissue explants with R181, the antiserum which specifically

detects degraded type II collagen. Experiments will be presented which directly demonstrate the effect(s) of IL-1, and implicate it in cartilage collagen breakdown.

B. Explant cultures of bovine articular cartilage

1. Immunohistochemistry

In these initial experiments using R181 and the immunohistochemical protocol described, the detection system was streptavidin labelled with fluorescein. Later experiments were performed using a method utilizing streptavidin-gold with silver enhancement which results in a permanent preparation.

Bovine articular cartilage was examined using antiserum R181 with immunofluorescence after various culture periods in the presence of absence of IL-1. In all experiments, tissue reacted with R181 was compared to tissue incubated with normal rabbit serum $F(ab')_2$ (NRS) under identical conditions to establish the specificity of antibody binding. The matrix of these cartilage sections treated with NRS $F(ab')_2$ did not stain. Only the cell nuclei were visible due to the counterstain, ethidium bromide (Figure 20a). As further controls for the immunostaining protocol, bovine articular cartilage sections were incubated omitting the primary antibody (R181 or NRS), testing the effects of the second step antibody, the donkey anti-rabbit $F(ab')_2$ (Figure 20b). As the positive control for the detection system (i.e. fluoresceinated streptavidin), identical sections of uncultured adult bovine articular cartilage were incubated with the rabbit antiserum R110 (Figure 20c). R110 reacts with bovine proteoglycan after chondrotinase ABC digestion and was raised and characterized in this laboratory. Figure 20 demonstrates the overall

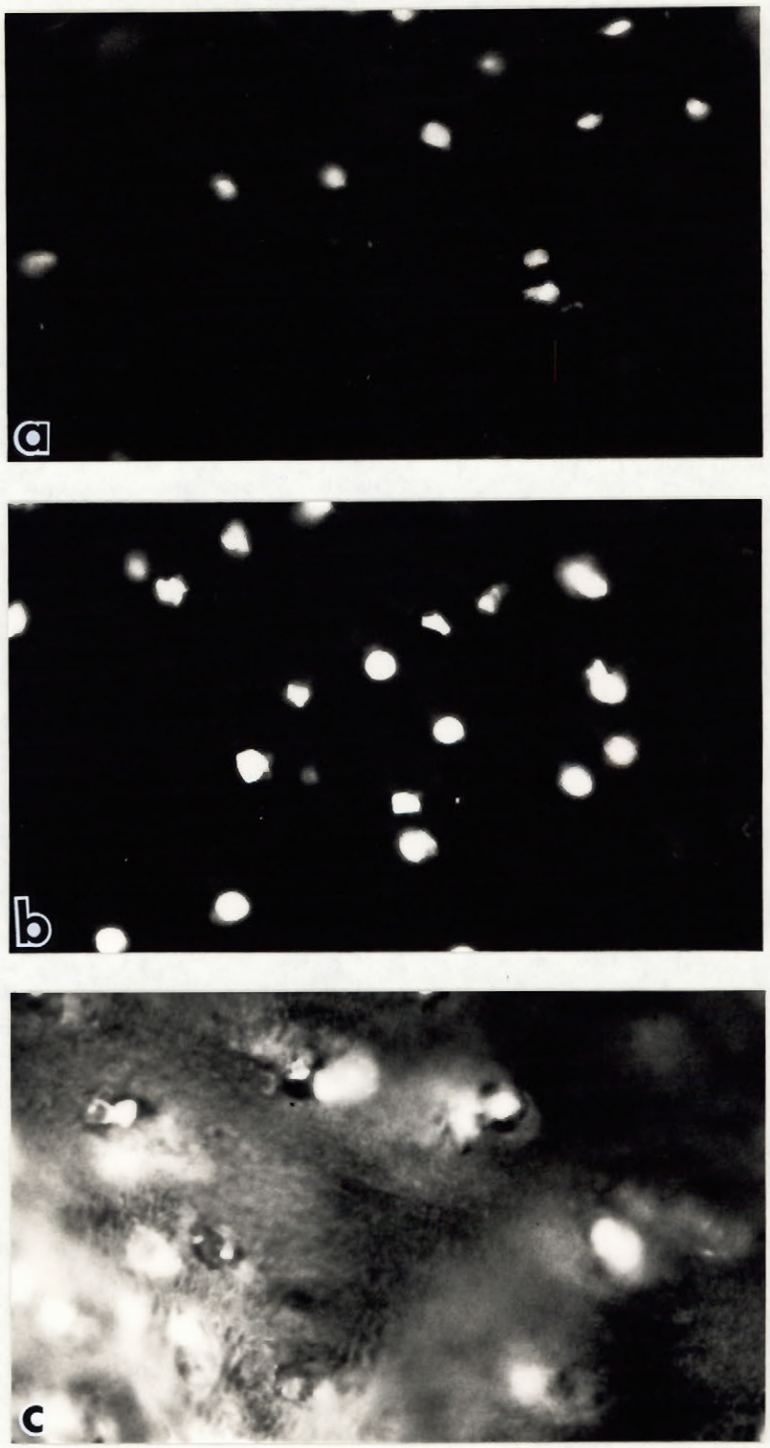


Figure 20 : Controls for Immunohistochemical Staining of Bovine Articular Cartilage.

Immunofluorescent staining of uncultured bovine articular cartilage, (a) without the primary antibody, (b) with NRS and (c) with R110 a rabbit antiserum to proteoglycan. Magnification 205x.

interterritorial and pericellular staining pattern typically seen with R110. The positive control was performed with each experiment to be certain that the system was working. This precluded the identification of any false negative reactions. Additional controls included the preabsorption of R181 $F(ab')_2$ with denatured type II collagen prior to use. Staining of normal bovine articular cartilage sections with preabsorbed R181 was considerably reduced when compared to sections identically treated with untreated immune R181 $F(ab')_2$, demonstrating the removal of antibodies to denatured type II collagen α chains.

When sections of normal bovine articular cartilage were pretreated with trypsin to remove proteoglycan and stained with R181, no increase in staining was observed after trypsin treatment (Figure 21 panels g and h) or in the low intensity staining pattern seen with NRS $F(ab')_2$ (Figure 21 panels e and f). Staining of similar sections with the monoclonal antibody AN9PI to keratan sulfate which was produced and characterized in this laboratory (261) showed a reduction in staining as compared to the staining detected in non-trypsin treated cartilage (Figure 22, panels c and d). Antibody reactions in these experiments were detected with the streptavidin-gold technique as described in the methods sections including silver enhancement for light microscopy. The procedure results in a permanent brown/black reaction product. Similar sections of cartilage were stained with the cationic dye, toluidine blue to demonstrate the presence of glycosaminoglycan side chains on the proteoglycan molecules. As shown in Figure 21a and b, trypsin pretreatment nearly completely depleted the cartilage of detectable glycosaminoglycans as detected by toluidine blue staining.

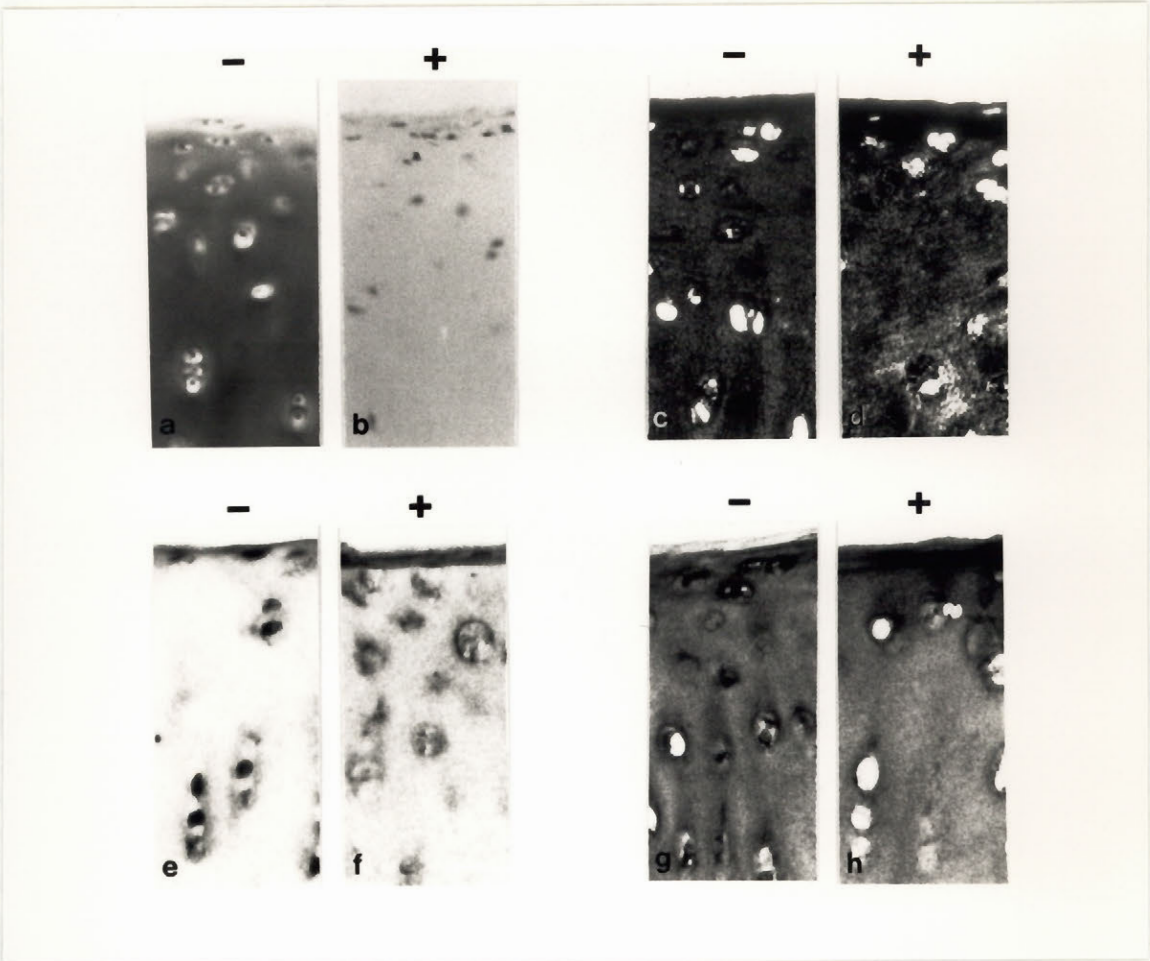


Figure 21 : Effects of Trypsin Pre-treatment on Immunolocalization of Type II Collagen Degradation and Detection of Proteoglycan in Bovine Articular Cartilage.

Adult bovine articular cartilage was pre-treated with trypsin, 0.125% (wt/vol) and processed as described in Materials and Methods. Sections were either pre-treated or not (+ or -) and stained with Toluidine blue (panels a and b); monoclonal AN9P1 (panels c and d); NRS $F(ab')_2$ (panels e and f) and R181 $F(ab')_2$ (panels g and h). Except for panels a and b, reactions were detected by using the streptavidin-gold system with silver enhancement. Magnification 450x.

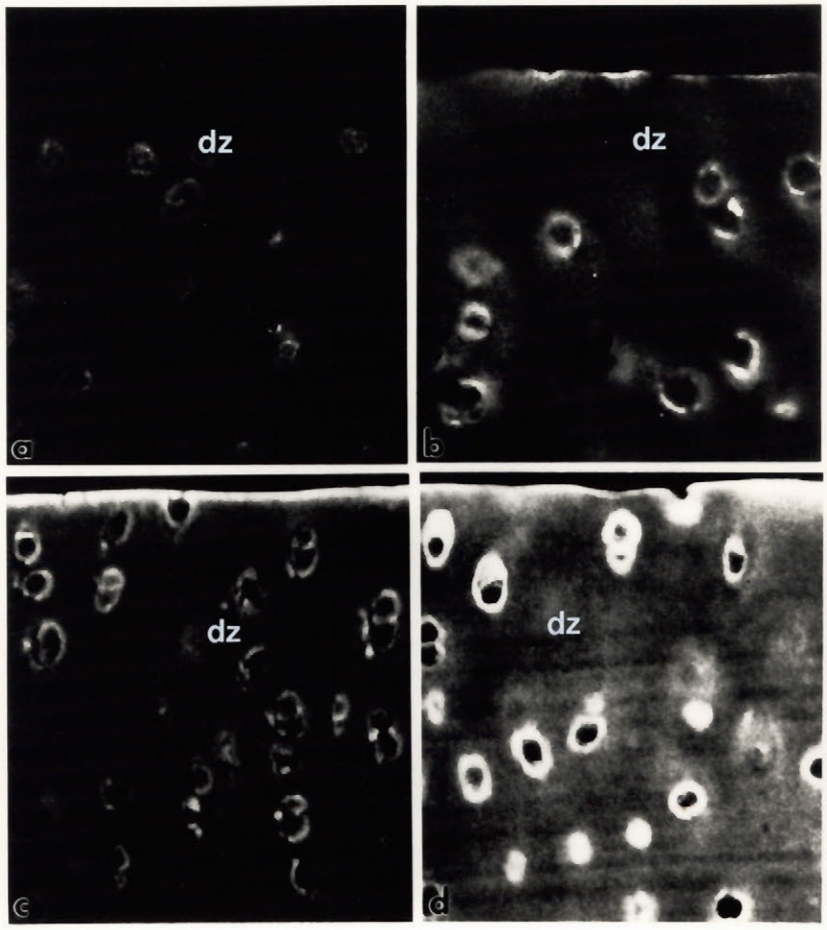


Figure 22 : Immunohistochemical Identification of Type II Collagen Degradation in Uncultured and Cultured Bovine Articular Cartilage.

Immunofluorescent staining with immune R181 $F(ab')_2$ of bovine articular cartilage, (b) uncultured, (c) cultured for 5 days, (d) cultured with IL-1 for 5 days. As a control for the antibody, (a) tissue cultured for 5 days was stained with control (NRS) $F(ab')_2$. Deep zone (dz) is identified. Magnification 730x.

