BIOSYNTHETIC RESPONSE OF YOUNG AND ADULT HUMAN ARTICULAR CARTILAGE TO GROWTH FACTORS

BY

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ABSTRACT

Adult articular cartilage of many species including humans has a limited capacity for repair following injury. The hypothesis that this might be related to a lack of responsiveness to growth factors involved in growth was investigated. Cartilage explants from 4 child and 5 adult human donors were cultured in the presence of various growth factors. Incorporation of $^{35}SO_4$ into proteoglycans and ^{3}H-thymidine into deoxyribonucleic acid used as measures of the biosynthetic response of cartilage.

Young cartilage showed the ability to behave in an autocrine or paracrine manner to stimulate its basal biosynthetic rate. Immature chondrocytes respond well to somatomedin C (insulin-like growth factor, IGF-I) and insulin but there was no significant stimulation in old cartilage. This data suggests a specific loss of responsiveness to IGF-I in mature cartilage. However, adult cartilage was stimulated by IGF-I and insulin after prolonged incubation times. Adult cartilage could also be stimulated by the addition of fetal calf serum and to some degree by platelet derived growth factor, indicating that adult chondrocytes still have the capacity to respond to external stimuli. The relevance of these results is discussed.

RESUME

Le cartilage articulaire adulte de nombre d'espèces incluant l'homme a, à la suite de blessures, une capacité de réparation limitée. Nous avons investigué l'hypothèse voulant que ce phénomène soit relié à un manque de réponse aux facteurs de croissance. Des explants de cartilage de quatre enfants et de cinq adultes ont été mis en culture en présence de divers facteurs de croissance. On a utilisé l'incorporation de $^{35}SO_4$ aux protéoglycanes et de ^{3}H-thymidine à l'acide deoxyribonucleique comme mesures de réponse biosynthétique du cartilage.

Le jeune cartilage présente la capacité de se comporter d'une manière autocrine ou paracrine pour stimuler son taux biosynthétique basal. Les jeunes chondrocytes répondent bien à somatomedin C ("insulin-like growth factor", IGF-I) et à l'insuline mais on n'observé aucune stimulation significative du vieux cartilage. Ces données suggèrent une perte spécifique de réponse à l'IGF-I dans le cartilage adulte. Cependant, après des temps d'incubation prolongés, celui-ci est stimulé par l'IGF-I et par l'insuline. Le cartilage peut également être stimuli par addition de serum bovin fetal et jusqu'à un certain point par facteur de croissance derivé des plaques (PDGF) ce qui indique que les chondrocytes adultes ont encore la capacité de répondre à des stimulis externes. La pertinence de ces résultats est discutée.

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1.0 INTRODUCTION

Cartilage is a highly specialized form of connective tissue consisting of chondrocytes and extracellular fibers embedded in a gel like matrix. Hyaline cartilage is the most common and most characteristic type and in childhood is found at the epiphyseal plates. In the adult the major location of hyaline cartilage is the lining of the articular surface of nearly all synovial joints. Adult cartilage is an "isolated" tissue in that it is aneural, alymphatic [1] and has no direct contact with the vascular system [2,3]. The chondrocytes derive their nutrition by diffusion of nutrients from the synovial fluid, thus in mature adults all nutrients must diffuse out of the synovial vascular plexus, traverse the synovial membrane to enter the synovial fluid, and pass through the dense hyaline matrix of the cartilage to reach the chondrocyte [2,4]. In immature animals this system is modified in that the basal layer may receive some nutrients by diffusion from the vascular tree of the underlying bone [5].

The articular cartilage matrix is synthesized by the chondrocyte and is composed mainly of water (65-80%). The other two major components of the extracellular matrix are collagen making up 15-25% of the wet weight and the proteoglycans constituting 10% of the wet weight [1]. Other matrix proteins account for an insignificant portion (1%). The extracellular components largely predominate over the chondrocytes and cartilage probably contains the largest amount of extracellular matrix produced per cell within the body.

Under the light microscope, the most obvious histological observation is that cell density decreases with increasing distance from the surface in adult human articular cartilage [6] and as a consequence the extracellular

matrix between neighbouring cells is more abundant. Chondrocytes are sparsely but homogeneously distributed throughout the matrix, appear small with crenated pyknotic nuclei and lie in lacunae. These characteristics have sugggested to investigators that adult chondrocytes are exhausted and for the most part inactive in any type of metabolism [7a]. It has more recently been demonstrated in rat articular cartilage that inadequate fixation leads to poor preservation of cellular detail [7b] and thus the morphologically degenerate appearance of adult articular cartilage may be incorrect.

Biochemical and morphological investigations have demonstrated that adult articular cartilage is in fact a heterogeneous tissue in that it is not uniform in its function, organization and composition [1,8-11]. Electron microscopy studies have revealed that adult articular cartilage is divided histologically into four zones; the superficial or tangential zone, middle or transitional zone, deep or radial zone, and the calcified zone [12]. The zones differ according to arrangement and diameter of collagen fibers [8], organizations and content of proteoglycan molecules [9, 10] and ultrastructural features of the chondrocytes [8].

The superficial zone is composed of tangentially oriented bundles of closely packed collagen fibers 320 Angstroms (Å) in diameter [8]. Metachromatic dye staining is reduced in this zone owing to the fact that it contains little negatively charged glycosaminoglycans [12,13]. This superficial zone also contains a special class of proteoglycan molecules, the dermatan sulfate proteoglycans [14]. The chondrocytes appear flattened and elongated resembling rather the morphology of fibroblasts [8]. The cytoplasmic volume is small containing a poorly developed granular

endoplasmic reticulum, a small Golgi complex and few mitochondria [8]. Signs of cell degeneration (myelin bodies, discontinuity of nuclear and plasma membrane, swollen mitochondria, and fragmentation of organelles) were occasionally seen but no more frequently than in deeper zones [8]. The characteristics of the chondrocytes of the superficial zone are not those of degenerating cells but rather those found in dormant or quiescent cell populations in which protein synthesis is reduced and metabolic activity is at a low level.

In the middle zone, collagen fiber bundles lose their tangential orientation and are replaced by randomly oriented widely spaced individual fibers with fibril diameter up to 600Å. At this level there exists more matrix between the collagen fibrils. In this region glycosaminoglycan content is the highest [10]. The concentration of chondroitin sulfate is highest in this region whereas keratan sulfate concentration increases with increasing depth from the articular surface [10, 13, 15, 16]. The chondrocytes from this middle zone are rounder, larger and surrounded by more matrix. These cells have an abundant cytoplasm containing an extensively developed endoplasmic reticulum heavily studded with ribosomes, a well developed Golgi complex and numerous mitochondria [8]. Numerous secretory vacuoles and lysosomal bodies are found in the cytoplasm. These morphological features are those of cells actively engaged in protein synthesis [8,17].

Collagen fibers of the deep zone have the largest variation in diameter (400Å to 800Å) but otherwise are arranged similarly to those of the middle zone [8]. Proteoglycans, link protein, hyaluronic acid and collagen in the deep zone are organized into a highly ordered lattice network not found in

other zones [9]. Chondrocytes in this zone have abundant granular endoplasmic reticulum, large Golgi apparatus and mitochondria, but these organelles are less extensive than those in cells of the middle zone. Occasionally degenerative cells are found [8]. Therefore, it appears the chondrocytes of the deep zone are also actively involved in protein synthesis.

In immature rabbit articular cartilage, there are two distinct anatomic zones based on the presence of chondrocyte replication [18,19]. In the superficial zone, five to twelve cells in depth lie paralliel to the gliding surface of the joint. The cells are ovoid with pale nuclei [19]. This zone is probably responsible for the gradual enlargement of the cartilage mass associated with skeletal growth [3, 18, 19].

The deeper zone is at a considerable depth from the gliding surface and approximately ten to fifteen cells in width. It is adjacent to the ossific epiphyseal nuclei but separated from it by a layer five to fifteen cells thick. The cells are spindle or ovoid with a non vacuolated but slighty granular cytoplasm and the nuclei are small and dark [9]. This zone most probably contributes to the growth of the bony nucleus of the underlying epiphysis [3, 18, 19].

As previously mentioned, the two major components of cartilage matrix are collagen and the proteoglycans. Ninety five percent of cartilage collagen is of the type II variety, consisting of 3 identical $[\alpha_1(II)]_3$ type II α chains. This type of collagen is heavily glycosylated with a high content of hydroxylysine [20]. Collagens type IX and XII have also been identified. Proteoglycan in cartilage consists of a core protein about 300 nm in length [9]. Radiating from the core protein are carbohydrates called

glycosaminoglycans. The principal glycosaminoglycans are chondroitin sulfate and keratan sulfate there being about 80 and 100 side chains respectively associated with each core protein molecule. At the N-terminal end of the core protein is a polypeptide segment relatively free of glycosaminoglycan side chains. This region binds to a very long hyaluronic acid molecule and is stabilized by link protein. The proteoglycans in adult cartilage matrix either aggregate with hyaluronic acid in situ or bind directly to collagen fibrils [1].

The molecular organization of the extracellular matrix is ideally suited to its function as the weight bearer of articular surfaces on bone. The collagen fibrils are embedded in a gel of proteoglycan and water and it is the properties and interaction of these molecules that permits load-bearing. The high negative charge created by the ionization of the sulfated and carboxyl groups of the glycosaminoglycan permits the attraction of a tremendous amount of water around the proteoglycan molecule. This property endows the tissue with a rigidity and an organization which can resist compression and shearing forces. As a load is applied, the cartilage deforms instantaneously, water is displaced and starts to leave the tissue. With the increased concentration of proteoglycan, swelling pressure is increased, balancing the applied force. When the load is taken off, the water can reenter into the cartilage until the original unloaded equilibrium is restored [1]. The rigid fibrillar scaffolding of collagen determines and maintains tissue shape restricting physically the swelling of the proteoglycan molecule and establishing an equilibrium between the swelling pressure of the proteoglycans and the tensile forces in the collagen. The tensile stiffness of cartilage is directly related to the collagen content.

When the articular surface is damaged, this stiffness is lost. The compressive stiffness of cartilage that is its resistance to deformation is due directly to the content of hydrated proteoglycan. Damage to the proteoglycan molecule probably results in increased traumatic insult to the more rigid collagen network [1].

During the normal aging phenomenon, macromolecules in cartilage matrix are gradually damaged and lose their essential function. Studies of proteoglycans reveals with increasing age, a decrease in size, increase in protein relative to glycosaminoglycan and an increase in proportion of proteoglycans of lower buoyant density [10, 21]. As well extracted link proteins are smaller in size [1]. This represents an increased accumulation within the matrix of damaged, less charged proteoglycan and damaged link protein suggesting increased proteoglycan degradation. There is a resultant aggregate instability predisposing towards degeneration. Collagen exhibits an increase in non-reducible cross links making the fibrils more brittle and predisposing the matrix to traumatic damage [1]. Because of the reduction in cellularity of cartilage with increasing age, the chondrocytes cannot handle the demands for maintainance of more matrix thus favoring net catabolic change.

Most cases of osteoarthritis result from a natural degenerative aging process. In early osteoarthritis the initial loss of proteoglycans is compensated for by an increased biosynthesis of proteoglycan relatively deficient in keratan sulfate [1]. Proteoglycans synthesized are of the higher buoyant density type and synthesis of fibronectin, collagen types I, II and IV are also found [1]. Water content is also increased in osteoarthritic cartilage. These features are characteristic of fetal and newborn cartilage.

Possibly the chondrocyte reverts to a chondroblastic state and switches synthesis towards that of an immature matrix which lacks the mechanical properties of mature cartilage. At areas where cartilage is superficially fibrillated and fissured as in early osteoarthritis, increases in chondrocyte number arranged in clumps or clones have been observed histologically [1,3]. This feature known as chondrocyte clustering is pathognomomic for osteoarthritis and probably represents an attempt at cellular proliferation. Experimentally producing osteoarthritis by sectioning the anterior cruciate ligament in dog, revealed hypertrophy of endoplasmic reticulum and cytoplasmic organelles of the chondrocytes after only 11 days. However, by 11 months, cellular degeneration was observed [22].

As the severity of osteoarthritis increases, there is a reduced rate of glycosaminoglycan synthesis, a decrease in chondroitin sulfate content accompanied by a relative decrease in chondroitin sulfate synthesis compared with keratan sulfate synthesis. Collagen gets degraded but apparantly not lost from the matrix [3]. Also at this stage reduction of cellularity is even greater than with aging and this favors a reduced matrix turnover as each cell becomes more responsible for more matrix. Consequently, there is an impairment of synthetic capacity. The message is clear, as disease activity increases, the reparative mechanism fails.

However, probably the most important process in the evolution of the osteoarthritic process is the degradation of cartilage collagen and proteoglycan by collagenase and neutral metalloproteinases. These enzymes are produced by the chondrocytes in response to stimulation by interleukin I, a mediator of the inflammatory response. Interleukin I can also inhibit the biosynthesis of matrix macromolecules thereby preventing a reparative

response [1]. What initiates interleukin I secretion is still not well understood. Therefore an important area when one discusses the eventual "healing" of osteoarthritic or traumatic cartilage which will not be mentioned in this study, are methods in which this enzyme degradation may be arrested.

The typical response to injury in vascularized mammalian tissue occurs in three general phases: necrosis, inflammation, and repair. However, because of the avascular status of cartilage response to trauma is likely to vary.

Depending on the extent and type of trauma, cartilage cells at the site of injury die and the matrix is disrupted, but since chondrocytes are relatively insensitive to hypoxia, there is probably less cell death than one might see in other tissues. The second phase, inflammation which is mediated by the vascular system and is responsible for transudation, exudation and production of fibrin to serve as the scaffolding for growth of repair tissue is entirely absent in cartilage. Since the inflammatory phase is absent and there is no growth of newly formed blood vessels, the inflammatory cells which accompany them are not present. Undifferentiated multipotential cells which could become fibroblasts or chondroblasts are not available and, as a consequence, there is a limitation on the number of cells that are available to respond to the trauma. This limitation puts a heavy burden on the existing chondrocyte [23]. Although, as will be discussed in the following section, these chondrocytes are capable of active synthesis of DNA and increased matrix synthetic activity, this task is an enormous one for a small number of cells with limited potential for metabolic activity. It is therefore not a surprise that numerous publications

have shown limited healing and regenerative powers of articular cartilage [3, 23, 24].

To better understand repair, Mitchell and Sheppard [25] created full thickness defects through femoral condyles into subchondral bone of adult rabbits. This would expose the cartilage to the vasculature of the subchondral bone and elicit a repair response characteristic of that seen in other vascularized tissues. Available to the injured cartilage would be the undifferentiated but pluripotential mesenchymal cells in the endosteum of the subchondral bone and similar cells in the bone marrow, including pericytes associated with blood vessels. Mitchell showed that repair of the full thickness defects occured by ingrowth of granulation tissue from the marrow. The initial fibrous tissue was converted to a hyaline like chondroid tissue which later became fibrocartilage. By one year the cartilage appeared somewhat degenerated and fibrillated. Therefore, despite the initial exposure to marrow elements, articular cartilage repair was not successful.

Bassett in 1962 [26] has shown that pluripotent mesenchymal cells in tissue culture differentiate into different phenotypes depending on physical factors and oxygen tension. Compaction and high O_2 tension caused these primitive cells to form bone. Compaction and low O_2 tension caused the cells to produce cartilage whereas high O_2 tension and a tensile force produced fibrous tissue. Using this principle, Mitchell and Sheppard [27] created intraarticular fractures in adult rabbits and reduced the fractures anatomically and with strong compression. One year later, the repaired cartilage appeared to be hyaline cartilage by light and electron microscopy Fractures reduced inadequately or with no compression healed with

fibrocartilage.

Salter [28] in his now classical article shows quite convincingly that continuous passive motion on full thickness defects in articular cartilage of adolescent and adult rabbits resulted in rapid and complete healing of the articular cartilage. The studies by Mitchell and Salter have shown that under certain conditions mature articular cartilage may undergo a satisfactory repair. However both studies implicate that neochondrogenesis in the healing of these full thickness defects occurred through the differentiation of the pluripotential cells of the subchondral tissue to chondrocytes. The chondrocytes located at the intact edges of the defect exhibited a short burst of mitotic activity which was short lived and essentially did not contribute in the healing process. Partial thickness defects that did not penetrate the subchondral bone, exhibited no evidence of healing even with continuous passive motion [28].

Ghadially [29] also revealed that the creation of partial thickness defects in articular cartilage of young and adult rabbits elicited a short lived reparative response but by two years the defect in the cartilage surface was identical to the appearance immeditely after injury. These studies strongly demonstrate the inability of existing mature chondrocytes to adequately repair structural defects in articular cartilage.

Repair is defined as the ability of a tissue to reproduce or regenerate a morphologically similar or more important functionally active tissue resembling the parent tissue. Repair is a cellular process in the sense that the cells must synthesize the repair material. The recruitment of these cells evolves by cell replication or transformation of existing cells or from cells that have migrated from the edges of the wound or from blood vessels

entering the tissue. Therefore superficial lacerations of articular cartilage rely on the ability of the existing chondrocytes to replicate and increase their synthesis of matrix molecules.

1.1 Metabolic Activity and DNA Synthesis of Articular Cartilage Chondrocytes

Despite the sparse cellularity and inert appearance of the chondrocyte as seen on light microscopy, articular cartilage is a metabolically active tissue [3, 11, 21, 23]. The chondrocyte may under favorable circumstances utilize the aerobic degradative pathway, but the majority of glucose breakdown occurs by the anerobic pathway [30,31]. As discussed in the previous section, electron microscopy studies have suggested chondrocytes in the middle and deep zone are actively engaged in the synthesis of protein and other components of the matrix.

The first biochemical evidence that adult chondrocytes were synthetically active was demonstrated nearly 2 decades ago by Collins and McElligott [12, 32]. Studies using labeled sulphate have shown this radioisotope to be a specific metabolic tracer for the proteoglycan molecules [12,32-38], and most investigators agree that the rate of incorporation of $^{35}SO_4$ is an ideal indicator of the rate of proteoglycan synthesis by chondrocytes. Metabolic studies using $^{35}SO_4$ and other radioisotopes have shown that chondrocytes synthesize proteoglycans and collagen, assemble them intracellulary and extrude them into the surrounding matrix [2,21,39,40]. Glycosylation and sulfation of the proteoglycan monomer occur principally in the Golgi appartas [41,42] wheras hydroxylation of proline and lysine as well as glycosylation of hydroxylysine of the collagen molecule occurs in the rough endoplasmic

reticulum [37,41].

