Modulation of Biosynthetic Patterns of Human Articular Chondrocytes by Cytokines and Growth Factors - Quantitative Evaluation at the mRNA Levels

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ABSTRACT

The modulation of biosynthetic patterns of human articular chondrocytes by cytokines and growth factors was investigated *in vitro* by analysing the gene expression of cartilage matrix components at the mRNA level. The effects of IL-1β, TGF-β, and IGF-1, individually or in combination on expression of mRNAs for type II collagen, aggrecan, stromelysin, collagenase, and tissue inhibitor of metalloproteinases (TIMP) were quantitatively determined by competitive PCR. The results revealed that each of these mRNA species had an unique response pattern to these external agents. TGF-β enhanced expression of mRNAs for type II collagen, aggrecan, and TIMP, while it depressed the expression of metalloproteinases. In contrast, IL-1 regulated expression of these mRNAs in an opposite fashion. The magnitude of response to IL-1 and TGF-β varies for different target mRNAs. A prolonged incubation with TGF-β effectively reversed the IL-1–induced depression of TIMP-1 mRNA level. IGF-1 increased the mRNA levels for aggrecan and type II collagen, and effectively reversed the inhibitory effects of IL-1 on these two genes. This study indicates how physiologically-relevant cytokines and growth factors regulate the expression of structural and degradative components by articular chondrocytes. The understanding of the factors regulating the biosynthetic behaviors of articular chondrocytes under various physiological and pathological conditions is important if effective repair of the tissue is to be achieved.

Résumé

Nous avons étudié in vitro, les changements biosynthétiques des chondrocytes articulaires humains induits par les cytokines et les facteurs de croissance, en analysant l'expression des gènes des protéines de la matrice du cartilage, au niveau de l'ARNm. A l'aide de la PCR compétitive nous avons déterminé quantitativement les effets combinés ou individuels de l'IL-1β, du TGF-β du IGF-1 sur l'expression des ARNm du collagène de type II, de l'aggrecan, de la stromelysine, de la collagénase et de TIMP. Les résultats indiquent que chacun de ces ARNm répond de manière distincte à ces différents facteurs. Le TGF-β augmente l'expression de l'ARNm du collagène de type II, de l'aggrecan et de TIMP, alors qu'il diminue l'expression au niveau de l'ARNm des métalloprotéinases. Par contre, après stimulation à l'IL-1, une réponse complètement opposée à celle du TGF-β est observée. L'ampleur de la réponse à l'IL-1 et au TGF-β varie selon l'ARNm analysé. Une incubation prolongée en présence du TGF-β contrecarre l'effet de l'IL-1 sur l'ARNm de TIMP-1. La stimulation par l'IGF-1 résulte dans l'augmentation des ARNm de l'aggrecan et du collagène de type II. De plus IGF-1 inverse l'effet inhibiteur de l'IL-1 sur l'expression des deux messagers. Cette étude indique l'importance des cytokines et des facteurs de croissance dans la régulation de l'éxpression des éléments biosynthétiques des chondrocytes articulaires. Si une meilleure connaissance du processus de régénération des maladies articulaires veut être obtenue, il est important de comprendre comment, dans différentes conditions physiologiques et pathologiques, les facteurs régissant le comportement biosynthétique des chondrocytes fonctionnent.

ABBREVIATION USED

cDNA	Complementary deoxyribonucleic acid
cRNA	Complementary ribonucleic acid
CS	Chondroitin sulfate
dCTP	2'-deoxycytidine-5'-triphosphate
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
D3PDH	Glyceraldehyde-3-phosphate dehydrogenase
DS	Dermatan sulfate
EDTA	Ethylenediamine tetraacetic acid
FCS	Fetal calf serum
HA	Hyaluronic acid
HBSS	Hank's balanced salts solution
IGF	Insulin-like growth factor
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
kDa	kilo Dalton
KS	Keratan sulfate
LP	Link protein
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
OA	Osteoarthritis
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RE	Restriction endonuclease
TIMP	Tissue inhibitor of metalloproteinases
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α

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Chapter 1. INTRODUCTION - REVIEW OF LITERATURE

Articular cartilage is a specialized form of avascular and aneural connective tissue covering the surface of the extremities of the bones in diarthrodial joints. It provides elasticity and resistance against compressive forces and ensures smooth articulation and cushioning of the subchondral bone during joint movements. The character and function of the cartilage are determined mainly by the metabolic balance between the various components of the extracellular matrix and the precise relationship among these components which depends on biosynthetic capability of chondrocyte, the cell within this tissue. In this chapter, the structure of human articular cartilage, the proteinases and their inhibitors in cartilage, and the cytokines and growth factors potentially involved in cartilage matrix turnover are reviewed.

1.1 Articular Cartilage

There are three different classes of cartilage: hyaline, fibrous and elastic. Articular cartilage belongs to the hyaline class, which is the most common type in the body and varies in thickness according to the shape of the articular surface on which it lies. Articular cartilage is composed of a matrix and has a small number of cells. The chondrocyte is the only resident cell which occupies about 5%, maybe even less than 1% to 2%, of the cartilage volume. The cells are embedded in a large volume of extracellular matrix and lack physical cell-to-cell contact [1-3]. Extracellular components largely predominate over the chondrocytes; the cartilage probably contains the largest amount of extracellular matrix produced per cell within the body. Articular cartilage matrix is composed of three major components: (1) water (65-80% of the tissue wet

weight) contributing to the turgor of the tissue; (2) collagen (10-30% of wet weight), a fibrous protein; and (3) aggregating proteoglycan or aggrecan (5–10% of wet weight), a glycoprotein found between collagen fibrils [4, 5]. The remainder is composed of other proteins, lipids, nutrients and inorganic materials. The water content of articular cartilage is extraordinarily important in maintaining the tissue's resiliency, as well as contributing to the almost frictionless movement associated with a hydrodynamic lubrication system [6]. The collagen molecules are arranged in a fibrillar network which gives cartilage its tensile strength and maintains its shape [1]. Proteoglycan molecules, in the form of aggregates with hyaluronate, are constrained within this collagen network.

The biomechanical properties of articular cartilage depend upon the composition of the extracellular matrix which changes during life to meet altered functional requirements. Articular chondrocytes are remarkable in their capacity to synthesize, organize, and regulate the deposition of their complex surrounding matrix in a highly ordered and efficient manner. At each stage of growth and development the relative rates of synthesis and degradation of cartilage matrix are adjusted to achieve net growth, remodeling, and a balanced equilibrium and to maintain normal functions of articular cartilage during life.

1.2 Articular Chondrocytes

The articular chondrocyte is a highly differentiated cell which, in the postmitotic state, has longevity. The distribution of chondrocytes and extracellular matrix components is not uniform in cartilage. The chondrocytes *in situ* vary in size, shape, distribution and the structure of individual cells according to which horizontal layer (zone) of the tissue is examined. However, the same basic features are encountered whatever the site and state of the cartilage sample. The most obvious histological observation is that cell density

decreases with increasing distance from the surface in adult human articular cartilage and, consequently, the extracellular matrix between neighboring cells is more abundant. In general, human articular cartilage can be divided into four zones: *superficial, middle, deep,* and *calcified zones* [6-8]. The cellular density is the greatest in the *superficial zone* (5-10% total thickness) where the chondrocytes are flattened and discoidal in shape, with little cytoplasm, small Golgi apparatus, and poorly developed, rough endoplasmic reticulum; these cells may be in a dormant or quiescent state. Chondrocytes in this zone have lower synthetic activity than that in other zones [8]. In the *middle zone* (40-45% total thickness) chondrocytes are larger than that in the superficial zone and possess a spherical shape, with abundant cytoplasm; they have a great deal of rough endoplasmic reticulum, enlarged Golgi apparatus, and many mitochondria, which may indicate that these cells actively synthesize cartilage matrix. The *deep zone* (40-45% total thickness) is characterized by a columnar arrangement of chondrocytes in groups of 4 - 8 cells, the cell density at its lowest. Underneath the deep zone is a *calcified zone* (5-10% total thickness) which separates hyaline cartilage from subchondraol bone; where there are few cells. Due to the avascular nature of cartilage, the chondrocytes depend primarily on anaerobic glycolysis for energy production. The cells receive their nutrients and oxygen requirements mainly from the synovial fluid by three potential mechanisms: simple diffusion, active transport, and the pumping action of the intermittent compression of cartilage matrix [8].

Although the number of chondrocytes in a unit volume of articular cartilage is small compared with other tissues, the chondrocytes show considerable metabolic activity in articular cartilage. Chondrocytes play an essential role in the manufacture and maintenance of integrity of the cartilage matrix since they are responsible for the continuous physiological "turnover" of the extracellular ground substances, such as collagen and proteoglycan, in articular cartilage, involving the synthesis and degradation of matrix structural

components. Typically, chondrocytes have cytoplasmic processes that may play an important role in releasing the enzymes, such collagenase and stromelysin, responsible for matrix remodeling [6]. In healthy articular cartilage, such precise regulation of tissue turnover may depend on production by the chondrocytes not only of the structural matrix macromolecules, but also of both degradative enzymes and enzyme inhibitors. Each cell can be thought of as a functional unit of cartilage, ultimately responsible for the maintenance of the extracellular matrix in its immediate vicinity.

1.3 Structural Components of Cartilage Matrix

In normal articular cartilage, the molecular organization of the cartilage matrix is ideally suited to its function as the weight bearer of articular surfaces on bone. Collagen and proteoglycan are two major macromolecules of cartilage matrix, and they are responsible for the biomechanical properties of the cartilage, which provides the tissue with its unique and resilient characteristics.

A brief review on the structure and functions of cartilage matrix components follows:

1.3.1 Collagens

Collagen is a large polydisperse family of related but genetically distinct molecules. The total genetic potential for collagen production has not yet been determined, however, to date, no less than thirteen collagen types have been identified [9, 10]. These different types can be classified according to their locations and functions.

Like most tissues, articular cartilage contains several genetically distinct types of collagen. About 90% to 95% of the cartilage collagen is type II [11-13]. Type II collagen is a specific product of chondrocytes and vitreous cells,

consisting of three identical polypeptide subunits, the α1(II) chains, which wind around a common axis to form a right-handed triple helix with a length of 300 nm and a diameter of 1.4 nm, forming a 3-dimensional fibrillar network of rope-like molecular aggregates [11, 14]. The tensile strength of collagen depends on covalent intermolecular cross-linking. The principle cross-linking residues in mature type II collagen are hydroxylysyl pyridinoline residues [15]. The initial structure of type II collagen is similar to that of type I, III, V and XI collagens. Intracellularly, the collagen is synthesized as precursors with nonhelical amino and carboxyextension peptides, forming as procollagen molecules which are released into the extracellular space. The nonhelical extensions are removed by specific proteinase to form fibrils which assemble in a quarter-staggered array and then readily organize into small–diameter fibrils. Fibers grow by aggregation with other small-diameter fibrils. A major modification of the collagen molecules, occurring after fibril formation, is the development of covalent interfibrillar cross–links [11, 16]. The type II collagen fibrillar network is essential for maintaining the tissue's volume and shape. The network of these fibrillar collagens gives articular cartilage its tensile strength; a property that is enhanced by the presence of crosslinks between the collagen molecules, to counteract the swelling pressure internally exerted by entrapped proteoglycan molecules and to resist external mechanical stresses and strains.

Up to 10% of the total collagen in articular cartilage is made up of other genetically distinct types (types IX, X and XI and, to a lesser extent, type VI) believed to make important contributions to the structure of the matrix. The type IX collagen, a short non-fibrillar collagen, has been demonstrated specifically in cartilaginous tissues, but, as yet, its function has not been determined. This molecule is assembled from three genetically distinct chains. One of these (α2 chain) becomes glycosylated with a chondroitin sulfate (CS) chain, making this collagen molecule a proteoglycan as well. Several studies have shown that type IX collagen binds covalently to type II collagen fibrils

and may help link fibrils together or bind fibrils to other matrix molecules [17-20]. Collagen type X, a short-chain collagen, is a specific biosynthetic product of growth plate hypertrophic chondrocytes and is found exclusively in the regions undergoing endochondral bone formation and it may play a role in the transition of cartilage to bone [21, 22]. Type XI collagen, another minor one, has recently been found to associate with both collagen types II and IX in fibrils and is probably involved in controlling the diameter of the type II fibrils [18]. Type VI collagen, representing only 1-2% of the total collagen in articular cartilage, seems to be primarily located in the vicinity of chondrocytes and the basket-like immediate surroundings of the chondrons [23]. Little is known about the specific functions of the other minor cartilage collagens. It appears, however, that they all play a role in the proper assembly of a cartilage matrix.

1.3.2 Proteoglycans

Although collagen is the major determinant of the shape and form of articular cartilage, the collagen meshwork requires the presence of proteoglycan to maintain cartilage function [23]. Proteoglycan is a diverse group of heterogeneous complex macromolecular glycoconjugates, which are defined as proteins containing one or more covalently attached glycosaminoglycan chains. These large, hydrated proteoglycan molecules surround and are loosely attached to the collagen fibrillar network, and, due to their osmotic properties, endure the tissue with the ability to withstand compressive forces [24-26] and to undergo reversible deformation. Proteoglycans in cartilage matrix are mainly of the large aggregating type, aggrecan, (50%-85%) and the large non-aggregating type (10%-40), with two distinct types of small non-aggregating dermatan sulfate proteoglycans, and a small non-aggregating keratin sulfate proteoglycan [27, 28]. The proteoglycan aggregate consists of a central filament of hyaluronate to which many aggrecan subunits are non-covalently attached. Each interaction between a aggrecan monomer and the hyaluronate

is further stabilized by a link protein. **Figure 1** shows a schematic representation of an aggrecan monomer and proteoglycan aggregate attached to a filament of hyaluronate, respectively.

Aggrecan A typical aggrecan monomer is composed of a core protein of 210 kDa to which over 100 chondroitin sulfate (CS) chains (20 kDa), about 20–50 keratan sulfate(KS) chains (8 kDa), and a smaller number of N-linked oligosaccharides chains, are covalently attached [29]. The core protein contains three globular domains (G1-G3) and can be divided into several distinct regions: an amino terminal region with two globular domains, G1 and G2; a KS-rich domain; a CS-rich domain, the largest region which may also contain some interspersed KS neutral oligosaccharide chains; and a C-terminal globular domain G3 [29]. Most of the glycosaminoglycan chains are clustered between G2 and G3. The G1 N-terminal region can interact noncovalently with hyaluronic acid (HA) and has thus been termed the HA binding region. Proteoglycan aggregates are formed by many proteoglycan monomers binding to a chain of hyaluronate at the G1 globular domain [30, 31], and each proteoglycan–hyaluronate bond is stabilized by a separate globular link protein (LP; 41-48 kDa) [32]. The interaction between proteoglycan and HA is reversible under physiological conditions. Little is known about the function of the G2 region of the aggrecan molecule. The G3 region has a high degree of homology with a hepatic lectin specific for galactose and glucose and may help anchor proteoglycan molecules within the extracellular cartilage matrix. At present, DNA analysis indicates a single gene codes for the aggregating proteoglycan, whose product has been termed "aggrecan" [33]. There is, however, some biochemical evidence to suggest the possible presence of additional aggregating proteoglycans [34].