These observations provide further evidence that there is no masking of native collagen epitopes by proteoglycans in as much as no increase of R181 staining was observed when the majority of proteoglycan/glycosaminoglycans were removed. Therefore, increased staining with R181 in experimental or pathological specimens is not due to an increased antibody accessibility, nor are the antibodies reactive with native collagen when large glycosaminoglycans are removed.

Experimentally, explants of adult bovine articular cartilage were cultured for up to 12 days with or without the addition of human recombinant IL-1 β. At two day intervals tissue was removed and cryosections were made for immunohistochemistry as described in methods sections. When crysosections of articular cartilage cultured for 5 days with IL-1 were examined (Figure 22d), intense fluorescence staining was observed with R181 as compared to cartilage cultured for the identical time and conditions except for the omission of IL-1 (Figure 22c) or control cartilage that was not cultured (Figure 22b). The staining for degraded type II collagen in cartilage cultured with IL-1 for 5 days (Figure 22d), shows both an increase in intensity of pericellular staining and an appearance of staining throughout the interritorial matrix. The reactions of R181 antibodies in these experiments were detected using the fluorescenated strepavidin system and specific controls with NRS $F(ab')_2$ were negative (Figure 22 panel a).

The most intense staining was always pericellular and was observed in cartilage from explants cultured with IL-1 for 3-5 days. Up until day 5 the number of cells identified with an area of intense broad pericellular staining, increased. After day 5, in most experiments, the percentage of chondrocytes with positive surrounding areas decreased, although the pericellular staining

that remained was always, as identified earlier at time points of 1-5 days, covering a broader zone than that seen in controls without IL-1. In sections from tissue which has been cultured for 12 days, the differences between cartilage cultured with or without IL-1 are less obvious (Figure 23). What is clear is that the staining pattern observed after long periods of culture with IL-1 is more diffuse and although still centralized in pericellular zones, these zones are more often around larger lucunae where cells reside. In almost all tissues with or without IL-1 present, the staining with R181 was overall reduced in cartilage from 12 days cultures in certain areas. Nonetheless, larger zones of "ghost-like" staining were observed in cartilage cultured for 12 days with IL-1, as is demonstrated by the Figure 23, panel b. Presumably the loss of staining in either case could be a result of a loss of epitope a result of progressive accumalative degradation of collagen.

Freshly isolated articular cartilage was characterized by weaker and smaller zones of pericellular staining and by low intensity interterritorial matrix staining (Figure 22, panel b). In general, the articular surface was lightly stained in all cartilages and this staining showed some small increase with culture time and/or the presence of IL-1. The cut edges in uncultured cartilages were not usually stained whereas after culture, cut edges stained brightly with R181. The experiments discussed and micrographs presented are of mid/deep zone cartilage. Cartilage explants studied represented full thickness cartilage slices removed just above the junction with subchondral bone.

2. Immunochemical analyses

Studies were performed to establish whether type II collagen α chains or fragments thereof could be detected in culture media and cartilage

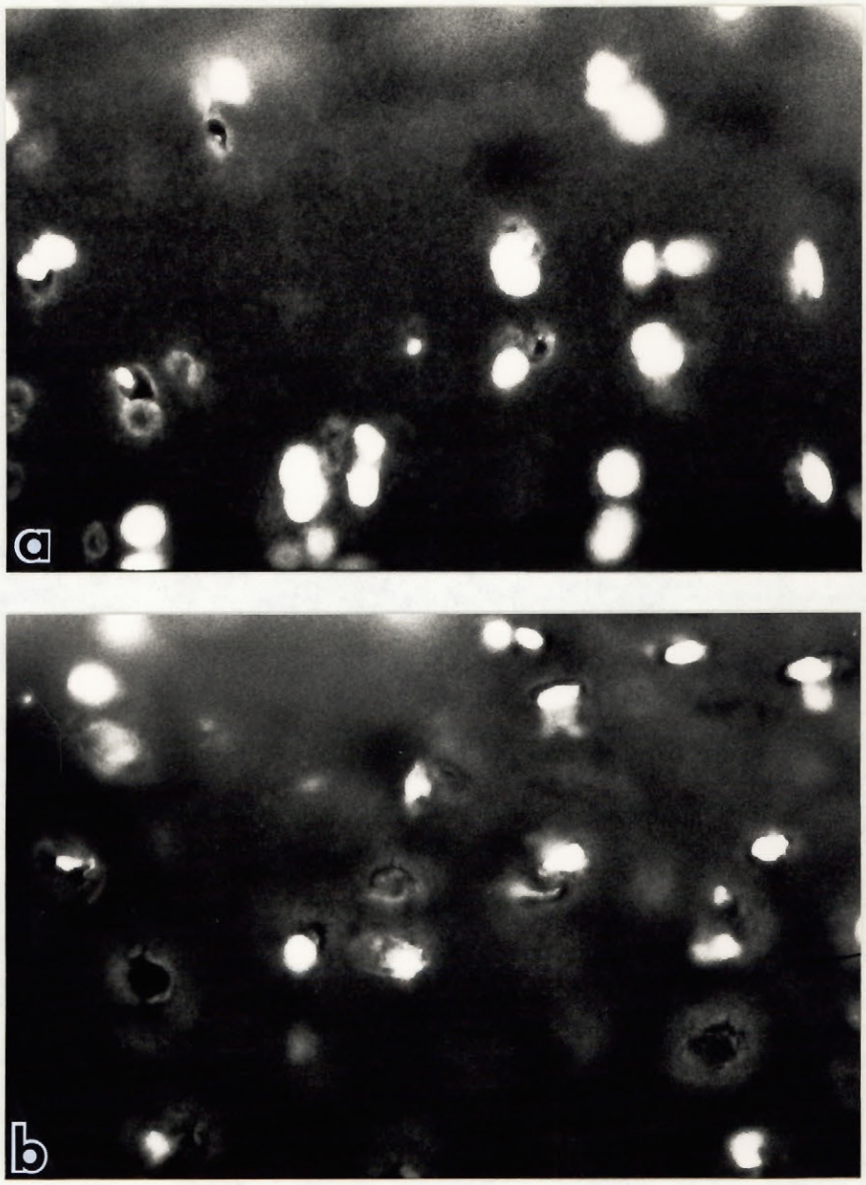


Figure 23 : Immunohistochemical Identification of Type II Collagen Degradation in Bovine Articular Cartilage Cultured for 12 Days With or Without IL-1.

Immunofluorescent staining with immune R181 $F(ab')_2$ of bovine articular cartilage cultured for 12 days in the presence (b) or absence (a) of IL-1. Magnification 205x.

extracts, using R181. Culture media removed at bi-daily intervals were lyopholized in equal aliquots, rehydrated and an aliquot was analyzed for the presence of type II collagen reactive components by electrophoresis and immunoblotting as described. Media removed from explant cultures treated with or without IL-1 at days 5,9 and 11 were examined by immunoblotting and by automated amino acid analysis. Immunochemically there was no evidence of reactivity of R181 with any protein smaller than the intact α chain of type II collagen from medium taken at day 5, 9 or 11 from either IL-1 treated tissue or control tissue not treated with IL-1, except in one experiment when a small fragment migrating ahead of the 14.3kd molecular weights standard appeared reactive in explant medium from day 9 IL-1 treated cultures (data not shown). This was not consistently observed. Therefore, in some cultures fragments of collagen-bearing immunoreactive epitopes were not present or were either not present in sufficient quantity in the culture media, to be detected by this methodology.

3. Amino acid analyses of explant culture media

Amino acid analysis of multiple samples of spent explant culture media revealed that no significant quantities of hydroxyproline could be detected and further, no correlation of trace amounts of hydroxyproline could be made with explant medium from IL-1 treated or untreated cartilage cultures (data not presented).

4. Immunochemical analyses of extracts of explant tissue

In contrast, when cartilage explant tissue was removed at 5,9 and 11 days and subjected to guanidine extraction, there were type II collagen reactive components detected with R181 and immunoblotting. Extracts after exhaustive dialysis were dried and analyzed by electrophoresis and

immunoblotting with R181 and compared to the nonspecific reaction profile of non-immune R181 (Figure 24). As can be seen, extracts of cartilage cultured with IL-1 for 11 days contained an immunoreactive band of a slightly smaller size than an intact α chain (upper arrow, Figure 24b) that was not detectable in extracts from cartilage for cultured identical periods without IL-1 or from normal uncultured bovine cartilage extracts (Figure 24b). This larger band could correspond to a cleaved or partially degraded α chain initiated by collagenase cleavage (285). This remains to be further clarified. Suprisingly, intact α chains were extractable from cartilage explants with some indication that with culture time and after culture with IL-1, an increase of intact α chains could be detected with R181. An additional smaller band migrating near the 30 kilodalton globular standard (Figure 24, lowest arrow) was observed in extracts from cartilage cultured for 11 days with IL-1.

R181 reactive bands also appear migrating slightly higher than the 30 kilodalton marker in both lanes D and E, (Figure 24) with noticably more in the untreated 11 day extract sample (lane D). These smaller collagenous bands could represent products of further proteinase action on the α chain, particularly since the R181-reactive determinants of the type II α chain are located towards the NH_2-terminal end (Figure 19). These R181-reactive bands were not seen in the control immunoblot which was incubated with preimmune serum (Figure 24a).

5. Collagenase activity

Media from bovine articular cartilage explant cultures were collected every second day and examined for collagenase activity. The spent culture medium was of equal volume, stored frozen until collectively assayed without

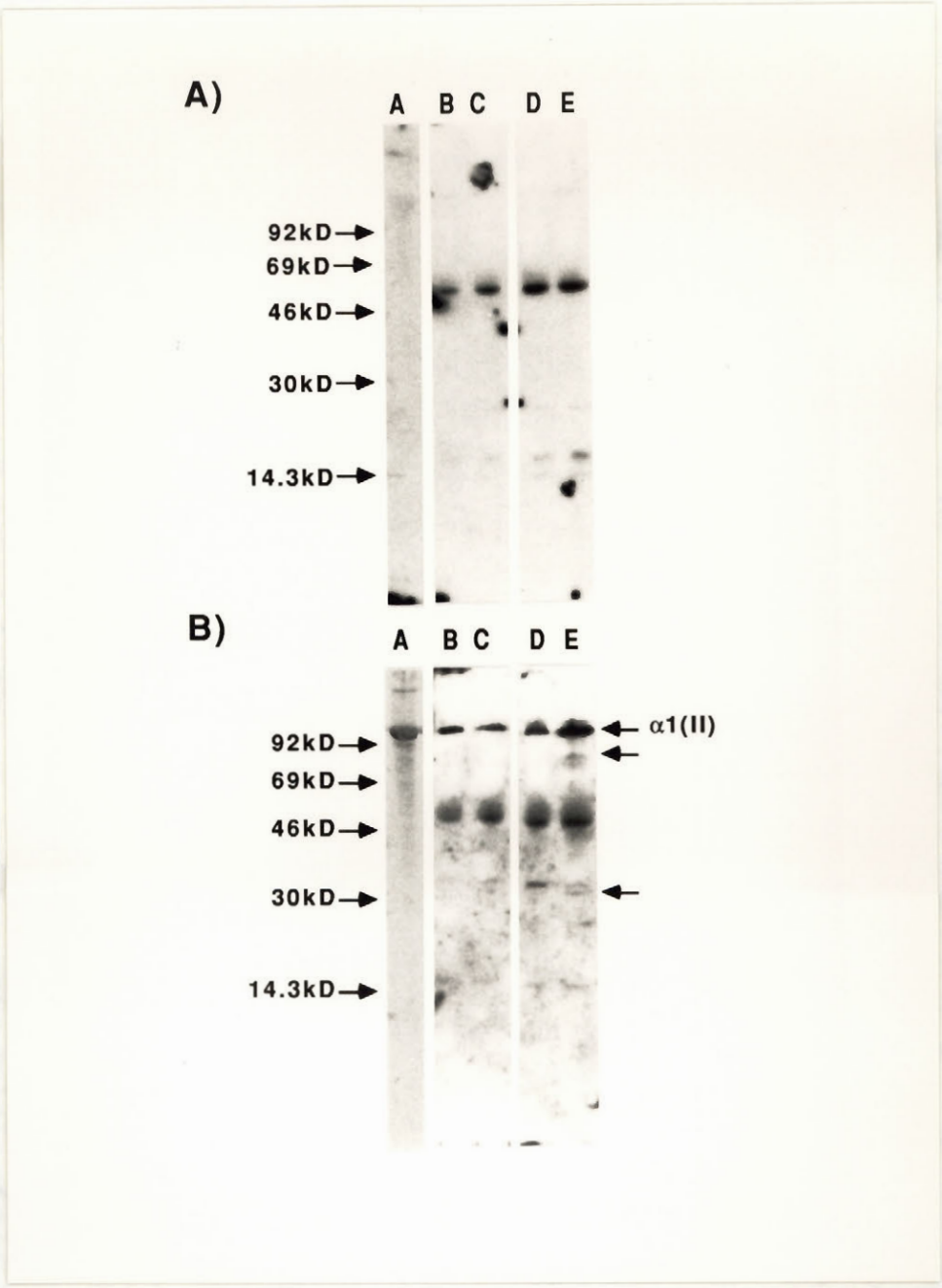


Figure 24 : Immunochemical Analyses of Bovine Cartilage Extracts After Culture With or Without IL-1

Guanidine extracts of bovine articular cartilage were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. Panel A was incubated with preimmune R181, panel B with immune R181. As a point of reference, 10μg of purified bovine type II collagen was run in lane A. Lanes B and C are extracts of articular cartilage after 5 days in culture without (lane B) and with (lane C) IL-1. Lanes D and E are extracts of cartilage after 11 days of culture without (lane D) and with (lane E) IL-1. Each cartilage extract was loaded at 30 μl representing 20% of the total extract prepared as described in Methods. The position of R181-reactive fragments are indicated on the right by an arrow, along with the position of the intact α (II) chain. Molecular weight standards were used as described and are indicated in kilodaltons to the left.

any dialysis or freeze-drying, at an appropriate dilution. Figure 25 is a representative experiment of 5 separate experiments showing cumulative collagenase activity in medium from explant cultures treated with or without IL-1 for a period of 2 to 9 days. With IL-1 the collagenase activity was progressively released into the medium over the 9 days culture period and was latent, and detected only after activation with APMA. Little or no active collagenase was ever detected in the medium from any experiment.

