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THE IDENTIFICATION OF COLLAGENASE-GENERATED CLEAVAGE PRODUCTS OF TYPE II COLLAGEN USING ANTI-NEOEPITOPE ANTIBODIES

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June 1997

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Doctor of Philosophy

PREFACE

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

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In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

A portion of this thesis includes text and figures from the following published manuscript and has been reproduced by copyright permission of the American Society for Clinical Investigation (Appendix A):

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

The contributions of each of the co-authors are as described below.

- Leif Dahlberg performed the studies to assess the effects of a synthetic inhibitor on collagen degradation in the human articular cartilage culture explants.
- Mirela Ionescu assisted in the characterization of the anti-neoepitope antibodies and in the development of the immunoassays using these antibodies, by incorporating an amplification system into the ELISAs to improve sensitivity and reproducibility.
- Agnes Reiner assisted in the immunochemical analyses of samples by improving the ELISA, used to measure type II collagen denaturation, and that was developed previously in this laboratory (Hollander *et al.*, 1994).
- Robert Bourne and Cecil Rorabeck supplied some of the human articular cartilage specimens used for immunochemical and immunohistochemical analyses.
- Peter Mitchell and John Hambor supplied the recombinant human enzymes, MMP-1 and MMP-13 and offered technical advise on the *in vitro* type II collagen cleavage studies.
- Oliver Diekmann and Harald Tschesche supplied the recombinant human MMP-8.
- Jeffrey Chen and Harold Van Wart supplied the synthetic inhibitors, RS 47,112 and RS 102,481, and determined their inhibition constants for use in the human and bovine cartilage explant culture studies.
- Robin Poole assisted in the design and development of all phases of the studies.

ABSTRACT

The degradation of type II collagen, the major collagen of articular cartilage, is thought to be initiated by a specific cleavage within its triple helical domain by one or more of MMP-1 (collagenase-1), MMP-8 (collagenase-2), and MMP-13 (collagenase-3). We demonstrate the direct involvement of increased collagenase activity in type II collagen cleavage, in osteoarthritic (OA) human femoral condylar cartilage, and in IL-1 stimulated mature bovine nasal and articular cartilages, by developing and using antibodies reactive to carboxy-terminal (COL2-3/4C_{short}) and amino-terminal (COL2-1/4N1) neoepitopes generated by cleavage of native type II collagen by these collagenases. A secondary cleavage followed the initial cleavage produced by recombinant human MMP-1, MMP-8 and MMP-13. This generated neoepitope COL2-1/4N2. These cleavage sites were first seen mainly around superficial chondrocytes in young cartilages, and then with increasing age (non-arthritic) and severity of OA, in deeper regions of human femoral articular cartilage. This pattern of cleavage was similar and correlated with that described for type II collagen denaturation. There were significantly higher neoepitope levels in OA compared to adult non-arthritic cartilages, determined by immunostain grading. Moreover, there was significantly more COL2-3/4C_{short} epitope in extracts of OA articular and IL-stimulated bovine nasal cartilages, compared to normal nonstimulated cartilages, determined by immunoassay. A synthetic preferential inhibitor of MMP-13 significantly reduced the spontaneous release of COL2-3/4C_{short} from human OA cartilage explant cultures. Moreover, this inhibitor and a broad spectrum MMP inhibitor significantly reduced the IL-1 stimulated generation of this neoepitope and the COL2-3/4m intrachain epitope in bovine nasal and articular cartilages. In contrast, the loss of proteoglycans from explants, which always preceded COL2-3/4C_{short} detection in conditioned media of stimulated cartilages, was not significantly altered by either inhibitor. These data suggest that collagenase(s) produced by chondrocytes is (are) involved in the cleavage and denaturation of type II collagen in cartilage, that these activities are increased in osteoarthritic and cytokine-stimulated articular cartilages, and that MMP-13 may play a significant role in these processes. The

COL2-3/4C$_{short}$ neoepitope has the potential to be a useful marker of type II collagen cleavage by mammalian collagenases *in vitro* and *in vivo*.

RÉSUMÉ

On pense que la dégradation du collagène de type II, collagène prédominant du cartilage articulaire, est initiée par un clivage spécifique à l'intérieur de sa triple hélice, dû soit à MMP-1 (collagènase-1), à MMP-8 (collagènase-2), à MMP-13 (collagènase-3) ou à plusieurs de ces métalloprotéinases. Nous démontrons le rôle direct d'une augmentation de l'activité de la collagènase dans le clivage du collagène de type II, dans le cartilage du condyle fémoral humain arthritique et dans le cartilage bovin nasal et articulaire, stimulé par IL-1. Cette démonstration est possible grâce au développement et à l'utilisation d'anticorps réagissants avec des néoépitopes du C-terminal (COL2-3/4C_{court}) et du N-terminal (COL2-1/4N1) générés par le clivage du collagène de type II par ces collagènases. Le clivage initial produit par MMP-1, MMP-8 et MMP-13 humaines recombinantes est suivi d'un clivage secondaire; ceci produit un néoépitope, COL2-1/4N2. Dans le cartilage de jeunes adultes on observe premièrement ces sites de clivage surtout autour des chondrocytes superficiels, puis chez les adultes plus âgés (non arthritiques) et chez les patients arthritiques, dans les régions plus profondes du cartilage du condyle fémoral. Ce type de clivage est semblable et correspond à ce qui a été décrit pour la dénaturation du collagène de type II. A l'aide d'une classification de l'immunocoloration, nous avons remarqué des niveaux de néoépitopes nettement plus élevés dans le cartilage arthritique comparé au cartilage adulte non arthritique. Qui plus est, l'épitope COL2-3/4C_{court} est plus abondant dans les extraits de cartilage ostéoathritique, de même que dans le cartilage bovin nasal stimulé par IL-1, comparé au cartilage normal non stimulé, ceci déterminé par immuno-essai. Dans des cultures d'explants de cartilage ostéoarthritique humain, la libération spontanée de COL2-3/4C_{court} est diminuée de façon significative par un inhibiteur synthétique préférentiel pour MMP-13. De plus, cet inhibiteur, de même qu'un inhibiteur de MMP à large spectre diminuent considérablement la production de ce néoépitope stimulée par IL-1 ainsi que l'épitope intrachaîne COL2-3/4m dans le cartilage bovin nasal et articulaire. En contraste, les deux inhibiteurs susmentionnés n'altèrent pas de façon significative la perte de protéoglycans des explants, qui précède toujours la détection de COL2-3/4C_{court} dans les milieux

conditionnés de cartilages stimulés. Ces données suggèrent que la (les) collagènase(s) produite par les chondrocytes est (sont) impliquée dans le clivage et la dénaturation du collagène de type II. Elles suggèrent aussi que ces activités sont augmentées dans les cartilages ostéoarthritiques et les cartilages stimulés par des cytokines et que MMP-13 pourrait jouer un rôle essentiel dans ces processus. Le néoépitope COL2-3/4C_{court} pourrait être un marqueur très utile du clivage du collagène de type II par des collagènases mammifères, in vitro et in vivo.

ACKNOWLEDGMENTS

The author would like to thank Dr. A. Robin Poole of the Department of Surgery, McGill University and the director of the Joint Diseases Laboratory of the Shriners Hospital (Montreal Unit) for his guidance and support in the development and execution of this research project, and for his advice in the preparation of the thesis. I would also like to express my appreciation to the other members of my Advisory Committee for their contributions, namely Dr. Anneliese Recklies, Dr. Peter Roughley, and Dr. Julius Gordon (Department of Surgery, McGill University).

I am most grateful to Mirela Ionescu and Agnes Reiner (Joint Diseases Laboratory) for their assistance in developing the immunoassays and adapting them for use with the various samples analyzed. I would also like to thank Isabelle Pidoux (Joint Diseases Laboratory) for her work in the immunohistochemical studies and Elisa de Miguel for the preparation of the synthetic peptides and for performing N-terminal sequencing analyses. Drs. Peter Mitchell, John Hambor and Ivan Otterness (Pfizer Central Research, Connecticut), Harald Tschesche and Oliver Diekmann (University of Bielefeld, Germany), and Harold Van Wart and Jeffrey Chen (Roche Bioscience, California) are thanked for their kind gifts of recombinant enzymes and proteinase inhibitors. The clinical collaboration of Drs. Cecil Rorabeck and Robert Bourne (Division of Orthopedic Surgery, University Hospital, University of Western Ontario) and Leif Dahlberg (Department of Orthopaedic, Malmo General Hospital, Sweden) are appreciated. Jane Wishart and Mark Lepik are thanked for their assistance with the figures included in the thesis.

Funding was provided by the Fonds de la Recherche en Sante du Quebec (to R.C. Billinghurst); the Medical Research Council of Canada, Searle Monsanto, St. Louis, MO, Pharmacia Diagnostics AB, Uppsala, Sweden, and the National Institutes of Health (to A.R. Poole); and the Shriners of North America (to R.C. Billinghurst and A.R. Poole).

Finally, I would like to express my sincere gratitude and love for my family. Their support and compassion in seeing this project to fruition are very much valued.

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LIST OF ABBREVIATIONS

α1(II)	alpha chain of type II collagen
ANOVA	analysis of variance
APMA	aminophenylmercuric acetate
BSA	bovine serum albumin
CB11B	intrachain hidden epitope of α1(II) chain (COL2-3/4m)
COL2-3/4C	neoepitope which is the C-terminus of the α1(II) chain three quarter TC^A fragment created by primary collagenase cleavage
$COL2/4C_{short}$	same as COL2-3/4C neoepitope
COL2-3/4m	intrachain hidden epitope of α1(II) chain (CB11B)
COL2-1/4N1	neoepitope which is the N-terminus of the α1(II) chain one quarter TC^B fragment created by primary collagenase cleavage
COL2-1/4N2	neoepitope which is the N-terminus of the α1(II) chain one quarter TC^B fragment due to secondary collagenase cleavage
C-terminus	carboxy-terminus
CNBr	cyanogen bromide
D	daltons
dH_20	distilled water
DMEM	Dulbecco's minimal essential medium
DMMB	dimethylmethylene blue
DMSO	dimethylsulfoxide
EDTA	ethylene diamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
$F(ab')_2$	bivalent antibody fragment made from pepsin digestion of IgG
HDC	heat-denatured collagen
Hyp	hydroxyproline
IgG	immunoglobulin G
IL-1	interleukin-1
IL-1ra	interleukin-1 receptor antagonist (IRAP)
IRAP	interleukin-1 receptor antagonist protein (IL-1ra)

I.T.S.	insulin-transferrin-sodium selenite culture medium supplement
kD	kilodaltons
K_i	dissociation constant of an enzyme-inhibitor complex
LIF	leukemia inhibition factor
MCP-1	monocyte chemotactic protein type 1
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1 matrix metalloproteinase
NO	nitric oxide
N-terminus	amino-terminus
OA	osteoarthritis
OVA	ovalbumin
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PGE_2	prostaglandin E_2
PVDF	polyvinylidene difluoride
RA	rheumatoid arthritis
rHu	recombinant human
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sGAG	sulphated glycosaminoglycan
sIL-1R	soluble interleukin-1 receptor
TC^A	collagenase-generated three quarter collagen α chain fragment
TC^B	collagenase-generated one quarter collagen α chain fragment
TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinases
tPA	tissue-type plasminogen activator
TPCK	N-tosyl-L-phenylalanine-chloromethyl ketone
TNF	tumour necrosis factor
uPA	urokinase-type plasminogen activator

1. INTRODUCTION

Osteoarthritis (OA) is a degenerative disorder of synovial joints that affects a large proportion of the human population and is the most common form of joint disease known. Synovial joints are complex structures consisting of distinct tissues including joint capsule, ligament, meniscus, subchondral bone, synovial tissue and hyaline cartilage. It is the articular cartilage that, despite a thickness of only a few millimeters, is able to resist compressive and tensile forces while distributing loads to provide normal joint function for many years. The degeneration of the articular cartilage, along with changes in other joint tissues, contributes to clinical OA. Unfortunately, cartilage degeneration is poorly correlated with clinical features of OA and with radiological imaging. The lack of objective measurements or markers of the osteoarthritic process has hindered clinical and therapeutic research and has been the impetus behind the work reported in this thesis.

Articular cartilage consists of cells, tissue fluid and an extracellular matrix of structural macromolecules. The only cells in normal articular cartilage are the chondrocytes, which contribute only 1% of the volume of adult human articular cartilage, yet are responsible for the maintenance of the cartilaginous matrix, synthesizing new matrix molecules and regulating degradation. The mechanisms controlling the balance between the catabolic and anabolic activities of chondrocytes are poorly understood. What is known is that disruption of this balance in favour of matrix degradation involves a complex cascade of catabolic factors that eventually results in articular cartilage degradation.

Within the matrix are two components: the tissue fluid and the macromolecular structural framework. Water makes up from 65 to 75% of the wet weight of cartilage

and, along with the structural macromolecules, determines the mechanical properties of the tissue. The matrix protects the chondrocytes from mechanical damage during normal joint use, and acts as a signal transducer for the chondrocytes. Moreover, the chondrocytes receive their nutrients and eliminate their waste products by diffusion through the matrix.

The two main structural macromolecular components of articular cartilage are the large aggregating proteoglycan monomer, called aggrecan, and the fibrils consisting mainly of type II collagen, that together contribute from 20 to 30% of the wet weight of this tissue. They provide the compressive stiffness and tensile strength, respectively, of articular cartilage. It has been well established that in arthritis, there is degradation of these supporting molecules leading eventually to cartilage destruction. It has been shown experimentally, that although the initial damage involves a fragmentation of aggrecan, the cartilage is able to rapidly resynthesize these molecules and restore near normal tissue proteoglycan levels (Tyler, 1985a; Page-Thomas *et al.*, 1991). Conversely, the degradation of the collagen network seems to signal the irreversibility of articular cartilage breakdown with a permanent loss of function that is a key feature of many human arthritides (Jubb and Fell, 1980). It is for these reasons that we have concentrated our efforts, in this project, on the proteolytic degradation of type II collagen.

Using polyclonal and monoclonal antibodies that react specifically to denatured type II collagen α chains, such as can result from intrahelical cleavage by collagenases, it has been shown that increased denaturation of this molecule can be detected in OA articular cartilages (Dodge and Poole, 1989; Hollander *et al.*, 1994, 1995). This implicates collagenases in the denaturation of type II collagen. However, to definitively

demonstrate a role for these proteinases in the cleavage of this molecule, a means of specifically identifying the collagenase cleavage site(s) *in situ* is required. This would be possible if we could prepare and use antibodies that react with the carboxy (C)- and amino (N)-terminal neoepitopes created by cleavage of collagen by collagenases and which do not react with the intact molecule or α chains thereof. Antibodies of this kind, prepared to cleavage site neoepitopes, have already proven valuable in defining MMP activity in aggrecan degradation (Hughes *et al.*, 1992; Mort *et al.*, 1993; Bayne *et al.*, 1995; Fosang *et al.*, 1995; Hughes *et al.*, 1995; Lark *et al.*, 1995; Singer *et al.*, 1995).

The primary focus of this research project was to develop and characterize antibodies to the neoepitopes that represent the C- and N-termini of the three quarter (TC^A) and one quarter (TC^B) fragments, respectively, of human type II collagen α1 chains. These fragments are produced by cleavage of the triple helical collagen molecule by any one of the three collagenases MMP-1, MMP-8 and MMP-13, and perhaps also MMP-14 (MT1-MMP; Ohuchi *et al.*, 1997). These anti-neoepitope antisera were used, in immunochemical and immunohistochemical analyses, to determine the involvement of collagenases and chondrocytes in the cleavage of type II collagen in healthy, aging and osteoarthritic human articular cartilages, and in IL-1 stimulated bovine cartilage explant cultures.

Aside from demonstrating MMP-specific cartilage degradation products, a second approach to demonstrate the role of chondrocyte-derived MMPs in cartilage degradation, and in particular type II collagen breakdown, is by investigating the effect of MMP-specific inhibitors on cartilage breakdown. We examine the efficacy of two synthetic MMP inhibitors, with differing inhibitory profiles, in preventing the IL-1 induced

breakdown of bovine nasal and articular cartilage explants, and in abrogating the *in vitro* spontaneous release of collagenase-specific type II collagen cleavage site neoepitopes from human OA articular cartilage explant cultures. Unlike inhibitors that have been used in the past, the two inhibitors chosen for this study allowed for the selective inhibition of one or more of the collagenases MMP-1, MMP-8 and MMP-13. As collagen release is believed to be the key factor in establishing irreversibility in cartilage degradation (Jubb and Fell, 1980), we focused our attention on the proteolytic cleavage and denaturation of type II collagen. The effectiveness of these inhibitors in preventing type II collagen degradation was assessed for collagenase cleavage, using an immunoassay that utilizes one of the anti-neoepitope antibodies developed in this study, COL2-3/4C_{short}, and for collagen denaturation using the COL2-3/4m inhibition ELISA also developed in this laboratory (Hollander *et al.*, 1994). The use of the COL2-3/4m immunoassay has been well described in the literature (Hollander *et al.*, 1994; Antoniou *et al.*, 1996; Hollander *et al.*, 1996; Xu *et al.*, 1996; Kozaci *et al.*, 1997). Both these immunoassays for collagen degradation products, as well as the determination of proteoglycan catabolism through the monitoring of sGAG levels, were used to quantitate the tissue and media content of these cartilage matrix breakdown products.

The significance of this work is that, in identifying the specific roles of the various MMPs in the pathophysiology of joint disease and, in particular, the collagenase(s) responsible for type II collagen degradation, we can potentially define effective therapeutic approaches in human arthritis. Ideally, it may be possible to block one specific enzyme and stop the destructive processes, while at the same time allowing normal turnover to proceed. It is a concept that has been well debated in the literature

(Cawston, 1991, 1993; Gordon *et al.*, 1993; Vincenti *et al.*, 1994, Buttle *et al.*, 1995). Moreover, by developing and using antibodies in immunoassays that allow for the detection of degradation products specific for articular cartilage matrix components, such as type II collagen, we will be better equipped to establish diagnostic and prognostic indices of joint disease.

The main hypothesis of this research project is that the degradation of type II collagen in cartilage is initiated by a specific intrahelical cleavage of the collagen alpha chains by mammalian collagenases. Moreover, this cleavage will be detectable in cartilage and in the conditioned media of cartilage explant cultures through the development and use of antibodies that recognize the individual collagen alpha chain termini (neoepitopes) created by the action of these endoproteinases. The intrahelical cleavage of type II collagen molecules will then lead to their unwinding or denaturation. The detection of cleaved and denatured collagen products will therefore be correlated and will be significantly decreased by collagenase-specific inhibition.

2. LITERATURE REVIEW

2.1 Arthritis and type II collagen degradation

2.1.1 Articular cartilage and type II collagen

The hyaline cartilage that lines the articulating surfaces of the bones of diarthrodial joints, despite its lack of vasculature and innervation, is a metabolically active tissue in both health and disease. The cellular components of cartilage, the chondrocytes, are embedded in a matrix that they must produce and maintain. The two major proteins and structural components of the extracellular matrix of cartilage are collagen and the large aggregating proteoglycan, aggrecan. Collagen fibrils provide the tensile strength and maintain the integrity of mammalian articular cartilage by forming a network that resists the swelling pressure resulting from the hydration of the polyanionic proteoglycan aggregates in the extracellular matrix (Kempson *et al.*, 1973; Poole, 1997). Damage to this fibrillar meshwork, made up of primarily type II collagen (~90 to 95%), may be a critical event in the pathology of many arthritides, due in part to the very slow rate of collagen turnover within the cartilage (McAnulty and Laurent, 1990).

The type II collagen molecule is a homotrimer, as it is composed of a triple helix of three identical α1(II) chains, each consisting of repeating $[Gly\text{-}X\text{-}Y]_n$ amino acid sequences (n = 338), where 10-12% of the X and Y residues are proline and hydroxyproline, respectively (Thomas *et al.*, 1994). There are short non-helical telopeptide regions, 18 and 27 amino acids in length, at both the carboxy- and amino-terminal ends, respectively, of the α1(II) chains. The collagen molecules form fibrils

which are stabilized mainly through intermolecular cross-links, one potential site in each telopeptide and two intrahelical sites, with other type II collagen molecules and with two minor collagens of articular cartilage, types IX and XI (Eyre, 1987). In early degeneration in articular cartilage, which may lead to arthritis, there is a loss of the tensile properties, indicative of damage to the fibrillar network (Kempson *et al.*, 1973).

2.1.2 Type II collagen degradation

There are three distinct ways in which tissue collagen is degraded in situ. One method involves the incorporation of extracellular collagen (type I) into phagolysosomes for destruction by acid proteases, such as cathepsin B (Burleigh *et al.* 1974). Such a system for type II collagen breakdown has not yet been identified. A less clearly defined pathway of collagen breakdown is that involving the rapid degradation of newly synthesized collagen that occurs either intracellularly or shortly after secretion. It is believed that this regulates the quality of the collagen synthesized, destroying abnormal molecules shortly after their production (Bienkowski *et al.*, 1978). The third, and perhaps most important system in connective tissue remodeling, is the extracellular degradation of the fibrillar collagens, types I, II, and III, that can occur both in non-helical sites (Liu *et al.*, 1995) and through a specific triple helical cleavage. Only the latter results in denaturation of the triple helix at physiological temperatures. This intrahelical cleavage is achieved by collagenases that belong to the group of neutral zinc-dependent endopeptidases called matrix metalloproteinases (MMPs), described in more detail below. These collagenases, with their unique role of initiating the degradation of type II collagen at neutral pH, are thought to define the rate-limiting step in normal tissue

remodeling events, such as bone resorption and growth, embryonic development, uterine involution, wound healing, and in degradative processes such as arthritis, tumour invasion and metastasis, and periodontitis.

2.1.3 Measuring type II collagen degradation in arthritis

There has been tremendous interest in the identification of markers of collagen metabolism that could be used as measures of disease activity. Such markers would be useful in understanding the pathophysiology of diseases in which collagen breakdown is a key component, as well as providing a method for monitoring disease progression, designing therapeutic strategies, and supplying prognostic indices. There are recent reviews of markers of cartilage degradation in the literature (Poole, 1994; Greenwald, 1996; Richardson and Emery, 1996) and this document will only discuss those markers of potential importance in identifying type II collagen breakdown.

The classical method for assessing generalized collagen breakdown is the determination of the hydroxyproline content of the sample being assayed (Dingle *et al.*, 1975). Despite the high hydroxyproline content of collagenous proteins, the measurement of this amino acid is non-specific, especially as a marker in body fluids of collagen breakdown, as levels may result from sources other than endogenous collagen breakdown, such as complement factor C1q, elastin, acetylcholine esterase or dietary collagens. Moreover, as hydroxyproline is a component of all collagens that require it for stabilization of the triple helix, its measurement does not allow for the identification of collagen type-specific degradation nor tissue-specific breakdown.

The tensile strength of collagen fibrils is due in large part to the covalent cross-links between collagen molecules. The levels of the pyridinium cross-links, which are present in mature collagen fibrils only, and are excreted in the urine as peptides after collagen degradation, have been shown to correlate with the severity of OA (Robins *et al.*, 1990). However, like hydroxyproline, these cross-links are not specific to type II collagen nor cartilage, as they are also found in the type I collagen of bone.

Antibodies have been developed which recognize epitopes only found in denatured type II collagen α1 chains (Dodge and Poole, 1989; Hollander *et al.*, 1994). As such, these immunoreagents are potentially powerful tools in the identification of type II collagen degradation. Immunolocalization studies in this laboratory, using these antibodies, have provided evidence for increased denaturation of the triple helix of type II collagen in human articular cartilage in OA and aging. This denaturation originates around chondrocytes, where the collagen fibrils are thinner (Poole, 1984), and progresses from the articular surface to the deeper layers of the matrix with advancing age and severity of disease (Dodge and Poole, 1989; Hollander *et al.*, 1994; 1995). Unfortunately, these antibodies have not been able to reproducibly detect denatured products of type II collagen degradation in body fluids.

2.2 Arthritis and collagenases

2.2.1 The collagenase family of matrix metalloproteinases

The family of enzymes known as the matrix metalloproteinases or MMPs has its origin with the discovery by Gross and Lapiere, in 1962, of a zinc-containing enzyme, that they called collagenase, that was responsible for the digestion of collagen-rich

tadpole tails. Thirty-five years and some 20 MMPs later, the importance of these proteinases in connective tissue turnover in health and disease has been firmly established. The MMPs have been simplistically categorized into three major groups, according to substrate specificity (Table 2.1). They are the collagenases, the gelatinases, and the stromelysins (reviewed in: Matrisian, 1992; Birkedal-Hansen *et al.*, 1993; Werb and Alexander, 1993; Rawlings and Barrett, 1995).

The collagenase subfamily of MMPs includes collagenase-1 or MMP-1 (interstitial collagenase), collagenase-2 or MMP-8 (neutrophil collagenase), and the recently cloned and characterized collagenase-3 or MMP-13 (Freije *et al.*, 1994; Knauper *et al.*, 1996; Mitchell *et al.*, 1996). These collagenases share greater than 50% amino acid sequence identity and, along with the other members of the MMP family of enzymes, all contain the functionally important propeptide, catalytic and zinc-binding domains. The collagenases are not constitutively synthesized in resident chondrocytes, but are produced upon demand. All are secreted as inactive zymogens that require activation through the disruption of the propeptide-active site Cys-Zn^{2+} bond, which is called "the cysteine switch" mechanism (Van Wart and Birkedal-Hansen, 1990). MMP-1, MMP-8, and MMP-13, along with MMP-14, which is also known as membrane type 1 matrix metalloproteinase (MT1-MMP; Ohuchi *et al.*, 1997), are the only mammalian enzymes known to be able to initiate the intrahelical cleavage of triple helical type II collagen at neutral pH. MMP-3 (stromelysin-1) can cleave type II fibrillar collagen, but only in the nonhelical amino-telopeptide domain (Wu *et al.*, 1991). Until now, there has been no direct evidence for the involvement of the mammalian collagenases in intrahelical cleavage, except for the inability of murine collagenase to cleave type I collagen *in vivo*

Table 2.1 The three major groups of matrix metalloproteinases based on substrate specificity

The chart depicts the three major groupings of the matrix metalloproteinases (MMPs), according to the substrates for each. They are the collagenases, the gelatinases, and the stromelysins. Shown in addition to their MMP numerical designation, are the number of the amino acids comprising each proenzyme (A.A.), the molecular weights (M.W.) of both the proenzyme (latent) and enzymatically active (active) enzyme forms, the human chromosomal location of the gene coding for each of the enzymes, and the respective substrates of each MMP.

Enzymes	MMP No.	A.A.	Latent M.W.	Active M.W.	Chromosome	Substrates
Interstitial collagenase	1	469	55 000	45 000	11q22.3-23	Collagens I, II, III, VII, X Gelatin, Aggrecan
Neutrophil collagenase	8	467	75 000 53 000	58 000 43 000	11q21-q22	Collagens I, II, III Gelatin, Aggrecan
Collagenase 3	13	471	65 000	55 000	11q22.3	Collagens I, II, III N-telopeptide of Collagen I
Gelatinase A	2	662	72 000	66 000	16q21	Gelatin, Aggrecan, Elastin Collagens IV, V, VII, X
Gelatinase B	9	707	92 000	86 000	16q20	Same as Gelatinase A
Stromelysin-1	3	477	55 000	45 000	11q22.3-23	Aggrecan core protein Fibronectin, Laminin, Elastin N-telopeptide of Col I, II Collagen IV, V, IX, X, XI Procollagenase
Stromelysin-2	10	476	60 000	55 000	-	Aggrecan, Collagen IV Fibronectin, Elastin
Stromelysin-3	11	488	65 000	45 000	-	Aggrecan Collagen IV Fibronectin, Laminin α1 Proteinase inhibitor α2 Macroglobulin

when the intrahelical site is mutated (Liu *et al.*, 1995). These collagenases cleave the fibrillar collagens type I, II and III at a single specific site, Gly_{775}-Leu/Ile_{776}, within each α chain of the triple helical collagen molecule (numbering from the beginning of the triple helical domain). This cleavage is approximately three quarters of the distance from the amino-terminal end of each chain, resulting in the generation of three quarter (TC^A) and one quarter (TC^B) length collagen fragments (Miller *et al.*, 1976; Hasty *et al.*, 1990; Mitchell *et al.*, 1996). It is believed that this site is uniquely sensitive to collagenase cleavage because here the triple helix is relaxed, and because it is preceded by a tight imino acid-rich (proline and hydroxyproline) helical region (Fields and Van Wart, 1992). Each of the collagenases cleaves one type of triple helical collagen more rapidly than the others. MMP-13 has been shown to be 5- to 10-fold more effective at cleaving native type II collagen than either MMP-1 or MMP-8 (Knauper *et al.*, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996). The cleaved collagen fragments spontaneously denature into nonhelical gelatin derivatives at physiological temperatures, thereby becoming susceptible to further degradation by these collagenases (Welgus *et al.*, 1982) and/or by other proteinases, such as stromelysin (MMP-3) and gelatinases (MMP-2 and MMP-9).

2.2.2 Collagenases in arthritic joint fluids and tissues

Collagenases belonging to the MMP group of endoproteinases have been detected in the synovial fluid of patients with traumatic arthritis, rheumatoid arthritis (RA) and OA (Walkovits *et al.*, 1992; Clark *et al.*, 1993; Lohmander *et al.*, 1993; Maeda *et al.*, 1995). In the synovial joint, there are three distinct tissue sources of the collagenases that may be involved in the degradation of articular cartilage. Cells in OA and RA synovium express

collagenase (Firestein *et al.*, 1991; Gravallesse *et al.*, 1991; Wolfe *et al.*, 1993; Keyser *et al.*, 1995). The expression of MMP-13 in the synovial membrane was identified in RA and OA tissues in one study (Wernicke *et al.*, 1996), but in other reports MMP-13 was not found in isolated synoviocytes from OA and RA patients (Borden *et al.*, 1996; Reboul *et al.*, 1996). Fibroblasts at the cartilage-pannus interface also secrete a collagenase that can degrade cartilage (Tetlow and Woolley, 1995). Chondrocytes express and secrete MMP-1 (Wolfe *et al.*, 1993; Borden *et al.*, 1996; Reboul *et al.*, 1996) and MMP-13 (Borden *et al.*, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996) in both normal and OA articular cartilages. Moreover, two recent studies describe the expression of MMP-8 (neutrophil collagenase) by human articular chondrocytes from normal donors (Chubinskaya *et al.*, 1996; Cole *et al.*, 1996). Finally, the expression of MT1-MMP (MMP-14) mRNA has been identified in human normal and OA cartilages (Buttner *et al.*, 1997).

2.2.3 Collagenase assays

Collagenases have been routinely assayed *in vitro* (Cawston and Barrett, 1979; Dean and Woessner, Jr., 1985) and *in vivo* (Nagai *et al.*, 1966; Karran *et al.*, 1995) using radiolabelled collagen fibrils or fluorogenic peptides (Knight *et al.*, 1992). These methods do not allow for the differentiation of the collagenases involved in the cleavage of these substrates. ELISAs have been developed using specific anti-collagenase antibodies allowing the detection of collagenase and tissue inhibitors of metalloproteinases (TIMP)-collagenase complexes (Clark *et al.*, 1992a, 1992b; Plumpton *et al.*, 1995). However, the presence of enzyme in body fluids and tissues does not

necessarily imply enzyme activity. The collagenases are secreted as zymogens, they require activation, and their catalytic potential is determined by the presence of natural specific MMP inhibitors such as TIMPs, which form irreversible 1:1 molar complexes with active collagenases, and the general proteinase inhibitor alpha$_2$ macroglobulin (reviewed in Twining, 1994).

2.3 Arthritis and proinflammatory cytokines

2.3.1 Proinflammatory cytokines in natural and experimental arthritis

Cytokines are soluble protein mediators that are involved in most biological processes and play an important role in the physiology of articular cartilage. It is the unregulated effects of cytokines that seem to play a pivotal part in the pathophysiology of many diseases, including human rheumatoid arthritis and osteoarthritis. In fact, the role for cytokines in RA and OA has been well researched and recently reviewed (Badolato and Oppenheim, 1996; Deleuran, 1996; Feldmann *et al.*, 1996; Westacott and Sharif, 1996). In particular, the proinflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor alpha (TNFα), which share many biological properties, have been shown to be key components in the degradative processes which are the hallmark of both these arthritides. Both cytokines are produced by chondrocytes (Shinmei *et al.*, 1991). Both have been assayed in synovial fluids and localized in synovia removed from arthritic joints (Buchan *et al.*, 1988; Hopkins and Meager, 1988; Hopkins *et al.*, 1988; Chu *et al.*, 1991). Membrane receptors for IL-1β (Martel-Pelletier *et al.*, 1992) and TNFα (Westacott *et al.*, 1994) are overexpressed on OA compared with normal chondrocytes. Increased levels of endogenous inhibitors of IL-1 and TNFα have been identified in sera,

synovial fluids, and synovia of arthritis patients (Suzuki *et al.*, 1990; Symons *et al.*, 1991; Cope *et al.*, 1992; Deleuran *et al.*, 1992; Firestein *et al.*, 1992; Arend *et al.*, 1994; Higgens and Postlethwaite, 1996).

IL-1 and TNFα are arthritogenic in several animal models as well, and the antagonism of their effects reduces and in some cases even prevents arthritis (see reviews in Brennan, 1994; Arend and Dayer, 1995; Weckmann and Alcocer-Varela, 1996). The intraarticular injection of IL-1 into rabbit knees was shown to produce a transient synovitis and proteoglycan depletion from the articular cartilage (Pettipher *et al.*, 1986). Neutralizing anti-IL-1 antibodies were able to prevent both inflammation and cartilage degradation in established antigen-induced (Van den Berg *et al.*, 1994) and collagen-induced (Joosten *et al.*, 1996) arthritis. A natural antagonist of IL-1 is the IL-1 receptor antagonist, IL-1ra (IRAP). The injection of this molecule (Wooley *et al.*, 1993; Van Lent *et al.*, 1995; Caron *et al.*, 1996) or the transplantation of cells expressing IL-1ra into joints (Otani *et al.*, 1996) has been shown to ameliorate certain features of arthritis in different animal models. The use of recombinant IL-1 antagonists, IL-1ra and soluble IL-1 receptors (sIL-1R), in human clinical RA trials is ongoing, and to date, results have not been as promising as with TNFα blockade by monoclonal antibodies (see review, Feldmann *et al.*, 1996; Weckmann and Alcocer-Varela, 1996). Interestingly, many effects of classical anti-rheumatic agents on inflammatory and immune responses are mediated by, or result in, cytokine modulation (Barrera *et al.*, 1996).

2.3.2 Interleukin-1 and MMPs

2.3.2.1 MMP expression

Catabolic cytokines, such as IL-1 and TNFα are potent stimulators of metalloproteinase gene expression. The MMPs, as previously discussed, are a family of neutral endoproteinases capable of degrading all protein components of the extracellular matrix of cartilage. IL-1 stimulates the expression of the collagenases MMP-1, MMP-8, and MMP-13 which are believed to play a pivotal role in initiating the breakdown of the supporting collagen framework.

A well described and reproducible *in vitro* system for investigating aggrecan and collagen degradation involves the co-culturing of cartilage explants or isolated chondrocytes with the pro-inflammatory cytokines IL-1α or IL-1β. The breakdown of cartilage proceeds in a well-defined and easily monitored fashion in this system. The increased expression of MMP-1, MMP-8, and MMP-13 has been identified in IL-1 stimulated and OA articular cartilages (Borden *et al.*, 1996; Chubinskaya *et al.*, 1996; Cole *et al.*, 1996; Martel-Pelletier and Pelletier, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996; Shlopov and Hasty, 1997). Stromelysin-1 (MMP-3) is capable of degrading aggrecan (Nguyen *et al.*, 1989), depolymerizing type II collagen fibrils by cleaving the individual α chains in their non-helical N-telopeptide regions (Wu *et al.*, 1991), and cleaving type IX collagen (Wu *et al.*, 1991). Moreover, MMP-3 can activate all three collagenases (Murphy *et al.*, 1987; Knauper *et al.*, 1993, 1996). Increased expression of MMP-3 often accompanies and exceeds that of MMP-1 in IL-1 stimulated and OA articular cartilages (Nguyen *et al.*, 1992), and increased levels of MMP-3 have been described in RA sera and synovial fluids (Walakovits *et al.*, 1992; Yoshihara *et al.*, 1995).

The gelatinases, MMP-2 and MMP-9, degrade unwound collagen α chains (gelatin). IL-1 induced the increased synthesis of the constitutively expressed MMP-2 (Arner and Tortorella, 1995) and the expression of MMP-9, which is not usually found in normal adult human articular cartilage, but is expressed in fibrillated OA cartilage (Mohtai *et al.*, 1993; Tsuchiya *et al.*, 1997). Moreover, increased collagenolytic and gelatinolytic activities were associated with type II collagen degradation in IL-stimulated nasal cartilage explants (Kozaci *et al.*, 1997).

IL-1 up-regulates the expression of plasminogen activators (uPA and tPA) in chondrocytes (Campbell *et al.*, 1988) which, through their role in the generation of plasmin, may play a key role in the physiological activation of the MMPs which are secreted as latent enzymes. In support of this claim, a recent study showed significant proteoglycan degradation in human articular cartilage explants only after co-culturing with IL-1β and plasminogen, and this could be prevented by inhibitors of plasmin and plasminogen activator (Oleksyszyn and Augustine, 1996).

2.3.2.2 MMP inhibition

The IL-1 stimulated cartilage explant culture system has been used successfully to monitor the effectiveness of various synthetic MMP inhibitors in preventing cartilage degradation (DiPasquale *et al.*, 1986; Nixon *et al.*, 1991; Andrews *et al.*, 1992; Mort *et al.*, 1993; Seed *et al.*, 1993; Ellis *et al.*, 1994; Cawston *et al.*, 1995; Chichester *et al.*, 1996; Kozaci *et al.*, 1997). These inhibitors are peptides that are designed to mimic the cleavage site of the substrate, which is collagen in the case of the collagenase-selective

inhibitors. There are restrictions on which amino acids are found in both the natural and synthetic substrates, relative to the potential collagenase cleavage site. For example, the S_2 amino acid in MMP-1 prefers interaction with hydrophobic amino acid residues in the P_2 position of the substrate (that is, the second amino acid N-terminal to the potential collagenase cleavage site). Moreover, the amino acid at P_1' (the first amino acid C-terminal to the collagenase cleavage site) is limited to leucine or isoleucine. These synthetic inhibitors are coupled to chelating agents, such as carboxylic acids (COOH), thiols (-SH), hydroxamic acids (CONHOH), and phosphonic acids (-P(O)(OH_2)-), which bind to the active site catalytic zinc atom, thereby preventing the enzyme from binding to and cleaving its natural substrate. Some features that these inhibitors must possess to be effective as therapeutics are that they must be stable in biological systems and small enough to penetrate the articular cartilage and reach the target enzyme. They must have high levels of activity with $K_i < 10^{-10}$ M, and they should not be immunogenic (Henderson and Davies, 1991). Very selective and effective inhibitors are now available, due in part to the determination of the crystal structures of the MMPs, which allows for consideration of enzyme topology, in addition to substrate cleavage site amino acid sequence, in the design of these inhibitors (Blundell, 1994). This is of particular importance when attempting to design inhibitors that allow for selective inhibition of one collagenase over another. An example of the utilization of a three-dimensional feature of an enzyme in producing selective inhibitors is with the hydrophobic S_1' pocket of the MMPs, which is of variable depth among these proteinases. This pocket, within which lies the P_1' amino acid of the substrate in the enzyme-substrate complex, is shallow in MMP-1 and deep in both MMP-8 and MMP-13 (Welch *et al.*, 1996).

2.3.3 Other catabolic effects of IL-1 on articular cartilage

The induction of collagenase and stromelysin activity in chondrocytes has been shown to be dependent upon and mediated by nitric oxide (NO) (Murrell *et al.*, 1995), and up-regulated by an IL-1-induced increase in chondrocyte integrin receptor expression (Arner and Tortorella, 1995). The IL-1 induced suppression of proteoglycan synthesis (Benton and Tyler, 1988) is in part due to NO generated by the induction of inducible nitric oxide synthase (iNOS) by IL-1 (Taskiran *et al.*, 1994). Moreover, IL-1 suppresses α1(II) and α1(IX) procollagen mRNA expression (Goldring *et al.*, 1988; Tyler and Benton, 1988) which is not NO-mediated, and type II procollagen protein synthesis, which, in contrast, is NO-mediated (Cao *et al.*, 1997). IL-1 induces PGE_2 synthesis through the induction of cyclooxygenase II (COXII) gene expression and NO (Blanco and Lotz, 1995). NO can also cause apoptosis in chondrocytes (Blanco *et al.*, 1995). IL-1 stimulates chondrocytes to produce chemotactic factors, such as MCP-1, PDGF, TGFβ and IL-8, which in vivo attract inflammatory leukocytes into the joint. IL-1 is also a potent inducer of IL-6, IL-11, LIF and oncostatin M in articular chondrocytes, which as a family stimulate TIMP expression in chondrocytes (Lotz and Guerne, 1991). It is the balance between TIMP and MMP levels that is considered to be a most important factor in both joint destruction and the progression of joint disease (Shingu *et al.*, 1993).

3. MATERIALS AND METHODS

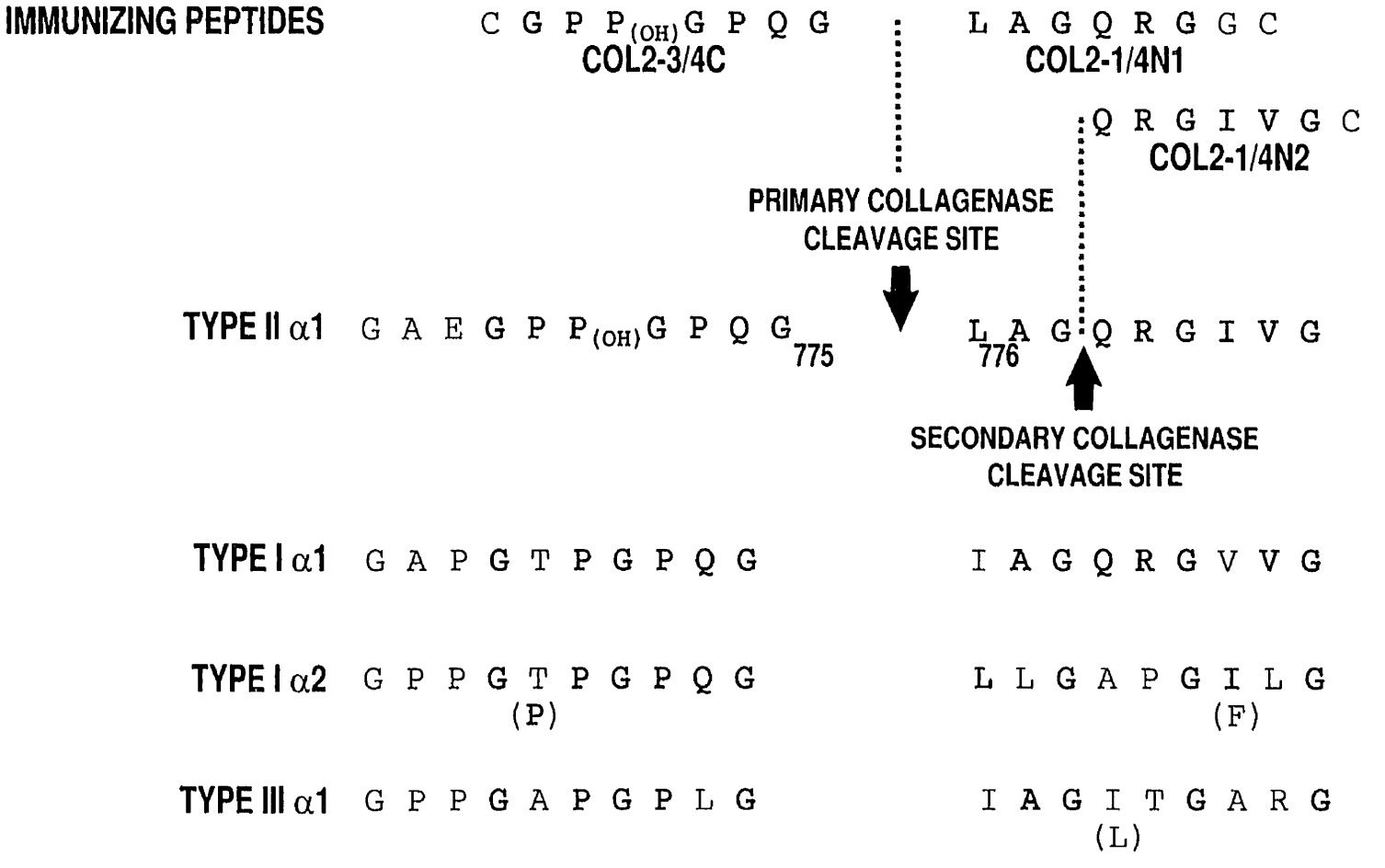
3.1 Anti-neoepitope antibody production and characterization

3.1.1 Identification and synthesis of collagenase cleavage site neoepitopes of type II collagen

The neoepitope peptides COL2-3/4C$_{short}$ (Gly-Pro-Hyp-Gly-Pro-Gln-Gly), and COL2-1/4N1 (Leu-Ala-Gly-Gln-Arg-Gly), correspond to the C-terminus of the three quarter fragment (TC^{A}) and to the N-terminus of the one quarter fragment (TC^{B}), respectively, produced by the known primary cleavage of native triple helical human type II collagen by collagenase-1 (MMP-1). The amino acid sequences for their synthesis were based on published amino acid sequences derived from cDNA (Birkedal-Hansen *et al.*, 1993) of fibroblast collagenase (MMP-1) cleaved human type II collagen α1 chain (Fig. 3.1), with the following exception. The assignment of the third residue of the COL2-3/4C$_{short}$ peptide (hereafter referred to only as COL2-3/4C), as a hydroxylated proline (Hyp), was based on the assumption that proline residues in the Y position of the Gly-X-Y triplets, that make up the helical portions of collagen molecules are potential hydroxylation sites within the collagen α chains. Moreover, we have shown that antisera generated to peptides containing a non-hydroxylated proline at this location, react poorly to the cleaved TC^{A} fragments of type II collagen. In turn, those peptides lacking Hyp, are recognized less effectively by antiserum generated from the COL2-3/4C peptide containing the Hyp residue (unpublished observations). The sequence for neoepitope peptide COL2-1/4N2 (Gln-Arg-Gly-Ile-Val-Gly), representing a secondary (N2) collagenase cleavage site at the N-terminus of the human α1(II) TC^{B} fragment, was

Figure 3.1 Sequence homology at the interstitial collagenase cleavage sites of human type I, II and III collagens and the immunizing peptides used to produce the COL2-3/4C, COL2-1/4N1 and COL2-1/4N2 anti-neoepitope antibodies.

