Growth Factors in Tissue Engineering the Knee Meniscus

by

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ABSTRACT

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Tissue engineering is a promising solution to creating a replacement meniscus. The structure and composition of the meniscus lends the tissue its ability to withstand tension and compression. Enhancing extracellular matrix production in an engineered construct, may improve mechanical properties of the construct. The goal of this study was to determine the growth factors that would most increase the production of collagen and glycosaminoglycans (GAGs) produced by meniscal fibrochondrocytes. Cells were studied in monolayer and in three dimensional cultures. The growth factors and concentrations evaluated were: TGF-β1 (1, 10, 100 ng/ml), IGF-1 (5, 12.5, 50 ng/ml), PDGF-AB (10, 25, 100 ng/ml), and bFGF (10, 25, 100 ng/ml). TGF-β1 (at 10 and 100 ng/ml) was the only growth factor that showed an increase in both collagen and GAG component uptake in both culture conditions as indicated by radiolabeling. TGF-β1 showed the most increase in component uptake over the control and over the other growth factors and is recommended for use in tissue engineering the knee meniscus.
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Knee Meniscus Background:

A review of the cellular, biochemical, and biomechanical variations within the tissue.

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Abstract

To fully understand the function of the knee meniscus, it is important to define the characteristics of its structure and composition, and how they vary spatially within the tissue. The meniscus is an excellent example of how structure can confer function in a mechanical device. The goal of this review is to summarize current findings related to meniscal properties. Cellular, biochemical, ultrastructural, and biomechanical characteristics are compared in the different zones, depths, and topographical regions of the knee meniscus. It is important to note the wide variety of species and testing methods that have been used to evaluate such properties. These variations render difficult direct comparisons of the properties of this anisotropic and inhomogeneous tissue. Understanding the structure-function relationships within the meniscus will lead to better design of replacements for this tissue.
Background and Motivation:

In the past, the meniscus of the knee was regarded as a tissue not crucial to the functioning of the tibio-femoral joint. Partial or complete meniscectomies were and are often the treatment of choice for a damaged knee meniscus. In 1996, there were 479,000 meniscectomies performed in the United States (as reported in the 1996 National Hospital Discharge Survey and the 1996 National Survey of Ambulatory Surgery). However, the meniscus protects the articular cartilage from extreme loads (36), and meniscectomies leave the articular cartilage susceptible to degeneration (14, 34), which can eventually lead to arthritis. In light of the growing amount of experimental data on the meniscus, and its role in the protection of articular cartilage, it is now thought that alternatives to meniscectomy should be explored (27). The significance of the meniscus in joint functioning, and therefore physical comfort, cannot be ignored. Strides are underway to engineer a suitable construct that could fully replace the meniscus. To accomplish this, a set of salient standard characteristics of the meniscus must be established. However, the tissue of the meniscus is quite intricate and its properties vary spatially within a given meniscus. By understanding these differences and how they relate to function, design of a replacement will be facilitated.

Basic Properties of the Meniscus:

Anatomy and Function:

The knee meniscus is a fibrocartilaginous tissue that sits between the rounded femoral head and the flat tibial plateau, enhancing the fit between the two by increasing the tibio-femoral congruence (8, 49). The unique composition of fibrocartilage (mainly collagen,
proteoglycans, and water) allows the meniscus to perform several significant duties in the diarthrodial joint. These duties include: load distribution (5, 10, 24, 27, 36, 37, 44, 54, 62), joint stability (62), shock absorption (6, 30, 66), friction reduction (6, 25, 66), and providing nutrition to the articular cartilage (21).

The entire meniscus is composed of two semi-lunar menisci: the medial and the lateral, each of which is concave on the superior surface to fit the femur and flat to convex on the inferior surface to fit the tibia (Fig. 1). Each meniscus is positioned in the joint so that the interior edge of the curve faces inward, towards the other meniscus. Going from the exterior to the interior, the meniscus gets thinner between its upper and lower surfaces, and therefore serves as a wedge, which has important mechanical consequences.

**Attachments:**

There are several key attachment points within the meniscus which are crucial for its functioning. Different species vary in the exact nature of their meniscal attachments, but there are several generalities. There is a posterior and anterior horn on each meniscus, and these are attached to the bone via insertional ligaments which are near the posterior and anterior crucial ligaments respectively (23, 57). The medial and lateral menisci are attached to each other at their anterior regions via the transverse ligament (23, 57).

Meniscofemoral ligaments serve as attachment to the medial femoral condyle (8, 57). There are two of these ligaments: the anterior is referred to as the ligament of Humphrey and the posterior is referred to as the ligament of Wrisberg (8). The presence of the ligaments of Humphrey and Wrisberg is noted to vary between species and individuals
Attachment of the meniscus to the joint capsule is through the periphery of the meniscus (25, 26). All of these attachments serve as anchors for the meniscus, and hence have important mechanical consequences.

**Vasculature and its Effect on Meniscal Repair:**

The vasculature of the meniscus is crucial in determining its healing capacity in response to an injury. The meniscus has blood supply to its periphery, but most of the tissue is avascular (8, 25, 26). Blood is delivered via the inferior and superior genicular arteries which branch in the perimeniscal capillary plexus that is located in the synovial and capsular tissue (8). The vessels of this capillary network work their way through the peripheral attachment of the joint capsule into the meniscus itself where they branch and then end in small loops (8). These branches only extend 10-30% into the interior of the meniscus, meaning the bulk of the tissue is left with no blood supply and must depend on diffusion for transport of nutrients and waste (8, 25). The anterior and posterior meniscal attachments are also well-vascularized, with blood supplied by the middle genicular artery (8). This uneven and limited distribution of blood means that the meniscus has a variable ability to heal itself (49).

Healing in the meniscus depends on the vascularization at the site of the tear (35, 49). If injured, the meniscus can repair itself only if there is access to the blood supply (34, 35). Therefore, injuries in the avascular zone, where there is no blood supply, are significant considering that less than 20% of the meniscus is vascularized by the time a person reaches the age of 40 years, a marked decrease from the 50% vascularization in a
newborn (46). If a tear is left untreated, or if no meniscal regrowth naturally occurs, loss of normal meniscal functioning can cause the articular cartilage covering the femur and tibia to be damaged (11). If regenerated tissue does form, its mechanical integrity may not be that of the natural tissue (34, 44). This is because tissue that forms after a tear usually shows randomness in collagen alignment (44). As will be described later in this review, it is the precise ultrastructure of the collagen fibers that confers mechanical integrity to the meniscus.

Besides vascularization, the organization of collagen fibers has a key role in determining the outcome of an injury. Injuries of the meniscus fall into two main categories: traumatic tears and degeneration (49). Tears occur in different forms (longitudinal, radial) and hence have different healing properties based on their orientation (Fig. 2). This difference in healing is due to the orientation of collagen fibers. Longitudinal tears (between circumferential fibers) heal more effectively than radial tears (across circumferential fibers), meaning the resulting tissue has better mechanical properties (44). Degenerative changes in the meniscus can also occur. This is a slower process of tissue destruction due to a change in tissue composition, but can ultimately lead to tears and articular cartilage degeneration. The difference in healing due to tissue architecture is an excellent indication of the role that structural properties play in meniscal functioning.

**Zones, Depths, and Regions in the Meniscus:**

In this review, several aforementioned properties of the meniscus will be compared in the different zones, depths, and regions of the meniscus. Zones of the meniscus can be
delineated in terms of vascularization (Fig. 3a). As mentioned above, the outer third (10-30% for the medial) of an adult human's meniscus is vascularized (8, 25). This outer third is called the red zone of the meniscus, or simply, the outer meniscus. The middle zone, or the red-white zone, of the meniscus represents the transition between the vascular and avascular zones. The inner zone of the meniscus, the edge nearest the interior, is completely avascular and is therefore referred to as the white zone or the inner zone of the meniscus. Topographical variations are also discussed in this review (Fig. 3b). Any regional variations in these properties of the meniscus will be discussed in terms of anterior, central, and posterior location. Material differences also exist between various depths of the meniscus (Fig. 3c). There is the superior surface with an associated lamellar layer (facing the femur), the middle/deep zone, and the inferior surface with an associated lamellar layer (facing the tibia).

Besides spatial variation within one meniscus, it is also important to note that the properties of the meniscus are dependent on factors such as species (59), degree of degeneration (29, 32), and age (32). For instance, it has been shown that the number of cells in the tissue decreases with age in humans (40). This same study also showed that the diameter of the collagen fibers changed and became less consistent as the subject aged. It is also known that the water content of menisci increases with the degree of degeneration within the joint (29). Different investigators use different animal models, methods and techniques to study the meniscus, rendering difficult comparisons between studies. Sweigart et al. (59) showed significant variations in mechanical properties that exist among different species. With all these possibilities for experimental variation, it is
often difficult to generalize properties of the meniscus. It is understood that the meniscus is not a homogeneous, isotropic material. There is great variation within the tissue as a result of its constituents and this leads to its unique functions. It is the aim of this review to compare meniscal properties on the following levels: cellular, biochemical/ultrastructural, and biomechanical. One will see that each category is dependent on the other categories. Cells make the biochemical components of the matrix, and these components and their architectural arrangement define the mechanical properties of the tissue.

**Cellular Variation in the Meniscus:**

Identifying categories, or sub-populations of cells, in the meniscus is relatively difficult. The shape of the cells and their phenotypic properties depend on the zone and region in which they are located, and also the species under consideration. It is now generally accepted that the cells of the meniscus are best categorized as fibrochondrocytes, although for some time researchers tried to strictly categorize the cells as either chondrocytes or fibroblasts (26). The term fibrochondrocyte is representative of the fact that these cells exhibit properties of both chondrocytes and fibroblasts. The cells are round with a territorial matrix, characteristic of chondrocytes, and they also secrete Type I collagen, characteristic of fibroblasts (39, 58). Yet, the exact nature of the cells varies according to their location in the tissue.

For some time, meniscal cells were put into two categories: those from the superficial layer, and those from the deeper zones. Although a more specific categorization has
hence been defined, these two classifications still represent the general cellular characteristics of the meniscus. The cells in the superficial layer are generally fusiform and parallel to the surface \((26, 28)\). These fusiform cells in the rabbit and the human have relatively little cytoplasm, which makes the nucleus appear prominent \((25, 26)\). For the most part, superficial cells either lack or have very few projections \((25, 26, 28)\). However, cells that seem more like fibroblasts have been detected in the surface of human menisci and these have long projections at their poles \((25)\). Superficial zone cells contain rough endoplasmic reticulum (RER), mitochondria, and Golgi, but glycogen and lipid particles are rare \((25, 26)\). There is no territorial matrix around most of these cells and therefore the collagen fibrils of the general matrix are able to touch their surfaces \((25)\).