The size, structural rigidity, and complex molecular conformation of normal proteoglycan aggregates make important contributions to the

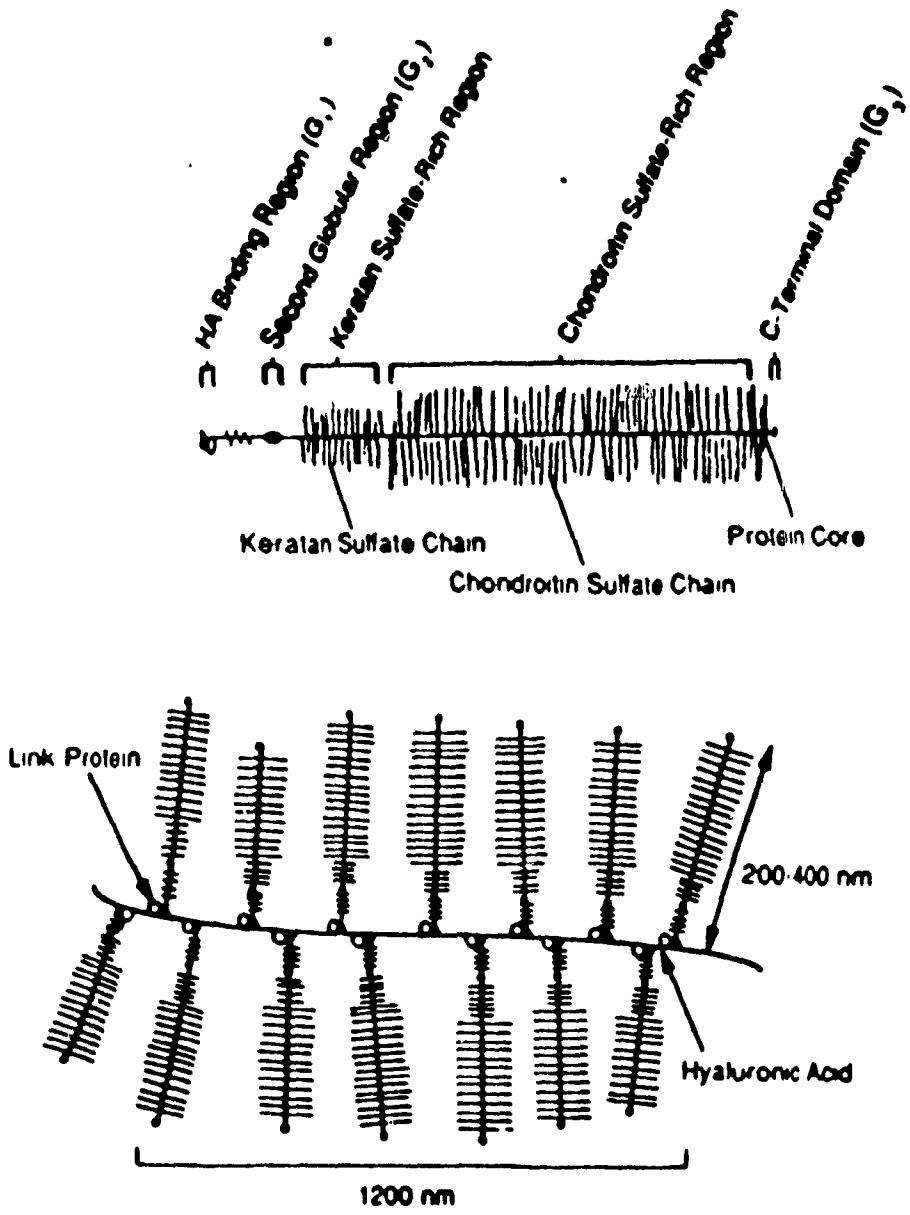


Figure 1. Schematic representation of an aggrecan monomer and a proteoglycan aggregate. Top: Schematic representation of an aggrecan monomer composed of keratan sulfate and chondroitin sulfate chains covalently bound to a protein core; bottom: representation of a proteoglycan aggregate composed of monomers noncovalently attached to hyaluronic acid, the interaction between the monomer and the hyaluronic acid core molecule being stabilized by link protein.

mechanical behavior of articular cartilage. Undoubtedly, the sheer size of proteoglycan macromolecules would arrest or minimize diffusion or hydrodynamic convective transport of the molecules through the extracellular matrix by steric occlusion or intermolecular frictional effects [35].

Non-aggregating proteoglycans Several other proteoglycans have been identified in articular cartilage [36, 37]. A proteoglycan similar in size and composition to aggrecan but lacking the ability to interact with HA has been identified. Two smaller dermatan sulfate (DS) containing proteoglycans are also present in varying amounts at different ages. The core protein of proteoglycan–I, also called biglycan, is substituted with 2 glycosaminoglycan chains, whereas proteoglycan-II contains only one DS chain. The latter has been localized along collagen fibers and has thus been named decorin [37]. It is present throughout the interterritorial articular cartilage matrix but in higher amounts in the superficial layer [37]. It may also influence the stability and organization of the collagen network.

1.3.3 Other cartilage matrix components

Articular cartilage contains numerous extracellular cartilage matrix proteins which are neither collagen nor proteoglycan. Although several noncollagenous proteins, including anchorin, chondronectin, have been identified, their composition and functions are poorly understood. Like the collagens and proteoglycans, these matrix proteins contribute to the cartilage matrix structure and may help organize and stabilize the other matrix component molecules. Link proteins, the best characterized noncollagenous protein, are involved in stabilizing proteoglycan aggregates and influences aggregate size and structure [38-40]. Anchorin-CII (34 kDa) has an amino acid composition that relates it to other cell surface receptors. It is associated with the chondrocyte membrane but also binds to collagen fibrils; anchorin-CII may thus act as a mechanoreceptor by transmitting signals to the cell and allowing it to recognize changes in

mechanical load [41]. Fibromodulin (59 kDa) is related to the core protein of decorin and may be involved in the formation of the fibrillar network [42]. In addition to the above non-collagenous proteins, cartilage also contains several other minor components: fibronectin, a very minor component in most cartilages; two chondrocyte surface binding proteins (36 and 50 kDa); a 21 kDa protein synthesized by hypertrophic chondrocytes and appearing to interact with type X collagen in the "tidemark" (the dense calcified plate of articular cartilage); and another specific cartilage oligomeric high molecular weight matrix protein [42]. All of these proteins' specific functions in cartilage are still under investigation.

1.4 Control of Cartilage Degradation

In normal articular cartilage, the structural integrity of articular cartilage is maintained by a dynamic balance between biosynthesis and degradation of cartilage matrix components. The matrix components are slowly, but continually, turning over to meet the functional requirement. As degradation products are released from the cartilage they are replaced by newly synthesized components. To replace the degraded matrix components, the chondrocytes must synthesize the complex and specific molecules at the appropriate rate. These carefully coordinated processes maintain the composition, structure, and function of articular cartilage. Any disturbance of the process may lead to deterioration of matrix, as observed in osteoarthritis or rheumatoid arthritis. Loss of cartilage function is a consequence of a gross imbalance of turnover in favour of degradative events, contributing further to degradation of the tissue leading to destruction of cartilage [39, 43]. It has been generally accepted that the degradation of cartilage matrix components is controlled by a group of

matrix metalloproteinases (MMPs) mainly secreted by the chondrocytes. Under normal conditions, the expression of these enzymes is tightly regulated at various levels including transcriptional, posttranscrptional and postranslational [44, 45]. The biological enzyme activity of metalloproteinases is modulated by specific secreted glycoprotein inhibitors, tissue inhibitor of metalloproteinases (TIMP), and thus the rates of degradation are determined by the levels of active MMPs and by the amount of TIMP. It has been speculated that the relative levels of TIMP *versus* MMPs in joint tissues may be critical in altering the balance between the maintenance of cartilage matrix and its destruction in diseased joints.

Although an obvious source of neutral proteinases participating in the degradation of cartilage matrix components is via secretion from the chondrocytes, proteinases derived from synovial tissue cells, haematopoietic cells, and blood plasma seem also to be involved in the destruction of cartilage, particularly under inflammatory conditions [46, 47]. In addition to MMPs, proteinases which have been implicated involved in the degradation of cartilage are summarized in **Table 1.** These proteinases are classified into four classes by catalytic mechanism [48]: *metallo-, serine, aspartic* and *cysterine proteinases.* The proteinases and their inhibitors, particularly, TIMP are briefly described as follows

1.4.1 Proteinases in cartilage

Metalloproteinases This class of proteinases contains the enzymes that are dependent on Zn^{++} ions for activity, and they are additionally stabilized and perhaps activated by Ca^{++}. Nine matrix metalloproteinases (MMPs) have been identifies by cDNA cloning and sequencing to date [49], and all members of this family share common features [50-53]. They contain an N-terminal

Table 1. Proteinases implicated in cartilage degradation

Class/Name	Matrix Components Degradated	pH optimum	Inhibitor	Made by Chondrocyte
Metallo				
Collagenase (MMP1)	Types II, X collagens; gelatin; proteoglycan	Neutral	TIMP	✓
Stromelysin-1 (MMP3)	Proteoglycan; procollagens; types II, IX and XI collagens fibronectin; gelatin	Neutral	TIMP	✓
Gelatinases (MMP2, MMP9)	Denatured collagens; elastin	Neutral	TIMP	✓
Serine				
Elastase	Types II, IX, X, XI collagens; proteoglycan	Neutral	α_1-PI[1]	—
Cathepsin G	TIMP; proteoglycan; elastin type II collagen	Neutral	α_1-AC[2]	—
Plasminogen activator	Plasminogen	Neutral	PN[3], PAI[4]	✓
Kallikrein	Proteoglycan	Neutral	?	✓
Plasmin	Proteoglycan	Neutral	?	✓
Cysteine				
Cathepsin B	Proteoglycan; Type II collagen (telopeptides)	5.0-6.5	Cystatins	✓
Cathepsin L	Proteoglycan; elastin	4.0-6.5	Cystatins	✓
Aspartic				
Cathepsin D	Proteoglycan; denatured collagens	3.0-6.0	?	✓

[1]α_1-proteinase inhibitor

[2]α_1-antichymotrypsin

[3]Protease nexin

[4]Plasminogen activator inhibitor

domain and a catalytic region containing two conserved histidine residues necessary for zinc binding [51, 54]. All metalloproteinases are synthesized as pre-proenzymes and secreted as latent proteinases. Activation of the different metalloproteinases appears to occur by a similar mechanism [55, 56]. These enzymes are secreted in a latent state and can be activated *in vitro* by the action of several proteinases (plasmin, kallikrein, mast cell tryptase, and trypsin etc.) and other nonproteolytic agents, such as organomercurial compounds, sodium thiocyanate, oxidized glutathione and N-ethylmaleimide. Latency of the proenzymes is thought to be due to the interaction between a cysteine residue in the pro-region with the zinc in the catalytic region, blocking the active site [56]. Activation can be achieved by either proteolytic removal of the pro-region or by chemical modification of the cysteine residue. The activation mechanism for these latent metalloproteinases *in vivo* is still not clear. It is believed that these related matrix-degrading neutral metalloproteinases, particularly collagenase, stromelysin, gelatinase, and pump (punctuated metalloproteinase, matrilysin) are in large part responsible for the deterioration of cartilage during arthritis and aging.

Collagenase (MMP1) has been considered as the best known and most specific of the metalloproteinases, which specifically cleaves all three chains of the triple helix at one susceptible point between residues 775 and 776 of the α1(I) chain. The bonds cleaved are between residues of glycine and isoleucine of collagens of types I, II, and III. Similar bonds occur elsewhere in the collagen molecules, but it is not clear why this region of the helix is particularly sensitive to proteolysis. Collagenase is also capable of cleaving collagen type VIII and makes two cleavages in collagen type X [57], but it does not degrade collagen types IX and XI [58]. The products of collagen cleavage by collagenase are two fragments, the larger consisting of 3/4 of the original collagen molecule from the N-terminal end, and a 1/4 length fragment containing the C-terminal end. These triple helices of each of the two products are more susceptible to

thermal denaturation than the native collagen molecule, and on denaturation, become susceptible to proteolytic degradation by gelatinase and other proteinases.

Stromelysin (MMP3) is a major metalloproteinase, other than collagenase, which has 55% sequence homology to collagenase [51]. Stromelysin has a wide substrate specificity, digesting cartilage proteoglycan, fibronectin, laminin, elastin, and gelatin etc. [59, 60]. When proteoglycan aggregates are cleaved by stromelysin, the primary cleavage appears to be between G1 and G2, producing a G1-hyaluronic-acid-binding-region domain [32]. Link protein is also cleaved [60]. Recently, it was shown that stromelysin also digests collagen types IV, IX and XI, and removes the telopeptides of type I and type II collagen that contain intra- and intermolecular crosslinks [61, 62]. Therefore, stromelysin has the potential to solubilize the fibrillar collagen network of cartilage. In addition, stromelysin is able to activate procollagenase by proteolytic cleavage, further enhancing its destructive potential [63, 64]. Because of this diversity of action, stromelysin was denoted by a variety of names in the earlier studies. These include proteoglycanase, matrix metalloproteinase-3, and procollagenase activator [65, 66]. It is not so clear, however, whether all proteoglycan–degrading activity in cartilage is due to stromelysin. The studies by Hughes et al [67] show that stromelysin degrades the proteoglycan protein core with major cleavages close to, but not within, the G1 domain, and extensive cleavage in other regions. Experiments with purified collagenase showed that it too cleaves proteoglycan at several sites within the glycosaminoglycan–rich region of the core protein [67]. Thus, metalloproteinase attack on proteoglycan not only occurs with stromelysin but also with collagenase. Another candidate for a connective tissue proteoglycanase would be stromelysin-2 [68], which has 78% sequence homology to stromelysin and is a dominant species in certain forms of human tumors [52]. However, human rheumatoid synovial cells appear to express little stromelysin-2 with respect to stromelysin-1 [68]. Little is known

about the substrate specificity of stromelysin-2.

Increases of collagen- and proteoglycan-degrading activity levels relative to normal cartilage have been found in human osteoarthritic cartilage and surrounding synovial tissues [69-71] and elevated amounts of prostromelysin have been detected in rheumatoid synovioblasts [72], suggesting the involvement of collagenase and stromelysin in the destruction of arthritic cartilage.

Gelatinases (MMP2, MMP9), with a molecular size of 72 kDa and 92 kDa, are produced by both macrophages and neutrophils as well as other cells. Although these enzymes are related, they are distinct gene products [64]. There is no apparent difference in the substrate specificity of these two forms. Like other metalloproteinases, the gelatinases are secreted as proenzymes that require limited proteolytic cleavage for activation. Synthesis of gelatinase is regulated by many of the same factors that regulate collagenase and stromelysin synthesis. These enzymes cleave native collagens types IV, V, VII and XI as well as gelatin [73, 74].

Pump (MMP7, punctuated metalloproteinase, matrilysin) is a more recently discovered matrix-degrading metalloproteinase, originally described as a putative metalloproteinase. It appears to be distantly related to stromelysin but lacks the C-terminal domain [49]. Pump has a sequence homology of 44% and 49% to collagenase and stromelysin, respectively [52]. The expression studies of pump cDNA revealed that it codes for a latent proteinase (28 kDa) which undergoes autolytic cleavages similar to those described for collagenase and stromelysin [75]. It degrades casein, denatured forms of collagen types I, III, IV and V, and fibronectin, and can activate procollagenase.

Serine proteinases There are a family of endopeptidases, with a catalytically essential serine residue at their active site, forming the largest class of mammalian proteinases. Several members of the serine proteinase family have

been implicated in the process of destruction of cartilage: Two major serine proteinases of the azurophil granules of polymorphs, elastase and cathepsin G, have been found to be involved in cartilage destruction [76]. Both enzymes degrade proteoglycan subunits, link proteins and type II collagen in the nonhelical domain, destroying the tensile properties of cartilage. It has been demonstrated that polymorphonuclear leukocyte elastase is the only serine proteinase capable of degrading tissue inhibitor of metalloproteinases into small peptides, abolishing its inhibitory activity. Thus, elastase is a very destructive proteinase [77]. In addition, two plasma proteinases, plasmin and kallikrein, may be involved in cartilage destruction by activating procollagenase [78]. Both plasmin and kallikrein are synthesized in latent forms - plasminogen and prekallikrein, which are activated by plasminogen activators, and factor XII, respectively. Bunning *et al.* [79] and Campbell *et al.* [80] found that human articular chondrocytes can produce plasminogen activators.

Cysteine proteinases Cysteine proteinases have also been associated with inflammatory processes. Cathepsin B and cathepsin L are the two best characterized members of the lysosomal cysteine proteinase family. Both proteinases can degrade proteoglycan and cleave the N-terminal peptides of collagen. Cathepsin L is much more effective in this action than cathepsin B [81]. Cathepsin B is capable of degrading several connective tissue macromolecules, including proteoglycan [82], elastin [83], and fibronectin [84]. The extracellular presence of cathepsin B in human rheumatoid synovial tissue [85], and a significant increase in cathepsin B-like activity in human osteoarthritic cartilage [86] and in chondrocytes and synoviocytes from arthritic rats [87], suggest that these cysteine proteinases may also be involved in the destruction of cartilage [88].