Bold letters indicate the amino acids in the alpha chains that are similar in both identity and location to those found in the natural immunizing peptide sequences, which are also highlighted by bold letters at the top of the figure. The primary collagenase cleavage sites were obtained from Birkedal-Hansen *et al.* (1993), while the secondary collagenase cleavage site was obtained by N-terminal sequencing of electrophoretically-separated and transferred collagen fragments obtained after rHuMMP-13 digestion of triple helical human type II collagen, as described in section 3.3.3.1.3. The assignment of hydroxylation of the proline residues in the immunizing peptides indicated as $P_{(OH)}$ is described in section 3.1.1. Letters in brackets represent the substitutions in the α chains of bovine collagens for the amino acids directly above each in the human α chain. Modified from Billinghurst, R.C. *et al.* (1997), with permission.


IMMUNIZING PEPTIDES
C G P P(OH) G P Q G
COL2-3/4C
L A G Q R G G C
COL2-1/4N1
Q R G I V G C
COL2-1/4N2
PRIMARY COLLAGENASE CLEAVAGE SITE
TYPE II α1
G A E G P P(OH) G P Q G
775
L A G Q R G I V G
776
SECONDARY COLLAGENASE CLEAVAGE SITE
TYPE I α1
G A P G T P G P Q G
I A G Q R G V V G
TYPE I α2
G P P G T P G P Q G
(P)
L L G A P G I L G
(F)
TYPE III α1
G P P G A P G P L G
I A G I T G A R G
(L)

determined using the methodology described recently (Mitchell *et al.*, 1996) and below in section 3.3.3.3.

These peptides (COL2-3/4C, COL2-1/4N1 and COL2-1/4N2) and those peptides used for epitope analyses and antisera characterization, as described below, were synthesized at a 0.25-mmol scale, using standard Fmoc (9-fluoroenylmethoxycarbonyl) chemistry, on a Model 431A solid-phase peptide synthesizer (Applied Biosystems, Foster City, CA). A cysteine was added to the N-terminus of peptide COL2-3/4C and to the C-termini of peptides COL2-1/4N1 and COL2-1/4N2 for conjugation of the peptides to the carrier proteins ovalbumin (OVA) and bovine serum albumin (BSA). This was accomplished using the bifunctional reagent N-hydroxy-succinimidyl bromoacetate (Sigma Chemical Co.; St. Louis, MO), as previously described (Hughes *et al.*, 1992). Successful coupling of the peptides to the carrier proteins was confirmed through the demonstration of reduced mobility of the conjugates in SDS-PAGE gels, relative to the cysteine-carrier protein conjugate prepared in the same manner. A glycine spacer was included in the synthesis of the COL2-1/4N1 peptide, between the added C-terminal cysteine and the neoepitope sequence, to improve both the immunogenicity of this peptide when conjugated to OVA, and the sensitivity of the assays employing the peptide conjugated to BSA.

3.1.2 Preparation of polyclonal antibodies from rabbit antisera

For anti-neoepitope antisera production, female New Zealand White rabbits weighing 2.5-3.0 kg (Ferme des Chenes Bleues Inc.; Montreal, QC) were initially immunized intramuscularly with 0.5 mg of neoepitope peptide conjugated to OVA in

0.25 ml of phosphate buffered saline (PBS) and emulsified with 0.25 ml of complete Freund's adjuvant (CFA; Difco, Detroit, MI) by repeated inspirations and aspirations through a 20 GA hypodermic needle attached to a 3 ml glass syringe. Booster injections of similar quantities of peptide-OVA emulsified with incomplete Freund's adjuvant (IFA; Difco) were given intramuscularly every 2 to 3 weeks. After the second booster, test bleeds were performed and antibody titres were determined by enzyme-linked immunosorbent assay (ELISA), as described below. In all cases, good titres were obtained after the two boosters. Animals were exsanguinated by cardiac puncture and approximately 50 ml of serum was obtained from each rabbit. For immunohistochemistry and ELISAs, $F(ab')_2$ preparations were obtained by pepsin digestion of each antiserum, as previously described (Poole *et al.*, 1980). The Fc portion and undigested IgG were removed by AH-Sepharose-Protein A chromatography (Pharmacia; Uppsala, Sweden).

3.1.3 Immunoanalyses for determining antibody titre and specificity

3.1.3.1 Preparation of collagenase cleavage site neoepitopes

3.1.3.1.1 Preparation of recombinant collagenases

Recombinant human MMP-1 (rHuMMP-1) and MMP-13 (rHuMMP-13) were kind gifts of Peter Mitchell and John Hambor (Pfizer Inc., Groton, CT). Both were purified from the culture medium of Sf9 cells that were infected with recombinant baculovirus, as described recently (Mitchell *et al.*, 1996). Briefly, MMP-1 was affinity purified using a monoclonal antibody column and MMP-13 was purified using heparin-agarose and SP-Sepharose Fast Flow columns. Both the enzymes were >95% pure as determined by Coomassie blue staining of SDS-PAGE gels. Recombinant human MMP-

8 (rHuMMP-8) was a kind gift of Harald Tschesche (University of Bielefeld, Germany). It was expressed in *E. coli* and purified as previously described (Tschesche, 1995).

3.1.3.1.2 Collagen purification

Human adult and fetal articular cartilages, and human fetal skin were collected at autopsy (within 15 h postmortem) and from therapeutic abortions. Human type II collagen was prepared by pepsin digestion and differential salt precipitation, using the method of Miller *et al.* (1971). Human types I and III collagen were prepared by pepsin digestion and differential denaturation and renaturation (ChandraRajan, 1978). Bovine type X collagen was provided by Dr. A. Marriott (Joint Diseases Laboratory, Montreal). Cyanogen bromide (CNBr) peptides of human type II were prepared as described by Dodge and Poole (1989).

3.1.3.1.3 Cleavage of purified collagens

Lyophilized human type I, II and III collagens were dissolved in 0.5 M acetic acid and then diluted to a final concentration of 2.5 mg/ml in digestion buffer consisting of 50 mM Tris, 10 mM $CaCl_2$, 0.5 M NaCl, 0.01 % Brij 35 (Sigma) and 0.02 % NaN_3, pH 7.6. Recombinant human MMP-1, MMP-8, and MMP-13 were activated by incubation with 2 mM (final concentration) aminophenylmercuric acetate (APMA; Sigma) in the same digestion buffer for 90 min at 37°C. Each activated enzyme solution was added to individual collagen solutions at a final molar ratio of collagenase to collagen of 1:5 (MMP-1/MMP-8:collagen) or 1:10 (MMP-13:collagen). Where necessary, the pH was

adjusted to 7.5. Controls contained collagen in digestion buffer with 2 mM APMA but with no collagenase.

For immunoanalyses by ELISA of cleavage neoepitopes, the samples were incubated for 24 h at 30°C and then the rHuMMPs were inactivated by the addition of 20 mM (final concentration) EDTA (Sigma). For studies of time-dependent collagenase cleavage of native type II collagen, aliquots of each of the digested collagen solutions were removed at the times indicated in the corresponding figure legends and the collagenase was inactivated in each aliquot with EDTA. The digestions were carried out at 30°C and the final aliquot was removed after 72 h of incubation.

3.1.3.2 Direct-binding ELISA

The immunizing peptides, conjugated to BSA, were diluted to 20µg/ml in 0.1 M carbonate buffer, pH 9.2, and 50 µl was added to each well (1 µg/well) of Immulon-2 flat bottom tissue culture microtitre plates (Dynatech Laboratories, Inc., Chantilly, VA). In other cases, plates were coated with 2 µg/well of either native, heat-denatured, or MMP-1 cleaved or MMP-13 cleaved human types I, II, III, and X collagen (see below). After 24 h at 4°C, the plates were washed three times with PBS containing 0.1% v/v Tween 20 (Sigma) (PBS-Tween). Non-coated binding sites were blocked by 150 µl/well of 1% w/v BSA in PBS (PBS-1% BSA) for 30 min at room temperature. The plates were washed once with PBS-Tween and 50 µl of serial dilutions of the appropriate polyclonal antiserum preparation were added to individual wells. After 90 min at 37°C, the plates were washed three times with PBS-Tween. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:30,000 in PBS-1% BSA-Tween was added at 50 µl/well.

After 1 h at 37°C, the plates were washed three times with PBS-Tween and once with distilled water. Finally, 50 µl of freshly prepared alkaline phosphatase substrate, disodium p-nitrophenyl phosphate (Sigma) at 0.5 mg/ml in 8.9 mM diethanolamine, 0.25 mM $MgCl_2$, pH 9.8 was added to each well for 20-30 min at 37°C. The absorbances were measured at 405 nm on a Multiskan plus MkII plate reader (ICN Flow; Mississauga, ON).

3.1.3.3 Inhibition ELISA

Linbro 96 well round bottom microtitre plates (ICN Flow) were pre-coated with 100 µl/well of PBS-1% BSA for 30 min at room temperature and washed once with PBS-Tween. Four non-specific binding wells each contained 100 µl of 50 µl PBS-1% BSA and 50 µl PBS-1% BSA-Tween. Each polyclonal $F(ab')_2$ antiserum preparation was diluted 1:200 in PBS-1% BSA-Tween (as determined by checkerboard analyses of antisera and peptide-BSA titrations, data not shown) and 50 µl was added to each of the remaining wells of these pre-incubation plates. Four wells with antisera were mixed with 50 µl/well of PBS-1% BSA to determine maximum binding in the absence of the inhibitory epitopes. To the remaining test wells containing 50 µl of diluted antiserum $F(ab')_2$ were added 50 µl/well of appropriate dilutions of the standard peptides COL2-3/4C, COL2-1/4N1 or COL2-1/4N2; native, heat-denatured (80°C for 20 min) or collagenase-cleaved type I, II and III collagen solutions. For antibody specificity analyses, standard peptides containing the epitope that had been used for immunization were used, as described above, but with amino acid residues either added to (+1, +2, +3) or removed from (-1, -2, -3) the end of the immunizing peptide corresponding to the

cleaved terminus of the α chain fragment. Also, two overlapping 16mer peptides representing the amino acid sequence bridging the primary and secondary collagenase cleavage sites of native type II collagen (Gly-Pro-Hyp-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val-Gly and Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val-Gly-Leu-Hyp-Gly) were used to demonstrate the specificity of the immunoreactivities of the antisera to the cleaved termini and not to intact α chains at the cleavage sites. All standards and samples were tested in duplicate wells. Analyses were always repeated at least once.

After incubation for 1 h at 37°C, 50 µl of each pre-incubated sample was transferred to the equivalent wells of Immulon-2 ELISA plates, pre-coated, as described above, with neoepitope peptide-BSA conjugates diluted to 50 ng/well for COL2-3/4C, 150 ng/well for COL2-1/4N1, and 120 ng/well for COL2-1/4N2, all in PBS, pH 7.2. Note that for the COL2-3/4C assay, the BSA-conjugate contained the peptide Cys-Gly-Gly-Glu-Gly-Pro-Hyp-Gly-Pro-Gln-Gly (COL2-3/4C_{long}), which included the natural in-sequence amino acid residue (Glu) and two glycine spacers added to the N-terminus of the immunizing COL2-3/4C peptide, was used to coat the plates. In preliminary experiments, the use of this longer peptide was found to increase the sensitivity of the COL2-3/4C assay over that obtained by using the shorter immunizing peptide-BSA conjugate (data not shown). These plates were incubated for 30 min at 4°C and then washed three times with PBS-Tween. Goat anti-rabbit IgG $F(ab')_2$ fragment conjugated to alkaline phosphatase (Sigma) was diluted 1:20,000 in PBS-1% BSA-Tween and added at 50 µl/well. After incubation for 1 h at 37°C, the plates were washed three times with PBS-Tween and three times with distilled water. From an ELISA amplification system

kit (Gibco BRL; Gaithersburg, MD), 50 µl of amplifier substrate (NADPH and iodonitrotetrazolium violet) was added to each well and after 15 min at room temperature, 50 µl of amplifier (ethanol, alcohol dehydrogenase and diaphorase) solution was added to each well. After a final 15 min at room temperature, the colour development was halted with 50 µl of 0.3 M H_2SO_4 and the absorbance was measured at 490 nm. For each plate the mean absorbance from the four non-specific binding wells was subtracted from the absorbance values of each of the other wells. The percentage inhibitions of binding by standards or samples were calculated relative to the mean absorbance from the four maximum binding wells which represented 0% inhibition (100% binding). Results were also expressed on a molar basis using a molecular weight for type II α chain of 98,291 D (Hollander *et al.*, 1994) and for the COL2-3/4C peptide of 608 D.

3.1.3.4 Electrophoresis and immunoblotting

SDS-PAGE of MMP-1, MMP-8 and MMP-13 cleaved purified native human collagens were performed as previously described (Laemmli, 1970) under denaturing conditions using 10%, 1 mm thick, 7 cm x 8 cm mini-Protean gels stained with Coomassie Blue R-250 (Bio-Rad Laboratories, Mississauga, ON) in 40% v/v methanol and 10% v/v acetic acid in distilled water. Electrophoretic transfers to nitrocellulose membranes (Bio-Rad) were performed as previously described (Dodge and Poole, 1989), with the following exceptions. The PBS-3% BSA blocked membranes were incubated overnight at 4°C with either anti-COL2-3/4C or anti-COL2-1/4N1 or anti-COL2-1/4N2 $F(ab')_2$ preparations diluted 1:200 in PBS-3% BSA-Tween. After three 10 min washes in PBS-1% BSA-Tween, the membranes were incubated for 1 h at room temperature with

alkaline phosphatase-conjugated goat anti-rabbit IgG $F(ab')_2$ fragment diluted 1:30,000 with PBS-3% BSA-Tween. The membranes were given 3 x 10 min washes in PBS-1% BSA-Tween and then rinsed well in distilled water, before adding alkaline phosphatase substrate solution from a commercial kit (Bio-Rad) using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. After optimal colour development (10-20 min), the reaction was stopped by washing off the substrate solution with distilled water.

3.2 Immunoanalyses of human articular cartilages

A summary cartoon depicting the methodology used to analyze the human articular cartilages is presented in Figure 3.2.

3.2.1 Immunohistochemical analysis

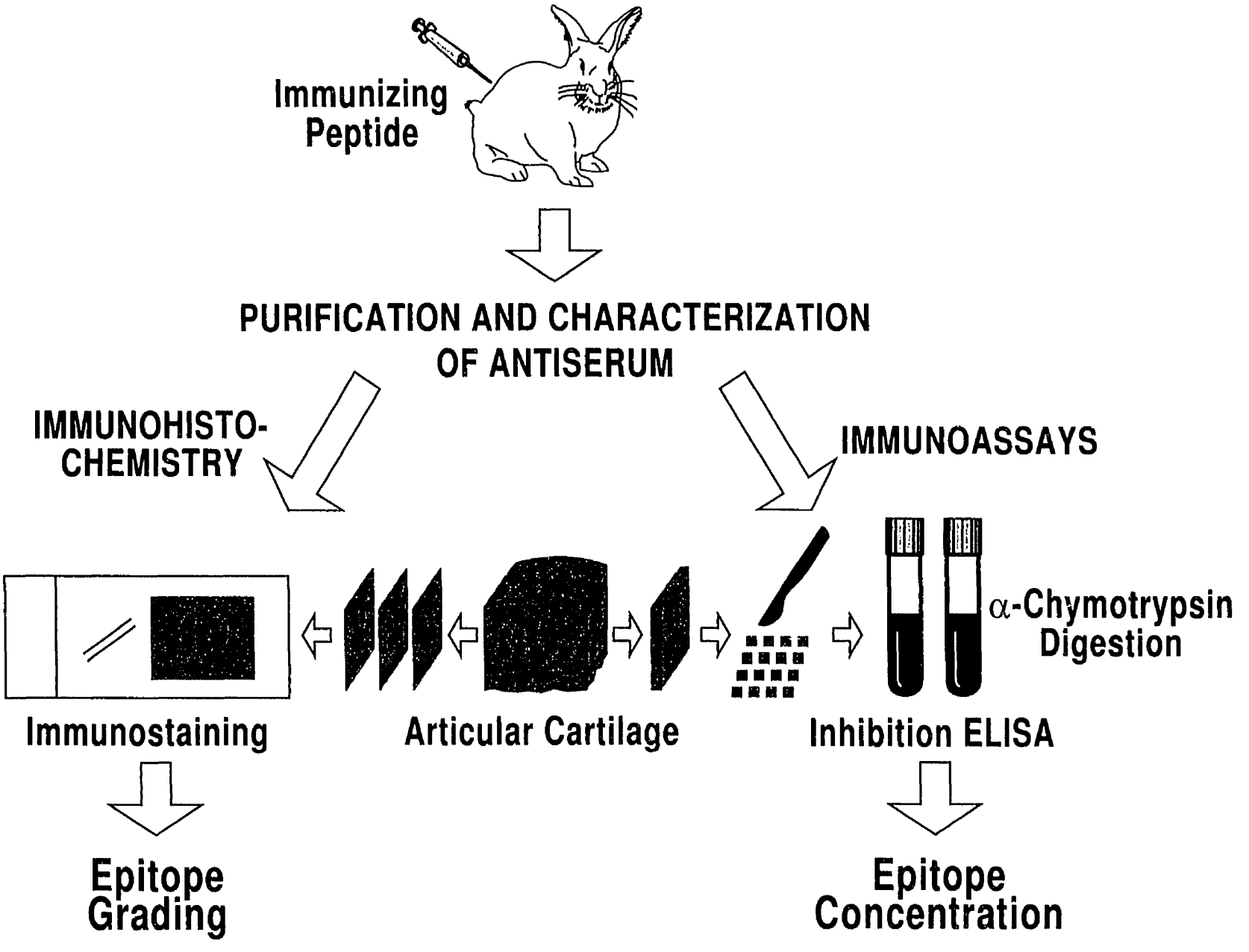
3.2.1.1 Preparation and immunostaining of cartilage sections

Full depth human osteoarthritic articular femoral condylar cartilage was obtained at arthroplasty from the knee joints of 10 patients with OA, as diagnosed using the criteria of the American College of Rheumatology for OA (Altman *et al.*, 1986). Osteophytic cartilages were never examined. Macroscopically normal full depth non-arthritic articular cartilages were obtained within 15 h postmortem from the femoral condylar regions of 15 individuals from sites where there were no observable joint abnormalities. None of these persons had recent chemotherapy.

Frozen articular cartilage sections (6 mm thick) were prepared and processed for immunoperoxidase histochemical analysis of denatured type II collagen using the COL2-3/4m antibody as described recently (Hollander *et al.*, 1994; 1995). This mouse monoclonal antibody recognizes an intrachain epitope COL2-3/4m (also known as

Figure 3.2 Methodology used to assess the presence and levels of collagenase-generated type II collagen neoepitopes in human articular cartilages.

The schematic depicts the formation of polyclonal antisera, through the immunization of rabbits with synthetic peptides representing amino acid sequences found at the termini of α1(II) chains created by the cleavage of triple helical type II collagen with MMP-1, MMP-8 or MMP-13. These antisera were characterized as anti-neoepitope antibodies and used to analyze human femoral condylar articular cartilage sections immunohistochemically, and the α-chymotrypsin digests of these tissues by immunoassay. Also examined in these samples, along with cleavage site neoepitopes COL2-3/4C and COL2-3/4N2, were the presence and levels of the intrachain hidden epitope, COL2-3/4m, indicative of type II collagen denaturation.


Immunizing Peptide
PURIFICATION AND CHARACTERIZATION OF ANTISERUM
IMMUNOHISTO-CHEMISTRY
IMMUNOASSAYS
α-Chymotrypsin Digestion
Immunostaining
Articular Cartilage
Inhibition ELISA
Epitope Grading
Epitope Concentration

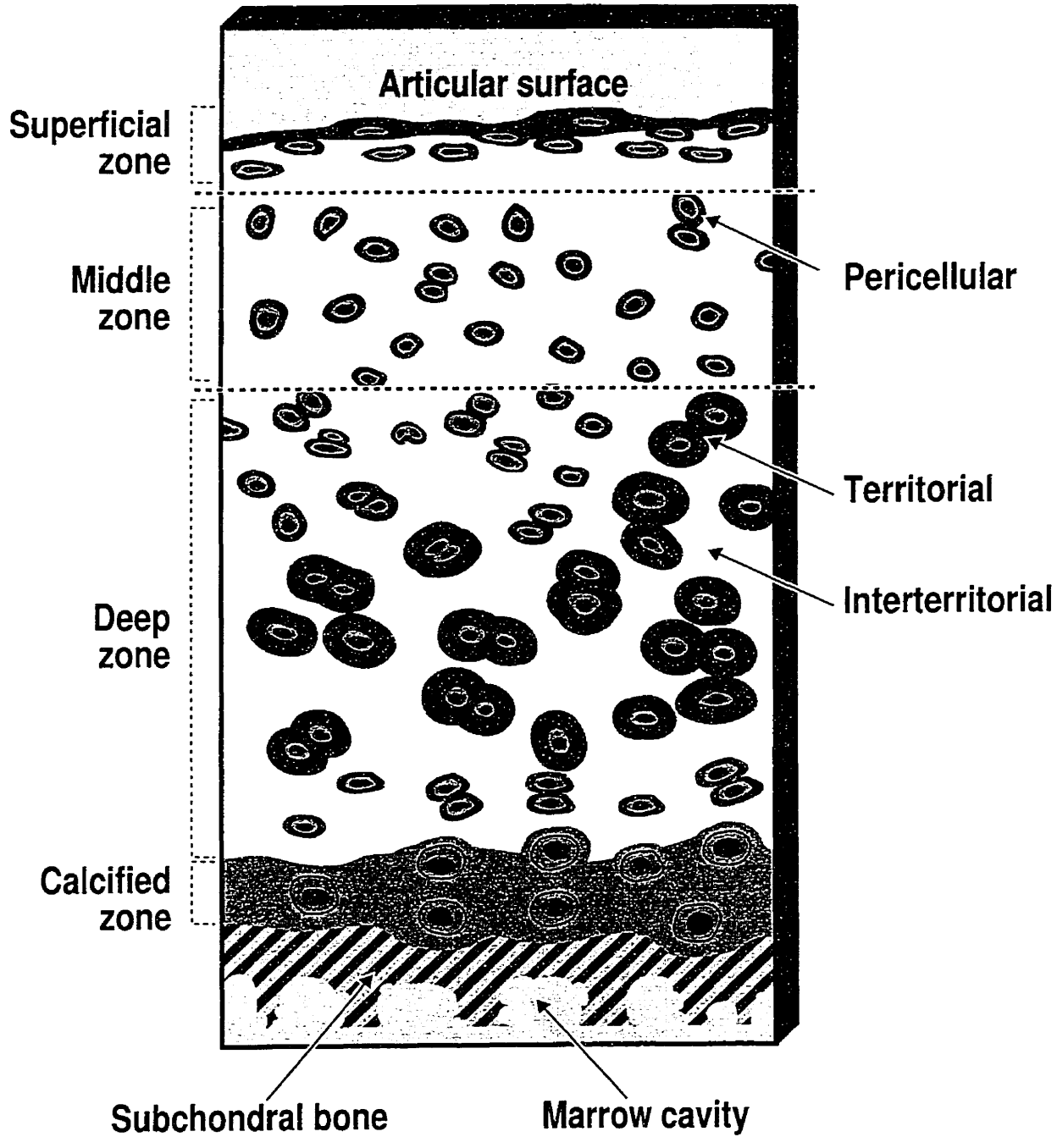
CB11B) in the TC^A fragment of human type II collagen. For neoepitope immunohistochemical analyses, the same methodology was applied with the following exceptions. Fifty µl/section of appropriately diluted anti-neoepitope $F(ab')_2$ preparations were added to tissue sections for 30 min at room temperature. Specific controls were prepared by incubating each antiserum, before use, for 1 h at 37°C with 100 µg/ml of the appropriate immunizing peptide. Sections were washed 3 x 10 min with PBS and incubated with biotinylated pig anti-rabbit IgG (DAKO; Denmark). After a further 3 x 10 min series of washes with PBS, a 1:200 dilution in PBS of peroxidase-conjugated streptavidin (Amersham Corp.; Arlington Heights, IL) was added for 20 min. A final 10 min wash with PBS was followed by demonstration of the peroxidase activity which was enhanced with copper H_2O_2/silver intensification as previously described (Gallyas and Merchanthaler, 1988). Permanent mounts were then prepared. In view of the lack of specificity of antibodies to epitope COL2-1/4N1 (as discussed below), only COL2-3/4C and COL2-1/4N2 epitopes were studied.

3.2.1.2 Grading of immunohistochemical staining

Immunostaining was recorded for full depth sections in the superficial (S), middle (M) and deep (D) zones of the articular cartilage. Staining in the pericellular, territorial and interterritorial sites (with respect to chondrocytes) within each zone (Fig. 3.3) was recorded as negative = 0, weak = 1, or moderate to strong = 2. Thus the maximum score attainable for each zone was 6 and the lowest (no staining) was 0. This grading system permitted the analysis of full depth changes in the different zones.

Figure 3.3 Zonal and site-specific classification system used for immunostain grading of articular cartilage sections.

Articular cartilage sections were arbitrarily divided into superficial, middle, and deep zones for the purpose of describing the localization, and for the subsequent grading of the immunostaining. Within each zone, the staining was recorded, as depicted, in pericellular, territorial, and interterritorial sites, relative to the chondrocytes. This staining was graded as 0 for no staining, 1 for weak staining, and 2 for moderate to strong staining. Thus, the maximum score attainable for each zone was 6. Modified from Poole, A.R. (1997), with permission.


Articular surface
Superficial zone
Middle zone
Deep zone
Calcified zone
Pericellular
Territorial
Interterritorial
Subchondral bone
Marrow cavity

Two observers (Robin Poole and Isabelle Pidoux) independently examined the same randomized blinded specimens (normal, n = 36; OA, n = 28). Data were then compared by Spearman rank correlation to determine inter-observer correlation in data recording for the immunohistochemical grading of the collagenase generated type II neoepitopes (COL2-3/4C and COL2-1/4N2) and the type II denaturation epitope (CB11B or COL2-3/4m). The results for the COL2-3/4C and COL2-1/4N2 neoepitope grading were r = 0.903, P = 0.0001, and r = 0.727, P = 0.0001, respectively. For staining with the COL2-3/4m antibody, the values were r = 0.852, P = 0.0001. The results of one blinded observer (I.P.) are presented in this study for whom correlations for duplicate analyses of the same specimens were as follows. For the COL2-3/4C and COL2-1/4N2 neoepitope gradings r = 0.895, P = 0.0001 and r = 0.854, P = 0.0001, respectively, and for the COL2-3/4m epitope, r = 0.936, P = 0.0001.

3.2.2 Immunochemical analyses

3.2.2.1 Extraction of denatured and cleaved collagens

Preliminary studies using MMP-1 and MMP-13 cleaved human type II collagens (not shown) established that the levels of the COL2-3/4C neoepitope, as detected by ELISA, were not significantly affected by α-chymotrypsin treatment under the conditions previously described to extract denatured collagen from articular cartilage (Hollander *et al.*, 1994). Therefore, femoral condylar articular cartilages from 19 normal and 26 osteoarthritic joints were diced and incubated overnight at 37°C with 1.0 mg/50-75 mg cartilage of α-chymotrypsin in 50 mM Tris-HCl, pH 7.6 (with the proteinase inhibitors: 1 mM EDTA, 1 mM iodoacetamide and 10 μg/ml pepstatin A; Sigma). The α-

chymotrypsin activity was inhibited with 20 µl (160 µg/ml final concentration) of *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK; Sigma) . After 20 min at 37°C, the samples were centrifuged and the supernatants were removed and assayed for the COL2-3/4C neoepitope, as described in section 3.1.3.3. The type II collagen hidden epitope, COL2-3/4m, was also assayed, using methodology described previously (Hollander *et al.*, 1994).

3.2.2.2 Cartilage digestion for total collagen content

The cartilage residues remaining after α-chymotrypsin extraction were digested overnight at 56°C with 1.0 mg/ml proteinase K (Sigma) in 50 mM Tris-HCl (pH 7.6) containing the same proteinase inhibitors and then boiled for 20 min to denature the enzyme. Total type II collagen content of each sample was determined from the collective amount of COL2-3/4m epitope in both the α-chymotrypsin extract and the proteinase K digestion. The percentage of collagen that was denatured was determined from the amount of COL2-3/4m epitope in the α-chymotrypsin extract expressed as a percentage of the total collagen content. The percentage of collagen that cleaved was calculated from the amount of COL2-3/4C neoepitope assayed in the α-chymotrypsin extract expressed as a percentage of the total collagen content.

3.2.3 Mankin grading

Frozen sections, that were prepared for immunohistochemistry and for extraction immunoassay, were stained with Safranin O and Fast Green and were graded for

degenerative changes as described by Mankin *et al.* (1971). The maximum grade was 13, as there was no calcified cartilage in the specimens used. These grades are summarized in Table 4.1 and in figure legends.

3.3 Cartilage explant cultures and MMP inhibitors

3.3.1 The synthetic MMP inhibitors

3.3.1.1 Determination of inhibition constants

Enzymatic assays on the MMPs were carried out by Dr. Hal Van Wart (Roche Bioscience, Palo Alto, CA) with catalytic domains of human MMP-1, MMP-2, and MMP-3 (Agouron Pharmaceutical, La Jolla, CA), and MMP-13 (Roche Bioscience), which were all expressed in *E. coli.* Human MMP-8 and MMP-9 were isolated and purified from neutrophils. The substrate used for the MMP assays was Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Bachem, Torrance, CA) [where Mca is (7-methoxycoumarin-4-yl)acetyl and Dpa is *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]. Initial rates of hydrolysis (v) were measured fluorometrically at 37°C using λ_{ex} 325 nm; λ_{em} 393 nm with a Perkin-Elmer LS-50B luminescence spectrometer. Values for v were determined from variable concentrations of the synthetic hydroxamate inhibitor RS 47,112 and the synthetic carboxylate inhibitor RS 102,481, and the K_i values shown in Table 3.1 were obtained from the equation:

$$v = \frac{-(K_i + [I]_o - [E]_o) + \{(K_i + [I]_o - [E]_o)^2 + 4 K_i [E]_o\}^{1/2}}{2 [E]_o}$$

where $[I]_o$ = initial inhibitor concentration, and $[E]_o$ = initial enzyme concentration.

Table 3.1 Inhibitory profiles of human MMP-selective inhibitors used in the cartilage explant culture studies

	K_i (nM)					
Compound	**MMP-1**	**MMP-8**	**MMP-13**	**MMP-2**	**MMP-3**	**MMP-9**
RS 47,112	0.40	0.62	0.53	0.46	0.15	2.3
RS 102,481	1,100	18	0.080	ND	19	32

All human MMPs shown in the chart were assayed by Roche Bioscience (Palo Alto, CA) using a synthetic fluorescent substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. Inhibition constants were determined as described in section 3.3.1.1 and are expressed as nM. ND = not determined.

All the assays were carried out in 50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 2.5% DMSO and 0.005% Brij 35, pH 7.5. All inhibitors were synthesized within the chemistry department of Roche Bioscience. An assumption was made in this study that has been made in previous ones which employ animal cartilage explant culture systems to evaluate synthetic inhibitors whose potencies have been evaluated to inactivate human enzymes. The assumption is that these inhibitors have similar activities against the bovine MMPs as that determined for their human counterparts. It should be noted, however, that there is a high degree of similarity between the catalytic domains of the human MMP-1, MMP-2, and MMP-3 with the corresponding bovine enzymes (Bottomley *et al.*, 1997).

3.3.1.2 Selective inhibition of type II collagen cleavage

Lyophilized human type II collagen was prepared by pepsin digestion and salt precipitation of femoral articular cartilage removed from autopsy cases, within 15 h of death, as described in section 3.1.3.1.2. The type II collagens were dissolved in 0.5 M acetic acid and then diluted to a final concentration of 2.5 mg/ml in digestion buffer consisting of 50 mM Tris, 10 mM $CaCl_2$, 0.5 M NaCl, 0.01% Brij 35 and 0.02% NaN_3, pH 7.6. In all cases, the pH was adjusted to 7.5 with NaOH. Recombinant human MMP-1 and MMP-13 were activated by incubation with 2 mM APMA (final concentration) in the same digestion buffer for 90 min at 37°C. Each of the 1 mM stock solutions of inhibitor in 100% DMSO was removed from storage at 4°C and diluted to concentrations that would allow for the selective inhibition of the collagenases MMP-1, MMP-8, and MMP-13, and that would subsequently be used in the cartilage explant cultures. From

the K_i values for each, it was decided that final concentrations of 1.0 and 10 nM for RS 102,481 should selectively inhibit MMP-13, and 20 and 200 nM for RS 47,112 should not allow for the selective inhibition of the three collagenases. Appropriate volumes of each inhibitor were added to aliquots of activated MMP-1 and MMP-13 for 10 min before being added to the type II collagen solution to yield a final molar ratio of 1:10 (enzyme:collagen). Controls contained type II collagen in digestion buffer with 2 mM APMA and 0.1% DMSO, but no enzyme. Aliquots were removed at 0, 1 and 3 h for the MMP-13 digestion and 0, 1 and 5 h for the MMP-1 digestion. To each was added 20 mM EDTA (final concentration) to completely inactivate the enzymes. These type II collagen digests were subjected to SDS-PAGE analyses according to Laemmli, 1970, by adding 20 µl of sample buffer to 20 µl of each collagen solution. This was then loaded to individual tracks of 10 %, 1 mm thick, 7 X 8 cm Mini-Protean gels and stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

3.3.2 Human OA articular cartilage explant cultures

3.3.2.1 Tissue preparation and culturing

Full depth human femoral condylar articular cartilages in serum-free culture media (see below) were removed from the subchondral bone within 6 h of its removal from three OA patients undergoing joint replacement surgery. The cartilage was diced into approximately 1 mm^3 pieces and placed for 1 h in basic culture medium consisting of DMEM with 3.6 mg/ml of Hepes (Gibco BRL), 100 units/ml penicillin and 100 µg/ml streptomycin to which was added 2.5 µg/ml amphotericin B (Fungizone; Gibco BRL). It was then transferred to the same basic culture medium (DMEM-Hepes) containing 1000

units/ml penicillin and 1 mg/ml streptomycin for 30 min. After a final wash in basic culture medium, 40-50 mg wet weight of cartilage was transferred to each well of a 24 well tissue culture plate (Falcon). Each well contained 1 ml of basic culture medium supplemented with 1 mg/ml BSA (Sigma), 50 µg/ml *L*-ascorbic acid (Sigma), and 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite (I.T.S.; Boehringer Mannheim, Germany). The cartilage was pre-cultured for 4 days at 37°C, 5% CO_2 and 95% relative humidity. The media were removed and quadruplicate samples were cultured either in the basic culture medium (+BSA, ascorbic acid, I.T.S.) supplemented with 10, 2.5, and 1.0 nM of RS 102,481 in 0.1% DMSO, or in medium with only 0.1% DMSO. Media and inhibitors were replaced every 2 days of culture and the conditioned media were frozen at -20°C until they were assayed for the COL2-3/4C neoepitope, as described above, except that the media were concentrated three-fold in a Savant Speed-Vac evaporator (Emerson Instruments, Scarborough, ON), before being assayed. Cultures were terminated after 12 days. The experiment was repeated twice with articular cartilage from two other patients.

3.3.2.2 Measurement of total collagen synthesis

To determine the effect of the inhibitor RS 102,481 on type II collagen synthesis, explants of human OA articular cartilage were cultured as described above, with or without the highest concentration (10 nM) of inhibitor. On days 14, 16 and 18 of culture, 15 µCi of ^{3}H-proline was added to each ml of medium. The conversion of radiolabelled proline into tritiated hydroxyproline within the explants, as an indicator of type II

collagen synthesis, was determined after day 20 in two independent cultures, using the method described by Tyler and Benton (1988).

3.3.3 IL-1 stimulated bovine cartilage explant cultures

3.3.3.1 Preparation and culture of cartilage explants

3.3.3.1.1 Articular cartilage

Articular cartilage was shaved from the distal condylar surfaces of the metacarpal and metatarsal bones and from the proximal articular surfaces of the first phalanges of adult cows, shortly after slaughter. The cartilage was diced into ~3 mm^2 sections, mixed and placed for 1 h in serum-free DMEM buffered with 3.6 mg/ml Hepes and 3.7 mg/ml sodium bicarbonate, and supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (all from Gibco BRL). After 30 min in DMEM-Hepes with 1,000 U/ml penicillin and 1 mg/ml streptomycin, and 30 min in DMEM-Hepes, the cartilage pieces were randomly assigned to 48-well tissue culture plates (Falcon Labware, Becton Dickinson & Co, Lincoln Park, NJ) at ~ 30 mg wet weight cartilage per well. Each well contained 0.5 ml of the basic culture medium of DMEM-Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin supplemented with 50 µg/ml L-ascorbic acid (Gibco), 0.1 mg/ml BSA (Sigma), and a solution of 5.0 µg/ml insulin, 5.0 µg/ml transferrin and 5.0 ng/ml sodium selenite (I.T.S.). The explants were pre-cultured for 2 days at 37°C in a humidified incubator with 5 % CO_2 / 95 % air. The explants were then divided into six groups of quadruplicate samples for each inhibitor, and cultured under the following conditions: control, basic culture medium; IL-1, basic medium + 5 ng/ml recombinant human IL-1α (R&D Systems, Minneapolis, MN); low

dose inhibitor, basic medium + 1 nM RS 102,481 or 20 nM 47,112 (both in 0.1% DMSO); low dose inhibitor + IL-1; high dose inhibitor, basic medium + 10 nM RS 102,481 or 200 nM RS 47,112 (both in 0.1% DMSO); and high dose inhibitor + IL-1. All media were replaced every 48 h with the fresh addition of IL-1α and/or inhibitors, where required. The conditioned media were stored at -20°C until assayed. Explants were removed at days 14 and 28 of culture for subsequent digestion and assay (see below) and all the articular cartilage explant cultures were terminated after 28 days.

3.3.3.1.2 Nasal cartilage

Nasal septa were removed from adult cows shortly after slaughter. All adhering tissue was trimmed from the cartilage and ~4 mm diameter cartilage plugs were removed with a sterile stainless steel punch. These plugs were cut into 1 mm thick discs, which were then processed exactly as described for the articular cartilage explants, in quadruplicate, with the following modifications. The discs were randomly assigned to wells of 48-well tissue culture plates at ~20 mg wet weight cartilage per well. The discs were weighed in sterile 1.5 ml centrifuge tubes before the start of, and after removal from, culturing. Explants were removed every 4 days for analysis and all the nasal cartilage explant cultures were terminated after 20 days.

3.3.3.2 Extraction and assay of cartilage explants

The explants from days 0, 4, 8, 12, 16, and 20 of culture for nasal, and from days 0, 14, and 28 of culture for articular cartilages were digested for the extraction of cleaved and denatured collagen as described above in section 3.2.2.1. The content of both the

COL2-3/4C and COL2-3/4m epitopes were expressed as pmoles of peptide per mg wet weight of cartilage, based on molecular weights of 608 and 2231, respectively. The use of cartilage wet weight to measure cartilage metabolic parameters has been shown to be valid for normal and OA human articular cartilage explants and correlates linearly with cartilage DNA content (Lafeber *et al.*, 1993).

Hydroxyproline content was also determined in both the α-chymotrypsin and proteinase K digests, and the sum of both gave an alternative measure of total collagen content in the cartilage explants. The methodology used was a modification of that previously described (Burleigh *et al.*, 1974). Since we were assaying enzyme digest solutions and not intact cartilages, concentrated HCl (~12 M) was added on a 1:1 basis with each of the digest solutions, to yield a final concentration of 6 M HCl. The samples were hydrolyzed for 20 h at 110°C, clarified with a charcoal resin decoloriser, consisting of equal parts of activated charcoal and AG-1 X8 anion exchange resin (Bio-Rad), centrifuged and the supernatants collected and neutralized with NaOH. Then 125 µl aliquots of each sample or standard (hydroxyproline at 0.5-40 µg/ml) was added to 125 µl of 50% saturated NaCl and 250 µl of freshly prepared chloramine T reagent. After 4 min at room temperature, 250 µl of a solution containing equal volumes of 5% p-dimethyl-aminobenzaldehyde in propan-1-ol and 32% perchloric acid was added to each sample and incubated for 12 min at 65°C. The samples were allowed to cool and the absorbances at 560 nm were determined. The content of hydroxyproline was expressed as either µg or pmoles per mg wet weight, based on a molecular weight of 114.14.

The conditioned media that were removed every other day from the nasal and articular cartilage explant cultures were assayed for COL2-3/4C neoepitope, COL2-3/4m

epitope and hydroxyproline content, as described above for the cartilage digests, with the following exception. The conditioned media that were assayed for COL2-3/4m epitope content were heated to boiling for 10 minutes to inactivate those proteases that were believed to be causing significant and spurious elevations in COL2-3/4m levels, especially in the media from cultures stimulated with IL-1.

3.3.3.3 Immunoblotting and sequencing analyses of media

Conditioned media (1.0 ml) that were removed from bovine nasal cartilages cultured for 0, 8, 14, and 20 days, and from bovine articular cartilages cultured for 0, 14, 20 and 28 days were concentrated using a Savant Speed-Vac. The media chosen were those from the explants cultured in medium alone, medium + IL-1α, and medium + IL-1α + 1 nM or 10 nM RS 102,481. To each of the dried samples was added 125 µl of SDS-PAGE sample buffer, and 25 µl/lane was electrophoresed in 1mm thick, 7 x 8 cm, Mini-Protean 4-20% gradient gels and stained with Coomassie Brilliant Blue R-250 (Bio-Rad). Duplicate gels were used for the transfer of the separated proteins onto nitrocellulose membranes (Bio-Rad). These were then blocked in PBS-3% BSA-Tween for 1 h and incubated overnight at 4°C in either a 1:500 dilution of affinity purified COL2-3/4C_{short} antiserum, or a 1:5000 dilution of COL2-3/4m monoclonal antibody, both in PBS-3% BSA-Tween. After 3x10 min washes in PBS-1% BSA-Tween, the blots were incubated for 1 h in either a 1:30,000 dilution of goat anti-rabbit IgG (for COL2-3/4C), or a 1:5000 dilution of goat anti-mouse IgG (for COL2-3/4m) alkaline phosphatase conjugated antibodies. The blots were washed 3x10 min in PBS-1% BSA-Tween, 10 min in distilled water (dH_20), and then alkaline phosphatase substrate solution was added which was

prepared from a commercial kit (Bio-Rad) using 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium. The blots were incubated until the optimal staining intensity was obtained and the reaction was stopped by washing in dH_2O.

To confirm the collagenous identity of the immunoreactive bands, 1.0 ml of conditioned media from day 14 of the bovine nasal cartilages, cultured with and without IL-1α, were dialyzed overnight in dH_2O to which had been added 20 mM EDTA and 0.05 % NaN_3, and then concentrated for N-terminal sequencing. Briefly, the dried samples were electrophoresed, as above, except that 10 % SDS-PAGE gels were used and the transfer of the separated proteins was onto polyvinylidine difluoride (PVDF) membranes. These membranes were stained with Coomassie Brilliant Blue R-250, and the N-terminal sequences of two distinct bands corresponding to one staining with the COL2-3/4C_{short} antiserum and another staining with the COL2-3/4m antibody on separately blotted membranes, were determined using a model 473A sequencer (Applied Biosystems Inc.).

3.3.3.4 Determination of proteoglycan degradation

The conditioned culture media and both the α-chymotrypsin and proteinase K cartilage digests were assayed for sulphated glycosaminoglycans (sGAG), considered to be a measure of proteoglycan content, by using a modification of the colorimetric 1,9-dimethylmethylene blue (DMMB) dye binding assay (Farndale *et al.*, 1986). The samples or standards (10 µl) were added to 190 µl of the dye reagent in microtitre plate wells and the absorbance was read at 525 nm within 10 min. The standard was shark chondroitin sulphate (Sigma) at concentrations from 1.56 to 200 µg/ml. Proteoglycan release (sGAG in the media) was expressed as mg sGAG per mg wet weight of cartilage.

3.4 Statistical analyses

Spearman rank correlations were employed for analyzing relationships between the different collagen degradation products assayed in this study. Mann-Whitney U tests were used to compare the non-arthritic (normal) and OA groups, in terms of the collagenase-generated neoepitopes detected by immunohistochemistry and by immunoassay. For the explant cultures, comparisons between two groups were done by paired t tests and for more than two groups by Kruskal-Wallis one-way ANOVA.

4. RESULTS

4.1 Polyclonal antibodies to mammalian collagenase-generated neoepitopes of collagen α1(II) TC^A and TC^B fragments

4.1.1 Direct-binding ELISAs for determination of antibody titres

Sera obtained from rabbits immunized with OVA conjugates of the neoepitope peptides and $F(ab')_2$ preparations of these sera were initially characterized by direct binding ELISAs. It should be noted that in both the immunohistochemical and immunochemical analyses, the $F(ab')_2$ preparations were used to ensure specific binding. Each of the anti-neoepitope antibodies reacted similarly with its own immunizing peptide (Fig. 4.1) which was conjugated to BSA and bound to the microtitre plates to improve immunoreactivity. None of the anti-neoepitope antibodies showed any significant immunoreactivity to BSA, native nor heat-denatured human type II collagen (Fig. 4.1), nor native nor heat-denatured human type I, III and X collagens (data not shown).

4.1.2 Inhibition ELISAs for neoepitope characterization

4.1.2.1 Defining the epitopes recognized by the antibodies

A panel of non-conjugated peptides with amino acid additions to (+1, +2, +3) and deletions from (-1, -2, -3) either the C-terminus of COL2-3/4C or the N-termini of COL2-1/4N1 and COL2-1/4N2 immunizing peptides were prepared. These were used in competitive ELISAs to determine the specific amino acid sequences recognized by each antibody and thereby verify that they were indeed anti-neoepitope antibodies. Removal or addition of one residue resulted in a loss of inhibition ranging from 80% to 100%,

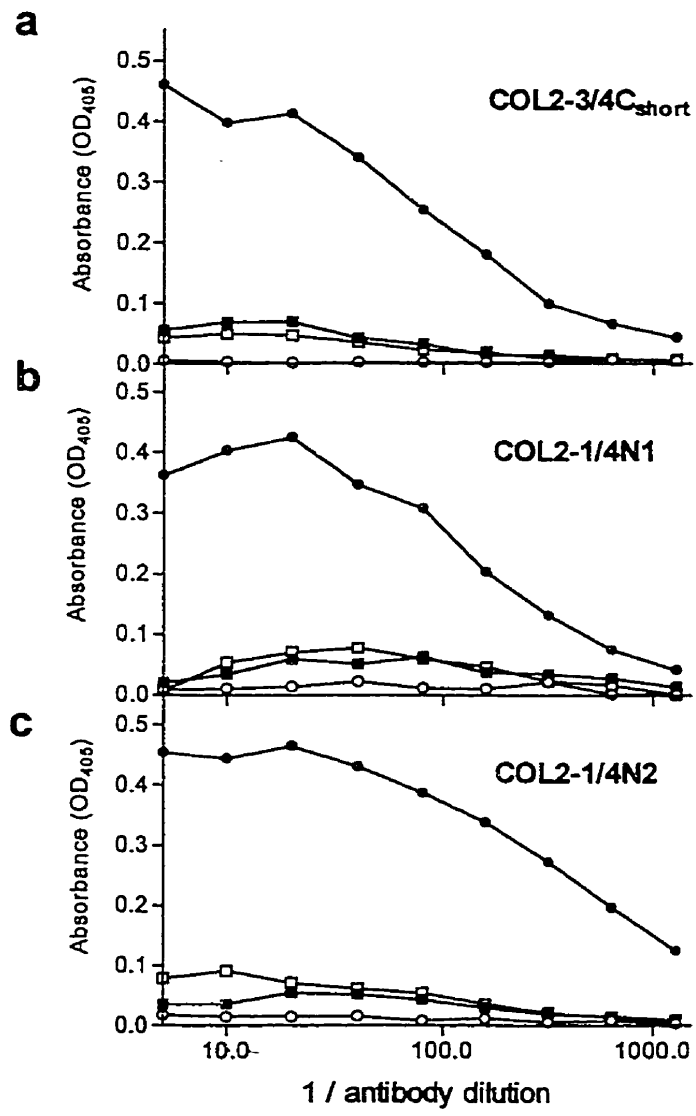


Figure 4.1 Immunoreactivities of collagenase anti-neoepitope F(ab')$_2$ antibody subunits with immunizing peptides and human type II collagen, as determined by direct-binding ELISA.