The cells of the deeper layers are polygonal and rounded \((3, 26)\). Some of these cells exhibit a territorial matrix \((3, 25)\). Clusters of these cells have been detected, although they are composed of only a few cells \((26)\). These deep zone cells have relatively numerous cell processes \((39)\). The RER and Golgi apparatus are well-developed, and mitochondria are also present in these cells \((26)\). Glycogen and lipid particles have also been detected in this zone \((25, 26)\).

 Recently, a more distinctive characterization of the cells in the rabbit meniscus has been elucidated. There are now four different morphologies, correlating to four different regions of the rabbit meniscus, that have been determined, indicating yet another facet of structure-function relationships in the meniscus \((28)\) (Fig. 4). Cells of the red zone (outer
rim) have many long, branching projections (28). It can be seen that the cytoskeleton of these cells extends into the projections. These projections allow interactions with the extracellular matrix (ECM) and also with other cells (28). Cells in the red and red-white regions are seen to align themselves in rows. Like the cells of the red zone, cells in the red-white zone have projections (which are fewer in number) (28). The projections of the cells in these two regions serve to connect the rows of cells, therefore forming connected sheets (28). These sheets are formed at different angles throughout the matrix (28). The cells rely on signals from their cytoskeleton about the state of the ECM, and the projections may facilitate this signaling process by creating a greater integration of the cytoplasm into the ECM. By bringing cells close to each other, the projections are also thought to help the formation of gap junctions, which are seen in these two zones (25, 28). Contrary to the random direction of projections in the red zone, projections from the cells of the red-white zone all point in the same direction, which may be a consequence of the tension that is present in this zone (28). Cells in the white zone of the meniscus are quite different from the two previous zones. These cells are round, evenly distributed, and do not have projections (28). Instead of a cytoskeleton that extends in projections, these cells have microtubules that form a complex network. These cells also lack gap junctions (25, 28). Because cells of the white zone are mostly under compression, and cells of the red and red-white zones are mostly under tension, gap junctions might therefore be more important in the cells’ ability to withstand tension than compression (25, 28). Cells found in the surface of the meniscus make up the fourth category of cellular classification. These cells, are fusiform in morphology and do not have projections (25, 28).
Other unique cell types have been detected in various areas of the meniscus. Mast cells, which are a form of leukocytes, have been found in the periphery (25). These are indicated by the presence of electron-dense granules and, at times, many cell processes (25). If a tear is present in the meniscus, quite often it is associated with the presence of myofibroblasts (3, 25). Accordingly, these cells have not been visualized in normal, uninjured portions of the tissue (25). It should be noted though that, using immunohistochemistry, it has been demonstrated that there seems to be an even distribution of smooth muscle actin (SMA)-containing cells (cells like myofibroblasts that are involved in contraction) throughout the matrix of the human meniscus (3). The authors suggest that only the polymerized form of actin, which would be made by a cell actively contracting (i.e., near a tear), has been detected by previous studies due to limitations of techniques like TEM. They propose that these cells are present throughout the matrix but may only contain SMA in its monomeric form which is undetectable by TEM (3). More research in this area is required to clarify this issue.

As would be expected of any tissue, there are also degenerate and necrotic cells found in fibrocartilage (25). In situ necrosis is thought to occur in both normal and injured portions of meniscal tissue (25). When cellular degradation occurs, the debris left behind is usually of an electron-dense and granular lipidic nature (25). This type of material has been found in lacunae and the general matrix of the tissue (25), indicating that if a cell undergoes in situ necrosis, the debris will migrate into the general matrix.
Several studies have found that some of the cells exhibit a territorial matrix (also called a pericellular matrix or juxtacellular matrix) (25, 26). This matrix consists of protein-polsaccharides, short filaments, and unbanded fibrils (26). The matrix of fibrochondrocytes is also more fibrous than that found in hyaline cartilage (25). Because it is not very abundant, this territorial matrix is usually seen to surround only part of the cell (25).

As mentioned previously, there is little to no blood supply to the meniscus tissue, consequently, the cells are sparse. The cells must rely on diffusion for receiving nutrients and disposal of waste. However, the cells are still capable of secreting an ECM. The fibrochondrocytes of the meniscus are obviously not uniform throughout the tissue. Different cell morphologies are seen in different locations. It is likely that these different cell types provide the proper ECM components required for withstanding the mechanical forces that are prevalent in their region of the meniscus.

**Biochemical and Ultrastructural Variations in the Meniscus:**

Even though the cells of the meniscus are scarce, the ECM they produce provides the meniscus with many of its unique mechanical properties. There are several major biochemical constituents in the meniscus ECM. These components include collagen, proteoglycans (PGs), and glycoproteins (GPs) (25, 39). The exact types and amounts of each of these molecules depend on the species being considered and also on the region or zone of the meniscus. As will be discussed in the biomechanical section of this review, the function of the meniscus relies heavily on the ultrastructure and distribution of its
biochemical constituents. Not only are the amounts of each biochemical component important, but also their precise arrangement and distribution allow the meniscus to fulfill its unique mechanical role within the knee joint.

The hydrated meniscus is approximately 70-75% water, 20% collagen, and 0.6% glycosaminoglycans (GAGs) (29, 32). Therefore, the major component of the ECM in the meniscus is fibrillar collagen which, as will be discussed in the biomechanics section of this review, is important in bearing tensile loads. Collagen and elastin are the two types of fibers present (25, 43). Of these two fibrillar components, collagen is the dominant one (43, 53). Making up 60-70% of the dry weight of the tissue, collagen has been found as Types I, II, III, V, and VI (although VI is not a fibrillar component) (18, 32, 39, 46). The orientation of the fibers varies throughout the meniscus and this ultrastructure is significant in it effects on the mechanical properties. Collagen fiber orientation is depth dependent, the largest differences being found between the surfaces and the middle/deep zone (25) (Fig. 5). The surface of the meniscus has collagen in random orientation (25, 47, 66). Underneath the surface layer is the lamellar layer. This layer also has randomly oriented collagen, but at the periphery of the posterior and anterior portions there are short fibers that are oriented in the radial direction (47). Within the next layer, the deep zone, the collagen fibers are aligned parallel to each other in a circumferential direction (47). These parallel fibers have amongst them a few fibers in the radial direction. These are called tie fibers and may be significant in the prevention of tears (10, 42). Although collagen fibers comprise the majority of the fibrillar component in the meniscus tissue, there are other fibers present.
Both immature and mature elastin fibers have been found in the general matrix (i.e., excluding the matrix immediately surrounding the cells), yet their function is less well understood and they comprise 1% or less of the dry weight of the tissue (25, 46). It is known that elastin is able to withstand large strains and can return to its original conformation after it has been stretched (38). Therefore, one suggestion for the purpose of elastin in the meniscus is that it might serve to return the meniscus to its original dimensions after deformation (38). It is also possible that elastin may contribute to the resiliency of the tissue (38). The fibrillar component of the tissue is necessary in order to support tension in the meniscus.

The ability of the meniscus to withstand compression is a consequence of the presence of PGs (43). PGs are basically protein cores that have GAGs attached to them and may or may not have the ability to form aggregates with hyaluronate via covalent attachment (1). Generally speaking, the meniscus has two types of PGs: large and small, which are further differentiated by their specific chains and binding capabilities. The large PGs have abundant chondroitin sulfate and keratan sulfate GAG chains attached to their protein core (1). These PGs form aggregates by means of binding at their globular ends with hyaluronic acid, which is an unsulfated GAG (1, 65). A link protein is usually used to stabilize these aggregates (1). The small PGs have only a few chondroitin sulfate or dermatan sulfate GAGs attached to their protein core and do not form aggregates (1, 39).

Overall, the purpose of the PGs and their GAGs is to provide compressive resistance (33) by attracting water to the tissue, and also to interact with the collagen (52). The dominant
GAG chains of the large PGs in meniscal tissue are chondroitin 6-sulfate and chondroitin 4-sulfate (2, 29, 56), although there is some amount of keratan sulfate (43). Because they have carboxyl and sulfate groups on their GAG side chains, these large PGs are polyanionic, and can interact with hyaluronic acid (39, 43). The hydrophilic characteristic and negative charges of the large PGs also attract the water needed to withstand compression (1, 21). The function of the small PGs is not exactly understood, but it is thought that they might regulate the diameter of the collagen bundles (52), aid in the prevention of soft tissue calcification (51), and also prevent fibrillogenesis of collagen (61). Biglycan and decorin are the two types of small PGs that exist in the meniscus (50, 53). These molecules differ in the number of GAG chains they have: decorin has one GAG chain, biglycan has two (1).

Besides PGs and collagen, there are non-collagenous matrix glycoproteins (GPs) in the ECM of the meniscus. These GPs include the link proteins (20), adhesive proteins (39), and a 116kDa protein of unknown function (20). The link proteins stabilize PGs with hyaluronic acid in articular cartilage and might serve similar roles in the meniscus (20). The adhesive GPs bind to other matrix molecules and cell surfaces (39). These adhesive molecules include: collagen Type VI (67) and thrombospondin (41). Thrombospondin has the RGD (arginine-glycine-aspartate) sequence that is shown to be important in binding (39). Because of these characteristic RGD adhesion sites, thrombospondin might be important in cell-matrix interactions (41). The 116kDa protein is found near collagen bundles, and it is hence thought to interact with them (20).
The arrangement and distribution of the fibers, PGs, and GPs within the matrix allows a variety of forces to be withstood by the meniscus. As Eyre et al. (17) point out, looking at differences in biochemical content between the meniscus and articular cartilage leads one to see a relationship between structure and function. The same comparison can be performed within the meniscus itself, showing that the distribution of matrix constituents varies throughout the different regions, zones, and depths of the tissue, therefore allowing different forces to be supported.

Several studies have shown regional variations in relative PG and collagen content within the meniscus. Nakano et al. (43, 53) looked at the collagen, PG and GAG content in the inner, middle, and outer zones of the pig meniscus. Their results showed that the meniscus is 76% collagen (dry weight) and 8% GAG in the inner zone and 93% collagen and 2% GAG in the outer zone, clearly indicating that collagen is the major component in the tissue. The middle zone was in between these two values, so that collagen and PG content have an inverse relationship that varies continuously across the width of the meniscus from the inner to outer zone (43). Because of its wedge shape, the meniscus bears its highest compressive load at the inner zone, and the highest tensile forces at the outer zone. These types of stresses seem to be correlated to the relative amounts of biochemical components at these sites: the highest relative GAG content and lowest relative collagen content is at the inner zone where compression is highest (43). More specifically, Nakano et al. (43) found that chondroitin sulfate is 5-6 times higher in the inner zone than the outer zone, and keratan sulfate is also higher at the inner zone, again suggesting that sulfated GAGs are important in compression (43). To support tension,
collagen is relatively higher and GAG content relatively lower in the outer zone where tension is dominant (43). Similar biochemical content relationships exist in the human meniscus, where significantly more sulfated PGs exist in the inner two thirds of the meniscus vs. the outer third (60). In the bovine meniscus, Eyre et al. (17) found differences in relative PG content that varied with depth and topographical location. About twice as many PGs were found on the surface than in the deeper zones, and there were more PGs in the posterior regions. Generally speaking, PGs/GAGs appear significant in compression and collagen is related to tension. These components also vary somewhat in their specific composition throughout the meniscus.