Aspartic proteinases These proteinases contain an aspartic acid residue on their catalytic site. The most prominent lysosomal proteinase acting at acid pH is cathepsin D, which is found in most mammalian cells. It degrades

proteoglycans by cleaving the hyaluronic acid-binding region and by producing a few cleavages in the polysaccharide–rich regions [89]. Cathepsin D can also cleave within the C-telopeptide of the α1(I) chain of collagen type I [81]. Similar to cathepsin B, it plays an important role in the intracellular protein turnover. In addition, cathepsin D has been shown to be released into the extracellular matrix of rheumatoid synovial tissue explants [90]. The circumstantial evidence suggests that cathepsin D may also play a role in the destruction processes of arthritis.

1.4.2 The proteinase inhibitors

Various proteinase inhibitors have been extracted from human articular cartilage and characterized by their specific actions [91-93], among them the tissue inhibitor of metalloproteinases (TIMP) is an effective and specific inhibitor playing a key role in control of the degradation of cartilage.

Tissue inhibitors of metalloproteinases The major physiologic inhibitors of the MMPs are α_2-macroglobulin, which is restricted in its sites of activity due to its large size (780 kDa), and a family of inhibitors that are specific for the MMPs and are produced by many cell types, including those of connective tissues. Two forms of TIMP have been fully characterized and cloned, both have closely related structures, inhibitory properties and similar cellular distribution [94]. TIMP-1 is a 30 kDa glycoprotein [95], and TIMP-2 a 23 kDa unglycosylated protein [96, 97]. The occurrence of TIMP-2 is not yet fully documented. It is possible that other related members of a TIMP family could be also present [98-100].

TIMP-1 appears to be a dominant species in this family, and it is a secreted 184-amino acid glycoprotein containing 12 cysteine residues participating in disulfide bonds and two potential N-linked glycosylation sites, both of which appear to be occupied [95, 101, 102]. The highly disulfide-bonded nature

probably confer stability to the TIMP molecule to changes in pH and temperature [102]. TIMP is capable of forming an equimolar, stoichiometric complex with metalloproteinases [94]. The essentially irreversible enzyme-inhibitor complex allows relatively small changes in levels of TIMP to lead to significant changes in net proteolytic activity. TIMP-1 has been found in every human body fluid examined [103], suggesting that TIMP is a fundamental and ubiquitous protein in human beings. The observation of significantly decreased levels of TIMP and serine proteinase inhibitors [91, 104] or relatively low levels of TIMP [105] in human osteoarthritic cartilage, in contrast to elevated levels of the proteinases, suggested that the excessive presence of the proteinases, coupled with lower levels of proteinase inhibitors in arthritic cartilage, contribute to the extensive loss of cartilage matrix [104]. In a recent report [106], Skup and Ponton show that increased collagenase activity in metastatic cells is the result of breakdown in regulation of TIMP-1 expression leading to decreased transcription of the TIMP gene. More recent studies by Hayakawa *et al.* [107, 108] implicated the possibility that TIMP-1 possesses growth-factor activity for a wide range of cells and that TIMP-1 is a new cell–growth factor in serum. It is intriguing that TIMP-1 appears to be a single glycoprotein with both protease inhibitory activity and growth-promoting activity because an homologous metalloproteinase inhibitor extracted from human articular cartilage and from human melanoma cell culture medium shows the properties compatible with TIMP.

Other proteinase inhibitors In addition to α_2-macroglobulin, a general proteinase inhibitor, several non-α_2-macroglobulin inhibitors have been detected in articular cartilage. A serine proteinase inhibitor of M_r 66 kDa has been partially characterized in human cartilage [91]. Its inhibitory activity was found similar to that of various plasminogen activator inhibitors (PAI-1,2,3) and protease nexin (PN), and therefore may regulate matrix degradation caused by the plasminogen/plasmin system. A member of the cysteine proteinase inhibitor

family named cystatins has also been found in mammalian cartilage, which shows inhibitory activity to cathepsin B [91].

1.5 Effects of Cytokines on Biosynthesis of Cartilage Matrix

The balance between the rates of synthesis and degradation of extracellular matrix components can be altered by the action of various cytokines which are involved in many aspects of cellular biological processes. Some cytokines have been thought to be important inflammatory mediators in a number of joint diseases. In fact, increased cytokine production by activated monocytes/macrophages, lymphocytes and synovial cells, as well as increased levels of these cytokines in joint fluids, has been found in rheumatoid arthritis. More and more evidence is emerging indicating that the cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) may play a crucial role in the destructive aspects of cartilage matrix turnover. Growth factors such as transforming growth factor-β (TGF-β) and insulin-like growth factor-1 (IGF-1) are the best candidates for activation of chondrocytes and induction of matrix synthesis.

1.5.1 Interleukin-1

IL-1 is a 17 kDa polypeptide produced by a variety of cells in response to infection, microbial toxins, inflammatory agents, products of activated lymphocytes and immune complexes. IL-1 has diverse effects on immunological, neurological, metabolic and endocrinological systems of many species. Monocytes/macrophages are the major source of IL-1. In addition, most connective tissue cells, notably synoviocytes and chondrocytes, are also capable of producing IL-1. Two forms termed IL-1α and IL-1β [109, 110], are encoded by separate genes, and have a limited sequence homology (26% for

human IL-1) between the two forms. IL-1α and IL-1β can be distinguished from each other by their isoelectric points of 5.0 and 7.0, respectively [111]. Neither form contains a signal peptide sequence that would indicate a cleavage site for the N-terminus. Both forms of IL-1 molecules bind to the same cell receptor, albeit with different affinities, but to elicit similar biologic responses [112]. IL-1 β is a prominent form of IL-1 found in body fluids and in cell supernates, and is only detected in human joint fluids from patients with a variety of joint diseases, including rheumatoid arthritis, osteoarthritis, traumatic arthritis, and psoriatic arthritis [113-115]. Some *in vitro* studies have shown that IL-1β stimulates the production of collagenase, and proteoglycan-degrading proteinase by connective tissue cells [116-117]. Incubation of cartilage explants with IL-1β results in a progressive release of large proteoglycan fragments and other structural protein components into the culture medium [118]. Thus, it is apparent that IL-1β can modulate the chondrocytes to enhance the catabolism of their extracellular matrix components [119, 120]. In addition to the induction of destructive proteinases, IL-1β suppresses the synthesis of aggregating proteoglycan [121], and collagen types II and IX [122] by human chondrocytes and inhibits chondrocyte growth as well [123]. Recent *in vivo* studies show that IL-1β can induce expression of metalloproteinases stromelysin and collagenase in articular cartilage following intra-articular injection in animals [124]. The mechanism by which IL-1β stimulates these proteinases had been partially understood. It appears that the stimulation of collagenase and stromelysin biosynthesis requires *de novo* synthesis of at least two proto-oncogene products, c-JUN and c-FOS, whose mRNA levels are transiently enhanced by IL-1β [125-127]. The catabolic properties of IL-1β and its effect on stimulating synthesis of proteinases by chondrocytes, suggest that IL-1β plays an important role in degradation of cartilage matrix. Moreover, recent evidence has indicated that IL-1β can stimulate its own transcription and expression in human synovial cells, skin fibroblasts and monocytes [128]. This

positive auto-regulation of IL–1β can, therefore, amplify, sustain, and further perpetuate the destructive action of IL-1β. Information derived from the *in vitro* effects of IL-1β on articular cartilage has been considered to be related to some of the biologic properties of IL-1β observed *in vivo*. It is increasingly clear that IL-1 is a major mediator of cartilage destruction in arthritis, such as RA and OA.

1.5.2 Tumor necrosis factor-α

This is another macrophage-derived 17-kDa cytokine which shares many similar biologic activities with IL-1, and is implicated in stimulating the synthesis of degradative enzymes in cartilage [129] and promoting the resorption of cartilage and inhibiting the synthesis of new proteoglycan in a similar manner to IL-1 [130]. But the cellular response to these two proteins appears to be independently regulated and controlled by different mechanisms. TNF-α binds to a separate receptor on target cells to induce collagenase production *in vitro* by chondrocytes and synovial fibroblasts, as well as bone resorption and fibroblast proliferation [130, 131]. Like IL-1 TNF-α is also capable of inducing the synthesis and secretion of various other cytokines such as IL-6 [132], IL-8 [133] and granulocye-macrophage colony stimulating factor (GM-CSF) [134]. These cytokines can augment the inflammatory process by promoting the accumulation and activation of lymphocytes and polymorphonuclear leukocytes in the joints. High levels of IL-6 and GM-CSF have been found in human rheumatoid synovial fluids [135, 136]. In addition, both IL-1 and TNF-α act in an autocrine manner on macrophages to stimulate their own transcription, as well as the production of each other, and therefore, may augment their tissue damaging potential and further amplifying their destructive action [129].

1.5.3 Transforming growth factor-β

Of the many polypeptide growth factors TGF-β appears to be relatively

unique in its global effects on cell growth and differentiation. At least five homodimeric polypeptides, with an apparent molecular weight of 25 kDa, sharing 70-80% homology and many biological activities, are represented in the highly conserved TGF-β family. Of these multiple forms, only TGF-β1, TGF-β2, and TGF-β3 have been identified in mammalian species [137]. TGF-β is abundant in bone and its mRNA has been detected in chondrocytes. Investigative studies have shown that TGF-β is locally synthesized by the chondrocytes and appears to stimulate the synthesis of extracellular matrix components, such as proteoglycan, collagen, fibronectin and osteonectin, in various cell types [138-140]. Furthermore, it inhibits the degradation of extracellular matrix by suppressing the synthesis of destructive enzymes, such as collagenase and stromelysin, and by stimulating the synthesis of TIMP [141, 142]. With these properties, TGF-β has been shown to antagonize the catabolic effect of IL-1 on chondrocytes [143, 144] and, therefore, can potentially ameliorate the destructive process occurring in arthritic joints. In addition, TGF-β has an enormous effect in stimulating wound repair [137, 145]. Recent reports showed a remarkable acceleration by TGF-β of fracture healing [146] and stimulation of chondrogenesis and cartilage formation [147].

1.5.4 Insulin-like growth factor-1

Insulin-like growth factors are, perhaps, the most well-studied of the growth factor family. There are two main forms of IGF, a basic (IGF-1) and an acidic (IGF-2) form; each with an apparent M_r of 7 kDa [148]. Insulin, IGF-1 and IGF-2 are three homologous peptides binding with varying affinity, to three distinct receptors on the cells [149, 150]. IGF-1 and IGF-2 are structurally homologous to proinsulin, almost the same size (70 and 67 amino acids) and share about 65% homology. IGF-1 exerts stronger effects on adult cartilage

explants and chondrocytes than IGF-2 [151, 152]. IGF-1, formerly known as somatamedin-C, has been found to increase the clonal proliferation of chondrocytes by stimulating DNA synthesis and enhancing the matrix synthesis and at the same time depressing or having little effect on the rate of proteoglycan catabolism [151-153], suggesting that IGF-1 is a systemic factor involved in the regulation of cartilage matrix metabolism. In addition, the anabolic effect of IGF-1 still persists even in the presence of IL-1 and TNF-α [154] and, therefore, IGF-1 could play an important role in repairing the proteoglycan-depleted matrix observed in arthritic joints.

1.6 Aims of the Thesis

Evidence has emerged to indicate that IL-1 can act as mediator of cartilage catabolism in inflammatory joint diseases; and therefore, the search for agents that could inhibit the local effects of this cytokine on cartilage matrix loss has become an approach to the potential control of joint erosion. TGF-β and IGF-1 have been implicated in playing a local role in modulating the functional behavior of articular chondrocytes, by activating their biosynthetic activity, and particularly, TGF-β has been considered the best candidate to antagonize the action of IL-1 in the degradation of articular cartilage. Since it is unlikely that tissue destruction in arthritis is mediated by any one cytokine and/or growth factor alone, synergistic interactions between factors and the priming by one factor of cellular responses to another factor represent the reality of cytokine/growth factor-triggered events in arthritis. Therefore, the purpose of this study was to investigate the individual effects of IL-1 and TGF-β or IGF-1, as well as interaction between them on biosynthetic activity of human articular chondrocytes, and to determine how these defined physiologically- or

pathologically-relevant cytokines precisely regulate the balanced expression of major cartilage matrix components at the mRNA levels.

The absolute quantification of mRNA levels will offer us an approach to obtain some information on the relative levels of response to these cytokines with respect to major components of cartilage matrix, in particular, the relative expression of metalloproteinases *versus* TIMP, which still remains unclear in human articular cartilage. A better understanding of the response of human articular cartilage to the effects of these cytokines may allow the development of conditions to favour tissue maintenance, moreover, it might be possible to prevent or reduce chondrocyte-mediated tissue destruction *in vivo*.

In order to achieve these aims, the following objectives were pursued in this study:

(1) To determine the steady-state expression of mRNAs for the major structural components type II collagen and aggrecan, and for the degradative components stromelysin and collagenase, as well as their inhibitor, TIMP-1 in human articular chondrocytes using the technique of competitive PCR which allows for acuate quantitation of number of mRNA transcripts, and thus to obtain a profile for the biosynthetic capacity of the cells at the mRNA levels.

(2) To quantify the mRNA transcripts for major component genes of cartilage matrix in response to IL-1β, TGF-β and IGF-1 stimulation, respectively, and to evaluate how these cytokines and growth factors mediate the biosynthetic patterns of human chondrocytes.

(3) To investigate the interaction between TGF-β and IL-1β or IGF-1 and IL−1β on the steady-state levels of mRNA for these components. Particularly, the relative levels of MMPs and TIMP-1 with respect to the interaction of cytokine and growth factors were to be determined.

Chapter 2. MEASUREMENT OF mRNA TRANSCRIPTS BY POLYMERASE CHAIN REACTION

How the chondrocytes transduce stimuli from their environment and alter their biosynthetic activities in response to the stimuli is currently a major subject of investigation. *In vitro* culture systems have provided an approach to study the mechanisms involved in the biosynthesis and turnover of matrix-specific macromolecules. In defined tissue culture media, the response of chondrocytes to various environmental or pharmacological stimuli can be investigated by the biosynthetic patterns of the cells. Although the biosynthetic activities of cells are controlled at many levels, changes in protein synthesis are very often preceded by changes in RNA levels, Recently developed biological techniques enable us to examine the biosynthesis of those matrix components at the mRNA level, and allow us to analyze and perhaps, better understand the normal and abnormal biosynthetic capabilities of chondrocytes isolated from heathy, arthritic, and degenerate cartilages.

Various methods have been developed to monitor the changes in transcription levels are allowing the quantitative detection of specific mRNAs [155-157]. Hybridization mediated detection of target sequences using labeled reporter molecules is one approach. In this method specificity is provided by the hybridization process. However, these conventional methods of mRNA analysis, such as Northern and "dotblot" hybridization and even nuclease protection mapping are not sensitive enough to detect mRNA in samples limited by either low cell number or low copy number per cell. Moreover, these methods permit only crude quantitation of mRNA. Another approach of *in situ* hybridization allows detection of mRNA in single cells but is insensitive or

nonquantitative. In recent years the amplification of target sequences using the polymerase chain reaction (PCR) has become widespread and has been shown to be considerably less sensitive to the sample degradation problem and provides high sensitivity detection of low abundant target sequences [158-159].

Amplification by PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primer by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Because the primer extension products synthesized in one cycle serve as the template in the next, the number of target DNA copies (approximately) doubles at every cycle. Thus, twenty cycles of PCR could yield about a million-fold amplification [160]. Application to the analysis of mRNA levels by PCR involves conversion of target RNA into complementary DNA (cDNA) by reverse transcription (RT) reaction with the enzyme RNA-dependent DNA polymerase. After PCR amplification of the cRNA, the initial rare mRNA can be efficiently amplified to easily detectable levels. This powerful technique has proved most useful in detecting specific mRNA species, especially those present in low copy number and those in a small amount of sample. [161-164].