Immulon-2 microtitre plates were coated with 1 µg/well of each of the immunizing peptides conjugated to BSA (●). As well, plates were coated with 1 µg/well of BSA (○) or 2 µg/well of either triple helical (■) or heat-denatured (80°C for 20 min) (□) human type II collagens. After blocking unbound sites with BSA, serial dilutions of the collagenase anti-neoepitope F(ab')$_2$ preparations [in PBS-1% BSA-Tween (from 1:5 to 1:1280)] were added in duplicate to the wells of their respective plates and processed as described in section 3.1.3.2. Shown are the results of the ELISAs for the COL2-3/4C$_{short}$ (**a**), COL2-1/4N1 (**b**) and COL2-1/4N2 (**c**) F(ab')$_2$ preparations. Modified from Billinghurst, R.C. *et al.* (1997), with permission.

depending upon the peptide concentration, for the COL2-3/4C_{short} antibody (Fig. 4.2a) and complete loss of inhibition for the COL2-1/4N2 antibody (Fig. 4.2b),. For the COL2-1/4N1 antibody, it was only after three amino acids were deleted from the N-terminus (which is then the N-terminus of the COL2-1/4N2 peptide) that there was a complete loss of inhibition (Fig. 4.2c). This is significant in that it shows that the COL2-1/4N1 antibody will not recognize the amino acids comprising the N-terminus of the secondary cleavage site produced by mammalian collagenases (Fig. 3.1). For this reason antibody COL2-1/4N1 was only used to analyze the secondary cleavage of type II collagen by collagenase and was not employed in immunohistochemistry.

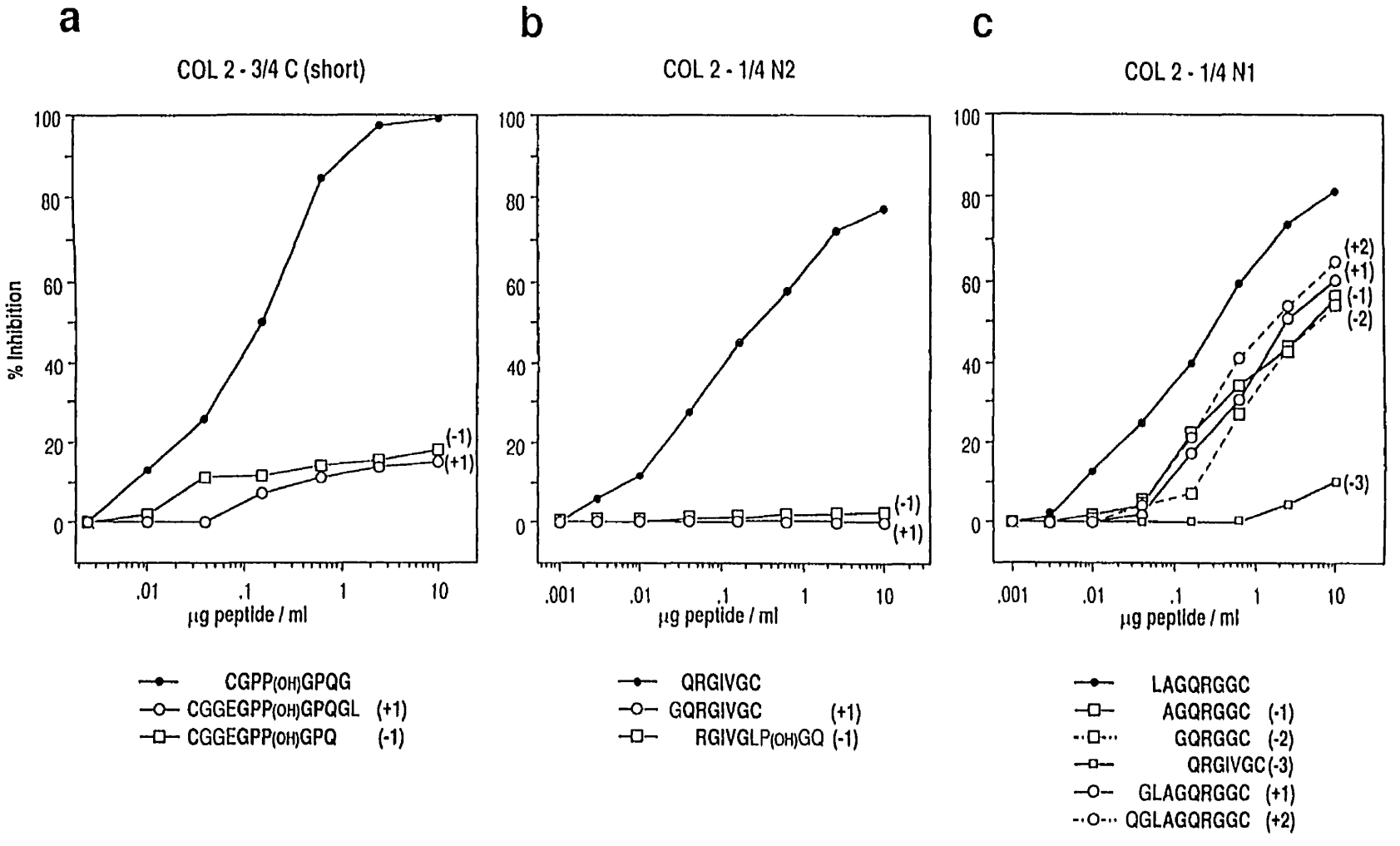
The two 16mer overlapping peptides designed to represent the amino acid sequences bridging both the primary and secondary collagenase cleavage sites of type II collagen α1 chains did not significantly inhibit any of the antibodies (data not shown).

4.1.2.2 Comparative reactivity of immunizing peptides and collagens

To further validate the specificity of the antibodies to the COL2-3/4C neoepitope, complete digests by MMP-1 and MMP-13 of collagen (verified by Coomassie staining of SDS-PAGE gels) and heat-denatured type I, II and III collagen solutions at concentrations of 29, 58, 115, 230, 460 and 920 μg/ml, were examined in inhibition ELISAs, along with the non-conjugated COL2-3/4C immunizing peptide. Typical standard curves plotted as the % inhibition against log of the competing antigen concentration (μg/ml) are shown in Figure 4.3a. Heat-denatured types I and II collagen showed no reactivity. Otherwise, positive immunoreactivity always exhibited excellent parallelity for each analysis. On a molar basis, the inhibition was very similar for MMP-1 and MMP-13 cleaved type II

Figure 4.2 Epitope specificities of the anti-neoepitope antibodies determined using competing synthetic peptides in an inhibition ELISA.

Varying dilutions of the non-conjugated immunizing peptides (●) were added to 1:200 dilutions of their respective $F(ab')_2$ preparations in 96 well round bottom microtitre plates for 1 h at 37°C. Then 50 µl was transferred from each well to the equivalent wells of Immulon-2 microtitre plates pre-coated with the respective immunizing peptides conjugated to BSA. The plates were then processed as described in section 3.1.3.3 for the development of a standard inhibition curve for each neoepitope antiserum. For the COL2-3/4C_{short} (**a**) and the COL2-1/4N2 (**b**) antibodies, shown are the results of ELISAs performed with synthetic peptides that had either 1 amino acid added to (○) or deleted from (□) the end of the immunizing peptide corresponding to the cleaved termini of the alpha chain fragments. The sequences of these peptides are shown in the individual legends for each anti-neoepitope antibody. For the COL2-1/4N1 antibody (**c**), residues were either added to (open circles) or deleted from (open squares) the N-terminus of the immunizing peptide. It was only when 3 residues were deleted from the N-terminus (▫) of the COL2-1/4N1 peptide and not 1 residue, as for the COL2-3/4C and COL2-1/4N2 peptides, that there was a complete loss of inhibition with the COL2-1/4N1 antibody. From Billinghurst, R.C. *et al.* (1997), with permission.


a
COL 2 - 3/4 C (short)
% Inhibition
100
80
60
40
20
0
.01
.1
1
10
µg peptide / ml
(-1)
(+1)
CGPP(OH)GPQG
CGGEGPP(OH)GPQGL (+1)
CGGEGPP(OH)GPQ (-1)
b
COL 2 - 1/4 N2
.001
.01
.1
1
10
µg peptide / ml
(-1)
(+1)
QRGIVGC
GQRGIVGC (+1)
RGIVGLP(OH)GQ (-1)
c
COL 2 - 1/4 N1
.001
.01
.1
1
10
µg peptide / ml
(+2)
(+1)
(-1)
(-2)
(-3)
LAGQRGGC
AGQRGGC (-1)
GQRGGC (-2)
QRGIVGC (-3)
GLAGQRGGC (+1)
QGLAGQRGGC (+2)

collagens and the COL2-3/4C peptide (Fig. 4.3b). The μM concentration of the COL2-3/4C epitope was calculated as described in section 3.1.3.3. Although there was parallelity between both the MMP-1 and MMP-13 cleaved type I collagen solutions and the COL2-3/4C peptide, the cleaved type II collagen was much more inhibitory than the cleaved type I collagen (Fig. 4.3b). There are two possible explanations for the disparity in immunoreactivity, despite the fact that the sequences in human type I and II collagen α chains for the five residues amino-terminal to the cleavage site are identical. We do not yet know how important hydroxylation is at Pro_{771}, nor do we know how important the threonine residue is at position 770 in the type I collagen α1 and α2 chains, which is a proline residue in the type II α1 chains.

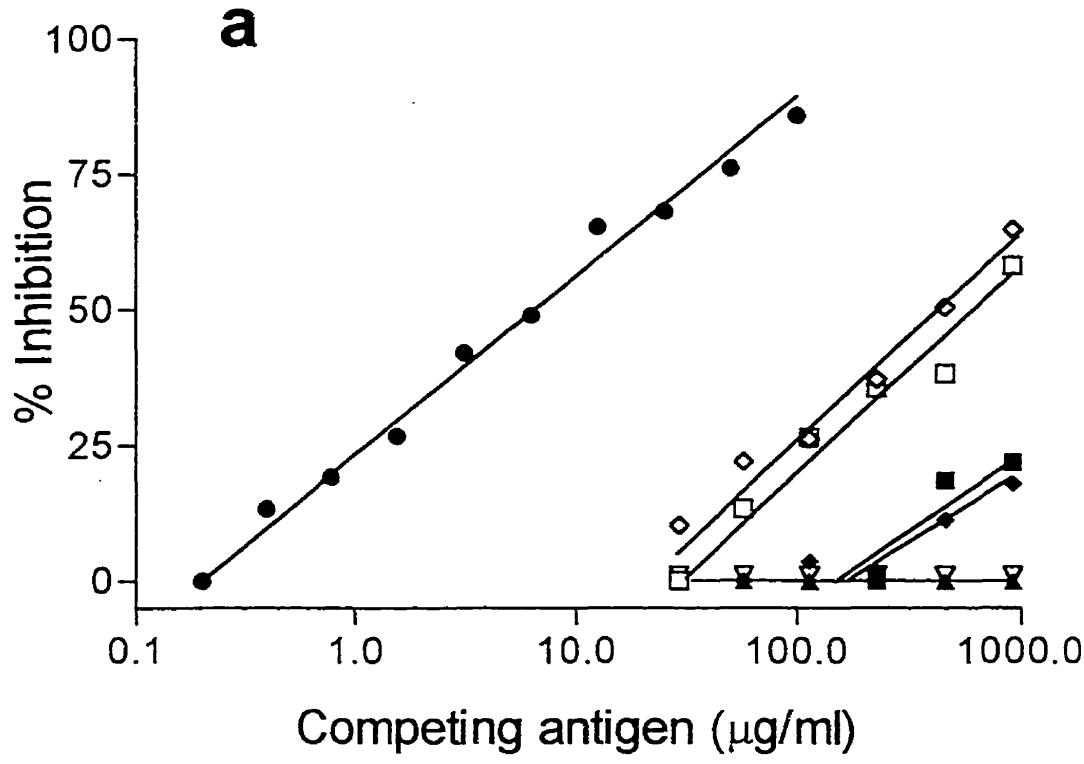
Neither the MMP-cleaved nor the heat-denatured type III collagen solutions showed inhibition (data not shown). The COL2-3/4C peptide was used as the standard in all subsequent ELISAs. Similar analyses for the antibodies to the COL2-1/4N2 epitope are not shown since they were not used in immunoassays in this study.

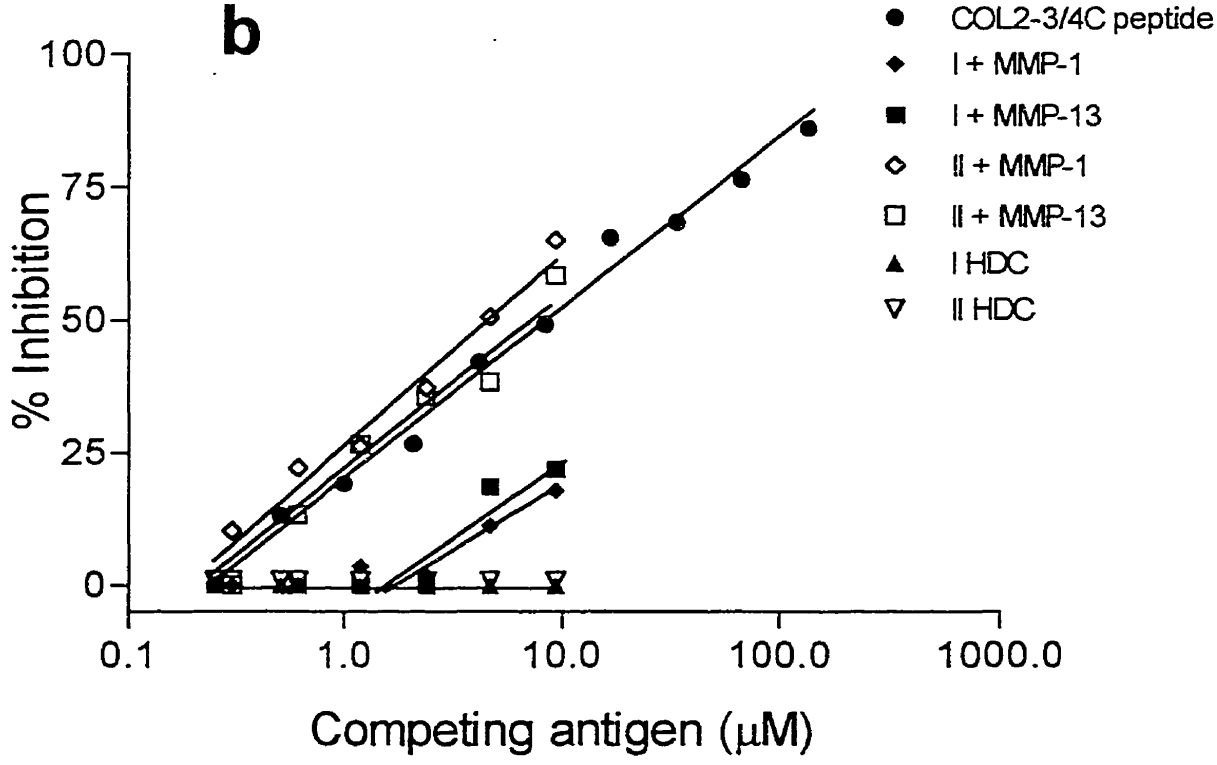
4.1.3 Sequential cleavages of type II collagen by collagenases

Acid soluble human type II collagen was cleaved with MMP-1 for up to 72 h, with aliquots removed and inactivated by the addition of EDTA at time 0, 0.5, 1, 3, 5, 24, 48 and 72 h. There was almost complete cleavage of the α1(II) chains by 5 h, as shown by Coomassie staining of SDS-PAGE separated digests (Fig. 4.4a). All the anti-neoepitope antibodies reacted with their respective cleaved fragments but showed no reactivity with α chains on Western blot analyses. The COL2-3/4C_{short} antibody stained both the single (αTC^A) and dimeric (βTC^A) α chain fragments created by MMP-1 cleavage of type II

Figure 4.3 Inhibition ELISA to show specificity of the COL2-3/4C antibody for the collagenase cleavage site C-terminal neoepitope of α1(II) collagen chains.

Heat-denatured and collagenase-cleaved (MMP-1 and MMP-13) human type I and II collagens (digestions were complete as shown by Coomassie staining of SDS-PAGE gels) were assayed in inhibition ELISAs at concentrations of 20, 58, 115, 230, 460 and 920 μg/ml of digestion buffer. A standard curve for the COL2-3/4C peptide (●) was also constructed using doubling dilutions from 100 to 0.1 μg of the peptide/ml of buffer in the same inhibition ELISA. Shown are typical standard curves plotted as % inhibition against the log of the competing antigen concentrations as μg/ml (**a**) and on a μM basis (**b**). Symbols show MMP-1 (◆, ◊) and MMP-13 (■, □) cleaved type I and type II collagens, respectively, and heat-denatured type I (I HDC; ▲) and type II (II HDC; ▽) collagens. From Billinghurst, R.C. *et al.* (1997), with permission.


a
100
75
50
25
0
% Inhibition
0.1
1.0
10.0
100.0
1000.0
Competing antigen (μg/ml)


b
100
75
50
25
0
% Inhibition
0.1
1.0
10.0
100.0
1000.0
Competing antigen (μM)
COL2-3/4C peptide
I + MMP-1
I + MMP-13
II + MMP-1
II + MMP-13
I HDC
II HDC

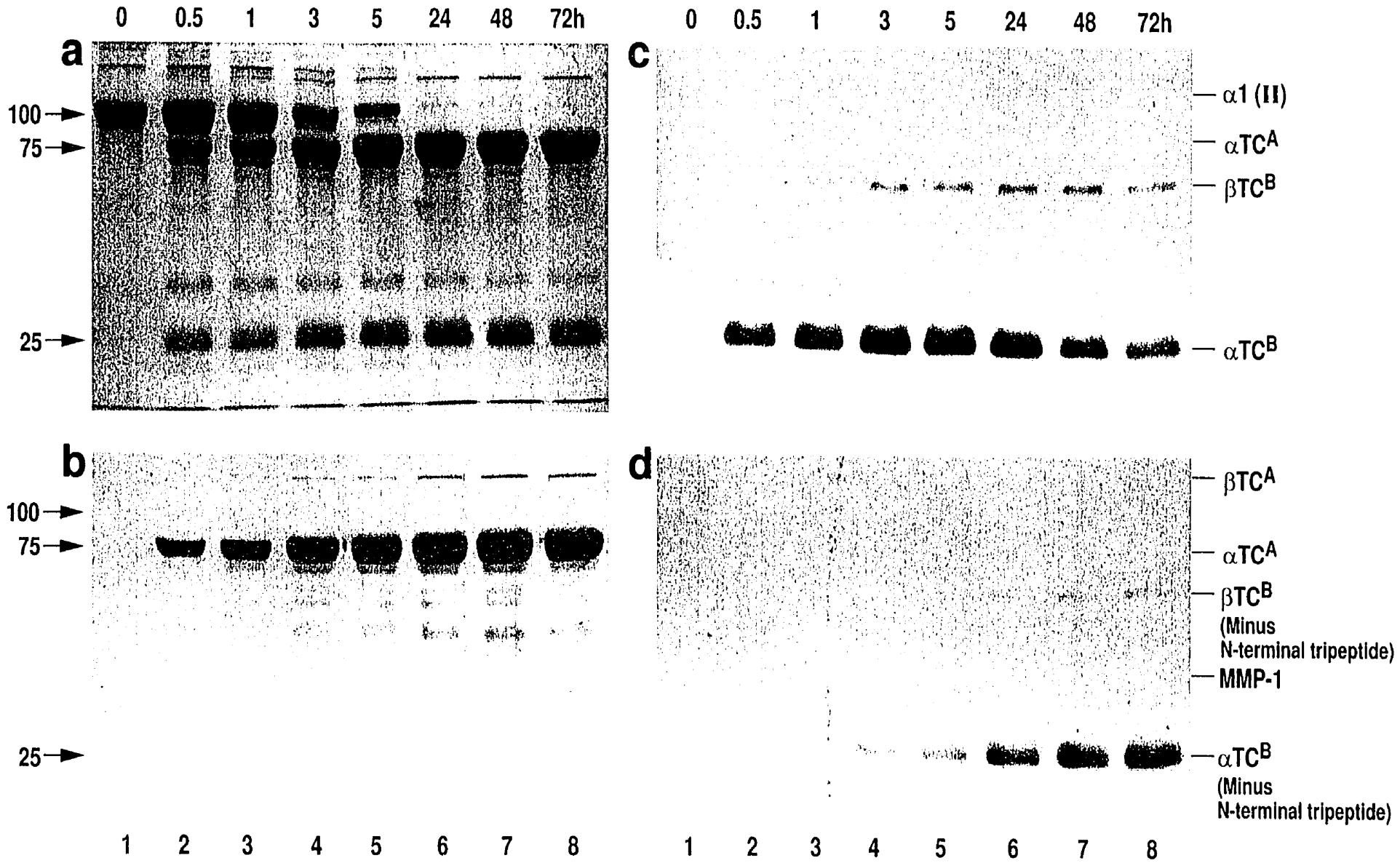
collagen with increasing intensity up to 48 h (Fig. 4.4b). The COL2-1/4N1 antibody stained both the single (αTC^B) and dimeric (βTC^B) fragments with increasing intensity up to 5 h and progressively declined thereafter (Fig. 4.4c). Moreover, the COL2-1/4N2 antibody first stained TC^B fragments weakly at 3 h and then with increasing intensity up to 72 h as the neoepitope COL2-1/4 N1 disappeared (Fig. 4.4d). This demonstrated a loss of the primary collagenase cleavage site neoepitope (COL2-1/4N1) and the appearance of the secondary cleavage site neoepitope (COL2-1/4N2) with MMP-1 digestion of type II collagen. This was confirmed by microsequencing (data not shown).

Type II collagen cleavage by MMP-8 was generally slower under the conditions of this study and the complete cleavage of $\alpha 1$(II) chains occurred only after 24 h of digestion (Fig. 4.5a). The COL2-3/4C_{short} antibody stained the TC^A fragments on Western blot analysis with increasing intensity up to 72 h (Fig. 4.5b). The COL2-1/4N1 antibody stained the TC^B fragment with increasing intensity up to 24 h and then progressively declined thereafter (Fig. 4.5c). The COL2-1/4N2 antibody stained TC^B fragments very weakly at 5 h and then increased in intensity up to 72 h, as the staining for the COL2-1/4N1 neoepitope started to weaken in intensity (Fig. 4.5d).

The primary cleavage of the $\alpha 1$(II) chains by MMP-13 was much faster than that by MMP-1 or MMP-8. Moreover, it was almost complete after only 30 min (Fig. 4.6a vs. 4.4a and 4.5a), confirming the results of recent studies comparing the rates of cleavage of type II collagens by MMP-1 and MMP-13 (Knauper *et al.*, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996). The primary cleavage site neoepitope (COL2-3/4C) was reduced in content after only 5 h digestion indicating further cleavage of the TC^A fragment (Fig 4.6b). Moreover, the N-terminal primary cleavage site neoepitope (COL2-1/4N1)

Figure 4.4 Time course of cleavage of triple helical human type II collagen by rHuMMP-1.

Purified human type II collagen was solubilized in digestion buffer and incubated at 30°C for 72 h with APMA-activated rHuMMP-1 at a final molar ratio of 1:5 (MMP-1:collagen). Aliquots were removed at 0, 0.5, 1, 3 5, 24, 48 and 72 h and the MMP-1 was inactivated in each sample by the addition of 20 mM EDTA (final concentration). The samples were separated under reducing conditions with SDS-PAGE (10%) and either stained with Coomassie Blue (**a**) or electrophoretically transferred to nitrocellulose and incubated overnight with 1:200 dilutions in PBS-3% BSA-Tween of COL2-3/4C_{short} (**b**), COL2-1/4N1 (**c**) and COL2-1/4N2 (**d**) anti-neoepitope F(ab')$_2$ preparations. Shown are undigested type II collagen (lane 1); type II collagen + APMA-activated MMP-1 for 0.5 (lane 2), 1 (lane 3), 3 (lane 4), 5 (lane 5), 24 (lane 6), 48 (lane 7) and 72 (lane 8) hours. Right margin indicates the positions of α1 (II) chains and the single (αTC^A) and dimeric (βTC^A) forms of the 3/4 and the single (αTC^B) and dimeric (βTC^B) forms of the 1/4 alpha chain fragments produced by MMP-1 cleavage of intact type II collagen. Left margin indicates the estimated molecular weights of the intact α1(II) chains (100 kD) and the αTC^A (75 kD) and αTC^B (25 kD) fragments of type II collagen. From Billinghurst, R.C. *et al.* (1997), with permission.


0 0.5 1 3 5 24 48 72h
a
100
75
25
0 0.5 1 3 5 24 48 72h
c
α1 (II)
αTC^A
βTC^B
αTC^B
b
100
75
25
d
βTC^A
αTC^A
βTC^B
(Minus
N-terminal tripeptide)
MMP-1
αTC^B
(Minus
N-terminal tripeptide)
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

60

Figure 4.5 Time course of cleavage of triple helical human type II collagen by rHuMMP-8.

The methodology used was as described for the rHuMMP-1 digestion (legend Fig.4.4). After SDS-PAGE (10%), gels were stained with Coomassie Blue (**a**) or the separated proteins were transferred to nitrocellulose and incubated overnight with 1:200 dilutions in PBS-3% BSA-Tween of COL2-3/4C_{short} (**b**), COL2-1/4N1 (**c**) or COL2-1/4N2 (**d**) anti-neoepitope $F(ab')_2$ preparations. Lanes and margins are as described in Figure 4.4. From Billinghurst, R.C. *et al.* (1997), with permission.

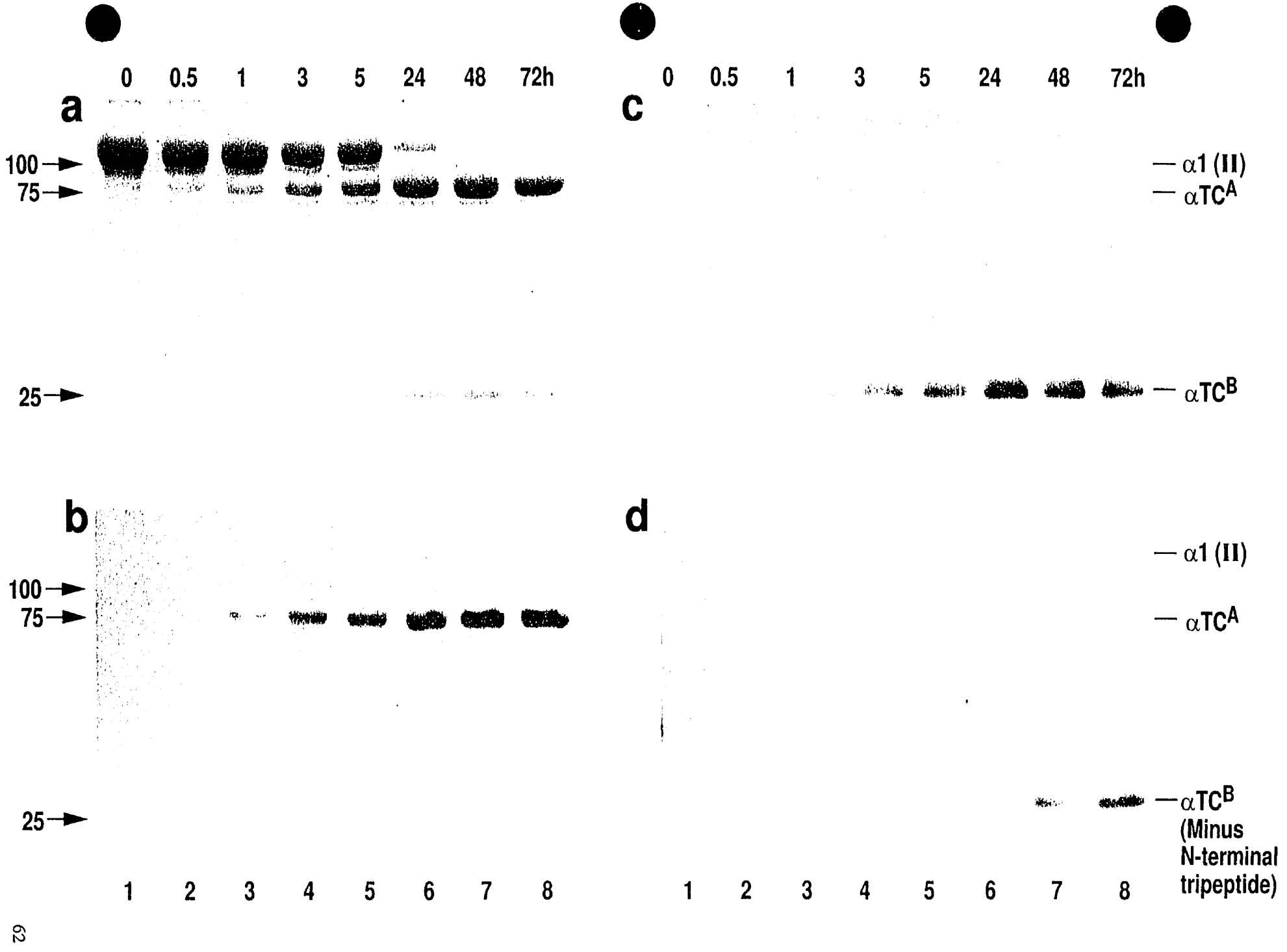
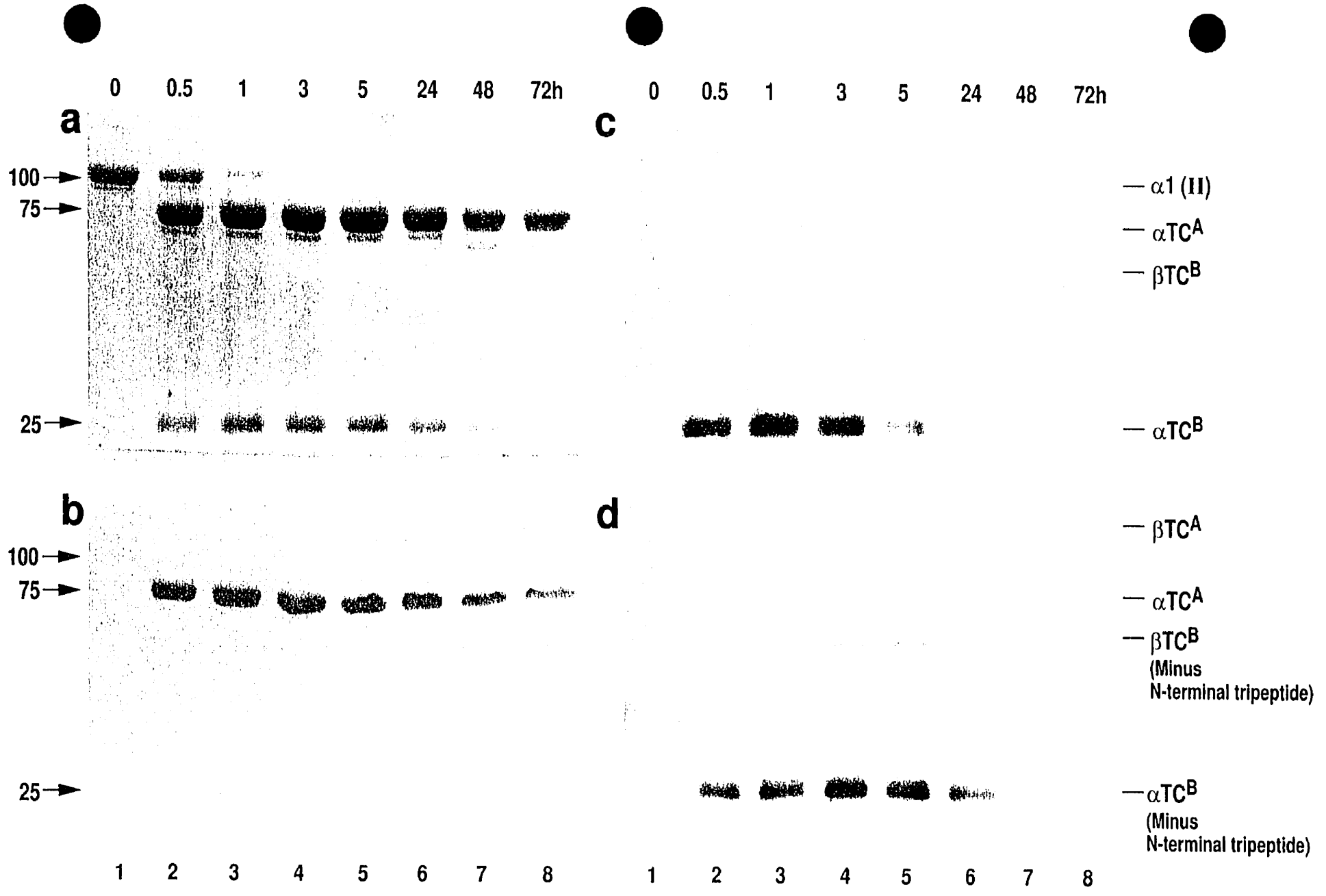

0 0.5 1 3 5 24 48 72h
a
100
75
25
c
— α1 (II)
— αTC^A
— αTC^B
b
100
75
25
d
— α1 (II)
— αTC^A
— αTC^B (Minus N-terminal tripeptide)
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

Figure 4.6 Time course of cleavage of triple helical human type II collagen by rHuMMP-13.

The methodology used was as described for rHuMMP-1 digestion (legend Fig. 4.4), except that a final molar ratio of 1:10 for MMP-13:collagen was used. After SDS-PAGE (10%), gels were stained with Coomassie Blue (**a**) or the separated proteins were transferred to nitrocellulose and incubated overnight with 1:200 dilutions in PBS-3% BSA-Tween of COL2-3/4C_{short} (**b**), COL2-1/4N1 (**c**) or COL2-1/4N2 (**d**) anti-neoepitope $F(ab')_2$ preparations. Lanes and margins are as described in Figure 4.4. From Billinghurst, R.C. *et al.* (1997), with permission.


0 0.5 1 3 5 24 48 72h
a
100
75
25
c
— α1 (II)
— αTC^A
— βTC^B
— αTC^B
b
100
75
25
d
— βTC^A
— αTC^A
— βTC^B
(Minus
N-terminal tripeptide)
— αTC^B
(Minus
N-terminal tripeptide)
1 2 3 4 5 6 7 8

increased up to 1 h but was then reduced and had disappeared after 3 h (Fig. 4.6c). In contrast, the secondary cleavage site neoepitope (COL2-1/4N2) was detectable within 30 min and increased up to 5 h before decreasing. After 24 h, it was no longer detectable (Fig. 4.6d).

This secondary cleavage produced by MMP-13 was recently described through the N-terminal sequencing of MMP-13 cleaved human type II collagen TC^B fragments (Mitchell *et al.*, 1996). To confirm this loss of the COL2-1/4N1 neoepitope (Fig. 4.6c), the MMP-13 digestion samples used for this immunoblot were assayed in an inhibition ELISA and no detectable COL2-1/4N1 epitope was found after 5 h of MMP-13 cleavage (data not shown). It is worth noting that the percentage recovery of this neoepitope, based on the amount of epitope present in the intact collagen, reached a maximum of only 21% after 60 min. This suggests a rapid removal of the N-terminal Leu-Ala-Gly tripeptide from the TC^B fragments. Further studies are in progress to compare, by immunoassay, the relative rates of digestion of type II and other collagens by MMP-1, MMP-8 and MMP-13.

None of the antibodies recognized the cyanogen bromide (CB) generated CB10 peptides of human type II collagen, which contain the intact collagenase cleavage site of triple helical collagen α1(II) chains (data not shown). This supports the ELISA results described above showing non-reactivity of the anti-neoepitope antisera for 16mer synthetic peptides representing the amino acid sequences bridging the primary and secondary collagenase cleavage sites in human type II collagen. Together these results confirm the classification of the COL2-3/4C_{short} and COL2-1/4N2 antibodies as anti-neoepitope antibodies recognizing only the termini of α1 chains of the TC^A and TC^B

fragments produced by the action of mammalian collagenases on human type II triple helical collagens.

All of the antibodies reacted to cleaved human type I collagen fragments produced by either MMP-1 (Fig. 4.7), MMP-8 or MMP-13 (not shown), confirming the results of the immunoassays that were mentioned above. None of the antibodies reacted to similarly cleaved fragments of human type III collagen. Type X collagen is a minor cartilage collagen that has recently been demonstrated in OA articular cartilage (Girkontaite *et al.*, 1996) and it is susceptible to cleavage by the mammalian collagenases. None of the anti-neoepitope antibodies showed reactivity to intact or MMP-1 cleaved alpha chain fragments of this collagen (data not shown).

4.2 Immunoanalyses of human normal and OA articular cartilages

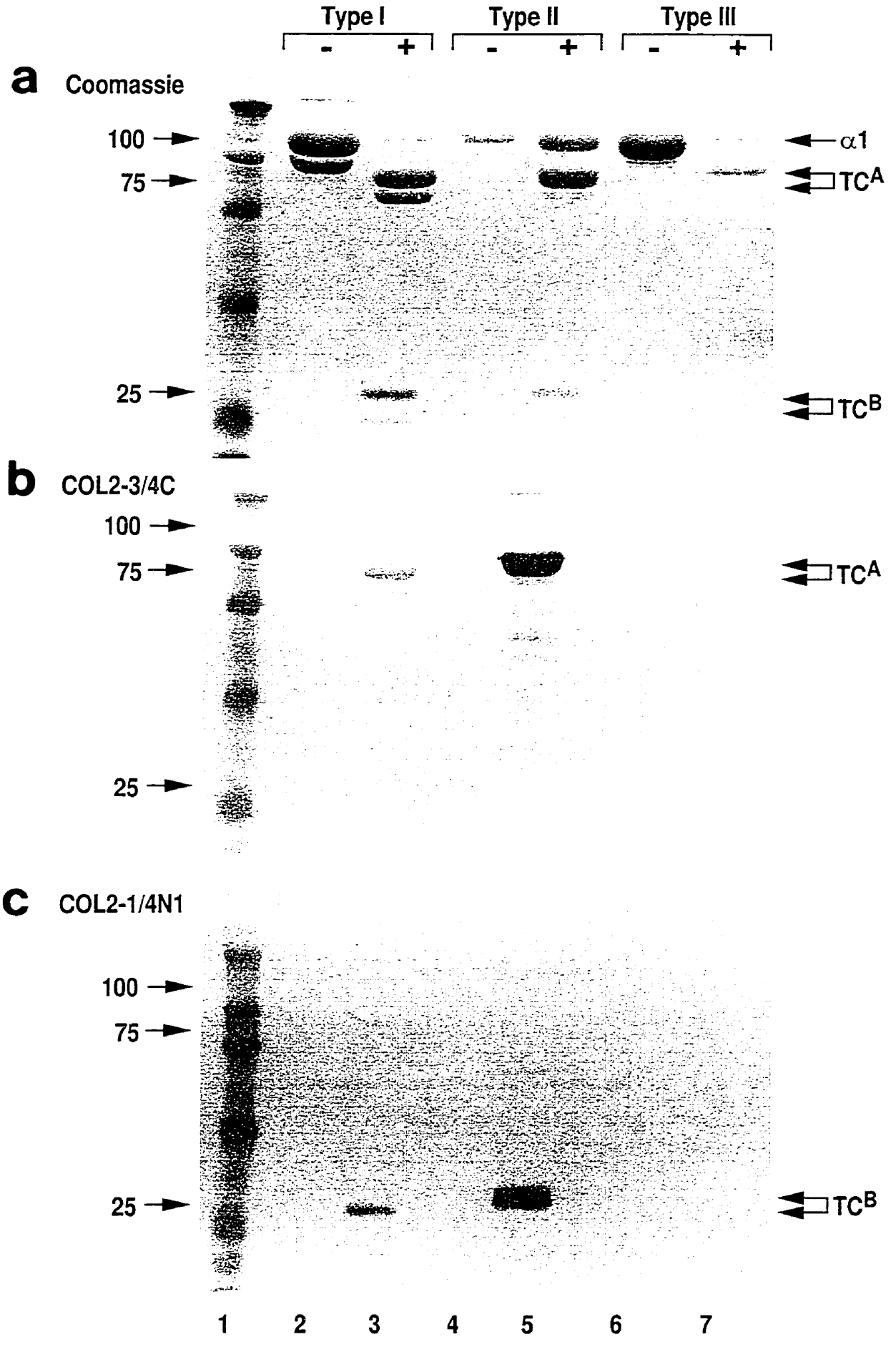
4.2.1 Immunohistochemical identification of cleavage site neoepitopes

Human articular femoral condylar cartilages from non-diseased (n = 15) and osteoarthritic (n = 10) joints were examined by immunohistochemistry for cleavage of type II collagen by mammalian collagenases using $F(ab')_2$ preparations of the COL2-3/4C_{short} and COL2-1/4N2 antibodies. The results obtained with immunostaining using the COL2-1/4N1 antibody are not reported here in view of the limited specificity of this antibody. The non-arthritic cartilage specimens were further arranged by age of patient into a young (20-41 years, n = 7) and an old (44-69 years, n = 8) group.

Shown in Figure 4.8 are immunostained femoral condylar articular cartilage sections from 2 non-arthritic joints (Mankin grades 1 and 2), which provide examples of staining of articular cartilage in the young (Fig. 4.8A, B and C) and old (Fig. 4.8D, E and

Figure 4.7 Comparative reactivities of the COL2-3/4C$_{short}$ and COL2-1/4N1 antibodies for MMP-1 cleaved human type I, II, and III collagens.

Purified acid-soluble triple helical collagen types I, II, and III were incubated with (+) or without (-) rHuMMP-1, as described in the legend of Fig. 4.4. Shown are the Coomassie blue stained SDS-PAGE (10%) gel (**a**), and the Western blots for COL2-3/4C (**b**) and COL2-1/4N1 (**c**) cleavage site neoepitopes. The lanes are molecular weight standards (lane 1), non-cleaved types I (lane 2), II (lane 4) and III (lane 6) collagens, and cleaved types I (lane 3), II (lane 5), and III (lane 7) collagens. The margins are as marked in Fig. 4.4.


Type I
Type II
Type III
- + - + - +
a Coomassie
100
75
25
α1
TC^A
TC^B
b COL2-3/4C
100
75
25
TC^A
c COL2-1/4N1
100
75
25
TC^B
1 2 3 4 5 6 7

F) groups. In the young (20 yr) non-arthritic articular cartilage specimen, there is evidence of type II collagen cleavage by collagenase pericellularly (Fig. 4.8B and C), especially in more superficial sites, with no evidence of collagen denaturation (Fig. 4.8A). In the older (68 yr) non-arthritic articular cartilage, there is more evidence of collagen cleavage and denaturation. The collagen denaturation shows similar staining intensity in a primarily pericellular location especially in more superficial sites, (Fig. 4.8D) to that for the COL2-3/4C neoepitope (Fig. 4.8E), but there is much less staining for the COL2-1/4N2 neoepitope (Fig. 4.8F). These differences probably reflect subsequent cleavages of the TC^A and TC^B fragments leading to differential loss of epitopes, as noted above in the analysis of purified type II collagen digestions.

In the less severe OA articular cartilage specimen (Fig. 4.9A-F; Mankin grade 6), there is similar localization and intensity of immunostaining for both the COL2-3/4C_{short} (Fig. 4.9B) and COL2-1/4N2 (Fig. 4.9C) antibodies as that for collagen denaturation, visualized with the COL-3/4m antibody (Fig. 4.9A). As described recently (Hollander *et al.*, 1995), there is usually a marked reduction or absence of staining for denatured collagen in these superficial sites in OA. This is probably due to excessive collagen cleavage in the superficial region of the cartilage matrix and loss of these molecules, as shown previously (Hollander *et al.*, 1994). Moreover, as also reported in Hollander *et al.* (1995), there is an increase in pericellular and territorial staining for denaturation in the middle and usually also the deep zones of OA cartilage, as compared to the non-arthritic cartilage specimens (Fig. 4.9A vs. 4.8A and D). This pattern of staining was also observed for collagen cleavage by collagenase. In the more severely affected OA articular cartilage (Fig. 4.9G-L; Mankin grade 10), the collagenase-generated neoepitopes

Figure 4.8 Immunostaining of non-arthritic femoral condylar articular cartilages to detect collagenase cleavage site neoepitopes and denatured type II collagen.

Specimens in (**A**), (**B**) and (**C**) are serial sections of articular cartilage from a 20 year old, Mankin grade 1; specimens in (**D**), (**E**) and (**F**) are serial sections from a 68 year old, Mankin grade 2. Sections (**A**) and (**D**) are stained with the COL2-3/4m monoclonal antibody detecting type II collagen denaturation; (**B**) and (**E**) are stained with the COL2-3/4C_{short} polyclonal antibody detecting the C-terminal neoepitope on the TC^A α1(II) fragments created by mammalian collagenase (MMP-1, MMP-8 and MMP-13) digestion of triple helical type II collagen molecules; and (**C**) and (**F**) are stained with the COL2-1/4N2 polyclonal antibody detecting the N-terminal neoepitope on the TC^B α1 (II) fragment created by secondary collagenase cleavage of triple helical type II collagen molecules. There is the appearance of pericellular staining by COL2-3/4m (**A** vs. **D**) and an increase in staining by COL2-3/4C_{short} (**B** vs. **E**) antibodies, as well as a decrease in staining by the COL2-1/4N2 antibody (**C** vs. **F**) within the cartilage sections from the older individual. The articular surface is at the top of each figure. Using section **E** as an example of how the immunostaining was graded, the superficial and middle zones were rated 3 (pericellular 2 + territorial 1 + interterritorial 0) and the deep zone was rated 2 (pericellular 2 + territorial 0 + interterritorial 0). Bar = 100 nm. Preparation and immunostaining of specimens were performed by Isabelle Pidoux, Joint Diseases Laboratory, Shriners Hospital, Montreal.