The synthetic activity of adult cartilage is almost entirely devoted to the renewal of the extracellular matrix and experimental work has shown turnover rates of the proteoglycan molecules to range from 3.5 - 20 days [22,38,43,44]. A possible explanation for these observations is that the proteoglycan pool is not metabolically homogenous but may in fact represent multiple pools with various turnover rates [41]. At any rate, the point is that the adult chondrocyte is constantly synthesizing components of its extracellular matrix and is thus far from being metabolically inert.

The collagen component of cartilage is considered stable, although there is some slight turnover and renewal of this molecule in normal adult articular cartilage [45, 46]. As we will be discussing later, not only does the articular chondrocyte constantly renew its matrix components, but it can also alter significantly its rate of synthesis in response to numerous endogenous and exogenous factors.

It has been well demonstrated by a large number of investigators that thymidine uptake by a cell indicates an imminent division and therefore serves as a highly specific quantitative index of cellular reproduction [47-49]. Despite countless studies, mitotic figures have never been seen in normal articular cartilage of adult animals [20,50-53] or humans [36,54,55]. However, chondrocytes from immature cartilage are capable of undergoing mitosis [19,20, 23,51]. The question therefore becomes obvious. Does the mature chondrocyte only "turn off the switch" for DNA synthesis or irreversible "break the switch"? There is now ample evidence that under certain circumstances, the mature articular chondrocyte can reinitiate DNA synthesis and cell division. ^{3}H-thymidine metabolic studies,

autoradiography and histological demonstration of mitotic figures from joints with osteoarthritis and lacerations have demonstrated evidence for DNA synthesis in animals [3,52,53,56] and in humans [12,23,24,36,54,55,57, 58]. However, even though this process of DNA synthesis eventually fails with increasing disease activity [3, 24], the fact remains that the mature chondrocyte can divide.

Inherent also to the discussion of repair is whether the chondrocyte synthetic apparatus has the capacity to vary the rate at which matrix components are synthesized, that is can the cartilage cell alter its rate of response to various stimuli to produce the increased quantities of matrix that are necessary for repair. Data obtained from a number of investigators have suggested that numerous endogenous and exogenous factors may modify significantly the rate of synthesis of matrix components by the articular chondrocyte [23,41]. Early osteoarthritis most definitely stimulates the cartilage cell to produce more proteoglycans as measured by increased $^{35}SO_4$ uptake [3,12,24,58,59]. Other diverse physical and pathological states such as altered hydrostatic pressure [60-62], pH changes [63], varied oxygen tension [26,30,64], calcium concentration [65], prostaglandins [66, 67], cortisol [68], salicylates and other nonsteroidal antiinflammatory drugs [69-71], hormones [72,73], polypeptide growth factors [74,75], and factors obtained from serum [75-77], can alter the rate of synthesis of proteoglycans. Therefore it is clear that adult articular chondrocytes can substantially increase their rate of matrix synthesis and the possibility exists for chondrocyte participation in repair.

Of utmost importance when dealing with repair reactions is to gain an understanding of the factors which influence the biosynthetic activity of the cell. Two levels of control are usually operative during tissue growth and differentation. One level is under genomic control that is inherent to that given cell type, the other extrinsic and influenced by growth factors, hormones, and other extracellular factors. Extrinsic factors seem to play a more important role in regulating the metabolic activity of the connective tissue cell in response to injury. It is now becoming clear that many repair reactions, particularly in connective tissues, are intimately associated with a variety of growth factors which may influence cell migration and division as well as their differentiation and synthesis of specific products. The presence of growth factors in platelets and the wound hematoma is thought to facilitate delivery of chemotactic and mitogenic factors to sites of injury where they may play a major role in wound healing [78-79].

Culture of most cell types in vitro requires the presence of serum. Consequently, investigators have spent much effort in a search to identify the various factors in serum that stimulate cell proliferation and differentiation. It is now well established that the addition of specific hormones and growth factors to synthetic media can fulfill the growth requirement of a number of cell lines for serum or plasma [80]. Chondrocyte culture systems are without exception and the effect of serum has dramatic effects on chondrocyte behaviour [75-77,81]. Growth factors present in serum no doubt play an important role in regulating the metabolic parameters of the chondrocyte. The following section will deal specifically with individual growth factors.

Growth factors may be defined as polypeptides that stimulate cell proliferation and differentiation through binding to specific high affinity cell membrane receptors. Growth factors do not usually act in an endocrine manner but rather act in a paracrine or autocrine fashion. Growth factors are found in plasma and a variety of adult and embroyonic tissues. Most cells have receptors for more than one growth factor [82-84]. Unless otherwise mentioned, the receptors of the growth factors we will be discussing operate through the tyrosine kinase pathway [78,82]. Most growth factors act synergistically with one another in that exposure of a cell to one growth factor can lower the threshold of mitogenicity of a second growth factor [78-85]. Besides their presumed involvement in neoplastic transformed cells and repair, growth factors seem to play an important role in fine tuning of proliferative and differentiation rates necessary for co-ordinated growth of cells to form tissues during development and to maintain tissues in the adult state [78].

1.2 Somatomedins/Insulin-Like Growth Factors

Is is now nearly 30 years since Salmon and Daughaday [86] hypothesized that stimulation of cartilage growth by growth hormone was mediated by a circulating "sulfation factor". This growth hormone-dependant plasma factor stimulates in cartilage not only the incorporation of sulphate in proteoglycans, but also the incorporation of thymidine into DNA [87-89], proline into collagen [87,90] and uridine into RNA [87,90]. Because of its importance in growth and its intermediary hormonal relationship with somatotropin (growth hormone), the operational term "sulfation factor" was replaced by a more general term "somatomedin" [91,92]. Metabolically the effects of somatomedins on muscle and adipose

tissue resemble those of insulin [93,94].

Meanwhile other investigators had isolated from serum a polypeptide with insulin-like activity on muscle and adipose tissue not suppressible by antibodies to insulin [95]. This nonsuppressible insulin-like activity (NSILA) from serum also exerts pronounced effects on growth of cultured cells and on sulfation, RNA, and protein synthesis of cartilage [96-99]. For these reasons along with the structural homology with proinsulin and the observation that these polypeptides are mitogenic for other cell types NSILA have been termed insulin-like growth factors [98,100]. With the advent of radioimmunoassays, more sophisticated purification techniques and amino acid sequencing there was no doubt that insulin-like growth factors I and II (IGF-I, IGF-II) corresponded to somatomedin C and somatomedin A respectively [101-104]. Therefore IGF's have both metabolic and growth promoting effects on many cell types including chondrocytes which are similar to those of insulin. IGF's exert their most pronounced effect on growth of cartilage where IGF-I is more potent than IGF-II [98,101,105].

Whereas the literature is abundant with regards to IGF's/Somatomedins stimulating cartilage growth and metabolism in rats [86,89,90], chick [97,106], pig [107] and bovine [75], it is quite scarce with respect to human cartilage. Asthon [108], using plasma as a source of somatomedins demonstrated that human articular cartilage incorporated more sulphate and thymidine when exposed to 10% human plasma with the greatest response being in the 12-17 year age group. IGF-I at 25 ng/ml is a potent stimulator for colony formation in 27 year old human articular cartilage [109].

Interestingly, plasma somatomedin levels do not correlate with cartilage growth activity suggesting young animals are more sensitive to somatomedins than is cartilage from older animals [107, 110] explaining possibly why young children grow more rapidly. It has been suggested this increased responsiveness might be due to a higher receptor affinity or to an increasing number of receptors [87,98, 111,112] for this growth factor. Receptors for IGF have beeen demonstrated in isolated chondrocytes from rat and bovine articular and growth plate cartilage [113-115]. No work has yet been done in detecting presence of receptors in chondrocytes of human articular cartilage.

Up until the early 80's, the somatomedin hypothesis which states that growth hormone stimulates chondrogenesis and subsequent growth indirectly through circulating peptide growth factors such as somatomedins originating in the liver [116] was traditionally accepted. However the theory is largely based on in vitro studies and despite work by Schoenele et al [117] demonstrating that chronic subcutaneous infusion of IGF-I in hypophysectomized rats led to an enlargement of the tibial epiphyseal growth plate, the evidence was still circumstantial. The most recent in vivo investigations have demonstrated that unilateral local injections of growth hormone into the tibia stimulates unilateral tibial epiphyseal growth [118-122] thereby not supporting the theory that increased plasma concentrations of somatomedins are necessary for accelerated longitudinal bone growth after growth hormone administration. The specific binding of growth hormone to isolated chondrocytes [123] further substantiates local and direct action of growth hormone.

However, IGF-I when injected directly into the tibial growth plate also caused an increase in the width of the tibial epiphyseal cartilage [117,120,121]. Immunohistochemical studies [124] have demonstrated that cells in the proliferative zone of the growth plate of normal rats exhibit a bright immunofluorescence with respect to IGF-I immunoreactivity. In hypophysectomized rats, the number of fluorescence cells are markedly reduced but quickly increased when the hypophysectomized animals were treated with growth hormone either systemically or locally. If an antibody to somatomedin C was injected along with growth hormone locally in the proximal tibial growth plate, the direct growth effect of growth hormone was completely abolished [125]. These studies strongly suggest that local production of somatomedins may be more important in promoting in vivo growth of cartilage than previously realized.

In vitro investigations have demonstrated IGF-I/somatomedin C production by cell types other than liver cells and the ability of the somatomedins produced by these cells to act in the microenvironment and promote mitogenesis [126-128] thereby acting in a paracrine or autocrine manner. The recent discovery [129] that growth hormone specifically stimulates the differentiation of cloned preadipose cells and myoblasts have led some to propose a dual effector theory with respect to growth hormone action on longitudinal bone growth [121,124]. The theory states that during the process of cell differentiation stimulated by growth hormone, genes that code for IGF-I are expressed. This gene activation results in an increased local production of IGF-I which promotes the clonal expansion of chondrocytes through paracrine or autocrine mechanisms. The final proof for this hypothesis has to await the positive identification of messenger

RNA for IGF-I in the growth plate. A recent in situ hybridization histochemistry study [130] using human fetal tissue has failed to localize mRNA for IGF-I in chondrocytes in costal cartilage. However mRNA for IGF-I was found in perichondrium and other cells of mesenchymal origin ideally situated to provide a source of IFG's for paracrine action on chondrocytes.

1.3 Platelet Derived Growth Factor (PDGF)

As previously mentioned, whole serum has historically been used to promote and study growth of many cells in culture and much of the mitogenic capacity of serum has been shown to be due to substances released from platelets [131-133]. One of these substances was a cationic polypeptide which induces DNA synthesis in quiescent mouse BALB/c 3T3 cells [134]. This basic polypeptide which is free of insulin-like activity was purified and named Platelet derived growth factor (PDGF) [135,136].

The process by which cells leave the quiescent state (G_0) and enter the cell cycle can be resolved into at least two discrete stages. The first stage, competence, is a prequisite for entry into the growth cycle while the second stage, progression, enables cells to leave the competent phase and progress through G_1 to enter the S phase of the cell cycle [137,138]. Mitogenic agents such as PDGF and fibroblast growth factors are potent inducers of competence [137,138] whereas somatomedins are potent mediators of progression [85].

The concentration of PDGF exerting half-maximal stimulation of DNA synthesis varies widely and this large variation may reflect interaction with other growth factors which may lower the cells threshold of response to PDGF [78]. Addition of small concentrations of PDGF to cultured human

fibroblast stimulated somatomedin production by the cultured cells [126]. Therefore, there certainly exists a synergistic effect between PDGF and somatomedins.

The exact role of PDGF in wound healing, connective tissue growth and development has not yet been precisely evaluated but it most probably has important mediating properties [139]. The fact the PDGF is one of the only growth factors which are chemotactic and usually only released within the microenvironment of traumatized tissue strongly supports its key role in wound repair [140].

There has been only a handful of investigations regarding the effect of PDGF on chondrocytes. Both platelet lysate [141] and purified PDGF [142] stimulated DNA synthesis two to four fold in rabbit articular chondrocytes in monolayer culture. However, there was a mixed picture with respect to stimulating proteoglycan synthsis. Not all experimental groups studied had a stimulatory effect [74] while other investigations noted a decrease in glycosaminoglycan synthesis [141]. Cell cultures of meniscal fibrochondrocytes exposed to PDGF showed a proliferative response [79]. Receptors for PDGF are found on a variety of mesenchymal cells [143] but their presence on chondrocytes has not yet been described.

1.4 Transforming Growth Factor β(TGF-β)

A potentially exciting candidate for control of chondrocyte growth might be the transforming growth factor β (TGF-β) which was first described by its unique ability to induce normal rat kidney fibroblasts to lose contact inhibition and undergo anchorage independent growth [144]. Since then it has been found in many neoplastic and nonneoplastic cell lines [145,146]. It has been purified from bovine kidney and human placenta but

it is human platelets which are a particularly rich source of TGF-β suggesting a possible role in repair [147]. Further evidence of the role of TGF-β in wound repair comes from studies which demonstrated that TGF-β stimulates collagen and fibronectin synthesis by fibroblasts [148, 149] as well as promoting the formation of granulation tissue when injected subcutaneously in mice [150].

TGF-β is considered by some to be the "master" growth factor since it regulates the actions of many other peptide growth factors as well as stimulating or inhibiting cellular proliferation, stimulating or inhibiting cell differentiation and imposing its multifunctional nature on other critical processes in cell function [146,151]. TGF-β receptors do not seem to operate via the tyrosine kinase system [78,82].

Implantation of demineralized bone matrix in a subcutaneous site in rats results in the induction of cartilage and bone. Cartilage inducing factor A (CIF-A) has been isolated from bovine demineralized bone and is thought to be responsible for this induction [152,153]. As TGF-β has been demonstrated to be identical to CIF-A [154,155] it is therefore an interesting candidate for control of chondrocyte growth.

1.5 Fibroblast Growth Factor (FGF)

Fibroblast growth factor (FGF) initially isolated and purified from bovine pituitary [156,157] and later from bovine brain [158] was first described as being a potent mitogenic agent for resting Balb/c 3TC mouse cells [156-158]. Since then FGF has also been shown to stimulate the division of a wide variety of mesoderm derived cells [159,160].

Early studies have demonstrated that FGF improves the proliferation of rabbit ear or articular chondrocytes exposed to serum supplemented

medium and that hydrocortisone potentiates the mitogenic effect of FGF [161,162]. More recent work has confirmed the previous finding that FGF is mitogenic for articular chondrocytes especially more so in the presence of serum [142,163]. However FGF has little effect on stimulation of proteoglycan synthesis by chondrocytes [74]. Receptors for FGF have not yet been clearly identified [78].

1.6 Epidermal Growth Factor (EGF)

Epidermal growth factor (EGF) was first isolated from the submaxillary gland of the adult male mouse [164] and is similar in structure to human urogastrone [165]. and is found in significant concentrations in the serum. EGF has been shown to be a mitogen for epidermal cells [166] as well as fibroblastic cells [159, 160]. As is the case for FGF, EGF is also mitogenic for cultured chondrocytes [142,162,163] and inhibitory for glycosaminoglycan synthesis [74].

1.7 Cartilage Derived Growth Factor (CDGF)

Since growth factors are thought to act in an autocrine or paracrine manner [167] a growth factor which can be isolated from a cartilage source would be ideally suited to have a functional role in chondrocyte behaviour. It has already been mentioned that there is strong evidence that somatomedins act locally [125, 127]. However a low molecular weight cationic polypeptide isolated from bovine scapular cartilage is found to stimulate DNA synthesis and cell division in quiescent mouse 3T3 cells and bovine chondrocytes [168,169]. This cartilage derived growth factor (CDGF) also stimulates endothelial cell proliferation [170] but has no effect on proteoglycan synthesis [168]. Recently it has been demonstrated that CDGF can stimulate formation of granulation tissue in rats in vivo

Table 1

Summary of growth factors, size, receptor activity and effect on chondrocyte metabolism

GROWTH FACTOR	SIZE AMINO ACIDS (aa)	MOLECULAR WEIGHT	RECEPTOR & ACTIVITY	EFFECT ON CHONDROCYTE BIOSYNTHESIS
Insulin like growth factor (IGF-I)	70aa	6000	two α and β chains tyrosine kinase	stimulates cell division stimulates proteoglycan synthesis
Insulin like growth factor (IGF-II)	67aa	6000	one polypeptide chain unknown activity	stimulates cell division stimulates proteoglycan synthesis but less potent than IGF-I
Insulin	51aa	5700	two α and β chains tyrosine kinase	stimulates cell division stimulates proteoglycan synthesis but at pharmacological concentrations
Platelet derived growth factor (PDGF)	Achain B chain 24 aa	31,000	one polypeptide chain tyrosive kinase	stimulates cell division no documented effect on proteoglycan synthesis
Transforming growth factor β (TGF-β)	2 x 112aa	25,000	2 α 280 KDA unknown activity but not via tyrosine kinase	possibly modulates both cell division and proteoglycan synthesis
Fibroblast growth factor (FGF)	146aa	18,000	unknown	stimulates cell division no documented effect on proteoglycan synthesis

Table 1, con't

Summary of growth factors, size, receptor activity and effect on chondrocyte metabolism

GROWTH FACTOR	SIZE AMINO ACIDS (aa)	MOLECULAR WEIGHT	RECEPTOR & ACTIVITY	EFFECT ON CHONDROCYTE BIOSYNTHESIS
Epidermal growth factor (EGF)	53aa	6000	one polypeptide chain tyrosine kinase	stimulates cell division no effect on proteoglycan synthesis
Cartilage derived growth factor (CDGF)	unknown	18,000	unknown	stimulates cell division possibly suppresses proteoglycan synthesis
Cartilage derived factor (CDF)	unknown	10,000	unknown	stimulates cell division stimulates proteoglycan synthesis

[171] and thus possibly may be involved in wound healing. The role of CDGF has not yet been elucidated but some feel that it may be involved in the invasion of capillaries into cartilage during bone formation [169]. Recent evidence suggests that CDGF is in fact identical to basic FGF.

1.8 Cartilage Derived Factor (CDF)

Kato and co-workers extracted a protease sensitive factor from fetal bovine cartilage which stimulated proteoglycan synthesis in rat and rabbit chondrocytes culture as shown by increased incorporation of ^{35}S-sulphate [172]. Later work revealed that this cartilage derived factor (CDF) also enhanced protein, RNA and DNA synthesis in cultured chondrocytes thus showing somatomedin-like activity [173]. CDF is 1000 times more effective than insulin and 1,000,000 times more effective than fetal calf serum in stimulating glycosaminoglycan synthesis [174]. CDGF differs from CDF in that CDGF is capable of stimulating ^{3}H-thymidine incorporation in bovine chondrocytes at much lower concentrations [174]. In addition, CDGF suppresses $^{35}SO_4$ incorporation into cultured chondrocytes whereas CDF stimulates proteoglycan synthesis [174].