C. Summary

The following is a summary of the salient observations and findings as a result of the experiments with bovine articular cartilage reported in this section.

1. Antibodies of immune serum R181 shown to be specific for fragmented and denatured type II collagen, were utilized in a immunohistochemical protocol to identify in situ type II collagen degradation in bovine articular cartilage.
2. R181 $F(ab')_2$ was shown to react with degraded/denatured type II collagen in tissue when compared to controls tissue incubated with NRS $F(ab')_2$.
3. Explants of bovine articular cartilage cultured with IL-1 showed a marked increase in staining for degraded type II collagen, particularly in pericellular sites.
4. Staining observed with R181 $F(ab')_2$ was not enhanced with further removal of proteoglycan with trypsin indicating that the reaction product was not a result of exposing epitopes or denatured collagen.

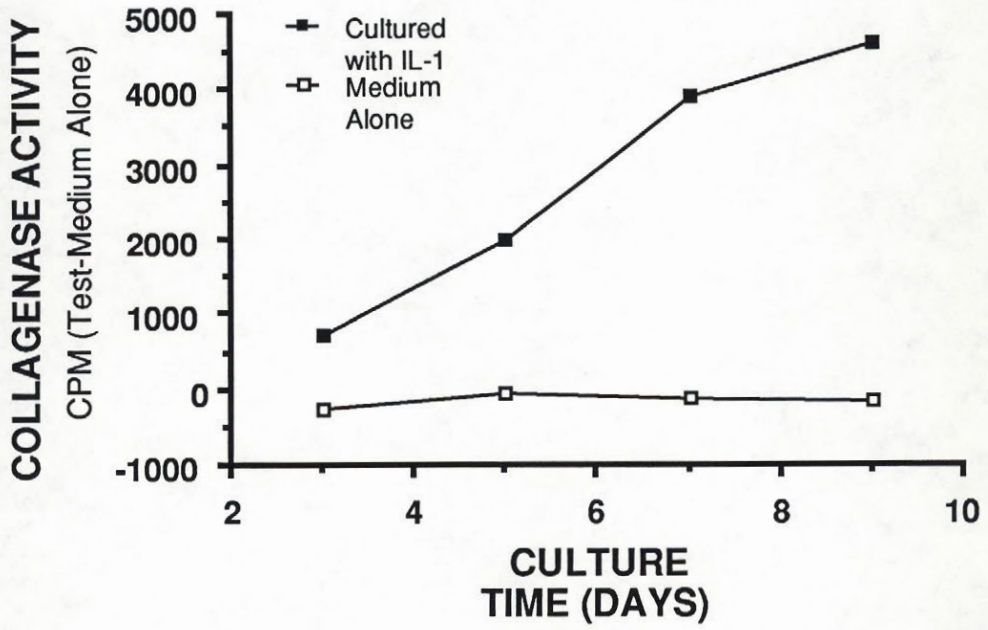


Figure 25 : Accumulation of Latent Collagenase Activity in Culture Media from Bovine Articular Cartilage Explants Cultured With and Without IL-1.

5. The increase of immunohistochemical staining observed in explants of bovine cartilage is maximal after 3-5 days in culture with IL-1 and the reactive epitopes begin to disappear with longer culture times, although staining is still present in cartilage cultured for 12 days with IL-1.
6. Latent collagenase activity can be detected in the culture media of bovine cartilage explants, and is significantly increased (approximately 40%) in cultures where IL-1 is included.
7. These experiments collectively demonstrate the utilization of a well-characterized antiserum (R181) in detecting collagen degradation in situ and provide direct evidence for the ability of the ubiquitous cytokine IL-1, to mediate/activate type II collagen degradation in the extracellular matrix of articular cartilage.
8. R181 was used to detect immunochemically type II collagen alpha chains and fragments of alpha chains in extracts of bovine articular cartilage explants after culture with IL-1. The R181-reactive collagen components which were smaller than alpha were chains detected only after 9 and 11 days in culture with IL-1.
9. Immunochemically detected alpha chains were present in extracts of bovine cartilage throughout the culture period of 5-11 days and an increase in extractable collagen type II α chains was detected.

IV. STUDIES OF HUMAN ARTICULAR CARTILAGE TYPE II COLLAGEN DEGRADATION

A. Preface

Human studies were made possible when the crossreactivity of the antiserum R181 with human denatured type II collagen was established, and antibodies were shown to specifically react with the CNBr derived type II collagen fragments, α1 (II)CB11 and α1(II)CB8 rather than intact native type II collagen. Studies were designed to utilize these antibodies to examine normal and diseased articular cartilage. Freshly obtained tissues were examined for in situ type II collagen degradation by the immunohistochemical method described in Materials and Methods (Chapter 2, VI), always utilizing the gold-streptavidin detection system with silver enhancement. Studies will be presented here of normal osteoarthritic and rheumatoid arthritic articular cartilages. In addition, similar in vitro studies to those described with bovine cartilage, were performed using freshly obtained tissue placed in explant culture with or without IL-1 to provide direct evidence of the cytokines role in human arthritic diseases. As an extension of these studies, a proteinase inhibitor (U24522) was used in this in vitro model of type II collagen degradation and its effect on IL-1 stimulated cartilage elucidated by immunohistochemistry. The results of these experiments will be presented.

B. Immunohistochemical identification of collagen degradation in human articular cartilage

1. Tissues

Human normal (non-arthritic) cartilage from healthy joints and diseased cartilage (osteoarthritic and rheumatoid arthritic) from adult patients were

examined for type II collagen degradation using antiserum R181. Arthritic patients were undergoing total knee arthroplastry whereas age-matched non-arthritic control tissues were removed at the time of autopsy within 10 hours of death. Each osteoarthritic and rheumatoid arthritic patient was diagnosed as having advanced forms of disease and is discussed in the Materials and Methods (Chapter 2). In the case of osteoarthritic cartilages, histological examination of the cartilage confirmed the diagnosis: gross irregularities in the articular surface with fibrillation and cleft formation were observed. All rheumatoid cartilages were from patients with advanced, chronic disease and were characterized by synovitis and by marked surface erosion. In some areas, cartilage was completely absent, eroded down to bone. Normal cartilages were from adult joints from age-matched individuals whose cause of death was known and completely unrelated to connective tissue abnormalities, except for those of the natural aging process. These cartilages appeared macroscopically normal with an intact articular surface with little or no irregularity.

2. Detection of degraded type II collagen in normal and diseased cartilage

Cartilages were all handled by identical procedures and reacted with the antibodies under the precise conditions described in the preceeding Methods section. Controls were performed to address questions regarding antiserum specificity and antigen (or epitope) accessibility. To increase the ability of antiserum, R181 to penetrate these adult human cartilages, tissues were all treated with both chondroitinase ABC and keratanase prior to antibody incubation. To control for antibody accessibility which may be impeded by proteoglycan, human rheumatoid cartilage was stained for proteoglycan using the monoclonal antibody AN9P1 and its staining pattern

compared to that of R181. Figure 26 demonstrates that in the case of a representative cartilage (patient A.D.) staining with AN9P1 (Figure 26, panels c and c') was intense throughout the superficial, mid and deep zones in a uniform pattern even when compared to the normal ascitic fluid control (Figure 26, panels d and d'). Less staining for proteoglycan was observed in rheumatoid cartilages in the superficial matrix than elsewhere. In comparison, the staining pattern seen with R181 was concentrated at the articular surface and in the pericellular and territorial areas of the deep zone (Figure 26, panels b and b') as compared to that in tissue reacted with the control normal rabbit serum $F(ab')_2$ (Figure 26, panels a and a'). In all cartilages examined, both normal and diseased, staining for proteoglycan was unrelated to the staining observed with R181. Another example of the lack of a relationship of staining for type II collagen degradation with proteoglycan localization is shown in Figure 27. This shows an age-matched normal (Figure 27, panels a and b) and rheumatoid arthritic cartilage (Figure 27, panels c and d). The pattern of staining is clearly different whereas the staining for type II collagen breakdown is mostly pericellular in this tissue specimen (patient DK) and that for proteoglycan is more uniform being present in pericellular, territorial and interterritorial matrix. From these studies it can be seen that there is no relationship between the staining with AN9P1 and R181. Therefore, there is no evidence to indicate that an increase in staining with R181 is due to an increase in antibody accessibility as a result of a loss of proteoglycan.

Osteoarthritic cartilages were examined from four patients (PS, CR, VM, CP). The example shown in Figure 28 is cartilage from patient PS and is representative of three of the four patients examined. The most striking

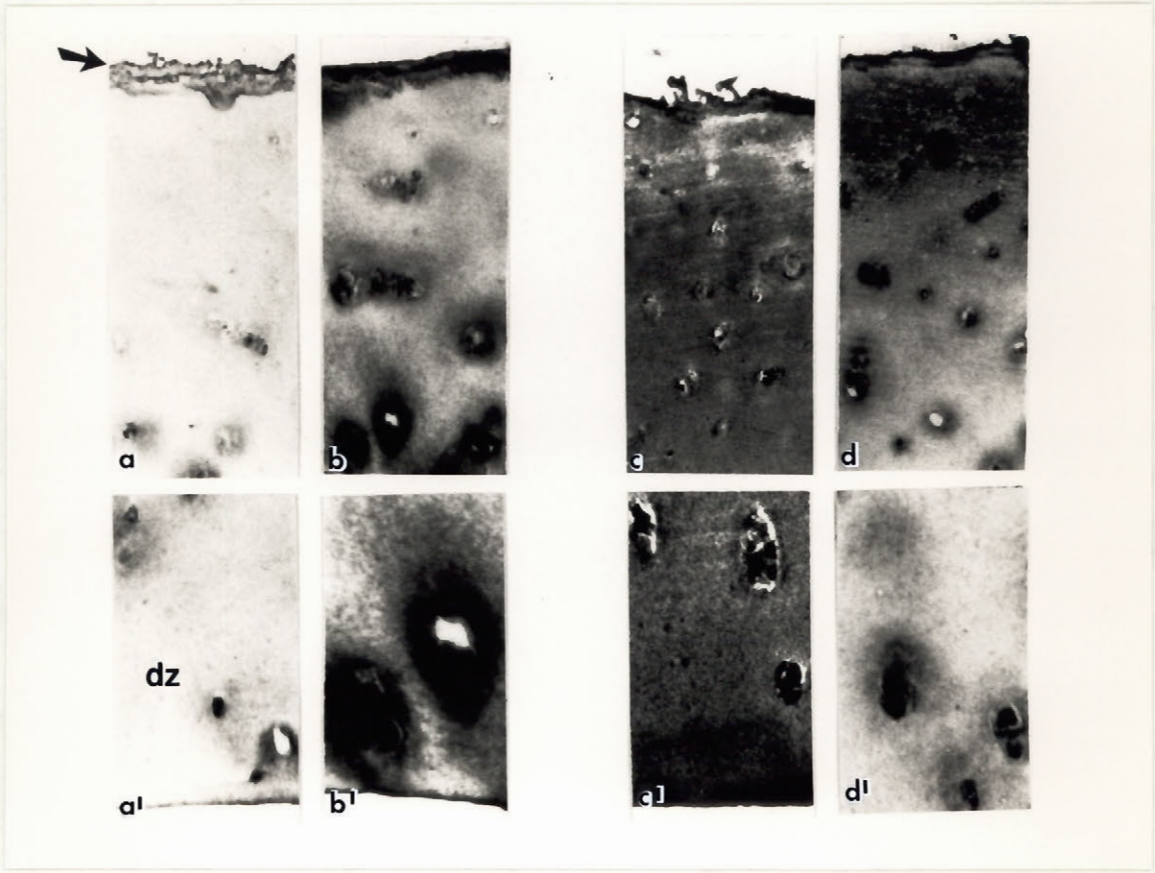


Figure 26 : Immunohistochemical Detection of Type II Collagen Degradation and the Presence of Proteoglycan in Rheumatoid Arthritic Human Articular Cartilage.

Sections of full thickness cartilage from patient AD were examined with NRS $F(ab')_2$ (panel a and a'); R181 $F(ab')_2$ (panel b and b'); AN9P1 (panel c and c') and normal ascitic fluid (panel d and d'). Reactions were detected with the streptavidin-gold system including silver enhancement for light microscopy. Deep zone (dz) and the articular surface (arrow) are indicated. Magnification panels a, b, c, d 173x, panels a', b', c', d' 480x.