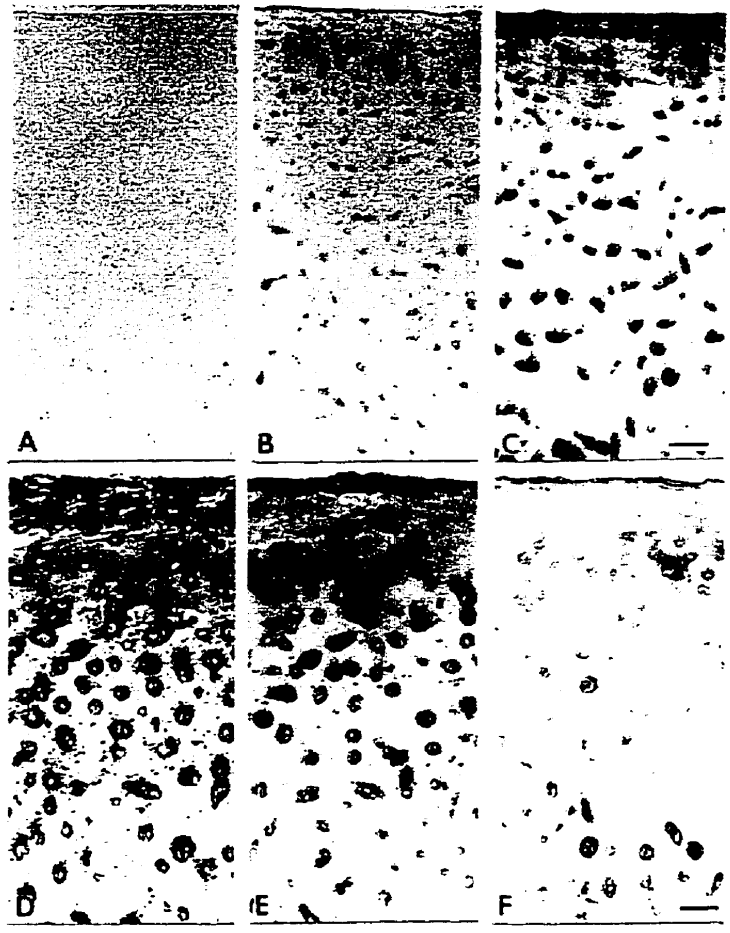
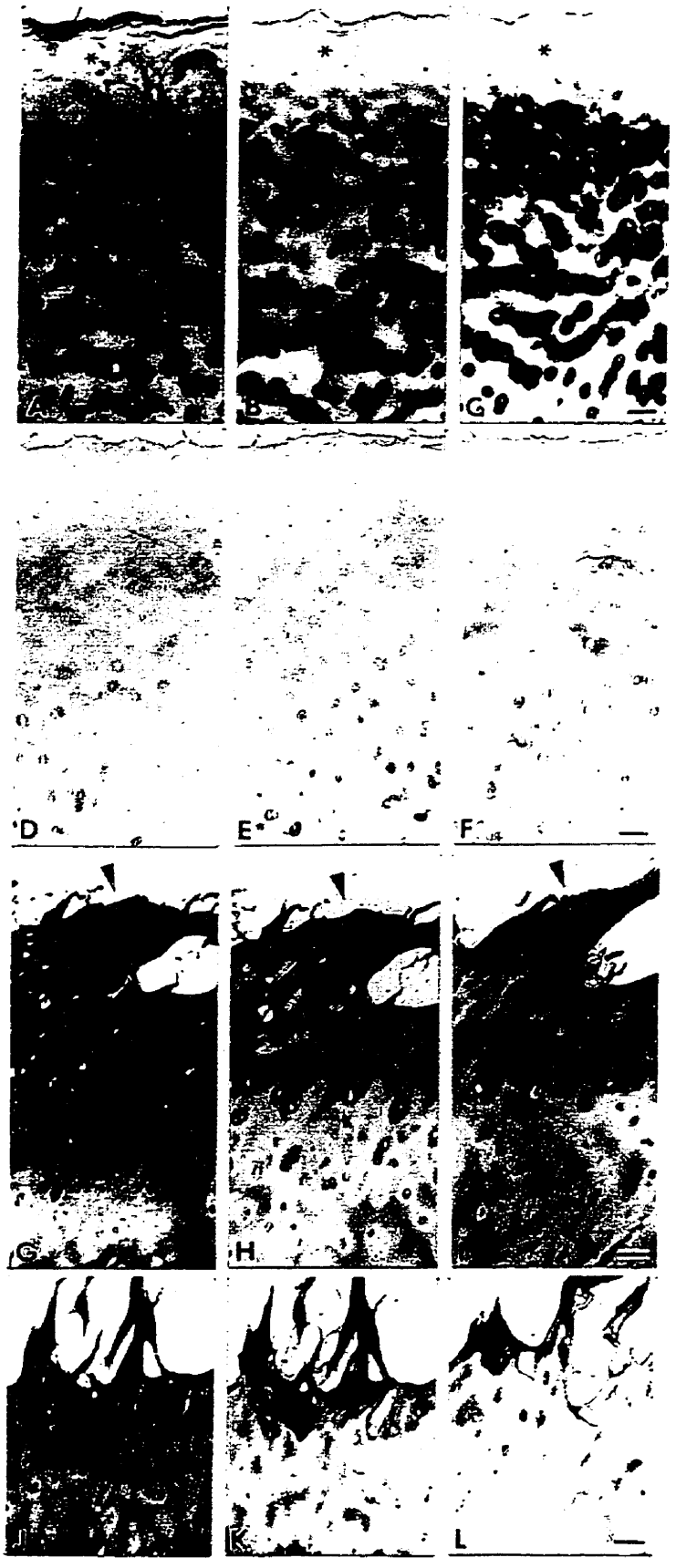

A
B
C
D
E
F

Figure 4.9 Immunostaining of osteoarthritic femoral condylar articular cartilages to detect collagenase cleavage site neoepitopes and denatured type II collagen.

Shown in A-F are serial sections of articular cartilage from a 76 year old, Mankin grade 6. Sections A and D are stained with the COL2-3/4m antibody without (**A**) and with (**D**) preincubation of the antibody with the immunizing peptide before staining; Sections B and E are stained with the COL2-3/4C_{short} antibody without (**B**) and with (**E**) preincubation with the COL2-3/4C immunizing peptide; and sections C and F are stained with the COL2-1/4N2 antibody without (**C**) and with (**F**) preincubation of the antibody with the COL2-1/4N2 immunizing peptide. In A, B and C, the superficial cartilage matrix shows little staining (asterisk) but there is intense pericellular staining by all antibodies throughout the middle and deep regions of the cartilage matrix. Staining is essentially abolished for all antibodies by preincubation with their respective immunizing peptides. Specimens G-L (58 year old, Mankin grade 10) show more advanced degeneration and fibrillation. Sections G, H and I are from one site and J, K and L are from another site of the same femoral condylar articular cartilage. Sections G and J are stained with the COL2-3/4m antibody detecting type II collagen denaturation. H and K are stained with the COL2-3/4C_{short} anti-neoepitope antibody; and I and L are stained with the COL2-1/4N2 anti-neoepitope antibody. There is intense staining of the fibrillated articular surface (indicated by arrowhead) by all antibodies and more diffuse staining extending down from this into the matrix with the COL2-3/4m antibody (**G**), which is less obvious with the COL2-3/4C_{short} (**H**) or COL2-1/4N2 (**I**) antibodies. Using section **B** as an example of how the immunostaining was graded, the superficial zone was rated 0 (no staining), the middle zone was rated 3 (pericellular 2 + territorial 1 + interterritorial 0), and the deep zone was rated 4 (pericellular 2 + territorial 2 + interterritorial 0). Bar = 100 nm. Preparation and immunostaining of specimens were performed by Isabelle Pidoux, Joint Diseases Laboratory, Shriners Hospital, Montreal.


A
B
C
D
E
F
G
H
J
K
L

are restricted to more superficial sites (Fig. 4.9H, I, K, L). Type II collagen denaturation is also observed deeper in the cartilage (Fig. 4.9G and J). The specificities of immunoreactivity of all the antibodies are shown by the loss of staining after pre-incubation of each antibody with its respective immunizing peptide (Fig. 4.9D, E and F), which was not observed after pre-incubation with other unrelated peptides (data not shown).

These results were analyzed by immunohistochemical grading for collagenase-generated cleavage site neoepitopes and for the type II collagen intrachain epitope, as described in the Methods, and summarized in Table 4.1, where the summary scores for staining in superficial (S), middle (M) and deep (D) zones are shown. There were significant differences between the OA and non-arthritic articular cartilage sections for immunostaining with the COL2-3/4C_{short} and COL2-1/4N2 anti-neoepitope antibodies. Significant differences were noted for immunostaining with both anti-neoepitope antibodies when comparing the OA with the non-arthritic articular cartilage sections for all the zones combined ($P = 0.013$ for COL2-3/4C_{short}; $P = 0.0004$ for COL2-1/4N2). When the immunostaining was analyzed for each of the three zones of the articular cartilage, significant increases in grading scores were noted for both the COL2-3/4C ($P = 0.041$) and the COL2-1/4N2 ($P = 0.028$) neoepitopes in the OA versus the older non-arthritic articular cartilages, in the deep zone only. Moreover, for the young non-arthritic versus OA cartilages, there was a significant increase in immunostaining score for the COL2-1/4N2 neoepitope in all three regions ($P = 0.032$ for S; $P = 0.027$ for M; $P = 0.01$ for D). This difference in immunostaining for the young vs. OA groups was not observed for the COL2-3/4C neoepitope.

Table 4.1 Summary of immunohistochemical analyses of non-arthritic and osteoarthritic (OA) articular cartilages for collagenase-generated cleavage site neoepitopes, COL2-3/4C and COL2-1/4N2, and the COL2-3/4m intrachain epitope detecting denaturation of type II collagen.

Age (years)	Mankin Grade	Zone	COL2-3/4m	COL2-3/4C	COL2-1/4N2
Non-arthritic (young)					
20-41 (32)	0-2 (1)	S	0-5 (2)	0-3 (1)	0-2 (0) *
n = 7		M	0-4 (1)	0-4 (2)	0-2 (0) *
		D	0-4 (1)	1-2 (2)	0-1 (1) *
Range (median):		all zones	0-5 (1)	0-4 (2)	0-2 (0) **
Non-arthritic (old)					
44-69 (65.5)	1-4 (2.5)	S	2-4 (4)	0-3 (1)	0-4 (1)
n = 8		M	0-4 (3.5)	0-3 (1.5)	1-4 (1.5)
		D	0-4 (2.5)	0-3 (0.5) *	0-3 (0) *
Range (median):		all zones	0-4 (3.5)	0-3 (1) *	0-4 (1) *
Osteoarthritic					
44-83 (68.5)	1-10 (6.5)	S	0-5 (3)	0-4 (3)	0-4 (3)
n = 10		M	0-6 (3)	0-5 (2)	0-4 (2.5)
		D	0-6 (3)	0-6 (2.5)	0-6 (2)
Range (median):		all zones	0-6 (3)	0-6 (3)	0-6 (2.5)

Serial sections of femoral condylar articular cartilage from non-arthritic (n = 15) and OA (n = 10) joints were stained with the COL2-3/4C$_{short}$ and COL2-1/4N2 anti-neoepitope antibodies and the COL2-3/4m intrachain antibody. The intensity and location of immunostaining were graded as described in section 3.2.1.2 and shown in Fig. 3.3. Shown are the ranges (medians in brackets) of the ages of the patients from which the samples were retrieved within each grouping (normal young, normal old and OA), the Mankin grade of the cartilage sections examined, and the scores for the COL2-3/4C, COL2-1/4N2, and COL2-3/4m immunostaining of each of the three zones, as well as all zones combined. Significant differences in overall immunostaining scores for the non-arthritic versus corresponding OA cartilages or within zones were determined by Mann-Whitney analyses and are indicated as * $P < 0.05$ and ** $P < 0.001$.

When comparing staining grades obtained with the COL2-3/4m antibody in different sites between non-arthritic and OA articular cartilages, no significant differences were noted. This appears to contradict the findings of our previous immunohistochemical study comparing non-arthritic and OA human articular cartilages, in which type II collagen denaturation, as determined using the same COL2-3/4m antibody, was directly related to Mankin grade in OA (Hollander *et al.*, 1995). However it is important to note that the grading systems used to score the immunohistochemical staining in that study differed from that utilized in the present work. In the previous study (Hollander *et al.*, 1995), grading of immunohistochemical staining for type II collagen denaturation was based on the increase and progression of staining from the articular cartilage surface to the deeper regions of the cartilage matrix. In this most recent study, we chose to grade both the intensity and location (pericellular, territorial and interterritorial) of immunostaining, separately, within each of three regions of the articular cartilage matrix (superficial, middle and deep). This allowed us to evaluate zonal variations and correlations in type II collagen cleavage and denaturation. Thus, it is not possible to compare the results of the grading of the immunostaining with the COL2-3/4m antibody in these two studies, as the grading systems were designed in each to investigate different aspects and interrelationships of type II collagen degradation.

Spearman rank correlations analyses were used to show significant correlations in the immunostain grading scores of COL2-3/4C and COL2-1/4N2 neoepitopes in all regions of the OA cartilages ($r = 0.915$, $P = 0.016$ for superficial; $r = 0.804$, $P = 0.016$ for middle; $r = 0.808$, $P = 0.015$ for deep) , but only in the non-arthritic cartilages in the deep regions of older individuals ($r = 0.834$, $P = 0.027$). Moreover, there were significant

correlations between the immunostaining scores for both COL2-3/4C and COL2-1/4N2 with COL2-3/4m for all regions (combined) in the OA cartilages ($r = 0.445$, $P = 0.025$ for COL2-3/4C and $r = 0.486$, $P = 0.012$ for COL2-1/4N2) and between COL2-3/4C and COL2-3/4m in the non-arthritic cartilages from the younger individuals ($r = 0.545$, $P = 0.015$). For the non-arthritic articular cartilage sections from the older individuals, there was more type II collagen denaturation throughout all regions of the matrix than there was cleavage by collagenase, based on the immunostaining scores (Table 4.1). As a result, there were no significant correlations between COL2-3/4m denaturation epitope and either the COL2-3/4C or COL2-1/4N2 cleavage site neoepitopes in the older non-arthritic samples.

4.2.2 Immunochemical identification of cleavage site neoepitopes

Human articular cartilage from the femoral condyles of 26 OA (Mankin grade 3-12) and 19 non-arthritic (Mankin grade 1-5) joints were treated with α-chymotrypsin to extract denatured type II collagen and to assay for COL2-3/4C neoepitope content. The median of the levels of COL2-3/4C epitope extracted from the OA specimens was significantly higher ($P = 0.0002$) than that of the non-arthritic articular cartilages (Fig. 4.10). Moreover, there were significant correlations found for both the non-arthritic ($r = 0.503$, $P = 0.028$; Fig 4.11a) and the OA ($r = 0.536$, $P = 0.0048$; Fig 4.11b) samples, when the collagenase-generated neoepitope (expressed as a percentage of total collagen present) was compared to the percentage denatured collagen, based on COL2-3/4m intrachain epitope content in each extract, These observations independently confirm the results of the immunohistochemical analyses reported above (Table 4.1), where the immunostaining

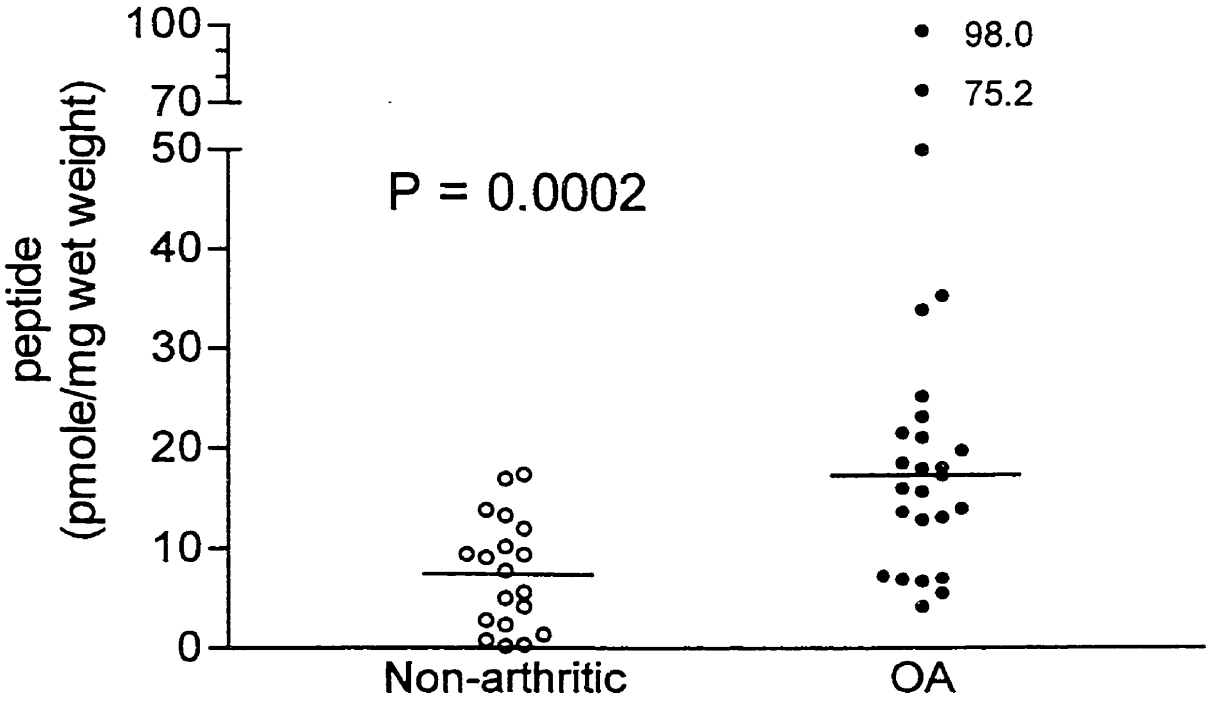
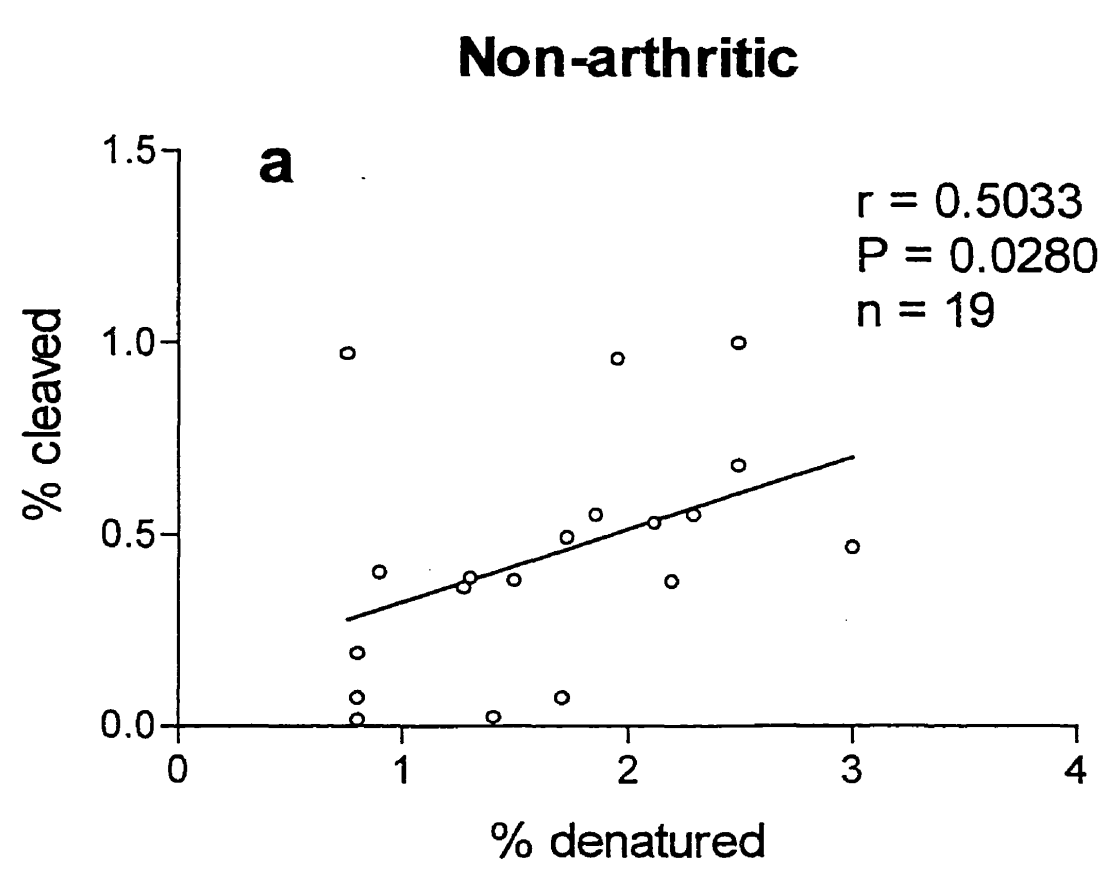


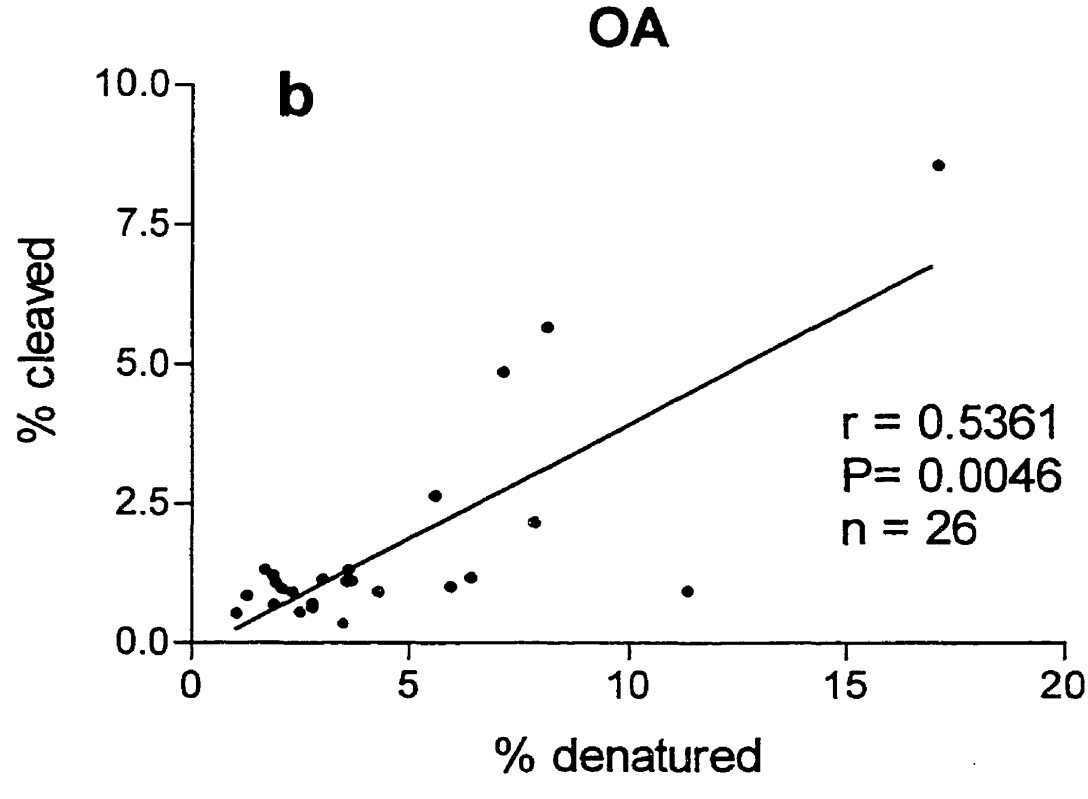
Figure 4.10 Levels of COL2-3/4C neoepitope in α-chymotrypsin extracts of human articular cartilages.

Articular cartilage was removed from the femoral condyles of 19 non-arthritic (patient ages: 20-69 yr; median, 60 yr) and 26 OA (patient ages: 44-85 yr; median 71 yr) joints and digested overnight at 37°C with 1 mg/50-75 mg wet weight of α-chymotrypsin in 50 mM Tris-HCl, pH 7.6 (+ the proteinase inhibitors: 1 mM EDTA, 1 mM iodoacetamide, 10 μg/ml pepstatin A). After inhibition of enzyme activity with 160 μg/ml TPCK, the samples were centrifuged and the supernatants were assayed for COL2-3/4C neoepitope as discussed in section 3.1.3.3. The levels of the neoepitope are expressed as pmole of peptide (based on a molecular weight of 608 D) per mg wet weight of cartilage. The absolute values of the two highest neoepitope concentrations for the OA extracts are shown in the figure. A significant difference between the median of neoepitope content in the non-arthritic (○) and the OA (●) cartilages was determined by Mann-Whitney analysis and the P value is shown in the figure. From Billinghurst, R.C. *et al.* (1997), with permission.

Figure 4.11 Correlation between the percent cleaved and the percent denatured type II collagen in α-chymotrypsin extracts of human articular cartilages.

The same articular cartilage specimens digested with α-chymotrypsin and described in the legend of Fig. 4.10, were further digested overnight at 56°C with 1 mg proteinase K in Tris-HCl, pH 7.6 (+ proteinase inhibitors) and then boiled for 20 minutes to inactivate the enzyme. The total type II collagen content was determined from the amount of COL2-3/4m epitope assayed in both the α-chymotrypsin extracts and the proteinase K digests, as described in the section 3.2.2.2. The percent denatured collagen represented the amount of COL2-3/4m and the percent cleaved collagen was the amount of COL2-3/4C in the α-chymotrypsin extracts and both are expressed as a percentage of the total type II collagen. Statistically significant relationships between percent cleaved and percent denatured type II collagen in (**a**) the non-arthritic (○) and (**b**) the OA (●) articular cartilage specimens were determined by Spearman rank correlational analysis and the corresponding n, r and P values are shown in the figure. From Billinghurst, R.C. *et al.* (1997), with permission.


Non-arthritic
a
r = 0.5033
P = 0.0280
n = 19
% cleaved
% denatured
1.5
1.0
0.5
0.0
0
1
2
3
4


OA
b
r = 0.5361
P= 0.0046
n = 26
% cleaved
% denatured
10.0
7.5
5.0
2.5
0.0
0
5
10
15
20

scores, when combined for all zones graded, showed significant correlations between COL2-3/4C and COL2-3/4m epitopes in the OA and in the young (< 42 yr) non-arthritic cartilages. Moreover, the content of intrachain epitope was about twice that of the collagenase-generated neoepitope (by immunoassay) suggesting an increased half-life of the former in both groups of cartilages.

No significant correlations (by Spearman rank analyses) were noted between Mankin grade and either percentage cleaved or percentage denatured collagen (data not shown). However, it is interesting to note that there was a tendency towards an increase in percentage collagenase cleaved collagen with increasing age ($P = 0.054$), that may reach significance with the sampling of more specimens.

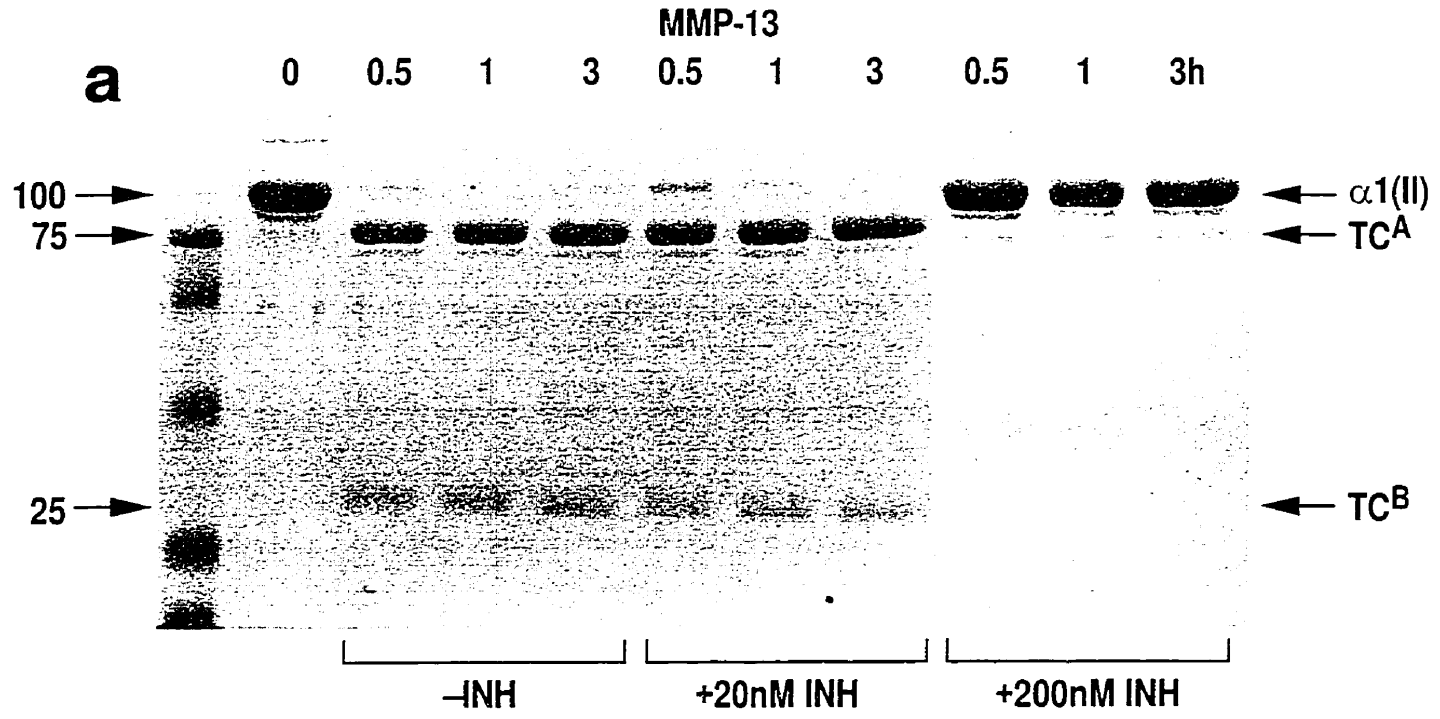
4.3 The effects of selective MMP inhibition

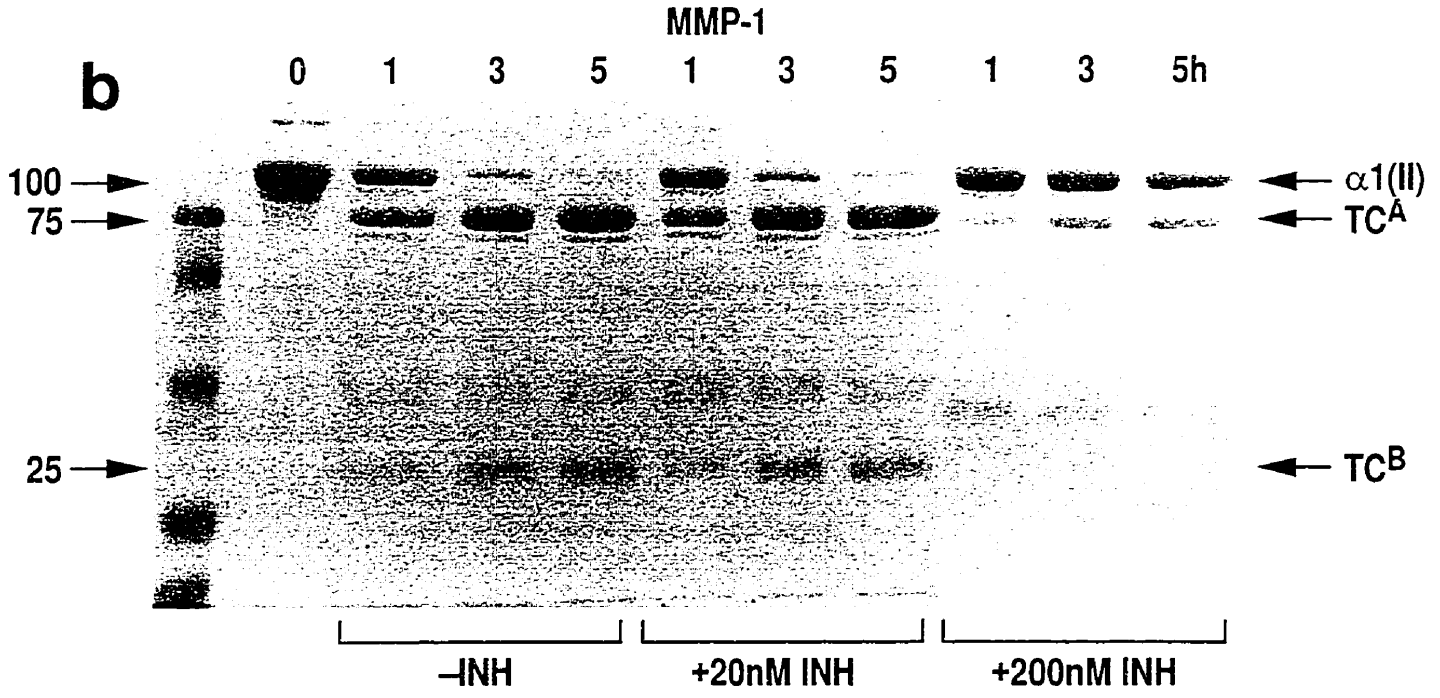
4.3.1 Cleavage of purified type II collagen by MMP-1 and MMP-13

The concentrations chosen for the synthetic inhibitors used in this study were based on the K_i values resulting from the inhibitory profiles of each compound in the enzymatic digestion of synthetic substrates (Table 3.1). The use of the broad-spectrum inhibitor RS 47,112 at 20 nM represents a concentration 9-fold and 133-fold higher than its K_i values for stromelysin and gelatinase, respectively. Moreover, based on its inhibitory profile, this peptide should not allow for the differential inhibition of the three collagenases, MMP-1, MMP-8, and MMP-13. This was confirmed by a similar lack of effect at 20 nM and a significant inhibition at 200 nM of RS 47,112 on the cleavage of purified type II collagen by both MMP-1 and MMP-13 (Fig. 4.12).

Figure 4.12 The effect of inhibitor RS 47,112 on the cleavage of human type II collagen by MMP-1 and MMP-13.

Purified acid-soluble triple helical human type II collagen was digested with APMA-activated rHuMMP-1 or rHuMMP-13, both in the absence and presence of 20 or 200 nM concentrations of synthetic inhibitor RS 47,112 (Roche Bioscience, Palo Alto, CA). An appropriate volume of inhibitor was added to aliquots of activated MMP-1 and MMP-13 for 10 min before adding both to the type II collagen solutions. The final molar ratio of enzyme:collagen was 1:10. Aliquots were removed at 0.5, 1, and 3 h for MMP-13, and at 1, 3, and 5 h for MMP-1, and both enzymes were inactivated by the addition of 20 mM EDTA. Shown are the Coomassie blue stained SDS-PAGE (10%) gels for the digestion with MMP-13 (**a**) and MMP-1 (**b**). The incubation times, with and without the inhibitor, are indicated across the top of each gel. The lanes are grouped and indicated at the bottom of the gels as no inhibitor (-INH), 20 nM inhibitor (+20 nM INH), and 200 nM inhibitor (+200 nM INH). The right hand margin indicates the locations of intact type II collagen α chains (α1(II)) and the collagenase-generated 3/4 (TC^A) and 1/4 (TC^B) α chain fragments.


MMP-13
a
0
0.5
1
3
0.5
1
3
0.5
1
3h
100
75
25
α1(II)
TC^A
TC^B
–INH
+20nM INH
+200nM INH


MMP-1
b
0
1
3
5
1
3
5
1
3
5h
100
75
25
α1(II)
TC^A
TC^B
–INH
+20nM INH
+200nM INH

RS 102,481 is a preferential inhibitor of MMP-13 at the 1 nM and 10 nM concentrations chosen for use in this study. These concentrations are 12.5 and 125 fold higher, respectively, than the K_i values for MMP-13, and 10^{-3} and 10^{-2} higher than the K_i for MMP-1. Using purified type II collagen as the substrate, RS 102,481 significantly inhibited only the cleavage by MMP-13 at the higher concentration of 10 nM and had no effect on the proteolysis by MMP-1 at either concentration tested (Fig 4.13).

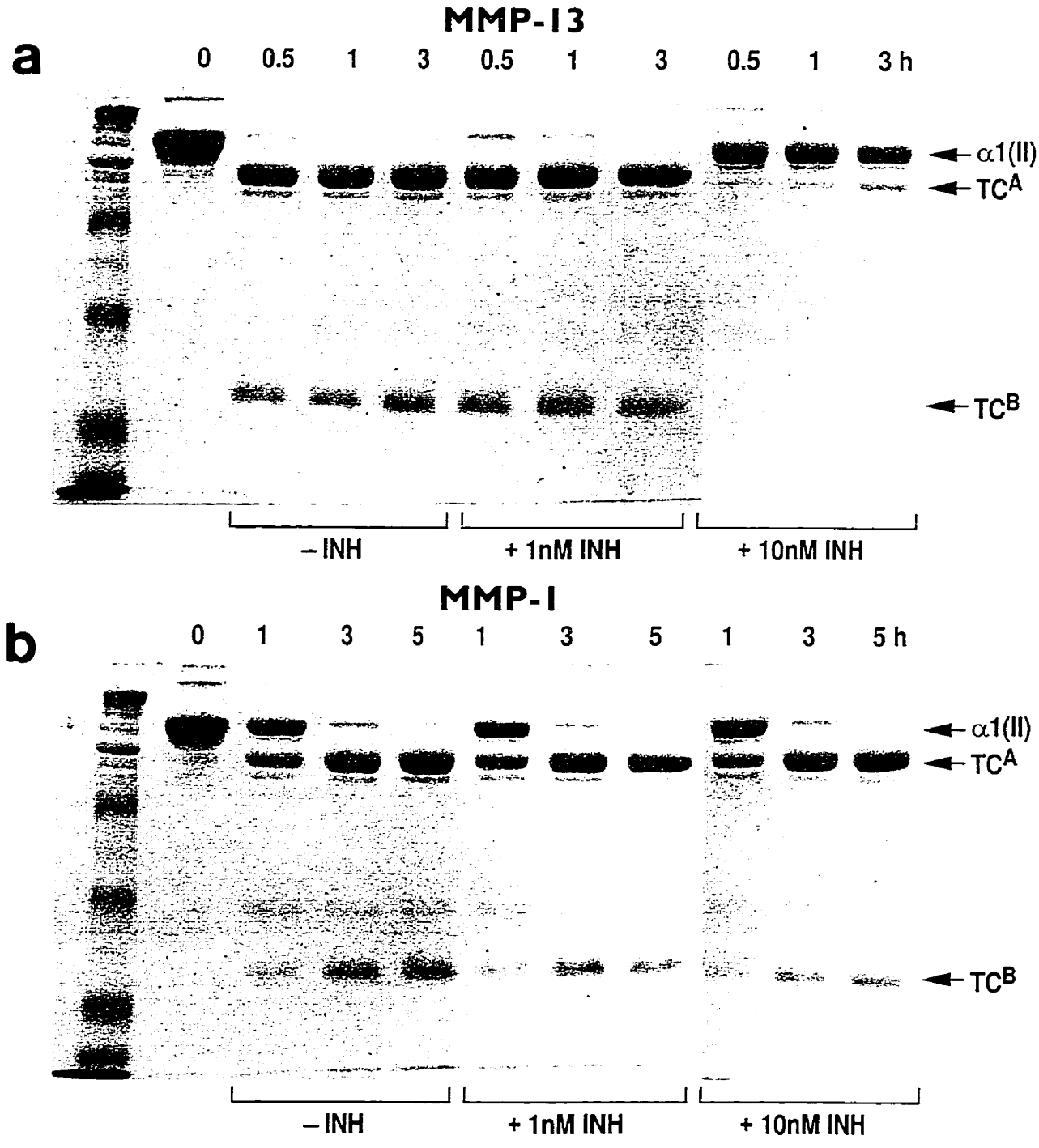
4.3.2 Human OA articular cartilage explant cultures

Explant cultures of human condylar articular cartilages from three different patients were analyzed for the release of COL2-3/4C neoepitope into culture media. There was a steady release of COL2-3/4C into culture media over the 12 days of culture. An example of one of the three studies is shown (Fig. 4.14). In an attempt to determine if and which of the collagenases MMP-1, MMP-8 and MMP-13 may be responsible for the generation and/or release of the COL2-3/4C neoepitope, explants from the same specimens were also incubated with different concentrations (1-10 nM) of the preferential inhibitor of MMP-13, RS 102,481. In all these studies, the inhibitor produced a significant reduction in the release of the COL2-3/4C neoepitope into the culture media over the entire 12 days of culture (Fig. 4.14).

That RS 102,481 did not have a detrimental effect on chondrocyte viability, was determined by the measurement of total collagen synthesis. In two separate experiments, there were no significant differences between the levels of tritiated hydroxyproline synthesized in OA cartilages cultured with or without the inhibitor at the highest concentration, namely 10 nM (Table 4.2).

Figure 4.13 The effect of inhibitor RS 102,481 on the cleavage of human type II collagen by MMP-1 and MMP-13.

Methodology used was as described in the legend of Fig. 4.12, except that inhibitor concentrations of 1 and 10 nM were used to allow for the selective inhibition of MMP-13. Shown are the Coomassie blue stained SDS-PAGE (10%) gels for the digestion with MMP-13 (**a**) and MMP-1 (**b**). All labels and margins are as indicated in Fig. 4.12.


MMP-13
a
0 0.5 1 3 0.5 1 3 0.5 1 3 h
α1(II)
TC^A
TC^B
– INH
+ 1nM INH
+ 10nM INH
MMP-1
b
0 1 3 5 1 3 5 1 3 5 h
α1(II)
TC^A
TC^B
– INH
+ 1nM INH
+ 10nM INH

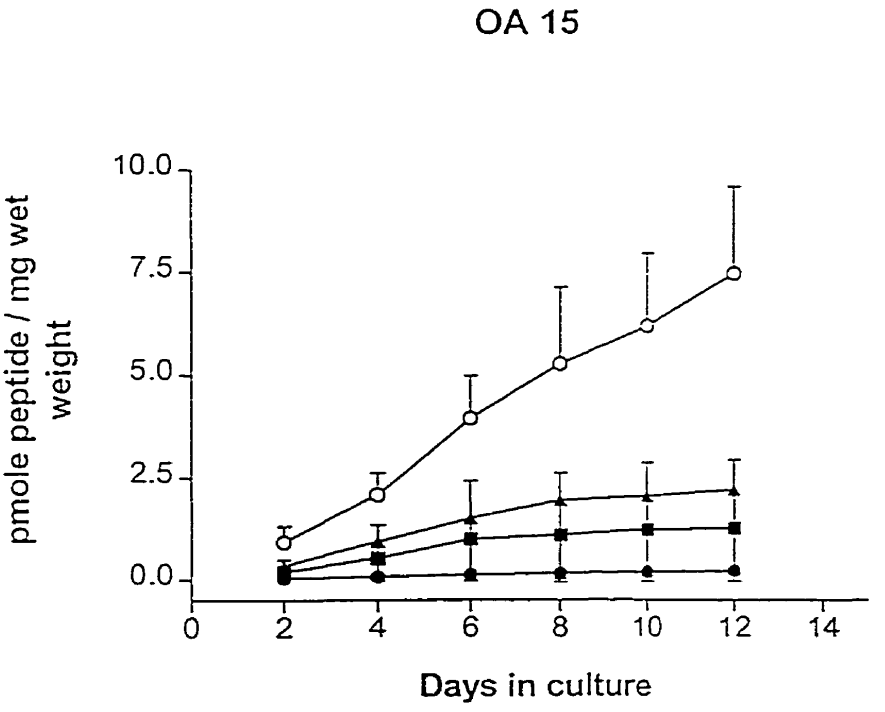


Figure 4.14 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on the release of COL2-3/4C neoepitope from human osteoarthritic articular cartilage explants.

The human OA articular cartilage explants were cultured in basic culture medium alone (○) or in the presence of RS 102,481, a preferential inhibitor of MMP-13, at 1.0 (▲), 2.5 (■) and 10 (●) nM. Media and inhibitor were replaced every two days and the conditioned media were assayed for COL2-3/4C neoepitope content. Shown is the cumulative release of the COL2-3/4C epitope over 12 days of a representative study (mean ± SD), using OA sample #15. From Billinghurst, R.C. *et al.* (1997), with permission.

Table 4.2 The lack of an effect of the synthetic collagenase inhibitor RS 102,481 (10 nM) on total collagen synthesis in explant cultures of human OA articular cartilage.

	CONTROL	RS 102,481
	cpm/mg wet weight	cpm/mg wet weight
Experiment #1	1968.3 (534.6)	1911.4 (770.7)
Experiment #2	941.4 (133.8)	1131.9 (38.7)

The amount of ^{3}H-hydroxyproline synthesized from ^{3}H-proline was determined in cartilage that had been labeled with ^{3}H-proline on days 14-20 of culture, as described in the section 3.3.2.2. Values are expressed as mean (SD). From Billinghurst, R.C. *et al.* (1997), with permission.