1.9 Insulin

In the early 50's it was demonstrated that if hypophysectomized rats were given slow-acting insulin in gradually increasing amounts and a high carbohydrate diet the width of the epiphyseal plate of the rat tibia increased 65% over control [175]. Further investigations revealed that insulin at only pharmacological concentrations was able to stimulate incorporation of ^{35}S-sulphate into proteoglycans and ^{3}H-thymidine into DNA of costal cartilage from hypophysectomized rats [72,86,93]. It is believed that the ability of pharmacological concentrations of insulin to

stimulate various anabolic responses in the chondrocyte is a result of interaction with the somatomedin receptor [98, 113]. However, in the chondrosarcoma chondrocyte of swarm rat, the ability of insulin to stimulate proteoglycan synthesis at physiological concentrations sugggests insulin is acting via a classic insulin receptor rather than as a result of interaction with a somatomedin receptor [176]. In the chondrosacoma model as is the case with other chondrocyte culture systems there is very little mitogenic effect by physiological concentrations of insulin. Using a monolayer culture system for rabbit articular chondrocytes Prins et al were able to show that insulin acted synergistically to varying degrees with PDGF, EGF and FGF in promoting ^{3}H-thymidine uptake in the chondrocytes in 1% fetal calf serum [142], but insulin alone increased proteoglycan production tremendously [74]. Table 1 summarizes the size, receptors and effect on chondrogenesis by the growth factors just mentioned.

Is is well known that once articular cartilage damage occurs be it by immunological, traumatic, or degenerative means adult articular cartilage in many species including humans has very limited capacities to repair itself. Consequently damage at the cartilagenous sites almost always leads to loss of joint function in the adult.

Salter's study [28] showed using a rabbit model, that under the influence of continuous passive motion, articular chondrocytes can be induced to undergo repair and reproduce what appears to be a grossly and histologically functionally active cartilage. But as Salter points out, the integrity of this newly reparative tissue when subjected to normal weight bearing activity for long periods is still unknown. Furthermore, the use of continuous passive motion clinically is reserved mostly for patients having

already undergone total knee replacement.

Many other studies mentioned in the previous paragraphs have exposed articular cartilage of different ages to a variety of external factors to better understand what governs the metabolic activity of the chondrocyte. Results have varied but generally immature cartilage has shown to be the most responsive. Most of the studies were performed on animal articular cartilage and only a few have looked specifically at growth factors.

Since we believe growth factors have a pivotal role in connective tissue repair and since human articular cartilage has hardly been studied, we chose to include both these factors in our present study. The hypothesis that the limited capacity of adult human articular cartilage to repair itself may be related to a lack of responsiveness to growth factors involved in cartilage growth was investigated. Cartilage explants from disease free human donors of various ages were cultured in the presence of various growth factors. Incorporation of $^{35}SO_4$ into proteoglycans and ^{3}H-thymidine into DNA were used as measures of the biosynthetic response of cartilage. The purpose of our study is to establish whether there is a metabolic response of human articular cartilage to certain growth factors and more precisely whether there is an age related difference. If there is a difference in response between young and old human cartilage, this may lead us to look more carefully at receptors on the chondrocytes and decide whether there is a qualitative or quantitative change. Ultimately we hope we will be able to manipulate chondrocytes such that they behave in a repair-oriented manner.

2.0 MATERIALS AND METHODS

2.1 Reagents and Media

Powdered Hank's balanced salt solution (HBSS), powdered Dulbecco-Vogt modified Eagle's medium (DMEM) (4.5 g/l glucose), and penicillin; streptomycin solution (10,000 units and 10,000 ug/ml, respectively) were from Grand Island Biological Company; Bovine serum albumin (RIA grade); gentamycin sulfate (crystallized glucuronolactone) were purchased from Sigma Chemical Company, 4-(2-hydroxyethyl)-1 piperazine-ethane sulfonic acid (HEPES) and dithiothreotol (Cleland's reagent) from Boehringer, and 3,5 diaminobenzoic acid dihydrochloride (DABA) from Aldrich. Carbazole was obtained from BDH chemicals and tricholoracetic acid, percholoric acid, sulfuric acid, sodium borate, sodium sulfate and sodium bicarbonate were from American Chemicals Ltd. ^{3}H-thymidine and ^{35}S-sodium sulphate were purchased as carrier-free solutions from ICN Radiochemicals.

2.2 Growth Factors

Recombinant insulin-like growth factor-I (IGF-I) was purchased from Toyobo Biochemicals, porcine insulin from Sigma. Platelet derived growth factor (PDGF) was purchased from Collaborative Research Incorporation. Fetal bovine serum (FCS) was obtained from Bocknek Laboratories.

2.3 Source of Tissue

Human articular cartilage was dissected at autopsy from the femoral condyles within 20 hours of death. The patients had no prior exposure to chemotherapeutic or immunosuppressive agents for at least three weeks prior to death. Only macroscopically normal cartilage was used, areas of fibrillation were left behind. Dissection of cartilage was performed as aseptically as conditions would permit. Tangential cuts to the femoral

Table 2

Characterization and Cause of Death in Individual Adult and Young Cartilage Samples

Patient	Sex	Age	Cause of Death	Post Mortem (hours)
1	M	59yrs	gastric carcinoma	7
2	M	63yrs	lung carcinoma	9
3	M	57yrs	lung carcinoma	12
4	M	51yrs	lung carcinoma	20
5	M	54yrs	melanoma	17
6	M	33yrs	cardiac surgery	12

Patient	Sex	Age	Cause of Death	Post Mortem (hours)
1	M	12mos	hydrocarbon poisoning	6
2	M	16mos	upper airway obstruction 2° to foreign body	19
3	F	newborn	respiratory distress syndrome	20
4	M	15yrs	pericardial effusion	7

condyles were employed to procure the articular cartilage. In the case of newborn children, complete knee joints were dissected out. Cartilage was transported to the laboratory in Hanks balanced salt solution (HBSS) [177] containing 1.0% penicillin streptomycin solution, 100 mg of crystalline gentamycin sulfate and .35 grams of sodium bicarbonate ($NaHCO_3$) per liter.

Adult autopsy samples were obtained from the Royal Victoria Hospital and the Montreal General Hospital. Child cartilage was procured at autopsies performed at Ste. Justines Hospital. Details of the individual specimens used in this work are listed in table 2 for adult and children.

2.4 Cartilage Culture

The cartilage pieces were chopped up sterily into approximately 2-4 mm^3 cubes. The cartilage slices were kept in HBSS during cutting to prevent them from drying. Fibrous tissue was carefully dissected away from the cartilage slices and HBSS was removed and all cartilage squares were placed in sterile 15mm Lab Teck (R) square plastic petri dishes containing 15 ml of culture medium supplemented with 0.2% bovine serum albumin (BSA). The culture medium consisted of DMEM, containing 25mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES), 1 gram of $NaHCO_3$, 0.5% of penicillin-streptomycin solution, adjusted to ph 7.4 and sterilized by filtration [178,179]. All cartilage explants were preincubated in an open air incubator at 37°C for a minimum of 24 hours.

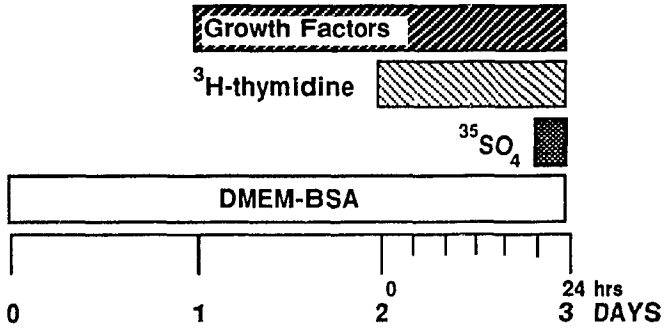
2.5 Experimental Protocol for Biosynthesis Experiments

Three general protocols were used. The major variable was culture time (see Figure 1).

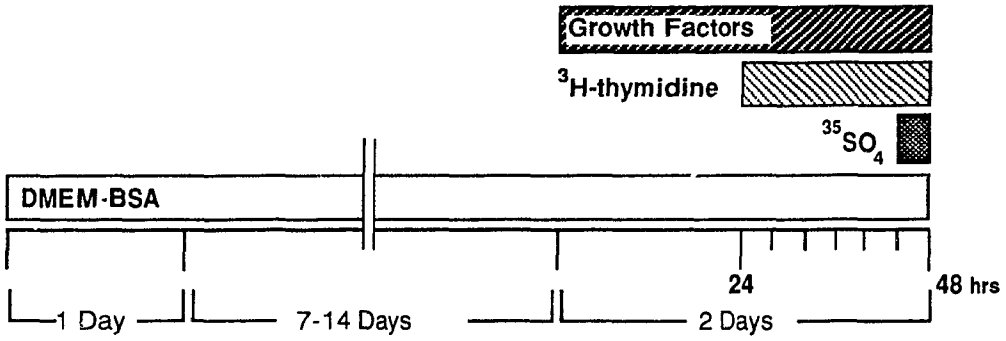
I) After 24 to 48 hour preincubation in DMEM-BSA, 50-100mg cartilage explants were placed in 12 x 75 mm polystyrene sterile culture tubes

A) Culture period

I



II



III

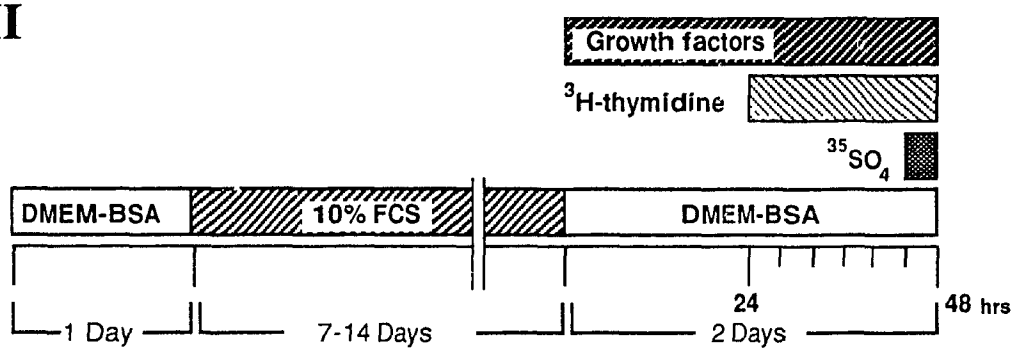


Figure 1 : Experimental Protocol for Biosynthesis Experiments

containing 0.5 mls of various concentrations of growth factors or hormones in DMEM-BSA. The concentration used were as follows: Insulin, 1 and 10μg/ml, IGF-I, 10-100 ng/ml, and PDGF, 1-5 units/ml. FCS at concentrations ranging from 2-20% was also tested. For each condition tested, triplicate cultures were set up.

II) Some of the cartilage explants were kept in DMEM-BSA for one to two weeks before addition of growth factors. The medium was replaced every 2 to 3 days throughout the entire incubation period. The response to growth factors was then tested as above.

III) Other cartilage explants after 24-48 hours of preincubation in DMEM-BSA were removed and placed in DMEM-BSA containing 10% FCS. The tissue was kept in 10% FCS for a period of 1-2 weeks replenishing the culture medium every 2 to 3 days. After the proposed time period, 10% FCS medium was removed and various concentration of growth factors diluted in DMEM-BSA were added as in I and II.

Regardless of the protocol used, growth factors, insulin, and FCS were added for a period of 48 hours (Figure 1). All cartilage explants were labelled for 24 hours with 1μCi of ^{3}H-thymidine after cartilage explants had already been exposed to growth factors for 24 hours (Figure 1). 5μCi $^{35}SO_4$ were added for the final 4 hours of incubation (Figure 1). Sulphate incorporation measures proteoglycan synthesis which therefore serves as a marker for extracellular matrix production. Cartilage explants remain in the 37°C incubator throughout exposure to factors and radioisotopes.

The culture period is promptly terminated after explants have been subjected to growth factors for 48 hours. Excess radioactivity was removed by washing the explants three times with HBSS. The cartilage was kept

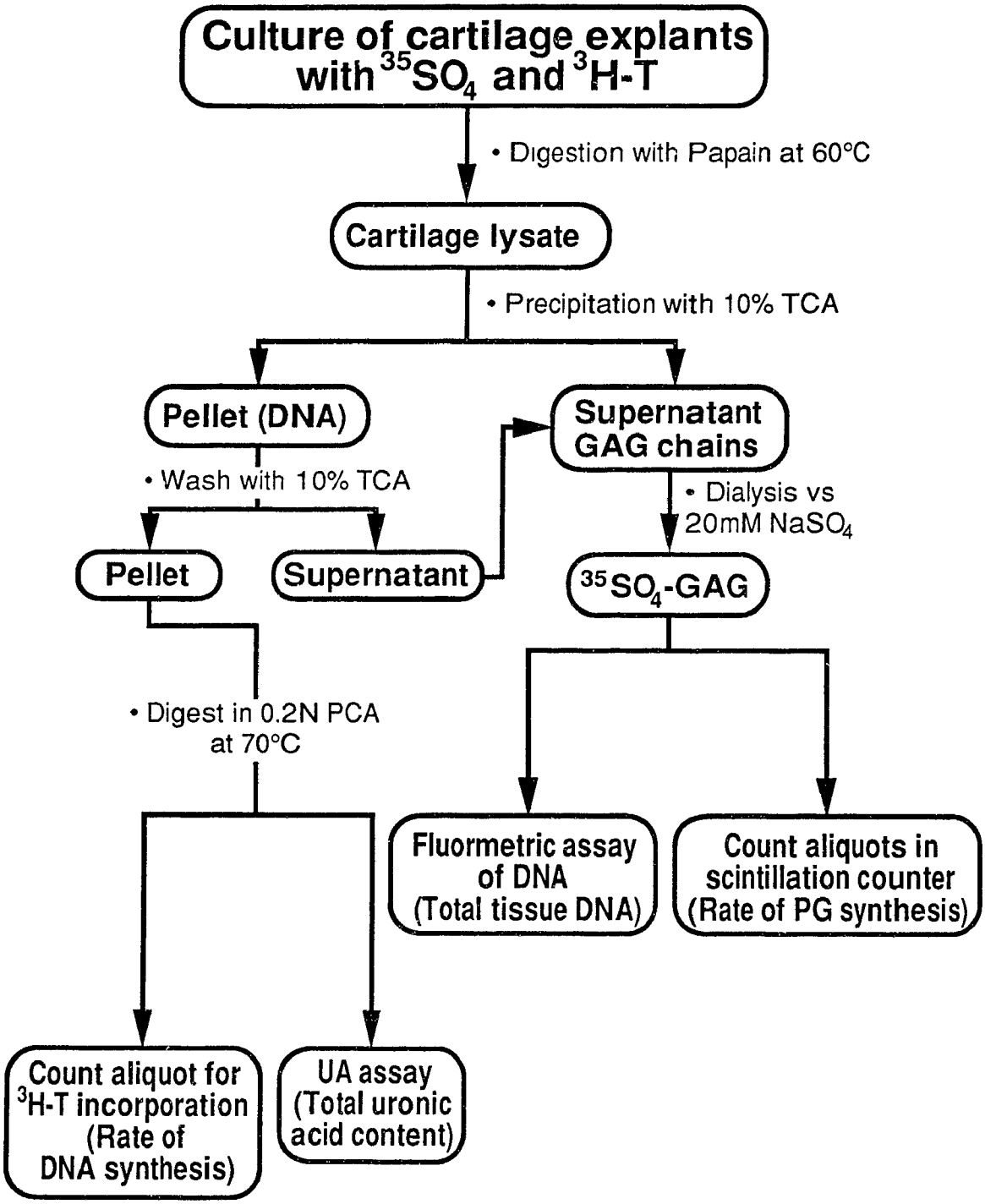


Figure 2 : Biochemical Analysis of DNA and Proteoglycan Synthesis

frozen at -20°C until analyzed further.

2.6 Analysis of DNA and Proteoglycan Synthesis (Figure 2)

Papain Digestion

The first step in analyzing the metabolic parameters of the recently incubated cartilage explants was subjecting the samples to vigorous digestion by papain.

100mg (250µl) of 0.4mg/ml papain solution made in 0.2M Tris-HCL buffer, containing 25mM EDTA, pH 7.0 and 5mM diothiothreitol (Cleland's reagent) was added to each test tube. The samples were incubated in a dry bath at 60°C for 2 hours. This treatment completely solubilized the cartilages.

DNA was precipitated from the cartilage lysates by addition of 250 µl of 20% cold tricholoracetic acid (TCA) giving a final concentration of 10%. The samples were then spun for 10 minutes at 4500 rpm in a Sorvall R.C.S. superspeed refrigerated centrifuge. The supernatant which contains keratan sulfate and chondroitin sulphate was saved and the pellet was washed with 300µL of 10% cold TCA and spun as above. The supernatant is then pooled with the previous one. The pellet which contains DNA amongst other molecules is saved frozen at -20°C for analysis of DNA content.

Determination of Radiosulphate Uptake

The entire supernatant was dialyzed using a Bethesda Research Laboratories microdialyzer (Spectrapor 12,000-14,000 molecular weight tubing) and against 20mM sodium sulphate at a rate of 1ml/min for 16-18 hours. The radioactivity of the dialysate always fell to background after this time period. This step removes the free $^{35}SO_4$ which has not been incorporated in the proteoglycan molecule. The volume of the dialysed

supernatants was weighed and adjusted to 1.0 ml with distilled water. 0.5ml of dialysed sample was then added to 10ml scintillation liquid (Ready Solv, EP, Beckman) in a plastic scintillation vial. After thorough mixing, radioactivity was determined in a Beckman Counter L5800, (Irvine, Ca). The remainder of the dialysate was stored at 4°C for uronic acid assay.

Determination of Thymidine Uptake

The pellet obtained from the TCA precipitation was digested in 0.5ml of 0.2N perchloric acid (PCA) for 1 hour at 70°C in a dry bath, and 0.25ml of the digest was added to 10ml scintillation liquid and counted on 2 channels to determine $^{35}SO_4$ and ^{3}H-thymidine content.