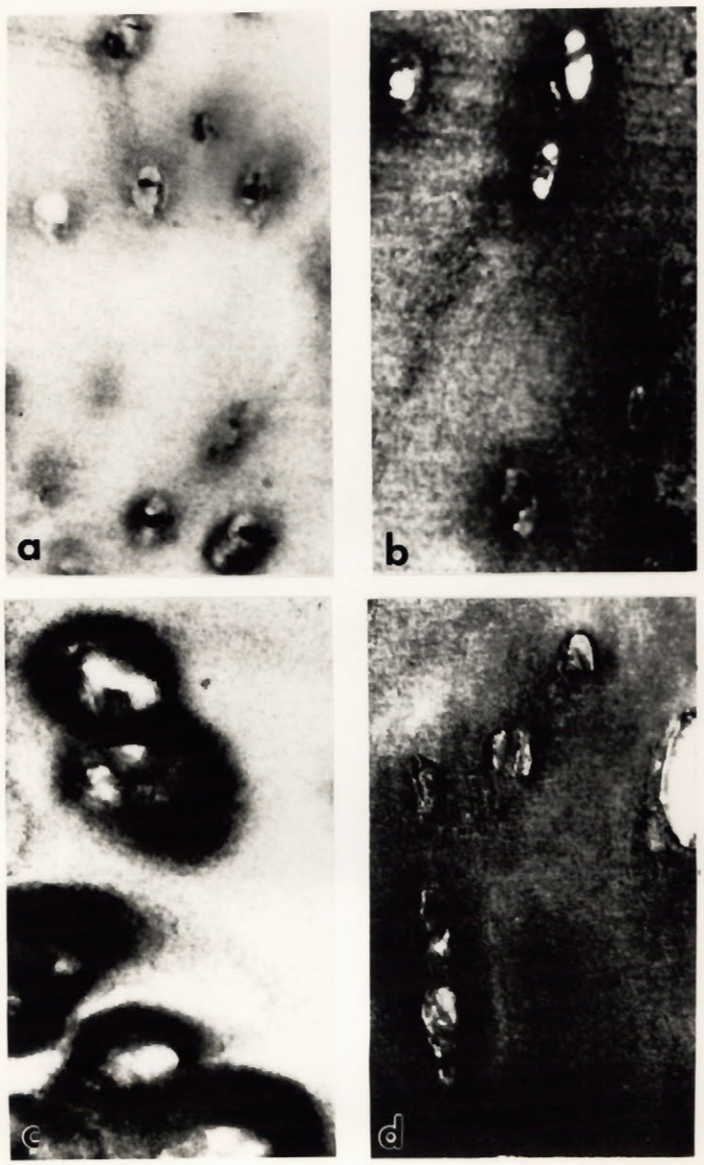


Figure 27 : Comparison of Type II Collagen Degradation and the Presence of Proteoglycan in Normal and Rheumatoid Arthritic Human Articular Cartilage

Deep zone normal adult articular cartilage (panels a and b) from an age-matched control with cartilage from a rheumatoid patient DK (panels c and d) were incubated with R181 $F(ab')_2$ (panels a and c) or AN9P1. (panels b and d) Reactions were detected with the streptavidin-gold system including silver enhancement for light microscopy. Tissues incubated with NRS $F(ab')_2$ (as a control for R181 $F(ab')_2$ or normal ascites fluid (as a control for monoclonal AN9P1) were negative in equivalent regions (data not shown). Magnification 490x.

staining was throughout the regions of the superficial fibrillation (Figure 28, panel b) and in the interterritorial matrix of the superficial and mid zones (Figure 28, panels b and d). Tissue incubated with NRS $F(ab')_2$ did not stain and is shown from similar regions of cartilage from patient PS as were those incubated with R181 $F(ab')_2$ (Figure 28, panels a,c and e). Evidence of pericellular degradation was also detected in the deep zone (Figure 28, panel f), although this was restricted, in this example, to the pericellular and territorial areas around the deep zone chondrocytes with little or no intercellular matrix staining elsewhere in the deep zone. The staining with R181 is specific and intense when compared to that seen in identical tissues stained with NRS $F(ab')_2$ (Figure 28, panel e). As mentioned, this staining profile was characteristic of three of the four osteoarthritic cartilages examined, while cartilage from patient CR was stained with R181 at the articular surface, not throughout the superficial zone and intensely stained in territorial sites around the chondrocytes in the deep zone.

Intense pericellular, territorial and interritorial matrix staining was detected in all rheumatoid articular cartilages examined. Shown in Figure 29 is a photomicrograph of articular cartilage from patient AD stained with R181 $F(ab')_2$ (Figure 29, panel b) and control NRS $F(ab')_2$ (Figure 29 panel a). In contrast to the osteoarthritic cartilage shown, the most striking evidence for collagen degradation was predominantly in the territorial and pericellular matrix of the deep zones and at the articular surface (Figure 29, panels b and d). The staining was concentrated around the deep zone chondrocytes with intense staining of the territorial matrix. This pattern of degradation was evident in three of four rheumatoid arthritic cartilages examined (patients LM, DS, AD). The staining was shown to be specific by incubating, in

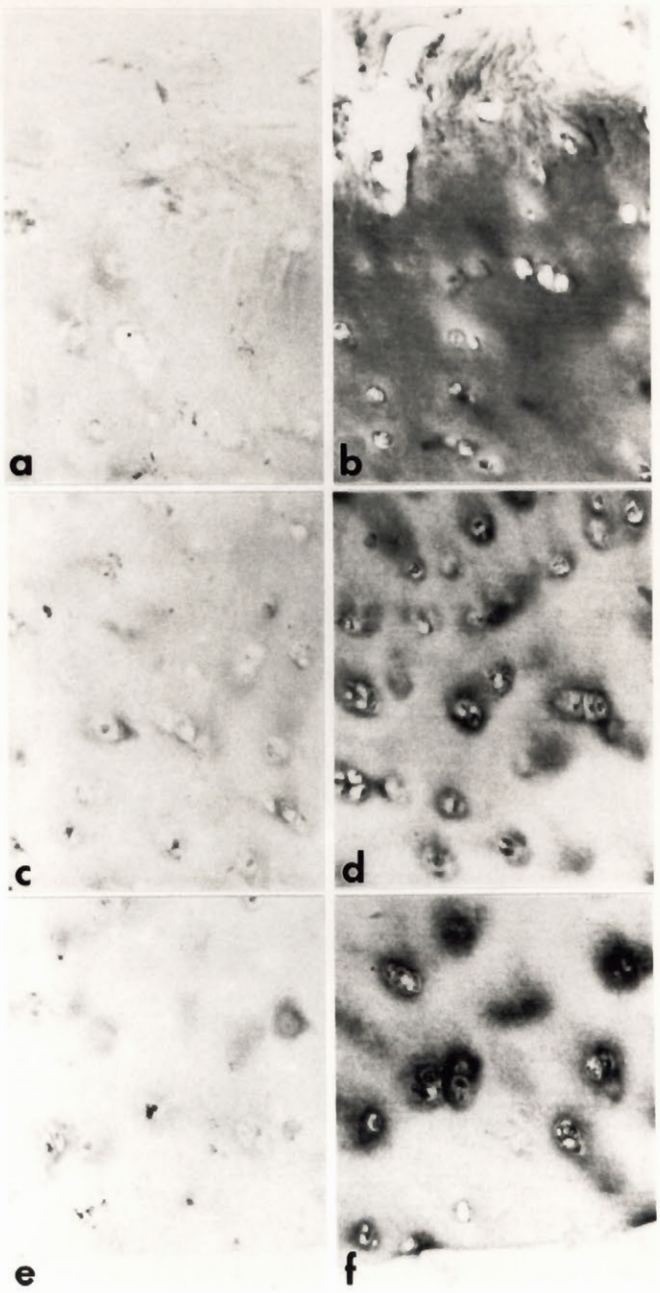


Figure 28 : Immunohistochemical Identification of Type II Collagen Degradation in Human Osteoarthritic Articular Cartilage.

Cryostat sections of femoral condylar articular cartilage from a 69-year-old male (patient P.S.) were stained with NRS $F(ab')_2$ (a, c, e) or immune R181 $F(ab')_2$ (b, d,f) and with the streptavidin-gold, as described. The superficial zone with a fibrillated articular surface (a, b), mid (c, d) and deep zones (e, f) are shown. Magnification 300x.

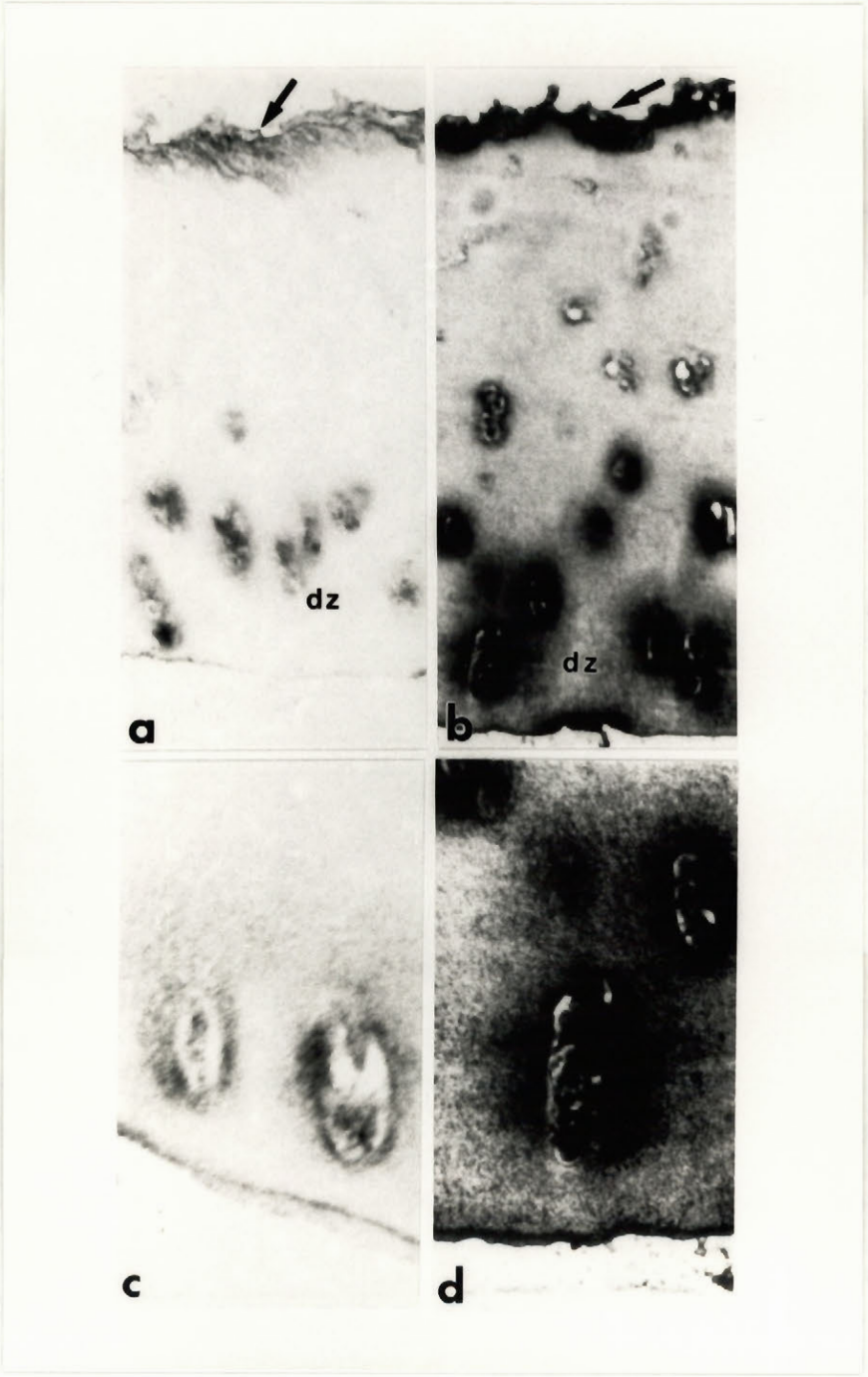


Figure 29 : Immunohistochemical Identification of Type II Collagen Degradation in Human Rheumatoid Articular Cartilage.

Cryostat sections of femoral condylar articular cartilage from a 60-year-old male (patient A.D.) stained with control NRS $F(ab')_2$ (a) or immune R181 $F(ab')_2$ (b) are shown (magnification 300x). Panels c and d are high power magnification (730x) micrographs of the deep zone cartilage of panels a and b, respectively. Antibody binding was as described using the streptavidin-gold method with silver enhancement. The articular surface (arrows) and deep zones (dz) are indicated.

parallel each cartilage tissue with control nonimmune $F(ab')_2$, as is demonstrated by the representative cartilage (patient AD) stained with NRS $F(ab')_2$ (Figure 29, panels a and c). All rheumatoid cartilages were boldly stained for collagen degradation and the staining seen at the articular surface was much more intense than that seen in osteoarthritic or normal cartilage. The exception to the pattern represented in patient AD was cartilage of patient KE, which was intensely stained throughout the matrix, indicating the more advanced destruction of articular cartilage.

Four nonarthritic (normal) cartilages were tested routinely with pathological tissues as age-matched controls. All normal cartilages presented weak staining both at the articular surface and in pericellular sites throughout the cartilage in all zones with light interterritorial staining (Figure 30). The staining seen in normal cartilage was always limited and not uniform, as is seen in the representative specimen of a 73 year old male (Figure 30), where staining is restricted to certain regions, both in certain pericellular sites, and in the territorial matrix.