4.3.3 IL-1 stimulated bovine cartilage explant cultures

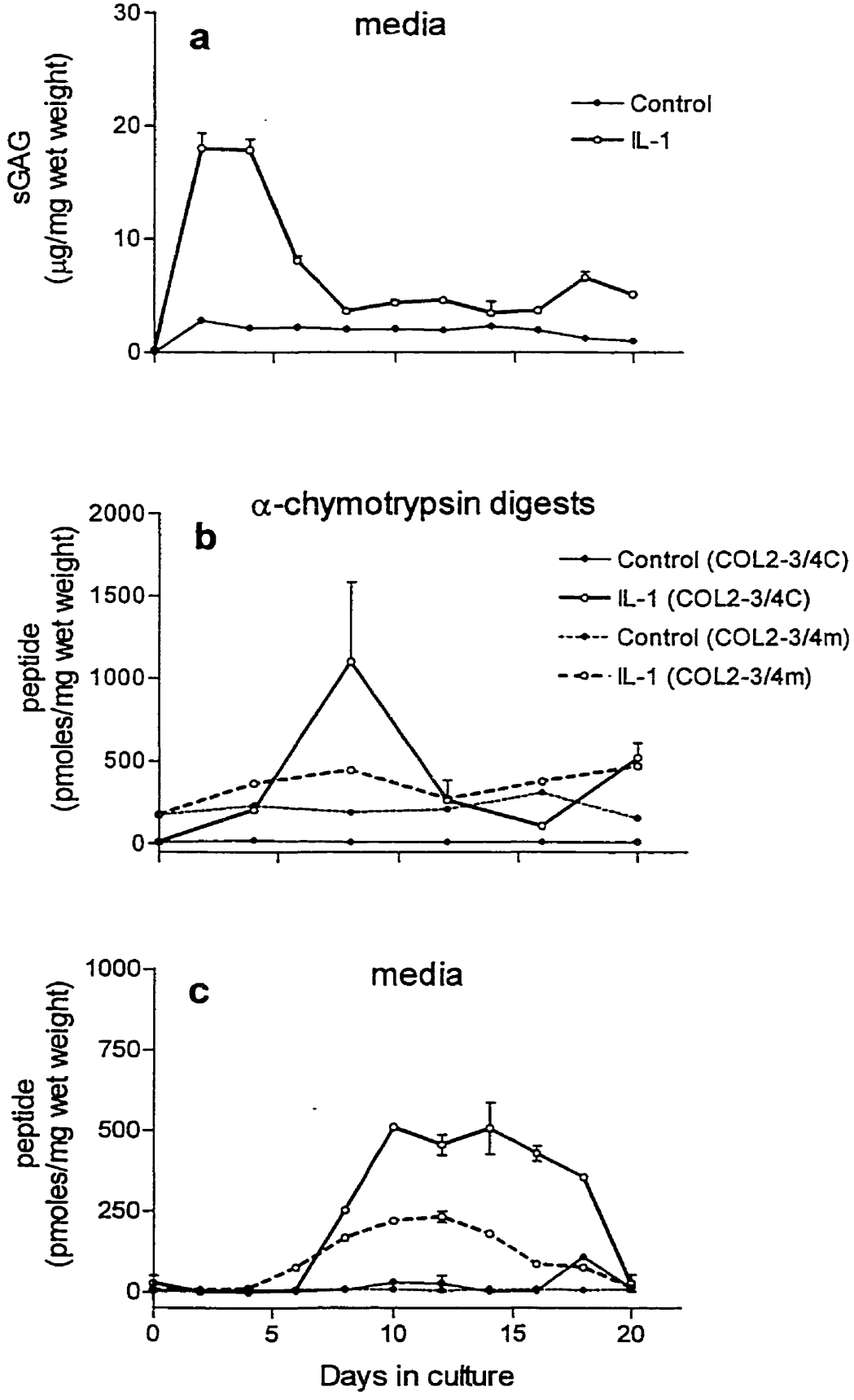
4.3.3.1 Effects on proteoglycans

The initial effect of IL-1α on the bovine cartilage explants was a rapid loss of sGAG from the tissue at levels of approximately 75% of the initial tissue levels by day 8 and day 10 for the nasal (Fig. 4.15a) and articular (Fig 4.16a) cartilages, respectively. Neither of the inhibitors was able to alter this dramatic release of proteoglycan degradative products. Moreover, when co-cultured with IL-1α, there was actually more sGAG released from the nasal cartilage with RS 102,481 (Fig. 4.17), and for the articular cartilage with RS 47,112 (Fig. 4.18a) during this initial period, at all concentrations of inhibitor used, than that induced by IL-1α alone.

After the initial dramatic release of proteoglycan fragments, there was a steady low level loss of sGAG from both the nasal and articular cartilage explants during the remainder of the culture period (Fig. 4.15 and 4.16). There was an apparent inhibitory effect (nasal) or no effect (articular) over this time period, and by the conclusion of the culture period there were no significant differences in the sGAG levels remaining in the IL-1 stimulated nasal cartilage explants cultured with or without the inhibitors (Fig. 4.17 and 4.18). When analyzing the amount of proteoglycans in terms of total sGAG released over the entire culture period combined with the sGAG remaining in the cartilages at the end of the culture period, there were no significant differences with either inhibitor at either concentration used and with the levels in control and IL-1 stimulated explants (Fig. 4.19). It should be noted that for the nasal cartilages, all cultures showed significant net proteoglycan synthesis, whereas only the control cultures did for the articular cartilage.

Figure 4.15 Time course of the effect of IL-1 on the generation and release of type II collagen cleavage site and intrachain epitopes and sGAG release in bovine nasal cartilage explant cultures.

Bovine nasal cartilage explants were prepared and cultured, as described in section 3.3.3.1.2., in the presence (○) or absence (●) of 5 ng/ml of recombinant human IL-1α (rHuIL-1α). Conditioned media were removed every 48 h and assayed for proteoglycan content, as sGAG (**a**), and for the collagen degradation epitopes (**c**), COL2-3/4C (solid lines) and COL2-3/4m (dashed lines). As well, cartilage explants were removed every 4 days, digested with α-chymotrypsin, as described in section 3.2.2.1, and assayed for both collagen epitopes (**b**). Each point on the graphs represents the mean (SD, error bars) of quadruplicate samples. The COL2-3/4C and COL2-3/4m collagen epitopes are expressed as pmoles of peptide based on molecular weights of 608 and 2231 kD, respectively.


a
media
sGAG
(μg/mg wet weight)
30
20
10
0
Control
IL-1
b
α-chymotrypsin digests
peptide
(pmoles/mg wet weight)
2000
1500
1000
500
0
Control (COL2-3/4C)
IL-1 (COL2-3/4C)
Control (COL2-3/4m)
IL-1 (COL2-3/4m)
c
media
peptide
(pmoles/mg wet weight)
1000
750
500
250
0
0
5
10
15
20
Days in culture

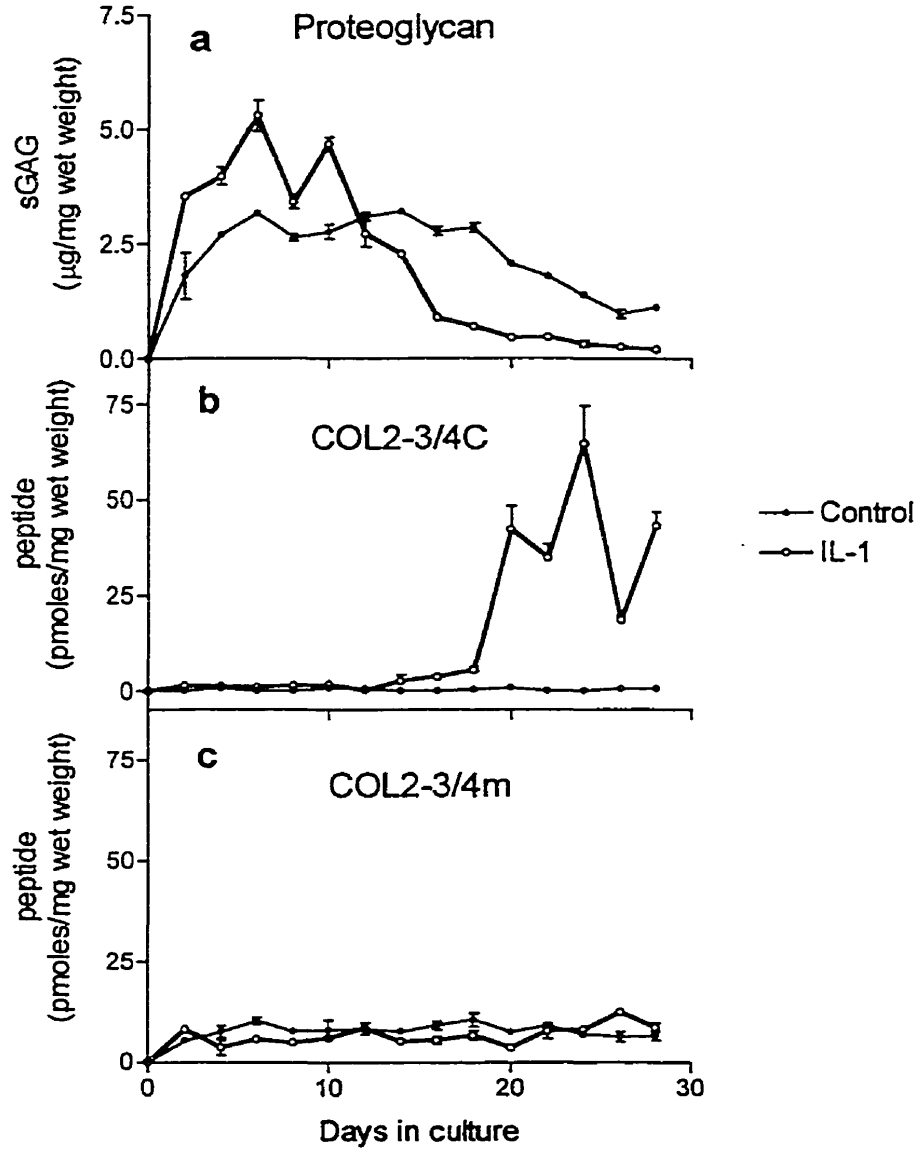


Figure 4.16. Time course of the effect of IL-1 on the release of the type II collagen cleavage site and intrachain epitopes and sGAG from bovine articular cartilage explants.

Bovine articular cartilage explants were prepared and cultured, as described in section 3.3.3.1.1, in the presence (○) or absence (●) of 5 ng/ml rHuIL-1α. The media was processed for proteoglycan, as sGAG (**a**), and the epitopes COL2-3/4C (**b**) and COL2-3/4m (**c**), as described in the legend of Fig. 4.15. The points on the graph represent the mean (SD, error bars) of quadruplicate samples. Units are as described in Fig. 4.15.

Figure 4.17 The effect of selective MMP inhibition on the release of proteoglycans from bovine nasal cartilage explant cultures.

Bovine nasal cartilage explants were cultured for 20 days in the presence (○) or absence (●) of 5 ng/ml of rHuIL-1α, and two different concentrations of two different MMP inhibitors (dashed lines), RS 47,112 (**a** and **b**) and RS 102,481 (**c** and **d**). Both the low concentrations ( ) of RS 47,112 (20 nM) and RS 102,481 (1 nM), and the high concentrations (Δ) of RS 47,112 (200 nM) and RS 102,481 (10 nM) were assessed in terms of their effects on the sGAG levels in the conditioned media (**b** and **d**) and in α-chymotrypsin digests (**a** and **c**) of the IL-1 stimulated nasal cartilages, as described in section 3.3.3.4. The individual points represent means (SD, error bars) of quadruplicate samples from a representative experiment. The control and IL-1 curves shown in this and subsequent figures are the same for both inhibitors, and they are duplicated to allow for a clearer demonstration of the effect of each of the inhibitors on epitope levels.

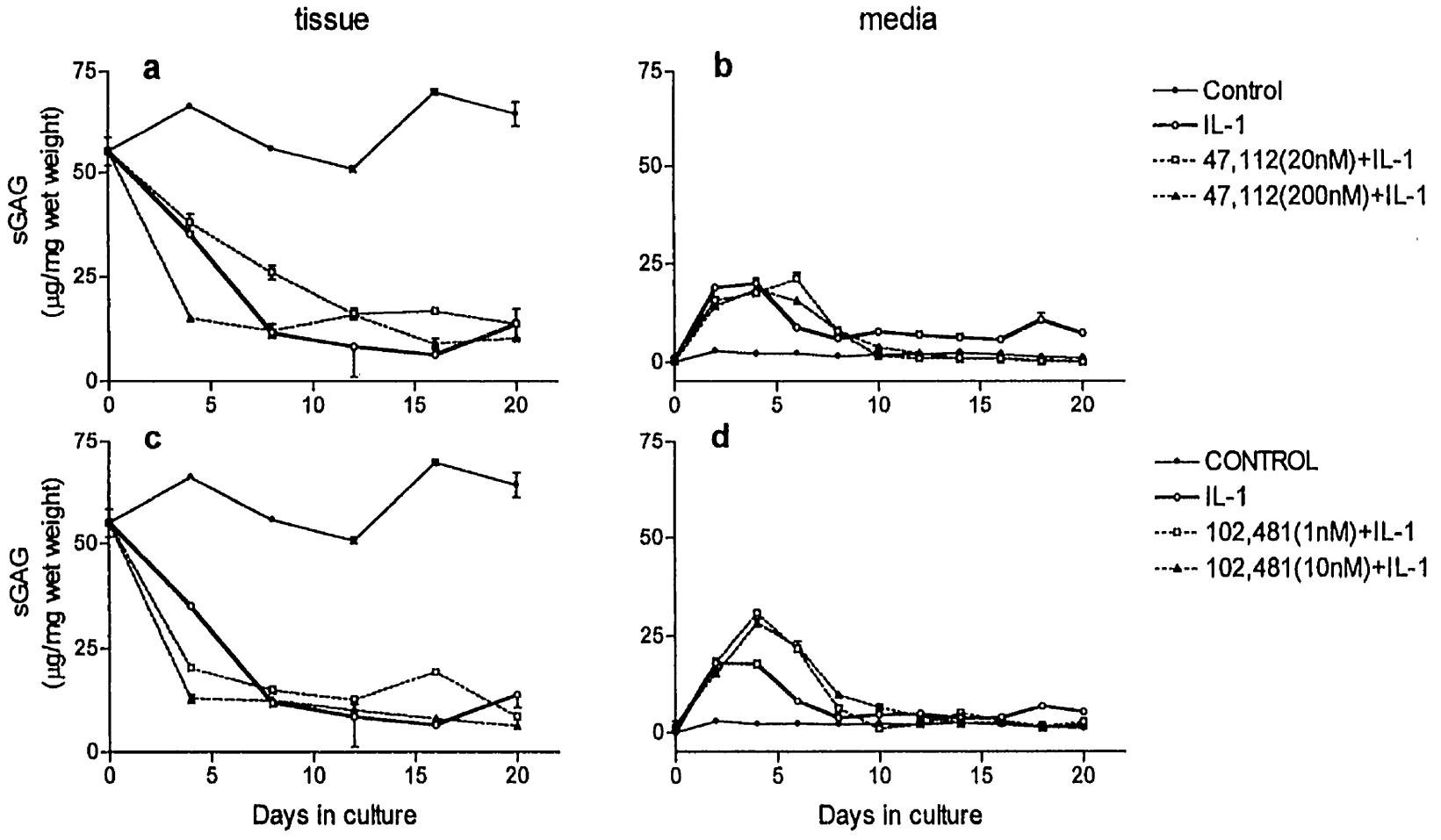
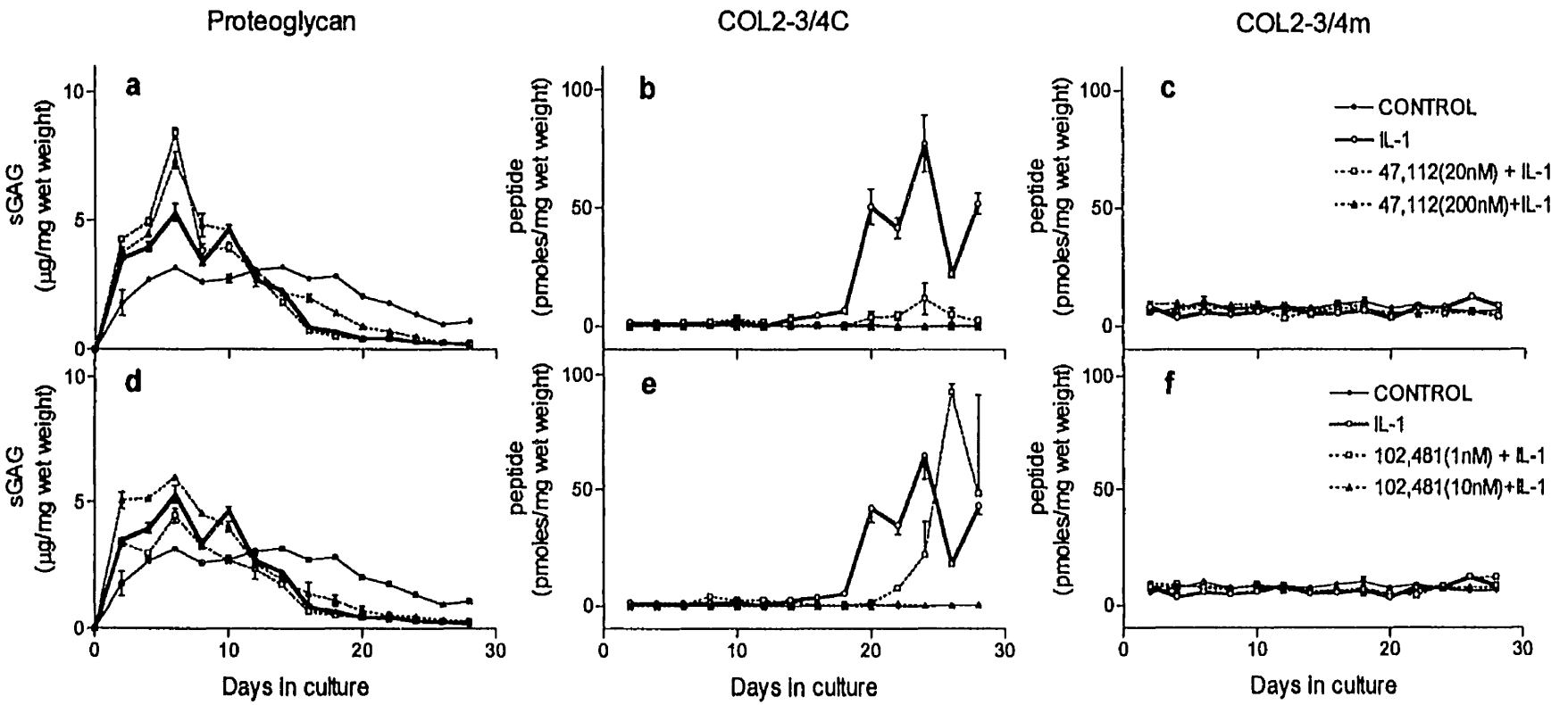

tissue
media
a
b
c
d
sGAG (µg/mg wet weight)
Days in culture
Control
IL-1
47,112(20nM)+IL-1
47,112(200nM)+IL-1
CONTROL
IL-1
102,481(1nM)+IL-1
102,481(10nM)+IL-1

Figure 4.18 The effect of selective MMP inhibition on the release of the type II collagen degradation epitopes, COL2-3/4C and COL2-3/4m, and proteoglycans, as sGAG, from bovine articular cartilage explants.

Bovine articular cartilage explants were cultured for 28 days in the presence (○) or absence (●) of 5 ng/ml rHuIL-1α and two different concentrations of two different synthetic MMP-selective inhibitors, RS 47,112 and RS 102,481 (dashed lines). Shown are the conditioned media levels of sGAG, representing proteoglycan loss (**a**), and the COL2-3/4C (**b**), and COL2-3/4m (**c**) epitopes, representing type II collagen cleavage and denaturation, respectively. Symbols are as described in Fig. 4.17.


Proteoglycan
COL2-3/4C
COL2-3/4m
a
b
c
d
e
f
sGAG (µg/mg wet weight)
peptide (pmoles/mg wet weight)
Days in culture
CONTROL
IL-1
47,112(20nM) + IL-1
47,112(200nM)+IL-1
102,481(1nM) + IL-1
102,481(10nM)+IL-1

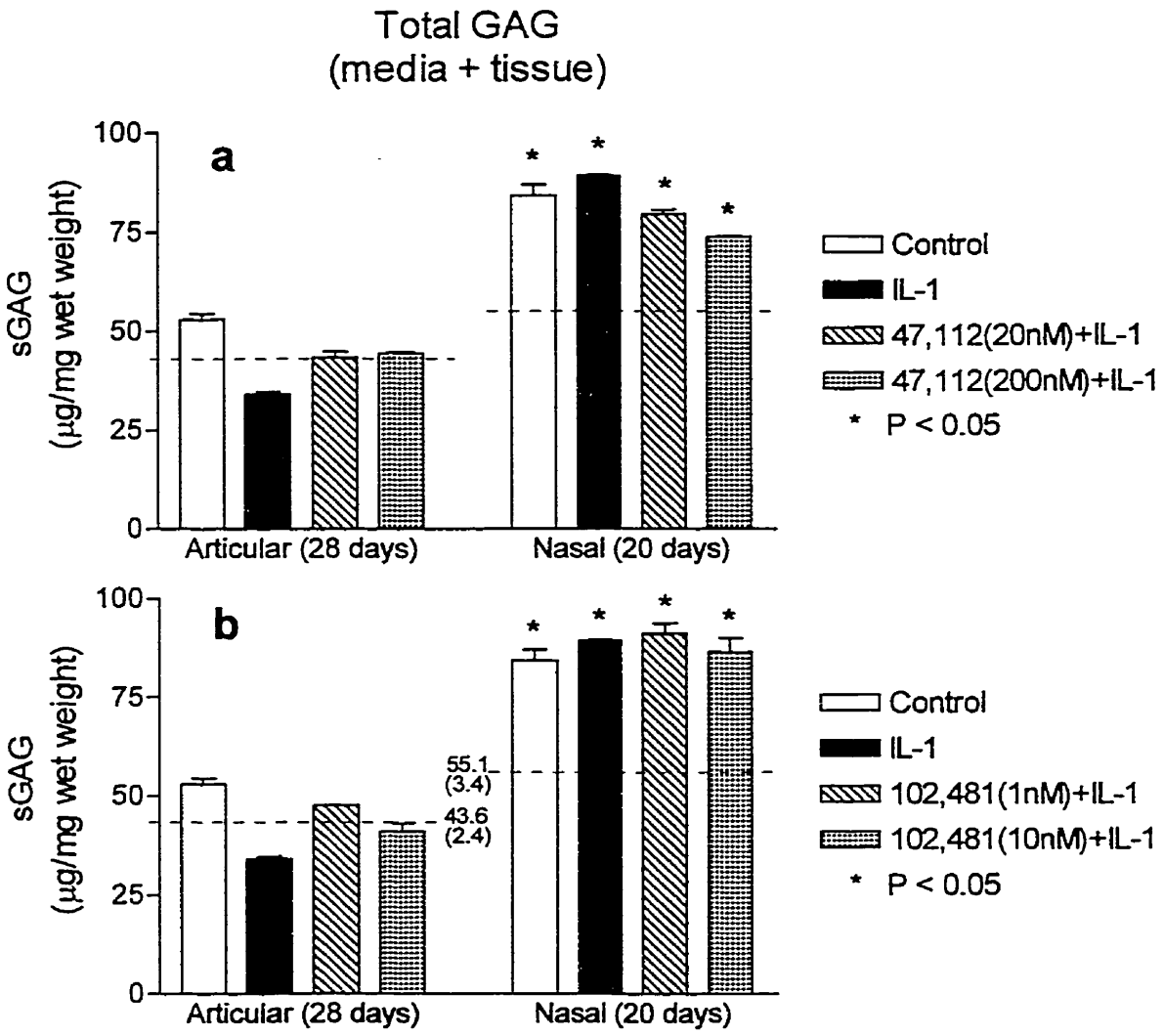


Figure 4.19 The effect of selective MMP inhibition on total sGAG levels (media + tissue) in IL-1 stimulated bovine cartilage explant cultures.

The cumulative release of sGAG into the media, for each of the articular and nasal cartilage explant cultures, were added to the sGAG levels measured in the respective tissue digests (α-chymotrypsin + proteinase K) of explants retrieved at the end of the culture periods. Shown are bar charts comparing control explants cultured without rHuIL-1α (open bars), rHuIL-1α stimulated explant cultures (solid bars), and explants cultured with the low (lined bars) and high concentrations (dotted bars) of RS 47,112 (**a**) and RS 102,481 (**b**), as indicated in the legends beside each chart. The values shown represent the means (SD, error bars) of quadruplicate samples. The dashed lines running through the bars indicate the sGAG levels assayed in the tissue digests of Day 0 cartilage explants, and the mean value (SD, in brackets) are shown beside each line in chart b. Significant differences from Day 0 sGAG tissue levels are indicated by * for $P < 0.05$.

4.3.3.2 Effects on collagenase cleavage

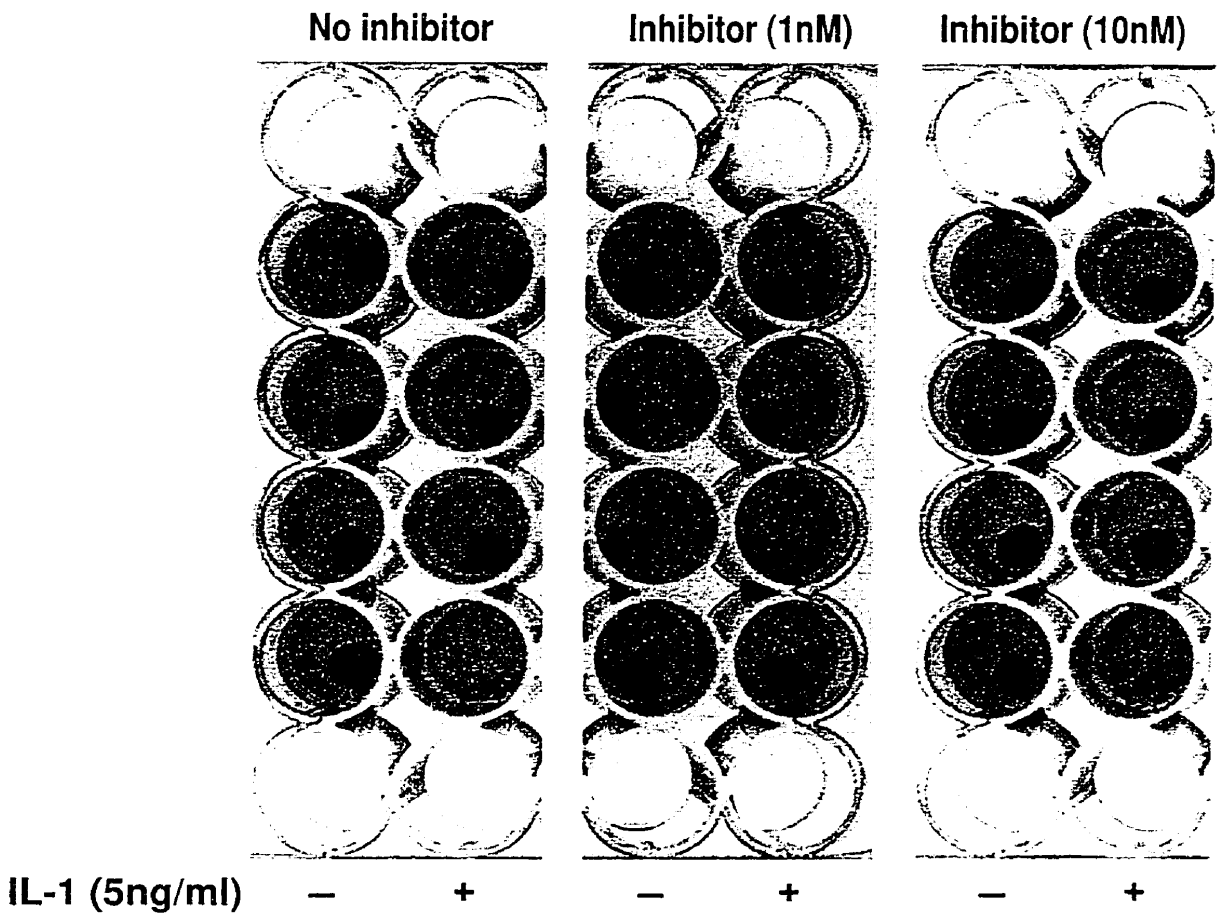
4.3.3.2.1 Immunoassay

The COL2-3/4C neoepitope created by the cleavage of native triple helical type II collagen by the collagenases MMP-1, MMP-8, and MMP-13 was only detectable in the media after the initial rapid loss of proteoglycans from the explants induced by IL-1α. For the nasal cartilage, this was after day 8 of culture and peaked at day 14, dropping thereafter (Fig. 4.15c). The situation for the articular cartilage was initially more subtle with a slow release from day 14 to 18 of culture, and then a dramatic release over the next 10 days (Fig. 4.16b). Comparing the two cartilage types in terms of absolute levels of neoepitope generated, there was approximately 6-fold more COL2-3/4C arising from the nasal cartilage than that from the articular cartilage. Moreover, macroscopic evidence of cartilage degradation induced by IL-1α was only apparent for the nasal cartilage explants and more importantly, its appearance coincided with the initial period of detection of the COL2-3/4C epitope in the media (Fig. 4.20). The articular cartilage explants displayed only microscopic changes in response to IL-1α and this was in the form of chondrocyte cloning (data not shown), discernible again only after the appearance of the COL2-3/4C epitope in the media. The clones of proliferating cells constitute a histological hallmark of the chondrocytic response to cartilage degeneration.

A result anticipated and worth noting is the fact that the COL2-3/4C neoepitope was detectable in the IL-1 stimulated tissue before its presence in the media. For the nasal cartilage explants, this was at day 4 of culture and levels peaked by day 8 (Fig. 4.15b). In the case of the articular cartilages, there was a significant increase in COL2-

Figure 4.20 Inhibition with RS 102,481 of the IL-1 induced macroscopic cartilage degradation of bovine nasal explants.

Shown are the effects of 5 ng/ml of rHuIL-1α on the macroscopic appearance of bovine nasal cartilage discs after 16 days in culture, and the abrogation of cartilage breakdown with the higher concentration (10 nM) of inhibitor RS 102,481. The discs are in the 48 well plates in which they were cultured, and are grouped in quadruplicates per condition. The culturing procedures are described in section 3.3.3.1.2.


Day 16
No inhibitor
Inhibitor (1nM)
Inhibitor (10nM)
IL-1 (5ng/ml) – + – + – +

3/4C neoepitope at day 14 in the IL-1α stimulated explants compared to the control tissues, which was not the situation in the media at the same time point (data not shown).

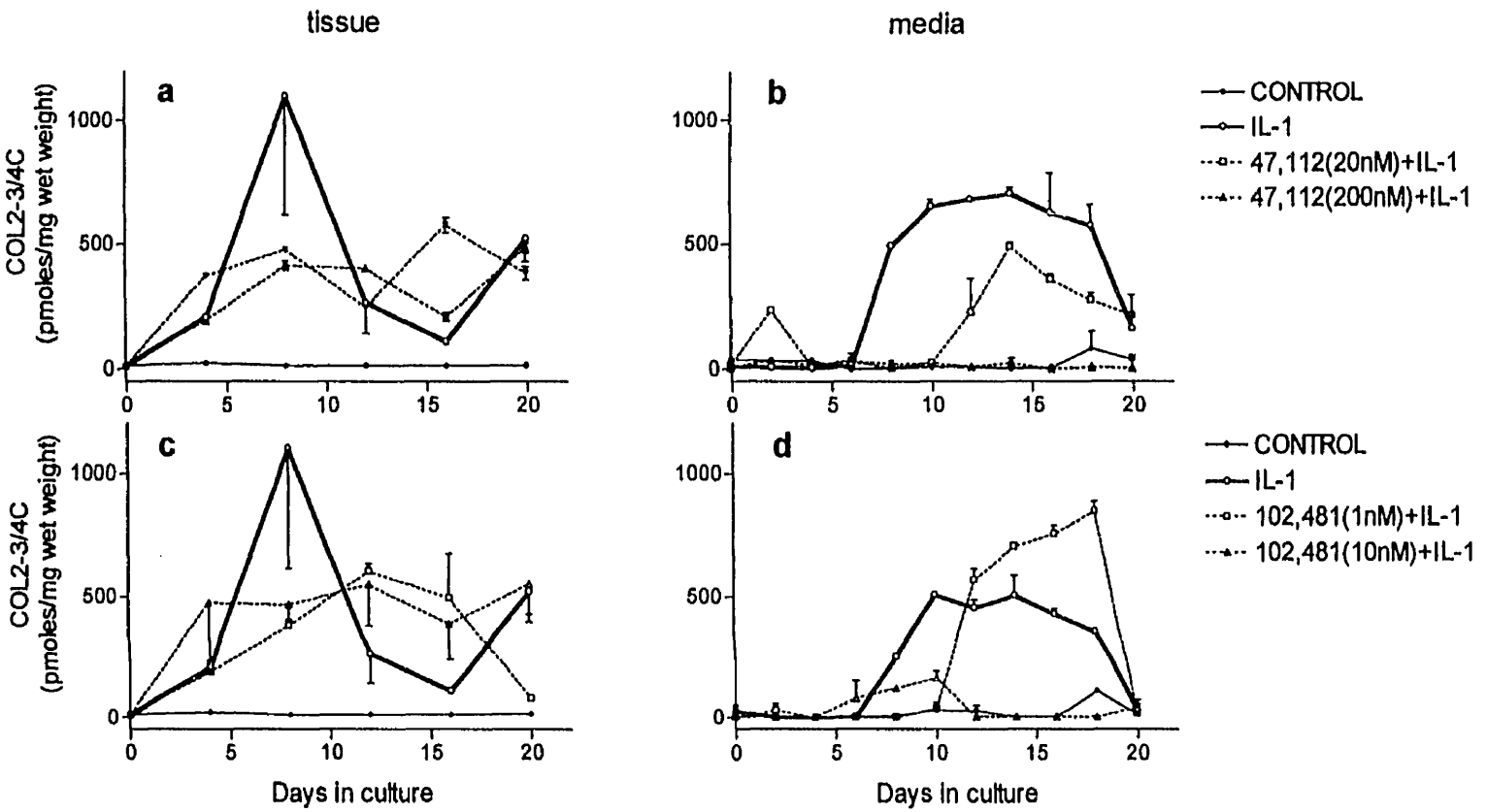
Inhibitor RS 47,112 showed dose-dependent reductions in total COL2-3/4C neoepitope generated (media + tissue) by IL-1α. Only the higher concentration of RS 102,481(10 nM) resulted in a significant drop in total epitope levels for both cartilage types (Fig. 4.21 and 4.22). Where the inhibitors did not completely prevent the generation of neoepitope by IL-1α, there was generally a 4 day delay in its detection in the media compared to that for the cartilages cultured with the cytokine alone (Fig. 4.18 and 4.21). For both the nasal and articular cartilages, RS 47,112 at 200 nM was the most effective at preventing the generation of COL2-3/4C neoepitope, but no inhibitor at the concentrations tested could restore levels to those of the nonstimulated control cultures (Fig. 4.22). It is important to note that for RS 47,112 (20 nM), there were actually increased levels of COL2-3/4C in the stimulated articular cartilage explants at day 14 and day 28 compared to those cultured with IL-1α alone (data not shown). This may reflect inhibition of the gelatinolytic degradation and release of the cleaved collagen fragments by these inhibitors and their subsequent build-up in the tissue.

4.3.3.2.2 Immunoblotting

The day 0, 8, 14, and 20 nasal cartilage conditioned media from the cultures with inhibitor RS 102,481 were lyophilized and subjected to Western blot analyses for the immunodetection of type II collagen fragments with the COL2-3/4C_{short} polyclonal antiserum used in the immunoassays. In agreement with the immunoassay results described above, there was the presence at day 8, of a distinct immunoreactive product in

Figure 4.21 The effects of selective MMP inhibition on the generation (tissue) and release (media) of the COL2-3/4C neoepitope in IL-1 stimulated bovine nasal cartilage explant cultures.

Bovine nasal cartilage explants were cultured for 20 days in the presence (○) or absence (●) of 5 ng/ml of rHuIL-1α, and two different concentrations of two different MMP-selective inhibitors, RS 47,112 (**a** and **b**) and RS 102,481 (**c** and **d**). Shown are the mean tissue levels (SD, error bars) of COL2-3/4C epitope assayed in α-chymotrypsin digests of cartilage explants retrieved on every fourth day of culture (**a** and **c**), and in the conditioned media retrieved on every second day of culture (**b** and **d**). Symbols and lines are as indicated in the legend of Fig. 4.17, as well as on this figure.


tissue
media
a
b
c
d
COL2-3/4C (pmoles/mg wet weight)
1000
500
0
0
5
10
15
20
Days in culture
CONTROL
IL-1
47,112(20nM)+IL-1
47,112(200nM)+IL-1
102,481(1nM)+IL-1
102,481(10nM)+IL-1

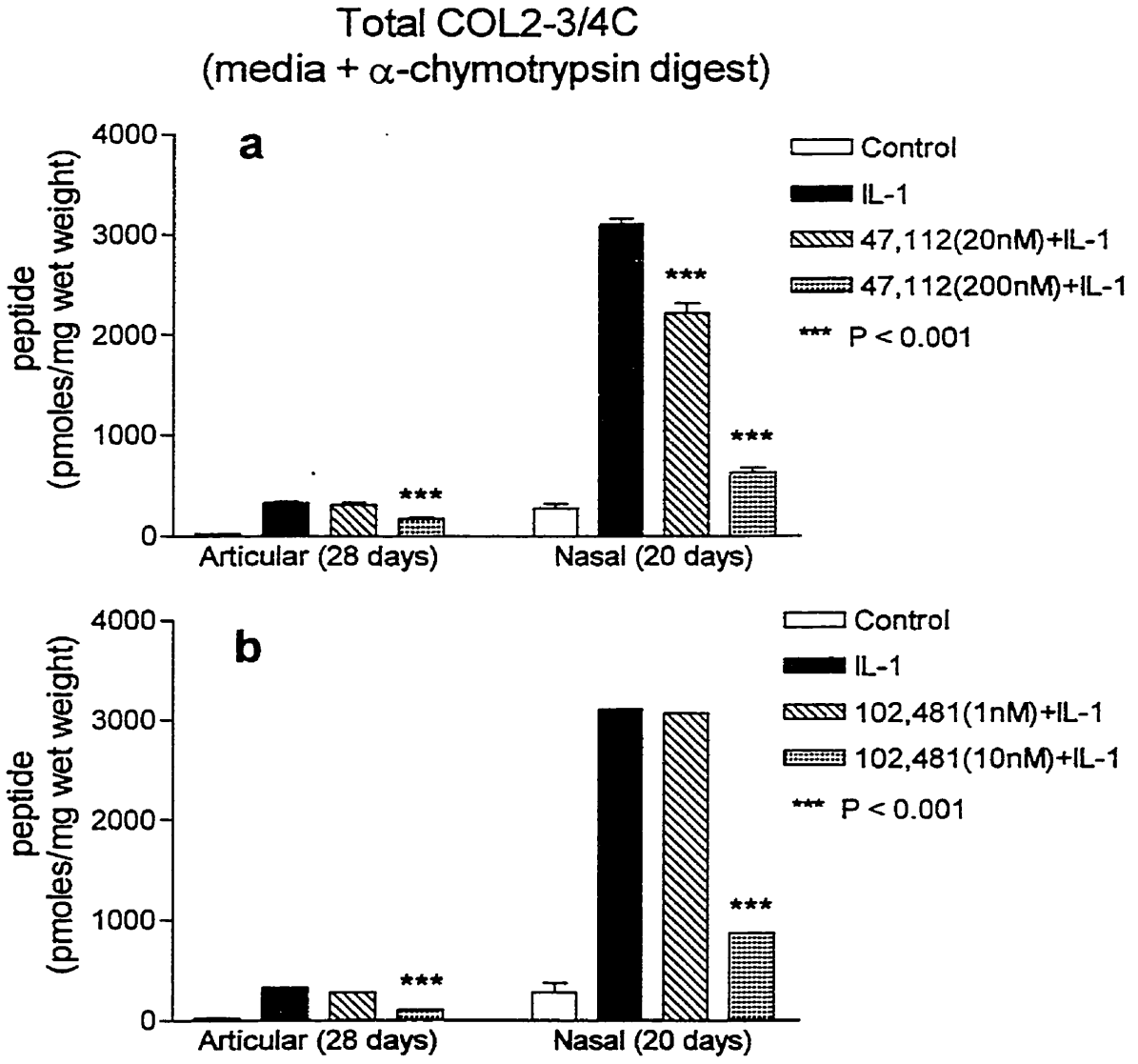


Figure 4.22 The effect of selective MMP inhibition on the total amount of COL2-3/C neoepitope generated in IL-1 stimulated bovine cartilage explant cultures.

The cumulative release of COL2-3/4C neoepitope into the media, for each of the articular and nasal cartilage explant cultures, were added to the COL2-3/4C levels in the respective α-chymotrypsin digests of day 20 (nasal) and day 28 (articular) explants. The bars are as described in the legend of Fig. 4.19 and as labelled on the figure. Shown are the results for both the RS 47,112 (**a**) and the RS 102,481 (**b**) inhibitors. Significant decreases in the mean neoepitope levels of cultures with inhibitor added, compared to those levels detected in the IL-1 stimulated cultures, are indicated by *** for $P < 0.001$.

the media from the IL-1α stimulated nasal cartilage, which stained less intensely in the media from the IL-1α stimulated cartilages co-cultured with 1 nM RS 102,481 (Fig. 4.23). In the day 14 media, again in complete agreement with the immunoassay results, there was greater immunostaining of fragments found in both the IL-1α and the IL-1α + RS 102,481 cultures (Fig. 4.24). In fact, the intensity of staining was greater for the media from the cultures with the inhibitor at 1 nM than with IL-1α alone, and there was the presence of more distinct and smaller immunoreactive fragments at this time point. The major immunoreactive product was ~35kD (based on cleaved collagen standards in Fig. 4.23 and 4.24), and there was some diffuse staining, which was evidence of less reactive and smaller fragments. N-terminal sequence analyses of the distinct COL2-3/4C positive staining band confirmed that it was indeed a fragment of type II collagen belonging to the TC^A or 3/4 fragment created by collagenase cleavage of the triple helical collagen molecule and bearing the COL2-3/4C neoepitope (Fig. 4.25b). Moreover, N-terminal sequencing of a distinct band on Coomassie staining (Fig. 4.25c), which was negative on COL2-3/4C Western blot analysis and seemed to correspond to the COL2-3/4m positive band in Fig 4.24c (based on gel mobility), suggested a cleavage between these two epitopes (see schematic in Fig. 4.25). As would be expected from the immunoassay results, which are recorded on Fig. 4.23 and Fig. 4.24 for each of the samples subjected to electrophoretic separation, there were no immunoreactive fragments in the Western blot of the day 20 media (Fig 4.24). Moreover, no fragments were detected in the immunoblotted articular cartilage media taken at similar points of culture as those for the bovine nasal cartilage explants (data not shown).

Figure 4.23 Immunoblot analyses of day 0 and day 8 conditioned media from bovine nasal cartilage explant cultures for COL2-3/4C and COL2-3/4m epitope-bearing molecules.

The day 0 and day 8 conditioned media from the bovine nasal cartilage cultures were lyophilized (1 ml/condition), reconstituted in 125 µl of sample buffer, separated on SDS-PAGE (4-20%) gradient gels, and transferred to nitrocellulose membranes for incubation with either the COL2-3/4C_{short} polyclonal or the COL2-3/4m monoclonal antibodies, as described in more detail in section 3.3.3.3. Shown are the Coomassie blue stained gels (**a**), and the immunoblots for the COL2-3/4C (**b**) and COL2-3/4m (**c**) epitopes. At the top of the figure are the conditions regarding the samples loaded in each lane, in respect to culturing without (-) or with (+) 5 ng/ml rHuIL-1α and either 1 nM (+ 1) or 10 nM (+10) concentrations of the RS 102,481 inhibitor. The end lane, marked II + 13, represents a control for both the COL2-3/4C_{short} and COL2-3/4m antibodies, and is a 3 h digest of triple helical type II collagen with MMP-13. At the bottom of each of the immunoblots are the approximate amounts of COL2-3/4C (**b**) and COL2-3/4m (**c**), expressed as µg of epitope loaded per lane. These amounts are based on the epitope levels that were determined by immunoassay for each sample. The right margin indicates the location of the 3/4 (TC^A) and 1/4 (TC^B) α1(II) fragments, and the BSA (0.1 mg/ml) used in the culture media.

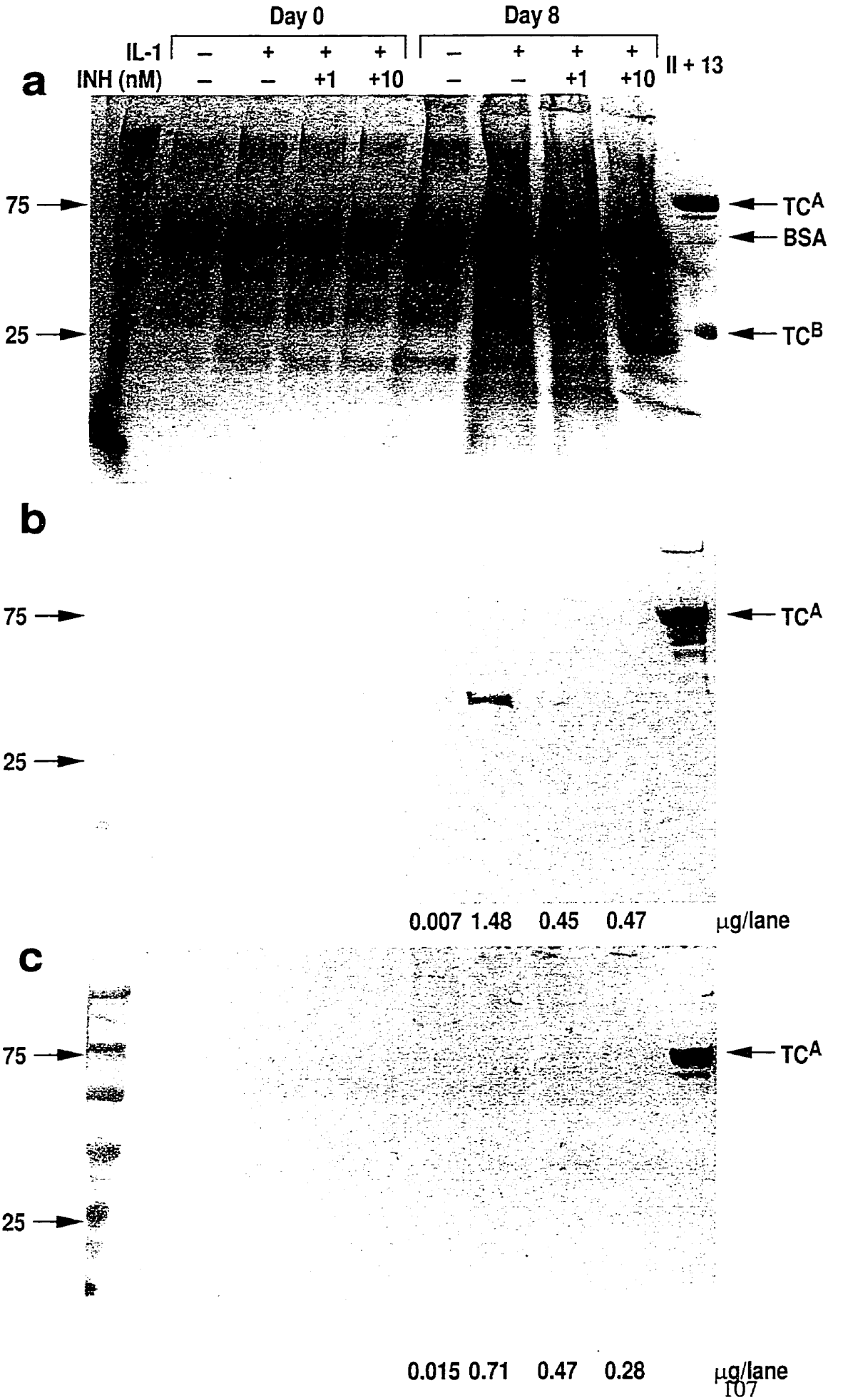

Day 0
Day 8
IL-1 – + + + – + + +
INH (nM) – – +1 +10 – – +1 +10
II + 13
a
75
25
TC^A
BSA
TC^B
b
75
25
TC^A
0.007 1.48 0.45 0.47 µg/lane
c
75
25
TC^A
0.015 0.71 0.47 0.28 µg/lane

Figure 4.24 Immunoblot analyses of day 14 and day 20 conditioned media from bovine nasal cartilage explant cultures for COL2-3/4C and COL2-3/4m epitope-bearing molecules.