Counting channels were adjusted such that no spillover occured from the ^{3}H-window. Spillover from the ^{35}S-window was determined using a standard with a known amount of ^{35}S and was generally found to be approximately 30%. (Please refer to calculations at end of chapter). The remainder of the PCA digest was saved at 4°C for DNA determination.

DNA Assay

The amount of DNA was determined by the fluorometric method of Kissane and Robins [180], including modifications by Switzer and Summer [181]. After the pellet was digested, with 0.2N PCA, 25µL and 50µl aliquots were assayed for child and adult cartilage respectively. 25µl volume were adjusted to 50µl with 0.2N PCA. 50µl of 0.2N PCA was used as a blank and the DNA standard was 25µl of a 40 µg/ml solution of calf thymus DNA in 0.2N PCA. All samples were assayed in duplicate.

A solution of 0.6g/ml 3,5-diamino benzoic acid dihydrochloride (DABA) in water was decolorized by extraction with charcoal until a colourless solution was obtained. For the assay, 50µl of colourless DABA was added to

each duplicate of blank, standard, and sample. The tubes were then heated in a dry bath at 60°C for 30 minutes and 0.9ml of 0.6N PCA were added to each tube since fluorescence yield is maximal in this concentration of acid. The fluorescent reaction product of DABA and deoxyribose is maximally excited at 420 nm and emits a fluorescent band with maximal intensity at a wave length of 500nm. The fluorescence was read in a Perkin-Elmer fluorescence spectrophotometer. The quantity of DNA in the sample is determined from the average fluorescence of the standards known to be 1.0µg. The amount of DNA is calculated for the specific sample volume used (25 or 50µl) and then corrected to the original volume of the pellet digest (0.5ml). Despite the sparse cellularity in human articular cartilage, this sensitive assay has proved superior to other methods used [182].

Uronic Acid Assay

The uronic acid content of the cartilage digest was determined by the modified carbazole reaction of Bitter and Muir [183]. Sample volumes used for assay were 50µl and 100µl for young and adult cartilage respectively. Aliquots were diluted to 500µl with distilled water, 500µl of water was used as a blank and the standards ranged from 100µl to 500µl of a 100µg/ml solution of glucuronolactone in H_2O. Sulfuric acid/borax reagent (3ml of 0.025M sodium tetraborate in concentrated sulfuric acid) was added to the diluted samples, mixed well and heated in a dry bath at 100°c for 10 minutes followed by 100µl of carbazole reagent (0.125% carbazole in absolute ethanol). The samples were mixed thoroughly and heated at 100°c for 15 minutes. After cooling, the absorbance at 530nm was determined for each specimen using a Gilford 250 spectrophotometer. Using the average of the standards, the uronic acid content was calculated for the specific sample

volume used (50 or 100µl) and then corrected to the original sample volume (1.0ml).

2.7 Characterization of Growth Factors, Insulin, and Fetal Calf Serum

Prior to subjecting our cartilage explants to the various growth factors, it was important to show that these test factors have the ability to stimulte cell proliferation in a standard culture system. The human fetal lung fibroblast (HFL-1) cell line was obtained from the American Type Culture Collection.

Cells were plated in 6- well Linbro plates at 10^5 cells/well in 2mls of DMEM containing 10% FCS and incubated at 37°C in a 5% CO_2 incubator for 3 days. Serum free medium (DMEM only) was added for two days. On the third day, serum free medium is removed and 2ml of serum-free medium containing various concentrations of growth factors were added. 24 hours later, 1µC of ^{3}H-thymidine was added for 6 hours. Unincorporated precursor was removed, the cell layers were washed three times with phosphate buffer saline, the cell layer was solubilized in 0.5ml of 0.2N NaOH and 0.4ml aliquots were counted in a Beckman scintillation counter. Results are expressed as a percentage of incorporation in serum-free medium (Table 3).

Table 3

DNA Synthesis of Human Fetal Lung Fibroblast in Response to Insulin, IGF-I, PDGF and FCS

Culture Conditions	^{3}H-thymidine incorporation (cpm)	% of Response in Serum-Free Medium (DMEM)
DMEM	2,713	100
Insulin 1μg/ml	5,908	218
10μg/ml	9,317	343
IFG-I 10μg/ml	10,681	394
20μg/ml	9,003	332
50μg/ml	18,153	669
PDGF 2unit/ml	9,156	337
FCS 10%	33,961	1,252

The table represents ^{3}H-thymidine incorporation in response to insulin, IGF-I, PDGF, and FCS expressed as cpm and as a percentage incorporation in serum-free medium (DMEM). The ^{3}H-thymidine incorporation represents an average of triplicate determinations.

Calculations

1) $^{35}SO_4$ and ^{3}H-Thymidine Incorporation

$^{35}SO_4$ incorporation consists of radioactivity in papain digest supernatant after TCA precipitation and residual activity in TCA pellet. The latter was usually less than 5% of the total sulfate incorporated. Dialysed TCA supernatants were routinely analyzed for ^{35}S only as no ^{3}H counts were found to be present. The solubilized TCA pellet was analyzed using 2 counting channels adjusted to give minimal spillover of ^{3}H (channel 1) into channel 2. The spillover of ^{35}S into channel 1 was determined for each experiment using a standard amount of ^{35}S only. Samples were counted using automatic quench correction.

For dual channel counting: cpm in channel 1 = CH1

cpm in channel 2 = CH2

constant (^{35}S-spillover) $= \frac{CH1}{CH2}$

For ^{35}S and ^{3}H: ^{35}S cpm=CH2 + CH2 x constant
=CH2(1 + constant)

^{3}H cpm=CH1 - CH2 x constant

Total $^{35}SO_4$ incorporation: (^{35}S in TCA soluble material + ^{35}S in TCA pellet) x 2

Total ^{3}H-Thymidine incorporation: (^{3}H in TCA pellet) x 2

All counts are expressed as cpm/µg DNA.

2) Statistical analysis

The statistical significance of observed differences in incorporation rates in the presence and absence of growth factors was determined using a single tailed student's t-test.

3) All calculations were performed on an IBM-AT personal computer, using a customized form of the Symphony spreadsheet software.

3.0 RESULTS

3.1 Introduction

The results chapter is divided into three sections. In the first section, data on the basal biosynthetic rate of the cartilage donors are presented. The second section of this chapter deals with the effect of prolonged culture time in unsupplemented DMEM-BSA on sulfate and thymidine incorporation by cartilage explants, and with the effect of fetal calf serum (FCS) on these parameters. The final section deals with the effect of various concentrations of insulin-like growth factor-I (IGF-I), insulin and platelet-derived growth factor (PDGF) on cell division and synthesis of extracellular matrix components by both immature and mature cartilage.

3.2 Basal Biosynthetic Rate of Cartilage

As mentioned in the methodology section, all cartilage specimens were always preincubated in DMEM-BSA supplemented with antibiotics for at least 24 hours to remove any possible endogenous contaminants. Some of the cartilage explants were then exposed to growth factors for a period of 48 hours while the control explants from the same speciman were kept in basal DMEM-BSA medium. To determine the extent of the basal biosynthetic rate of cartilage, thymidine and sulphate incorporation as indices of cell division and synthesis of extracellular matrix components were studied without the addition of any possible stimulants (growth factors, insulin, or fetal calf serum). It was also important to examine these parameters at the earliest time after the 24 hour preincubation period since this would simulate more closely the basal biosynthetic activity of the donor's cartilage. The basal rates of sulphate and thymidine incorporation of cartilage explants were studied after three or five days of incubation in

unsupplemented DMEM-BSA. These time points were chosen, because we planned to investigate the response of cartilage explants to growth factors and it was felt that an exposure time of 2-3 days was necessary to allow saturation of the cartilage matrix.

The results demonstrate a wide variation of activity within the adult age group as well as within the child age group (see Table 4). In the adults, sulphate incorporation ranged from 882 to 3952 counts per minute per µg DNA and in the child, the values were 1074 to 2125 cpm/µg DNA. Thymidine incorporation also varied quite significantly in both groups. The adults ^{3}H-thymidine cpm/µg ranged from 719 to 2385 whereas the child's cartilage incorporated 326 to 1680 cpm/µg DNA. These results point out that after 3 or 5 days of incubation mature cartilage is as, if not more metabolically active than immature cartilage.

To ensure that the radioactive counts we obtained were in fact due to chondrocyte cell division and matrix synthesis, chondrocytes of cartilage explants were killed by 3 cycles of freeze-thawing in liquid nitrogen prior to culture. Results for sulphate and thymidine incorporation were 75 and 6 cpm/µg DNA respectively. This quite amply demonstrates that non-specific incorporation of radioactivity in our experimental protocol was kept at a minimum, and the incorporation observed reflects the biosynthetic activity of the cartilage.

3.3 Effect of Prolonged Culture Time on ^{35}S-Sulphate and ^{3}H-Thymidine Incorporation by Cartilage Explants

Culture In Unsupplemented DMEM-BSA

Since the effect of growth factors on cartilage metabolism was to be studied over a prolonged culture period, it was important to determine the

TABLE 4

Comparison of Basal $^{35}SO_4$ and ^{3}H-Thymidine Incorporation in Old and Young Cartilage Over Time in Unsupplemented DMEM-BSA Medium

	Early Culture Time 3-5 Days		Late Culture Time 9-21 Days	
	$^{35}SO_4$	^{3}H	$^{35}SO_4$	^{3}H
	cpm/µg DNA		cpm/µg DNA	
ADULT	882-3952	719-2385	679-3174	425-656
CHILD	1074-2125	326-1680	1422-6398	528-4235

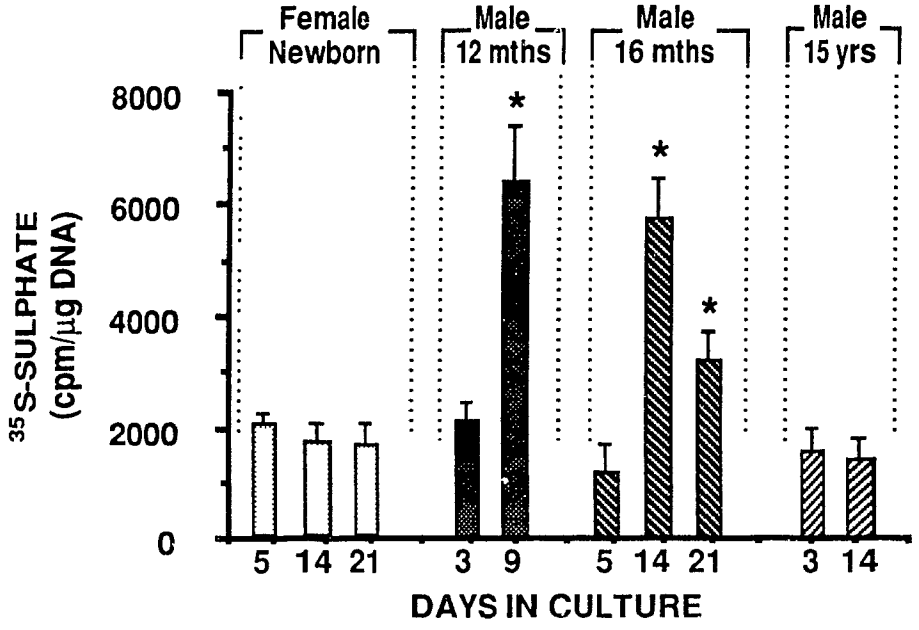
Table 4 The incorporation of ^{35}S-Sulphate and ^{3}H-Thymidine is expressed as counts per minute per µg DNA. The values in the table represents the range of counts observed from five adult and four child donors of all cartilage explants cultured in unsupplemented DMEM-BSA medium.

change in basal biosynthetic rate of cartilage explants with time in culture. Basal incorporation refers to the amount of ^{3}H-thymidine and $^{35}SO_4$ the cartilage explants incorporate without the addition of growth factors. In this set of data we investigate the metabolic parameters of the chondrocyte at 3 or 5 days and compare these to days 9, 15, and 21,when applicable. The cartilage explants in these cases are exposed only to DMEM-BSA. Our results demonstrate a clear cut difference between child and adult cartilage. In section 3.2 it was shown that at the early culture period (3 or 5 days), there was no difference between adult and young cartilage incorporation values, however as culture time was increased we obse"ved that immature cartilage became metabolically more active than mature cartilage (see Table 4)

More specifically with respect to $^{35}SO_4$ incorporation, as culture time increases, the chondrocytes of the young donors either increase their $^{35}SO_4$ uptake or maintain at least the same level of activity. Figure 3 depicts the effect of prolonged culture on proteoglycan synthesis by each individual young sample. The percentage increase in sulphate incorporation with time is also included. In the adult however as culture time increases the mature chondrocyte either decreases its $^{35}SO_4$ incorporation or maintains the same level of activity. Figure 4 reveals these data more closely.

Generally the same basic trend is seen in the cartilage explants with regard to ^{3}H-thymidine incorporation. Therefore, in the young, as cartilage explants spend more time in unsupplemented DMEM-BSA, ^{3}H-thymidine uptake either increases or remains the same. Figure 5 examines each young individual donor's ability to incorporate ^{3}H-thymidine over time. In the adult cartilage the situation is reversed in that cell division in most

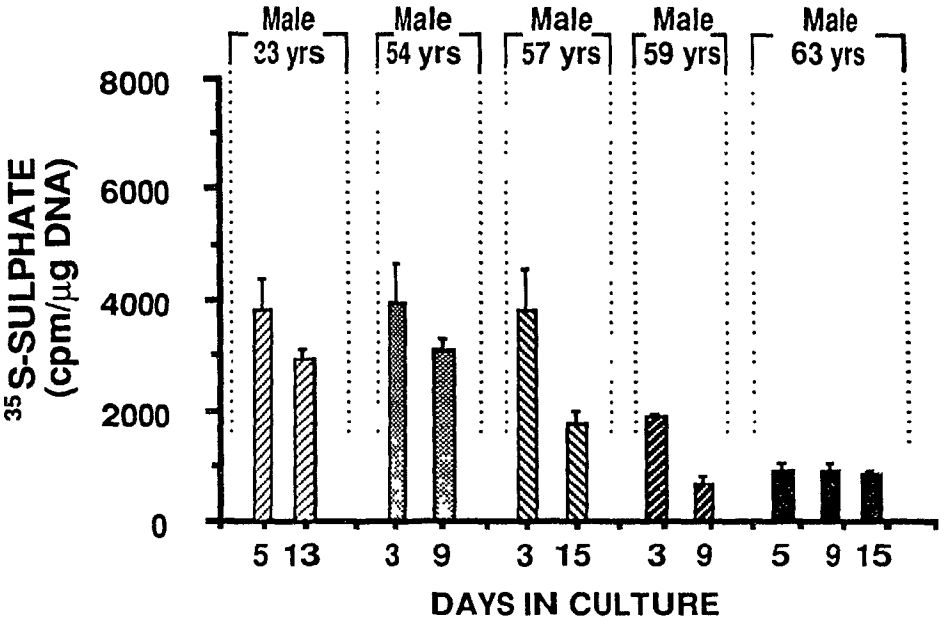
EFFECT OF PROLONGED CULTURE TIME IN DMEM-BSA ON PROTEOGLYCAN SYNTHESIS BY YOUNG ARTICULAR CARTILAGE



	Day 9	Day 14	Day 21
Newborn	310%*	83%	83%
12 mths			
16 mths		488%*	274%*
15 yr-old		91%	

Figure 3 ^{35}S-Sulphate incorporation by cultured cartilage explants from young donors. ^{35}S-Sulphate incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations The table below the graph represents incorporation data expressed as percentage of earliest culture time which was 3 days for the 12 month and 15 year-old donors and 5 days for the newborn and the 16 month-old donors. Day 3 and day 5 sulphate incorporation values are defined as 100%. The asterix indicates a statistically significant difference at $p < 0,05$.

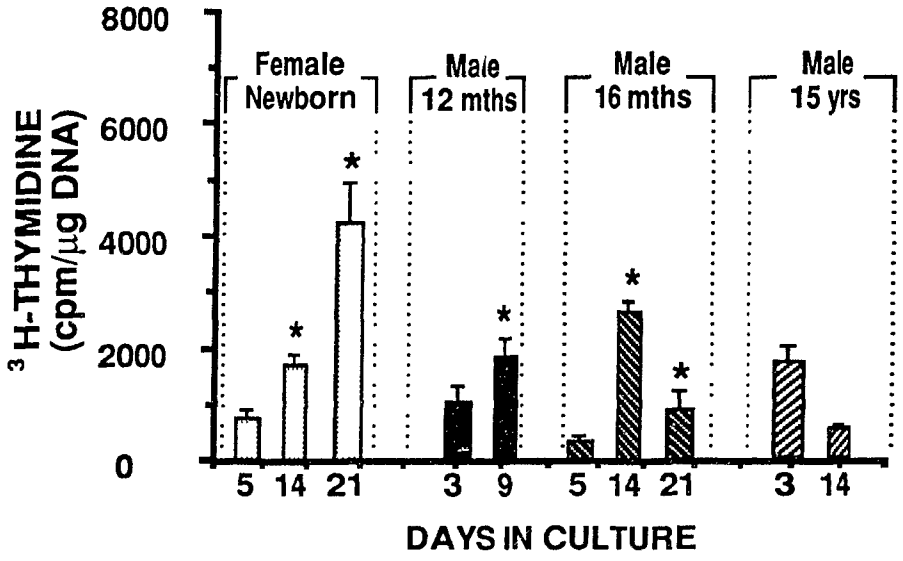
EFFECT OF PROLONGED CULTURE TIME IN DMEM-BSA ON PROTEOGLYCAN SYNTHESIS BY ADULT ARTICULAR CARTILAGE



	Day 9	Day 13	Day 15
33 yr-old		77%	
54 yr-old	79%		
57 yr-old			46%
59 yr-old	36%		
63 yr-old	103%		95%

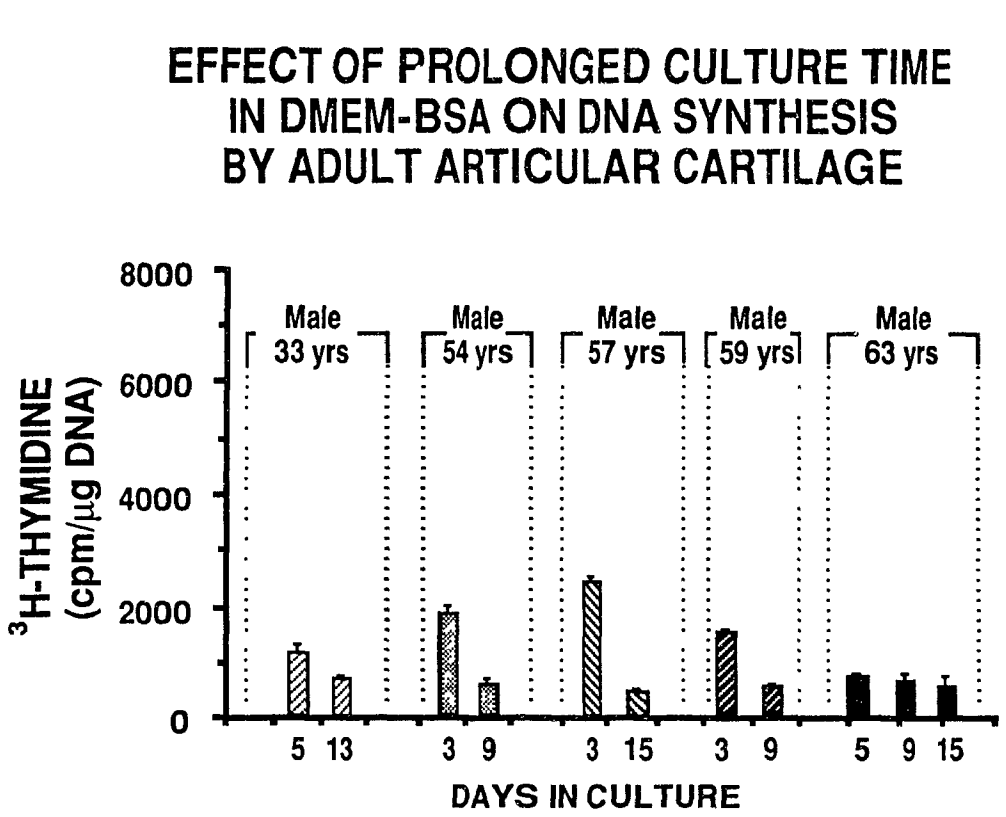
Figure 4 ^{35}S-Sulphate incorporation by cultured cartilage explants from adult donors. ^{35}S-Sulphate incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations.The table below the graph represents incorporation data expressed as percentage of earliest culture time which was 3 days for the 54, 57, and 59 year-old donors and 5 days for the 33 and 63 year-old donors Day 3 and day 5 sulphate incorporation values are defined as 100%.