C. Cartilage explant culture experiments

Explants of human articular cartilage asceptically removed from autopsy patients (within 10h post mortem), was cultured in serum-free medium with or without recombinant human IL-1β in modified DMEM as described in Materials and Methods. The medium was collected at 2 day intervals and replaced with fresh medium containing IL-1 where necessary, and in later experiments the compound, U24522, a hydroxamic acid with known metal metalloproteinase inhibiting activity (293,297). These human explant cultures were an extension of the work of Dr. John S. Mort, and

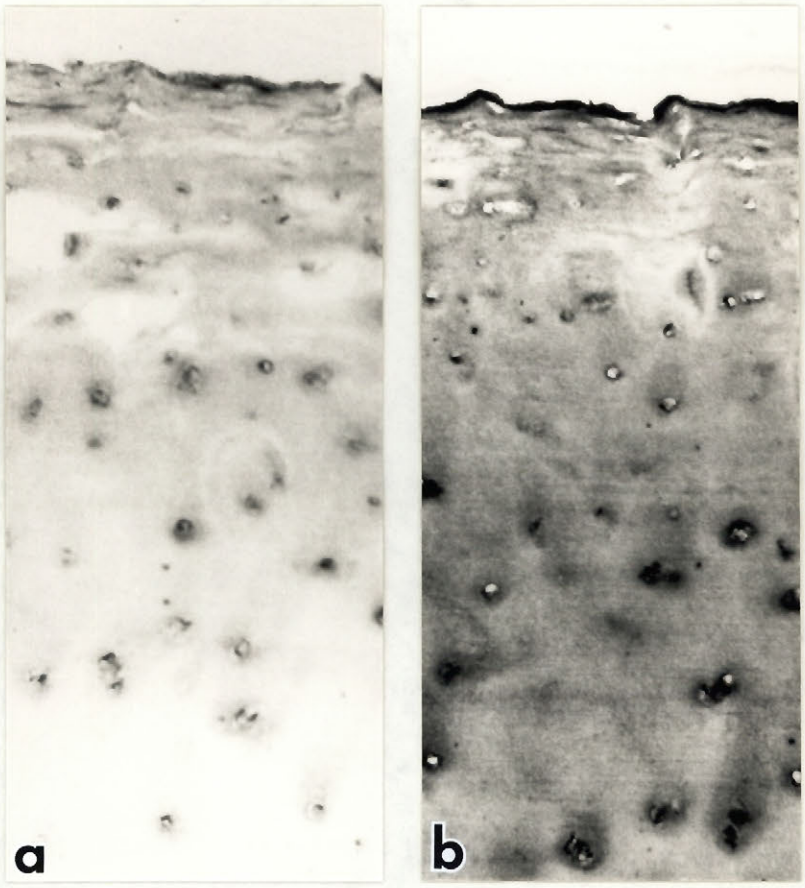


Figure 30 : Immunohistochemical Idendification of Type II Collagen Degradation in Normal Human Articular Cartilage.

Sections of cartilage from a nonarthritic (normal) 73 year old patient were stained with NRS $F(ab')_2$ (panel a) or R181 $F(ab')_2$ (panel b). The reaction was detected using the streptavidin-gold system with silver enhancement. Magnification 295x.

others in the Joint Diseases Laboratory (J.S. Mort et al. manuscript in preparation) and were based on previous studies investigating proteoglycan degradation and the effects of IL-1 (204). Tissues were removed at various times intervals including days 5, 9, and 12, and handled as described for human cartilage in Materials and Methods and stained immunohistochemically for type II collagen degradation with antiserum R181.

Initial experiments were performed to immunohistochemically detect the state of type II collagen in human cartilage after culture with IL-1. Figure 31 demonstrates the increase in collagen degradation after culture with IL-1 for 9 days (panel b), as compared to the basal level of degraded collagen present in cartilage cultured for the identical time under the same conditions except without IL-1 (panel a). Cartilage for studies shown here was obtained from a non-arthritic 47 year old male taken at autopsy 6 h post mortem. Cartilage appeared macroscopically normal. The specifity of the reaction is demonstrated by incubation of tissue sections with NRS $F(ab')_2$ (panel c). In tissue stained with R181 $F(ab')_2$ striking (Figure 31, panels a, b) striking amount of collagen degradation is detected in pericellular and territorial zones around chondrocytes or clusters of chondrocytes in the deep zone where the most staining was observed and is where these micrographs are taken. Interleukin-1 stimulated type II collagen degradation was immunohistochemically detected most clearly after 9 days of culture whereas little increase in staining was observed in cartilage examined after 5 days in culture. The tissues examined after 12 days in culture were stained more intensely for collagen degradation in IL-1 treated explant cultures (Figure 32, panel c) than those untreated tissues cultured for the same time. As was seen in this photomicrograph, there is much type II collagen degradation

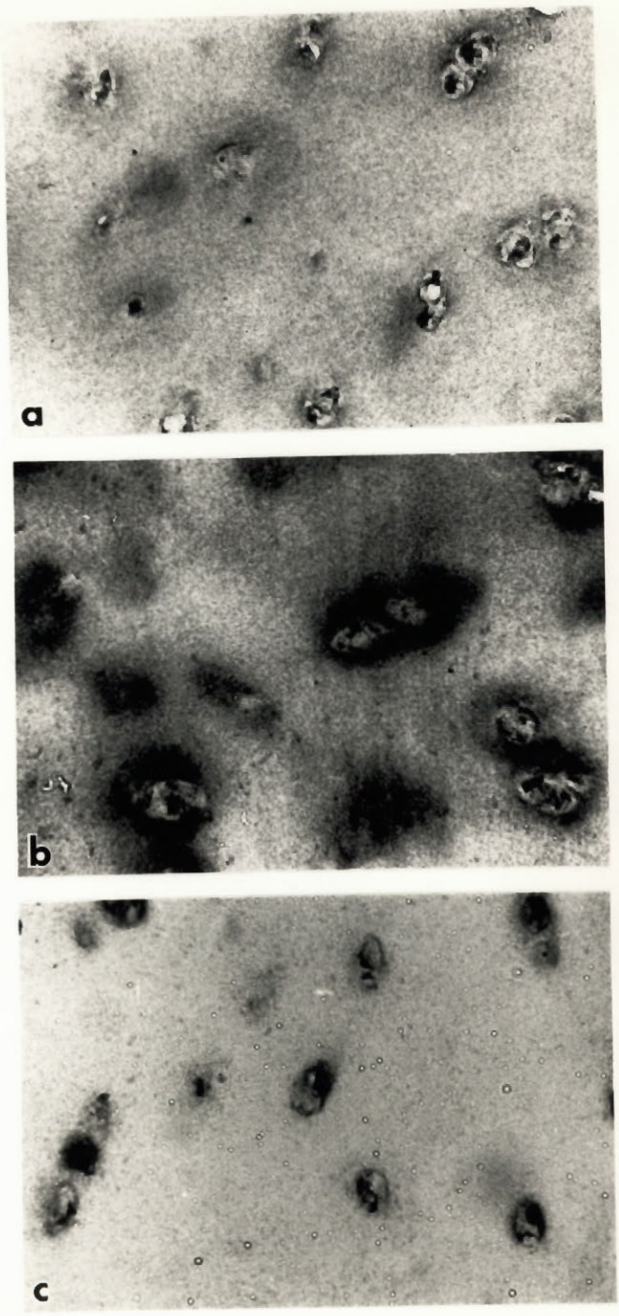


Figure 31 : Immunohistochemical Identification of IL-1 Induced Type II Collagen Degradation in Explant Cultures of Adult Human Articular Cartilage

Human articular cartilage was cultured without IL-1 (panel a) or with IL-1 (panels b and c) for 9 days. Control amount of DMSO was added to each explant culture condition. Sections of explant cartilage were stained with R181 $F(ab')_2$ (panels a and b) or with NRS $F(ab')_2$ (panel c). Reaction of antibody binding is detected using the streptavidin-gold system as described. Cartilage is representative of the deep zone. Magnification 375x.

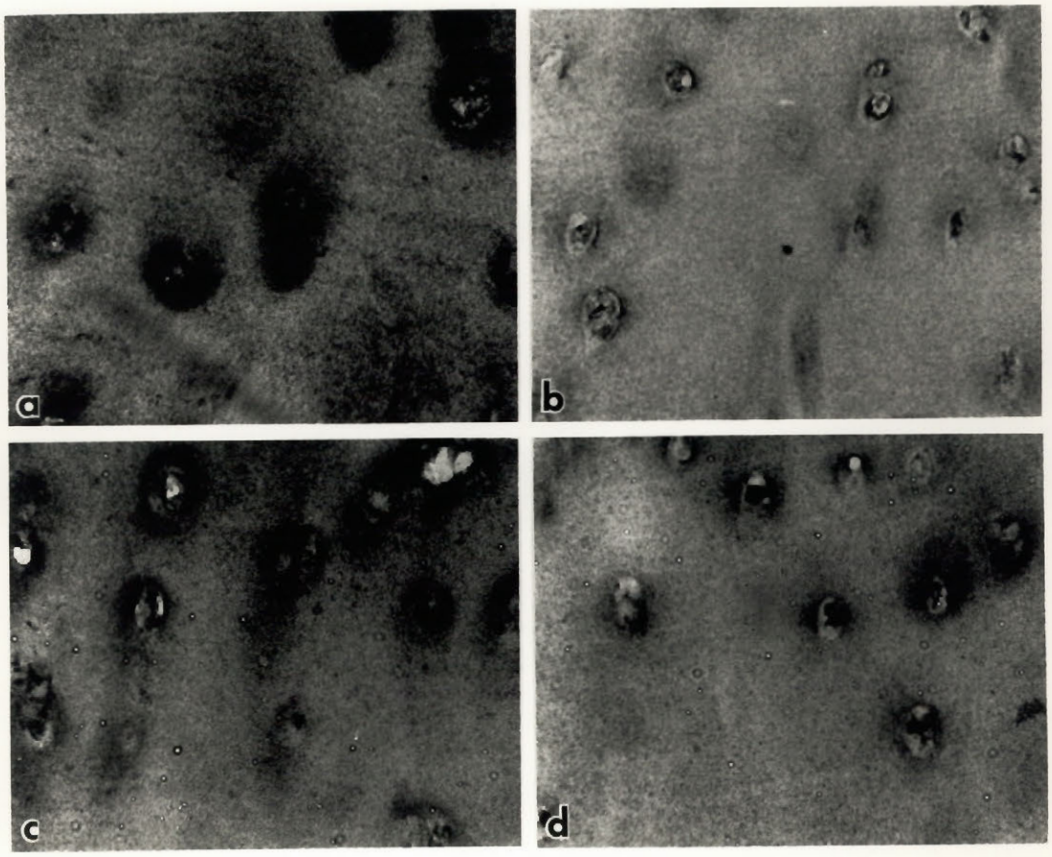


Figure 32 : Inhibition of IL-1 Induced Type II Collagen Degradation By U24522

Explants of adult human articular cartilage were incubated for 9 days (panels a and b) or for 12 days (c and d). Panels a and c represent sections of cartilage from cultures incubated with IL-1 (including the vehicle control for U24522) and stained with R181 $F(ab')_2$ for type II collagen degradation. Panels b and d represent those cartilages cultured with 1×10^{-5} M U24522 in addition to IL-1. Control cartilage from this experiment cultured without IL-1 is represented in Figure 31 panel a. Reactions were detected using the streptavidin-gold system with silver enhancement for light microscopy. Cartilage is representative of the deep zone. Magnification 375x.

detected in both tissue taken from day 9 cultures (panel a) and from day 12 cultures (panel c) with only slightly less interterritorial matrix staining in this later time period. In contrast to the bovine explant cultures experiments discussed previously, the effects of IL-1 and stimulation of collagenase secretion in these studies with human cartilage is slightly slower on onset and longer in duration.

Collagenase activity was measured in the culture medium from the explant culture treated with or without IL-1. Interleukin-1 was present at a concentration of 5 units/ml where a 1-unit is as that which gives $^{1}/2$ maximal stimulation of IL-1 responsive cells and was determined by the supplier (Genzyme). Figure 33 shows data representative of three separate experiments where explants of human articular cartilage were stimulated with IL-1 for up to 14 days. Data is presented as accumulated latent collagenase activity which was measured by the ^{3}H-collagen fibril assay. No active collagenase was detectable in these media. Interleukin-1 stimulated the production/secretion of latent collagenase. Enzyme activity was detected in increasing amounts over a culture period of 14 days.

As an extension of these studies and in an attempt to explore the relevance of the R181-immunohistochemical technique to detect degraded type II collagen, experiments were designed to include the metalloproteinase inhibitor (U24522). These studies were carried out in collaboration with Drs. A.R. Poole, J.S. Mort, P.J. Roughley, of the Joint Diseases laboratory, Montreal and Dr. G. DiPasquale of Stuart Pharmaceuticals, Wilmington DE, supplier of the research compound U24522. Because of the special solubility properties of this synthetic compound, a hydroxamic acid, a stock solution of 20mM of U24522 was prepared and added to cultures when necessary in a

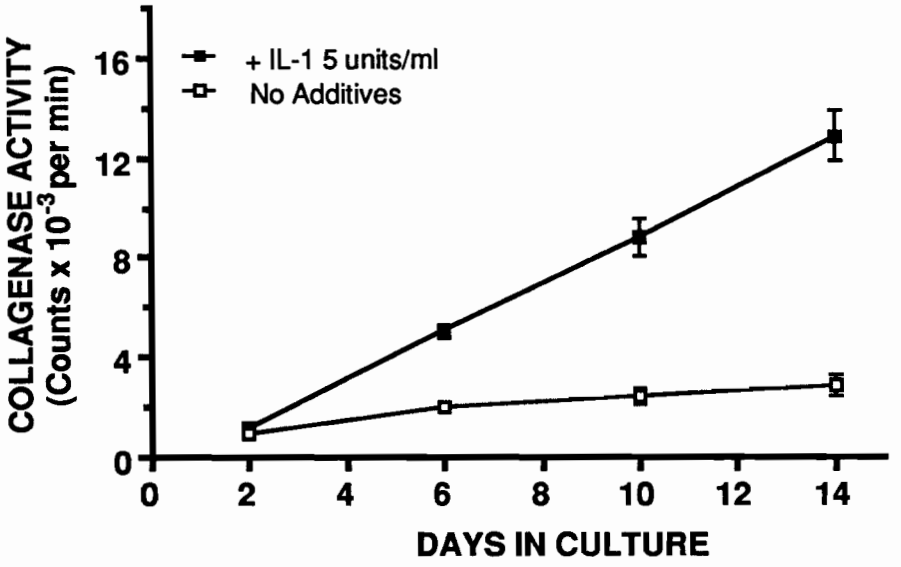


Figure 33: Accumulative (Latent) Collagenase Activity in Medium of Human Articular Cartilage Explants Stimulated with Il-1.