The specific collagen types show spatial variation throughout the meniscus. Major collagen differences are found when comparing the inner third of the tissue to the outer two thirds. In the bovine meniscus, collagen types I and II are found in the inner region, although type II dominates (60%) (12). However, tissue in the outer two thirds is almost entirely type I (80%), with trace amounts of types III and V (12). Differences in types of collagen have also been found in the different depths of the meniscus: type V collagen is seen to be about three times higher in the surface than in the interior (17). The different proportions of collagen types in the various sites of the meniscus may indicate the importance of specific fibers in bearing different amounts of tension.

Looking at the distribution of the specific types of PGs/GAGs, only some regional variation is seen. In the canine, Adams et al. (2) found that chondroitin 6-sulfate is the dominant GAG (55-60%), while there are lesser amounts of chondroitin 4-sulfate (25-
27%) and non-sulfated chondroitin (9-13%)(2). Although the absolute amounts of GAGs varied throughout the meniscus, the proportions of the GAGs in each region did not appear to vary (2). The proportion of hyaluronic acid to chondroitin sulfates was also found constant for the regions (2), which further supports the fact that these two molecules form aggregates. However, derman sulfate is seen to vary in concentration throughout the meniscus (2). The inner third of the porcine meniscus has been shown to have a ratio of derman:chondroitin sulfate in the range of 1:5 to 1:6, while the outer third shows a ratio of 1: 3/2 (43), showing a dramatic increase in the relative amount of derman sulfate in the outer region. Scott et al. (53) looked at the distribution of the small PGs decorin and biglycan in the porcine meniscus. They found that biglycan is the more abundant of the two and that the highest amount is in the inner two thirds of the meniscus, indicating that it may be important in compression. The functions of the different GAGs, large and small, may be better understood if their relative amounts are related to where they are located in the tissue.

**Biomechanical Function and Variations in the Meniscus:**

**The Functions of the Meniscus:**

The meniscus is an excellent example of a tissue that uses its structure to perform specific functions. Because of its wedge shape, the meniscus experiences tensile, shear, and compressive forces. These forces are transmitted through the matrix of the tissue, which is largely composed of collagen fibers, dispersed in different orientations depending on their location within the meniscus, and also proteoglycans. When the femur presses down on the meniscus, the compressive force is translated into tensile stress in the
circumferential direction which is supported by the collagen fibers (10, 66). This tensile force is the result of the meniscus being displaced, while at the same time being anchored by its attachments in its anterior and posterior regions (27). Therefore, hoop stresses in the circumferential direction are caused by a compression of the joint (36, 54, 66), as are shear forces between the fibers (57). The ways in which these forces are distributed in the tissue are directly related to the geometry of the meniscus, and the resulting loads are withstood by a unique arrangement of molecular constituents. As previously described, it is the unique molecular constituents and ultrastructural arrangement of the meniscus that cause regional and zonal variations in its mechanical properties. A description of the various meniscal functions, followed by a comparison of the mechanical properties of the different regions and zones of the meniscus is provided in this section.

The meniscus tissue is biphasic, containing a solid component and a fluid component (19, 48). The fluid portion is water and salts, while the solid portion is a porous permeable matrix of mostly collagen and proteoglycans (48). The meniscus is approximately 75% water by weight (19, 21, 48). This composition lends fibrocartilage viscoelasticity, which means it is compliant and that its deformation is dependent on the size of the load and the rate of loading (36, 68). The negatively-charged GAGs in the tissue attract water. When a force is applied, some of the water is forced to leave the matrix. Fluid flow results from this applied load, and the tissue deforms accordingly (19, 21) (Fig. 6). After the force is removed, the water is allowed to once again associate with the negative charges, rehydrating the tissue.
Creep and stress-relaxation are also seen to result from frictional drag that is caused by this fluid flow (21). When exposed to a constant shear strain, the stress in the meniscus relaxes (68). Creep under confined compression is due to fluid leaving the tissue (48). Therefore, even if there is an increase in stress, the fluid will redistribute in order to relieve it (21). Zhu et al. (68) analyzed the bovine meniscus and found that frequency and both shear and compressive strains affect the viscoelastic shear properties. They found the dynamic shear modulus increased with compressive strain, meaning the tissue got stiffer. This stiffening behavior was also seen by Krause et al. (36) in their compression studies of canine and human menisci. However, Zhu et al. (68) saw that as the shear strain increased (in the range of 0.5-5% strain), the dynamic shear modulus decreased almost two times, meaning the tissue got softer. This softening of tissue under shear is needed in order for the meniscus to conform to changes in the position between the femur and tibia as the joint moves (68). Compared to articular cartilage, the meniscus has a dynamic shear modulus about ten times less, which also supports the idea that softening under shear is needed to accommodate changes in shape in the joint (68). Therefore, the biphasic nature of the meniscus allows it to increase congruency within the joint. Congruency is not the only purpose of the fluid-solid interactions. The inward and outward flow of fluid provides lubrication and nutrition transport to the articular cartilage (21). It is evident that the structural characteristics of the meniscus interrelate and provide various functions within the joint.

Not only is the meniscus able to change shape in accordance with the demands of the joint, its wedge shape and position within the joint allow it to improve the fit between the
femur and the tibia (54). Joint stability is a result of this improved fit between the femoral condyles and the tibial plateau (49, 54). With the presence of the meniscus, stresses are placed on the edge of the tibia, where the articular cartilage is not covered by the meniscus. Without the meniscus, this stress would be on the center of the tibial plateau. This would mean a decrease in the lever arm through which loads act, and therefore, less stability (62).

Another main function of the meniscus is to bear a significant amount of the load in the joint. The exact amount reported varies but values have been given in the range of 45-75% (4, 5, 54). Reported values show variation due to animal models used, degree of knee flexion/extension, and degree of degeneration. This load-bearing function is partially achieved by the increase in surface area which the meniscus provides the two articulating surfaces, with the meniscus allowing up to 70% contact within the joint (54, 62). Therefore, the meniscus can protect the articular cartilage by increasing the surface contact area and allowing less stress on the tibial plateau. Contact area between the bones in the joint depends on the angle of flexion and rotation, decreasing 4% for every 30° of flexion (63), and this will cause the amount of stress applied to the joint to vary. A meniscectomy decreases contact area and puts 2-3 times more stress on the articulating surfaces (6, 36), which is why alternative healing approaches are desirable. Walker and Erkman (62) showed the importance of contact area on the load bearing properties of the human meniscus. They found that with no load applied, only 10% of the articular cartilage on the ends of the femur and tibia was in contact with each other, and that there is an average space of 1mm between most of the articular cartilage. In this study, the
medial meniscus was shown to bear loads equally with the exposed cartilage beside it, while the lateral meniscus supported almost all the load exposed to it, up to about 100-150kg. The net result is that the meniscus bears the majority of applied loads up to a certain loading point. Articular cartilage covered by the meniscus will be under some load most of the time, however, exposed articular cartilage (i.e., not covered by the meniscus) will only experience increased force when higher (~150kg) loads are applied. It has also been shown that the meniscus geometry is crucial in it mechanical function. Proper geometry allows the meniscus to function as a spacer between the femur and tibia and to also evenly distribute loads (6, 21). Load-bearing is not only a function of contact area and geometry, it also depends on the composition of the tissue. The meniscus is able to withstand compressive loads also because of its PG content (21). Some of the PGs are large and have a negative charge. These PGs are hydrophilic and therefore attract water (21). It is the presence of water that allows the meniscus to bear its compressive loads.

Because it is exposed to large loads, the meniscus must also function as a shock absorber. This property may be due to the structure of the collagen bundles which are composed of collagen fibers of different diameters (25, 26). This variation in fiber size allows for a range of different vibration frequencies to be absorbed (25). The shock absorption properties of the meniscus have been indicated by studies that show a 2-3 times increase in the elasticity modulus (E) of the joint when the meniscus is removed (24, 37), as well as an increase in the amount of energy dissipation (37). It has also been shown from compression studies that bovine meniscus cartilage is 1/2 as stiff and 1/6 as permeable as articular cartilage (19, 48, 66). The relatively low stiffness and permeability of the
meniscus tissue indicates that it acts to absorb most of the shock in the joint and is able to easily deform to accommodate changes in shape in the joint (21). A study by Hoshino and Wallace (30) showed an increase in peak impact force with an increase in damage to the human knee joint. When they looked at the knee joint with menisci that had been cut radially and vertically, they saw a 113% increase in peak force in the joint. Looking at the joint after meniscectomy, there was a peak force increase of 123%. If there is less absorption of load, more fractures can occur in the articular cartilage, possibly leading to osteoarthritis (30). Therefore, it can be seen that an intact meniscus (i.e., intact collagen fibers) is necessary for proper shock absorption.

This importance of intact menisci clearly demonstrates that function and structure are related in this tissue. This relationship is further proved by a comparison of regional variations in structural and functional characteristics of the meniscus. Different regions have been shown to support different kinds of forces. By looking at the structure of the tissue at these locations, it can be seen that the meniscus function relies on a precise architecture of particular components.

*Variations in Mechanical Properties:*

Various mechanical tests (uniaxial tension, compression, shear) have shown that the cartilage of the meniscus is anisotropic and inhomogeneous (7, 10, 21, 22, 44, 48, 55, 59, 66, 68). Even though there is variation in experimental results because of the variety of animal models used (59) and the variation in methods, important structure-function relationships can still be seen in the meniscus.
Tension:

Numerous tensile tests have shown that the dependence of material properties on position in the meniscus relies heavily on the orientation of the collagen fibers and also on their spatial location in the tissue (10, 22, 48, 55, 59, 66). Differences in tissue tensile stiffness and strength seem to be directly correlated with fiber orientation, and not with biochemical composition (22, 48). Proctor et al. (48) performed tensile tests on normal bovine medial menisci. They found that the surface of the meniscus is actually isotropic, but that specimens from deeper within the meniscus are anisotropic, a finding supported by numerous other studies (48, 59, 66). The isotropic properties found on the surface are thought to be a direct result of the random fiber arrangement found there (66). On the surface, the tissue is found to fail at high stresses and low strains (48), indicative of this fiber arrangement.