EFFECT OF PROLONGED CULTURE TIME IN DMEM-BSA ON DNA SYNTHESIS BY YOUNG ARTICULAR CARTILAGE



	Day 9	Day 14	Day 21
Newborn	180%*	219%*	553%*
12 mths			
16 mths		805%*	275%*
15 yr-old		31%	

Figure 5 ^{3}H-Thymidine incorporation by cultured cartilage explants from young donors. ^{3}H-Thymidine incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. The table below the graph represents incorporation data expressed as percentage of earliest culture time which was 3 days for the 12 month and 15 year-old donors and 5 days for the newborn and the 16 month-old donors Day 3 and day 5 thymidine incorporation values are defined as 100%. The asterix indicates a statistically significant difference at $p < 0,05$.



	Day 9	Day 13	Day 15
33 yr-old		58%	
54 yr-old	32%		
57 yr-old			17%
59 yr-old	32%		
63 yr-old	86%		74%

Figure 6 ^{3}H-Thymidine incorporation by cultured cartilage explants from adult donors.^{3}H-Thymidine incorporation is expressed as counts per minute per μg DNA and bars represent an average of triplicates with respective standard deviations. The table below the graph represents incorporation data expressed as percentage of earliest culture time which was 3 days for the 54, 57, and 59 year-old donors and 5 days for the 33 and 63 year-old donors. Day 3 and day 5 thymidine incorporation values are defined as 100%.

cases decreases with prolonged incubation time (Figure 6). These data demonstrate that generally the rate of proteoglycan as well as DNA synthesis in immature cartilage explants increases with prolonged culture in the absence of any added growth factors while the opposite is true for adult or mature cartilage explants. This suggests that immature cartilage might have the ability to synthesize growth factors such as IGF-I which maintain the rates of biosynthesis of matrix molecules and that this capacity is lost in adult cartilage.

Culture in DMEM-BSA Supplemented with 10% FCS

In this situation we studied the effect of prolonged culture on the incorporation of $^{35}SO_4$ and ^{3}H-thymidine by the cartilage explants in DMEM-BSA supplemented with 10% FCS. We chose to study FCS since it generally contains a mixture of growth factors and has been reported to optimally stimulate proteoglycan synthesis in immature cartilage of various species (74-77). Therefore the effect of FCS was investigated to determine whether biosynthetic rates in articular cartilage could be stimulated by what was assumed to be an optimal mix of growth factors as was shown to be the case for bovine articular cartilage [76]. In addition we wanted to determine whether the decrease in sulfate and thymidine uptake observed in mature cartilage at later culture time (9-15 days) in unsupplemented medium could be attenuated or reversed by FCS.

As in the previous section, we examined these metabolic parameters over at least two different time periods. All cartilage explants regardless of experimental protocol were preincubated in DMEM-BSA for 1 to 3 days (refer to figure 1) after which they were exposed to 10% FCS in DMEM-BSA for the remaining culture period (figure 1, line III).

Only two child donors were subjected to this experimental protocol. The 12 month old cartilage demonstrated as it did with unsupplemented medium an increase in proteoglycan synthesis with prolonged incubation time. In addition FCS stimulated sulfate incorporation during the same time frame compared to unsupplemented medium. Figure 7 demonstrates these two points. The 15 year old donor which did not increase its $^{35}SO_4$ incorporation with time when the explants were maintained in unsupplemented DMEM-BSA showed a significant stimulation when incubated in 10% FCS for 14 days, but not after short exposure to FCS (figure 7).

The three adult donors which were subjected to 10% FCS all increased their proteoglycan synthesis by about two-fold over prolonged incubation time. However, similar to the 15 year old this stimulation was only seen at the later culture times (9-15 days), and no stimulation was observed after a shorter exposure to FCS. Figure 8 exhibits these points.

The pattern of thymidine uptake shows a response similar to proteoglycan synthesis. Each sample of immature and mature cartilage exposed to 10% FCS dramatically increased their ^{3}H-thymidine incorporation over time (Figure 9 and 10). There was no ^{3}H-thymidine incorporation observed in either the 15 year old sample or the adults that were kept in unsupplemented DMEM-BSA (Figure 5 and 6). These results also demonstrate that stimulation of both $^{35}SO_4$ and ^{3}H-thymidine uptake in adult cartilage by FCS is greater than in young cartilage when compared to the same time points without FCS.

Generally then it would seem that adult cartilage requires extended culture periods to become responsive to the growth promoting activity of

EFFECT OF PROLONGED CULTURE TIME IN DMEM-BSA SUPPLEMENTED WITH 10% FCS ON PROTEOGLYCAN SYNTHESIS BY YOUNG ARTICULAR CARTILAGE

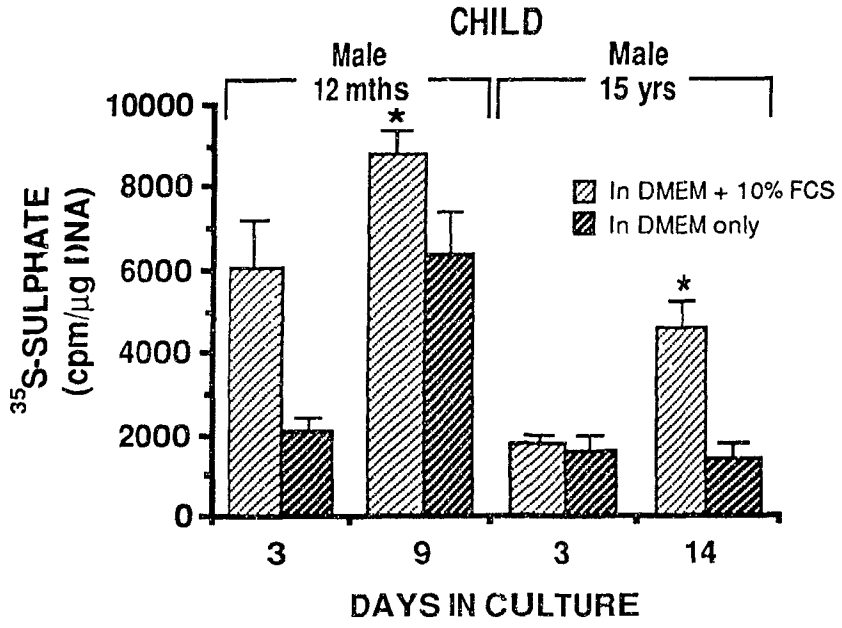


Table A

	Day 9	Day 14
12 months	145%*	
15 years		253%*

Table B

	Day 3	Day 9	Day 14
12 months	331%*	137%	
15 years	115%		320%*

Figure 7 ^{35}S-Sulphate incorporation by cultured cartilage explants from young donors.^{35}S-Sulphate incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations.Table A represents incorporation data expressed as percentage of earliest culture time which was 3 days for the 12 month and 15 year-old donors of explants incubated in DMEM-BSA supplemented with 10% FCS. Day 3 sulphate incorporation values are defined as 100%. To demonstrate stimulation by 10% FCS table B represents incorporation data expressed as a percentage increase over unsupplemented DMEM-BSA at the same day The asterix indicates a statistically significant difference at $p<0.05$.

EFFECT OF PROLONGED CULTURE TIME IN DMEM-BSA SUPPLEMENTED WITH 10% FCS ON PROTEOGLYCAN SYNTHESIS BY ADULT ARTICULAR CARTILAGE

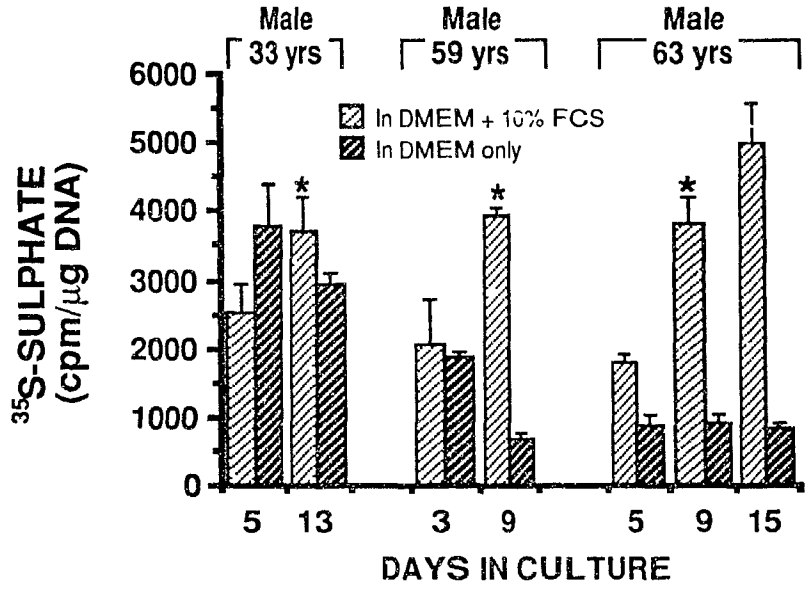


Table A

	Day 9	Day 13	Day 15
33 yr-old		148%*	
59 yr-old	191%*		
63 yr-old	213%*		280%*

Table B

	Day 3	Day 5	Day 9	Day 13	Day 15
33 yr-old		66%		130%	
59 yr-old	110%		577%*		
63 yr-old		203%*	419%*		597%*

Figure 8 ^{35}S- Sulphate incorporation by cultured cartilage explants from adult donors. ^{35}S- Sulphate incorporation is expressed as counts per minute per μg DNA and bars represent an average of triplicates with respective standard deviations Table A represents incorporation data expressed as percentage of earliest culture time which was 3 days for the 59 year-old donor and 5 days for the 33 and 63 year-old donors of explants incubated in DMEM-BSA supplemented with 10% FCS Day 3 and day 5 sulphate incorporation values are defined as 100%. To demonstrate stimulation by 10% FCS table B represents incorporation data expressed as a percentage increase over unsupplemented DMEM-BSA at the same day. The asterix indicates a statistically significant difference at $p<0.05$.

EFFECT OF PROLONGED CULTURE TIME IN DMEM-BSA SUPPLEMENTED WITH 10% FCS ON DNA SYNTHESIS BY YOUNG ARTICULAR CARTILAGE

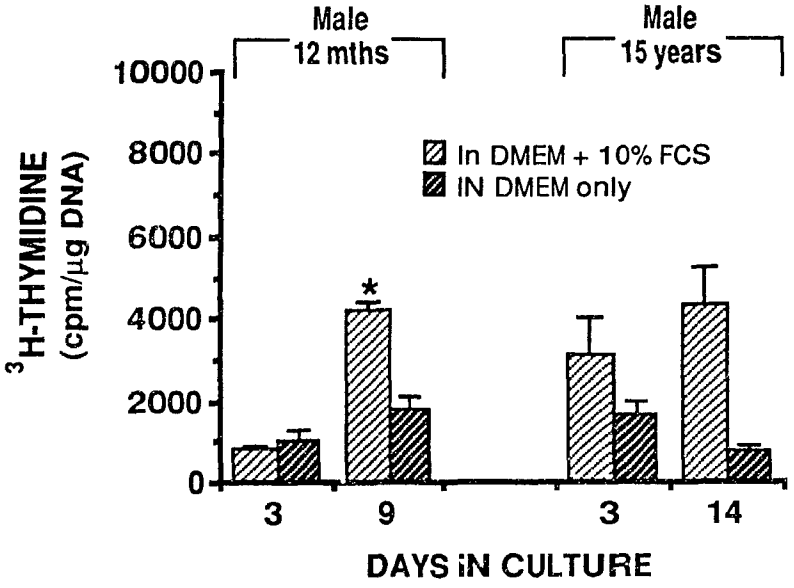


Table A

	Day 9	Day 14
12 mth-old	509% *	
15 yr-old		138%

Table B

	Day 3	Day 9	Day 14
12 mth-old	89%	236%*	
15 yr-old	187%*		593%*

Figure 9 ^{3}H-Thymidine incorporation by cultured cartilage explants from young donors. ^{3}H-Thymidine incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. Table A represents incorporation data expressed as percentage of earliest culture time which was 3 days for the 12 month and 15 year-old donors of explants incubated in DMEM-BSA supplemented with 10% FCS Day 3 sulphate incorporation values are defined as 100%. To demonstrate stimulation by 10% FCS table B represents incorporation data expressed as a percentage increase over unsupplemented DMEM-BSA at the same day. The asterix indicates a statistically significant difference at $p<0,05$.

EFFECT OF PROLONGED CULTURE TIME IN DMEM-BSA SUPPLEMENTED WITH 10% FCS ON DNA SYNTHESIS BY ADULT ARTICULAR CARTILAGE

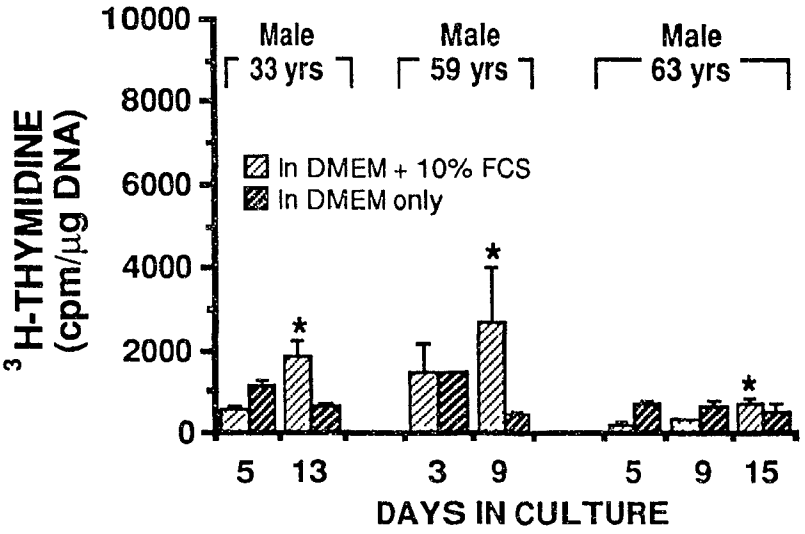


Table A

	Day 9	Day 13	Day 15
33 yr-old		323%*	
59 yr-old	183%*		
63 yr-old	132%		315%*

Table B

	Day 3	Day 5	Day 9	Day 13	Day 15
33 yr-old		50%*		280%*	
59 yr-old	100%*		562%*		
63 yr-old		30%	47%		130%*

Figure 10 ^{3}H-Thymidine incorporation by cultured cartilage explants from adult donors ^{3}H-Thymidine incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. Table A represents incorporation data expressed as percentage of earliest culture time which was 3 days for the 59 year-old donor and 5 days for the 33 and 63 year-old donors of explants incubated in DMEM-BSA supplemented with 10% FCS. Day 3 and day 5 thymidine incorporation values are defined as 100%. To demonstrate stimulation by 10% FCS table B represents incorporation data expressed as a percentage increase over unsupplemented DMEM-BSA at the same day The asterix indicates a statistically significant difference at $p < 0,05$.

FCS, and that FCS and thus addition of exogenous factors can reverse the decrease in the rates of proteoglycan and DNA synthesis observed on culture in unsupplemented medium.

3.4 Effect of Insulin-Like Growth Factor (IGF-I), Insulin and Platelet Derived Growth Factor (PDGF) on Proteoglycan and DNA Synthesis by Cartilage Explants

Insulin-Like Growth Factor I (IGF-I)

The somatomedins or the IGF family have long been implicated as the key growth factors involved in cartilage growth with IGF-I being the most potent of the insulin-like growth factor family [99]. The effect of IGF-I was investigated to determine whether our cartilage explant system incorporates more $^{35}SO_4$ and ^{3}H-thymidine in response to various concentrations of IGF-I. In addition we wanted to determine whether there is a difference in responsiveness between young and old cartilage to IGF-I since previous studies have only looked at young immature specimens.

Young Cartilage

The effect of IGF-I on proteoglycan synthesis of immature articular cartilage was investigated to determine if young chondrocytes would respond to this growth factor and whether the magnitude of the response was similar to that obtained in the presence of FCS. Only two age groups (12 month and 15 year old) were cultured in the presence of IGF-I. In both cases a stimulatory response was observed at all concentrations used. Increases of up to 250% over unsupplemented DMEM-BSA were observed in explants from a 12 month old cartilage, while the response was less in the 15 year old cartilage (see Fig. 11 and table 5).