Although PCR allows the detection and amplification of small numbers of specific mRNA transcripts, it has been more difficult to quantitate the absolute copy number present in the starting material. PCR amplification is an exponential process, where the extent of amplification $Y = N(1 + R)^n$, where N and Y is the number of copies before and after amplification, respectively,

and R is the reaction rate, and n is the cycle number. It is clear that minute differences in any of the variables controlling the reaction rate will dramatically alter the yield of the PCR product. Variables that influence the rate of the PCR include the concentrations of primers and other reaction components; reaction temperature; cycle length and number; rate of "primer-dimer" formation; and presence of contaminating DNA, etc. Even when these parameters are controlled precisely, there is sometimes a tube–to–tube variation that precludes accurate quantification. To circumvent this problem, several methods have been developed to quantitatively measure mRNA transcripts levels by PCR. Two main approaches have been developed: Quantitative PCR and Competitive PCR.

Quantitative PCR This is the first approach to quantify mRNA using PCR [165]. Under carefully controlled conditions keeping a constant exponential synthesis rate allowing for correlation between the number of target molecules and specifically synthesized product (as standard), the PCR was performed with a limited number of cycles to exploit the logarithmic phase of amplification [166-170]. The initial mRNA copy number and the amount of PCR product were to be close to a linear relationship within a range of magnitude [166]. The absolute quantification of target nucleic acids is achieved with the aid of a calibration curve established in an independent experiment through a dilution series of either synthetic RNA or standardized cells containing a known copy number of integrated target DNA. Therefore, it is necessary to first titrate the specific mRNA to find the range of concentrations that gives exponential amplification over a defined range of cycle numbers to reliably quantify the mRNA by PCR [171-172]. In addition, as a prerequisite for valid results, the time course of the target as well as the co–amplified standard must be established.

Since it is crucial to keep the reaction within the exponential phase, the co-amplified control sequence needs to be tested for its own kinetics of amplification [163, 173]. Moreover, the establishment of *R* values for both the target and the standard is absolutely required. If these *R* values turn out to be different, then the amplification has to be analyzed kinetically. This assumes that at least there is no quality variation between the samples. However, for many types of samples it is very difficult to reproducibly prepare RNA free of inhibitors, and is therefore unsatisfactory for quantification from clinical samples.

Competitive PCR Becker-Andre and Hahlbrock [174] as well as Gilliland *et al.* [175] described a method that overcomes the disadvantages of quantitative PCR and allows for absolute quantitative evaluation. It is based on the co-reverse transcription and co-amplification of target mRNA with an *in vitro*-generated RNA transcript (as a internal standard). The standard RNA contains the identical primer regions to that of target mRNA, but flanking an fragment differing from the target RNA by either a single base exchange which creates a new restriction endonuclease recognition site or by a small insert for size differentiation of the product. Thus, if the target mRNA and known amount standard cRNA are subjected to the first strand cDNA synthesis and the PCR process using the same primer pair in a reaction, differences in primer efficiency are minimized. In addition, a change in any of the variables affecting the rate of PCR amplification will affect the yield of both templates equally, and the target and the standard behave identically during the whole process of amplification. Therefore, the ratio of both PCR products remains precisely constant through the amplification. The size difference in the PCR products permits easy separation of the that from standard and target by gel

electrophoresis. **Figure 2** shows a diagrammatic representation of competitive PCR. The relative amount of each product can be readily determined either by direct densitometric scanning of ethidium-bromide-stained gels or by incorporation of radio-labeled dNTPs. Because the starting concentration of the internal standard is known, the initial amount of the target mRNA can be determined. It might be concluded that a single dilution of standard could be sufficient in theory for quantitation of unknown target by ratio of both products. However, because the precise analysis of two template species present in very different amounts may be difficult and imprecise in practice, competitive PCR amplification using three or more internal standard dilutions within the expected range of target molecules adds reliability.

Recently, a series of complementary RNA (cRNA) transcribed from plasmid constructs had been synthesized as internal standards for quantitatively studying the major components of human cartilage matrix at the mRNA levels in the Joint Diseases Laboratory of Shriners Hospital in Montreal. **Figure 3** shows a diagrammatic representation of standard cRNA transcript formation. Initially, DNA constructs were made for each standard by PCR using specific primers consisting of a restriction site (to allow subsequent cloning), the specific target mRNA primer sequences and a bacteriophage lambda overlap region. Using bacteriophage lambda DNA as template, the appropriate DNA inserts were amplified and cloned into the vector pSP6(polyA) which carrys a SP6 promoter for bacteriophage-encoded DNA-dependent RNA polymerase transcription. For cRNA production the plasmids were linearized and used to direct the synthesis of poly A-cRNAs *in vitro* using SP6 RNA polymerase. The cRNA transcripts were purified, and their concentrations measured by the spectrophotometric determination at 260 nm.

The specificity of PCR amplification of nucleic acids sequences is provided

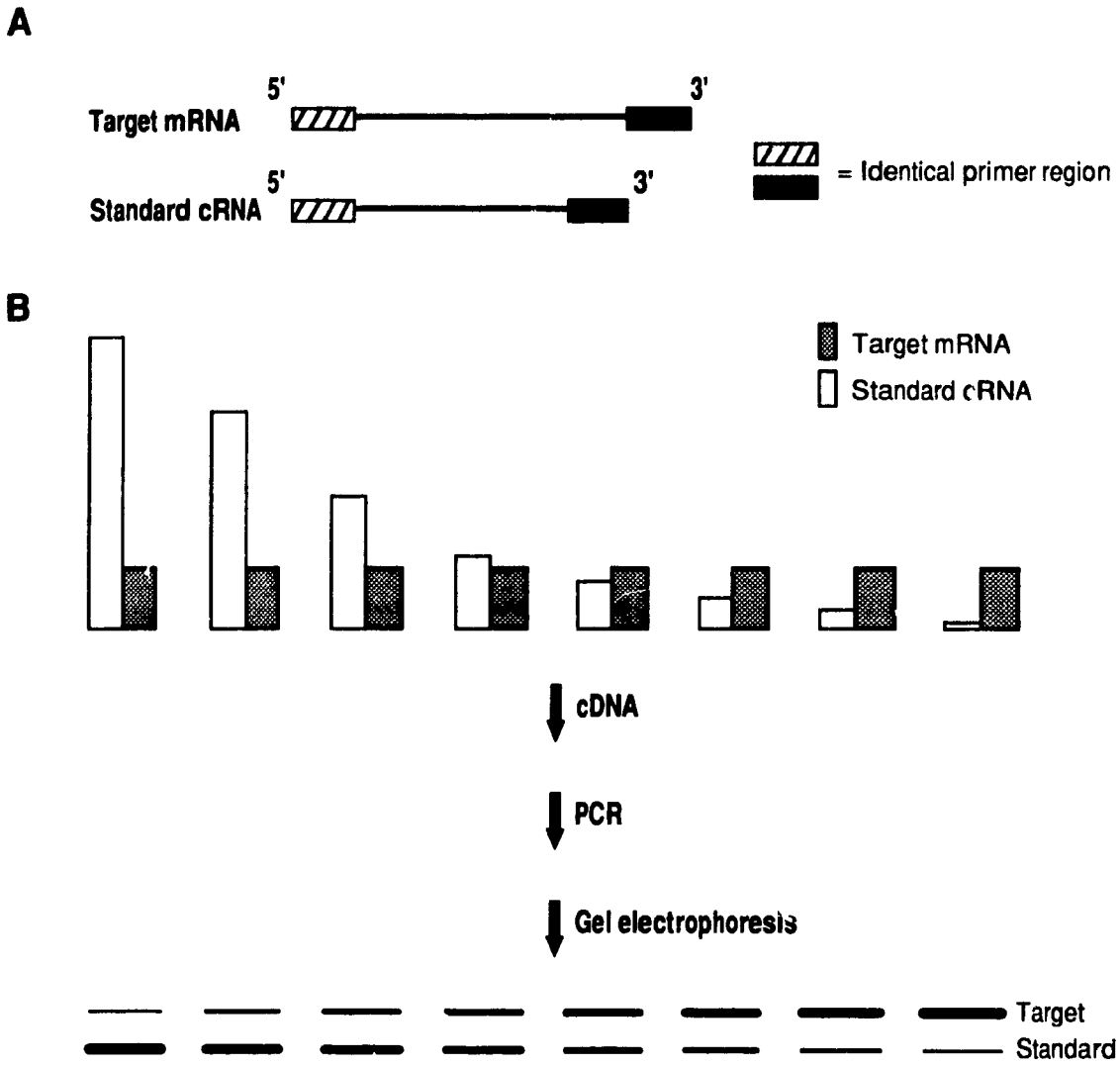
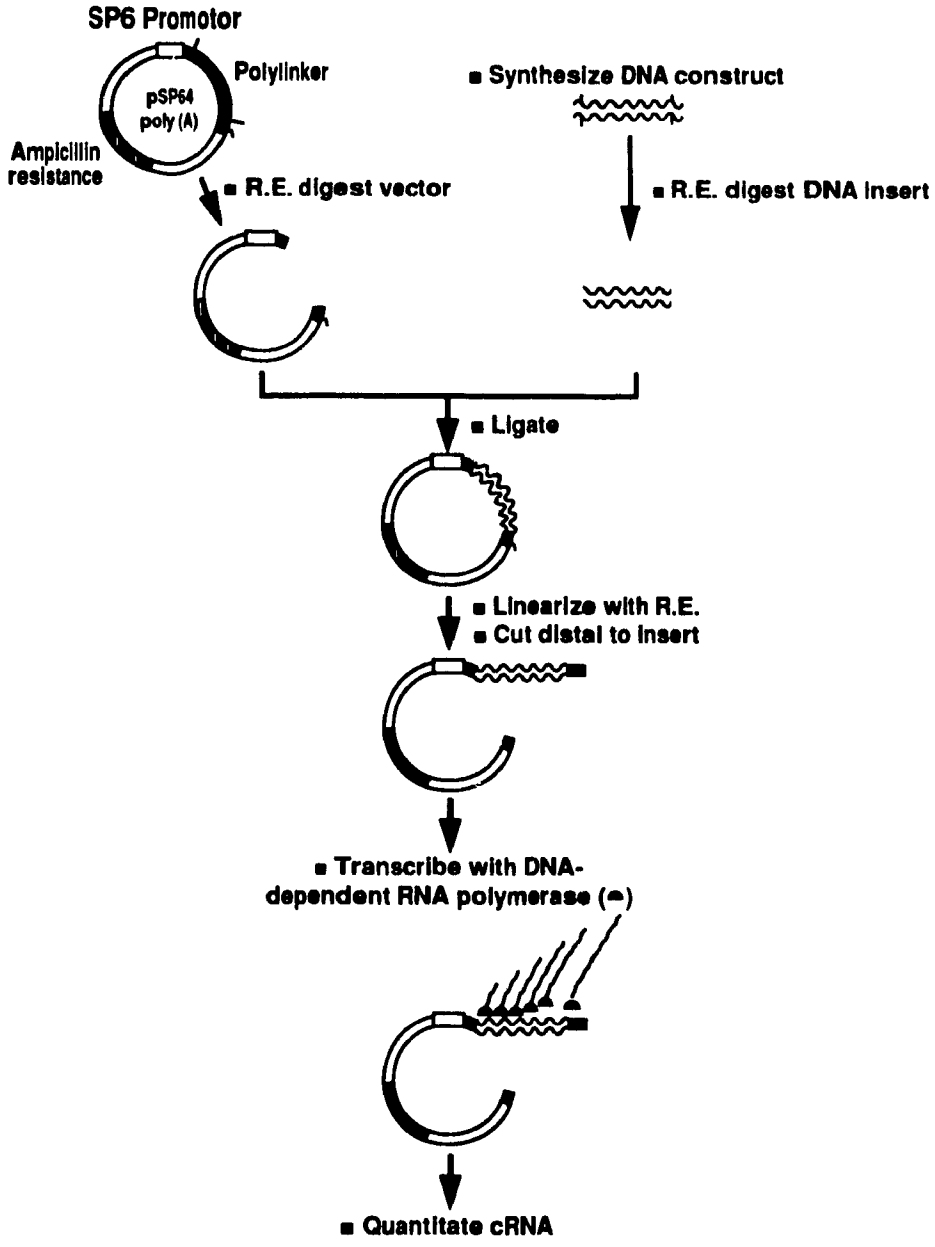


Figure 2. Diagrammatic Representation of Competitive Polymerase Chain Reaction. **A**: The standard RNA contains the identical primer regions to that of target mRNA, but flanks a fragment differing from the target RNA in size. **B**: Aliquots of one total RNA sample are titrated with decreasing known amounts of standard RNA. After conversion to cDNA and PCR the amplified products are analyzed by gel electrophoresis for the relative presence of target RNA- and standard RNA-specific DNA fragments which are distinguished by their difference in size. (Adapted and modified from Becker-Andre and Hahlbrock, 1989.)



R.E.: Restriction enzymes; ⌐ : R.E. cut sites.

Figure 3. Diagrammatic Representation of Standard cRNA Transcript Preparation

by the primers. The efficient and specific primers used for target mRNA and the standard RNA are generally selected based on following guidelines [172]: (1). an average G+C content of around 50% and random base distribution is chosen; (2). primers with stretches of polypurines, polypyrimnidines, or sequences with significant secondary structure are avoided; (3). the primers are checked against each other to avoid complementarity, in particular, for 3' overlaps which would allow formation of "primer-dimer".

Competitive PCR allows for not only specific detection of small amount of RNA but also actual quantitation of this amount. It provides us with a useful approach to precisely investigate the expression of mRNAs for various components of cartilage matrix under normal physiologic or pathologic conditions.

Chapter 3. MATERIALS AND METHODS

3.1 Growth Factors/Cytokines

Recombinant human IL-1β and porcine platelet TGF-β were purchased from R & D Systems, Minneapolis, MN. Recombinant human IGF-1 was purchased from Boehringer Mannheim, Laval, Canada.

3.2 Materials

DulbeccoVogt modified Eagle's medium (DMEM) (4.5 g/L glucose), and Hank's balanced salts solution (HBSS) were from Grand Island Biological Company (GIBCO, Burlington, Ontario). Fetal calf serum (FCS) was obtained from Bocknek Laboratories. Tissue culture supplies were from Flow Laboratories (Toronto, Ontario), or from GIBCO. SeaPlaque and NuSieve agarose gel were from FMC BioProducts (Rockland, ME). PCR Microtubes containing a screw cap featuring a built in shaft which seals the tube directly above the sample were from BIO/CAN SCIENTIFIC (Mississauga Ontario). This design permits running the PCR reaction without the need for mineral oil to eliminate sample evaporation during the reaction.

3.3 Cell and Cell Culture

Human articular chondrocytes were from disease-free femoral condylar cartilage obtained from neonates (3 month old) and adult (18 year old) at autopsy. Chondrocytes were isolated by digestion with trypsin-collagenase, and stored frozen after passage. Frozen cells were thawed and washed with DMEM, and then plated at 10^6 cells/75 cm^2 flask and maintained as monolayer culture in 15 ml DMEM supplemented with 10% FCS, 2 mM L-glutamine, and

penicillin (100 units/ml) and streptomycin (100 µl/ml) at 37°C in a humidified atmosphere of 5% CO_2/95% air for 10 to 14 days. The media were changed every three days. At confluence, the cells were subcultured to 12-well tissue culture plates at a density of 5 x 10^4 cells/cm^2 in DMEM containing 10% FCS, and incubated for 48 hours to allow attachment. Subsequently, the FCS-containing medium was replaced by serum-free DMEM, and the cells were exposed to IL-1β (2 units/ml), TGF-β1 or TGF-β2 (2 ng/ml), and IGF-1 (100 ng/ml), respectively, or combinations of IL-1β (2 units/ml) with TGF-β1 (2 - 20 ng/ml) or IGF-1 (100 ng/ml). The cells were incubated for 24 or 48 hours under experimental conditions as above, and total cellular RNA was extracted. For all experiments, the cells at passage levels 3 or 4 were used.