Tensile properties of samples taken from deeper zones depend on the orientation of the sample and also on the region, meaning this portion of the tissue is anisotropic and inhomogeneous. Anisotropy is demonstrated by testing the meniscus in different orientations (Fig. 7). When a sample from the deep zone of the meniscus is tested parallel to the main fiber orientation, the stiffness is 30 times as great as when the sample is taken perpendicular to the main fiber orientation (66). Overall, circumferential samples (aligned with the predominant fiber orientation) are stiffer (48, 66), have a higher failure stress (48) and lower failure strain (48, 66) than the perpendicular samples taken from the radial direction. Consequently, the main function of these fibers is to withstand tension (10). The importance of circumferential collagen fibers in preventing
degeneration of articular cartilage was shown by Burr and Radin (11). All of their human subjects showed meniscus rim regeneration after total meniscectomy and were free of articular cartilage degeneration upon 10-14 year follow-ups (11). They then hypothesized that these circumferential fibers in the rim were necessary in meniscus tensile functioning and in the prevention of joint degeneration. Although radially-oriented specimens are weaker than those of circumferential orientation, the importance of radial fibers is not to be ignored. Radial fibers originate at the periphery and travel inward at right angles to the circumferential fibers. The presence of more radial fibers in the posterior region than the anterior of the bovine meniscus has been shown by tensile tests (55). If a radial specimen is taken that contains either a partial or full radial fiber, the stiffness and strength of that specimen is greater than one that lacks radial fibers (55). These radial fibers may serve as ties between the circumferential fibers and help to prevent tears between them (10, 42).

As mentioned earlier, fiber arrangement is crucial in the healing of tears. This healing property is a direct result of the anisotropic characteristic of the tissue in tension. A cut in the radial axis results in the same load distribution that results from a meniscectomy, causing higher joint stress (54). When the meniscus is cut radially and then allowed to form scar tissue, the joint suffers more degeneration than if the cuts were in the circumferential direction (44). Healed radial cuts have been shown to decrease the displacement in the joint for a given load when under compression, thereby increasing the stiffness, leading to poorer load distribution (44). These same trends are seen to occur after experimental medial meniscectomy followed by varus rotation of the joint, a process
that is thought to naturally occur in vivo after the loss of a meniscus (6). The mechanically inferior repair tissue resulting from radial tears has been shown to have a higher content of randomly-arranged collagen fibers than the tissue of a circumferential cut (44). The meniscus's function relies heavily on its distinct fiber arrangement. Any discontinuities in this structure will undoubtedly hinder proper meniscal performance.

Comparing different regions and zones in the meniscus, one also finds inhomogeneity in the tissue, which corresponds to inhomogeneity in tensile properties. Underneath the surface of the meniscus, the tensile properties show great variation due to fiber orientation and location. Circumferential samples from the middle zone are approximately four times stiffer than those in the surface, while the deep zone circumferential samples are only about three times as stiff as the surface samples (48, 66). This pattern is seen in circumferential samples from medial human menisci (66) and from bovine menisci (48). Variation in tensile properties between the anterior and posterior regions of the meniscus is not consistent between studies. It is not currently possible to absolutely conclude that the posterior region is stiffer than the anterior, or vice versa, because there is a wide variation in experimental results. However, it is agreed that the elastic modulus does not vary between anterior and posterior regions for surface samples because of the isotropy of the surface, but the deeper zones are seen to vary in their relative stiffness (48, 66). In one study by Proctor et al. (48), circumferential samples from the middle zone of the bovine meniscus were found to be two times stiffer and the deep zone three times stiffer in the posterior than the anterior region. Greater stiffness in the posterior region of the bovine meniscus was also found by Skaggs et al. (55) and
Whipple et al. (66). Yet, Fithian et al. (22) looked at human menisci, and found that the posterior medial meniscus has a significantly smaller modulus than the anterior, which correlates with the fact that more injuries occur in this weaker medial posterior region. Although this is also the region where tears are most likely to occur, it is not determined whether this correlates directly with collagen content and organization in this region, or if this is merely a result of how specimens were prepared (42). Besides variations with depth and region, differences in tensile properties are also seen between the inner and outer zones of the meniscus. It has been shown that the inner one third of the bovine meniscus is less stiff than the outer periphery when the specimens are oriented circumferentially (21). More work needs to be done on the various species that have already been tested before conclusions can be drawn about which regions of the meniscus are stiffer and might be the most important in meniscus functioning and injury. Owing to the diverse nature of joint mechanics in different species, this structure-function relationship may not be consistent from species to species.

*Compression:*

Compression tests of the meniscus have also shown variation between regions and zones of the meniscus (21, 48, 59). As was noted earlier, the wedge shape geometry of the meniscus indicates that compression is highest in the inner zone. This compressive property is also correlated with a higher relative amount of PGs in the inner zone, as was discussed in the biochemical section of this review. As for regional variations in compression, it is once again important to note that different investigators have obtained different results for these variations, perhaps due to methods and species used. Proctor et
al. (48) performed confined compression tests on medial bovine meniscus samples in order to determine material variations in the different regions and zones. Their findings revealed that the aggregate modulus ($H_A$) is up to one third higher in the deep posterior area of the meniscus, compared to the deep anterior and deep central regions and that permeability ($k$) did not vary by two-way analysis of variance of region or depth (48). Water content was also found to vary, depending on the region (not depth) with the posterior region having the highest proportional content (48). In contrast to these results, are the findings by Sweigart et al. (59). Using a creep indentation apparatus to test various animal models (bovine, porcine, canine, baboon, lapine, and human), it was found that $H_A$ was greatest in the anterior region for all species, except the baboon (59). It was also seen that permeability did not vary by region, except in the case of the rabbit, where the anterior region was more permeable (59).

Shear:

Variations in shear properties also exist in the meniscus due to fiber orientation and loading conditions, and these results are seen to vary among studies (7, 59, 68). Zhu et al. (68) looked at the effects of collagen fiber orientation on shear moduli. They demonstrated that the viscoelastic shear properties of the meniscus are not only anisotropic, but also dependent on dynamic torsional frequency, shear strain, and compressive strain. The authors found that when dynamic shear tests are performed on circumferential samples (Fig. 7C) from bovine menisci at low strains (<10%), the results show a 20-36% (depending on the amount of compressive strain) greater stiffness than radial samples (Fig. 7R) (68). For example, at 7% strain the circumferential samples
were 36% stiffer than the radial samples. These differences may be due to the fiber organization: when the circumferential specimens were sheared, the major fiber orientation was perpendicular to the face of the specimen, and were therefore stretched in tension. The radial specimens contained fibers that experienced shear between the collagen layers. It was hypothesized that the circumferential samples are able to withstand the tension that develops when they are stretched and therefore resist shear. The radial samples had to bear the force within weaker planes of shear between the fibers. This demonstrates that the fibers are more likely to tear along this inter-fiber plane (longitudinal tears). However, the effectiveness of circumferential samples in resisting shear increases at a slower rate as compressive strain increases. The slackening of the circumferential fibers as they are compressed may lead to this decreased ability to resist tension. Similar findings of anisotropy and inhomogeneity are presented by Anderson et al. (7) for the equine model. Shear testing along the direction of the fibers on equine models has indicated that the shear modulus is highest at the posterior region on the superior surface and also increases with the shear rate (7). In their study on various species, Sweigart et al. (59) found that the shear modulus was greater in the anterior region for all species tested, which is reasonable due to the fact that most injuries occur in the posterior region where the shear modulus was found to be the lowest. It should be noted that this study did not include an equine model. These discrepancies in results highlight the necessity for establishing model standards in order for data to be compared in a fair manner.
The biomechanical properties of the meniscus are anisotropic and inhomogeneous, as has been shown by numerous tensile, compressive, and shear tests. However, it is because of these spatial variations that the meniscus can perform many important mechanical functions. The intricacy of this tissue’s structure-function relationships provides researchers and tissue engineers with a challenge. To create a properly functioning replacement for the fibrocartilage of the meniscus, the native tissue must be thoroughly studied and characterized in terms of mechanical properties. The goal of a tissue-engineered construct would be to replace the protective function of the meniscus by mimicking the mechanical functions of the native tissue.

**Conclusion:**

There is overwhelming evidence that the meniscus serves an indispensable role within the knee joint. Without the meniscus, articular cartilage and its underlying bone are susceptible to biomechanics-induced degradation. Current therapies for repair of meniscal injuries do not result in adequate mechanical properties of the repaired tissue. Without proper tissue composition, both biochemically and ultrastructurally, the mechanical properties of the tissue will not be sufficient to carry out the required meniscal functions.

The goal of tissue engineers is to design an implantable tissue that can replace lost function in a damaged one. Tissue engineering of meniscal fibrocartilage is well under way to elucidate the optimal combination of cells, biochemical signals, and mechanical stimulation needed to produce an efficacious replacement. Development of such a
construct is likely to involve several key factors: cells, growth factors, scaffolds, and mechanical stimulation. Choosing the proper cell type is important so that the proper ECM components will be made. The cells also need some kind of structure to which they can adhere and which will provide them with a framework for making a tissue. Natural or synthetic materials can be used to make such a scaffold. Researchers are currently attempting to find the best material for this application. The material’s degradation profile, mechanical integrity, and interaction with the cells are only a few of the considerations that must be taken when choosing a scaffold. Growth factors can also be incorporated into the scaffold in order to prompt the cells to secrete higher levels of ECM. Considering that the cells of the meniscus are under constant mechanical stimulation in their natural environment, it is highly likely that culturing the cell-scaffold constructs under various mechanical stimuli will also aid in ECM production and organization.

To design and evaluate a tissue-engineered meniscus, a set of criteria must be established by studying properties of the native tissue. These criteria must be the cellular, biochemical, ultrastructural, and biomechanical properties discussed in this review. Once an appropriate standard is created, researchers will have a set of characteristics that can be used to design an engineered meniscus. These criteria can also be used to evaluate the final engineered construct. To achieve successful function, an engineered meniscus must mimic the native tissue. This will require obtaining the same anisotropic and inhomogeneous properties that are seen throughout the meniscus. The meniscus is
uniquely designed to bear the loads to which it is exposed. Any engineered design will have to demonstrate the all important concept that structure confers function.
Effects of growth factors on meniscal fibrochondrocytes in monolayer cultures

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Abstract

To tissue engineer the knee meniscus, our laboratory follows a paradigm that includes biomaterial scaffolding, mechanical stimulation, and growth factor addition. The aim of this study was to study extracellular matrix (ECM) component uptake by meniscal fibrochondrocytes when stimulated by platelet-derived growth factor-AB (PDGF-AB), transforming growth factor-β1 (TGF-β1), insulin-like growth factor I (IGF-I), and basic fibroblastic growth factor (bFGF) at various concentrations (low, medium, and high levels for each). Growth factors were applied to monolayer cultures for three weeks in a soluble form as part of the culture media. Radiolabeling with $^3$H-proline and $^{35}$S-sulfate was performed to indicate collagen and glycosaminoglycan (GAG) production, respectively. TGF-β1 is the only growth factor that increased the uptake of both components. It showed the most consistent behavior and the highest response. There is not conclusive evidence whether the high concentration of TGF-β1 (100 ng/ml) is better over the medium (10 ng/ml). Therefore the results of this study demonstrate that TGF-β1 at either 10 or 100 ng/ml be used to upregulate ECM production in monolayer cultures of meniscal fibrochondrocytes.
**Introduction:**

The knee meniscus is an important tissue in the functioning of the knee joint. Situated between the distal femur and proximal tibia, the meniscus distributes loads (5, 10, 24, 27, 36, 37, 44, 54, 62), reduces friction (6, 25, 66), and absorbs shock (6, 30, 66) between these two articulating surfaces. Because it has limited ability to heal itself, developing a replacement may be necessary to address the many complications caused by injuries to the meniscus. One current tissue engineering approach is to seed fibrochondrocytes of the meniscus onto a scaffold and use various factors, both biochemical and mechanical, to stimulate the production of extracellular matrix (ECM) by the cells (57). Biochemical factors, such as growth factors, serve to signal the cells to perform various parts of their cell cycle: proliferate, migrate, and synthesize ECM (57). For tissue engineering the meniscus, it is desirable to upregulate the production of ECM components in a given construct. Increased ECM synthesis would then render the generated tissue more biomechanically suitable to function in the strenuous environment of the knee. The addition of various growth factors, at various concentrations, to cell cultures may result in an optimal increase in matrix production.