PROTEOGLYCAN SYNTHESIS OF YOUNG ARTICULAR CARTILAGE IN RESPONSE TO IGF-I

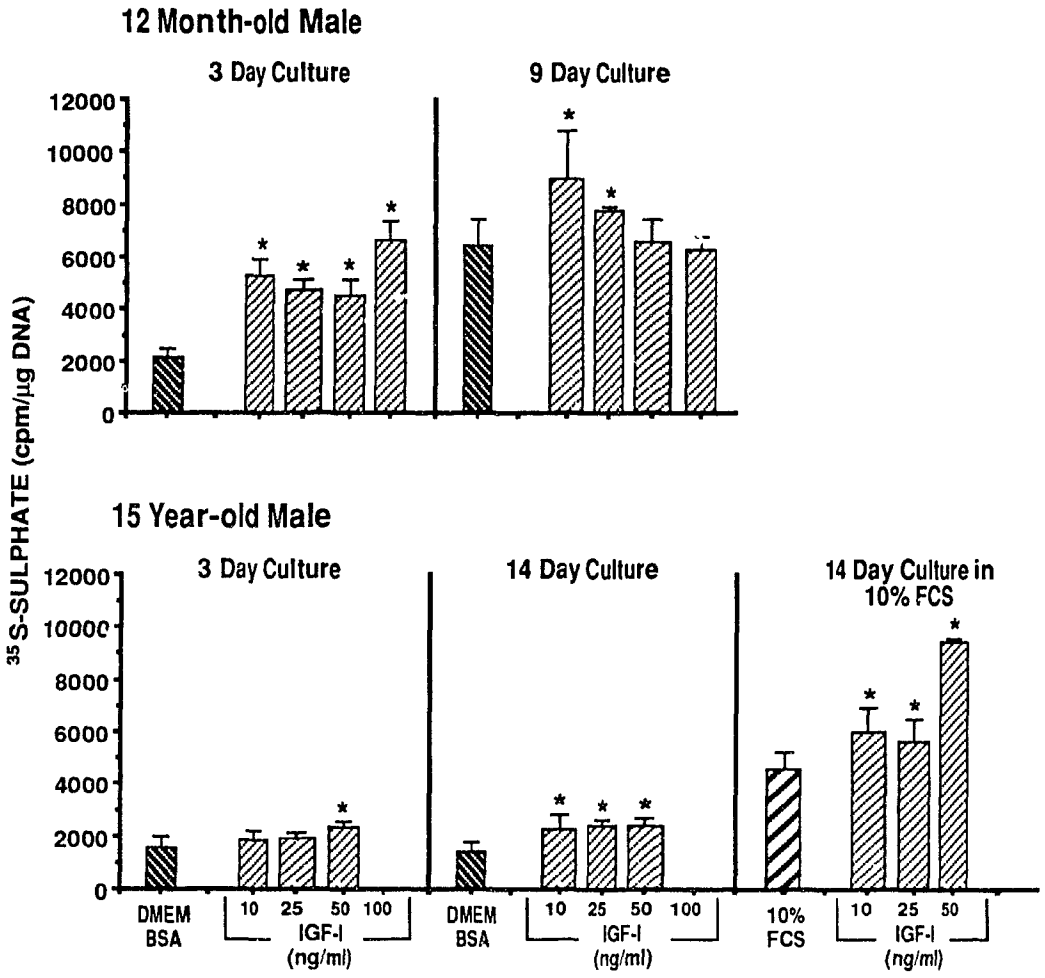


Figure 11 ^{35}S-Sulphate incorporation by cultured cartilage explants from young donors in response to concentrations of 10-100 ng/ml of IGF-I. ^{35}S-Sulphate incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. The asterix indicates a statistically significant difference at $p < 0,05$.

TABLE 5

Proteoglycan Synthesis of Young Articular Cartilage in Response to IGF-I

Donor	Days in Culture		
	3	9	14
12 month-old	249%*	115%	
15 year-old	136%*		164%*/155%*

Table 5 The table represents ^{35}S-Sulphate incorporation in response to IGF-I expressed as a percentage of incorporation in unsupplemented DMEM-BSA medium. The value of 155% is the response of the cartilage explants from the 15 year-old to IGF-I after a 14 day preculture period in DMEM-BSA supplemented with 10%FCS. The values represent the average response of all the concentrations of IGF-I used (10-100 ng/ml). The asterix indicates a statistically significant difference at $p < 0,05$.

Adult Cartilage

In nearly all situations when adult cartilage explants were exposed to IGF-I no stimulation of proteoglycan synthesis was observed as compared to controls (DMEM-BSA only). Figure 12 shows the rates of sulphate incorporation of adult cartilage explants from 3 donors, exposed to 10, 25, 50 ng/ml IGF-I for 48 hours. Synthesis rates were measured after 3 and 9 or 15 days culture in unsupplemented medium. Table 6 illustrates that there is no substantial percentage increase over basal incorporation rate in unsupplemented DMEM-BSA. The only adult sample which showed a consistent stimulation by IGF-I cartilage was the 33 year old donor after 13 days of culture in DMEM-BSA (figure 13). The culture of the 33 year old explants in unsupplemented medium for 12 days prior to exposure to IGF-I increased the magnitude of the response, as was observed in explants from the 15 year old donor using the same approach (figure 11). An increase of 219% over basal incorporation was observed. The explants which were supported in DMEM-BSA containing 10% FCS showed a marginal stimulation in response to addition of IGF-I (figure 13).

With respect to ^{3}H-thymidine incorporation, both child age groups that were studied showed a significant increase in ^{3}H-thymidine at 3 and 9 or 14 days of culture. Figure 14 illustrates this point. Increases of up to 180% over control values were achieved (table 7). The effects of IGF-I on thymidine incorporation in adult cartilage show the same pattern as was observed for $^{35}SO_4$ incorporation. There was essentially no stimulation of cell division in the mature chondrocyte (figure 15). Table 8 illustrates these results by expressing responses as percent of that in basal medium. The only positive response was evoked in the 33 year old after 13 days incubation in

PROTEOGLYCAN SYNTHESIS BY ADULT ARTICULAR CARTILAGE IN RESPONSE TO IGF-I

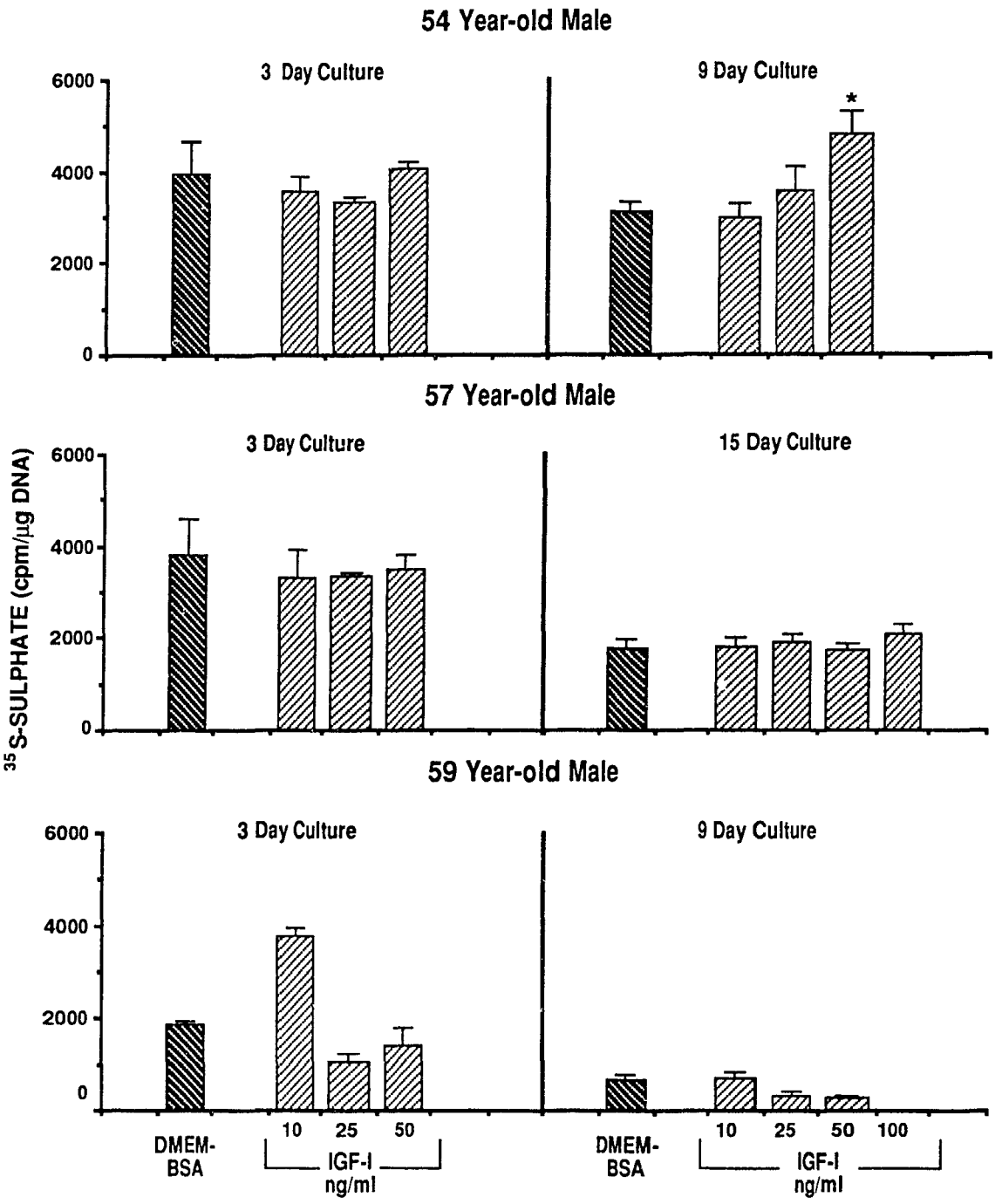


Figure 12 ^{35}S-Sulphate incorporation by cultured cartilage explants from adult donors in response to concentrations of 10-100 ng/ml of IGF-I. ^{35}S-Sulphate incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. The asterix indicates statistically significant difference at $p < 0,05$.

TABLE 6

Proteoglycan Synthesis of Adult Articular Cartilage in Response to IGF-I

Donor	Days in Culture		
	3	9	15
54 yr-old	92%	118%	
57 yr-old	89%		107%
59 yr-old	112%	64%	

Table 6 The table represents ^{35}S-Sulphate incorporation in response to IGF-I expressed as a percentage of incorporation in unsupplemented DMEM-BSA medium. The values represent the average response of all the concentrations of IGF-I used (10-100 ng/ml).

PROTEOGLYCAN SYNTHESIS BY THE 33 YEAR-OLD ARTICULAR CARTILAGE IN RESPONSE TO IGF-I

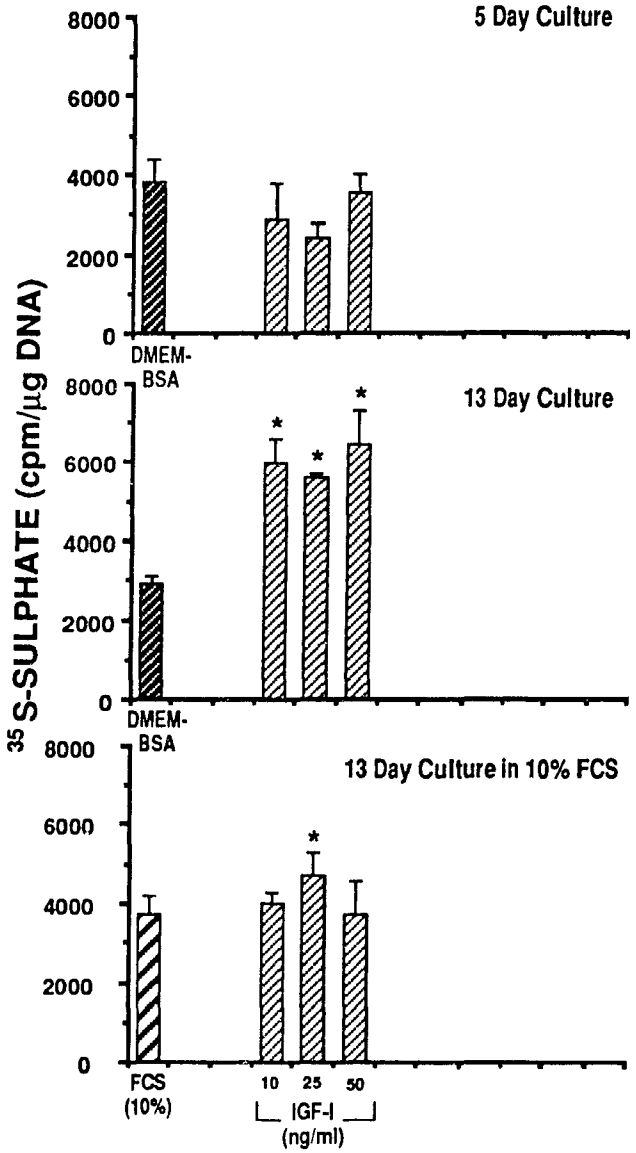


Figure 13 ^{35}S-Sulphate incorporation by cultured cartilage explants from a 33 year-old donor in response to concentrations of 10-50ng/ml of IGF-I. The response of the 33 year-old cultured cartilage explant at 13 days to 50 ng/ml of IGF-I represents 219% of the response observed in unsupplemented DMEM-BSA of the same culture period. ^{35}S-Sulphate incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. The asterix indicates statistically significant difference at $p < 0,05$.

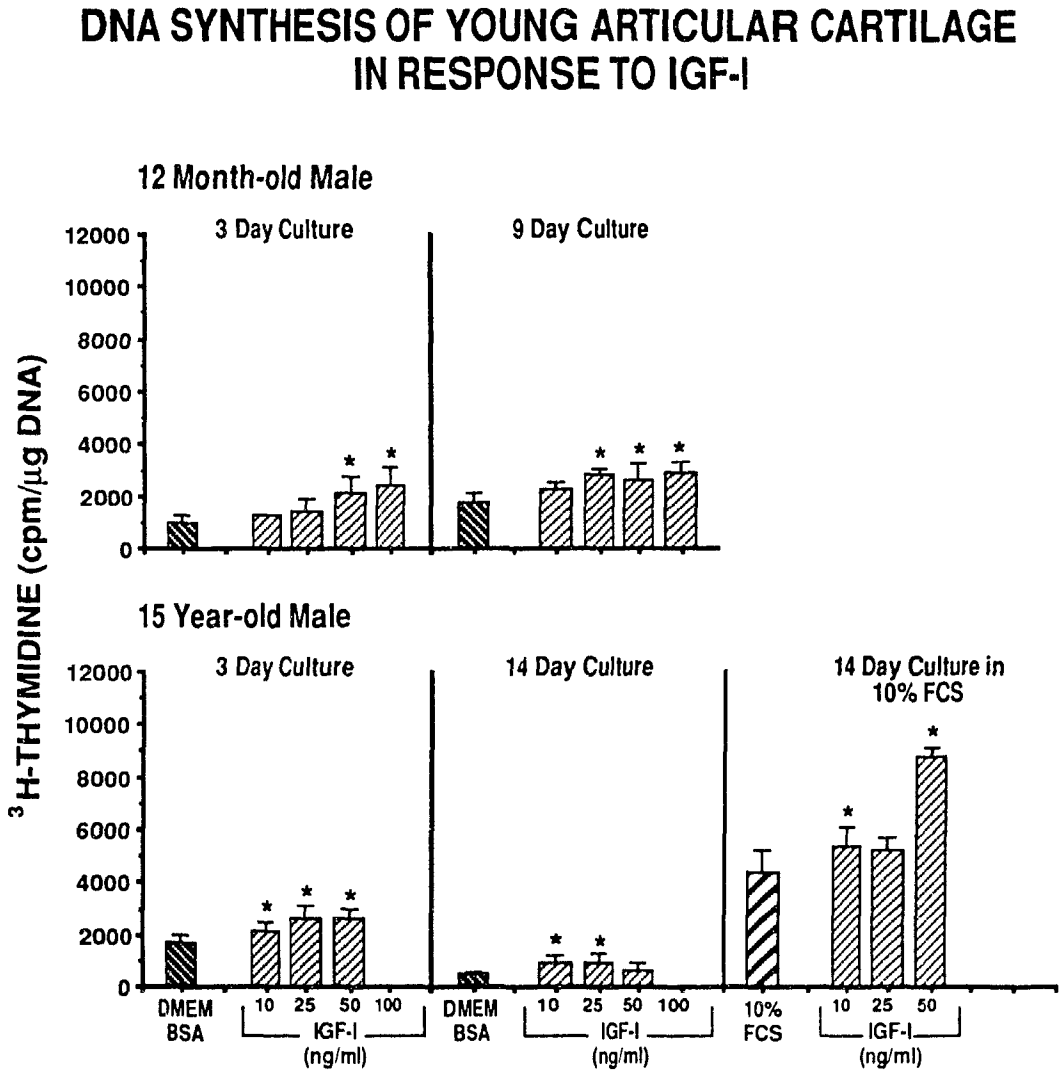


Figure 14 ^{3}H-Thymidine incorporation by cultured cartilage explants from young donors in response to concentrations of 10-100 ng/ml of IGF-I. ^{3}H-Thymidine incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. The asterix indicates a statistically significant difference at $p < 0,05$

TABLE 7

DNA Synthesis of Young Articular Cartilage in Response to IGF-I

Donor	Days in Culture		
	3	9	14
12 month-old	180%*	149%*	
15 yr-old	146%*		158%*/ 148%*

Table 7 The table represents ^{3}H-Thymidine incorporation in response to IGF-I expressed as a percentage of incorporation in unsupplemented DMEM-BSA medium. The value of 148% is the response of the cartilage explants from the 15 year-old to IGF-I after a 14 day preculture period in DMEM-BSA supplemented with 10% FCS. The values represent the average response of all the concentrations of IGF-I used (10-100 ng/ml). The asterix indicates a statistically significant difference at $p < 0,05$.