small aliquot to reach a final concentration of 0.01mM. Due to the toxicity of DMSO control cultures were routinely used containing the identical concentration of DMSO (0.05%). Even though the compound was dissolved as a stock solution in DMSO, there was evidence that in culture the compound would come out of solution and appear as lipid-like droplets deposited on the bottom of the culture dish. This phenomenon was detected during routine microscopic examination of cultures. This problem of solubility and resultant drug availability was minimized since the compound was freshly added each 2 days when the culture media were changed. The effects of the compound U24522 on detectable collagenase activity is shown in Figure 34, where accumulative latent collagenase activity is shown both before dialysis and after dialysis to remove this inhibitor. In the upper panel it is demonstrated that the addition of (5 units/ml) IL-1 significantly increases the amount of collagenase released into the medium of explant cultures as compared to the amount detected in unstimulated cultures. All activity detected was latent and was activated with APMA. The inclusion of U24522 at a concentration of 0.01mM in cultures with IL-1 resulted in a marked decrease (approximately 45%) in detectable collagenase. In the presence of the inhibitor the levels of collagenase were reduced to nearly those levels detected in media from unstimulated cultures. In order to study the effect of the inhibitor on the metalloproteinase itself present in the medium, the media were exhaustively dialyzed against assay buffer and reassayed for collagenase activity in the presence of APMA. Shown in Figure 34 in the lower panel, is the level of collagenase measured after dialysis to remove the inhibitor. The activity was approximately restored to that of the IL-1 stimulated values. The data suggest that the effect of the inhibitor is reversible. Although the inhibitor

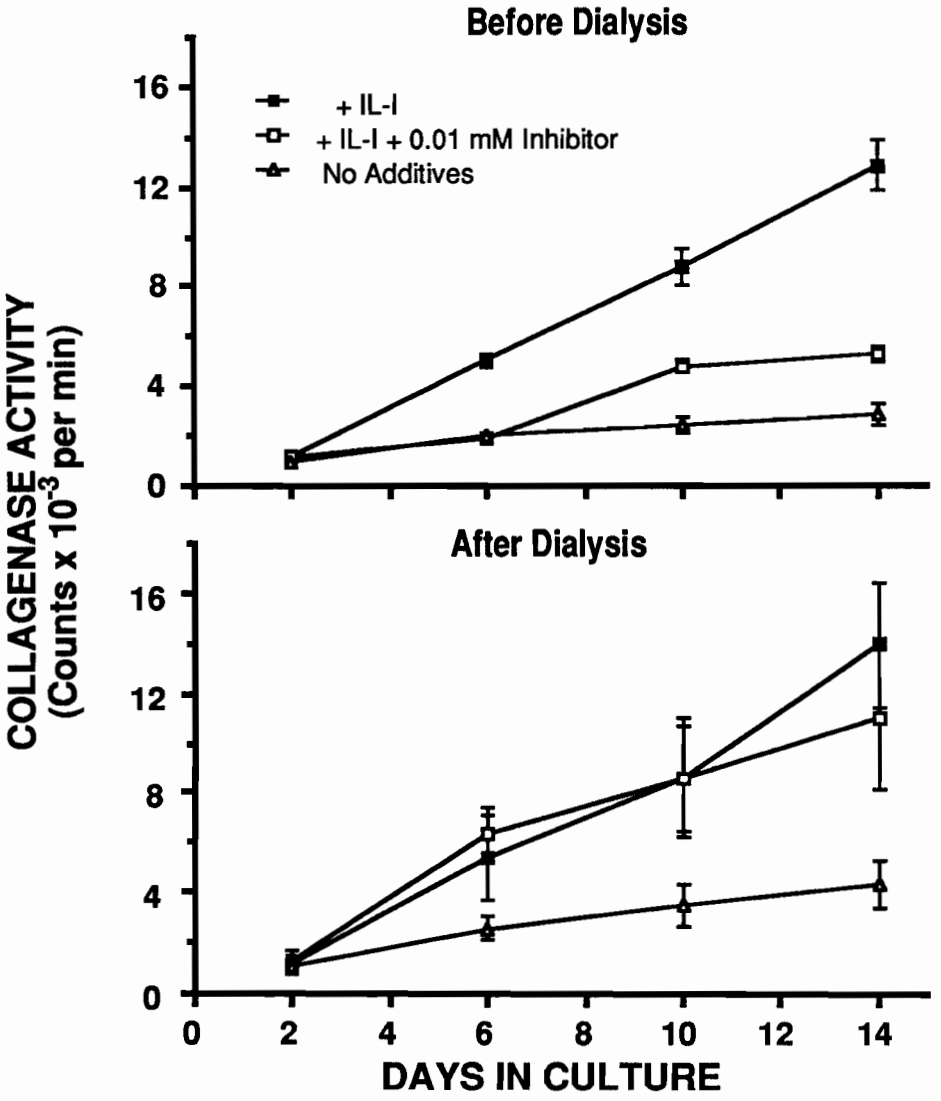


Figure 34 : Accumulative (Latent) Collagenase Activity in Medium of Human Articular Cartilage Explants Cultured With and Without IL-1 and The Inhibitor U24522

was removed before activation with APMA, no definitive conclusion regarding the interaction of the inhibitor and enzyme can be made.

Tissue sections of these human explant cultures were examined with the antiserum R181 using the immunohistochemical protocol described for human cartilage in Materials and Methods. As mentioned previously, cartilage cultured with Il-1 for 9 days showed a marked increase in staining for degraded type II collagen (Figure 31, panel b) over that seen in cartilage cultured for the same period without IL-1 (Figure 31, panel a). In this same experiment this predominately pericellular and territorial reaction with some overall interterritorial matrix staining can be seen as being maintained at comparable intensity from day 9 (Figure 32, panel a) to day 12 (Figure 32, panel c) culture, as compared to the control (no IL-1) in Figure 31, panel a. All tissue sections were taken from tissue which had been cultured with Il-1 including the control for the DMSO vehicle at a final concentration of 0.05%. This level of DMSO has little or no effect on the stimulatory effects of Il-1 on cartilage explants. The most striking results were seen in the ability of the inhibitor to negate or reduce the amount of collagen degradation in situ produced by IL-1 (Figure 32, panels c and d). Although effects of IL-1 and the effects of the inhibitor U24522 could not be detected earlier in explant culture, the effects were most dramatic at day 9 as shown in Figure 32, panels a and b. The effect of the inhibitor is still evident when day 12 tissue is examined. In Figure 32 cartilage stimulated with IL-1 (panel c) is compared to cartilages cultured for 12 days with IL-1 and the inhibitor (panel d). The region studied and represented here in these photomicrographs is tissue from the deep zone of human femoral condylar cartilage.

It was of importance to ascertain the viability of chondroyctes after extensive exposure to the compound U24522. This was addressed by determining the ability of cartilage from 14 day explant cultures to incorporate ^{3}H-leucine as described in Materials and Methods. The results from this experiment are tabulated in Table 2. They show the incorporation of ^{3}H-leucine expressed in CPM per culture where the incorporation of ^{3}H-leucine was corrected for the actual amount of cartilage in each culture. As can be seen, there is no evidence for a loss of viability after as long as 14 days in culture with the inhibitor. One can see that comparing the incorporation in the culture with Il-1 and that of Il-1 with the control level of DMSO, that this has no significant effect on viability. These data provide further evidence that this compound exhibits a direct inhibitory effect on the action of a collagen degrading enzyme.

D. Immunoelectron microscopic studies

Normal, osteoarthritic and rheumatoid arthritic cartilages were examined using R181 and the streptavidin-gold system of detection and were further processed for examination at the electron microscopic level. Tissue sections of articular cartilage obtained as serial frozen sections were either used for electron microscopic studies or were examined with the light microscope for collagen degradation and proteoglycan content. Since the same cartilage sections could be utilized for both light and electron microscopy a substantial amount of information could be obtained from the same specimens (identical patients). These studies were used not only to elucidate further the location and intensity of reaction products, but could also be used to demonstrate the specifity of the reaction. As can be seen in

Explant Culture	Normalized CPM/culture (~200 µg cartilage)
Unstimulated (medium alone)	**197,000**
+IL-1	**295,000**
+IL-1 + 0.25%DMSO	**250,000**
+ IL-1 + U24522 0.05 mM	**265,000**

Table 2 : Viability of Adult Human Articular Cartilage During Explant Culture with IL-1 and U24522

Due to the slight variation in the distribution of cartilage in the explant cultures at the onset of the experiment, the data presented have been normalized to the amount of cartilage in the cultures at day 2 when all cultures are identical. Prior to the addition of IL-1, inhibitor U24522, or vehicle control on day 2, all cultures were the same. The data has been normalized to the amount of proteoglycan released into the medium at day 2 using the Dimethylmethylene Blue binding assay as described elsewhere (125). cpm represents counts per minute of incorporated ^{3}H-Leucine.

Figure 35, rheumatoid cartilage (from patient AD) which was incubated with R181 $F(ab')_2$ showed distinct focal concentrations of gold particles associated with broken collagen fibrils or with fibrils which appear as less electron dense. Regions shown in the micrograph are representative of the territorial matrix of the deep zone. In comparison, similar cartilage sections incubated with NRS $F(ab')_2$ and carried through the identical prodedures for electron microscopy, were negative. In these tissues one detects few or no colloidal gold particles, yet there is a disordered matrix and an abundance of broken collagen fibrils. This negative reaction with NRS is an additional control for the immunohistochemistry method. This further demonstrates the specificity of the R181 reaction for degraded collagen fibrils. In each diseased tissue examined, staining with R181 was observed associated with disrupted collagen fibrils or what appeared to be material derived from collagen fibrils, rather than with intact fibrils which show the classic banding pattern. In fact, in diseased cartilage there is a marked decrease in the number of fibrils present with the typical banding pattern.

Normal age-matched cartilage was examined in parallel with rheumatoid articular cartilage for the presence of type II collagen degradation. In Figure 36, two such cartilages are presented where marked staining is observed in the rheumatoid cartilage commonly associated with amorphous, less electron dense material which appear as remnants of collagen fibrils or as unravelled fibrils. Normal tissue examined from similar territorial sites remote from the articular surface, showed very little staining. When staining was observed in normal non-arthritic cartilage it was more often detected in pericellular zones and was associated with 'fractured' collagen fibrils or less electron dense material, perhaps an indication of basal

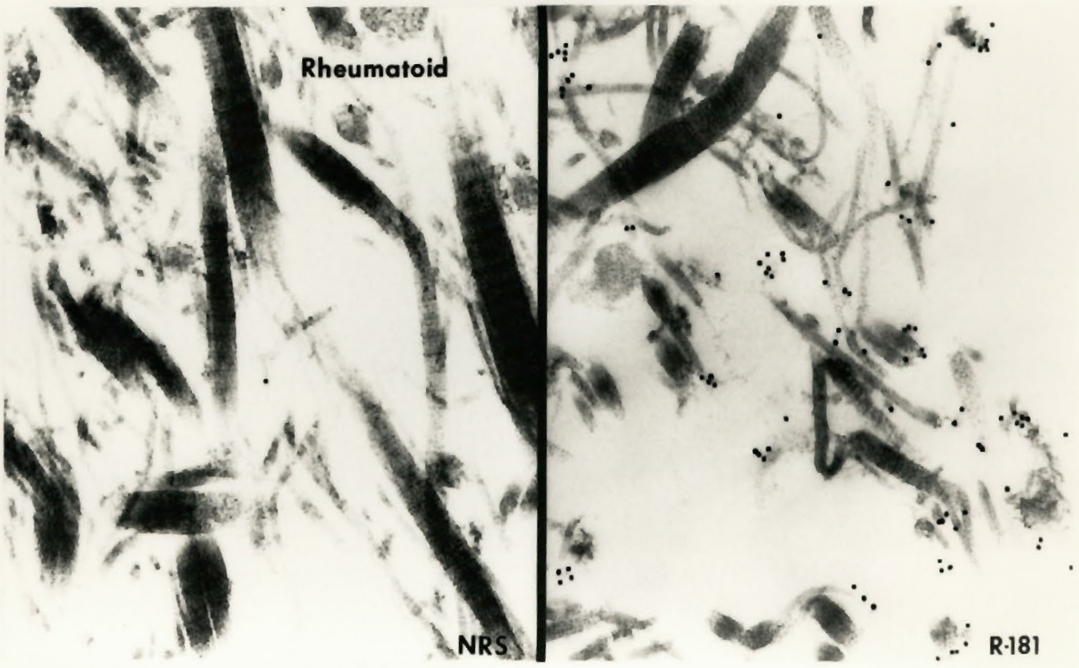


Figure 35 : Immunoelectron Microscopic Demonstration of Type II Collagen Degradation in Human Rheumatoid Articular Cartilage

Rheumatoid articular cartilage from patient A.D. was incubated with NRS $F(ab')_2$ (left panel) or R181$F(ab')_2$ (right panel). Antibody binding was detected using the streptavidin-gold system. Regions of cartilage shown are representative of the territorial matrix of the deep zone. Magnification 46,000x.