The ECM of the meniscus is comprised mostly of glycosaminoglycans (GAGs) and collagen (mostly types I and II) (25, 39). Collagen comprises 25% of the wet weight and 70% of the dry weight of the tissue and provides the tissue with tensile strength (10, 66). Making up 5% of the wet weight and 0.6% of the dry weight, GAGs contribute to the compressive strength of the tissue (43). The meniscus must withstand both types of forces when it undergoes compression in the joint.
Several growth factors have shown promise in increasing the amounts of ECM that comprise the matrix of the meniscus or stimulating proliferation and migration of the cells (Table 1). It appears that growth factors elicit their maximum effectiveness at a certain concentration and their effect will plateau or decline once this concentration is reached (9, 31). Particular growth factors also show differences in which matrix component (GAG or collagen) they stimulate to be produced to a greater extent. Imler et al. (31) have shown that meniscus explants produce more GAG than collagen when treated with different concentrations of platelet-derived growth factor-AB (PDGF-AB), transforming growth factor-β1 (TGF-β1), insulin-like growth factor I (IGF-I), and basic fibroblastic growth factor (bFGF). These investigators found that TGF-β1 increased sulfate uptake over the control group nearly 8-fold and proline uptake nearly 2-fold. However, using a high TGF-β1 concentration of 5 ng/ml did not show a saturation effect for production of either ECM component. Imler et al. (31) also found increased sulfate and proline uptake induced by IGF-I and PDGF-AB. IGF-I showed a saturation effect with its higher concentrations (>200ng/ml) producing approximately a 5-fold and 3-fold increase in sulfate and proline uptake, respectively. PDGF-AB also showed a plateau effect at its higher concentrations (>100 ng/ml), stimulating sulfate and proline incorporation nearly 6-fold and 2-fold respectively. bFGF showed a 3-fold increase in sulfate uptake, but no significant increase in proline uptake. There have also been studies on application of growth factors to monolayer cultures. Collier and Ghosh (13) treated monolayer cultures with TGF-β1 and found an increase in proteoglycan production and cell proliferation. An increase in migration and proliferation in monolayer cultures has also been seen with PDGF-AB, hepatocyte growth factor/scatter factor, and bone
morphogenic protein-2 (9). Detamore and Athanasiou (16) looked at the effects of bFGF, PDGF-AB, and IGF-I on cells from the temporomandibular joint (TMJ) disc, which are somewhat comparable to meniscal cells. They found that bFGF (10 and 100 ng/ml) stimulated the most proliferation and GAG production and that IGF-I (100 ng/ml) stimulated the most collagen production. The higher concentration (100 ng/ml) of these growth factors generally produced an increase in proliferation while the lower concentration (10 ng/ml) produced an increase in ECM production. In tissue engineering applications, it is important to evaluate the effects growth factors have on collagen and GAG production, since both of these components are essential to withstand the environment in the knee joint.

To fabricate an engineered construct suitable for meniscus replacement, both collagen and GAG must be produced in quantities capable of withstanding the forces applied to the tissue. Within the knee joint, the meniscus must be able to withstand 45-75% of the total applied load (4, 5, 54). This capability is largely dependent on the biochemical content of the tissue. To determine how growth factors affect ECM production in three-dimensional cultures, which is required for their use in tissue engineering, the response of cells in monolayer cultures must first be observed. By studying monolayer cultures, a baseline may be established for growth factor effects on fibrochondrocytes. Growth factors may exhibit synergistic effects when used combinatorially. However, the effect of each factor alone must first be established as a standard. By looking at how different growth factors at different concentrations can affect cells, these factors can be optimally
incorporated into the design of an engineered construct to regenerate the fibrocartilage of the knee meniscus.

This study was designed to evaluate the effects of TGF-β1, PDGF-AB, IGF-I, and bFGF on monolayer cultures of meniscal fibrochondrocytes. Each growth factor was applied at a low, medium, and high concentration level. TGF-β1 was applied at 1, 10, and 100 ng/ml. PDGF-AB was applied at 10, 25, and 100 ng/ml. IGF-I was applied at 5, 12.5, and 50 ng/ml. bFGF was applied at 10, 25, and 100 ng/ml. These values were based on previous fibrochondrocyte studies, yet there are no current studies comparing all of these growth factors on monolayer cultures. The motivation for using these different concentration levels was to achieve a saturation effect in ECM component uptake. The hypothesis was that at least one growth factor would stimulate GAG and collagen production in comparison to the control (no growth factor treatment), and that this growth factor would be most effective at a particular concentration or range of concentrations.

Methods:

Cell harvest and culture:

Six healthy New Zealand white rabbits, ranging in weight from 3.74-3.96 kg, were anesthetized with ketamine/xylozone and euthanized with beuthanasia. Medial menisci were harvested under aseptic conditions. Tissue was digested in 0.3% collagenase for 48 hours. Cells were cultured on T285 flasks in a combination media of Ham’s F12 and DMEM, supplemented with ascorbic acid (50 μg/ml), fetal bovine serum (10%), penicillin/streptomycin/fungizone antibiotics (1%), and non-essential amino acids (1%).
Media were changed completely every three days. Cells were passaged three times with trypsin/EDTA.

**Seeding:**

Third passage cells were seeded at approximately 12,600 cells/cm² onto 48-well tissue culture-treated plates with 0.3 ml of media (F12/DMEM). These static cultures were kept in sterile, 37°C, 5% CO₂ incubators. Cells were allowed to reach confluency (5 days) before the addition of growth factors. Media were changed fully every other day.

**Growth factor addition:**

After confluency was reached, the addition of growth factors was initiated (week 1 and week 3 refer to one and three weeks of growth factor addition, respectively). Media containing growth factors were added every other day in the amount of 0.15 ml to obtain the desired growth factor concentrations (this same volume was first removed from each culture). Four different growth factors were administered: TGF-β1, PDGF-AB, IGF-I, and bFGF. For PDGF-AB and bFGF concentrations of 10, 25, and 100 ng/ml were used. For TGFβ1 concentrations of 1, 10, and 100 ng/ml were used. For IGF-I concentrations of 5, 12.5, and 50 ng/ml were used. Each sample was run in triplicate for radiolabeling. Controls for this study were cultures treated with the same media, but without growth factors.
**Radiolabeling assay:**

A radiolabeling assay was performed once a week for three weeks on the samples. Samples were labeled either with $^3$H-proline, to indicate collagen production, or $^{35}$S-sulfate, to indicate GAG production. Cultures were labeled at a concentration of 10 $\mu$Ci/ml, except at week 1 when the $^3$H-proline label was applied at 5 $\mu$Ci/ml. Cells were incubated with the labels for approximately 12 hours. Due to the manual processing time, some groups were actually incubated for longer (up to two hours). This difference was kept constant from week to week. To process the cultures, media were removed and cells were rinsed with phosphate buffered saline (PBS). Cells and ECM remaining on the plate were extracted using 4M guanidine hydrochloride. Wells from each sample were again rinsed with PBS. Cells/ECM and the second wash were placed together in a scintillation vial with 10 ml of scintillation fluid. Scintillation counts of the samples were then performed.

**Statistical Analysis:**

Data for radiolabeling (n=3) are reported as mean ± standard error. To determine the significance of the effects of concentration, a single factor analysis of variance (ANOVA) was performed, followed by a *post hoc* test, if necessary. For all statistical analysis, p<0.05.
Results:

**Gross appearance:**
The most notable visible difference in cultures was seen after three weeks of growth factor addition with the use of a light microscope (100x). Although some cultures showed a lower level of confluency than others, the most dramatic distinction between samples was shown between TGF-β1 (medium and high concentrations) and the other cultures. TGF-β1-treated cells had several distinguishing features: cells were tightly packed and were extremely elongated, forming a swirled pattern (Fig. 8). There was also a fibrous-looking covering, possibly indicating matrix production.

**Collagen:**
TGF-β1 and IGF-I show similar patterns in \(^3\)H-proline uptake at week 1 (one week after the beginning of growth factor addition). All concentrations of both growth factors show a significant increase in activity over the control (Fig. 9). TGF-β1 at 100 ng/ml shows nearly a 3-fold increase over the control. At this time point, TGF-β1 also shows a trend of dose-dependent response with the low concentration having significantly less activity than the two higher concentrations, almost two times less than the highest concentration. There is no significant difference between the medium and high concentrations of TGF-β1, although they are both significantly greater than all concentrations of IGF-I. There are no significant differences between any of the concentration levels for IGF-I. At week 3, the lowest concentration of IGF-I is trending towards actually lowering uptake compared to the control and the medium concentration appears to be producing the most activity (not significant over the control). For IGF-I at week 3, the medium concentration
is significantly higher than both the lower and higher concentrations. In fact, the medium concentration of IGF-I is nearly twice as active in $^3$H-proline uptake as is the high concentration. In terms of $^3$H-proline uptake, neither PDGF-AB nor bFGF show significant activity over the control at any concentration at any time point, nor do they show any notable trends (Table 2a-c).

**GAG:**

Growth factors have a significant effect on $^{35}$S-sulfate uptake at week 1 (Table 2a-c). TGF-$\beta$1 and bFGF show similar trends at week 1: both at medium and high concentrations they show significantly more activity than the control (Fig. 10). The high concentrations of TGF-$\beta$1 and bFGF show approximately a 2-fold increase over the control, and are not significantly different from each other. These two growth factors also exhibit a dose-dependent response at this time, showing activity increasing with dose (the low concentration of each is not significant over the control). The high concentrations of TGF-$\beta$1 and bFGF are significantly greater in uptake than the medium concentrations. The medium and high concentrations of PDGF-AB also show a significant increase over the control and dose-dependent response at week 1, but to a lesser extent than TGF-$\beta$1 and bFGF. Both TGF-$\beta$1 and bFGF have a significant increase in $^{35}$S-sulfate uptake between their medium and high concentrations at week 1; PDGF-AB does not show such a significance. IGF-I does not show any significant increases in $^{35}$S-sulfate uptake at any time point.
Discussion:

The objective of this study was to analyze effects of TGF-β1, PDGF-AB, IGF-I, and bFGF at low, medium, and high concentrations on monolayer cultures of meniscal fibrochondrocytes. Cell cultures were assessed for increases in collagen and GAG production over the control using a radiolabeling assay and also visual observations.