DNA SYNTHESIS OF ADULT ARTICULAR CARTILAGE IN RESPONSE TO IGF-I

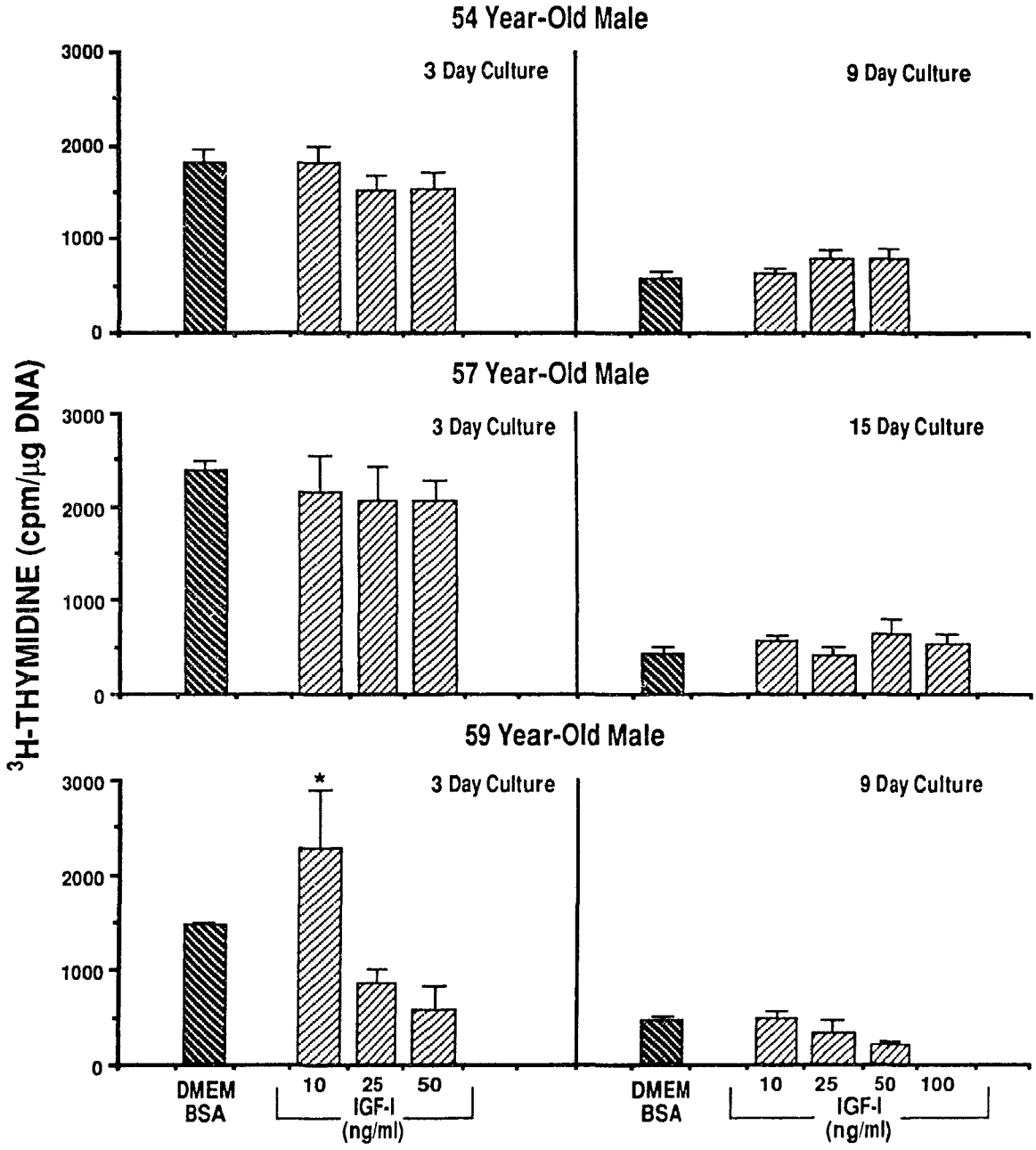


Figure 15 ^{3}H-Thymidine incorporation by cultured cartilage explants from adult donors in response to concentrations of 10-100 ng/ml of IGF-I. ^{3}H-Thymidine incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. The asterix indicates a statistically significant difference at $p < 0,05$

TABLE 8

DNA Synthesis of Adult Articular Cartilage in Response to IGF-I

Donor	Days in Culture		
	3	9	15
54 yr-old	89%	128%	
57 yr-old	88%		107%
59 yr-old	84%	73%	

Table 8 The table represents ^{3}H-Thymidine incorporation in response to IGF-I expressed as a percentage of incorporation in unsupplemented DMEM-BSA medium. The values represent the average response of all the concentrations of IGF-I used (10-100 ng/ml).

DMEM-BSA (figure 16). A 170% increase was observed similar to the effect on proteoglycan synthesis.

From these observations we conclude that there is a general decrease in the capacity of adult articular chondrocytes to respond to IGF-I. The responsiveness of adult chondrocytes to IGF-I can, in some cases be unmasked by prolonged preculture in unsupplemented medium suggesting the presence of factors in uncultured tissue which can modulate the response of adult cartilage to IGF-I.

Insulin

^{35}S-Sulphate Incorporation

Insulin at pharmacological concentrations has been shown to have the same effect as IGF-I on articular chondrocyte metabolism. The effect of insulin at high concentrations should therefore mimick the action of IGF on our explants. In addition, insulin is readily available and relatively inexpensive.

The young cartilage responded in a peculiar way to insulin. When the young explants were incubated for short periods (3-5 days), all samples treated with insulin were stimulated to incorporate significantly more sulphate than controls (figure 17). In two cases (16 month and 15 year old specimen) insulin at the higher dose (10μg/ml) was less effective than at the lower dose used (1μg/ml). As culture time in unsupplemented DMEM-BSA increased, the addition of insulin no longer enticed an increase in proteoglycan synthesis (figure 18). However, the control explants in this latter situation also showed a significant increase in their basal rate of proteoglycan synthesis (figure 3). The only exception was the 15 year old donor (figure 18), where stimulation of proteoglycan synthesis was observed

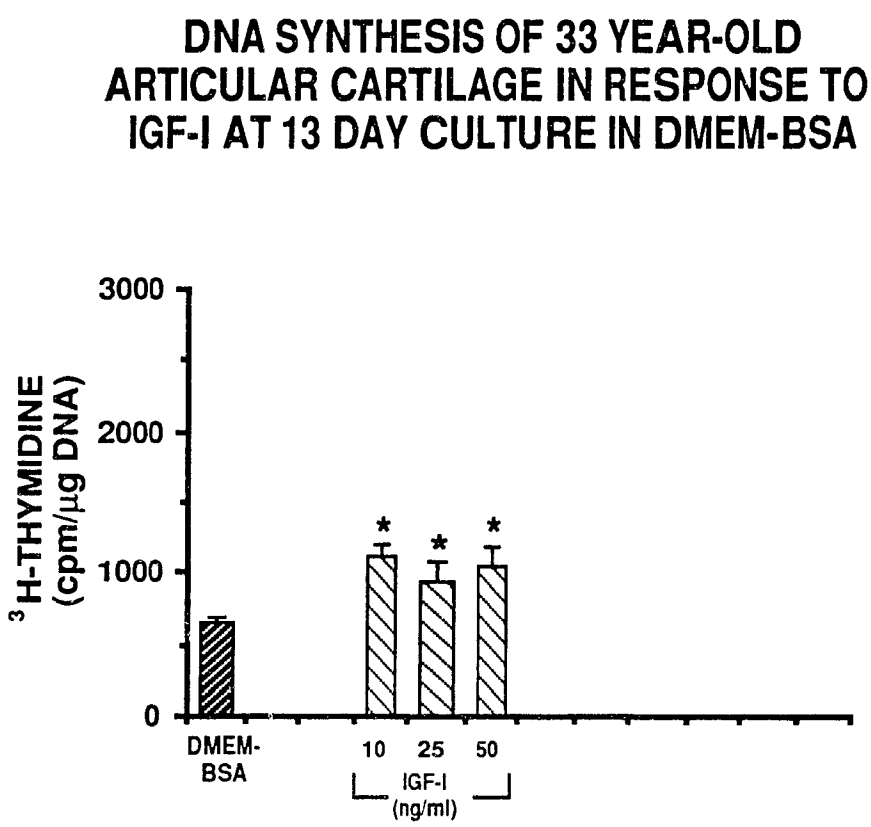


Figure 16 ^{3}H-Thymidine incorporation by cultured cartilage explants from a 33 year-old donor in response to concentrations of 10-50 ng/ml of IGF-I. The response of the 33 year-old cultured cartilage explant at 13 days to 10 ng/ml of IGF-I represents 170% of the response observed in unsupplemented DMEM-BSA of the same culture period. ^{3} H-Thymidine incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations.The asterix indicates a statistically significant difference at $p < 0,05$.

PROTEOGLYCAN SYNTHESIS OF YOUNG ARTICULAR CARTILAGE IN RESPONSE TO INSULIN (Early Culture Period)

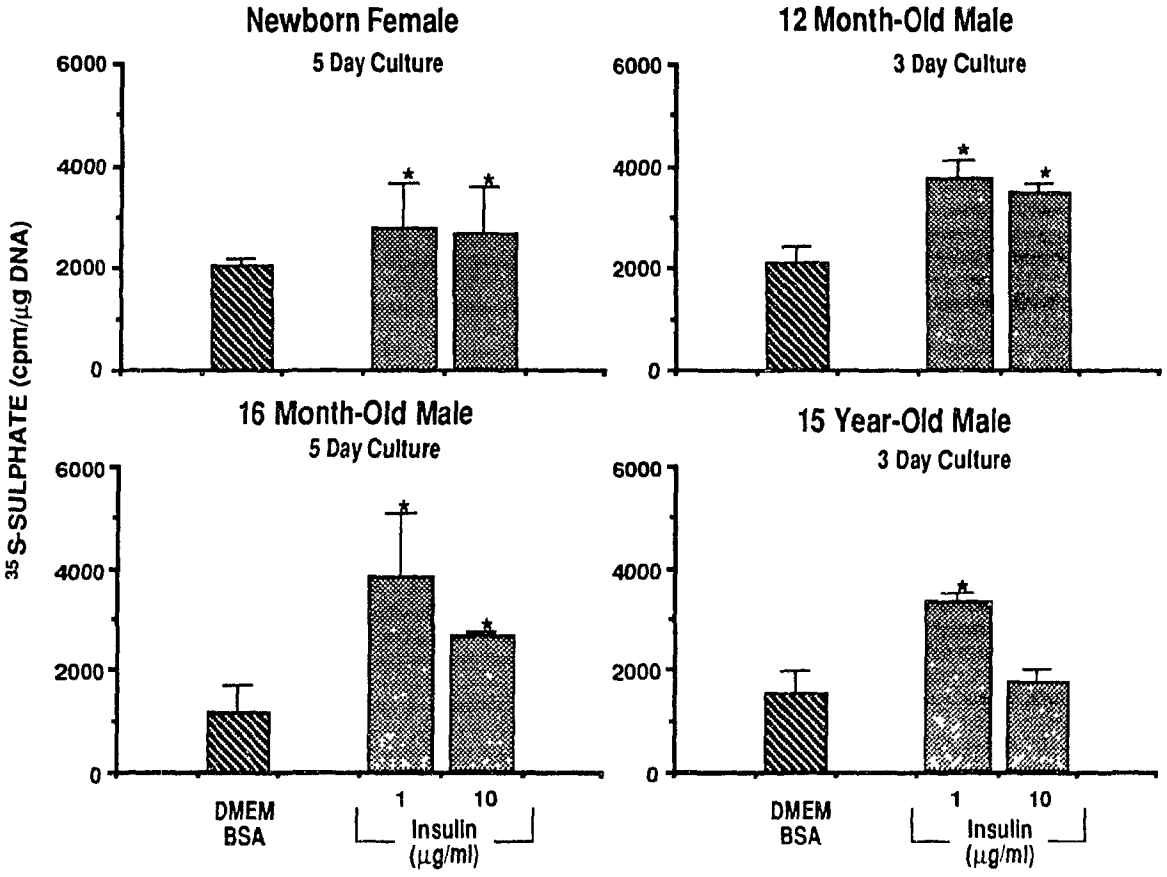


Figure 17 ^{35}S-Sulphate incorporation by cultured cartilage explants from young donors in response to concentrations of 1-10 µg/ml of insulin measured after a short preculture period (3-5 days). ^{35}S-Sulphate incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations The asterix indicates a statistically significant difference at $p < 0,05$.

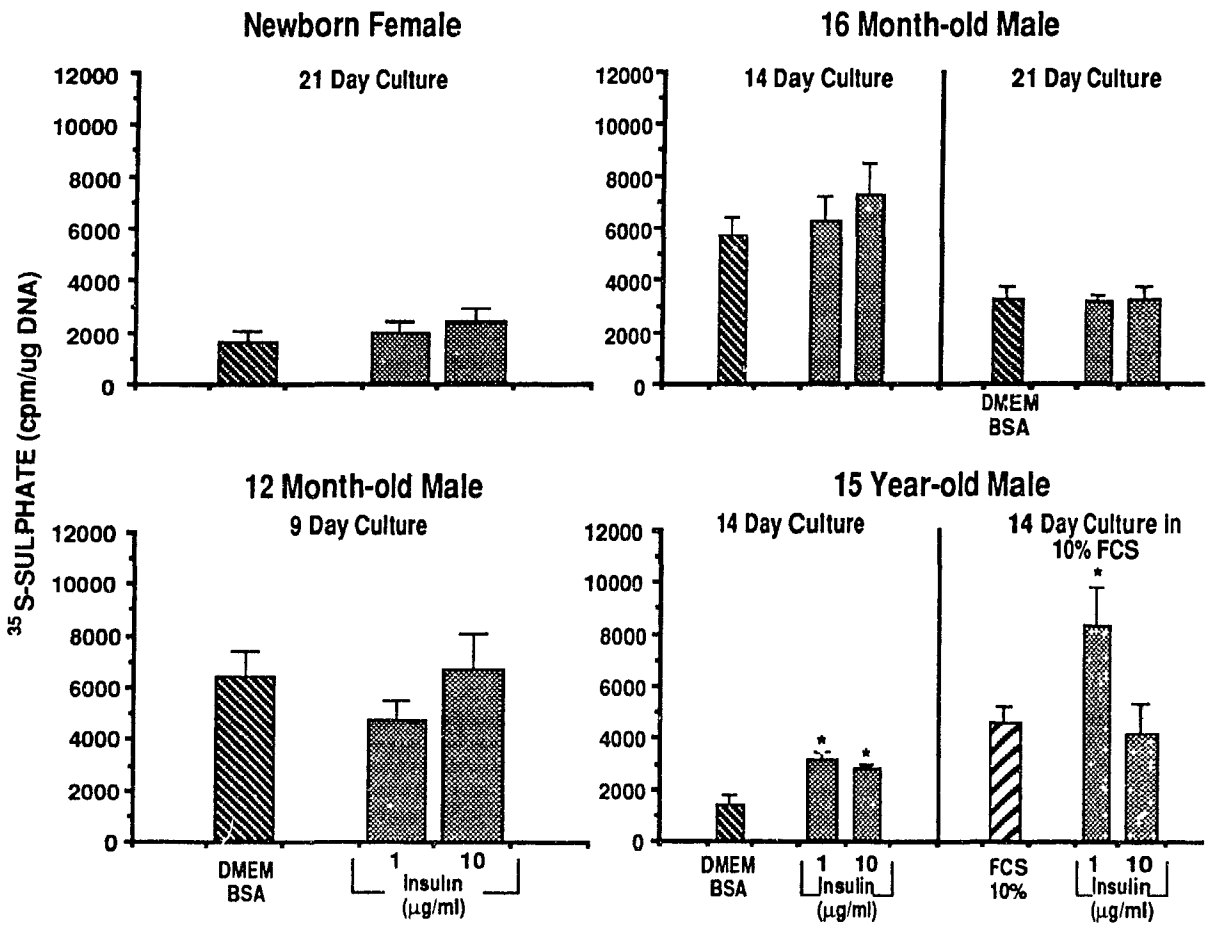


Figure 18 ^{35}S-Sulphate incorporation by cultured cartilage explants fromyoung donors in response to concentrations of 1-10 µg/ml of insulin measured after a long preculture period (9-21 days). ^{35}S-Sulphate incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. The asterix indicates a statistically significant difference at $p < 0.05$.

both early and late in the culture period, similar to the behavior of explants from a 33 year old donor. The responses of young cartilage to insulin at early and late culture periods are summarized in table 9, and in general are similar to those in the presence of IGF-I.

The adult explants exposed to pharmacological concentrations of insulin (1 and 10μg/ml) showed a response pattern slightly different than those with IGF-I. The 54 and 33 year old donors responded to insulin early in the culture period (table 10). The amplitude of the response increased after 9 or 13 days of preculture respectively. In contrast, cartilage explants from the 33 year old specimen did not respond to IGF-I early in the culture period, but responded after 9 days of preculture, similar as was observed for insulin.

^{3}H-Thymidine Incorporation

With respect to thymidine incorporation, the only adult samples which responded with any type of consistency were the 33 and 54 year old at 13 and 9 day cultures in DMEM-BSA respectively. Increases of 165% and 171% over basal medium was observed (data not shown). Thus in these specimens both proteoglycan and DNA synthesis is more stimulated by insulin than IGF-I.

In the child, ^{3}H-thymidine incorporation was sporadic in response to insulin. However in the 15 year old, there was an increase in thymidine incorporation at all culture times and conditions. Increases of 209% and 158% were observed at 3 and 14 days of culture respectively. In the experimental protocol where 10% FCS in DMEM-BSA was utilized throughout the culture period, a stimulation of 217% by insulin was observed.

TABLE 9

Proteoglycan Synthesis of Young Articular Cartilage in Response to Insulin

Days in Culture

	Early		Late		
Donor	**3**	**5**	**9**	**14**	**21**
Newborn		135%*			130%
12 month-old	171%*		89%		
16 month-old		278%*		118%	100%
15 year-old	165%*			210%*/137%+	

Table 9 The table represents ^{35}S-Sulphate incorporation in response to insulin expressed as a percentage of incorporation in unsupplemented DMEM-BSA medium. A comparison between early and late preculture time is made. The value of 137%* is the response of the cartilage explants from the 15 year-old to insulin after a 14 day preculture period in DMEM-BSA supplemented with 10% FCS. The values represent the average response of all the concentrations of insulin used (1-10 µg/ml). The asterix indicates a statistically significant difference at $p < 0,05$.