3.4 RNA Extraction

The chondrocyte monolayers were washed twice with HBSS solution and detached with 0.25% trypsin (Sigma, St Louis, MO) in phosphate-buffered saline, and subsequently suspended in FCS containing-DMEM. The cell pellet was obtained by centrifugation. Total cellular RNA was extracted by using an acid guanidinium thiocyanate-phenol-chloroform extraction method based on method of Chomczynski and Sacchi [176]: the cells were lysed in 100 µl of 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2–mercaptoethanol, pH 7.0 (lysis buffer). Sequentially, 10 µl of 2 M sodium acetate, pH 4, 100 µl of phenol (water saturated), and 20 µl of a chloroform–isoamyl alcohol mixture (49:1) were added to the extract, with thorough mixing by inversion after the addition of each reagent. The extraction mixture was vortexed throughly for 10 seconds, then cooled on ice for 15 minutes. Samples were centrifuged at 10,000g for 20 minutes at 4°C. After centrifugation the aqueous phase containing RNA was transferred to a fresh

tube, mixed with 100 µl of isopropanol, and then placed at -20°C for at least 2 hours and then at -70°C for 15 minutes to precipitate RNA. After centrifugation, the RNA pellet was reconstituted in 100 µl of lysis buffer and reprecipitated with an equal volume of isopropanol for 1 hour, and centrifugated. The RNA was washed with 75% ethanol, sedimented, vacuum dried, and consequently redissolved in 100 µl diethylpyrocarbonate (DEPC)-treated water. The RNA concentration was measured spectrophotometricclly by absorbance at 260 nm (Beckmann spectrophotometer, Beckmann Instruments DU-40, Fullerton, CA). The cellular RNA preparations were stored at -70°C for further determination of specific mRNA transcript numbers.

3.5 Oligonucleotide Synthesis

The nucleotide sequences of 5'-primers (upstream) and 3'-primers (downstream) were selected for type II collagen, aggrecan, collagenase, stromelysin, TIMP-1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), according to their published DNA sequences. The primers were between 20-27 bases in length and were chosen to give a melting temperature at around 60°C. The sizes of target mRNAs, internal standard cRNAs and their corresponding primers used for reverse transcription and PCR are listed in **Table 2**. The size difference of the PCR products between targets and standards are from 80 to 90 base pairs, allowing easy separation by gel electrophoresis. All of the oligonucleotides were synthesized on an Applied Biosystems Model 392 RNA/DNA synthesizer in the biotechnology facility of Shriners Hospital of Montreal. The primers were purified using an oligonucleotide purification cartridge (OPC) purification protocol (Applied Biosystems). Each primer was diluted to 20 pmol/µl in DEPC-treated water. The oligonucleotide pairs were demonstrated to generate products of the predicted size by PCR amplification

mRNA species	5' primers	3' primers	Size of PCR product, bp	
			mRNA	cRNA
• G3PDH	5'-GCTCTCCAGAACATCATCCCTGCC-3'	5'-CGTTGTCATACCAGGAAATGAGCTT-3'	346	436
• Aggrecan	5'-TGAGGAGGGCTGGAACAAGTACC-3'	5'-GGAGGTGGTAATTGCAGGGAACA-3'	350	427
• Collagen α1(II)	5'-CTGGCTCCCAACACTGCCAACGTC-3'	5'-TCCTTTGGGTTTGCAACGGATTGT-3'	414	334
• Collagenase	5'-CTGTTCAGGGACAGAATGTGCT-3'	5'-TTGGACTCACACCATGTGTT-3'	403	322
• Stromelysin 1	5'-CCCTCCAGAACCTGGGAC-3'	5'-ATAAAAGAACCCAAATTCTTCAAAA-3'	505	429
• TIMP-1	5'-TACTTCCACAGGTCCCACAACC-3'	5'-GGCTATCTGGGACCGCAGGGACTGCCA-3'	339	409

Table 2. Polymerase Chain Reaction Primer Pairs Used in This Study. Nucleotide sequences of 5'- and 3'-primers and expected sizes of the amplified PCR products.

for 25 cycles using cellular RNA as template.

3.6 Reverse Transcription (RT) of RNAs

The internal standard cRNAs for various target mRNAs under study had been prepared by Dr. BE Hakala in the Joint Diseases Laboratory of the Shriners Hospital. Each cRNA was stored at -70°C in DEPC-treated water. A precise dilution series were prepared, ranging from 1 x 10^4 to 1 x 10^8 copies of cRNA/10 µl in relatively large volumes (e.g. 1 ml) so that the same dilution series can be used for multiple determinations.

The first strand cDNAs were prepared by reverse transcription of RNA for further PCR amplification. Both mRNA and cRNA were heated at 80°C for 5 minutes followed by quick chilling to overcome secondary structure. Three or four serial dilutions of the internal standard cRNA (1 x 10^4, 1 x 10^5, 1 x 10^6 and 1 x 10^7 copies/µl) and a constant amount of the extracted total cellular RNA (20 or 80 ng) was mixed in a final volume of 20 µl of 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% m/v gelatin, pH 8.3 buffer (1 x PCR buffer) containing 20 units of the RNase inhibitor RNasin (Boehringer Mannheim Biochemica), 200 µM each of the four deoxyribonucleoside triphosphates (dNTPs), 100 µM dithiothreitol, 100 units of the recombinant Molony murine leukemia virus (MMLV) reverse transcriptase (GIBCO) and 20 pmols of 3' oligonucleotide primer. The use of specific antisense primers (complementary to the corresponding mRNAs and cRNAs) rather than oligo(dT) for the reverse transcription reaction added additional specificity to the subsequent PCR and ensured that only the target RNAs were amplified. The reverse transcriptase reaction was carried out by incubation at room temperature for 10 minutes, then 45 minutes at 42°C. The reaction was stopped by heating at 90°C for 5 minutes, and the samples were chilled on ice for further competitive PCR amplification.

3.7 Competitive PCR

The cDNA strands, transcribed from mRNAs and cRNAs in a final volume of 100 µl of 1 x PCR buffer containing 40 pmols each of 5' and 3' primer, 2 units of *Thermus aquaticus* DNA polymerase (GIBCO) and 1 µCi (37 GBq) of [α^{32}P]-dCTP (Amersham), were co-amplified by PCR for 40 cycles using a Hybaid Thermal Reactor (Bio/Can Scientific). The following temperature profile was used: denaturation at 94°C for 30 seconds; primer annealing at 60°C for 60 seconds; primer extension at 72°C for 60 seconds. The cycling concluded with a final extension at 72°C for 5 minutes. In all experiments, negative controls containing no template RNAs were subjected to the same procedures for monitoring false positive reaction product and to determine background incorporation levels.

3.8 Agarose Gel Electrophoresis

A 20-µl portion of the PCR products was electrophoresed on 3% NuSieve-1% SeaPlaque agarose composite gels in 40 mM Tris-acetate pH 7.5 buffer containing 1 mM EDTA. The separated PCR products were stained with ethidium bromide (0.5 µg/ml), visualized under ultraviolet light, and photographed. The ^{32}P–labeled–bands corresponding to standard and target were excised from the gel and radioactivity was determined by scintillation counting in a Liquid Scintillation Analyzer (Model 1900 CA, Packard Instrument Co.)

3.9 Data Analysis

As the radio of the amount of PCR products is directly related to the ratio of the initial templates, and as all other reaction conditions are identical a linear

relationship is expected when these parameters are plotted. The cpm counts obtained for the internal standard product and the mRNA product were corrected for differences in length and G+C content, and the ratios were plotted against the copy number of internal standards using a double log scale for convenience. The data were fitted to a line by least equate line regression analysis and the initial target number was calculated from the equation parameters. The point of equivalence (i.e., where there is a 1:1 ratio of cRNA/mRNA) is where target equals internal standard and represents the initial amount of target mRNA in the unknown.

3.10 Calculation of Relative mRNA Levels

Quantification of G3PDH, considered as a "house keeping gene" was used for standardizing each cell culture and RNA preparation, expressed at relatively constant levels. The levels of specific mRNA under study were standardized against G3PDH mRNA levels. Standardization against G3PDH mRNA levels enabled the quantitative evaluation of specific mRNA induction, without differences due to variations in cell number, cell extraction, or in the amounts of total cellular RNA used in PCR amplification. To verify the reproducibility of the method, determinations of G3PDH mRNA were performed in triplicate. The same amount of total cellular RNA as that used in quantification of G3PDH mRNA levels was used for other target mRNA determinations.

Chapter 4. RESULTS

4.1 PCR Detection of mRNA Phenotypes by Using Appropriate Primers

An initial study was carried out to determine whether the primers selected allowed detection of specific mRNAs and whether qualitative changes were detectable under different experimental conditions. By PCR amplification, the initial screen for the expression of the mRNA species under study was performed on neonate human chondrocyte treated with or without IL-1β (2 units/ml) in serum-free DMEM for 24 hours. Various mRNA species in a limited amount of sample were reverse-transcribed and amplified by PCR for 25 cycles using specific primer pairs [shown in Table 2] for type II collagen, aggrecan, stromelysin, collagenase, TIMP-1 and G3PDH. Thus, target mRNAs, including those present in low numbers, should be amplified sufficiently for detection on agarose gels, and G3PDH should provide consistent bands to confirm the similar rate of conversion of RNA to cRDA in different RNA preparations. The modulating effects of IL-1 on expression of these mRNAs should be detectable qualitatively.

The results of agarose gel separation of the PCR products for these mRNA species are shown in **Figure 4**, presenting pairs of cultures incubated in the presence and the absence of IL-1β (2 units/ml). It is noted that twenty-five cycles of PCR resulted in sufficient amplification for the detection of the expected products. The PCR products are easily distinguished for all of the above species by the their difference of the sizes as predicted by each primary sequence. A clear signal for G3PDH in the control is similar to that in IL-1β-treated cell cultures. Results indicate that levels of expression of type II collagen and TIMP-1 are relatively higher than those of aggrecan and collagenase in unstimulated cell cultures. There is an obvious effect of IL-1 on the expression of these mRNAs, in particular, it enhanced the expression of

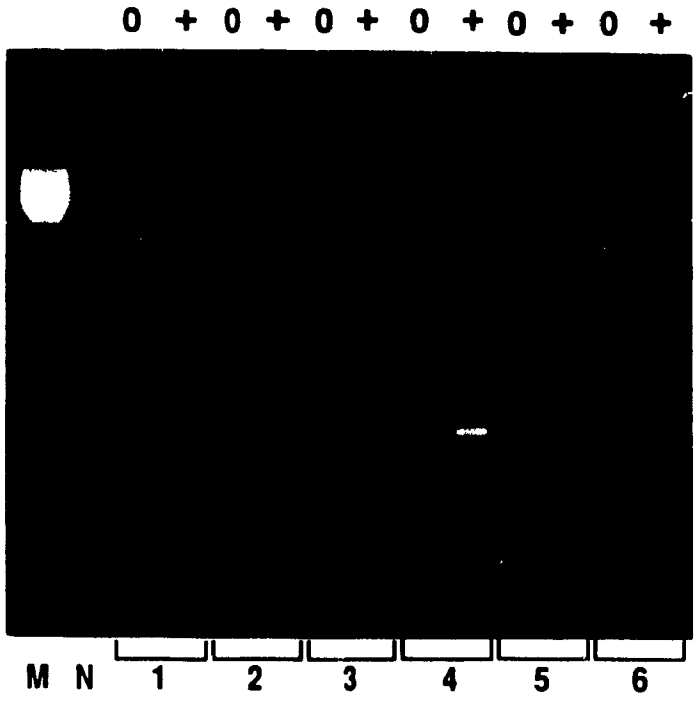


Figure 4. PCR Detection of Various mRNA Phenotypes in Human Chondrocytes Using Appropriate Primer Pairs. Total cellular RNA was extracted from neonatal human chondrocyte cultured in the presence (+) and absence (0) of IL-1β (2 units) for 24 h. Various target mRNA species under study were reverse transcribed, and amplified by PCR for 25 cycles using specific primers as listed in **Table 2**. PCR products were then electrophoresed in a 1.8% agarose gel and visualized with ethidium bromide under UV light. Lane M: 100-base pair DNA ladder marker, Lane N: negative control, Lane 1: G3PDH, Lane 2: type II collagen, Lane 3: aggrecan, Lane 4: stromelysin, Lane 5: collagenase, Lane 6: TIMP-1.

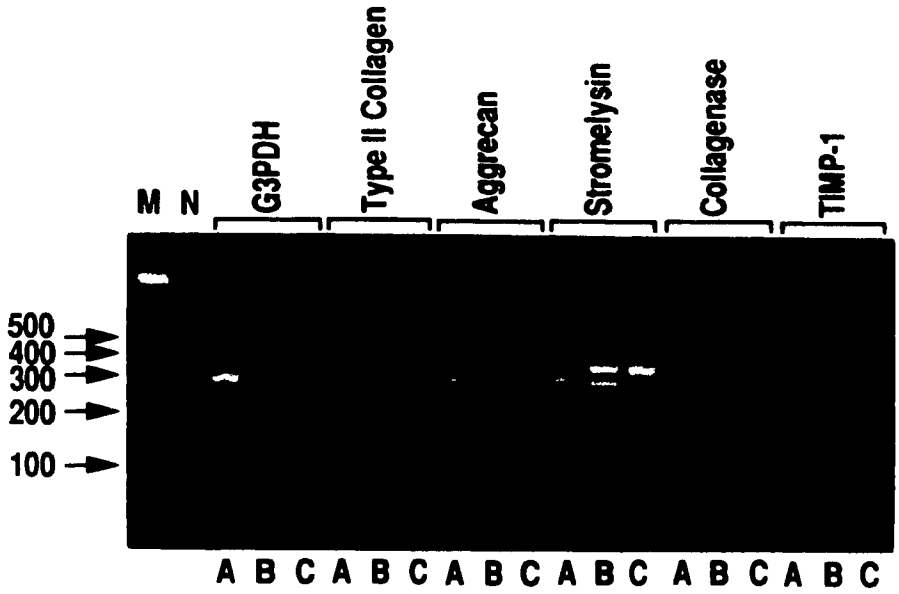
stromelysin and collagenase while it depressed the expression of aggrecan mRNA. These findings are consistent with the generally accepted action of IL–1 on the synthesis of the cartilage matrix components or metalloproteinases.

Because the PCR technique alone provides qualitative rather than quantitative results, it is important to note that although expression of type II collagen was not notably depressed, it may still be reduced to some degree. This has to be verified using the quantitative method of competitive PCR. A quantitative analysis of the affects of cytokines and growth factors on the mRNA levels of the structural genes and metalloproteinases will allow us to determine and compare the effects on the expression of these individual components as a whole.

4.2 Measurement of the Steady-State mRNA Levels by Competitive PCR

In order to obtain an accurate estimate of the steady-state levels of the mRNA species under study in unstimulated human chondrocyte culture, which will be used as a base line for further investigations of effects of cytokines on the cells, the above mRNA levels were quantitated by competitive PCR.

With three dilutions of each cRNA, constant amounts of the RNA prepared from unstimulated chondrocytes, were co–reverse transcribed into cDNAs by using the appropriate downstream primer for each mRNA, Competitive PCR was carried out for 40 cycles in the presence of ^{32}P-dCTP. A portion of each PCR reaction mixture was then subjected to gel electrophoresis to separate the the amplified products from the cRNA and mRNA. The result are shown in **Figure 5**. The PCR products resulting from the internal standard and from the target mRNA are clearly separated and in all cases the observed sizes corresponded to those predicted from the cDNA sequenced and from the corresponding internal standard construct. The appropriate bands were excised



Lane M : Molecular weight markers
Lane N : No template negative control
Lane A : 5 x 10^6 copies of internal cRNA standards
Lane B : 5 x 10^5 copies of internal cRNA standards
Lane C : 5 x 10^4 copies of internal cRNA standards

Figure 5. Measurement of the Steady-state mRNA Levels By Competitive PCR. Total cellular RNA was extracted from unstimulated human chondrocyte cultures using acid guanidinium thiocyanate. Constant amounts of total RNA were co-reverse-transcribed and co-amplified with three dilutions (5 x 106, 5 x 105, and 5 x 104 copies) of internal standard cRNA using specific primer pairs for G3PDH, type II collagen, aggrecan, stromelysin, collagenase, and TIMP-1. The competitive PCR was performed for 40 cycles. A 20 µl aliquot of each PCR incubation mixture was electrophoresed in 3% NuSieve-1% SeaPlaque agarose gel, stained with ethidium bromide, visualized under UV light and photographed. The numbers on the left indicate the size of molecular weight markers. The results represent one of three similar experiments.

from the agarose gels for radioactivity determination and data were analyzed to determine each initial mRNA level. The quantitation of G3PDH mRNA is illustrated here as an example. Results of three separate analyses are shown in **Table 3.** which presents the raw counts obtained for the internal standard and target mRNA PCR products, corrected for background and the ratio of these values. A plot of these ratios versus cRNA copy-numbers for easy determination is shown in **Figure 6.** The mean of these three determinations is $1.07 \pm 0.05 \times 10^5$ copies/ng total RNA. The results demonstrate the reproducibility of this quantitative method. Each copy number of the other mRNAs under study was quantitated by competitive PCR, and standardized to G3PDH level as copies mRNA per 1 copy G3PDH mRNA. The results are shown on **Figure 7.** The difference of three quantitations for each mRNA level is less than 5%. The results clearly indicate that the copy number of type II collagen mRNA transcripts was about 3.5-fold higher than that of aggrecan, while stromelysin mRNA was about 3.2-fold higher than that of collagenase. The mRNA level of TIMP-1 was also relatively high. Based on the number of cells cultured and the amount of total RNA used, the copy numbers calculated for these mRNA species are all around 10^3 copies/cell. The data basically correspond to the typical mRNA population in eukaryote. Generally, there may be 10^3 to 10^5 copies of mRNA for an abundant or moderately abundant gene whose protein is the principal product of the cell [177].