The conditioned media from the day 14 and day 20 bovine nasal cartilage cultures were processed exactly as the day 0 and day 8 cultures, and the conditions have been previously described in the legend of Fig. 4.23. This figure shows the Coomassie blue stained gel (**a**), and the immunoblots for the COL2-3/4C (**b**) and COL2-3/4m (**c**) epitope-bearing molecules found in the day 14 and day 20 conditioned media. All labelling is as described in Fig. 4.23.

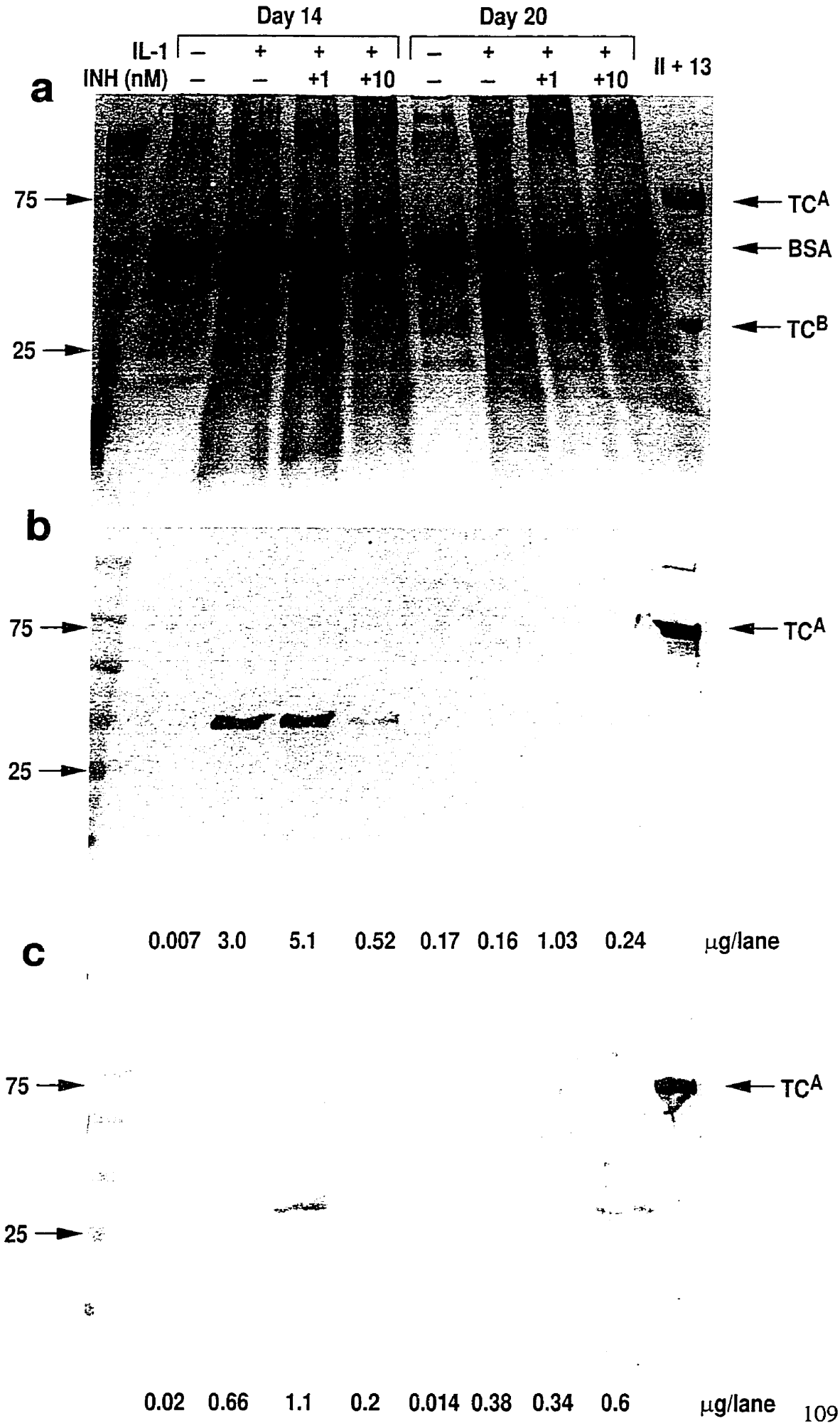
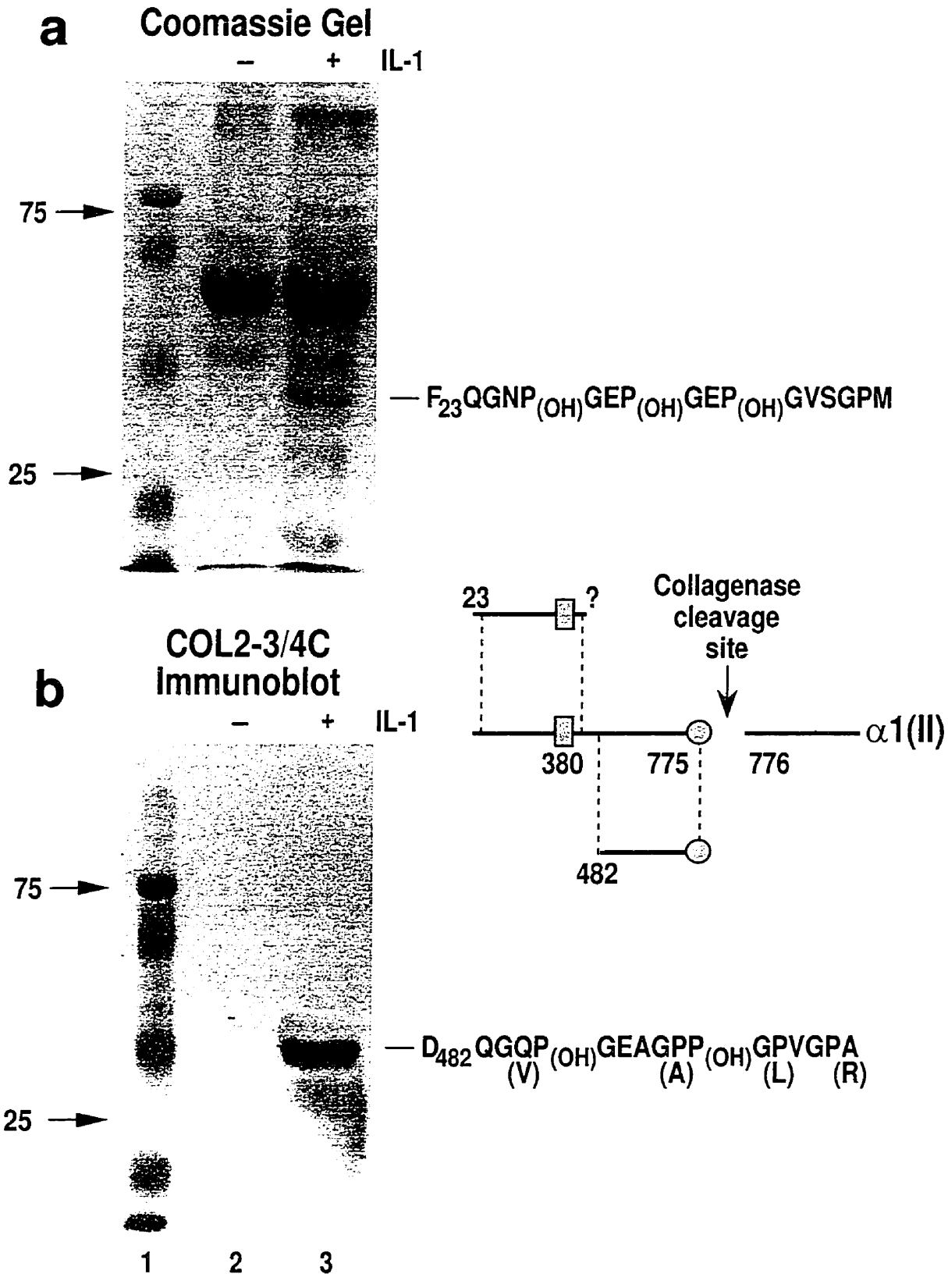

Day 14
Day 20
IL-1 – + + + – + + +
INH (nM) – – +1 +10 – – +1 +10
II + 13
a
75
25
TC^A
BSA
TC^B
b
75
25
TC^A
0.007 3.0 5.1 0.52 0.17 0.16 1.03 0.24 µg/lane
c
75
25
TC^A
0.02 0.66 1.1 0.2 0.014 0.38 0.34 0.6 µg/lane

Figure 4.25 N-terminal sequencing of immunoreactive collagen degradation products in the conditioned media of IL-1 stimulated bovine nasal cartilage cultures.

The conditioned media from the day 14 bovine nasal cartilage cultures, in the presence (+) or absence (-) of 5 ng/ml rHuIL-1α, were dialyzed in dH_20, lyophilized, separated on SDS-PAGE (10% gels), stained with Coomassie blue (**a**), and transferred to nitrocellulose membranes for immunostaining with the COL2-3/4C_{short} antibody (**b**). A duplicate gel was transferred onto a PVDF membrane for N-terminal sequencing, as described in section 3.3.3.3. The results of the sequencing are indicated to the right of both a distinct Coomassie staining band in the IL-1 conditioned media (**a**), and a distinct COL2-3/4C immunoreactive band in the Western analysis (**b**). Both the sequences shown are found in the 3/4 (TC^A) α chain fragment of collagenase-cleaved type II collagen, and likely represent products of cleavage of the α1(II) collagen chains between the COL2-3/4C (shaded circle) and COL2-3/4m (shaded box) epitopes, as shown in the schematic appearing between the two sequences. The numbering of the N-terminal residue of each of the fragments corresponds to the number of amino acids C-terminal to the beginning of the triple helical domain of bovine type II collagen α1 chains. Residues appearing in brackets indicate the amino acids in the human α1(II) chain that correspond to the residues immediately above each in the sequenced bovine α1(II) chain fragment (**b**).


a
Coomassie Gel
– + IL-1
75
25
— F23QGNP(OH)GEP(OH)GEP(OH)GVSGPM
b
COL2-3/4C
Immunoblot
– + IL-1
75
25
1 2 3
— D482QGQP(OH)GEAGPP(OH)GPVGPA
(V) (A) (L) (R)
23
?
Collagenase
cleavage
site
α1(II)
380
775
776
482

4.3.3.3 Effects on collagen denaturation

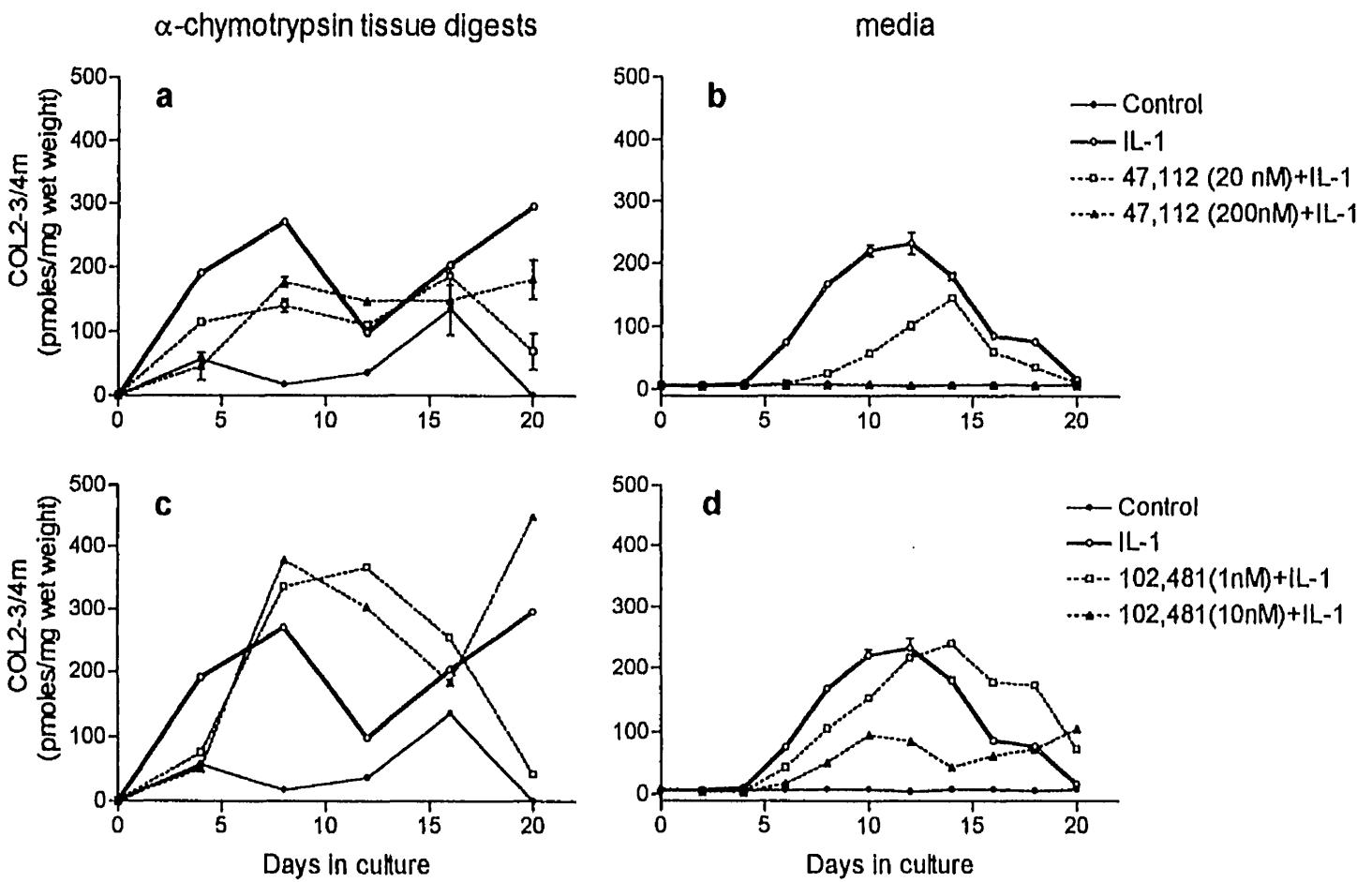
4.3.3.3.1 Immunoassay

The detection of denatured type II collagen was accomplished through the use the COL2-3/4m immunoassay developed in this laboratory (Hollander *et al.*, 1994). There was significantly more COL2-3/4m epitope in the media of IL-1α stimulated nasal cartilage cultures than that from control cultures (Fig. 4.15c). Media levels rose dramatically from day 6 to day 12 and then dropped to control levels by day 20. Moreover, similar to the situation with the COL2-3/4C neoepitope, the COL2-3/4m epitope was detected at higher levels in the nasal cartilage at an earlier time point (day 4) than its presence was noted in the media (day 6) (compare Fig. 4.15b and c). Only RS 47,112 at both concentrations tested (20 and 200 nM) significantly reduced the overall generation of the COL2-3/4m epitope and percentage of denatured collagen by IL-1α. As well, as noted for the COL2-3/4C neoepitope, where there was not complete inhibition, the inhibitors delayed the generation and release of the COL2-3/4m epitope by up to 4 days (Fig. 4.26). RS 102,481 at 10 nM significantly reduced the media levels of COL2-3/4m, but there were higher levels of this epitope in the cartilage cultured with the inhibitor, resulting in an overall lack of an effect by this inhibitor.

There was approximately three-fold more total COL2-3/4m generated by the IL-1 stimulated nasal than the stimulated articular cartilages (Fig. 4.27). For the articular cartilage explants, most of this was retained within the tissue and very little was detected in the media (Fig. 4.16c). Thus the peak levels of COL2-3/4m in the media from the stimulated articular cartilage explants were 20-fold lower than the peak levels in the media from the nasal cartilages. Moreover, surprisingly, there was generally less COL2-

Figure 4.26 The effect of selective MMP inhibition on the generation (α-chymotrypsin tissue digests) and release (media) of the COL2-3/4m epitope in bovine nasal cartilage explant cultures.

The conditions, lines and labels are exactly as described in the legend of Fig. 4.21, except that these figures represent the levels of the COL2-3/4m intrachain epitope, which is specific for triple helical type II collagen α chain denaturation or unwinding. Shown are the α-chymotrypsin tissue digest (**a** and **c**) and media (**b** and **d**) levels of the COL2-3/4m epitope in cultures with the inhibitors RS 47,112 (**a** and **b**) and RS 102,481 (**c** and **d**).


α-chymotrypsin tissue digests
media
a
b
c
d
COL2-3/4m (pmoles/mg wet weight)
Days in culture
Control
IL-1
47,112 (20 nM)+IL-1
47,112 (200nM)+IL-1
102,481(1nM)+IL-1
102,481(10nM)+IL-1

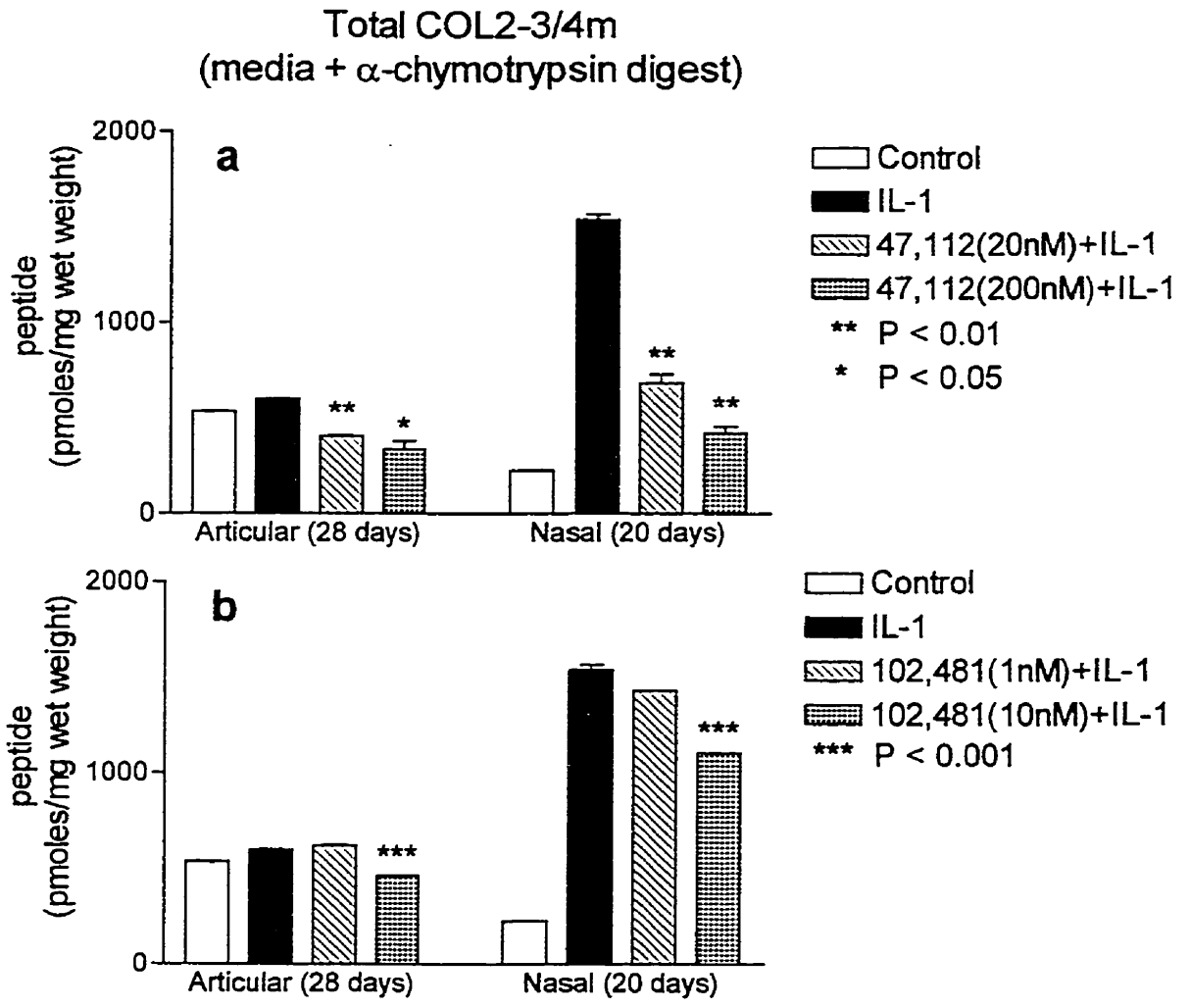


Figure 4.27 The effect of selective MMP inhibition on total type II collagen denaturation (unwinding) in IL-1 stimulated bovine cartilage explant cultures. The cumulative release of the COL2-3/4m intrachain epitope into the media, of each of the articular and nasal cartilage explant cultures, was added to the COL2-3/4m levels in the respective α-chymotrypsin tissue digests of the day 20 (nasal) and day 28 (articular) explants. The bars and labelling are as described in the legend of Fig. 4.19. Shown are the results for cultures with the RS 47,112 (**a**) and RS 102,481 (**b**) inhibitors. Significant inhibition of epitope generation, compared to total epitope levels for the IL-1 stimulated cartilages, are indicated by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3/4m epitope in the media from IL-1α stimulated articular cartilage than that in the control culture media (compare Fig. 4.16c and 4.15c). As was the case for the nasal cartilages, inhibitor RS 47,112 was the most effective at preventing the generation of the COL2-3/4m epitope by IL-1α, to levels even below those found in the nonstimulated cartilages (Fig. 4.27a). Only with the higher concentration of RS 102,481 (10 nM), was there a significant lowering of the articular cartilage tissue levels of COL2-3m epitope at the end of the culture period (Fig 4.27b).

4.3.3.3.2 Immunoblotting

Western blot analyses with the COL2-3/4m monoclonal antibody used in the immunoassay of the media from the nasal cartilages co-cultured with RS 102,481, revealed, at day 8 of culture, the presence of similar sized collagen fragments as those detected with the COL2-3/4C_{short} antiserum. The intensity of staining however was much less with the COL2-3/4m antibody (Fig. 4.23b vs. c). The day 14 media showed a similar response for immunoreactivity with the COL2-3/4m antibody as that reported above for the COL2-3/4C_{short} antiserum, in that there was greater staining of fragments in the media from IL-1 stimulated cartilages that had been co-cultured with the lower concentration of inhibitor (Fig 4.24b and c). However, the most distinct and intense staining band ran at a faster rate on the gel than that which stained the most with the COL2-3/4C_{short} antiserum. Moreover, there was a faintly staining band corresponding to intact type II collagen released by the control cartilage at day 14, that was not detected for any of the other media at any time point. By day 20, the only staining noted for the COL2-3/4m epitope was with the media from the cartilages co-cultured with IL-1α and the higher

concentration (10 nM) of RS 102,481. This was in complete agreement with the results of the immunoassay (results included in Fig. 4.23 and 4.24).

4.3.3.4 Correlations between COL2-3/4C, COL2-3/4m, and hydroxyproline levels

There was a strong correlation between the levels of epitopes COL2-3/4C and COL2-3/4m in the media and α-chymotrypsin digests of the nasal cartilages (Fig. 4.28). Moreover, there was a strong correlation between each of these epitopes and the hydroxyproline levels in the nasal cartilage conditioned media (Fig. 4.29). There was a rapid increase in the media levels of hydroxyproline in the IL-1 stimulated nasal cartilage cultures from day 4 to day 12 (Fig. 4.30a), just as noted for the COL2-3/4C and COL2-3/4m epitopes (Fig. 4.15). Similar patterns of inhibition by RS 102,481 of hydroxyproline release were noted for the nasal cartilage cultures, as observed for both the COL2-3/4C and COL2-3/4m epitopes. There was no effect, however, with IL-1α +/- RS 102,481 at day 14 or day 28 in terms of hydroxyproline levels in the articular cartilage explant cultures (data not shown). In agreement with the results of the COL2-3/4C and COL2-3/4m immunoassays, there were more than 10-fold greater levels of hydroxyproline in the media from the stimulated nasal than the stimulated articular cartilage cultures (Fig. 4.30a vs. b). When comparing COL2-3/4C and COL2-3/4m levels on a pmole/mg wet weight of cartilage basis, there was approximately 10-fold more of the former than the latter in the conditioned media from the stimulated articular cartilages (Fig. 4.16b vs. c). The inverse was true for the tissue levels (data not shown). In the IL-1 stimulated nasal cartilage cultures, there was 2-3 fold more COL2-3/4C than COL2-3/4m assayed in the media and α-chymotrypsin tissue digests (Fig. 4.15).

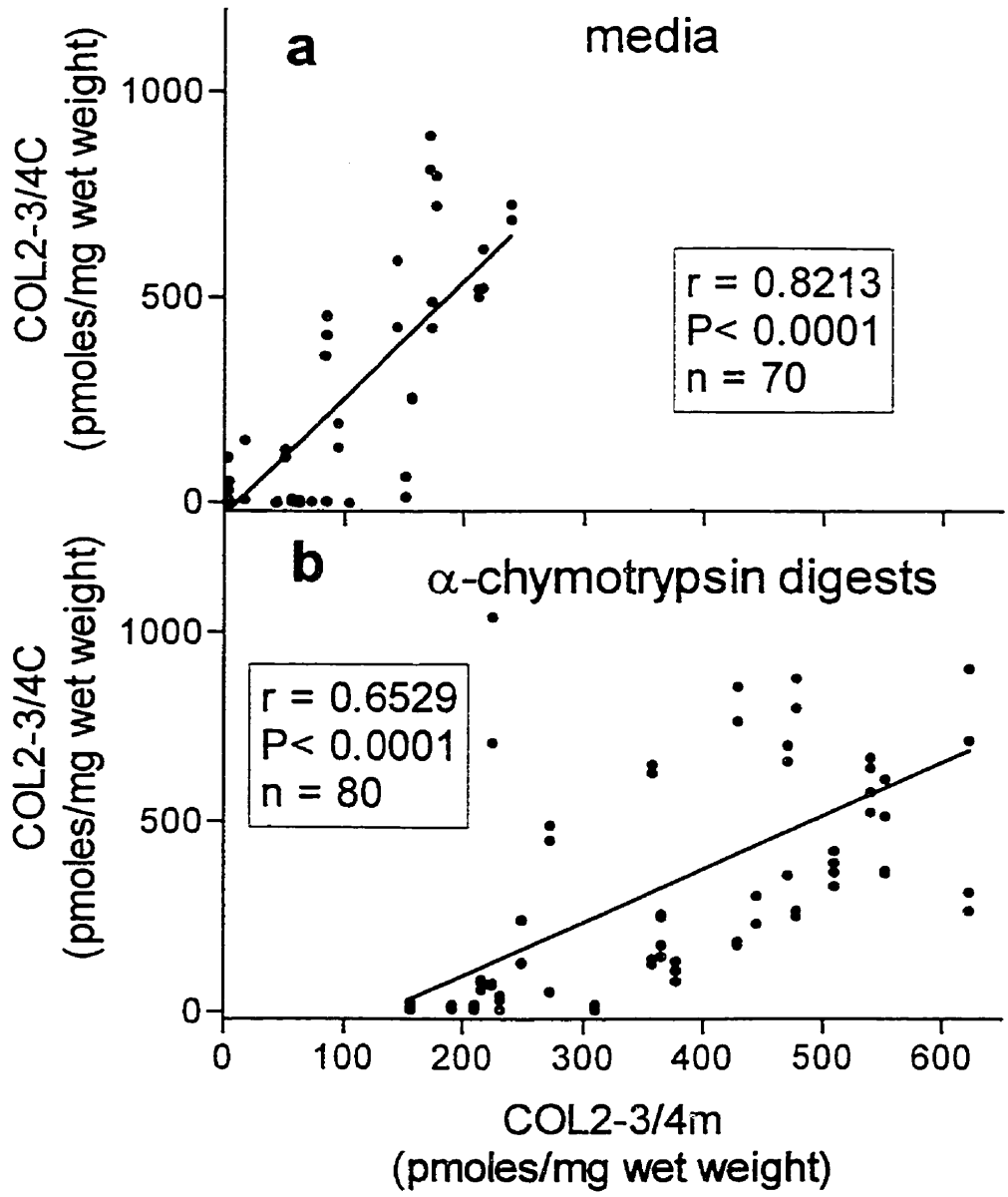


Figure 4.28 Correlation between the collagenase-generated cleavage site neoepitope, COL2-3/4C, and the type II collagen intrachain epitope, COL2-3/4m, in bovine nasal cartilage explant cultures.

The COL2-3/4C and COL2-3/4m epitope content in both the media (**a**) and α-chymotrypsin tissue digests (**b**) of bovine nasal cartilage explants, cultured in the presence or absence of 5 ng/ml rHuIL-1α, were compared by Spearman rank correlational analyses. The corresponding r, P, and n values, and lines generated by linear regression analyses, are shown on each of the plots.

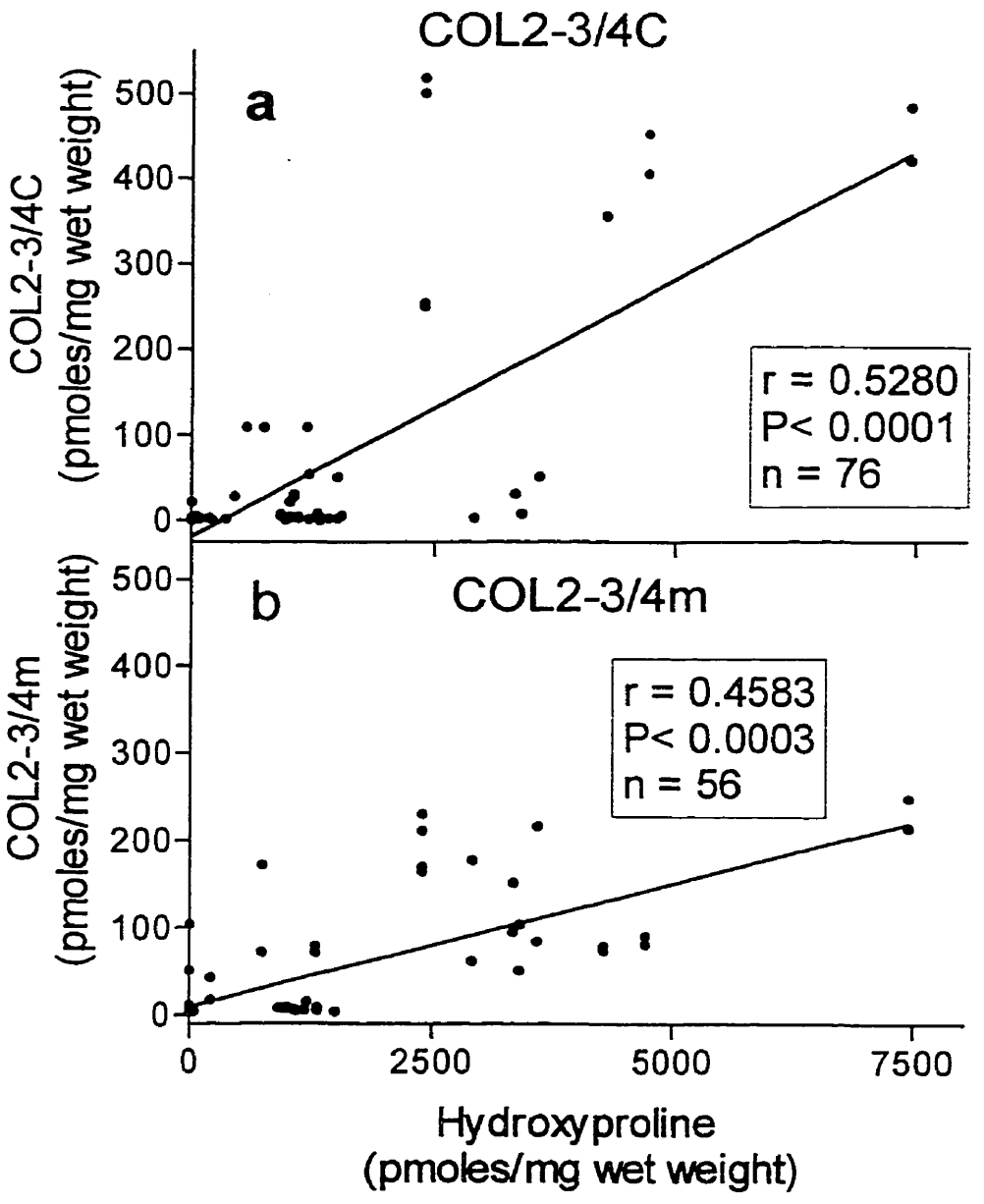


Figure 4.29 Correlation between each of the type II collagen degradation epitopes and total collagen levels, measured as hydroxyproline, in the media of bovine nasal cartilage explant cultures.

Hydroxyproline levels in the conditioned media of bovine nasal cartilages, cultured in the presence or absence of rHuIL-1α, were determined as described in section 3.3.3.2. These values are expressed as pmoles, to allow a direct comparison with the pmole levels of each of the COL2-3/4C (**a**) and COL2-3/4m (**b**) epitopes. The results of Spearman rank correlational analyses are shown, and the r, P, and n values are indicated, along with the linear regression lines, on each of the plots.

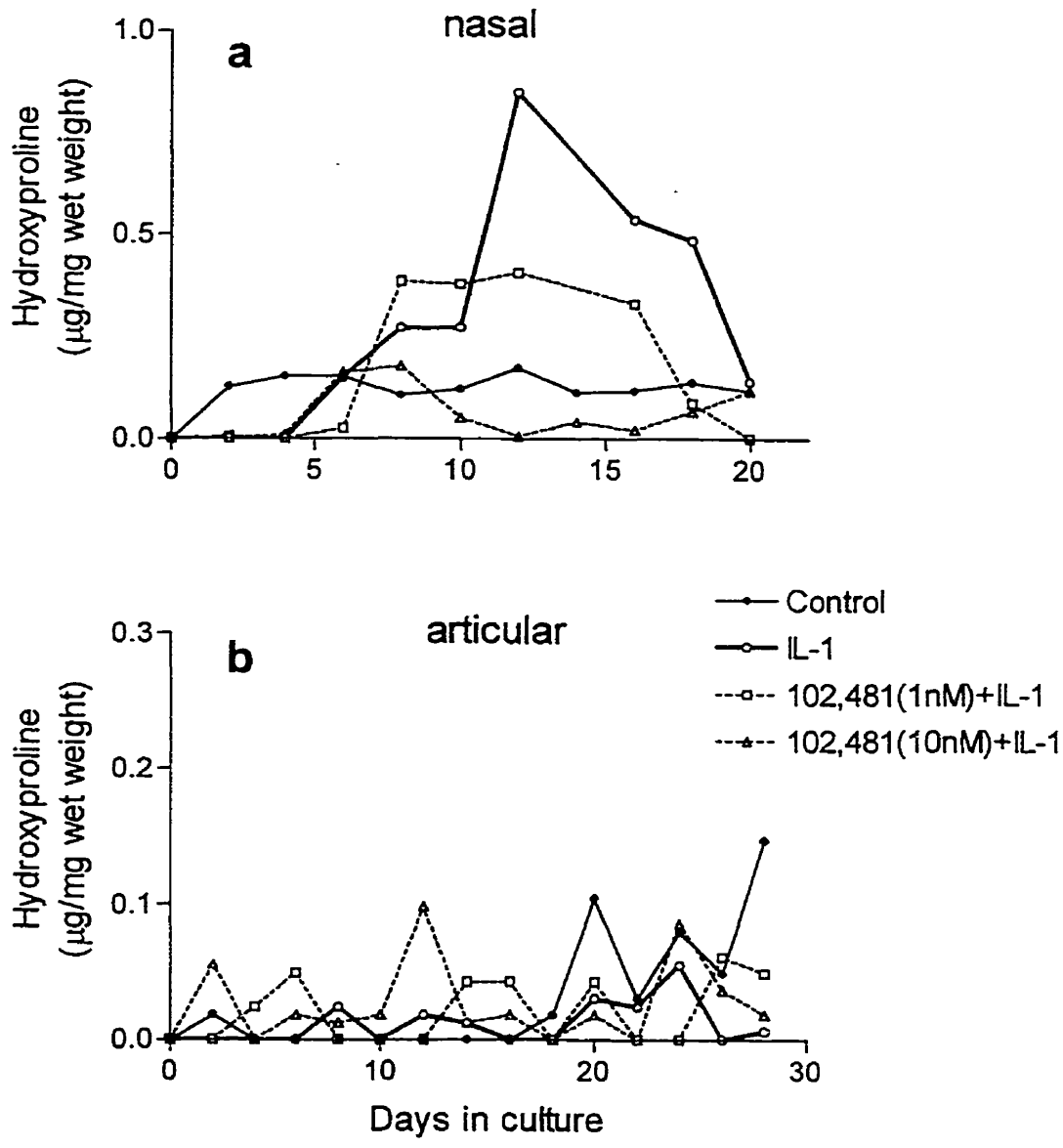


Figure 4.30 The effect of the inhibitor RS 102,481 on the release of hydroxyproline from IL-1 stimulated bovine cartilage explant cultures.

The hydroxyproline levels in the conditioned media of bovine nasal (**a**) and articular (**b**) cartilage explant cultures were determined as described in section 3.3.3.2. Shown are the values for explants cultured in the presence (○) or absence (●) of 5 ng/ml rHuIL-1α, and for those cartilages co-cultured with IL-1 and either 1 nM ( ) or 10 nM (Δ) concentration of RS 102,481. The hydroxyproline content was determined for 50-75 mg (wet weight) samples, at each time point and for each condition.

It is important to note that the inhibitors or vehicle alone (0.1% DMSO) were also added to cartilage explants without IL-1α stimulation. In all cases there were no significant alterations in the levels of COL2-3/4C, COL2-3/4m, sGAG or hydroxyproline from those of control explants cultured without IL-1α and inhibitors (data not shown). Moreover, in many cases, the levels of COL2-3/4C and COL2-3/4m were less in the explants cultured with the inhibitors alone than the control explants, suggesting inhibition of the proteolytic activity involved in the normal turnover of collagen molecules in cartilage.

4.3.3.5 Effects on total collagen

The cartilage explants responded to IL-1 stimulation +/- the inhibitor RS 102,481 in a similar manner, in terms of total collagen levels (Fig. 4.31), as they did for total sGAG levels (Fig. 4.19). The nasal cartilage explants cultured without IL-1 demonstrated significant increases in total collagen levels compared to day 0 tissue levels, when assessed as total hydroxyproline released into the media throughout the culture period, plus the amount remaining in the tissue at the end of the culture period. There were also increases in total collagen for the nasal explants co-cultured with IL-1 and a 10 nM concentration of RS 102,481, however the increases were not significant as with total sGAG. The articular cartilages showed no significant differences in total collagen levels compared to day 0 levels, for any of the conditions tested (Fig. 4.31).

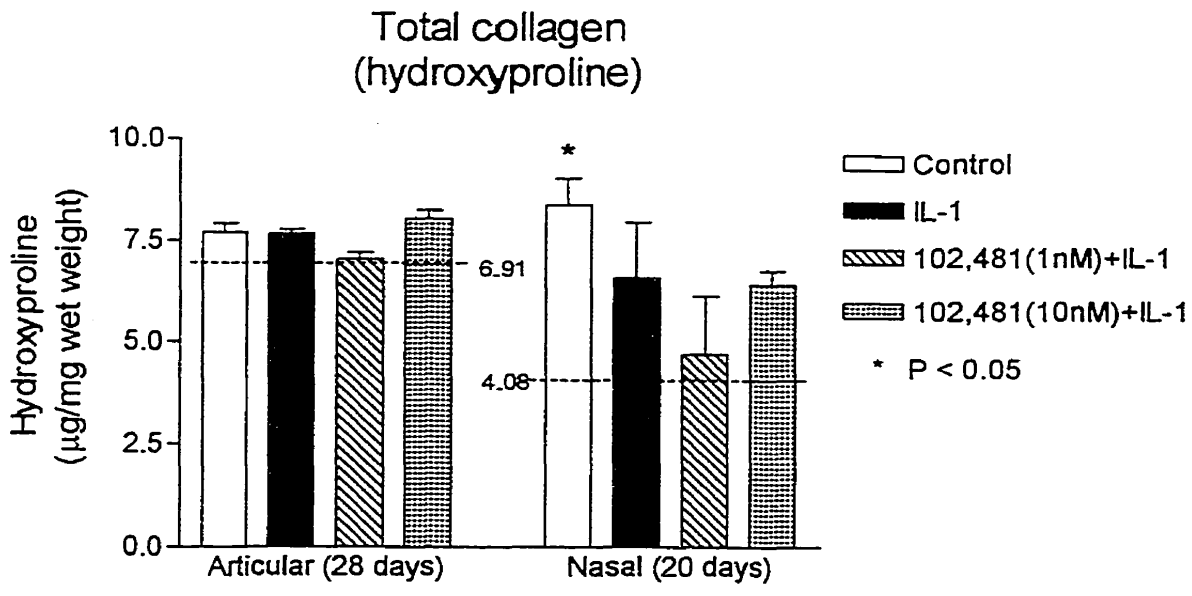


Figure 4.31 The effect of selective MMP inhibition on the total amount of collagen, measured as hydroxyproline, in IL-1 stimulated bovine cartilage explant cultures.

The total collagen levels shown represent the cumulative amount of hydroxyproline released into the media of both the nasal and articular cartilage explant cultures, added to the hydroxyproline levels in the respective α-chymotrypsin and proteinase K digests of day 20 (nasal) and day 28 (articular) explants. The bars and labels are as described in the legend of Fig. 4.19. Shown are the total levels of collagen for cultures with the RS 102,481 inhibitor. The dashed lines and accompanying values represent the mean levels of hydroxyproline in α-chymotrypsin and proteinase K digests of day 0 explants (50-75 mg wet weight of tissue). Significant increases over Day 0 hydroxyproline tissue levels are indicated by * for $P < 0.05$.

5. DISCUSSION

Different approaches have been used to evaluate cartilage breakdown (Poole *et al.*, 1993). The method that we selected was the identification of products generated by the proteolytic activities of specific matrix degrading enzymes, MMPs, on a specific cartilage matrix molecule, type II collagen. Furthermore, the effect of the selective inhibition of MMP activity on the generation of these products was examined. It was previously shown in this laboratory that osteoarthritis, which involves the progressive degeneration of articular cartilages, is characterized by increased denaturation of type II collagen (Hollander *et al.*, 1994, 1995). This molecule is essential for the maintenance of the tensile properties of this tissue, its molecular architecture and thus, its survival. The collagenases MMP-1, MMP-8 and MMP-13, along with MT1-MMP, are the only proteinases currently known to be capable of initiating the denaturation of fibrillar collagens, such as type II, by first cleaving the triple helix of this molecule. Primary cleavage occurs at a single site within the triple helix, producing approximately three quarter and one quarter length denatured α chain fragments.

We have produced antibodies that recognize the amino- and carboxy-termini (neoepitopes) of the collagenase cleavage sites in native triple helical type II collagen. We used these antibodies to further characterize the cleavage of type II collagen by MMP-1, MMP-8 and MMP-13. These studies show that there is continued processing at the amino-terminus of the one quarter fragment, by all these collagenases, to produce first a secondary cleavage site at Gly_{778} -Gln_{779}, and then further cleavage(s) leading to the release and/or cleavage of these neoepitopes. An antibody was prepared against the

secondary amino-terminal neoepitope of the one quarter fragment, and was used to demonstrate its production by MMP-1, MMP-8 and MMP-13. There was evidence for continued processing at the C-terminus of the three quarter fragment by MMP-13, albeit at a much slower rate compared to that for the N-terminal processing of the one quarter fragment by the same enzyme. Moreover, the apparent greater resistance of the C-terminus of the three quarter collagen fragment to secondary proteolysis, as compared to the N-terminus of the one quarter fragment, suggested to us that it would be more useful to assay for the C-terminal COL2-3/4C neoepitope as an indicator of collagenase cleavage of type II collagen. This was done for the immunochemical analyses in this study.

Our immunohistochemical analyses using the anti-neoepitope antibodies revealed that collagen cleavage primarily originates around the chondrocytes of the cartilage matrix. In aging, this is most pronounced at and close to the articular surface, and progresses into the deeper regions with increasing age of the individual and in OA. These observations implicate the chondrocyte as the source of collagenase(s) producing this damage, as was also indicated in our recent studies of collagen II denaturation (Hollander *et al.*, 1995). Moreover, the immunochemical analysis showing significantly higher levels of COL2-3/4C neoepitope in human OA articular cartilages, compared to non-arthritic articular cartilages, supports the semi-quantitative findings of immunostaining showing increased type II collagen cleavage in OA.

Observations worth noting are the significant direct correlations between the COL2-3/4m intrachain epitope, indicative of type II collagen denaturation, and both the COL2-3/4C and COL2-1/4N2 collagenase-specific cleavage site neoepitopes, as determined through the grading of immunostaining of human articular cartilages by the

antibodies specific to these epitopes. This was supported by the significant correlations noted between percentage cleaved collagen (as determined by immunoassay for the COL2-3/4C neoepitope) and percentage denatured collagen (calculated from immunoassay for the COL2-3/4m intrachain epitope) in the immunoassays of extracts of human OA articular cartilage. These correlations were evident in both non-arthritic (patients <42 yr for immunohistochemistry) and OA specimens. Moreover, as will be further discussed later in this section, there were significant correlations between both these epitopes in terms of media and tissue digest levels in IL-1 stimulated bovine nasal cartilage explant cultures. Taken together, these findings support the dogma that collagenase activity plays a key role in the denaturation and subsequent degradation of type II collagen in normal and OA articular cartilages, as well as cytokine (IL-1) stimulated cartilage breakdown. However, as epitope content was usually lower for the collagenase-generated epitope, COL2-3/4C, compared to the hidden denaturation epitope, COL2-3/4m, in both the immunochemical and the semi-quantitative immunohistochemical analyses of the human articular cartilages, it would seem that the former is preferentially lost from the cartilage. This loss of the epitope could be detected in cultures of both human OA articular cartilages and IL-1 stimulated bovine cartilages. Moreover, since this loss was abrogated by a preferential MMP-13 inhibitor, there is thus evidence presented here to suggest that MMP-13 may play a significant role in the cleavage of type II collagen.