Significant visible changes in meniscal fibrochondrocyte monolayer cultures were difficult to discern at almost four weeks of total culturing (three weeks of growth factor addition). However, TGF-β1 was able to produce obvious changes in the cells that it stimulated at medium and high concentrations. The shape and packing of the cells may indicate that TGF-β1 acts as a proliferative agent on these cells. It may also alter the morphology or even phenotype of the cells, causing them to become more fibroblastic in nature. The fibrous-looking nature of what appears to be a coating on the cells may be a collagen sheet. It has been noted by our laboratory that it is a common occurrence for a collagen sheet to form when an abundance of collagen is being produced by the cells. If this change in cell morphology is correlated to an increase in matrix production, it may be a beneficial effect of TGF-β1.

In terms of collagen production, as indicated by $^3$H-proline uptake, both TGF-β1 and IGF-I are effective on fibrochondrocytes. TGF-β1 exhibits a dose-dependent effect at week 1 in terms of $^3$H-proline uptake which is significantly higher than both IGF-I and the control. TGF-β1 at medium and high concentrations is higher than the control at week 1, but this difference is not significant at weeks 2 and 3. Also at week 1, the high
concentration of TGF-β1 is not significantly higher than the medium concentration, indicating that a saturation effect on $^3$H-proline uptake has been reached at 10 ng/ml. The temporal trend shown by IGF-I is more inconsistent than that shown by TGF-β1, and it is inconclusive as to which concentration would be most effective in longer culture times. Because of the more consistent behavior of TGF-β1, it is likely that it is more effective than IGF-I at producing collagen over time. Neither bFGF nor PDGF-AB show significant increases over the control in $^3$H-proline uptake.

TGF-β1 and bFGF show the most significance over the control at week 1 in terms of $^{35}$S-sulfate uptake. Although there is not much difference between TGF-β1, bFGF, and PDGF-AB at their medium concentrations, the high concentrations of TGF-β1 and bFGF are both approximately 1.5 times higher in $^{35}$S-sulfate uptake than the high concentration of PDGF-AB. At this time point, TGF-β1 behaves in a dose dependent manner, increasing uptake with concentration level. This same dose-dependent trend is shown in the third week of growth factor addition, although not significant. It is likely that this is a trend that would continue with further time points. At week 1, the high concentration of TGF-β1 is significantly higher than the medium concentration. This indicates that a saturation effect on $^{35}$S-sulfate uptake has not been reached with 100 ng/ml. A saturated level of $^{35}$S-sulfate uptake also was not achieved with the tested concentrations of bFGF. Because its medium and high concentrations were not significantly different from each other, PDGF-AB does exhibit a saturation effect in terms of $^{35}$S-sulfate uptake. As with $^3$H-proline incorporation, in comparison to the other growth factors, TGF-β1 shows more
consistent trends that indicate overall higher GAG production. IGF-I showed no significant increase in $^{35}$S-sulfate uptake.

To select a growth factor from this study to be used in tissue engineering the knee meniscus, as a final step it is important to select the growth factor(s) that shows the strongest trends and that shows the most dramatic upregulation of ECM production in monolayer cultures. The chosen growth factor must also increase the uptake of both collagen and GAG components. Because of the importance of collagen in providing tensile integrity to the meniscus, growth factors that stimulate proline uptake should be used in engineering the meniscus. GAGs lend compressive strength to the meniscus and attract water, keeping the tissue hydrated. Therefore, growth factors that increase sulfate uptake should also be used in meniscus applications. The results from this study indicate that certain growth factors, such as bFGF, IGF-I and PDGF-AB, may stimulate production of only one type of ECM component, while others like TGF-β1 can stimulate both collagen and GAG. TGF-β1 is the only growth factor of the ones studied in this experiment that increased the uptake of both collagen and GAG components. Imler et al. (31) found that IGF-I and PDGF-AB also stimulated uptake of both components. However, the study used meniscus explants. Cells in their native three-dimensional cultures (explants) may have different signaling abilities than those in monolayers. Cells in explants have a different morphology and may be better suited to receive signals from different growth factors. Imler et al. (31) also used higher concentrations of IGF-I and PDGF-AB. It is possible that higher concentrations that those tested in the current study would also be necessary in monolayer cultures. Imler et al.(31) found that both of these
growth factors reached a saturation level in their effectiveness, while TGF-β1 showed potential for further effectiveness with higher concentrations (>5 ng/ml). The current study tested concentrations of TGF-β1 (1-100 ng/ml) and PDGF-AB (10-100 ng/ml) that achieved saturation levels for proline and sulfate uptake respectively.

Because both collagen and GAG are important in the functioning of the meniscus, a growth factor that shows the capability of stimulating the production of each should be selected for fibrochondrocyte culture in tissue engineering. The results of this study suggest TGF-β1 is the only growth factor that meets these criteria. TGF-β1 generally shows the greatest amount of increase in uptake for each ECM component. Although it stimulates significantly more $^{35}$S-sulfate uptake at a high over a medium concentration, this is shown only at the first time point, and it is therefore inconclusive as to whether or not the high concentration is actually more effective than the medium over longer culture periods. It is recommended that TGF-β1 at either 10 or 100 ng/ml be used to upregulate synthesis of ECM in monolayers of meniscal fibrochondrocytes.
Effects of growth factors on meniscal fibrochondrocytes in three-dimensional cultures

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Abstract

Four growth factors, transforming growth factor-β1 (TGF-β1), platelet-derived growth factor-AB (PDGF-AB), insulin-like growth factor I (IGF-I), and basic fibroblastic growth factor (bFGF), were tested at different concentrations for their effects on extracellular matrix (ECM) production in three-dimensional cultures of meniscal fibrochondrocytes. Cells from New Zealand white rabbits were seeded on poly-glycolic acid (PGA) scaffolds and were stimulated with growth factors for three weeks. $^3$H-proline and $^{35}$S-sulfate labels were used to measure uptake of collagen and glycosaminoglycan (GAG) components respectively. Biochemical assays were performed to measure the total collagen, GAG, and DNA present in the scaffolds at the end of the study. TGF-β1 (10 and 100 ng/ml) stimulated both $^3$H-proline and $^{35}$S-sulfate uptake, showing a dose-dependent response for both and a temporal response for $^{35}$S-sulfate uptake. IGF-I (5 ng/ml) and bFGF (25 and 100 ng/ml) showed an increase in $^3$H-proline uptake by the third week of growth factor addition. PDGF-AB did not show notable increases in uptake. Biochemical data fell at or below the sensitivity of the assays used and the only significant data was for collagen production by IGF-I (5 ng/ml) and bFGF (100 ng/ml). Because TGF-β1 (10 and 100 ng/ml) had visibly denser scaffolds at 100x, and the strongest uptake responses to both $^{35}$S-sulfate and $^3$H-proline, it is the most effective growth factor for use in scaffold-based approaches to tissue engineer the knee meniscus.
**Introduction:**

Positioned between the articulating surfaces of the tibia and the femur, the knee meniscus in a piece of fibrocartilage that is indispensable to the function of the knee. The meniscus provides several basic functions: weight distribution (5, 10, 24, 36, 37, 44, 54, 62), friction reduction (6, 25, 66), joint stability (62), and shock absorption (6, 30, 66). Without the meniscus, the articular cartilage on the ends of the bones would become susceptible to damage which can lead to osteoarthritis. Once torn, the meniscus is unable to fully repair itself in the inner region due to its lack of vascularity (35, 49) and low cellularity. Although it has been common practice to remove all or some of a damaged meniscus, this leaves the underlying cartilage exposed and therefore alternative procedures are being sought. One such promising solution is that of tissue engineering: replacing the entire meniscus with a new piece of fibrocartilage that has been generated from a patient’s own cells. This method involves seeding cells on a biocompatible scaffold, stimulating the cells with growth factors, and enhancing physical properties of the construct using mechanical stimulation (57). Although growth factors have been studied on monolayer cultures of fibrochondrocytes, meniscal explants, in alginate beads, as well as open defects and wounds (9, 13, 31, 57, 58, 64), there are no studies that look at the effects of growth factors on fibrochondrocytes seeded on scaffolds.

Growth factors have been previously shown to increase extracellular matrix (ECM) production, migration, and proliferation of meniscal cells (9, 13, 31, 64) (Table 1). Different growth factors at different concentrations can have varying effects on the type of stimulation they provide. Imler et al.(31) looked at four different growth factors:
(transforming growth factor-β1 (TGF-β1) in doses of 0.05-5 ng/ml, platelet-derived growth factor-AB (PDGF-AB) in doses of 3-300 ng/ml, insulin-like growth factor I (IGF-I) in doses of 30-1000 ng/ml, and basic fibroblastic growth factor (bFGF) in doses of 1-300 ng/ml. The growth factors were applied to meniscal explants for either 4 days (to determine dose response) or up to 14 days (to determine temporal effects). The investigators noted that each growth factor increased the production of glycosaminoglycans (GAGs) more than they stimulated the production of collagen, and each to a different extent. Saturation of proline and sulfate incorporation was seen with the higher concentrations of IGF-I (>200 ng/ml) and PDGF-AB (>100 ng/ml). IGF-I showed nearly a 5-fold and 3-fold increase in sulfate and proline incorporation, respectively. PDGF-AB showed nearly a 6-fold and 2-fold increase in sulfate and proline incorporation respectively. The range of concentrations tested for TGF-β1 did not show saturation for proline nor sulfate incorporation. However, at 5 ng/ml, TGF-β1 stimulated sulfate incorporation nearly 8-fold and proline incorporation nearly 2-fold. bFGF only stimulated sulfate incorporation (3-fold) at its highest concentrations (>100 ng/ml). In explants cultured for 14 days, TGF-β1 showed the greatest amount of sulfate incorporation, indicating it was producing the most GAG. The highest increase (approximately 5-fold) was shown during the first 4 days of culture. TGF-β1 has also been shown to increase proteoglycan production in explants (4 ng/ml TGF-β1), monolayers (1 ng/ml TGF-β1), and alginate beads (5 ng/ml TGF-β1) (13). TGF-β1 has also increased proteoglycan synthesis in a dose-dependent manner in fibrochondrocytes (60). Bhargava et al. (9) showed a dose-dependent increase in proliferation of meniscal
fibrochondrocytes caused by PDGF-AB, hepatocyte growth factor/scatter factor, and bone morphogenic protein-2.