4.3. Quantitative Analysis of the Effects of IL-1β and/or TGF-β on the mRNA Expression

Using the above approach, the effects of IL-1 or TGF-β as well as the interaction of IL-1 and TGF-β on the expression of these mRNAs under study were determined in a quantitative manner. It is important to know how this growth factor precisely modulates the actions of IL-1 with respect to expression

Total cellular RNA used	cRNA copies	cpm in PCR products from cRNA	cpm in PCR products from mRNA	Ratio (cRNA/mRNA)
	50,000,000	111904	14823	7.549
A 20 ng	10,000,000	107117	34588	3.097
	5,000,000	80113	44754	1.791
	5,000,000	42873	28287	1.516
B 20 ng	500,000	24557	38903	0.631
	50,000	12140	40341	0.301
	5,000,000	69652	92902	0.749
C 80 ng	500,000	24917	89796	0.277
	50,000	6179	123528	0.050

Table 3. Quantitation of G3PDH mRNA Levels by Competitive PCR. Total RNA was extracted from unstimulated human chondrocytes. Three quantitations (A, B, C) of G3PDH mRNA were performed. 20 ng (in A and B, from one RNA preparation) and 80 ng (in C) of total RNA were used for co-reverse transcription and co-amplification, respectively, along with different serial dilutions of internal standard G3PDH cRNA as described in Materials and Methods. A portion of each PCR product was separated by agarose gel electrophoresis, and appropriate radioactive bands reflecting the products amplified from cRNA and mRNA were excised and radioactivity was determined. The ratio of the PCR products derived from the cRNA (P_c) and the mRNA (P_m) was obtained from each sample. These data were used in the analysis shown in Figure 6.

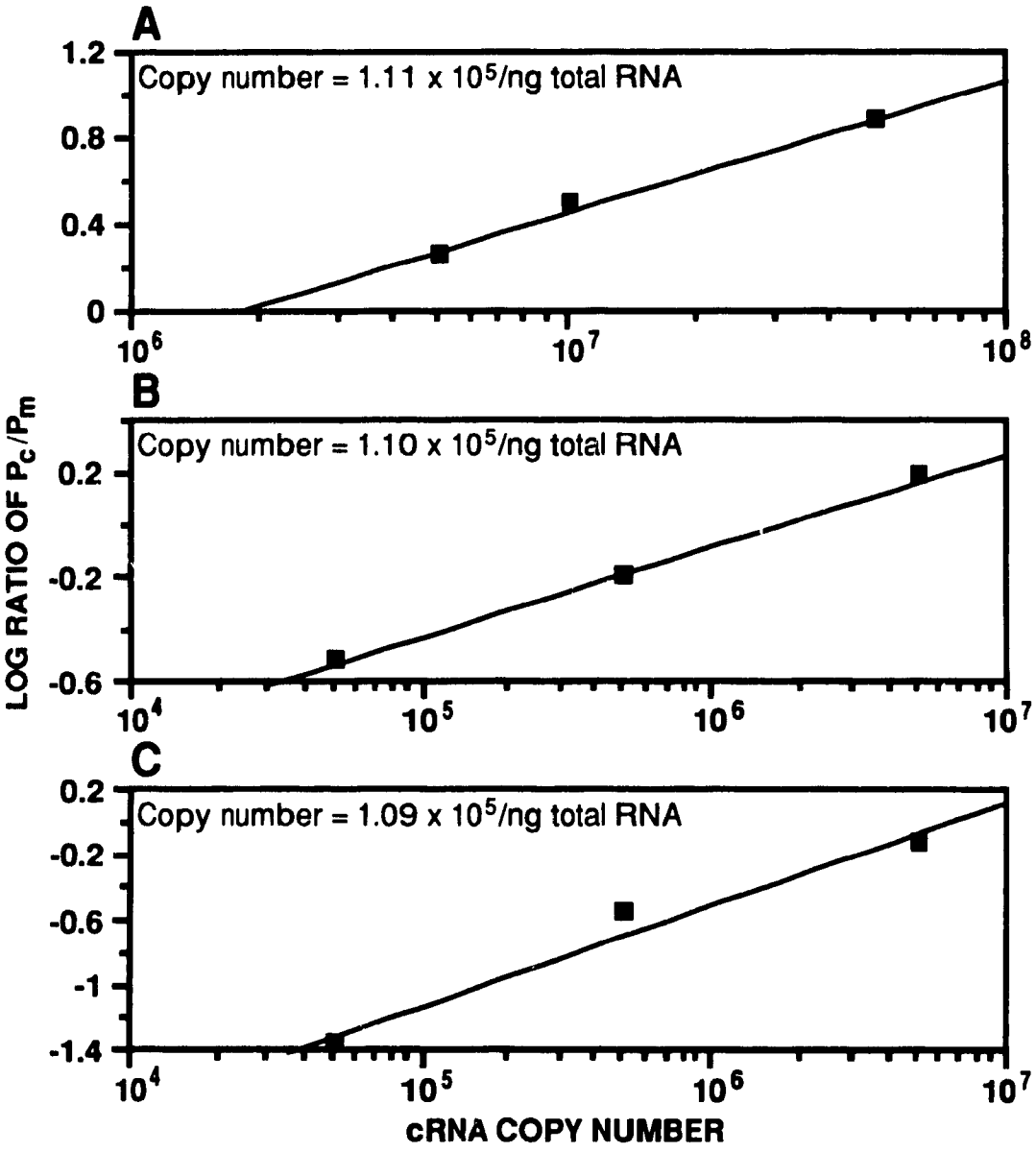


Figure 6. Calculation of Copy Number of G3PDH mRNA. From the data in Table 3, the ratios of cRNA and mRNA were multiplied by 220/172 (G+C content of standard/target) to correct for the difference of G/C-content. Data were plotted as log ratio of P_c/P_m vs. initial copy number of the G3PDH cRNA. The points were fitted to the equation $y = a + bx$, and the copy number was calculated from the constants a and b. The results are shown as copies of mRNA/ng total RNA: A = 1.11 x 10^5, B = 1.10 x 10^5, C = 1.09 x 10^5, giving a mean copy number of 1.10 x 10^5/ng total RNA.

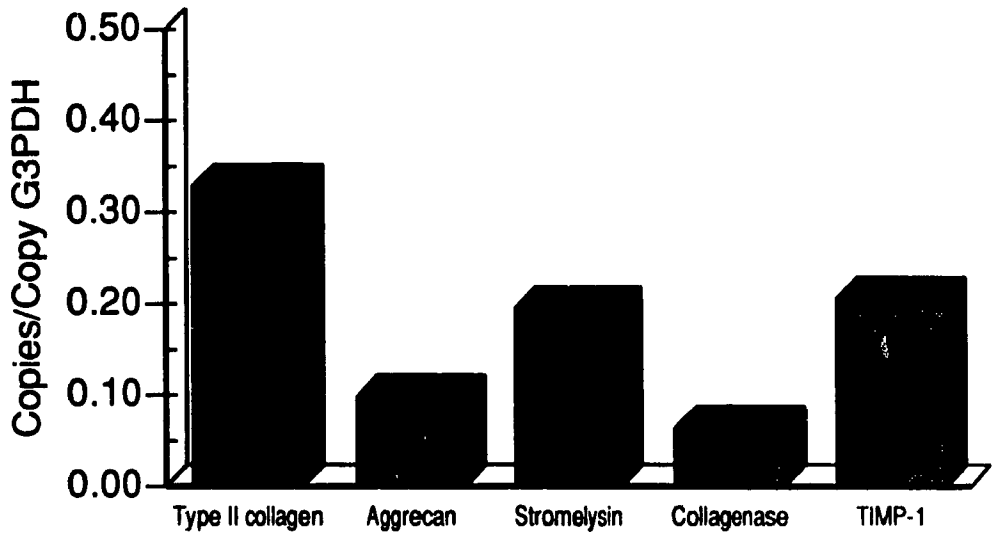


Figure 7. Quantitative analysis of steady-state mRNA levels for type II collagen, aggrecan, stromelysin, collagenase, and TIMP-1 in unstimulated human chondrocyte cultures. The copy numbers of these mRNA species were calculated from the ratios of the PCR products from the cRNA and mRNA, and standardized to the G3PDH mRNA level as copies mRNA per 1 copy G3PDH mRNA. The results represent one of three similar experiments. The difference of three quantitations was less than 5% in each mRNA determination.

of mRNA for the structural components and degradative components. The human chondrocytes were cultured in the presence of IL-1β (2 units/ml) and/or TGF-β1 (2 ng/ml) and β2 (2 ng/ml) for 24 hours under serum-free conditions, and total RNA prepared from these cell cultures was analyzed for mRNA levels of type II collagen, aggrecan, stromelysin, collagenase, and TIMP-1 by competitive PCR. The results are shown in **Figure 8**, presenting the mRNA levels of the structural components type II collagen and aggrecan, and **Figure 9**, showing MMP and TIMP-1 mRNA levels, respectively.

As expected, IL-1β reduced the expression of the structural components and induced the expression of the MMPs, as demonstrated quantitatively in the previous PCR detection (Figure 4). But it reveals that the mRNA level of type II collagen was reduced by IL-1 to 66% of control levels. Moreover, the results clearly indicate that the extent of the response pattern was unique for each component. There was a strong induction of stromelysin (7-fold) and collagenase (6-fold), while TIMP–1 mRNA, which is relatively abundant in unstimulated chondrocytes, was depressed by stimulation with IL-1β to about 50% of control levels. The depressing effect of IL-1 was very pronounced in the case of aggrecan with more than a 10-fold reduction, while type II collagen mRNA levels were only slightly reduced.

Following treatment with TGF-β, the results reveal that TGF-β enhanced mRNA levels for type II collagen and aggrecan with a 2-fold increase of type II collagen and 3-fold increase of aggrecan in comparison with control levels, respectively. In addition, it is noted that both TGF-β1 and TGF-β2, at the same concentration of 2 ng/ml were equally potent in inducing the expressions of type II collagen and aggrecan, and reducing the mRNA levels for stromelysin (60% decreased) and collagenase (40% decrease) while significantly inducing the mRNA level of TIMP-1 with a striking 6.5-fold increase.

The results clearly indicated that response patterns of structual components *versus* those of degradative enzymes were regulated in an

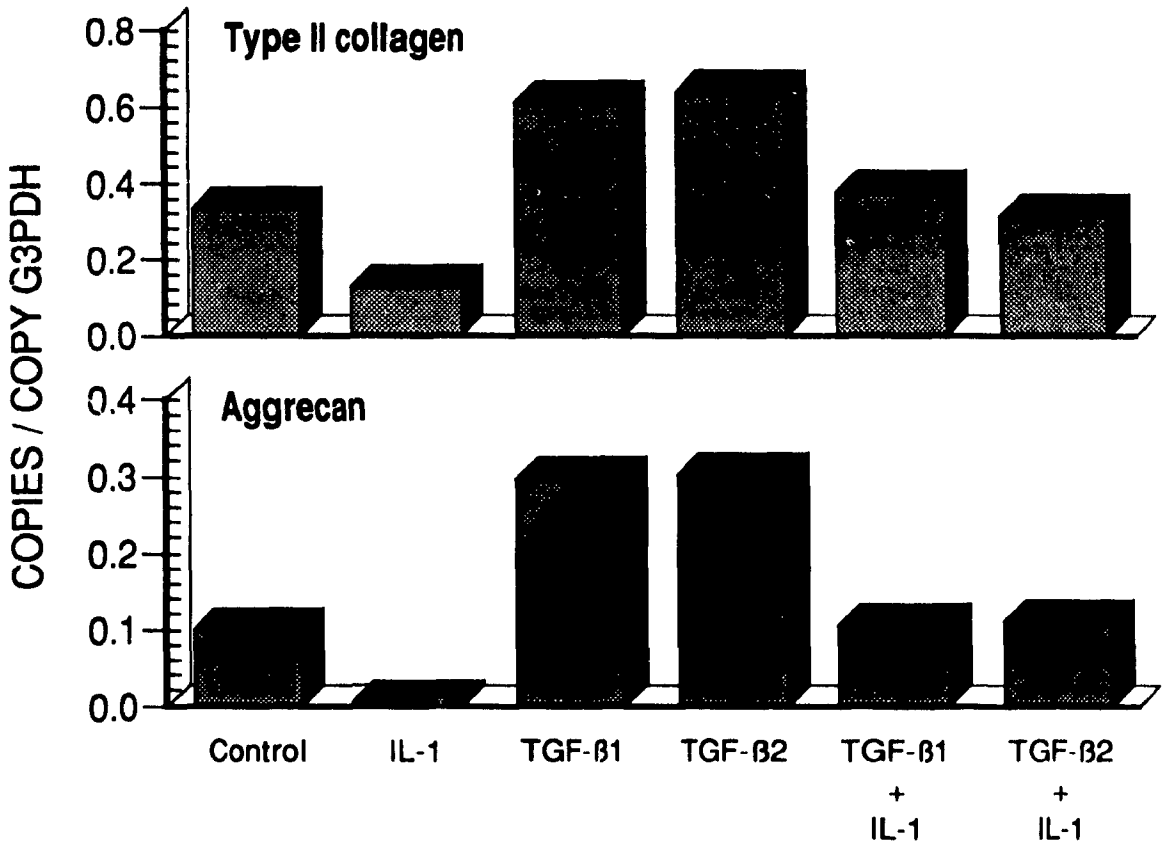


Figure 8. mRNA Levels for Type II Collagen and Aggrecan in Human Chondrocytes Treated with IL-1β and/or TGF-β1 or TGF-β2. The total RNA prepared from human chondrocyte cultures treated with IL-1βs (2 units/ml), TGF-β1 (2 ng/ml) or TGF-β2 (2 ng/ml) or a combination of IL-1β (2 units/ml) and TGF-βs (2 ng/ml). The mRNA levels for type II collagen, aggrecan and G3PDH were quantitated using competitive PCR with specific primers for each mRNA as described in Materials and Methods. Each mRNA induction from different cell cultures was calculated and standardized with respect to corresponding G3PDH mRNA level. The results are represented as copies mRNA per 1 copy G3PDH.