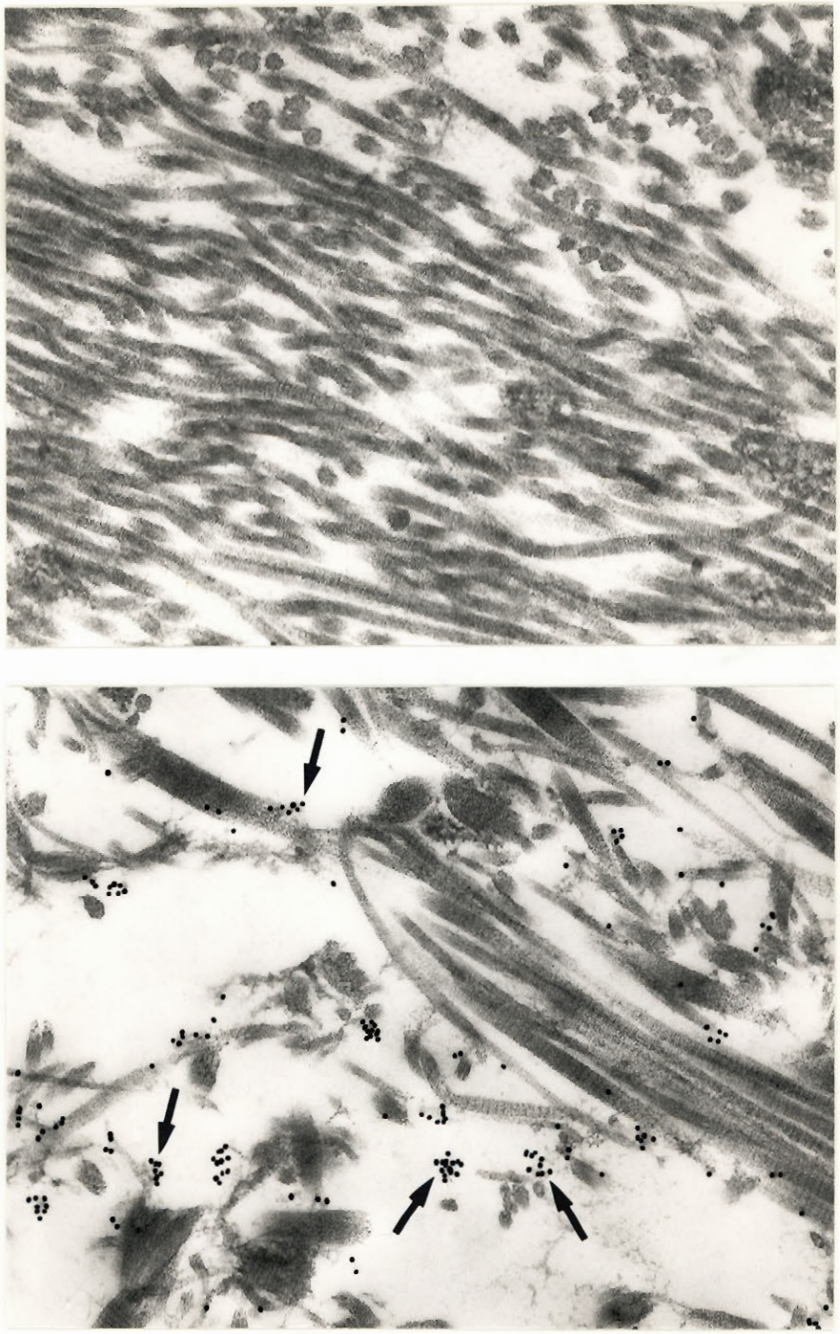


Figure 37 : Immunoelectron Microscopic Detection of Type II Collagen Degradation At the Articular Surface of Normal and Rheumatoid Articular Cartilage

Articular cartilage from patient AD (lower panel) and an age-matched non-arthritic normal (top panel) are presented after immunostaining with R181 $F(ab')_2$ and streptavidin-gold. Areas represent matrix at the articular surface. Arrows indicate reaction at disrupted collagen fibrils. Magnification 52,000x.

pericellular space. The intensity of staining for collagen degradation is indicative of the local effects, presumably of enzymes such as collagenase produced by the resident chondrocyte. Antibody binding in pericellular material from deep zone cartilage, is to the ends or fragments of collagen fibrils or fibrils which appear less electron dense and partly degraded.

These observations have been made in cartilage from this patient (AD) and others. Figure 39 shows another example from a different rheumatoid patient (patient DK). Throughout the arthritic tissues studied, collagen fibrils appeared to be of a smaller diameter in rheumatoid cartilages than non-arthritic. This is also seen in cartilage of Figure 39. These thinner fibrils may be newly synthesized or immature fibrils formed as a repair response.

E. Summary

The following summary is a compilation of the most prominent findings of the studies reported in this section.

1. Antiserum R181 was demonstrated to react specifically with degraded or denatured type II collagen in human articular cartilage.
2. Staining detected in normal and arthritic human articular cartilage was not related to the presence of proteoglycan: type II collagen degradation could be detected in human rheumatoid without a significant loss of proteoglycan.
3. All human arthritic cartilage examined contained areas of pronounced pericellular and territorial staining for collagen degradation as compared to non-arthritic age-matched control cartilage.

4. Most rheumatoid cartilages exhibited intense staining for degraded type II collagen at the articular surface and in the pericellular and territorial matrix in the deep zones.

5. Osteoarthritic cartilage was stained for type II collagen degradation in a different pattern, most staining being observed in the interteritorial and territorial matrix of the superficial and mid zones, in part, associated with surface irregularities and fibrillation. Although less intense than rheumatoid tissues, staining was observed around chondrocytes of the deep zone.

6. Although some distinct staining was observed in age-matched controls (themselves from patient 36-73 years old) it was of a low intensity and usually at the articular surface, presumably where stress and aging effects are most evident, and in dispersed, tight pericellular sites throughout the cartilage matrix.

7. Human cartilage explants cultured with IL-1 secreted significant levels of collagenase in the culture medium. This was associated in a time dependent manner, with the degradation of type II collagen detected immunohistochemically in situ.

8. Even though the amount of degradation and collagenase secretion stimulated with IL-1 was striking, it could be almost completed abrogated by the synthetic metalloproteinase inhibitor U24522.

9. Electron microscopic studies of human cartilage revealed that the staining of arthritic cartilages with R181 was localized to broken collagen fibrils and/or remnants of collagen which appear less electron dense. As compared to non-arthritic cartilage, the staining was seen in pericellular and territorial areas of the deep zone and the matrix of the

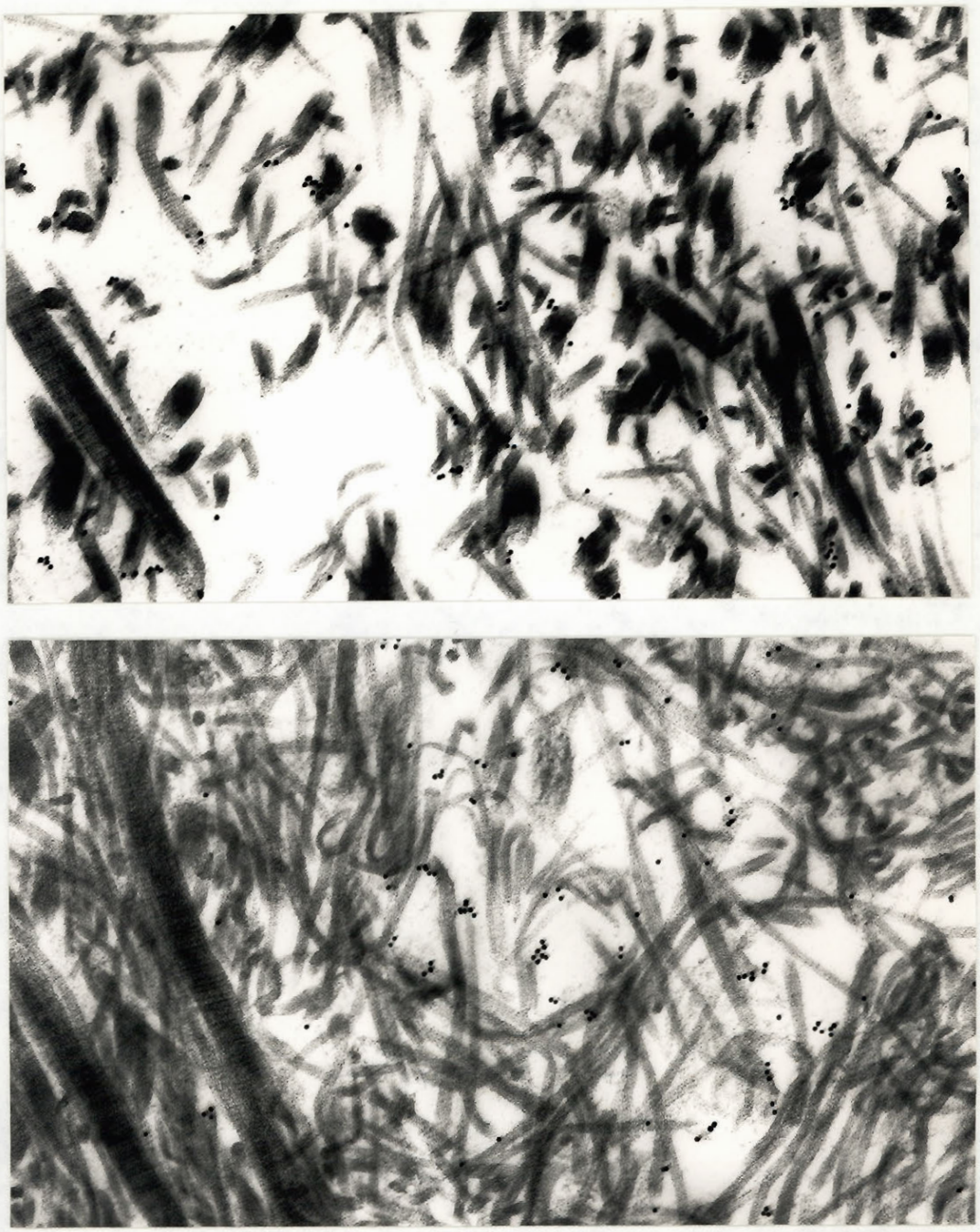


Figure 39 : Immunoelectron Microscopic Detection of Type II Collagen Degradation in Human Rheumatoid Articular Cartilage.

Rheumatoid articular cartilage from patient DK was stained with R181 $F(ab')_2$ (both panels) and the reaction detected with the gold-streptavidin system. Regions represented in these electron micrographs are of deep zone cartilage. Magnification 52,000x.

articular surface where there was a loss of collagen organization and where fibrils were disordered and lacked the classic banding pattern.

CHAPTER 4

I. DISCUSSION

The chapter will include a general discussion of the findings, which have already been summarized at the end of each Results section. A general discussion of the novel immunohistochemical and immunochemical approach to collagen breakdown utilized in these studies will draw attention to the new information and original contributions of this thesis.

The biochemical, chemical and biological properties of articular cartilage depend intimately on the molecules which comprise the extracellular matrix, collagen and proteoglycan. These large polymeric molecules provide articular cartilage with the resiliency required to withstand compressive forces and the stresses of motion and successfully protect the underlying skeletal bone (46,294,295). Perturbation of one or all of these extracellular matrix components resulting in damage or loss, causes dramatic changes in the functional properties leading to pathological conditions, such as arthritis. Many studies have been designed to determine the physical and enzymic mechanisms which are involved in the destruction of articular cartilage and subsequent loss of function. Whenever cartilage is damaged mechanically or biochemically, there is damage and loss of matrix and new molecules are synthesized in an attempt to restore the tissue. The balance between synthesis and breakdown (degradation) is a critical and a determining factor in the restoration of the cartilage with little or no loss of integrity or function (223).

One of the limiting factors in the study of cartilage degradation has been the inability to detect collagen breakdown in articular cartilage other than by detecting the release of hydroxyproline (294). A variety of studies

have addressed cartilage degradation from the standpoint of changes in proteoglycan in relation to size, type of glycosaminoglycan side chains and an altered proportion of protein relative to glycosaminoglycan (296-298). In addition to these types of biochemical studies, proteoglycan, due to its unique charge properties, can be detected using cationic dyes, such as safranin O and toludine blue (26,299,300). In contrast, the study of collagen changes in degenerative diseases has been limited by the technology and experimental approaches available which are primarily morphological at the electron microscopic level. From the studies presented in this thesis, one can conclude that the inability of previous studies to identify collagen degradation as an early event in in vitro cartilage experiments (289,290,294) was a result of a lack of a sensitive and specific detection method with which to detect the degradation of type II collagen.

This thesis has been concerned with the development of a technique which could immunohistochemically and immunochemically detect collagen degradation in experimental animal and human articular cartilage. By using a highly specific polyclonal antibody directed against fragmented and denatured type II collagen, I have developed methods for the detection of collagen degradation in situ produced both experimentally and in human arthritis. Based on earlier work on the immunology and antigenicity of collagens (63,75,150,228,229) a monospecific antiserum (R181) was prepared to antigenic determinants on the α chains of the type II collagen molecule which are only exposed upon unwinding of the triple helix (i.e. central determinants). Theoretically, this unwinding can result from cleavage of the collagen molecule either in the triple helix by collagenase (161) or by cleavage at the non-helical telopeptide ends, such as can be produced by

elastase (176,301). A method to recognize collagen post-cleavage (denatured) could provide a valuable tool to elucidate further events in cartilage catabolism. A primary goal of this work was completed with the generation and characterization of the rabbit antiserum, designated R181. This enabled the design of experiments to further study and understand the status of collagen in articular cartilage.

To generate an antiserum for these studies type II collagen, peptides generated by cyanogen bromide cleavage of bovine type II tropocollagen which do not recombine to form helical collagen were used as an immunogen. Although the immunogenicity of tropocollagen (native or denatured) is often weak, selective antibody responses to central determinants have previously been observed (67).

Even though type II collagen has been identified as the major molecular form of collagen in cartilage (15), cartilage is now known to contain several collagen types. In addition to type II, Eyre and his colleagues have identified types V, VI, IX and XI, each accounting for 1-2% of the total collagen (284). To rule out any possible cross-reaction with these minor collagens, antiserum R181 was tested in ELISA and shown not to react with the native or denatured forms of bovine type V, VI, IX and XI collagens. The specificity of R181 for degraded type II collagen coupled with its demonstrated lack of reactivity with other cartilage molecules in bovine and human cartilage makes it possible to detect specifically the degradation of type II collagen.