The temporomandibular joint (TMJ) disc is similar to the meniscus in that it is fibrous cartilage. Detamore et al. (16) studied PDGF-AB, bFGF, and IGF-I at low (10 ng/ml) and high (100 ng/ml) concentration levels. bFGF stimulated the most proliferation and GAG synthesis, and also increased collagen production by TMJ cells. IGF-I showed the highest collagen production by the TMJ cells.

Besides choosing a growth factor, a proper biocompatible scaffolding material must be selected for three-dimensional culturing of cells. Cells need a surface on which they can attach and a framework that will encourage proper tissue formation. One advantage of using a synthetic scaffolding material over a natural material is the ability to alter the material’s properties (degradation time, porosity) (15). Poly-glycolic acid (PGA) is a polymer that promotes cell adhesion and degrades into glycolic acid, a naturally-occurring biological product. After cells are seeded on a scaffold, they will hopefully begin to produce ECM. The growing tissue should compensate for the degradation of polymer, resulting in a completely cell-formed tissue. By using a non-woven mesh of PGA, a porous network of fibers allows attachment and migration of cells to the interior of the scaffold. The porosity also allows diffusion of media throughout the scaffold. Scaffolds can be cut into desired shapes and sizes to mimic the natural tissue.
Based on current growth factor literature, the following growth factors and concentrations were chosen for this study: TGF-β1 (1, 10, 100 ng/ml), IGF-I (5, 12.5, 50 ng/ml), PDGF-AB (10, 25, 100 ng/ml), and bFGF (10, 25, 100 ng/ml). Each growth factor was tested at a low, medium, and high concentration level in order to see any saturation effects. These various growth factors have been shown to have effects on the synthetic output of meniscal fibrochondrocytes, yet they have not been applied to PGA scaffolds. It is hoped that at least one growth factor will prove successful in stimulating ECM production under these conditions.

The dry weight of the meniscus is 60-70% collagen (mostly types I and II) and 5% GAGs (25, 39). The optimal growth factor for use in tissue engineering the meniscus will be one that can increase the production of these ECM components, as compared to a control, when the cells are seeded on scaffolds. The increased ECM is needed to lend the engineered construct proper mechanical functionality. The knee meniscus must withstand 45-75% of the load applied to the joint (4, 5, 54). The exact load depends on the species under consideration, the extent of flexion or extension in the joint, and the health of the joint. Collagen provides the tissue ability to resist tension and GAGs provide the tissue with compressive resistance. As the meniscus is compressed, it distends outward, while remaining anchored at its ends, creating a hoop stress circumferentially. Since the meniscus is exposed to both tension and compression, it is important to promote both collagen and GAG production in tissue-engineered constructs of the meniscus. The focus of this study is aimed at the interaction of growth factors and
fibrochondrocytes that are seeded on PGA scaffolds, and how the growth factors affect ECM production by the cells.

Methods:

Cell harvest and culture:
Fibrochondrocytes were harvested from the menisci of six New Zealand white rabbits. The rabbits were healthy and ranged in weight from 3.74-3.96kg. Ketamine/xylazine was used to anesthetize and beuthanasia was used to euthanize the rabbits. Harvesting of menisci was performed under aseptic conditions and only medial menisci were used. After removal, the tissue was digested in 0.3% collagenase for 48 hours. Cells were cultured in T-285 flasks with a combination Ham’s F12/DMEM media (50 μg/ml ascorbic acid, 10% fetal bovine serum, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids) and stored in sterile, 5% CO₂, 37°C incubators. All media were changed fully every three days. After confluent, cells were passaged with trypsin/EDTA. A total of three passages were performed.

Seeding:
PGA (Albany International, molecular weight: unknown, porosity: 95%) scaffolds were first sterilized using ethylene oxide. Scaffolds were then pre-wetted with ethyl alcohol, rinsed with phosphate-buffered saline (PBS), and then soaked in media overnight. Scaffolds were 3mm in diameter and approximately 1.5mm in height. Third passage cells were seeded onto the scaffolds at an approximate seeding density of 25 million cells/ml scaffold. Scaffolds were placed in 48-well tissue culture treated plates which
had been coated with poly HEME to prevent attachment of cells to the plate itself. A
total volume of 0.5 ml media (F12/DMEM) was added to each well and plates were
placed on an orbital shaker in a 37°C incubator for three days. The orbital shaker
facilitated the initial attachment of the cells to the scaffolds. Plates were rotated so that
optimal media flow was achieved without noticeable scaffold movement within the well.
Scaffolds were subsequently left in static culture for nine days and half the media was
changed every other day. The static culture period allowed the cells to stabilize and
attach firmly to the scaffolds.

_Growth factor addition:_

After the static culture period, addition of growth factors began. Every other day, half the
volume of media was exchanged for a new dose of growth factor-supplemented media.
Growth factors used were TGF-β1, PDGF-AB, IGF-I, and bFGF. Each growth factor
was tested at a low, medium, and high concentration level: TGF-β1 at 1, 10, and 100
ng/ml; IGF-I at 5, 12.5; and 50 ng/ml, and PDGF-AB and bFGF at 10, 25, and 100 ng/ml.
Three samples of each group were prepared for radiolabeling assays and six samples of
each were prepared for biochemical tests. Cells seeded on scaffolds cultured in media
without growth factors served as the control.

_Radiolabeling assay:_

Once a week, for three weeks (with week 1 being one week after the start of growth
factor addition) a radiolabeling assay was performed. $^{35}$S-sulfate was used to indicate the
amount of sulfate being taken up by the cells for eventual GAG production. $^{3}$H-proline
was used to measure how much proline the cells were consuming as an indicator of collagen production. The labels were applied at concentrations of 10 μCi/ml, except at week 1 where only 5 μCi/ml of ³H-proline was used. The incubation time for the cells with the label was approximately 12 hours, although it was several hours longer for some groups due to constraints of processing time (the difference was held constant from week to week). To analyze each scaffold, media were removed and the scaffolds were rinsed with PBS. Scaffolds were placed in scintillation vials with 10 ml of scintillation fluid and measured for activity.

**Biochemical analysis:**

After three weeks of culture in the growth factor-supplemented media, scaffolds were rinsed with PBS and lyophilized. Scaffolds were then digested with 1 ml papain digest solution and stored at -20°C to await biochemical analysis. Actual amounts of collagen, GAG, and DNA (to indicate cell number) were then measured. After hydrolyzation with NaOH, scaffolds were assessed for total collagen content using a hydroxyproline assay. GAG was measured with a dimethylmethylene blue dye association reagent kit. A PicoGreen™ assay was used to quantify DNA in each scaffold.

**Histology:**

Scaffolds were rinsed with PBS at the end of the culture period and then fixed in 10% phosphate buffered formalin. Samples were cryosectioned into 14 μm thick slices at the surface and middle of the scaffolds. Slides were first treated with hematoxylin/eosin to
stain for cell nuclei. A picrosirious red dye was used to stain for collagen, and saffronin-o and fast green were used to indicate GAG distribution.

**Statistical analysis:**

For radiolabeling analysis, samples were run in triplicate and data are reported as mean ± standard error. For biochemical analysis, n=6 was used as the sample size and data are reported as mean ± standard deviation. A one-factor analysis of variance (ANOVA) was used to determine significance of concentration for one growth factor compared to the control. If such a difference was detected, a *post hoc* test was performed to determine which concentrations of the given growth factor had an effect. All statistical tests employed a significance level of 95%.

**Results:**

**Gross appearance:**

Cells could be seen with a light microscope (100x) attached to the PGA fibers after seeding and as they gradually proliferated over time. Until about the third week of culture, there was not a noticeable visible difference among samples in terms of cell density. During the third week of culture, samples treated with medium and high concentrations of TGF-β1 (10 and 100 ng/ml respectively) were quite different from the other treatment groups. These scaffolds were densely packed with either cells and/or ECM (Fig. 11). Individual PGA fibers could not be distinguished as they were for the scaffolds under other treatments.
**Histology:**

Histological results were inconclusive as to what growth factor produced the highest amount of ECM. Several individual samples showed some GAG, collagen, and/or cells spread between the PGA fibers, but there was too little of each component remaining in the scaffolds to accurately describe the distribution. Most of what these pictures show is the staining of the PGA fibers (Fig. 12).

**Radiolabeling:**

**Collagen**

Significant $^3$H-proline uptake occurs in week three. TGF-β1 and bFGF both show a significant increase in uptake (Fig. 13). TGF-β1 shows a dose response with both the medium and high concentrations being significantly higher than the control and the low concentration, and the high having a significant increase over the medium. Compared to the control, TGF-β1 is nearly 1.8 and 2.4 times more active at its medium and high concentrations respectively. For bFGF, the medium and high concentrations both show a significant increase over the control, and the medium is significant over the low. bFGF at its medium and high concentrations is approximately 1.7 and 1.6 times as active, respectively, as the control. The high concentration of TGF-β1 is significantly higher in uptake than the high concentration of bFGF at this time point. All data for $^3$H-proline uptake are included in Table 3a-c. Neither IGF-1 nor PDGF-AB show significant increases in $^3$H-proline uptake at any time point.
GAG –

A strong trend for $^{35}$S-sulfate uptake is shown by TGF-β1 (Fig. 14). In week 2, the highest concentration is significant over the lower levels and the control (approximately 2.6 times greater than the control), and the medium concentration is trending towards being higher than the lower concentration as well as the control. By week three, the medium and high concentrations are both significantly higher than the low concentration and the control. The medium and high concentrations are 4 and 5 times higher than the control respectively, but are not significantly different from each other. After two weeks of growth factor addition, PDGF-AB actually shows a significant decrease in $^{35}$S-sulfate uptake. Most notably, the control is nearly 1.6 times greater than the medium concentration (25 ng/ml) of PDGF-AB at this time point. The other growth factors do not show significant differences from the control or notable trends in $^{35}$S-sulfate uptake (Table 3a-c).

Biochemistry:

When the total amounts of collagen were measured for each scaffold and normalized by cell number, only two growth factors showed significantly more production than the control: IGF-I and bFGF (Table 4). The high concentration of bFGF showed approximately a 5.6-fold increase over the control while the low concentration of IGF-I showed approximately an 8.4-fold increase over the control. All concentrations of IGF-I and bFGF showed an increase in cell number over the control.
Once normalized by cell number, none of the scaffolds contained significantly more GAG than the control.

**Discussion:**

By seeding meniscal fibrochondrocytes on PGA scaffolds and applying different growth factors at different concentrations, the aim of this study was to select the optimal growth factors for tissue engineering the knee meniscus. The ideal growth factor and concentration would stimulate the cells to upregulate both collagen and GAG production when compared to a control (no growth factors). The production of these components was evaluated by gross appearance, histology, biochemical analysis, and radiolabeling.