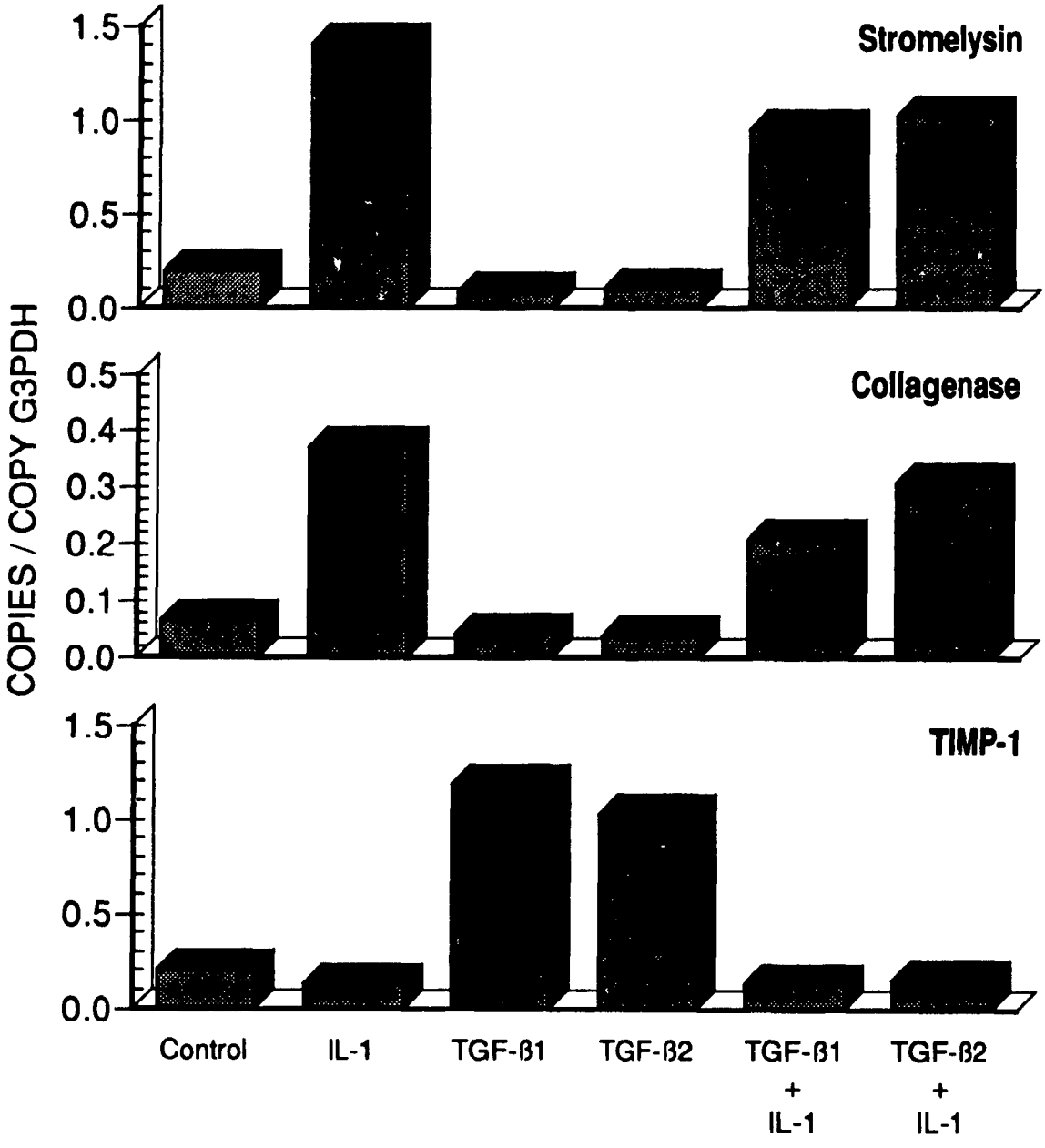


Figure 9. Quantitation of mRNA Levels for Stromelysin, Collagenase, and TIMP-1 in Human Chondrocytes Treated with IL-1β and/or TGF-β1 or TGF-β2. The total RNA prepared from human chondrocyte cultures treated with IL-1β (2 units/ml), TGF-β1 (2 ng/ml) or TGF-β2 (2 ng/ml) or a combination of IL-1β (2 units/ml) and TGF-β (2 ng/ml). The mRNA levels for stromelysin, collagenase and G3PDH were quantitated using competitive PCR with specific primers for each mRNA as described in Materials and Methods. Each mRNA induction from different cell cultures was calculated and standardized with respect to the corresponding G3PDH mRNA level. The results are represented as copies mRNA per 1 copy G3PDH.

opposite fashion by IL-1 and TGF-β.

An analysis of mRNA levels reveals that the stimulatory or inhibitory effects of IL-1β could be reversed by addition of either TGF-β1 or TGF-β2, but again there were markedly quantitative differences in the degree of the rescueing action with respect to each of the components investigated. In addition, the stimulatory effects of either TGF-β1 and β2 on mRNA expression for type II collagen and aggrecan were more effective than their inhibitory effect on collagenase and stromelysin expression when combined with IL-1β. The observation that the expression of aggrecan mRNA, which was essentially inhibited by IL-1β, was effectively restored to control levels by TGF–β, indicates that this gene is very sensitive to regulation by both agents. However, during a 24-hour incubation period TGF-β at the concentration used did not reduce mRNA levels of either metalloproteinase to control levels in the presence of IL-1. Moreover, TIMP-1 mRNA levels, which were obviously depressed by IL-1β were not effectively restored to control levels by TGF-β at a concentration of 2 ng/ml, although TGF-β alone, at the same concentration, had a very pronounced effect on the expression of this mRNA.

4.4 The Dose-Effect of TGF-β on IL-1 Modulation of Aggrecan, Stromelysin and TIMP-1 mRNA Expression

To address the question whether the differences in the action of TGF-β with respect to the structural components and TIMP-1, in the presence of IL-1, is due to the differences in the sensitivity of the regulatory mechanism., additional experiments were carried out using adult human chondrocytes to investigate the dose-response of this aspect. The cells were incubated with TGF-β1(2 ng/ml) or IL-1β (2 units/ml), and combinations of IL-1β (2 units/ml) with TGF-β1 at various concentrations ranging from 2 to 20 ng/ml for 24 hours and 48 hours, respectively, The total cellular RNA prepared from these cell cultures was

quantitatively analyzed for aggrecan, stromelysin, and TIMP-1 mRNA levels by competitive PCR using each specific primer pairs and three orders of dilutions of standard cRNA as described in Materials and Methods.

The results revealed that the steady-state mRNA levels of TIMP-1 and stromelysin, relative to the G3PDH are relatively higher than that of aggrecan, with a relative copy number of 0.7 and 0.6 for stromelysin and TIMP, and 0.2 for aggrecan, respectively. These findings from adult chondrocyte cultures are similar to that relative levels of these three component in neonatal chondrocyte cultures under the same conditions. The respective stimulatory or inhibitory effects of TGF-β or IL-1 had reached a maximum by 24 hours with only slight increases or decreases in mRNA levels at 48 hours. In combination with IL-1, aggrecan mRNA levels increased with increasing concentrations of TGF-β [shown in **Figure 10**]. In contrast, IL-1β -induced stromelysin mRNA expression was effectively reduced with a trend towards control levels only at 10 ng/ml [shown in **Figure 11**]. The patterns of expression between 24–hour and 48–hour incubations were similar for aggrecan mRNA and stromelysin mRNA in response to the dose-effects of TGF-β, suggesting that a new steady state level is established by 24 hours. However, it reveals that the IL-1–reduced TIMP-1 mRNA expression was not effectively restored after a 24–hour incubation period even in the presence of TGF-β at the highest concentration of 20 ng/ml [**Figure 12**]. It is noted that treatment with TGF-β alone at a concentration of 20 ng/ml further increases TIMP-1 mRNA levels. After 48-hour of co-incubation, TIMP-1 mRNA levels were markedly increased relative to the values determined for 24 hours. These observations suggest that the mechanisms which regulate TIMP-1 expression are different to those regulating expression of aggrecan or stromelysin.

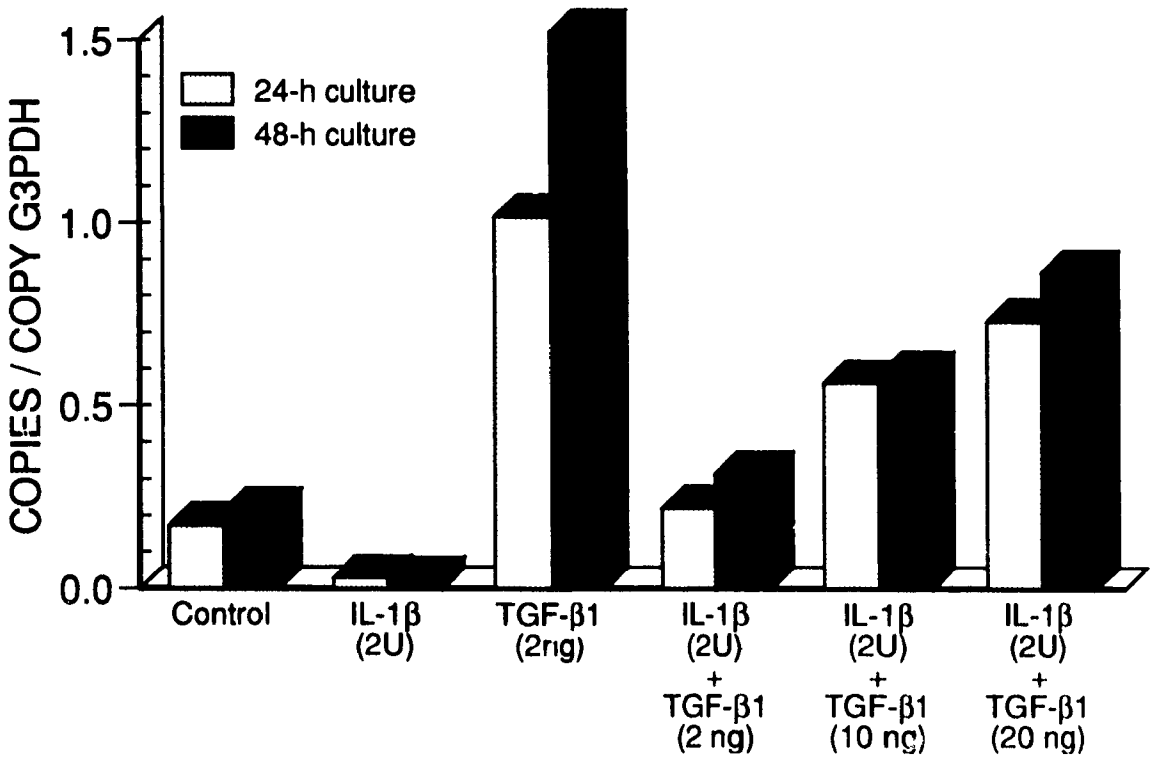


Figure 10. The Dose-Effect of TGF-β1 on IL-1β Modulation of Aggrecan mRNA Expression. The total RNA prepared from adult human chondrocytes cultured with IL-1β (2 units/ml), TGF-β1 (2 ng/ml) or a combination of IL-1β (2 units/ml) and TGF-β1 (2 ng/ml, 10 ng/ml, 20 ng/ml) for 24 h and 48 h, respectively. Aggrecan mRNA transcripts were co-reverse transcribed and co-amplified with three dilutions of aggrecan cRNA by competitive PCR using appropriate primers, and copy numbers of mRNA were calculated as described in Materials and Methods. The copy number was standardized with respect to the corresponding G3PDH mRNA level for each RNA preparation. The results are shown as copies mRNA per 1 copy G3PDH mRNA.

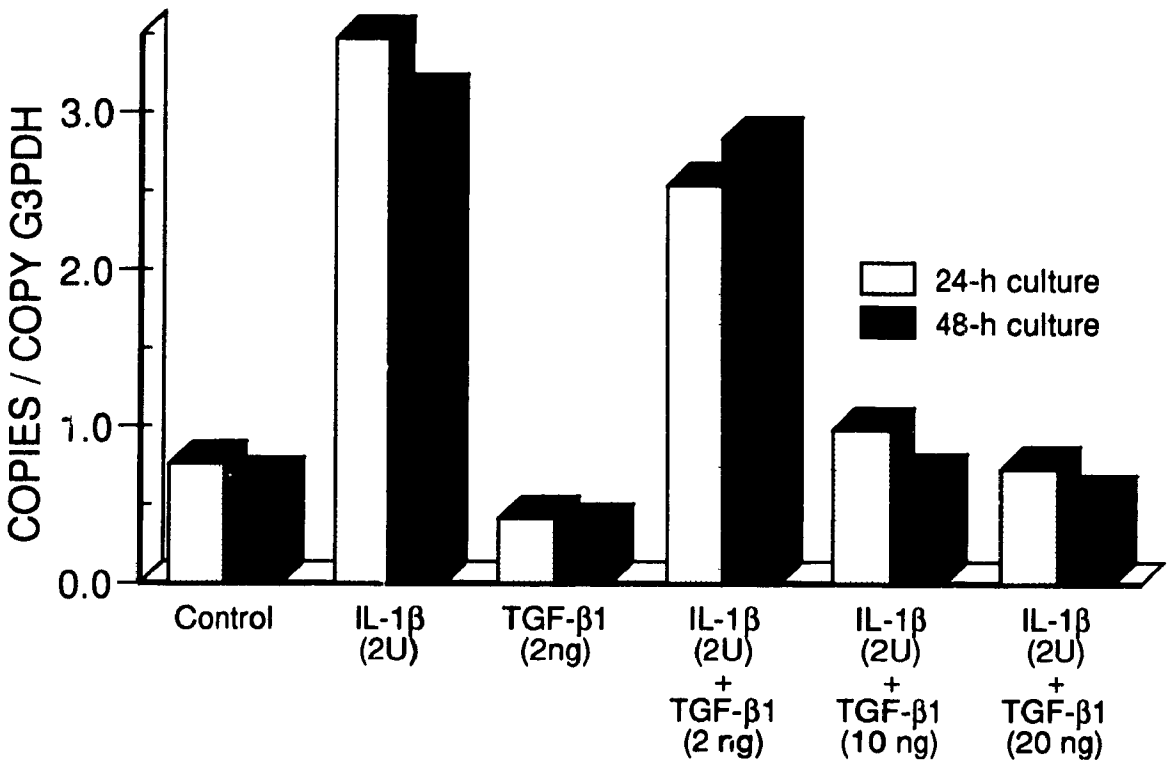


Figure 11. The Dose-Effect of TGF-β1 on IL-1β Modulation of Stromelysin mRNA Expression. The total RNA prepared from adult human chondrocytes cultured with IL-1β (2 units/ml), TGF-β1 (2 ng/ml) or a combination of IL-1β (2 units/ml) and TGF-β1 (2 ng/ml, 10 ng/ml, 20 ng/ml) for 24 h and 48 h, respectively. Stromelysin mRNA transcripts were co-reverse transcribed and co-amplified with three dilutions of stromelysin cRNA by competitive PCR using appropriate primers, and copy numbers of mRNA were calculated as described in Materials and Methods. The copy number was standardized with respect to corresponding G3PDH mRNA level for each RNA preparation. The results are shown as copies mRNA per 1 copy G3PDH mRNA.