Paramount to the interpretation of any experiments when antibodies are utilized, is the precise specificity of the antiserum, whether it be a polyclonal or monoclonal antibody preparation. The identification of the location of the R181 reactive epitopes on the type II collagen alpha chains

was made possible using CNBr-derived peptides, immunoblotting and microsequencing. The major immuno-reactive peptides, α1(II)CB8 and α1(II)CB11, are located well within the helical region and are adjacent to the region of the molecule containing the collagenase cleavage site (Figure 19), where the initial disruption of the collagen molecule is thought to occur. In the experimental bovine cartilage study a high molecular weight fragment of type II collagen α chain was identified in extracts of bovine cartilage undergoing collagen degradation induced by IL-1. This fragment is similar in size to the 3/4 length collagenase cleavage product (161) and implicates more directly mammalian collagenase in type II collagen degradation. The smaller collagenous fragments detected could derive from further degradation of the alpha chain or represent further degradation of the 3/4 length collagenase-derived fragment. It could be presumed that in situ a reaction with R181 indicates that an initial cleavage has occured by the most likely candidate, collagenase. Moreover, the intensity of the reaction could relate to the amount of unwinding and exposure of the epitopes which exist downstream to the collagenae cleavage site. When a polyclonal antiserum can be shown to be specific for the molecule of interest, the benefit of a polyclonal antiserum over a monoclonal antibody is that the the former contains a heterogenous pool of antibodies which react with a variety of epitopes in the same molecule. This has the potential of enhancing the reaction and increasing the sensitivity, although the reaction is critically dependent on specificity.

The experimental studies of cartilage degradation induced by IL-1 served to demonstrate for the first time the degradation of type II collagen in situ. In these experiments it was shown that treatment of chondrocytes with IL-1 (in explant culture) stimulates the release of collagenase and that this is

accompanied by collagen degradation, mainly around chondrocytes in the cartilage explants. Since it is reasonable to assign the role of normal matrix maintenance to the chondrocyte it is also reasonable to, blame the chondrocyte for the damage caused by enzymes and factors produced by these cells. Although discussed further in this section, it is important to add here that these studied directly implicate the chondrocyte and stimulation by cytokines such as IL-1 in the destruction of articular cartilage. These cells may not only affected by the factors released by macrophages/monocytes which are resident in the inflammed synovium and circulate in blood, (211,302) but by chondrocytes themselves. In the deep zone, in particular, chondrocytes must be in communication with or affected by mediators from the cells in the inflammed subchondral bone marrow (220,221). Since collagenase has been detected biochemically in elevated amounts in osteoarthritic and rheumatoid arthritic cartilage (150,303) and immunohistochemically, in the matrix in cartilage from the synovial pannus junction of rheumatoid arthritic cartilage (151), the high degree of collagen degradation around and near to chondrocytes in arthritic cartilage is not completely unexpected.

Experimentally, IL-1 was used to stimulate collagenase secretion in both human and bovine articular cartilage. Explants of bovine and human cartilage cultured with IL-1 showed early signs of collagen degradation when stained with the antiserum R181 as compared to controls. Marked staining, in particular pericellular, for type II collagen degradation was immunohistochemically detected in bovine cartilage after only 3 days in culture with IL-1, whereas in human cartilage collagen degradation was detected most strikingly on or after 9 days in culture with IL-1. These data,

from explant culture studies, suggest a direct effect of IL-1 on resident chondrocytes and provide strong evidence of the role of the chondrocytes and the mediators such as Il-1 in cartilage degradation.

In the studies with bovine cartilage, increased extractable degraded collagen was detected immunochemically after exposure to IL-1 at 9-10 days when immunohistochemical staining for collagen degradation in situ was less pronounced. The increased staining in cartilage cultured for only 3-5 days with IL-1 suggests that unwound type II collagen may react in situ with antibody but the cartilage at these early time intervals may not contain significant amounts of extractable α-chains or α-chain fragments.

New evidence of possible alternative mechanisms of collagen degradation as an early event in cartilage breakdown is presented in the studies of extractable collagen. Extractable α chains of type II collagen were detected in explants of bovine cartilage cultured with or without IL-1. Although these studies were not designed to be quantitative, one can make the general observation that more α chains were extractable from cartilage explants which were cultured with IL-1 (Figure 24). Moreover, immunochemical detection of α chains as seen in Figure 24 revealed that there was an increase in extractable α chains with increasing culture time. These findings suggest that there is in addition to collagenase, another proteinases present and stimulated by IL-1 which is capable of cleaving collagen molecules in non-helical regions. Obvious candidates in vivo are elastase and cathepsin G both which have been shown to be capable of degrading collagen in the non-helical regions where intermolecular cross links occur. In addition there may be new or yet to be described proteinases or "cross linkases" which are present and stimulated by IL-1 in cartilage, adding

to the molecules which mediate the breakdown and destablization of the collagen fibril. The action of specific enzymes for cleaving cross-links between α chains is one explanation of why intact α chains are present in guanidine extracts of explant cartilage. Another possibility is that the collagen which is readily guanidine extractable after explant culture represents newly synthesized collagen although this arguement would be inconsistent with the finding that IL-1 can inhibit new collagen synthesis (304). The presence of extractable collagen α chains in cartilage after explant culture requires further study.

Studies of collagen degradation in human cartilages revealed that arthritic cartilages exhibit much more evidence of collagen degradation than the corresponding normal adult cartilages. The staining of degraded collagen observed in arthritic cartilage did not correspond to any increased permeability due to loss of proteoglycan since there was no significant staining in the collagen staining sites. This staining with R181 for degraded collagen was independent of a loss of proteoglycans or glycosaminoglycans and there was no evidence for staining of intact helical collagen.

Both normal bovine and human adult articular cartilage exhibit low level staining for degraded collagen and which was present in certain pericellular sites and throughout the matrix. Since cartilage, like most connective tissues, is constantly undergoing turnover and remodelling for proteoglycans one would presume the pericellular staining may reflect basal collagen turnover. In all four normal human cartilages examined, the matrix in the superficial zone is also lightly stained. This suggests that there may be a more active process of collagen remodelling of the articular surface or that collagen degradation per se has taken place at or near the articular surface.

In the human cartilages examined, differences in staining of degraded collagen were not only in intensity, but also in distribution. In 3 out of the 4 osteoarthritic cartilages examined, staining was observed mainly in the superficial and mid zones, often where fibrillation was occurring, with intense staining in both pericellular sites and moderate staining in territorial sites which may vary in location depending upon the joint pathology of each patient. The pericellular and strong diffuse staining associated with fibrillated superficial cartilage clearly indicates collagen degradation in these sites and explains in part the appearance of fibrillated cartilage.

In contrast, in rheumatoid cartilage there was very intense staining of the articular surface. This collagen degradation may be caused by proteolytic enzymes originating from the synovial fluid which are capable of degrading collagen. Obvious candidates are the collagen-degrading proteases, elastase and cathepsin G (175,305) which can be released by polymorphonuclear leucocytes upon attachment to articular cartilage, and which result in the degradation of the more superficial articular cartilage (306,307). In one specimen (KE), degradation was detected throughout the matrix. In the others it was concentrated in the deep and mid zones. Degraded type II collagen was detected around chondrocytes in pericellular and territorial sites in the deep zone close to the calcified cartilage. Most rheumatoid cartilage was examined from sites at or close to the cartilage-synovial junction. The data indicate that not only may type II collagen degradation occur at the cartilage-synovial junction, associated with mononuclear cell infiltration and pannus formation (151,257,258,308) but that it also occurs around the chondrocytes more remote from the articular surface and remote from the synovium and synovial fluid. Earlier,

ultramorphological studies of rheumatoid articular cartilage, using cationic dyes, also showed a loss of staining for proteoglycans and evidence for damage to collagen fibrils in the deep zone cartilage (259). Since much of the degradation in human cartilage was centered around cells, the chondrocyte is clearly implicated as the primary source of enzymes degrading the type II collagen, as in bovine cartilage. Experimently, these studies have shown in bovine cartilage that molecules such as IL-1 can diffuse into bovine cartilage and activate chondrocytes to degrade the collagen matrix throughout the matrix. The degradation of collagen around and between cells appears to result from chondrocyte activation resulting from cytokines, such as IL-1.

In addition to such cytokines originating from synovium, pannus and synovial fluid (223) they could also arise from the inflammed subchondral bone marrow. Inflammatory changes are well recognized in subchondral sites in rheumatoid arthritis and are associated with bone resorption and loss which can result from the action of cytokines such as IL-1 and tumour necrosis factor (197,262,309-312). Cellular infiltration from subchondral bone into cartilage has also been observed in rheumatoid arthritis (263-265). Earlier studies by Fell and co-workers demonstrated that stimulation of subchondral bone marrow with complement sufficient antiserum can cause degradation of the adjacent articular cartilage (220,221). The present observations thus indicate that degradative changes occurring in the deep zone cartilage may be initiated by the release of cytokines released from inflammatory cells not only in the joint cavity but also from underlying inflammed subchondral marrow cavities.

There is a large body of evidence that would link both collagen and proteoglycan-degrading enzymes to the loss of integrity of articular cartilage in arthritic diseases (223). The study described here has permitted the identification of cartilage collagen degradation in experimental and human pathology and has produced important observations and raised questions concerning the pathology of these diseases. Further studies utilizing these techniques should help to elucidate the destructive process involved and the mediators that regulate the control of cartilage destruction.

In addition to the investigations into collagen degradation and its assessment in tissue utilizing the immunohistochemical protocol with R181, the in vitro studies with human articular cartilage explants and IL-1, produced important findings. Although the data are circumstantial, the fact that explant cultures of human articular cartilage were stimulated with IL-1 to result in increased type II collagen degradation, implicates IL-1 as a mediator of cartilage type II collagen destruction in vivo. This provides new evidence that IL-1 is, in part, responsible for the collagen damage observed in diseased cartilage. Previously, the large body of evidence linking IL-1 to the stimulation of collagenase or activators of collagenase, was provided by the measurement of enzyme activity in vitro. The detection of low intensity staining for collagen degradation in normal, aging cartilages suggests that these processes are a natural part of maintaining cartilage integrity and are under strict control. The balance between inhibitor of enzyme activity or activator of latent enzyme is critical to the avoidance of excess degradation (or synthesis).

An important feature of these studies is that although it is well accepted that collagen fibrils are the supporting network or scaffold for

entrapping the highly charged, highly hydrophilic proteoglycans, it is not as clear as to which is degraded (or lost first) in cartilages incapable of fulfilling there intended function. In these experiments, collagen degradation could be detected with marked intensity without a significant net loss of staining for proteoglycan (Figure 27) and most assuredly not with a total loss of proteoglycan.

The pharmacological effect of the metalloproteinase inhibitor, U24522, on arresting type II collagen degradation in explants of bovine articular cartilage explants stimulated with IL-1, was striking. These data demonstrates the utility of the immunohistochemical technique described within this thesis in studies where the effects of pharmocologic compounds and their mechanism of action are investigated. These studies represent the first demonstration that type II collagen degradation can be arrested in vitro by a non-toxic inhibition of metalloproteinase.

The experiments described in this thesis have permitted the identification of cartilage collagen degradation in experimental and human pathology and have produced several new observations and raised questions concerning the pathology of these arthritic diseases. Further studies utilizing these techniques should help elucidate the destructive processes and the mediators involved, that regulate and control cartilage destruction.

II. EPILOGUE

The development of the antiserum specific for fragmented and denatured type II collagen and the immunochemical applications described here in this thesis, make possible future studies which examine collagen degradation and synthesis on a new basis. The uniqueness of the antibodies allowed, in these studies with cartilage and type II collagen, detection of fragmented (or unwound) type II collagen in situ in human and bovine cartilages for the first time. Collectively, these experiments suggest that collagen degradation in cartilage is an early event when chondrocytes are stimulated by mediators such as IL-1. When cartilage was in explant culture, IL-1 stimulated cartilage showed distinct staining for type II collagen degradation which if compared with the degradation seen in diseased cartilage, could suggest that this is an operative mechanism in vivo. Further studies utilizing this immunochemical approach to collagen degradation and well defined model systems where other factors (i.e. monokines, inhibitors of proteinases, inhibitors of mediators such as IL-1), could elucidate the precise control mechanism(s) which regulate the destruction and or synthesis of collagen in normal and abnormal cartilage.

The immunohistochemical study which identified extractable alpha chains and fragments of type II was the first observation of its kind and draws attention to the major role that collagen plays in cartilage integrity. It requires further study. This initial observation does not clarify the mechanism(s) involved which releases intact alpha chains from cartilage in explant culture. The existence of a novel enzyme which has its action on the collagen cross-links which stabilize the collagen molecules is a distinct possibility and would be the basis for a very interesting and important new

set of experiments. These types of experiments would be quite difficult, although one approach could be to start by examining and characterizing molecules which might inhibit this phenomenon and approach the identification of the enzyme(s) involved by understanding the regulation of the enzyme(s) activity or production by using well defined proteinase inhibitors. Utilization of a bovine or human explant culture sysem (with IL-1) as described here could be used and provides a valid basis from which to interpret the results.

The studies have revealed collagen type II degradation to be an early event in contrast to the previously held notion that proteoglycan is initially degraded and a significant part of early cartilage destruction. These observations could extend a more detailed comparison of proteoglycan (and specific proteoglycan components) and collagen changes under various experimental conditions and pathological states. Similar antiserum could be generated to other collagen components of cartilage and their degradation in cartilage evaluated to understand the involvement of and the condition of these "minor collagens" in articular cartilage pathology.

The technology and application described within this thesis provides new and unique information regarding the ongoing events in articular cartilage and have opened new possibilities to analytically study collagen changes in cartilage homeostasis and pathology. Distinct patterns of degraded type II collagen were identified in arthritic cartilages which suggests that although some of the same mechanism(s) may be involved in cartilage destruction in osteoarthritis and rheumatoid arthritis, the results described here suggest that different mechanisms, mediators or regulation is involved in cartilage damage and loss of function. Other studies which would

be a natural extension of this work would be in situ hybridization with cDNA probes for certain well described major components of cartilage destruction such as collagenase or an activator of collagenase, stromelysin. Correlation of these types of molecular studies with the immunohistochemical and immunochemical data provided in these studies described here would bring us closer to understanding the pathobiology and clinical condition and make possible the design of novel and effective therapies.

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