Recent studies have provided evidence for the expression in normal, and the increased expression in OA articular cartilages, of MMP-1, MMP-8 and MMP-13 (Borden *et al.*, 1996; Chubinskaya *et al.*, 1996; Cole *et al.*, 1996; Mitchell *et al.*, 1996;

Reboul *et al.*, 1996). With the new technology described in this thesis, we can now show that the increased expression of any or all of MMP-1, MMP-8 and MMP-13 is associated with increased cleavage by collagenase(s) of triple helical type II collagen molecules in OA articular cartilage. Other collagenases responsible for this cleavage remain to be identified in human OA articular cartilage, however similar intrahelical cleavage has been recently described for MT1-MMP (Ohuchi *et al.*, 1997), the mRNA expression of which has been identified in human normal and OA cartilages (Buttner *et al.*, 1997). By demonstrating the production of the cleavage site produced only by collagenases, our study is the first to definitively implicate the activity of a collagenase in the cleavage of type II collagen in human articular cartilage, in addition to its increased activity in OA. Whether MMP-13 does in fact play a major role in this cleavage, as is suggested by the inhibitor studies, would require further investigation. In view of the K_i values, it is conceivable that the preferential MMP-13 inhibitor, RS 102,481, may also block MMP-8 activity at the higher concentrations. However, it is unlikely that MMP-1 would be affected, as supported by the in vitro cleavage studies, reported here, using recombinant enzyme and purified type II collagen. In the studies of cultured bovine nasal and articular cartilage explants stimulated with IL-1, we show a similar significant reduction in the detection of the COL2-3/4C neoepitope in media when these cultures included the same collagenase inhibitor as that used with the human OA articular cartilage explants. Thus, combined with its capacity to preferentially cleave type II collagen, as demonstrated here and by others (Knauper *et al.*, 1996; Mitchell *et al.*, 1996), MMP-13, and possibly MMP-8, are identified as potential targets for therapeutic intervention in OA.

In parallel studies that will be published separately, we have shown that in cultured OA cartilage, there is an increased release over normal tissue of the COL2-3/4C neoepitope (L. Dahlberg, R. C. Billinghurst *et al.*, manuscript in preparation). This, combined with our ability to detect type II collagen degradation products bearing the COL2-3/4C neoepitope in body fluids from arthritic patients (unpublished observations), means that the increased degradation of type II collagen by collagenase in OA is a fundamental feature of this arthritis. Its measurement *in vivo* may be of value in studying disease activity and in its clinical management.

This study is the first to describe the IL-1 induced degradation of type II collagen in cartilage, in terms of both the cleavage by specific collagenases and the subsequent denaturation of its triple helical molecular structure. Monitored in this investigation were both the tissue and media levels of the type II collagen collagenase-generated neoepitope COL2-3/4C and the hidden intrachain epitope COL2-3/4m. This study demonstrates the usefulness of an *in vitro* culture system, utilizing the COL2-3/4C and COL2-3/4m immunoassays, in assessing the efficiency of inhibitory agents that target specific degradative processes in the breakdown of cartilage and its structural components, specifically type II collagen with these two assays.

As has been previously reported, IL-1α initially induces the release of proteoglycans from the matrix of cultured bovine nasal (Nixon *et al.*, 91; Buttle *et al.*, 1993; Ellis *et al.*, 1994; Wright *et al.*, 1994; Cawston *et al.*, 1995; Bottomley *et al.*, 1997; Kozaci *et al.*, 1997) and articular (Buttle *et al.*, 1993; Oleksyszyn and Augustine, 1996; Xu *et al.*, 1996; Kozaci *et al.*, 1997) cartilage explants. Such were the findings in this study. It appears that the proteoglycans serve to protect the collagen network from

proteolytic breakdown, as it was only after this initial period of proteoglycan degradation, in which the bulk of sGAG was released from the cartilage matrix, that there was the simultaneous appearance of both type II collagen cleavage and denaturation epitopes. Moreover, the appearance of both collagen degradation epitopes (COL2-3/4C and COL2-3/4m) in tissue digests always preceded their detection in media. Together these observations suggest that the intrahelical cleavage generating COL2-3/4C neoepitope is directly responsible for the unwinding of type II collagen α1 chains, which exposes the COL2-3/4m epitope and is indicative of type II collagen denaturation. The delayed release of cleaved and denatured collagen molecules, from the matrix and into the media, may be due, in part, to the necessity for secondary proteolytic activity to free the collagen fragments from their cross-links in the fibril, such as through the telopeptidase activity of MMP-3 (Wu *et al.*, 1991) and/or MMP-13 (Knauper *et al.*, 1997), and/or the gelatinase activity of various enzymes, including MMP-1, -2, -3, -8, -9 and -13 (see below).

To lend support to the claim that the irreversibility of cartilage degradation is due to the breakdown of the collagen network (Jubb and Fell, 1980), it was only after the detection of type II collagen cleavage and denaturation epitopes that the IL-1 stimulated nasal cartilage explants began to dissolve macroscopically. In a recent report, it was shown that this macroscopic degradation of nasal cartilage cultured with IL-1α was correlated to a large increase in MMP activity, both collagenolytic and gelatinolytic (Kozaci *et al.*, 1997).

This study expands upon previous investigations in which the loss of collagen from bovine nasal cartilage explants stimulated with IL-1α was prevented by the selective inhibition of collagenases +/- gelatinases (Nixon *et al.*, 1991, Ellis *et al.*, 1994; Cawston

et al., 1995; Chichester *et al.*, 1996; Bottomley *et al.*, 1997; Kozaci *et al.*, 1997). In these earlier studies, the inhibitory profiles of the compounds used against some MMPs were not reported, either because they were unknown or because the MMP had not been identified at that time, such as the recently cloned and characterized MMP-13. The inhibitors used in these previous studies did not permit selective inhibition of the collagenases now known to be expressed by chondrocytes, namely MMP-1, MMP-8 and MMP-13. Moreover, it is likely that compounds shown to inhibit MMP-1, also inhibited MMP-13, as it is the activity of the latter enzyme that is more readily blocked by synthetic inhibitors. This is based on the unpublished observation that there is usually a lower K_i for MMP-13 than MMP-1 with most inhibitors, where the inhibition of both MMPs has been examined.

In our study, we demonstrated, with selective inhibition of MMP-13 (RS 102,481) and with the more broad spectrum inhibition of MMP-1, MMP-8 and MMP-13 (RS 47,112), that it is likely that MMP-13 is a key collagenase involved in the cleavage of type II collagen in IL-1 stimulated bovine cartilage explants. This conclusion is based upon the fact that, in this study, both these inhibitors prevented the generation of the collagenase-generated COL2-3/4C neoepitope, more or less to the same degree. The only collagenase that would be inhibited by these compounds, based on the K_i values, at the concentrations used, would be MMP-13. Moreover, this enzyme has been shown to be from 5- to 10-fold more effective at cleaving native triple helical type II collagen than either MMP-1 or MMP-8 (Knauper *et al.*, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996). This supports a key role for MMP-13 in the breakdown of articular cartilage, which is rich in type II collagen. The increased expression of MMP-13 in human articular

cartilage stimulated with IL-1 has been reported (Borden *et al.*, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996; Shlopov and Hasty, 1997). IL-1α has also been shown to induce the production of MMP-13 in bovine nasal (Kozaci *et al.*, 1997) and articular cartilage explants (W. Wu *et al.*, manuscript in preparation). Although the more selective MMP-13 inhibitor, RS 102,481, at 1 nM showed no significant effect on the overall generation of COL2-3/4C by IL-1α, it was used at concentrations that were 20- and 200-fold lower than those used for RS 47,112. This was designed to minimize the potential for inhibition of MMP-8 by RS 102,481. Moreover, the K_is have been determined using peptide substrates that have a much higher K_m than that of the high molecular weight type II collagen substrate. The Western blot analyses of nasal cartilage conditioned culture media confirmed that RS 102,481 was able, in a dose-dependent fashion, to inhibit the release and/or generation of fragments bearing the collagenase-generated COL2-3/4C neoepitope.

The complete degradation of cleaved triple helical type II collagen molecules is believed to be dependent upon the unwinding of the α chains. Then as gelatin, it is a substrate for many enzymes including the gelatinases MMP-2 and MMP-9, stromelysin MMP-3, and the collagenases MMP-1, MMP-8, and MMP-13 (Knauper *et al.*, 1996). The COL2-3/4m epitope is specific for the denaturation of type II collagen molecules, and in this study, the generation of this epitope seemed to be dependent upon both collagenase and gelatinase activity. Only RS 47,112, at both concentrations used in this study (20 and 200 nM), significantly reduced the generation of the COL2-3/4m epitope by IL-1α in the media and tissue digests of both the nasal and articular cartilages. At the concentrations used in this study and based on the K_i values for each, RS 47,112 would be inhibitory for

both gelatinases, MMP-2 and MMP-9, and RS 102,481 would not be inhibitory for MMP-9 (MMP-2 not determined),. RS 102,481 had no significant effect on the tissue digest levels of COL2-3/4m, but did significantly reduce the release of this epitope into the media at the higher concentration of 10 nM. This suggests that MMP-13 may also have a role in the release of collagen degradation products from IL-1 stimulated cartilages, perhaps, as suggested earlier in this section and by Knauper *et al.* (1997), through its gelatinolytic and/or telopeptidase activities.

Unlike previous reports (Xu *et al.*, 1996; Kozaci *et al.*, 1997), we have shown, with our sensitive immunoassay for the COL2-3/4C neoepitope, that there is indeed type II collagen degradation, in the form of collagenase-generated cleavage and denaturation, in bovine articular cartilage explants that have been stimulated with IL-1α. It is, however, at a much lower level than that which occurs in bovine nasal cartilage explants, as there was 10-fold less COL2-3/4C epitope in the media and digests of the articular cartilage explants than that found with the nasal cartilage explants. This may be explained, in part, by the recent observation that there was an absence of MMP activity (collagenolytic and gelatinolytic) in bovine articular cartilage explants stimulated with IL-1α (Brown *et al.*, 1996; Kozaci *et al.*, 1997). Another difference noted between the nasal and articular cartilages is that, although there was 2- to 3-fold more of the COL2-3/4m epitope generated by IL-1α in the nasal compared to articular cartilage explant cultures, the majority of the epitope detected with the articular cartilage explants was associated with the tissue digests. Very little was found in the media. Denatured collagen was assessed at the tissue level only after 28 days of culture in the Xu *et al.* (1996) study, and, similar to our investigations, they found no significant increase in COL2-3/4m epitope at

this time point in the digests of IL-1α stimulated bovine articular cartilage, compared to control nonstimulated digests. However, at 14 days of culture, we detected 2- to 3-fold more COL2-3/4m epitope in the IL-1 stimulated articular cartilage digests than in the control digests. Tissue levels of denatured collagen were not assessed in the Kozaci *et al.* (1997) study. This coupled with our similar findings of trace amounts of COL2-3/4m epitope in the media throughout the culture period, probably accounts for their conclusion of a lack of IL-1-induced type II collagen degradation in bovine articular cartilage explants.

It has been shown that bovine articular and nasal chondrocytes, as pellet cultures, degrade type II collagen in a similar fashion after stimulation with IL-1α (Xu *et al.*, 1996). The difference in collagen degradation noted, between the two different tissues as explant cultures, may then be more the result of differences within the matrix of each. Important may be the higher level of cross-linking of collagen in articular compared to nasal cartilage (Eyre *et al.*, 1984). The more protracted release of proteoglycans from the articular cartilage, compared to nasal cartilage explants, may serve to retain high tissue levels of the denatured collagen molecules. However, if it was merely a matter of prolonged retention of epitope in the tissue, one would expect articular cartilage levels of the COL2-3/4m and COL2-3/4C epitopes to be relatively similar. There was, in fact, 10-fold more COL2-3/4m than COL2-3/4C epitope in the articular cartilage digests suggesting a selective retention of the former and release of the latter, perhaps through a proteolytic cleavage between the two epitopes. This is supported indirectly by the observation that no fragments bearing the COL2-3/4C neoepitope could be identified in the Western blots of articular cartilage media that were positive for this epitope on

immunoassay. This suggests that fragments bearing the COL2-3/4C epitope, in the electrophoresed articular cartilage conditioned media, were too small to be retained on the gel, which could result from a cleavage between the COL2-3/4m and COL2-3/4C epitopes of the type II collagen TC^A α chain fragments. It is also possible that the levels of the COL2-3/4C epitope in the articular cartilage media were simply below the sensitivity level for detection by Western blotting. There was 10-fold less COL2-3/4C epitope identified by immunoassay in the articular compared to the nasal cartilage culture media which had fragments bearing the COL2-3/4C neoepitope identified by similar immunoblot analyses.

In a previous study using bovine articular cartilage explant cultures (Dodge and Poole, 1989), there was no evidence of collagen fragments in the media of explants that were stimulated with IL-1β, despite the fact that fragments were identified in the stimulated cartilage digests. It must be noted that in this earlier investigation, the conditioned media examined were from explants that had been cultured with IL-1 for 11 days, and in our present study, there was immunochemical detection of significant levels of the COL2-3/4C epitope only after 18 days of IL-1α stimulation.

In the nasal cartilage cultured with IL-1α, despite similar tissue digest levels of COL2-3/4C and COL2-3/4m, significant correlations between the two epitopes in both media and tissue, and significant correlations between the two epitopes and hydroxyproline levels in the media, there was 2- to 3-fold more of the COL2-3/4C neoepitope than the COL2-3/4m epitope in the conditioned media. This may be the result of further proteolytic degradation, as described above for the articular cartilage explants, with the subsequent loss within the media of the COL2-3/4m epitope. This was

suggested by the Western blotting analyses of the nasal cartilage conditioned media, in which fragments ranging from 25-40 kD were identified that were positive for COL2-3/4C neoepitope, and fewer and less reactive fragments were shown to retain COL2-3/4m immunoreactivity. In fact, the major band that reacted with the COL2-3/4C_{short} antiserum appeared to not be the same band, based on its mobility within the gel, as the major band that stained with the COL2-3/4m antibody. This would again support the idea of a cleavage of the type II collagen α chain between the two epitopes. Type II collagen fragments of a similar size range have previously been identified in the conditioned media of bovine nasal cartilage explant cultures stimulated with IL-1 (Chichester *et al.*, 1996). Further studies are necessary to define the exact cleavage site(s) within the α1(II) chains that would be responsible for the generation of the fragments identified in the present investigation. Such work was however beyond the focus of this project.

The lack of an inhibitory effect on IL-1 induced proteoglycan release from cartilage, by either of the compounds used, was not totally unexpected. This has been reported previously using both synthetic selective MMP inhibitors (Nixon *et al.*, 1991; Ellis *et al.*, 1994; Bottomley *et al.*, 1997), and naturally occurring tissue inhibitor of metalloproteinases (TIMP) (Andrews *et al.*, 1992; Ellis *et al.*, 1994). Taken together, these results suggest that the breakdown of the cartilage proteoglycan aggrecan is dependent upon proteinases other than the MMPs, such as cysteine proteinases (Buttle *et al.*, 1993) and/or the yet to be identified "aggrecanase" (Sandy *et al.*, 1992). These enzymes appear not to be inhibited by those compounds targeting the known MMPs. In support of this concept, it was recently shown that MMP activity did not correlate with

proteoglycan degradation in cartilage explant cultures (Brown *et al.*, 1996; Kozaci *et al.*, 1997).

The failure of both inhibitors used in this study to block proteoglycan release from the cartilage, supports a minor role, at best, for MMP-13 in the degradation of cartilage aggrecan (Fosang *et al.*, 1996). The failure of RS 47,112, which would be inhibitory against MMP-8 (based on the K_i values) at the concentrations used in this study, to prevent the degradation of proteoglycans in the cartilage explants, supports studies discrediting MMP-8 as being the "aggrecanase" (Arner *et al.*, 1997). As well, the failure of RS 47,112 to inhibit proteoglycan breakdown in the bovine explants suggests little, if any, involvement of any of the known collagenases in this process, despite the recent claim that a novel collagenase-like proteinase(s) may be involved in cartilage proteoglycan breakdown (Brown *et al.*, 1996). Moreover, the fact that there were, in some cases, increases in GAG release when the inhibitors were added to the IL-1 stimulated cartilages, may suggest that the MMPs may be involved more in feedback mechanisms controlling the level of proteoglycan degradation.

Stromelysin-1 (MMP-3) has been shown to cause significant proteoglycan loss both *in vitro* from bovine articular cartilage explant cultures to which it was added (Bonassar *et al.*, 1995a), and *in vivo* from lapine articular cartilage of joints into which it was injected (Bonassar *et al.*, 1995b). Moreover, inhibition of stromelysin-1 with selective synthetic inhibitors was shown to markedly reduce IL-1 induced proteoglycan release from nasal (Bottomley *et al.*, 1997) and articular cartilage explants (Mort *et al.*, 1993; Bonassar *et al.*, 1995a). It is important to note that it has been shown that the inhibitor concentrations necessary to prevent cartilage, and specifically, proteoglycan

degradation, are substantially higher (from 10- to 1000-fold) than those concentrations effective against the isolated MMPs and reflected in the K_i and IC_{50} values (Caputo *et al.*, 1987; Andrews *et al.*, 1992, Ellis *et al.*, 1994; Bottomley *et al.*, 1997). The highest concentration used for RS 47,112 of 200 nM represents an inhibitor level 87-fold greater than its K_i value against stromelysin. Nevertheless, although this enzyme may have been only weakly inhibited, at best, by the concentrations of RS 47,112 used in the current study, and not at all by RS 102,481, the role of stromelysin as the proteinase directly responsible for GAG release *in vivo* has been argued, based upon the characteristics of proteoglycan cleavage products identified in arthritic joints (Sandy *et al.*, 1992). Moreover, the inhibition of proteoglycan degradation induced by IL-1α in the cartilage explants was not to be the focus of this study, and, as such, the choice and concentrations of inhibitors used were determined with the intent to preferentially inhibit the collagenases and type II collagen cleavage and denaturation.

One surprising finding in this study was that the total amount of sGAG assayed from the IL-1 stimulated bovine nasal cartilage explants (media + tissue), after 20 days of culture, was significantly elevated over the tissue levels of day 0 nasal cartilage explants. This was not the situation with the articular cartilage explants, where there was a non-significant decrease in levels of total sGAG (media + tissue) in the IL-1 stimulated explants at day 28 of culture, compared to the day 0 articular cartilage explant tissue levels of sGAG. Moreover, a similar pattern was identified for the total collagen levels, however the increase for IL-1 stimulated nasal cartilage explant cultures was not significant.

It has been well documented that there is an inhibition of the mRNA expression and synthesis of both proteoglycan (Tyler, 1985a, 1985b; Benton and Tyler, 1988; Morales and Hascall, 1989; Seed *et al.*, 1993), and type II collagen (Tyler, 1988; Frazer *et al.*, 1994) in articular cartilage chondrocytes, in response to IL-1. It appears that, although there was a significant reduction in the tissue levels of sGAG in our investigations, due likely to the increased proteolytic activity induced by IL-1 (Ratcliffe *et al.*, 1986), the chondrocytes are continuing to synthesize replacement proteoglycans which are not being retained by the matrix. There is experimental evidence showing that considerable loss and replacement of proteoglycans in articular cartilage may occur before becoming irreversible, as long as the collagen network is undamaged (Tyler, 1985a; Pettipher and Henderson, 1988; Page-Thomas *et al.*, 1991; Rizkalla *et al.*, 1992). Moreover, proteoglycan synthesis is usually up-regulated during the early stages of osteoarthritis, where cytokines may be more involved in the pathological processes (Muir, 1992). It should also be noted that the cultures in the current studies were supplemented with insulin, and it has been shown that insulin-like growth factor can increase the synthesis of proteoglycans in cartilage exposed to IL-1 (Tyler, 1989).

The decrease in total collagen levels in the nasal cartilage explants stimulated with IL-1α, as compared to the control cultures without IL-1α, coupled with the lack of such a response in the articular cartilages, may help to explain the maintenance of the structural integrity of the articular cartilages and the complete degradation of the nasal cartilages after culturing with IL-1. Moreover, it was recently shown that there was no difference in the tissue levels of total collagen, after 4 weeks, in bovine articular cartilage cultured with or without 10-fold more IL-1α (50 ng/ml) than used in our investigations (Kozaci *et al.*,

1996). However, they did not compare total collagen levels (media + tissue) to day 0 tissue levels, as was done in our study, so it is difficult to compare their results with ours.

The reasons behind the differential responses of articular and nasal cartilages to IL-1 stimulation, in terms of both proteoglycan and type II collagen metabolism, are not known. A recent report showed that articular cartilage chondrocytes, as pellet cultures, deposit much more matrix than nasal cartilage chondrocytes (Xu *et al.*, 1996). This suggests an inherent difference in metabolic capabilities between these two chondrocyte populations. Other possible explanations include a difference in the number and/or type of interleukin-1 receptors, and thus IL-1 responsiveness, between these two cartilages. It has been shown, when comparing human ankle and knee joint articular cartilages, that there are fewer IL-1 receptors and thus, differences in response to IL-1 in the ankle cartilage (Hauselmann, 1994). There may be differences in MMP and/or TIMP expression and synthesis between nasal and articular cartilages. A differential expression of MMP-8, again between ankle and knee joint articular cartilage chondrocytes, has been described (Chubinskaya *et al.*, 1996). A similar situation may exist between bovine nasal and articular cartilage chondrocytes, as suggested by a recent report that bovine nasal cartilage chondrocytes synthesize high levels of MMPs, in response to IL-1, while bovine articular chondrocytes do not (Kozaci *et al.*, 1996). The articular cartilage chondrocyte may not possess the cascade of enzymes necessary for initiation of collagen breakdown (Buttle *et al.*, 1995). For example, the urokinase-type plasminogen activator (uPA)/ plasminogen activator inhibitor (PAI) balance, generated by chondrocytes in response to IL-1, and which may be important in the activation of MMPs in vivo, may differ between tissue types (Oleksyszyn and Augustine, 1996). Moreover, there may be differences in

integrin receptor expression and interaction with matrix ligands, between the two tissues, and this may influence IL-1 mediated MMP expression (Arner and Tortorella, 1995).

6. CONCLUSIONS

This work has demonstrated that it is possible to produce antibodies that will recognize the cleaved termini of collagen α chains, generated by the proteolytic activity of collagenases belonging to the MMP family of endoproteinases, on triple helical collagen molecules. As a result of the investigations, which utilized these antibodies and were reported in the preceding sections of this document, the following conclusions can be made concerning the interrelationships between arthritis, type II collagen degradation, matrix metalloproteinases and proinflammatory cytokines:

- There is continued processing of the cleaved termini of type II collagen α chains by the proteinases responsible for generating these neoepitopes; i.e. MMP-1, MMP-8 and MMP-13.
- There is a significantly correlated increase in the cleavage and denaturation of type II collagen in human articular cartilage with normal aging, based upon immunostain grading; in osteoarthritis, based upon immunostain grading and immunoassays; and in IL-1 stimulated bovine nasal cartilage, based upon immunoassays. This lends support to the dogma that it is the intrahelical cleavage of the type II collagen molecule that leads to its denaturation (unwinding) and the subsequent irreversible breakdown of the collagenous framework of cartilage, which are characteristic of arthritis.
- The initial pericellular location of cleavage-site and intrahelical (denaturation) epitopes in normal aging and low grade OA articular cartilages, coupled with the spontaneous release of cleavage-site neoepitopes in cultured OA articular cartilages, implicates the chondrocyte in the production and/or activation of the collagenases responsible for the cleavage of native triple helical type II collagen in situ.

- The spontaneous release from human OA cartilage explant cultures, and the IL-1-induced production in bovine articular cartilage explant cultures, of the COL2-3/4C cleavage site neoepitope, could be significantly reduced with a synthetic preferential inhibitor of MMP-13. This suggests a role for MMP-13 in type II collagen cleavage in both natural (OA) and cytokine-induced (IL-1) articular cartilage degradation.
- The proteoglycans of cartilage may protect native type II collagen from proteolytic attack, for it is only after the bulk of tissue proteoglycan has been lost, that there is the appearance of cleaved and denatured collagen epitopes in the media and in the α-chymotrypsin tissue digests of IL-1 stimulated cartilage explant cultures. The inhibitor studies suggest that the known MMPs may play, at best, a minor role in the breakdown of proteoglycans in this in vitro system of cytokine-induced cartilage degradation.
- There is a differential response to IL-1, in terms of collagen and proteoglycan metabolism, between articular and nasal cartilages as explant cultures, which may help to explain the differential responses of both cartilages in terms of cartilage degradation. The articular cartilages appear to be less responsive to IL-1 compared to the nasal cartilages.
- The COL2-3/4C_{short} antibody can be used in immunochemical and immunohistochemical analyses to evaluate the effect of various agents, both therapeutics (e.g. MMP inhibitors) and potential pathophysiological mediators (e.g. IL-1), on the cleavage of native type II collagen in articular cartilage both in health and disease.

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8. APPENDIX

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

Enhanced Cleavage of Type II Collagen by Collagenases in Osteoarthritic Articular Cartilage

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Abstract

We demonstrate the direct involvement of increased collagenase activity in the cleavage of type II collagen in osteoarthritic human femoral condylar cartilage by developing and using antibodies reactive to carboxy-terminal (COL2-3/4C$_{short}$) and amino-terminal (COL2-1/4N1) neoepitopes generated by cleavage of native human type II collagen by collagenase matrix metalloproteinase (MMP)-1 (collagenase-1), MMP-8 (collagenase-2), and MMP-13 (collagenase-3). A secondary cleavage followed the initial cleavage produced by these recombinant collagenases. This generated neoepitope COL2-1/4N2. There was significantly more COL2-3/4C$_{short}$ neoepitope in osteoarthritis (OA) compared to adult nonarthritic cartilages as determined by immunoassay of cartilage extracts. A synthetic preferential inhibitor of MMP-13 significantly reduced the unstimulated release in culture of neoepitope COL2-3/4C$_{short}$ from human osteoarthritic cartilage explants. These data suggest that collagenase(s) produced by chondrocytes is (are) involved in the cleavage and denaturation of type II collagen in articular cartilage, that this is increased in OA, and that MMP-13 may play a significant role in this process. (*J. Clin. Invest.* 1997. 99:1534–1545.) Key words: matrix metalloproteinases • arthritis • chondrocytes • antibodies

Introduction

Type II collagen is composed of a triple helix of three identical α chains. These molecules associate to form a fibril that is stabilized by intermolecular crosslinks (1). The fibrils provide the tensile strength and maintain the integrity of mammalian articular cartilage by forming a network that resists the swelling pressure resulting from the hydration of the polyanionic proteoglycan aggregates in the extracellular matrix (2, 3). Damage to this fibrillar meshwork, made up of primarily type II collagen (~ 90–95%), may be a critical event in the pathology of many arthritides, due in part to the very slow rate of collagen turnover within the cartilage (4). In early degeneration in articular cartilage, which may lead to osteoarthritis (OA)[1], there is a loss of the tensile properties, indicative of damage to the fibrillar network (2). Recent studies in this laboratory have provided evidence for increased denaturation of the triple helix of type II collagen in human articular cartilage in OA (5–7).

The extracellular degradation of fibrillar types I, II, and III collagens can occur both in nonhelical sites (8) and through a triple helical cleavage. Only the latter results in denaturation of the triple helix at physiological temperatures. This is achieved by collagenases which belong to the family of zinc-dependent endopeptidases called matrix metalloproteinases (MMPs). Collagenase-1 or MMP-1 (interstitial collagenase), collagenase-2 or MMP-8 (neutrophil collagenase), and the recently cloned and characterized collagenase-3 or MMP-13 (9, 10) are the only mammalian enzymes known to be able to initiate the intrahelical cleavage of triple helical collagen at neutral pH. MMP-3 (stromelysin-1) can cleave type II fibrillar collagen, but only in the nonhelical amino–telopeptide domain (11). These collagenases are thought to define the rate-limiting step in normal tissue remodeling events such as bone resorption and growth, embryonic development, uterine involution, wound healing, and in degradative processes such as arthritis, tumor invasion and metastasis, and periodontitis.

Until now, there has been no direct evidence for their involvement in intrahelical cleavage, except for the inability of murine collagenase to cleave type I collagen in vivo when the intrahelical site is mutated (8). These three collagenases cleave the fibrillar collagens type I, II, and III at a single site (Gly$_{775}$–Leu/Ile$_{776}$) within each α chain of the triple helical collagen molecule, approximately three quarters of the distance from the amino-

A preliminary report of this study was presented at the Gordon Research Conference on metalloproteinases, June 1995.

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Received for publication 7 July 1996 and accepted in revised form 17 January 1997.

J. Clin. Invest.

0021-9738/97/04/1534/12 $2.00
Volume 99, Number 7, April 1997, 1534–1545

1. *Abbreviations used in paper:* APMA, aminophenyl mercuric acetate; COL2-3/4C, type II collagen three-quarter fragment primary collagenase cleavage site carboxy-terminal neoepitope; COL2-1/4N2, type II collagen one-quarter fragment secondary collagenase cleavage site amino-terminal neoepitope; COL2-3/4m, type II collagen three-quarter fragment intrachain epitope; F(ab')$_2$, pepsin-generated divalent antibody fragment; MMP, matrix metalloproteinase; OA, osteoarthritis; OVA, ovalbumin; TC^A, three-quarter collagen fragment; TC^B, one-quarter collagen fragment; TIMP, tissue inhibitor of metalloproteinases.

terminal end of each chain, resulting in the generation of three-quarter (TC^A) and one-quarter (TC^B) length collagen fragments (12–14). The cleaved collagen fragments spontaneously denature into nonhelical gelatin derivatives at physiological temperatures, thereby becoming susceptible to further degradation by these collagenases (15) and/or by other proteinases, such as MMP-3, and the gelatinases MMP-2 and MMP-9.

Collagenases have been detected in the synovial fluid of patients with traumatic arthritis, RA, and OA (16–19). In the synovial joint, there are three distinct tissue sources of the collagenases that may be involved in the degradation of articular cartilage. Cells in OA and RA synovium express collagenases (20–23). The expression of MMP-13 in the synovial membrane was identified in RA and OA tissues in one study (24), but in other reports MMP-13 was not found in isolated synoviocytes from OA and RA patients, nor could it be induced in these cells by IL-1 (25, 26). Fibroblasts at the cartilage-pannus interface also secrete a collagenase that can degrade cartilage (27). Chondrocytes express and secrete MMP-1 (22, 25, 26, 28) and MMP-13 (14, 25, 26) in both normal and OA articular cartilages. This is upregulated by IL-1 (14). Moreover, two recent studies describe the expression of MMP-8 (neutrophil collagenase) by human articular chondrocytes from normal donors, and its upregulation by IL-1 (29, 30).

Collagenases have been routinely assayed in vitro (31, 32) and in vivo (33, 34) using radiolabeled collagen fibrils or fluorogenic peptides (35). These methods do not allow for the differentiation of the collagenases involved in the cleavage of these substrates. ELISAs have been developed using specific anticollagenase antibodies allowing the detection of collagenase and tissue inhibitor of metalloproteinase (TIMP)-collagenase complexes (36–38). However, the presence of enzyme in body fluids and tissues does not necessarily imply activity, as the collagenases are secreted as zymogens, they require activation, and their catalytic potential is determined by the presence of inhibitors such as tissue inhibitor of metalloproteinase and $alpha_2$ macroglobulin.

Using polyclonal and monoclonal antibodies that react specifically to denatured type II collagen α chains, such as can result from intrahelical cleavage by collagenases, we have shown that increased denaturation of this molecule can be detected in OA articular cartilages (5–7). This implicates collagenases in the denaturation of type II collagen. However, to definitively demonstrate a role for these proteinases in the cleavage of this molecule, a means of specifically identifying the collagenase cleavage site(s) in situ is required. This would be possible if we could prepare and use antibodies that react with the carboxy- (COOH-) and amino- (NH_2-) terminal neoepitopes created by cleavage of collagen by collagenases and which do not react with the intact molecule or α chains thereof. Antibodies of this kind prepared to cleavage site neoepitopes have already proven valuable in defining MMP activity in aggrecan degradation (39–45).

In this study, we have developed, for the first time, antibodies to the neoepitopes that represent the COOH- and NH_2 termini of TC^A and TC^B, respectively, of human type II collagen α chains, produced by cleavage of the triple helical collagen molecule by any one of the three collagenases: MMP-1, MMP-8, and MMP-13. Here we describe how these antineoepitope antisera were used to provide direct evidence for the involvement of collagenases in the cleavage of type II collagen in osteoarthritic human articular cartilages. Moreover, we show that there is a direct association between this cleavage and the denaturation of type II collagen. Finally, through the use of a synthetic preferential inhibitor of MMP-13, we provide evidence that indicates that the in vitro release of collagenase-specific type II collagen cleavage site neoepitopes from OA articular cartilage is usually mediated by MMP-13.

Methods

Tissue. Human adult and fetal articular cartilages, and human fetal skin were collected at autopsy (within 15 h postmortem) and from therapeutic abortions. Full-depth human osteoarthritic articular femoral condylar cartilage was obtained at arthroplasty from the knee joints of 26 patients with OA, as diagnosed using the criteria of the American College of Rheumatology for OA (46). Osteophytic cartilages were never examined. Macroscopically normal full-depth nonarthritic articular cartilages were obtained within 15 h of postmortem from the femoral condylar regions of 19 individuals from sites where there were no observable joint abnormalities. None of these persons had recent chemotherapy.

Collagen purification. Human type II collagen was prepared by pepsin digestion and differential salt precipitation, using the method of Miller (47). Human types I and III collagen were prepared by pepsin digestion and differential denaturation and renaturation (48). Bovine type X collagen was provided by Dr. A. Marriott (Joint Diseases Laboratory, Montreal, Canada). Cyanogen bromide (CNBr) peptides of human type II were prepared as described by Dodge and Poole (5).

Preparation of recombinant human proMMP-1, proMMP-8, and proMMP-13. Recombinant human proMMP-1 and proMMP-13 were purified from the culture medium of Sf9 cells that were infected with recombinant baculovirus, as described recently (14). Briefly, proMMP-1 was affinity purified using a monoclonal antibody column and proMMP-13 was purified using heparin-agarose and SP-Sepharose Fast Flow columns. Both the enzymes were > 95% pure as determined by Coomassie blue staining of SDS-PAGE gels. Recombinant human proMMP-8 was expressed in *Escherichia coli* as inclusion body protein, refolded, and purified as previously described (49).

Identification and synthesis of type II collagen collagenase cleavage site neoepitopes. For both the neoepitope peptides COL2-3/4C_{short} (Gly-Pro-Hyp-Gly-Pro-Gln-Gly), which corresponds to the COOH terminus of TC^A, and COL2-1/4N1 (Leu-Ala-Gly-Gln-Arg-Gly), corresponding to the NH_2 terminus of TC^B of the primary cleavage of native triple helical human type II collagen produced by collagenase-1 (MMP-1), amino acid sequences for their synthesis were based on published amino acid sequences (50) of fibroblast collagenase (MMP-1) cleaved human type II collagen alpha 1 (α1) chains (Fig. 1), with the following exception. The assignment of the third residue of the COL2-3/4C_{short} peptide (hereafter referred to only as COL2-3/4C), as a hydroxylated proline (Hyp) was based on the assumption that proline residues in the Y position of the repeating Gly-X-Y triplets that make up the helical portions of collagen molecules are potential hydroxylation sites within the collagen α chains. Moreover, we have shown that antisera generated to peptides containing a nonhydroxylated proline at this position react poorly to the cleaved TC^A of type II collagen and in turn, these peptides lacking hydroxylated proline, are recognized less effectively by antiserum generated from the COL2-3/4C peptide containing the hydroxylated proline residue (unpublished observations). The sequence for neoepitope peptide COL2-1/4N2 (Gln-Arg-Gly-Ile-Val-Gly), representing a secondary (N2) collagenase cleavage site at the NH_2 terminus of the human α1(II) TC^B, was determined using the methodology described recently (12).

These peptides (COL2-3/4C, COL2-1/4N1, and COL2-1/4N2) and those peptides used for epitope analyses and antisera characterization, as described below, were synthesized at a 0.25-mmol scale, using standard Fmoc (9-fluoroenylmethoxycarbonyl) chemistry, on a solid-phase peptide synthesizer (model 431A; Applied Biosystems, Foster City, CA). A cysteine was added to the NH_2 terminus of pep-

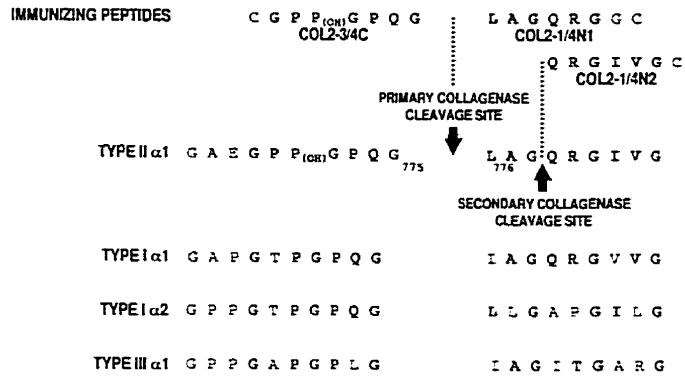

Figure 1. Sequence homology at and close to the interstitial collagenase cleavage sites of human type I, II, and III collagens and the immunizing peptides used to produce the COL2-3/4C, COL2-1/4N1, and COL2-1/4N2 antineoepitope antibodies. Bold letters indicate the amino acids in the alpha chains which are similar in both identity and location to those found in the natural immunizing peptide sequences, which are also highlighted by bold letters at the top of the figure. The primary collagenase cleavage sites were obtained from Birkedal-Hansen (50) while the secondary collagenase cleavage site was obtained by NH_2 terminal sequencing of electrophoretically separated and transferred collagen fragments obtained after MMP-13 (rHuMMP-13) digestion of triple helical human type II collagen, as described in Methods. The assignment of hydroxylation of the proline residues in the immunizing peptides indicated as $P_{(OH)}$, is also described in Methods.

tide COL2-3/4C and to the COOH termini of peptides COL2-1/4N1 and COL2-1/4N2 for conjugation of the peptides to the carrier proteins ovalbumin (OVA) and BSA using the bifunctional reagent *N*-hydroxy-succinimidyl bromoacetate (Sigma Chemical Co., St. Louis, MO), as described by Hughes et al. (39). A glycine spacer was included in the synthesis of the COL2-1/4N1 peptide, between the added COOH terminal cysteine and the neoepitope sequence, to improve both the immunogenicity of this peptide when conjugated to OVA and the sensitivity of the assays employing the peptide conjugated to BSA.

Preparation of polyclonal antibodies from rabbit antisera. For antineoepitope antisera production, female New Zealand White rabbits weighing 2.5–3.0 kg (Ferme des Chenes Bleues Inc., Montreal, Quebec, Canada) were initially immunized intramuscularly with 0.5 mg of neoepitope peptide conjugated to OVA in 0.25 ml PBS and emulsified with 0.25 ml of CFA (Difco Laboratories, Inc., Detroit, MI). Booster injections of similar quantities of peptide-OVA emulsified with incomplete Freund's adjuvant (IFA; Difco Inc.) were given intramuscularly every 2–3 wk. After the second booster, test bleeds were performed and antibody titers were determined by ELISA, as described below. In all cases, good titers were obtained after the two boosters. Animals were exsanguinated by cardiac puncture and ~ 50 ml of serum was obtained. For immunoassays, $F(ab')_2$ preparations were obtained by pepsin digestion of each antiserum, as previously described (51). The Fc portion and undigested IgG were removed by AH-Sepharose-Protein A chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden).

The preparation of a mouse monoclonal antibody (COL2-3/4m) to an intrachain epitope (CB11B) in the TC^ of human type II collagen was described by us recently (6).

ELISAs for determining antibody titer and specificity. The immunizing peptides, conjugated to BSA, were diluted to 20 μg/ml in 0.1 M carbonate buffer, pH 9.2, and 50 μl was added to each well (1 μg/well) of Immulon-2 flat-bottom tissue culture microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). In other cases, plates were coated with 2 μg/well of either native, heat-denatured, or MMP-1–cleaved or MMP-13–cleaved human type I, II, III, and X collagens (see below). After 24 h at 4°C, the plates were washed three times with PBS containing 0.1% vol/vol Tween 20 (PBS-Tween; Sigma Chemical Co.). Noncoated binding sites were blocked by 150 μl/well of 1% wt/vol BSA in PBS (PBS-1% BSA) for 30 min at room temperature. The plates were washed once with PBS-Tween and 50 μl of serial dilutions of the appropriate polyclonal antiserum preparation were added to individual wells. After 90 min at 37°C, the plates were washed three times with PBS-Tween. Alkaline phosphatase-conjugated goat anti–rabbit IgG (Sigma Chemical Co.) diluted 1:30,000 in PBS-1% BSA-Tween was added at 50 μl/well. After 1 h at 37°C, the plates were washed three times with PBS-Tween and once with distilled water. Finally, 50 μl of freshly prepared alkaline phosphatase substrate, disodium *p*-nitrophenyl phosphate (Sigma Chemical Co.) at 0.5 mg/ml in 8.9 mM diethanolamine, 0.25 mM $MgCl_2$, pH 9.8 was added to each well for 20–30 min at 37°C. The absorbances were measured at 405 nm on a plate reader (model Multiskan™ MkII; ICN/Flow, Mississauga, Ontario, Canada).

Inhibition ELISAs for analyses of type II collagen neoepitopes produced by collagenase cleavage. Linbro 96-well round bottom microtiter plates (ICN Flow) were precoated with 100 μl/well of PBS-1% BSA for 30 min at room temperature and washed once with PBS-Tween. Four nonspecific binding wells each contained 100 μl of 50 μl PBS-1% BSA and 50 μl PBS-1% BSA-Tween. Each polyclonal $F(ab')_2$ antiserum preparation was diluted 1:200 in PBS-1% BSA-Tween (as determined by checkerboard analyses of antisera and peptide-BSA titrations, data not shown) and 50 μl was added to each of the remaining wells of these preincubation plates. Four wells with antisera were mixed with 50 μl/well of PBS-1% BSA to determine maximum binding in the absence of the inhibitory epitopes. To the remaining test wells containing 50 μl of diluted antiserum $F(ab')_2$ were added 50 μl/well of appropriate dilutions of the standard peptides COL2-3/4C, COL2-1/4N1, or COL2-1/4N2; native, heat-denatured (80°C for 20 min) or collagenase-cleaved type I, II, and III collagen solutions; concentrated (threefold) culture media from human OA articular cartilage explants; or α-chymotrypsin extracts of human OA articular cartilage. For antibody specificity analyses, standard peptides containing the epitope that had been used for immunization were added, as described above, but with amino acid residues either added to (+1, +2, +3) or removed from (−1, −2, −3) the end of the immunizing peptide corresponding to the cleaved terminus of the α chain fragment. Also, two overlapping 16mer peptides representing the amino acid sequence bridging the primary and secondary collagenase cleavage sites of native type II collagen (Gly-Pro-Hyp-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val-Gly and Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val-Gly-Leu-Hyp-Gly) were used to demonstrate the specificity of the immunoreactivities of the antisera to the cleaved termini and not to intact α chains at the cleavage sites. All standards and samples were tested in duplicate wells. Analyses were always repeated at least once.

After incubation for 1 h at 37°C, 50 μl of each preincubated sample was transferred to the equivalent wells of Immulon-2 ELISA plates, precoated, as described above, with neoepitope peptide-BSA conjugates diluted to 50 ng/well for COL2-3/4C, 150 ng/well for COL2-1/4N1, and 120 ng/well for COL2-1/4N2, all in PBS, pH 7.2. Note that for the COL2-3/4C assay, the BSA-conjugate used to coat the plates contained the peptide Cys-Gly-Gly-Glu-Gly-Pro-Hyp-Gly-Pro-Gln-Gly ($COL2\text{-}3/4C_{long}$), which included the natural in-sequence amino acid residue (Glu) and two glycine spacers added to the NH_2 terminus of the immunizing COL2-3/4C peptide. In preliminary experiments, the use of this longer peptide was found to increase the sensitivity of the COL2-3/4C assay over that obtained by using the shorter immunizing peptide-BSA conjugate (data not shown). These plates were incubated for 30 min at 4°C and then washed three times with PBS-Tween. Goat anti–rabbit IgG $F(ab')_2$ fragment conjugated to alkaline phosphatase (Sigma Chemical Co.) was diluted 1:20,000 in

PBS-1% BSA-Tween and added at 50 μl/well. After incubation for 1 h at 37°C, the plates were washed three times with PBS-Tween and three times with distilled water. From an ELISA amplification system kit (GIBCO BRL, Gaithersburg, MD), 50 μl of amplifier substrate (NADPH) was added to each well and after 15 min at room temperature, 50 μl of amplifier (alcohol dehydrogenase and diaphorase) solution was added to each well. After a final 15 min at room temperature, the color development was halted with 50 μl of 0.3 M H_2SO_4 and the absorbance was measured at 490 nm. For each plate the mean absorbance from the four nonspecific binding wells was subtracted from the absorbance values of each of the other wells. The percentage inhibition of binding by standards or samples were calculated relative to the mean absorbance from the four maximum binding wells which represented 0% inhibition (100% binding). Results were also expressed on a molar basis using a molecular weight for type II α chain of 98,291 D (6) and for the COL2-3/4C peptide of 608 D.

Proteolytic cleavage of native type I, II, and III collagens by human recombinant collagenases MMP-1, MMP-8, and MMP-13. Lyophilized human type I, II, and III collagens were dissolved in 0.5 M acetic acid and then diluted to a final concentration of 2.5 mg/ml in digestion buffer consisting of 50 mM Tris, 10 mM $CaCl_2$, 0.5 M NaCl, 0.01% Brij 35 (Sigma Chemical Co.) and 0.02% NaN_3, pH 7.6. Recombinant human proenzymes MMP-1 (rHuMMP-1), MMP-8 (rHuMMP-8), and MMP-13 (rHuMMP-13) were activated by incubation with 2 mM (final concentration) aminophenyl mercuric acetate (APMA; Sigma Chemical Co.) in the same digestion buffer for 90 min at 37°C. Each activated enzyme solution was added to individual collagen solutions at a final molar ratio of collagenase to collagen of 1:5 (MMP-1 or MMP-8/collagen) or 1:10 (MMP-13/collagen). Where necessary, the pH was adjusted to 7.5. Controls contained collagen in digestion buffer with 2 mM APMA but with no collagenase.

For immunoanalyses by ELISA of cleavage neoepitopes, the samples were incubated for 24 h at 30°C and then the rHuMMPs were inactivated by the addition of 20 mM (final concentration) EDTA (Sigma Chemical Co.). For studies of time-dependent collagenase cleavage of native type II collagen, aliquots were removed at times indicated in the figure legends, over 72 h of digestion at 30°C and the collagenase was inactivated with EDTA.

Electrophoresis and immunoblotting. SDS-PAGE of MMP-1, MMP-8, and MMP-13 cleaved purified native human collagens were performed as previously described (52) under denaturing conditions using 10%, 1-mm thick, 7 × 8 cm mini-Protean gels stained with Coomassie Blue R-250 (Bio-Rad Laboratories, Mississauga, Ontario, Canada) in 40% vol/vol methanol and 10% vol/vol acetic acid in distilled water. Electrophoretic transfers to nitrocellulose membranes (Bio-Rad) were performed as previously described (5), with the following exceptions. The PBS-3% BSA blocked membranes were incubated overnight at 4°C with either anti-COL2-3/4C or anti-COL2-1/4N1 or anti-COL2-1/4N2 $F(ab')_2$ preparations diluted 1:200 in PBS-3% BSA-Tween. After three 10-min washes in PBS-1% BSA-Tween, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG $F(ab')_2$ fragment diluted 1:30,000 with PBS-3% BSA-Tween. The membranes were given 3 × 10 min washes in PBS-1% BSA-Tween and then rinsed well in distilled water, before adding alkaline phosphatase substrate solution from a commercial kit (Bio-Rad) using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. After optimal color development (10–20 min), the reaction was stopped by washing off the substrate solution with distilled water.