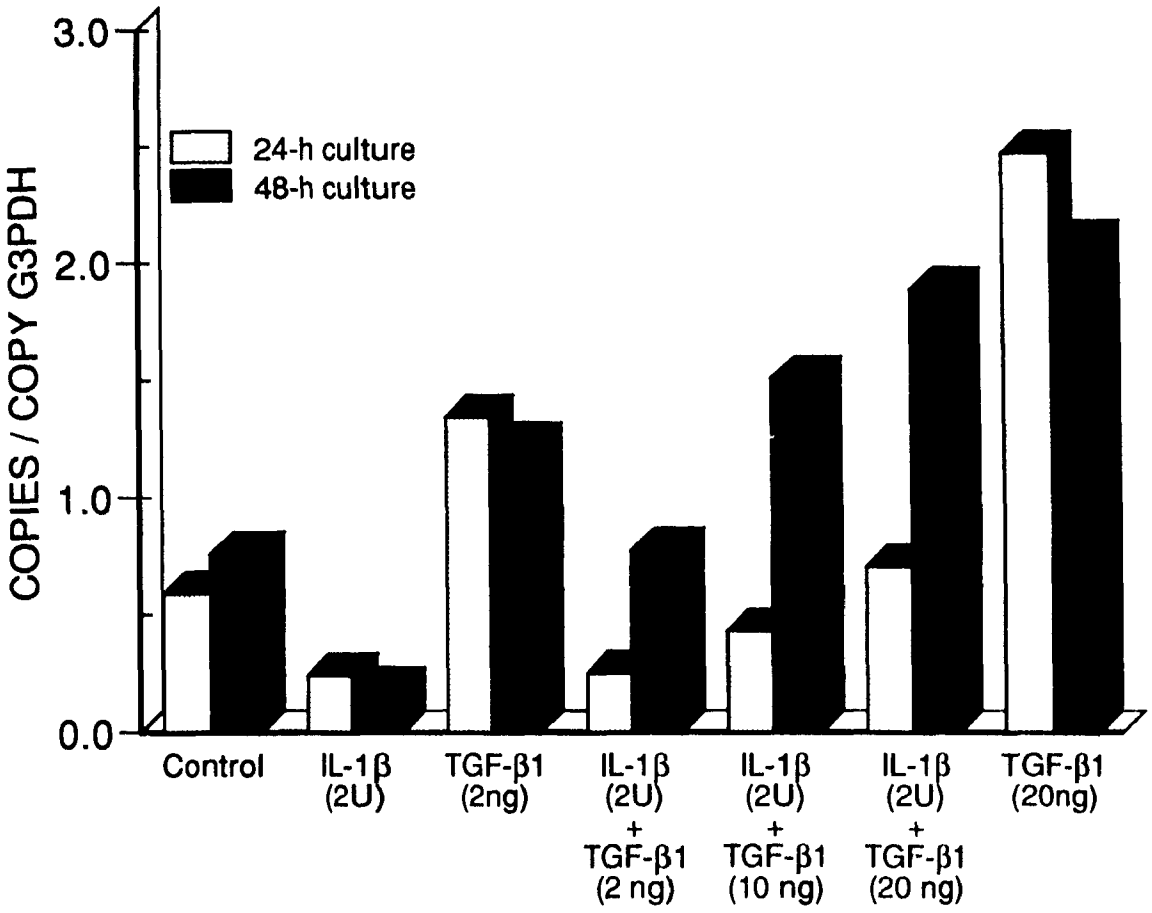


Figure 12. The Dose-Effect of TGF-β1 on IL-1β Modulation of TIMP-1 mRNA Expression. The total RNA prepared from adult human chondrocytes cultured with IL-1β (2 units/ml), TGF-β1 (2 ng/ml, 20 ng/ml) or a combination of IL-1β (2 units/ml) and TGF-β1 (2 ng/ml, 10 ng/ml, 20 ng/ml) for 24 h and 48 h, respectively. TIMP-1 mRNA transcripts were co-reverse transcribed and co-amplified with three dilutions of TIMP-1 cRNA by competitive PCR using appropriate primers, and copy numbers of mRNA were calculated as described in Materials and Methods. The copy number was standardized with respect to corresponding G3PDH mRNA levels for each RNA preparation. The results are shown as copies mRNA per 1 copy G3PDH mRNA.

4.5 Quantitative Analysis of Expression of the mRNA induced by IGF-1 and/or IL-1β

IGF-1 is a growth factor with potent anabolic effects on chondrocytes, and, similar to TGF-β, has been shown to stimulate the biosynthesis of cartilage matrix components. It is not yet clear whether IGF-1 directly antagonizes the inhibitory effects of cytokines in human chondrocytes at the level of mRNA production. Since IGF-1 is potent stimulator of RNA synthesis, it may stimulate not only the synthesis of structural components, but that of degradative components. In this study early passage level neonatal human chondrocytes were cultured in the presence of IGF-1 (100 ng/ml) or IGF-1 (100 ng/ml) plus IL-1β (2 units/ml) for 24 hours. Expression of mRNA levels for type II collagen, aggrecan, stromelysin, collagenase, and TIMP–1 were determined by competitive PCR as above, where the copy numbers of G3PDH/ng RNA were similar for control, IL-1 and IGF treated cells. Thus IGF-1 does not appear to greatly effect the level of expression of this house-keeping gene. The results of this analysis are shown in **Figure 13.** It reveals that mRNA levels for type II collagen and aggrecan were enhanced, with a 1.5- to 2-fold increase after IGF-1 stimulation. Even in combination with IL-1β the levels of both RNAs still remained elevated. IGF-1 also increased the mRNA levels for both stromelysin and collagenase. In the presence of IL-1 a further increase was observed, although this was not additive. The TIMP-1 mRNA levels were significantly induced after treatment with IGF-1, respectively, in a 4-fold increase over control, in the presence or absence of IL-1 in this 24–hour cell culture. Thus, at the concentrations used, the regulation of mRNA levels by IGF-1 appears to be dominant when compared to IL-1β, such that the negative effects of interleukin-1 on matrix component and TIMP-1 expression are completely overcome.

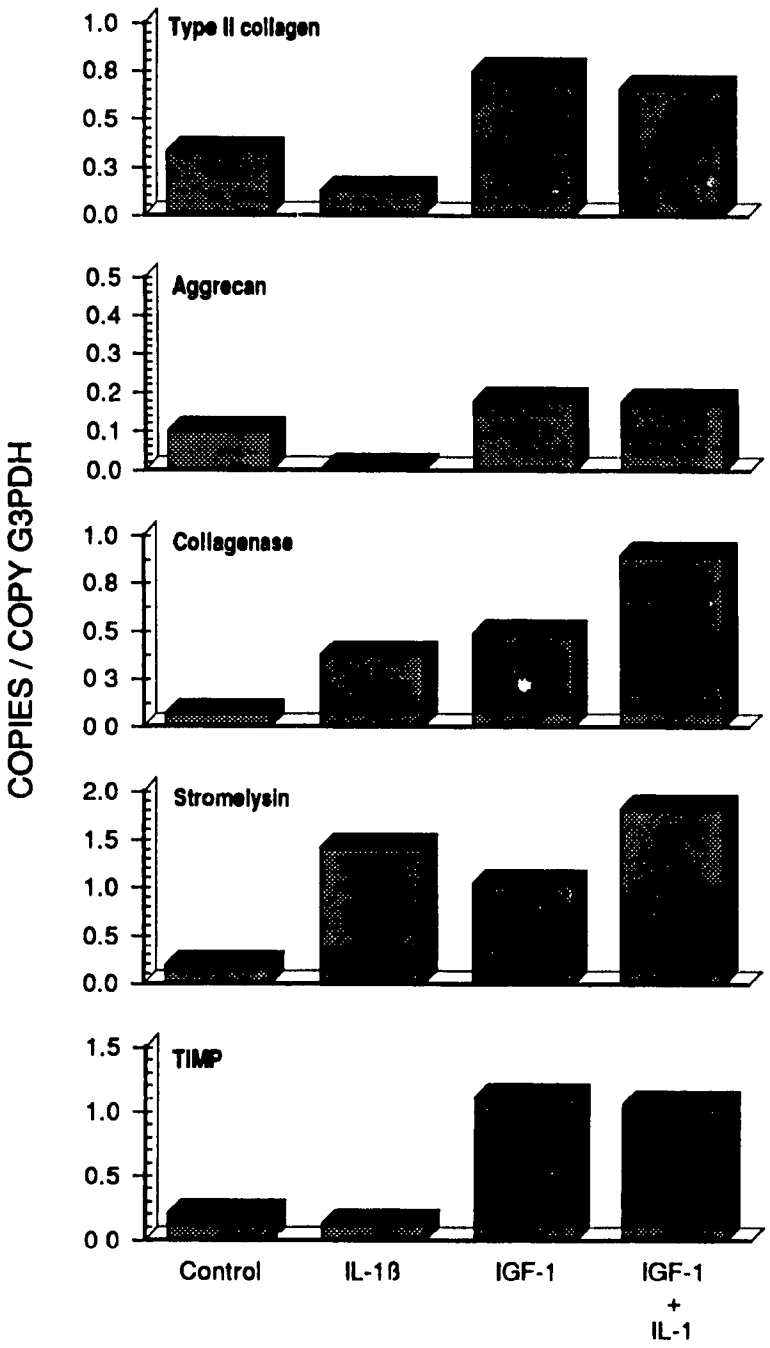


Figure 13. Quantitative Analysis of the Effects of IGF-1 Alone or Plus IL-1 on mRNA Expression. The total RNA prepared from the neonatal human chondrocytes treated with IGF-1 (100 ng/ml), IL-1ß (2 units/ml) alone or a combination of IGF-1 (100 ng/ml) and IL-1ß (2 units/ml), respectively, for 24h. The mRNA levels for type II collagen, aggrecan, stromelysin, collagenase, and TIMP-1 were quantitated using competitive PCR as described in Materials and Methods. All copy numbers of mRNA were standardized to corresponding G3PDH mRNA levels for each RNA preparation. The results are shown as copies mRNA per 1 copy G3PDH mRNA.

Chapter 5. DISCUSSION

Taking advantage of the competitive PCR technique, we were able to concurrently quantitate the various mRNA species in RNA preparations derived from low numbers of human articular chondrocytes, such as would be expected to be present in adult cartilage samples. To our knowledge this is the first time that the competitive PCR method has been used to characterize the biosynthetic patterns of human chondrocytes for multiple components of cartilage matrix under different experimental conditions. With the sensitivity and rapidity of this methodology, the relative levels of change of mRNA expression among those components under study were analyzed in different cell cultures with respect to responses to stimulation by cytokines and growth factors. Absolute quantitation of mRNA copy numbers allows us to study and compare the responses of multiple components to changes in the extracellular environments. In this system the responses of individual components to IL-1 and growth factors had been established by more conventional methods both at the message and protein levels, but comparision between different components had not been possible previously.

The study clearly demonstrated that TGF-β induces the expression of structural components and TIMP-1 mRNA, in human articular chondrocytes, while depressing the expression of the metalloproteinases stromelysin and collagenase. In contrast, IL-1β affected these mRNA levels in an opposite fashion. These findings generally agree with previously reported observations using conventional methods of analysis, such as Northern blotting, and further support the currently accepted concepts about the key roles these individual cytokines play in the erosion of articular cartilage matrix [178-180]. Quantitative determination, however, revealed that the extent of the response pattern was unique for each component investigated. Aggrecan and

collagenase mRNAs responded more dramatically to external modulation by TGF-β or IL-1β than did those of stromelysin and type II collagen. It has been suggested that cytokines induce proteoglycan depletion in cartilage *in vitro* and *in vivo* by decreasing synthesis in the presence of an unchanged rate of turnover. In a recent report Dingle *et al.* [181] showed that inhibition of proteoglycan synthesis in human articular cartilage is more sensitive to IL-1 by three orders of magnitude compared to its catabolic action, implicating that the cytokine's major role in the development of the characteristic matrix loss in the arthritic joint is to impair the replacement of aggrecan. The observation that mRNA levels for type II collagen are relatively much less effected by IL-1β after such exposure suggests that levels of type II collagen are initially maintained at nearly normal levels. This agrees with the generally accepted temporal pattern of proteoglycan and collagen loss observed from early to late stages of degenerative joint disease [182, 183].

The elevated levels of stromelysin relative to collagenase mRNA in human chondrocytes treated with/without IL-1 is consistent with recent reports demonstrating the relative abundance of stromelysin message compared to collagenase [179]. It has also been shown that stromelysin (protein) concentration in synovial fluids of patients with RA [184] is 20-fold higher than that of collagenase. The reasons for this differential pattern of expression are not clear, but these observations suggest gene specific mechanisms of regulation for these two closely related metalloproteinases. Studies on human fibroblasts suggests that collagenase and stromelysin may be regulated independently [185, 186]. It is possible that there may be differences in half-life of the mRNA molecule or the transcription of the gene may remain active for a longer time [184].

It is, therefore, postulated that different patterns in gene expression observed after TGF-β or IL-1 stimulation reflect different regulatory mechanisms for each of these genes in human articular chondrocyte. While IL-1 and TGF-β

responsive elements have been described in the promoter regions of a number of genes [187], additional regulation could occur at the level of message stability as well as, at an as yet uncharacterized additional regulatory elements of the gene.

Results from the study on interaction between TGF-β and IL-1 were also consistent with those which had been previously documented, showing that TGF-β is able to inhibit the action of IL-1 in cultured chondrocytes or in cartilage explants [143, 180]. But, again, quantitative analysis clearly indicated that the interactive effects of growth factors and cytokines can vary in magnitude for different targets. It was believed that the inhibitory effect of TGF-β was a direct consequence of a reduction in the steady-state mRNA levels for stromelysin and collagenase, with a corresponding reduction in the synthesis of the protein. Recent reports [180, 188] have shown that TGF-β-treated chondrocytes display a reduction in IL-1β receptor number with no apparent change in affinity, suggesting that TGF-β may inhibit IL-1β responsiveness by down-regulating IL-1β receptors on chondrocytes and thus may exert a regulatory influence on cartilage matrix degradative mechanisms.

The MMPs, and the balance between levels for these enzymes and their inhibitor, TIMP, are thought to be important in destruction of articular cartilage as seen in RA and OA [184, 189]. In a number of recent studies *in situ* hybridization demonstrated that the level of TIMP gene expression was decreased compared with metalloproteinase levels in highly inflammatory or highly severe arthritis [190, 191], suggesting that this imbalanced expression is critical for destruction of articular cartilage. In this study the observation that IL-1 induced collagenase and stromelysin mRNA expression in parallel with a decline in TIMP-1 mRNA levels support the importance of IL-1 in the etiology of cartilage degradation. The findings are correlated to those of a recent study [191], at the translation level, which showed that IL-1 increased collagenase and stromelysin protein production in a dose dependent manner and at the same

time decreases TIMP production, suggesting that IL-1 inversely coregulates these proteins. Thus, imbalanced mRNA expression between the MMPs and TIMP may define a pivotal role for IL-1 in the pathogenesis of arthritis.

Furthermore, the observation of different time course- and dose-effect of TGF-β on IL-1 modulation on TIMP-1 mRNA expression in this study reveals that the mechanism which regulates TIMP-1 mRNA expression is different to those regulating expression of aggrecan and stromelysin. This implies that a lack of effect of TGF-β on counteracting the depression of TIMP gene expression by IL-1 and the only partial reduction of metalloproteinase mRNA levels could still result in a net increase in the degradative capacity of these cells, which will eventually lead to cartilage destruction. The dose-response curve shows that the final outcome in terms of net matrix accumulation or loss may depend on the relative levels of cytokines and growth factors.

In this study it was shown that IGF-1 had general effects on increasing the mRNA levels for all those components under study. In particular IGF-1 effectively induced TIMP-1 mRNA levels even in the presence of IL-1. Compared to the responses to TGF-β in the presence of IL-1 with respect to aggrecan or metalloproteinase mRNA levels, the results would suggest a different mechanism of action of IGF-1. It may indicate the important role of IGF-1 in the regulation of biosynthesis of the chondrocytes to maintain the dynamic balance between synthesis and degradation of cartilage matrix components.

Chapter 6. CONCLUSIONS

Application of competitive PCR enables us to concurrently quantitate multiple mRNA species in a limited amount of the cells or tissues. Thus, the relative levels of multiple mRNAs manifest quantitatively the biosynthetic activity of the cells as well as the responsiveness of the cells with respect to environmental stimuli. The individual and interactive effects of cytokines and growth factors on biosynthetic patterns of chondrocytes are precisely studied. This methodology, therefore, offers an useful approach to the investigation of the regulation of biosynthesis of articular cartilage.

Significances of this study for future investigation:

1. The quantitation of mRNA species for various components can be expanded to the study of intact articular cartilage under physiologic and pathologic conditions

Future questions to be addressed are: How are these genes express *in vivo* in normal and degenerate cartilage ? Do human chondrocytes in degenerated tissues respond to cytokines and growth factors similarly to those in healthy tissue ? In addition, the quantitative analysis would provide us with possibility to determine whether of not a repair response is evident in specific areas of articular cartilage, if the analysis of mRNA level were carried out in a site-specific manner. A better understanding of modulation of cytokine and growth factors on biosynthetic patterns of cartilage matrix components involved in the deposition and maintenance of a functional cartilage matrix, offers us an approach to design therapies for the reduction of pathological cartilage destruction and the enhancement of repair of this tissues.

2. Investigation of the relationship between mRNA levels and biosynthesis of the protein product in human articular chondrocytes under normal and pathological conditions.

We can evaluate the regulation of biosynthesis of human chondrocytes for those components at the transcriptional and translational levels to obtain a better understanding the regulatory mechanism for cartilage matrix synthesis by these cytokines and growth factors. Analysis of rates of biosynthesis at the protein level would require antibodies specific for each of the components studied.

3. Potential clinical applications of competitive PCR in arthritis

The sensitivity of mRNA quantitation by competitive PCR, allowing analysis of small tissue samples, together with an understanding of the normal site-specific expression pattern of various cartilage components could be applied in clinical diagnosis and response to therapy.

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