TABLE 10

Proteoglycan Synthesis of Adult Articular Cartilage in Response to Concentrations of Insulin

Donors	Days in Culture				
	3	5	9	13	15
33 yr-old		146%*		208%*/146%*	
54 yr-old	137%*		208%*		
59 yr-old	124%		59%		
63 yr-old		87%	96%		113%

Table 10 The table represents ^{35}S-Sulphate incorporation in response to insulin expressed as a percentage of incorporation in unsupplemented DMEM-BSA medium. The value of 146%* is the response of the cartilage explants of the 33 year-old to insulin after a 13 day preculture period in DMEM-BSA supplemented with 10% FCS. The values represent the average response of all the concentrations of insulin used (1 and10 µg/ml). The asterix indicates a statistically significant difference at $p < 0,05$.

Platelet Derived Growth Factor (PDGF)

PDGF is generally present in serum as it is released from platelets during the clotting process and thus could account for part of the stimulatory activity of FCS.

Only 3 donors (newborn, 16 month old and 63 year old) were subjected to PDGF. PDGF did not stimulate any proteoglycan synthesis as measured by sulphate incorporation in any of the samples. DNA synthesis machinery on the other hand was turned on by PDGF. More specifically the 63 year old cartilage increased its cell division proportionally to rising concentrations of PDGF at 5 day culture time (figure 19). At this point cartilage explants from the same donor did not respond to insulin with respect to DNA synthesis. The young cartilage used responded to PDGF also after 5 days in culture. Later culture times (14 and 21 days) showed no significant stimulation (data not shown). Thus PDGF may account for some of the effects of FCS on DNA synthesis observed in some cartilage explants. However stimulation of proteoglycan synthesis by serum is most likely due to factors other than PDGF.

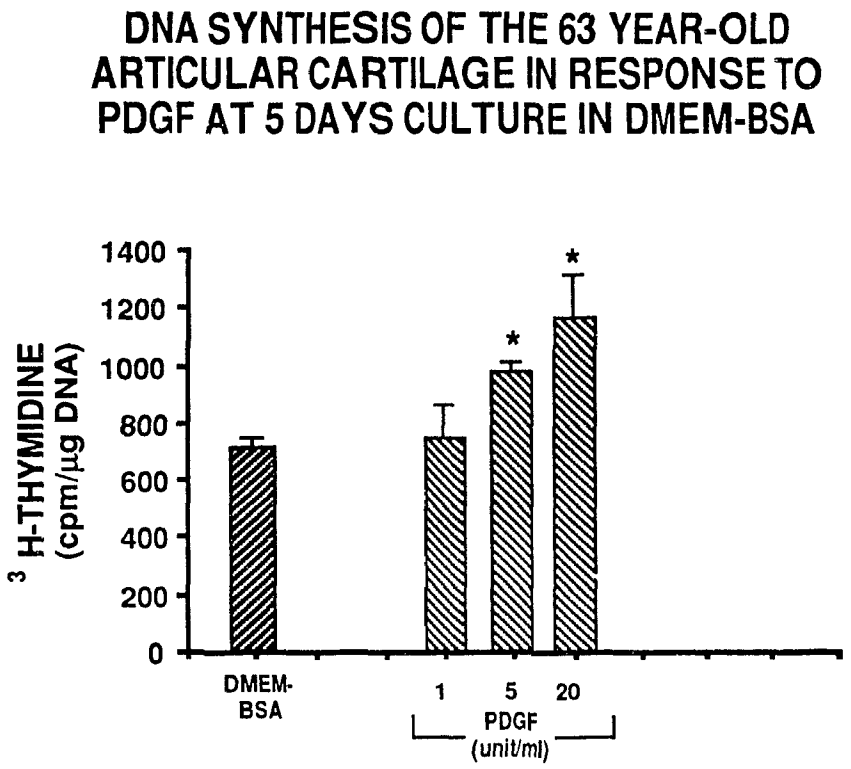


Figure 19 ^{3}H-Thymidine incorporation by cultured cartilage explants from a 63 year-old donor in response to concentrations of 1-20 units/ml of PDGF. The response of the 63 year-old cultured cartilage explant at 5 days to 5 and 20 units/ml of PDGF represents 147% and 152% respectively of the response observed in unsupplemented DMEM-BSA of the same culture period. ^{3}H-Thymidine incorporation is expressed as counts per minute per µg DNA and bars represent an average of triplicates with respective standard deviations. The asterix indicates a statistically significant difference at $p < 0,05$.

4.0 DISCUSSION

Adult articular cartilage in many species including humans is noted for its inability to repair itself following traumatic or degenerative injury. Despite the fact that much work has adressed this question over the years, our understanding for the reasons of this deficiency at the cellular level is still very limited. While mature chondrocytes appear to be able to divide and revert to a chondroblastic state under some circumstances [12,23,24,36,54,55,58], this effort seems to be inadequate for the regeneration of a properly functional cartilage matrix [3,24, 25,29]. Salter [28] and Mitchell [27] have shown that repair of full thickness cartilage defects induced in normal rabbits is possible when continuous passive motion and compression are supplied. Both, however suggested that the neochondrogenesis in the healing of these defects occured through the differentiation of the pleuripotential cells of the subchondral tissue to chondrocytes. Superficial cartilage defects did not heal implying the inability of existing mature chondrocytes to adequately repair structural defects [29]. Even though electron microscope studies show morphological evidence of mature chondrocyte activity [8] this would seem to be entirely devoted to renewal of the extracelluar matrix which is constantly being turned over. It would appear that the mature chondrocyte machine may be too exhausted to participate in the added burden of repair.

Since we believe growth factors which influence cell migration and division as well as the differentiation and synthesis of specific products have a crucial role in connective tissue repair, we chose to study them as a possible clue to the enigma of cartilage repair. IGF-I, the best known mediator of cartilage growth has been shown to stimulate both cell division

and matrix protein synthesis in immature animal chondrocytes [86,87,98,99,108,109]. Very little is known about the response of chondrocyte in adult articular cartilage, particularly humans. We undertook this study to establish whether there is a metabolic response of human articular cartilage to certain growth factors and more precisely whether there is an age related difference.

Histologically, the cartilage is different in the child since the child is involved in growth. There is also a close proximity of the immature chondrocytes to the vascular supply [5,18,19]. These structural differences may play a role in modifying responses to injury but our experimental protocol does not account for this possible variable.

We chose to study cartilage explants since we feel this would simulate most closely an in vivo system. Monolayer cell cultures have been used but the rigorous enzymatic treatment they are subjected to during isolation may alter the plasma membrane of the chondrocyte which may alter the binding characteristics of the cells. Despite the potential for the differentiation of chondrocytes studies have shown that bovine articular chondrocytes can remain phenotypically stable in primary culture for several weeks if they are placed at high density of 1-2 x 10^5 cells/cm^2 [115,184,185]. Therefore if monolayer chondrocyte cultures don't dedifferentiate when placed at high cell densities, one would not expect cartilage explants to do so. Furthermore we chose cartilage explants since the chondrocytes remain in contact with their extracellular matrix. It is well recognized that chondrocyte environmental conditions may exert a significant influence on chondrocyte behaviour [63,186] possibly via a feedback mechanism to stimulate or inhibit metabolism.

The biosynthetic activity of the chrondrocytes was measured using ^{3}H-thymidine and ^{35}S-sulphate as an index of cell division and synthesis of glycosaminoglycan chains of proteoglycans respectively. The relation between sulphate and thymidine uptake and proteoglycan and DNA synthesis respectively has been confirmed by numerous previous studies [12,32-36,47-49]. We tested each of the growth factors in a standard culture system consisting of human fetal lung fibroblast. Table 3 reveals that all concentrations of growth factors along with 10% fetal calf serum (FCS) showed a dramatic stimulatory response, indicating their biological activity. Therefore a negative response to a certain growth factor by the articular chondrocytes would suggest a defect in the chondrocyte mechanism and not the growth factor in question.

Our first set of data (please refer to Table 4) reveals a wide variation of basal metabolic activity in both young and adult cartilage. At the early culture period which simulates most closely basal biosynthetic rate of the chondrocytes, the data demonstrates a 3 to 5 fold difference in thymidine and sulphate uptake between the donors.

Morphological [8], biochemical [187, 188] and metabolic [11] studies all have confirmed that articular cartilage contains a heterogeneous population of cells. Our results demonstrate that within the same age groups cartilage explants showed different rates of thymidine and sulphate uptake. Whether factors such as sex, body structure and state of health are responsible for this variation is not known. Our system however does not permit us to say whether the chondrocytes from the same individual differ metabolically but one must assume they do if we base ourselves on earlier studies [11]. Because of the differing biosynthetic rates of the samples, in

analyzing our results we can only compare them within the same donor and not cross reference between them even though they may be of the same age.

A second point which can be extracted from the data in table 4, is that at early culture period, there is no great difference in the basal biosynthetic rate between the adult and the child. There appears to be no age related difference at early culture period. This is important because it partially eliminates fibrocytes as a source of increased metabolic rate in the young cartilage. It is generally difficult to dissect out fibrous tissue from the joint capsule in children, especially newborns. This could potentially mean our explants may contain some fibrocytes, but our results from our early culture period do not demonstrate that these fibrocytes play a significant role.

In vivo [121,125] and immunohistochemical studies [124] on immature cartilage have suggested that local production of IGF-I may be an important source of growth in cartilage. Our work has demonstrated that as young cartilage explants are cultured longer in unsupplemented media, there is an increase in thymidine and sulphate incorporation. It seems as though the basal biosynthetic rate of the young chondrocytes has increased with increasing culture time. On the other hand, the adult cartilage not only did not show an increase in activity but increasing culture time resulted in a net decrease in metabolic rate. There are two possible explanations for these findings. The first is that the young cartilage cells have the ability to produce an endogenous factor which acts in a paracrine or autocrine fashion to turn itself on. This factor may well be IGF-I. Possibly also with increasing culture time an inhibitor is washed out thus permitting expression of chondrocyte metabolism. This could explain the increased rate we see in

the immature cartilage but not the decreased rate we observed in adult cartilage. Maybe this inhibitor is binding to adult chondrocytes irreversibly and with the possibility that in adult chondrocytes there is a decreased turnover of receptors, there isn't much chance for new receptors to be acted upon. Alternatively maybe to maintain the adult basal biosynthetic rate, some type of endogenous factor needs to be present which we wash out with increasing culture time. The reason we don't see this in young chondrocytes is that these immature cells are capable of producing their own endogenous stimulatory factor. There is also the possibility that with increasing culture time, the adult chondrocytes just die.

It is also apparent from our data that DNA synthesis is the more sensitive parameter. With increasing culture time and without addition of any growth factors, DNA synthesis is more dramatically elevated or depressed in young and old chondrocytes respectively than is proteoglycan synthesis. An explanation for this heightened sensitivity is that the endogenous factor produced is extremely mitogenic. Alternatively, there may be two factors produced, the one responsible for DNA synthesis being more potent. It is well known that dividing chondrocytes do not produce proteoglycans while in the cell division cycle.

The only child donor which did not follow this pattern was the 15 year old whose metabolic rates closely resembled the adult's. The most logical explanation is that the 15 year old cartilage is more mature and displays characteristics similar to those of the adult.

The addition of 10% FCS to culture medium dramatically increased ^{35}S-sulphate and ^{3}H-thymidine incorporation in both young and adult cartilage with increasing culture time. It is not surprising to see the

metabolic activity of young cartilage increase with time in 10% FCS since its activity rose in unsupplemented DMEM. However the 15 year old sample which had shown no increase in unsupplemented medium with time increased its DNA and proteoglycan synthesis by 38% and 153% respectively when FCS was added. Even more impressive was that all the adult donors exposed to 10% FCS increased their DNA and proteoglycan biosynthesis (see Figure 9 and 11). These results are in direct accordance with McQuillan's study [76] which showed that FCS stimulated proteoglycan synthesis in adult bovine articular cartilage. There must be some substance or substances in FCS which are able to stimulate metabolic activity in the adult cartilage. The mode of action of substances in FCS may be by direct binding to chondrocyte receptor and therefore turning on the biosynthetic machinery, or indirectly by binding to an inhibitor and allowing expression of the chondrocyte. By increasing culture time, we may also be washing out an inhibitor and allowing FCS to work.

FCS contains a host of nutrients, amino acids, and growth factors all of which may be responsible for increasing the synthetic rate of the chondrocyte. McQuillan in another study [75] showed that addition of a monoclonal antibody against insulin like growth factor (IGF-I) prevented FCS from stimulating proteoglycan biosynthesis suggesting quite strongly that the presence of IGF-I may be very responsible for the increased proteoglycan metabolism seen in adult human articular cartilage exposed to FCS.

However, the most important feature drawn from these results with FCS is that adult human cartilage has the capacity after 9 to 15 days in culture to be metabolically turned on. The adult human chondrocyte still has

the biosynthetic machinery at its disposal to synthesize DNA and proteoglycans provided the appropriate stimulus is present. This situation might be analogous to the increased biosynthesis of proteoglycans observed in early osteoarthritis.

IGF-I evoked an increase in ^{3}H-thymidine and ^{35}S-sulphate uptake in young articular cartilage at early culture period. Longer culture periods did not show as pronounced stimulatory effect. This discrepancy could be explained by the fact that young cartilage at prolonged incubation without addition of growth factors is already stimulated. Therefore since this young cartilage is already responding to some type of endogenous factor it may already be maximally stimulated and further addition of IGF-I would serve only to increase its rate slightly.

In the adult there is basically no stimulation by IGF-I. However, three of our adult donors (33,54,57 years old) after 9 or 15 days in unsupplemented media, responded somewhat to the addition of IGF-I. It would appear the adult chondrocytes are more responsive at this later culture time period. A possible explanation would be that with increasing culture time, we are washing out the presence of a protein inhibitor binding to IGF-I surface cell receptor or IGF-I binding proteins thereby permitting action of IGF-I on the adult chondrocyte at this later time period.

Many investigations have revealed that immature chondrocytes readily increase their metabolic rate in response to IGF-I [86,87,98,99,108,109]. Our data has also revealed this, expecially at early culture period and a logical explanation would be that the young chondrocyte has more functional surface cell IGF-I receptors than the adult. However, studies have shown that plasma IGF-I levels are lower in infant and young children yet more

rapid skeletal growth occurs [87]. Since cartilage from young animals is more sensitive to IGF-I than is cartilage from older animals [107, 110], it is possible young chondrocytes are more responsive to IGF-I. This possiblity is supported by the observation of increased binding of IGF's to human newborn mononuclear cells [111]. Therefore it may appear that the difference in response to IGF-I in young vs adult chondrocytes may reside in a qualitative and/or quantitative change in IGF-I receptors.

Insulin at pharmacological concentrations has traditionally stimulated various anabolic responses in chondrocytes as do physiological concentrations of IGF-I [72,86,93]. This is believed to be a result of weak insulin interaction with the IGF-I receptor [98,113]. However the chondrosarcoma rat model revealed the ability of insulin to act at physiological concentrations suggesting insulin is acting via a classic insulin receptor [176]. The increased sensitivity of the chondrosarcoma chondrocyte may be a property of the transformed phenotype since malignant cells in culture show a greater sensitivity to insulin than control cells with respect to stimulation of growth. Our data shows that cultured cartilage explants behave the same way to pharmacological concentrations of insulin as they do to IGF-I. Some of our adult donors were more responsive to insulin at prolonged culture time which would satisfy the hypothesis that increasing culture time may be washing out an inhibitor binding to surface cell receptors. The young cartilage was more responsive at early culture time than late culture time because perhaps the young chondrocytes are maximally stimulated at later culture time and cannot sustain more of a response to an already heightened response. Our results would tend to suggest that insulin is acting weakly via an IGF-I receptor.

The interesting result obtained from our insulin studies is that both sets of cartilage showed some mitogenic response to insulin. Prins et al [142] showed insulin when added along with other growth factors, stimulated DNA synthesis, suggesting a synergistic action. Despite the fact we did not externally add any growth factors, this does not rule out the possibility that growth factors were endogenously produced in our culture system.

Finally platelet derived growth factor (PDGF) was barely used secondary to cost but when it was utilized the adult and young cartilages responded by increasing its DNA synthesis. There was no stimulation of proteoglycan synthesis and along with the mitogenic response observed, this correlates well with previous results [74,141,142]. The stimulation observed in the 63 year old cartilage explants in response to PDGF suggests this growth factor may be one of the components responsible for the stimulatory effects of FCS. However the magnitude of the response to FCS as compared to PDGF suggests that other factors are required as well.

4.1 Conclusion

1. Despite the fact our experimental protocol is an in vitro system our results are very much in accordance with prior in vivo animal studies suggesting our cartilage explant system is a reliable indicator of in vivo studies.

2. There is a marked variation of basal metabolic activity in articular chondrocytes of the same age group.

3. Young human articular cartilage seems to have the ability to act in an autocrine or paracrine manner to stimulate its growth in vitro as demonstrated by its ability to increase its basal incorporation of sulphate

and thymidine in an unsupplemented culture medium over time. This may be due to the young chondrocytes ability to secrete an endogenous stimulating substance or an unknown inhibitor may have been washed out with time.

4. Young human articular cartilage explants are stimulated by IGF-I, insulin, and FCS to incorporate both sulphate and thymidine at early culture periods. Even though the stimulatory effect is not as apparent at later culture time, one must realize that the young cartilage may already be responding to the presence of an endogenously synthesized factor.

5. Adult human cartilage is not significantly stimulated by insulin or IGF-I. Whether this lack of response is due to a lack of receptors or an inhibitory protein binding to a surface receptor remains to be established.

6. Adult human articular cartilage explants can be stimulated by FCS to synthesize an extracellular matrix. Whether the effect of FCS is due to a single component such as PDGF, or multiple components which act synergestically is not known at the present.

7. Adult articular cartilage explants appear to become slightly more responsive to either insulin, IGF-I, and FCS after a prolonged culture period in the absence of growth factors. Whether this is due to the presence of a protein binding to surface cell receptor which is washed out with time or some other mechanism remains to be established.

8. Adult human cartilage is not totally refractory to external factors.

Suggestions for future work could include analyzing the culture media after prolonged incubation time to try to determine what is being produced by the young chondrocyte which may be stimulating their metabolic activity. Fetal calf serum should be further analysed to try to determine which components are responsible for this increased acitivity. Possible

addition of specific monoclonal antibodies to a preparation of FCS may help solve this puzzle.

Growth factors are known to act in synergy, therefore synergistic studies should also be undertaken to see if one growth factor potentiates the effect of another. The addition of transforming growth factor β to the explant system which is proving to be a very exciting growth factor should also be investigated.

Receptor studies on the chondrocytes will enable us to take a giant step in understanding the control mechanisms in the biomachinery of the chondrocyte. Ultimately we hope to manipulate the chondrocytes such that they behave in a repair oriented manner.

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