Extraction of human articular cartilage for the measurement of collagenase-cleaved, denatured, and total type II collagen contents. Preliminary studies using MMP-1 and MMP-13 cleaved human type II collagens (not shown) established that the levels of the COL2-3/4C neoepitope, as detected by ELISA, were not significantly affected by α-chymotrypsin treatment under the conditions previously described to extract denatured collagen from articular cartilage (6). Therefore, femoral condylar articular cartilages from 19 normal and 26 osteoarthritic joints were diced and incubated overnight at 37°C with 1.0 mg/50–75 mg cartilage of α-chymotrypsin in 50 mM Tris-HCl, pH 7.6 (with the proteinase inhibitors: 1 mM EDTA, 1 mM iodoacetamide, and 10 μg/ml pepstatin A; Sigma Chemical Co.). The α-chymotrypsin activity was inhibited with 20 μl (160 μg/ml final concentration) of *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK; Sigma Chemical Co.). After 20 min at 37 °C, the samples were centrifuged and the supernatants were removed and assayed for the COL2-3/4C neoepitope (as described above) and COL2-3/4m hidden epitope (6). The remaining cartilage residues were digested overnight at 56°C with 1.0 mg/ml proteinase K in Tris solution containing the same proteinase inhibitors and then boiled for 20 min to denature the enzyme. Total type II collagen content of each sample was determined from the collective amount of COL2-3/4m epitope in both the α-chymotrypsin extract and the proteinase K digestion. The percent denatured collagen was determined from the amount of COL2-3/4m epitope in the α-chymotrypsin extract expressed as a percentage of the total collagen content. The percent cleaved collagen was calculated from the amount of COL2-3/4C neoepitope assayed in the α-chymotrypsin extract expressed as a percentage of the total collagen content.

Mankin grading. Frozen sections that were prepared for extraction immunoassay were stained with Safranin O and Fast Green and were graded for degenerative changes as described by Mankin et al. (53). The maximum grade was 13, as there was no calcified cartilage in the specimens used.

Human OA articular cartilage explant cultures. Full-depth human femoral condylar articular cartilages in serum-free culture media (see below) were removed from the subchondral bone within 6 h of its removal from three OA patients undergoing joint replacement surgery. The cartilage was diced into ~ 1-mm^3 pieces and placed for 1 h in basic culture medium consisting of DME with 3.6 mg/ml of Hepes (GIBCO BRL), 100 U/ml penicillin and 100 μg/ml streptomycin to which was added 2.5 μg/ml amphotericin B (Fungizone; GIBCO BRL). It was then transferred to the same basic culture medium (DME-Hepes) containing 1,000 U/ml penicillin and 1 mg/ml streptomycin for 30 min. After a final wash in basic culture medium, 40–50 mg wet weight of cartilage was transferred to each well of a 24-well tissue culture plate (Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ) containing 1 ml/well of basic culture medium supplemented with 1 mg/ml BSA (Sigma Chemical Co.), 50 μg/ml L-ascorbic acid (Sigma Chemical Co.), and 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite (ITS; Boehringer Mannheim, Mannheim, Germany). The cartilage was precultured for four days at 37°C, 5% CO_2 and 95% relative humidity. The media were removed and quadruplicate samples were cultured either in the basic culture medium (+ BSA, ascorbic acid, ITS; Boehringer Mannheim) supplemented with 10, 2.5, and 1.0 nM of RS 102,481 (Roche Bioscience, Palo Alto, CA), a synthetic, preferential MMP-13, carboxylate inhibitor (K_i [nM]: 1,100, MMP-1; 32, MMP-2; 19, MMP-3; 18, MMP-8; 0.08, MMP-13) in 0.1% DMSO, or in medium with only 0.1% DMSO, the inhibitor vehicle. Media and inhibitors were replaced every 2 d of culture and the conditioned media were frozen at −20°C until assayed for the COL2-3/4C neoepitope (as described above). Cultures were terminated after 12 d. The experiment was repeated twice with the articular cartilage from two other patients.

The measurement of total collagen synthesis. To determine the effect of inhibitor RS 102,481 on type II collagen synthesis, explants of human OA articular cartilage were cultured as described above, with or without the highest concentration (10 nM) of inhibitor. On days 14, 16, and 18 of culture, 15 μCi of [^{3}H]proline was added to each ml of medium. The conversion of radiolabeled proline into tritiated hydroxyproline within the explants, as an indicator of type II collagen synthesis, was determined after day 20 in two independent cultures, using the method described by Tyler and Benton (54).

Statistical analysis. Spearman rank correlations were used for analyzing relationships between different parameters used in the study. Mann-Whitney U tests were used to compare the nonarthritic (normal) and OA groups, in terms of the collagenase-generated neoepitopes detected by immunoassay.

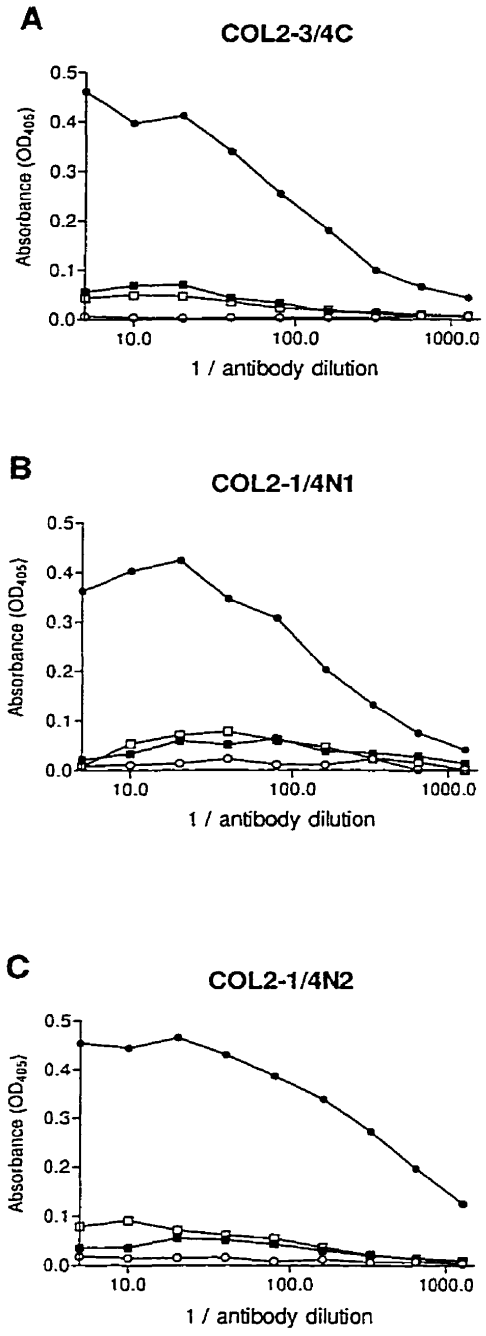

Figure 2. Immunoreactivities of collagenase antineoepitope $F(ab')_2$ antibody subunits with immunizing peptides and human type II collagen as determined by direct-binding ELISAs. Immulon-2 microtiter plates were coated with 1 μg/well of each of the immunizing peptides conjugated to BSA (●). As well, plates were coated with 1 μg/well of BSA (○) or 2 μg/well of either triple helical (■) or heat-denatured (80°C for 20 min) (□) human type II collagens. After blocking unbound sites with BSA, serial dilutions of the collagenase antineoepitope $F(ab')_2$ preparations (in PBS-1% BSA-Tween [from 1:5 to 1:1280]) were added in duplicate to the wells of their respective plates and processed as described in Methods. Shown are the results of the ELISAs for the COL2-3/4C (*A*), COL2-1/4N1 (*B*), and COL2-1/4N2 (*C*) $F(ab')_2$ preparations.

Results

Polyclonal antibodies to mammalian collagenase-generated neoepitopes of α1 (II) collagen TC^A and TC^B

Direct-binding ELISAs for determination of antibody titers. Sera obtained from rabbits immunized with OVA conjugates of the neoepitope peptides and $F(ab')_2$ preparations of these sera were initially characterized by direct binding ELISAs. In the immunochemical analyses, the $F(ab')_2$ preparations were used to ensure specific binding. Each of the antineoepitope antibodies reacted similarly with its own immunizing peptide (Fig. 2) which was conjugated to BSA and bound to the microtiter plates to improve immunoreactivity. None of the antibodies showed any significant immunoreactivity to BSA, native or heat-denatured human type II collagen (Fig. 2), or native or heat-denatured human type I, III, and X collagens (data not shown).

Inhibition ELISAs for neoepitope characterization. A panel of nonconjugated peptides with amino acid additions to (+1, +2, +3) and deletions from (−1, −2, −3) either the COOH terminus of COL2-3/4C or the NH_2 termini of COL2-1/4N1 and COL2-1/4N2 immunizing peptides were prepared. These were used in competitive ELISAs to determine the specific amino acid sequences recognized by each antibody and thereby verify that they were indeed antineoepitope antibodies. Removal or addition of one residue resulted in a loss of inhibition ranging from 80 to 100%, depending upon the peptide concentration, for the COL2-3/4C antibody (Fig. 3 *A*) and complete loss of inhibition for the COL2-1/4N2 antibody (Fig. 3 *B*). For the COL2-1/4N1 antibody, it was only after three amino acids were deleted from the NH_2 terminus (which is then the NH_2 terminus of the COL2-1/4N2 peptide) that there was a complete loss of inhibition (Fig. 3 *C*). This is significant in that it shows that the COL2-1/4N1 antibody will not recognize the amino acids comprising the NH_2 terminus of the secondary cleavage site produced by mammalian collagenases (Fig. 1).

The two 16mer overlapping peptides designed to represent the amino acid sequences bridging both the primary and secondary collagenase cleavage sites of type II collagen α1 chains did not significantly inhibit any of the antibodies (data not shown).

Inhibition ELISAs using antibodies to COL2-3/4C neoepitope demonstrating comparative reactivity to immunizing peptide and collagenase-cleaved and uncleaved fibrillar collagens

To further validate the specificity of the antibodies to the COL2-3/4C neoepitope, complete digests by MMP-1 and MMP-13 of collagen (verified by Coomassie staining of SDS-PAGE gels) and heat-denatured type I, II, and III collagen solutions at concentrations of 29, 58, 115, 230, 460, and 920 μg/ml were examined in inhibition ELISAs along with the nonconjugated COL2-3/4C immunizing peptide. Typical standard curves plotted as the percent inhibition against log of the competing antigen concentration (μg/ml) are shown in Fig. 4 *A*. Heat-denatured type I and II collagens showed no reactivity. Otherwise, positive immunoreactivity always exhibited excellent parallelity for each analysis. On a molar basis, the inhibition was very similar for MMP-1 and MMP-13 cleaved type II collagens and the COL2-3/4C peptide (Fig. 4 *B*). The μM concentration of the COL2-3/4C epitope was calculated as described in Methods. Although there was parallelity between both the MMP-1 and MMP-13 cleaved type I collagen solutions and the COL2-

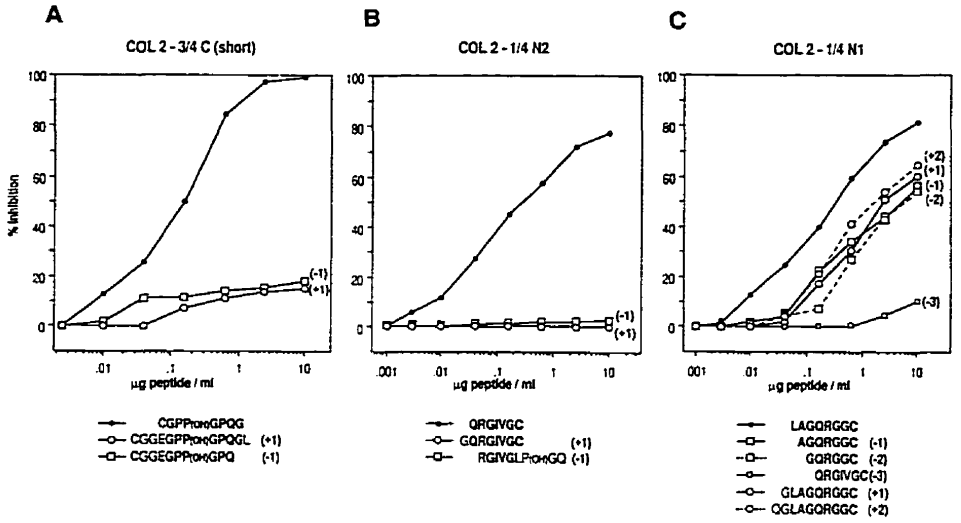

Figure 3. Epitope specificities of the anti-neoepitope antisera determined using competing synthetic peptides in inhibition ELISAs. Varying dilutions of the nonconjugated immunizing peptides (●) were added to 1:200 dilutions of their respective $F(ab')_2$ preparations in 96-well round bottom microtiter plates for 1 h at 37°C. Then 50 µl was transferred from each well to the equivalent wells of Immulon-2 microtiter plates precoated with the respective immunizing peptides conjugated to BSA. The plates were then processed as described in Methods for the development of a standard inhibition curve for each neoepitope antiserum. For the COL2-3/4C (*A*) and the COL2-1/4N2 (*B*) antibodies, shown are the results of ELISAs performed with synthetic peptides that had either 1 amino acid added to (○) or deleted from (□) the end of the immunizing peptide corresponding to the cleaved termini of the alpha chain fragments. The sequences of these peptides are shown in the individual legends for each antineoepitope antiserum. For the COL2-1/4N1 antibody (*C*), residues were either added to (*open circles*) or deleted from (*open squares*) the NH_2 terminus of the immunizing peptide. It was only when three residues were deleted from the NH_2 terminus (□) of the COL2-1/4N1 peptide and not one residue, as for the COL2-3/4C and COL2-1/4N2 peptides, that there was a complete loss of inhibition with the COL2-1/4N1 antibody.

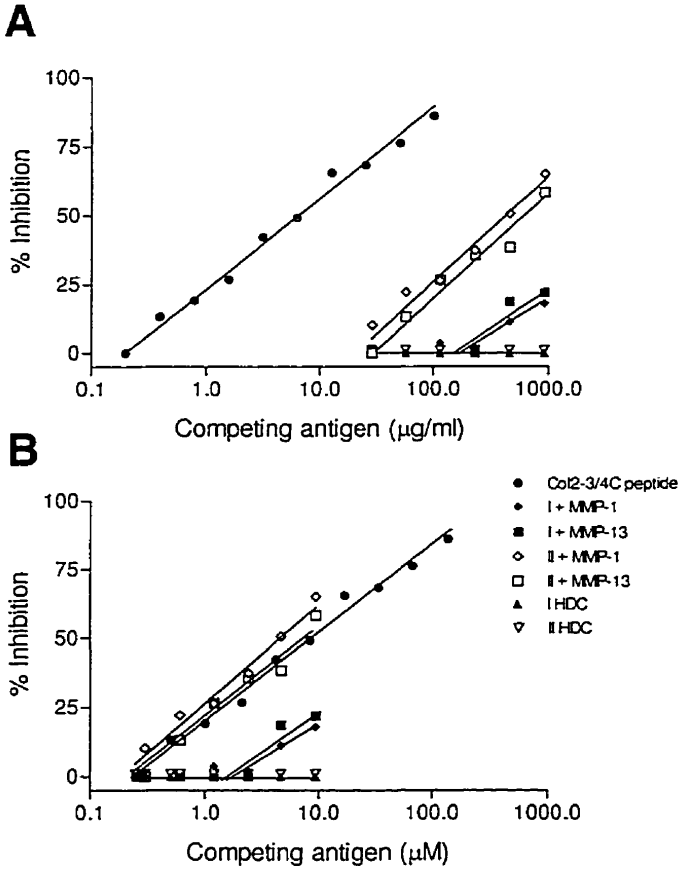

Figure 4. ELISA to show specificity of the COL2-3/4C antibody for the COOH terminal neoepitope of α1(II) collagen chains created by the digestion of triple helical type II collagen by the collagenases MMP-1 and MMP-13. Heat-denatured and collagenase-cleaved (MMP-1 and MMP-13) human type I, II, and III collagens (digestions were complete as shown by Coomassie staining of SDS-PAGE gels) were assayed in inhibition ELISAs at concentrations of 20, 58, 115, 230, 460, and 920 µg/ml of digestion buffer. A standard curve for the COL2-3/4C peptide (●) was also constructed using doubling dilutions from 50 to 0.1 µg of the peptide/ml of buffer in the same inhibition ELISA. Shown are typical standard curves plotted as percent inhibition against the log of the competing antigen concentrations as µg/ml (*A*) and on a µM basis (*B*). Symbols show MMP-1 (◆, ◇) and MMP-13 (■, □) cleaved type I and type II collagens, respectively, and heat-denatured type I (I HDC; ▲) and type II (II HDC; ▽) collagens.

3/4C peptide, the cleaved type II collagen was much more inhibitory than the cleaved type I collagen (Fig. 4 *B*). This may in part be due to the fact that the sequence in human type I and II collagen α chains for the five residues amino-terminal to the cleavage site is identical. We do not yet know how important hydroxylation is at Pro_{771}, nor do we know how important the threonine residue is at position 770 in the type I collagen α1 and α2 chains, which is a proline residue in the type II α1 chains. Studies are in progress to address these issues. Neither the MMP-cleaved nor the heat-denatured type III collagen solutions showed inhibition (data not shown). The COL2-3/4C peptide was used as the standard in all subsequent ELISAs. Similar analyses for the antibodies to the COL2-1/4N2 epitope are not shown because they were not used in immunoassays in this study.

MMP-1, MMP-8, and MMP-13 produce sequential cleavages in type II collagen

Acid-soluble human type II collagen was cleaved with MMP-1 for up to 72 h, with aliquots removed and inactivated by the addition of EDTA at time 0, 0.5, 1, 3, 5, 24, 48, and 72 h. There was almost complete cleavage of the α1(II) chains by 5 h, as shown by Coomassie staining of SDS-PAGE separated digests (Fig. 5 *A*). All the antineoepitope antibodies reacted with their

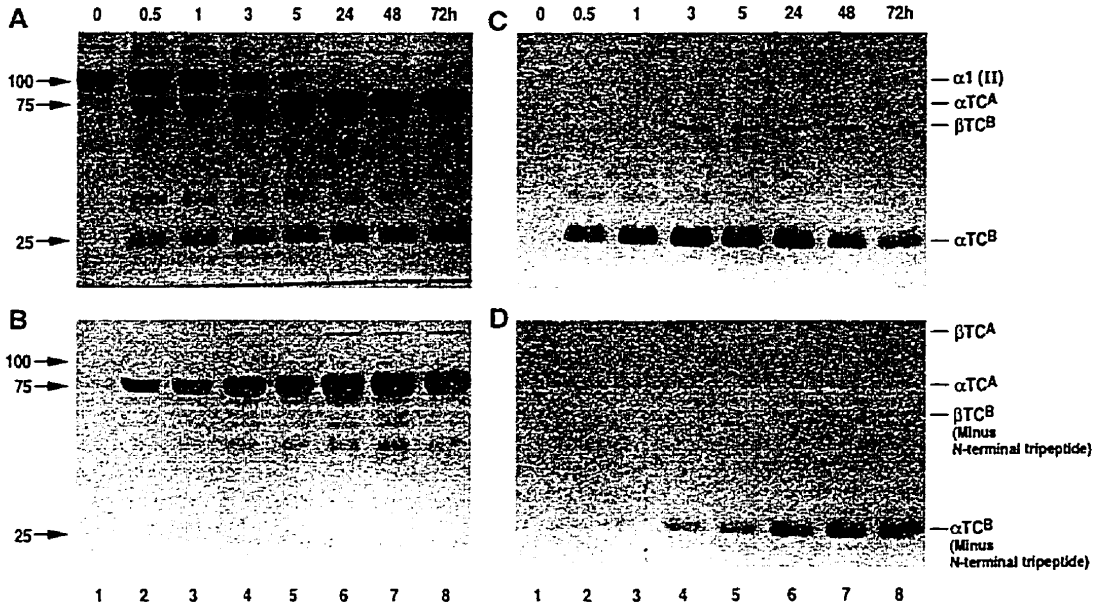

Figure 5. Time course of cleavage of triple helical human type II collagen by rHuMMP-1. Purified human type II collagen was solubilized in digestion buffer and incubated at 30°C for 72 h with APMA-activated rHuMMP-1 at a final molar ratio of 1:5 (MMP-1:collagen). Aliquots were removed at 0, 0.5, 1, 3, 5, 24, 48, and 72 h and the MMP-1 was inactivated in each sample by the addition of 20 mM EDTA (final concentration). The samples were separated under reducing conditions with SDS-PAGE (10%) and either stained with Coomassie Blue (*A*) or electrophoretically transferred to nitrocellulose and incubated overnight with 1:200 dilutions in PBS-3% BSA-Tween of COL2-3/4C (*B*), COL2-1/4N1 (*C*), and COL2-1/4N2 (*D*) antineoepitope $F(ab')_2$ preparations. Shown are undigested type II collagen (lane *1*); type II collagen + APMA-activated MMP-1 for 0.5 (lane *2*), 1 (lane *3*), 3 (lane *4*), 5 (lane *5*), 24 (lane *6*), 48 (lane *7*), and 72 (lane *8*) h. Right margin indicates the positions of α1 (II) chains and the single (αTC^A) and dimeric (βTC^A) forms of TC^A and the single (αTC^B) and dimeric (βTC^B) forms of TC^B alpha chain fragments produced by MMP-1 cleavage of intact type II collagen. Left margin indicates the estimated molecular weights of the intact α1(II) chains (100 kD) and αTC^A (75 kD) and αTC^B (25 kD) of type II collagen.

respective cleaved fragments but showed no reactivity with α chains on Western blot analyses. The COL2-3/4C antibody stained both the single (αTC^A) and dimeric (βTC^A) α chain fragments created by MMP-1 cleavage of type II collagen with increasing intensity up to 48 h (Fig. 5 *B*). The COL2-1/4N1 antibody stained both the single (αTC^B) and dimeric (βTC^B) fragments with increasing intensity up to 5 h and progressively declined thereafter (Fig. 5 *C*). Moreover, the COL2-1/4N2 antibody first stained TC^B weakly at 3 h and then with increasing intensity up to 72 h as the neoepitope COL2-1/4 N1 disappeared (Fig. 5 *D*). This demonstrated a loss of the primary collagenase cleavage site neoepitope (COL2-1/4N1) and the appearance of the secondary cleavage site neoepitope (COL2-1/4N2) with MMP-1 digestion of type II collagen. This was confirmed by microsequencing (data not shown).

Type II collagen cleavage by MMP-8 was generally slower under the conditions of this study and the complete cleavage of α1(II) chains occurred only after 24 h of digestion (Fig. 6 *A*).

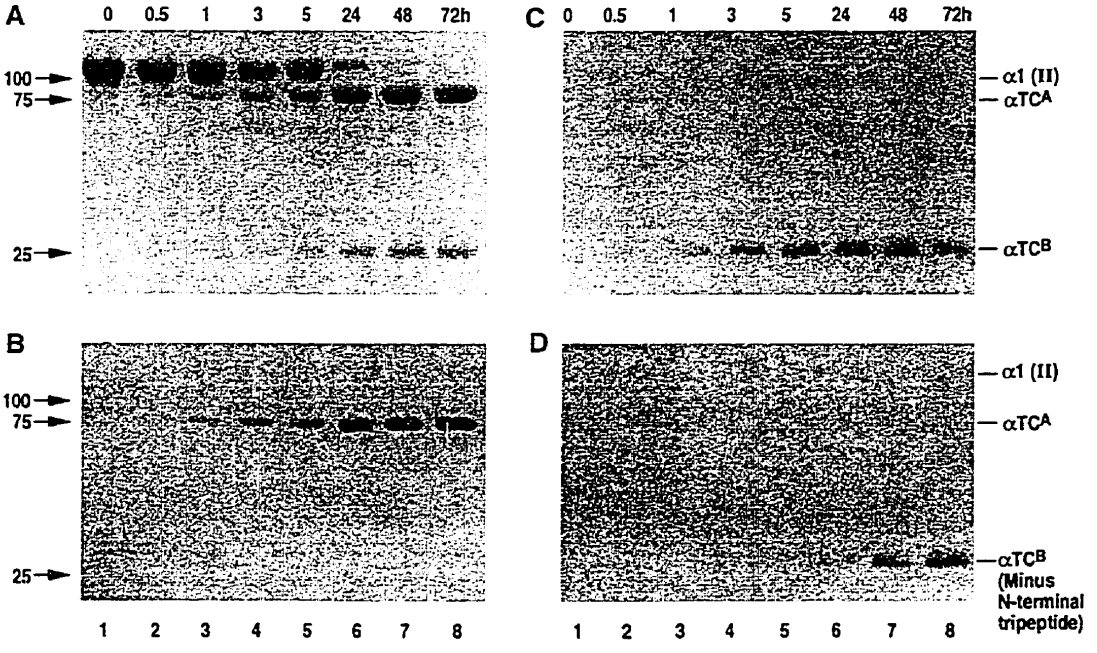

Figure 6. Time course of cleavage of triple helical human type II collagen by rHuMMP-8. The methodology used was as described for the rHuMMP-1 digestion. After SDS-PAGE (10%), gels were stained with Coomassie Blue (*A*) or the separated proteins were transferred to nitrocellulose and incubated overnight with 1:200 dilutions in PBS-3% BSA-Tween of COL2-3/4C (*B*), COL2-1/4N1 (*C*), or COL2-1/4N2 (*D*) antineoepitope $F(ab')_2$ preparations. Lanes and margins are as described in Fig. 5.

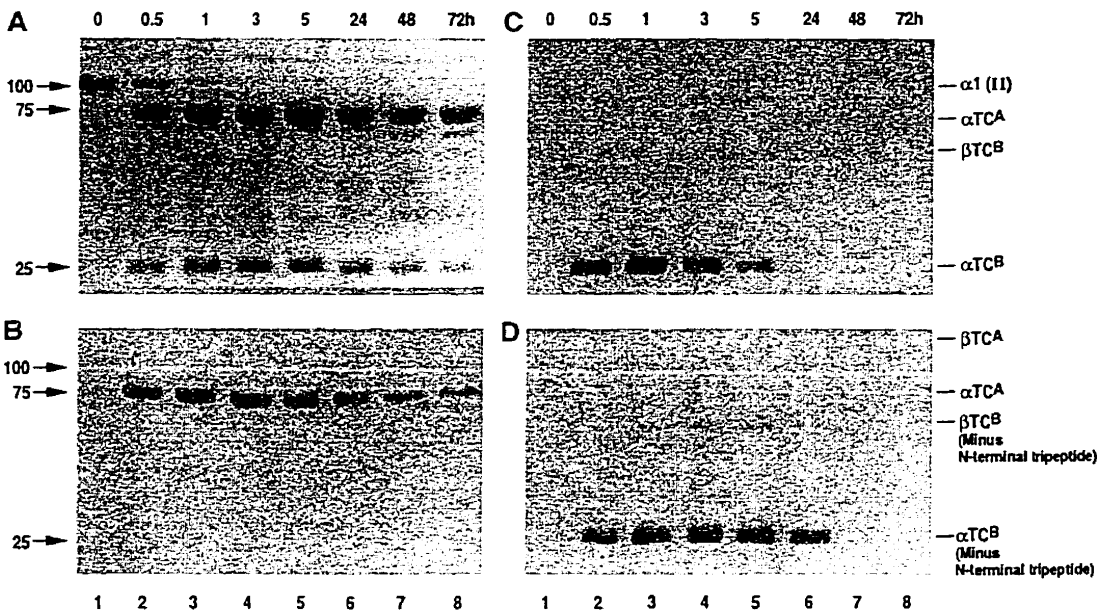

Figure 7. Time course of cleavage of triple helical human type II collagen by recombinant human MMP (rHuMMP-13). The methodology used was as described for rHuMMP-1 digestion (see legend, Fig. 5), except that a final molar ratio of 1:10 for MMP-13:collagen was used. After SDS-PAGE (10%), gels were stained with Coomassie Blue (*A*) or the separated proteins were transferred to nitrocellulose and incubated overnight with 1:200 dilutions in PBS-3% BSA-Tween of COL2-3/4C (*B*), COL2-1/4N1 (*C*), or COL2-1/4N2 (*D*) antineoepitope $F(ab')_2$ preparations. Lanes and margins are as described in Fig. 5.

The COL2-3/4C antibody stained TC^A on Western blot analysis with increasing intensity up to 72 h (Fig. 6 *B*). The COL2-1/4N1 antibody stained TC^B with increasing intensity up to 24 h and then progressively declined thereafter (Fig. 6 *C*). The COL2-1/4N2 antibody stained TC^B with increasing intensity from 5–72 h, as the staining intensity for the COL2-1/4N1 neoepitope weakened (Fig. 6 *D*).

The primary cleavage of the α1(II) chains by MMP-13 was much faster than that by MMP-1 or MMP-8 and was almost complete after only 30 min (compare Fig. 7 *A* with Fig. 5 *A* and Fig. 6 *A*), confirming the results of recent studies comparing the rates of cleavage of type II collagens by MMP-1 and MMP-13 (10, 14, 26). The primary cleavage site neoepitope (COL2-3/4C) was reduced in content after only 5 h digestion indicating further cleavage of TC^A (Fig. 7 *B*). Moreover, the NH_2 terminal primary cleavage site neoepitope (COL2-1/4N1) increased up to 1 h but was then reduced and had disappeared after 3 h (Fig. 7 *C*). In contrast, the secondary cleavage site neoepitope (COL2-1/4N2) was detectable within 30 min and increased up to 5 h before decreasing. After 24 h, it was no longer detectable (Fig. 6 *D*).

This secondary cleavage produced by MMP-13 was recently described through the NH_2 terminal sequencing of MMP-13 cleaved human type II collagen TC^B (14). To confirm this loss of the COL2-1/4N1 neoepitope (Fig. 7 *C*), the MMP-13 digestion samples used for this immunoblot were assayed in an inhibition ELISA and no detectable COL2-1/4N1 epitope was found after 5 h of MMP-13 cleavage (data not shown). It is worth noting that the percentage recovery of this neoepitope, based on the amount of epitope present in the intact collagen, reached a maximum of only 21% after 60 min. This suggests a rapid removal of the NH_2 terminal Leu-Ala-Gly tripeptide from TC^B. Further studies are in progress to compare, by immunoassay, the relative rates of digestion of type II and other collagens by MMP-1, MMP-8, and MMP-13.

None of the antibodies recognized the cyanogen bromide generated CB10 peptides of human type II collagen, which contain the intact collagenase cleavage site of triple helical collagen α1(II) chains (data not shown). This supports the ELISA results described above showing nonreactivity of the neoepitope antisera for 16mer synthetic peptides representing the amino acid sequences bridging the primary and secondary collagenase cleavage sites in human type II collagen. Together these results confirm the classification of the COL2-3/4C and COL2-1/4N2 antibodies as antineoepitope antibodies recognizing only the termini of α1 chains of TC^A and TC^B produced by the action of mammalian collagenases on type II triple helical collagens.

None of the antibodies reacted to cleaved human type III collagen fragments produced by either MMP-1, MMP-8, or MMP-13, but all reacted to similarly cleaved fragments of human type I collagen (data not shown), confirming the results of the immunoassays that were mentioned above. Type X collagen is a minor cartilage collagen that has recently been demonstrated in OA articular cartilage (55) and it is susceptible to cleavage by the mammalian collagenases. None of the antibodies showed reactivity to intact or MMP-1 cleaved alpha chain fragments of this collagen (data not shown).

The collagenase-generated neoepitope (COL2-3/4C) is significantly elevated in OA articular cartilage and is correlated to the amount of type II collagen denaturation

Human articular cartilage from the femoral condyles of 26 OA (Mankin grade 3–12) and 19 nonarthritic (Mankin grade 1–5) joints were treated with α-chymotrypsin to extract denatured type II collagen and to assay for COL2-3/4C neoepitope content. The median of the levels of COL2-3/4C epitope extracted from the OA specimens was significantly higher ($P = 0.0002$) than that of the nonarthritic articular cartilages (Fig. 8). Moreover, when the collagenase-generated neoepitope (expressed as a percentage of total collagen present) was compared to the percentage denatured collagen, based on COL2-3/4m intrachain epitope content in each extract, there were significant correlations found for both the nonarthritic ($r = 0.503$, $P = 0.028$; Fig. 9 *A*) and the OA ($r = 0.536$, $P = 0.0048$; Fig. 9 *B*) samples. The content of intrachain epitope was about twice that of the collagenase-generated neoepitope (by immunoas-

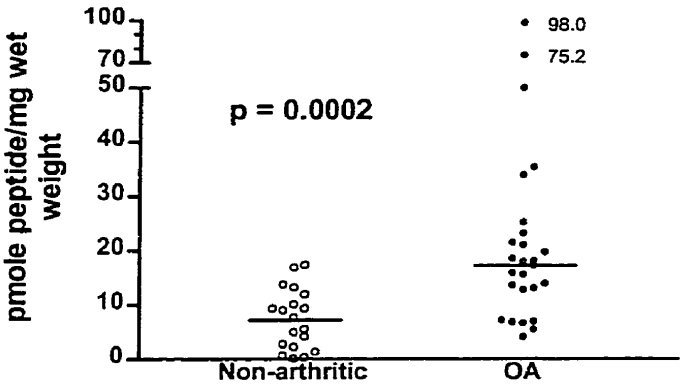

Figure 8. Levels of COL2-3/4C neoepitope in α-chymotrypsin extracts of human articular cartilage. Articular cartilage was removed from the femoral condyles of 19 nonarthritic (patient ages: 20–69 yr; median, 60 yr) and 26 OA (patient ages: 44–85 yr; median 71 yr) joints and digested overnight at 37°C with 1 mg/50–75 mg wet weight of α-chymotrypsin in 50 mM Tris-HCl, pH 7.6 (+ the proteinase inhibitors: 1 mM EDTA, 1 mM iodoacetamide, and 10 μg/ml pepstatin A). After inhibition of enzyme activity with 160 μg/ml TPCK, the samples were centrifuged and the supernatants were assayed for COL2-3/4C neoepitope as discussed in Methods. The levels of the neoepitope are expressed as pmole of peptide (based on a molecular weight of 608 D) per mg wet weight of cartilage. The absolute values of the two highest neoepitope concentrations for the OA extracts are shown in the figure. A significant difference between the median of neoepitope content in the nonarthritic (○) and the OA (●) cartilages was determined by Mann-Whitney analysis and the *P* value is shown in the figure.

say) suggesting an increased half-life of the former in both groups of cartilages.

No significant correlations (by Spearman rank analyses) were noted between Mankin grade and either percentage cleaved or percentage denatured collagen (data not shown). However, it is interesting to note that there was a tendency towards an increase in percentage collagenase cleaved collagen with increasing age ($P = 0.054$), that may reach significance with the sampling of more specimens.

Preferential inhibition of MMP-13 abrogates the release of the collagenase-generated COL2-3/4C neoepitope from human OA articular cartilage explants

Explant cultures of human condylar articular cartilages from three different patients were analyzed for the release of COL2-3/4C neoepitope into culture media. There was a steady release of COL2-3/4C into culture media over the 12 d of culture. An example of one of the three studies (OA sample 15) is shown (Fig. 10). In an attempt to determine if and which of the collagenases MMP-1, MMP-8, and MMP-13 may be responsible for the generation and/or release of the COL2-3/4C neoepitope, explants from the same specimens were also incubated with different concentrations (1–10 nM) of a preferential inhibitor of MMP-13, RS 102,481 (see Methods for inhibition profile). In all these studies, the inhibitor produced a significant reduction in the release of the COL2-3/4C neoepitope into the culture media over the 12 d of culture (Fig. 10).

That RS 102,481 did not have a detrimental effect on chondrocyte viability was determined by the measurement of total collagen synthesis. In two separate experiments there were no significant differences between the levels of tritiated hydroxyproline synthesized in OA cartilages cultured with or without the inhibitor at the highest concentration, namely 10 nM (Table I).

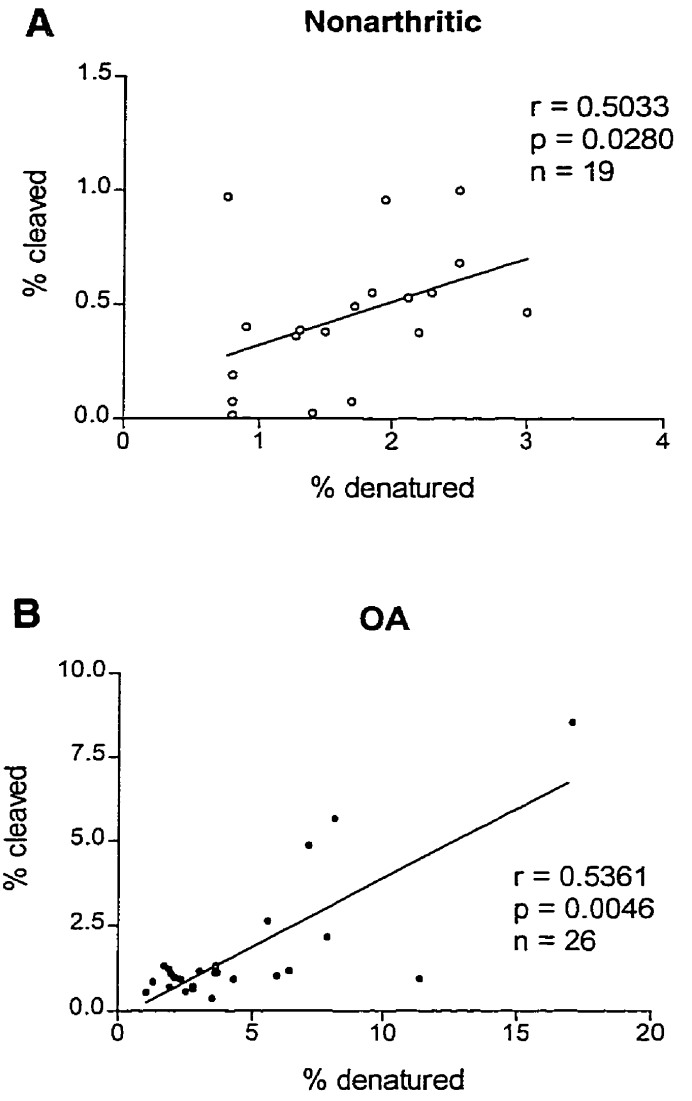

Figure 9. Correlation between the percent cleaved and the percent denatured type II collagen in α-chymotrypsin extracts of human articular cartilage. The same articular cartilage specimens digested with α-chymotrypsin and described in the legend of Fig. 8, were further digested overnight at 56°C with 1 mg proteinase K in Tris-HCl, pH 7.6 (+ proteinase inhibitors) and then boiled for 20 min to inactivate the enzyme. The total type II collagen content was determined from the amount of COL2-3/4m epitope assayed in both the α-chymotrypsin extracts and the proteinase K digests, as described in Methods. The percent denatured collagen represented the amount of COL2-3/4m and the percent cleaved collagen was the amount of COL2-3/4C in the α-chymotrypsin extracts and both are expressed as a percentage of the total type II collagen. Statistically significant relationships between percent cleaved and percent denatured type II collagen in (*A*) the nonarthritic (○) and (*B*) the OA (●) articular cartilage specimens were determined by Spearman rank correlational analysis and the corresponding *n*, *r* and *P* values are shown in the figure.

Discussion

We have previously shown that OA, which involves the progressive degeneration of articular cartilages, is characterized

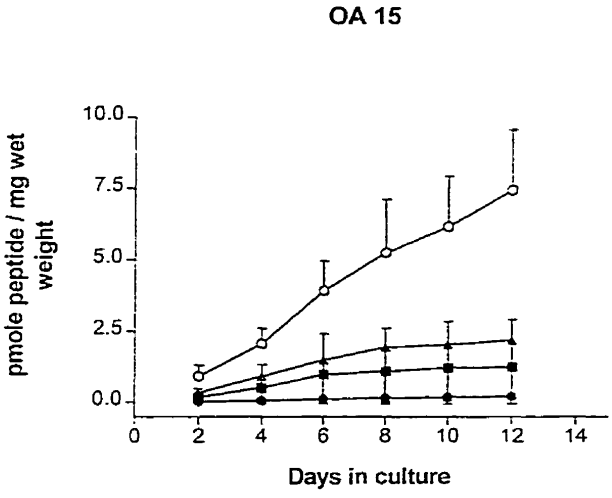

Figure 10. The effect of a synthetic preferential inhibitor of MMP-13 on the release of COL2-3/4C neoepitope from human osteoarthritic articular cartilage explants. The explants were cultured in basic culture medium (±DMSO) (○) or in the presence of RS 102,481, a preferential inhibitor of MMP-13, at 1.0 (▲), 2.5 (■), and 10 (●) nM. Media and inhibitor were replaced every 2 d and the conditioned media were assayed for COL2-3/4C neoepitope content. Shown is the cumulative release of the COL2-3/4C epitope over 12 d of a representative study (mean±SD), using OA sample 15.

by increased denaturation of type II collagen (6, 7), a molecule essential for the maintenance of the tensile properties of this tissue, its molecular architecture, and its survival. The collagenases MMP-1, MMP-8, and MMP-13 are the only proteinases currently known to be capable of initiating the denaturation of fibrillar collagens, such as type II, by first cleaving the triple helix of this molecule. Primary cleavage occurs at a single site within the triple helix, producing approximately 3/4 and 1/4 length denatured α chain fragments TC^A and TC^B, respectively. We have produced antibodies that recognize the amino- and carboxy-termini (neoepitopes) of this cleavage site in type II collagen. We used these antibodies to further characterize the cleavage of type II collagen by MMP-1, MMP-8, and MMP-13. These studies show that there is continued processing at the amino terminus of TC^B by all these collagenases to produce first a secondary cleavage site at Gly_{778}–Gln_{779}, and then further cleavage(s) leading to the release or cleavage of these neoepitopes. An antibody was prepared against the secondary amino-terminal neoepitope of TC^B and was used to demonstrate its production by MMP-1, MMP-8, and MMP-13. There

Table I. The Lack of an Effect of the Synthetic Collagenase Inhibitor RS 102,481 (10 nM) on Total Collagen Synthesis in Explant Cultures of Human OA Articular Cartilage

	Control	RS 102,481
	cpm/mg wet weight	*cpm/mg wet weight*
Experiment 1	1968.3 (534.6)	1911.4 (770.7)
Experiment 2	941.4 (133.8)	1131.9 (38.7)

The amount of [^{3}H]hydroxyproline synthesized from [^{3}H]proline was determined in cartilage that had been labeled with [^{3}H]proline on days 14–20 of culture, as described in Methods. Values are expressed as mean (SD).

was evidence for continued processing at the COOH terminus of TC^A by MMP-13, albeit at a much slower rate compared to TC^B NH_2-terminal processing. Moreover, the apparent greater resistance of the COOH terminus of TC^A to secondary proteolysis, as compared to the NH_2 terminus of TC^B, suggested to us that it would be more useful to assay for the COL2-3/4C neoepitope as an indicator of collagenase cleavage of type II collagen, as was done for the immunochemical analyses in this study.

Significant correlations were noted between percentage cleaved collagen (as determined by immunoassay for the COL2-3/4C neoepitope) and percentage denatured collagen (calculated from immunoassay for the COL2-3/4m intrachain epitope) in the immunoassays of extracts of human OA articular cartilage. These correlations were evident in both nonarthritic and OA specimens. These findings support the dogma that collagenase activity plays a key role in the denaturation and subsequent degradation of type II collagen in normal and OA articular cartilages. However, in view of the differences in epitope content being usually lower for the collagenase-generated epitope, it would seem that this epitope is preferentially lost from the cartilage. This loss of the epitope could be detected in culture. Because it was inhibited by a preferential MMP-13 inhibitor, there is thus evidence presented here to suggest that MMP-13 may play a significant role in the cleavage of type II collagen.

Recent studies of normal and OA cartilages have provided evidence for the expression of MMP-1 in normal and OA cartilages (26) and the increased expression and protein content of MMP-8 (29) and MMP-13 (14, 26) in OA cartilages. With the new technology described in this paper, we can now show that the increased expression of MMP-13 and MMP-8 is associated with increased cleavage by collagenase(s) of type II collagen in OA. Other collagenases responsible for this cleavage remain to be identified in human OA articular cartilage, but clearly all three are candidates. By demonstrating the production of the cleavage site that is only produced by collagenases, our study is the first to definitively implicate the activity of a collagenase in the cleavage of type II collagen in human articular cartilage and its increased activity in OA. Whether MMP-13 does in fact play a major role in this cleavage, as is suggested by the inhibitor studies, is presently being investigated by us. In view of the K_i values, it is conceivable that the inhibitor may also block MMP-8 activity at the higher concentrations. However, it is unlikely that MMP-1 would be affected. In separate studies of cultured bovine articular cartilage explants stimulated with IL-1, we can show a similar significant reduction in the detection of the COL2-3/4C neoepitope in media when these cultures included the same collagenase inhibitor (Billinghurst, R.C., M. Ionescu, A. Reiner, W. Wu, J. Chen, H. Van Wart, and A.R. Poole, manuscript in preparation). Thus, combined with its capacity to preferentially cleave type II collagen as demonstrated here and by others (10, 14), MMP-13, and possibly MMP-8, are identified as potential targets for therapeutic intervention in OA.

In parallel studies that will be published separately, we have shown that in cultured OA cartilage there is an increased release over normal tissue of the COL2-3/4C neoepitope. This combined with our ability to detect type II collagen degradation products in body fluids (Billinghurst, R.C., M. Ionescu, and A.R. Poole, manuscript in preparation) means that the increased degradation of type II collagen by collagenase in OA

is a fundamental feature of this arthritis, and that its measurement in vivo may be of value in studying disease activity and its clinical management.

Acknowledgments

This manuscript is dedicated to Dr. J. Gross on the occasion of his 80th birthday. The authors wish to thank Elisa de Miguel for the preparation of the synthetic peptides, Ivan Otterness of Pfizer Central Research, Connecticut, for his generous assistance and support in establishing the collaborative studies on the recombinant collagenases, and Jane Wishart for her assistance with the figures.

Funding was provided by the Fonds de la Recherche en Sante du Quebec (to R.C. Billinghurst); the Swedish Society of Medicine, Wenner-Gren Center Foundation, and Swedish Center for Research in Sports (to L. Dahlberg); the Shriners of North America, the Medical Research Council of Canada, Searle Monsanto, St. Louis, MO, Pharmacia Diagnostics AB, Uppsala, Sweden, and the National Institutes of Health (to A.R. Poole).

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IMAGE EVALUATION TEST TARGET (QA-3)

150mm

6"

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