Scaffolds treated with TGF-β1 (medium and high concentrations) visually appeared to either have more cells and/or ECM coating their fibers than scaffolds treated with other growth factors. Results from the radiolabeling assay support this observation in that TGF-β1, at medium and high concentrations, increased the uptake of both collagen (³H-proline) and GAG (³⁵S-sulfate) components more than the control as well as the other growth factors. The gross appearance of the scaffolds concurs with our previous investigation of the same growth factors and concentrations on monolayer cultures: TGF-β1 (10 and 100 ng/ml) produced monolayer cultures that had densely packed cells and appeared to have a sheet of matrix covering them (45). It is evident that these scaffolds, treated with TGF-β1, have substantially more matrix production or cell proliferation than the other treatment groups.
Although microscopic visualization reveals overall differences in some TGF-β1-treated scaffolds, histology does not show notable trends or differences between different scaffold groups. There is too little stained collagen and GAG to reliably portray ECM or cellular distribution. There are several possibilities to explain the lack of staining. The total culture time of this experiment was slightly less than five weeks (three weeks with growth factor addition), perhaps not enough time for substantial ECM production in static culture conditions. Using dynamic mechanical stimulation (direct compression, hydrostatic pressure, and bioreactors) to culture the scaffolds, ECM production would likely be enhanced even more and this may affect the building of a more stable ECM, making a clearer distribution visible. This is the subject of further studies in tissue engineering by our group. However, the gross appearance in this study shows unmistakable differences in the TGF-β1 (10 and 100 ng/ml) scaffolds. This indicates that histological results are not accurate representations of true scaffold content. Only one scaffold was tested histologically for each group, and this may not have been a representative sample in several cases. Each scaffold was first treated with formalin. After this treatment, scaffolds looked notably void of cells and/or matrix. If there were too little cells and/or matrix on the scaffolds, the exposure to fluid flow during manipulation may have caused the cells to fall of the scaffold, further affecting histological analysis.

After three weeks of growth factor addition, differences in \(^3\)H-proline uptake in comparison to the control could be seen. At week 3, TGF-β1 and bFGF showed higher \(^3\)H-proline uptake than the control at various concentrations. It is TGF-β1 that shows the
highest increase at the high concentration level. This correlates well with the visual observations that indicate considerably more matrix in these scaffolds. TGF-β1 at 10 and 100 ng/ml is the best inducer of ³H-proline uptake and consequently, collagen production. Since the high concentration (100 ng/ml) is 1.4 times greater in uptake over the medium concentration (10 ng/ml), saturation has not been achieved for ³H-proline uptake. However, monetary considerations must also be given to using even higher doses of TGF-β1. The concentrations of bFGF used do show a saturation effect in terms of ³H-proline uptake.

The biochemical data for collagen are inconclusive. The only significant results were for collagen production by IGF-I (5 ng/ml) and bFGF (100 ng/ml). Most of the raw data from biochemical analysis are at the lower limit of, and generally below, the standards used. Because these levels of ECM content are so low, they fall below the sensitivity of the assays and give large variation for most samples. It is possible some of the collagen produced was washed out of the scaffolds. However, since the culture time of this study was relatively short and there was no mechanical stimulation, it is unlikely enough collagen was produced to be accurately measured by the hydroxyproline assay. Due to the limitations of the assay, and the large amount of variation seen within sample groups, only radiolabeling data are weighted as a factor in the determination of growth factor effects on collagen production. Radiolabeling is more sensitive and in this study, a more accurate indicator of growth factor effectiveness.
TGF-β1 shows a consistent temporal and dose response in terms of $^{35}$S-sulfate uptake. It is also the only growth factor to show any significance over the control in terms of $^{35}$S-sulfate uptake. The medium and high concentrations increase the cell-seeded scaffolds' effectiveness in $^{35}$S-sulfate uptake as a function of time, while the low concentration is not higher than the control. After three weeks of growth factor addition, the medium and high concentrations of TGF-β1 are not significantly different from each other. This indicates a plateau effect of concentration over time. These results for TGF-β1 coincide with data from meniscus explants: TGF-β1 stimulated the most sulfate uptake and did not reach a saturation effect with 5 ng/ml used as the high concentration (31). As with the collagen biochemical assays, the biochemical assays to detect GAG fell below or nearly below the detectable limit of the standards. This lack of substantial ECM content further supports the idea that longer culture times and biomechanical stimulation are needed. However, the radiolabeling assay is sensitive enough to detect molecular uptake and can provide a valid assessment of which growth factor(s) most stimulates a cell to upregulate ECM production.

Because of the higher content of DNA in all scaffolds treated with IGF-I and bFGF, these growth factors seem to have a proliferative effect on meniscal fibrochondrocytes. This finding may indicate increases in uptake shown in these treatment groups may be due to an increase in cell number. As in the native meniscus, cell number in tissue-engineered constructs is limited by diffusion. It is therefore more advantageous to use a growth factor that increases ECM production per cell, as is indicated by the increased uptake of
$^3$H-proline and $^{35}$S-sulfate induced by TGF-β1 which is not correlated to an increase in cell number.

TGF-β1 shows the strongest uptake response for both collagen and GAG components. This response is dose dependent and, for $^{35}$S-sulfate uptake, increases temporally relative to the control. Since GAG and collagen and both important in the meniscus, TGF-β1 is the recommended growth factor for subsequent use in scaffold engineering for the knee meniscus. Cells stimulated by TGF-β1 are activated to drastically increase their uptake of ECM components, indicating an increase in ECM production. As far as the optimal concentration of TGF-β1 to be used, either 10 or 100 ng/ml are be valid choices. There seems to be a plateau effect in terms of $^{35}$S-sulfate uptake over time, with these two concentrations not being significantly different from each other in uptake at week 3. There is about a 100% increase in the amount of $^3$H-proline uptake at week 3 using the high concentration over the medium. However, there is not a consistent significant difference between the medium and high concentrations to warrant using one over the other. It is therefore suggested that a concentration of 10 ng/ml of TGF-β1 be used for scaffold-based tissue engineering of the meniscus, due to economical benefits.
Figure 1. The two semi-lunar menisci as they sit on the tibial plateau. Posterior (P), anterior (A), medial (M), and lateral (L) orientations are depicted.
Figure 2. Circumferential and radial orientations in the meniscus. Radial tears occur when circumferential fibers are torn mid-fiber, as shown by the red line in (A). Longitudinal tears occur when circumferential fibers are pulled apart from each other in the plane shown by the red line in (B). This schematic also shows how samples for testing are often taken as radial specimens (A) and circumferential specimens (B).
Figure 3. Spatial variations within the meniscus. Varying degrees of vascularization distinguish different zones of the meniscus (a). These zones are the red (R), red-white (R-W), and white (W) zones. Topographical variation is defined in three different regions (b). These regions are the anterior (A), central (C), and posterior (P) regions. Several different depths can also be distinguished (c). There are the surfaces (S), lamellar layers (L), and the deep zone (D).
Figure 4. Fibroblasts of the meniscus have different morphologies depending on where they are located. Fusiform cells without cell processes are found on the surface (S). Cells with numerous, randomly-aligned cell processes are found in the red zone nearer the surface (R). Cells with one or two unipolar cell processes are found in the red-white zone near the deep zone (R-W). Rounded cells lacking cell processes are found in the white zone (W).
Figure 5. Collagen fiber orientations in the meniscus depend on depth. The lamellar layers have collagen fibrils that are randomly aligned, but there are radial fibers in the anterior and posterior regions (a). In the deep zone, which is the bulk of the meniscus, the fibers are oriented circumferentially with several radial tie fibers (b). The surface has randomly aligned fibers (c).
Figure 6. Viscoelastic properties of the meniscus. The GAGs (-) of the ECM are negatively charged and attract water to the tissue (a). Upon application of a load (F), some of the water molecules are forced to leave the tissue, and the tissue deforms accordingly (b). Once the load is removed, the water, which has been extruded into the synovial space, is once again drawn into the tissue to associate with the negative charges (c).
Figure 7. Shear tests of circumferential (C) and radial specimens (R). Samples were taken from the meniscus and then sheared as shown. Sample orientation determines fiber alignment during shear. Fibers in circumferential samples are more able to resist shear between individual fibers. Radial samples experience shear between sheets of fibers.
Figure 8. Cells treated with the high concentration of TGF-β1 (b) are more elongated than cells treated with other growth factors (a, c, d) and the control (e). All pictures were taken during week 3. (100x)
$^3$H-Proline Uptake in Monolayers at Week 1

Figure 9. At week 1, TGF-β1 and IGF-I show significantly more $^3$H-proline uptake than the control at all concentrations. * indicates significance over the control. $p<0.05$
Figure 10. TGF-β1, PDGF-AB, and bFGF all show significantly more $^{35}$S-sulfate uptake over the control at week 1. * indicates significance over the control. p<0.05
Figure 11. The gross appearance (100x) of several scaffolds is greatly different after three weeks of growth factor addition, as evidenced by a comparison of all growth factors at medium concentration levels and the control. TGF-β1 treated scaffolds are the most densely packed and individual PGA fibers can not be distinguished.
Figure 12. Collagen (a, c) and GAG (b, d) distribution in a TGF-β1 scaffold (100 ng/ml) and a bFGF (25 ng/ml) scaffold. In a and c, the PGA fibers are stained red and the collagen is the sparse fibrous material. In the right picture, the PGA fibers are the darkly stained material and the GAG is the material that is spread between the fibers. All histology was performed at week 3.
Figure 13. After three weeks of stimulation, TGF-β1 and bFGF show significant $^{3}$H-proline uptake over the control. * indicates significance over the control. $p<0.05$
35S-Sulfate Uptake in Scaffolds for TGF-β1

Figure 14. TGF-β1 is the only growth factor to show significant increases over the control in 35S-sulfate uptake. This increase occurs in week 3. An increase in activity is seen temporally and with dose. * indicates significance over the control. p<0.05.
Table 1. Summary of growth factor studies on meniscal fibrochondrocytes.

<table>
<thead>
<tr>
<th>Author, Date</th>
<th>Growth Factors Tested (relevant to present study)</th>
<th>Culture Type</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Webber et al. 1985</td>
<td>Pituitary FGF</td>
<td>Monolayer</td>
<td>Proliferation increased, $^{35}$SO$_4$ uptake decreased</td>
</tr>
<tr>
<td>Bhargava et al. 1999</td>
<td>PDGF-AB</td>
<td>Monolayer</td>
<td>Increased DNA synthesis</td>
</tr>
<tr>
<td>Collier&amp;Ghosh 1995</td>
<td>TGFβ</td>
<td>Explants</td>
<td>Increased proteoglycan (PG) production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer</td>
<td>Increase PG production and proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alginate beads</td>
<td>Increased PG production</td>
</tr>
<tr>
<td>Imler et al. 2003</td>
<td>TGF-β1, IGF-I, PDGF-AB, bFGF</td>
<td>Explants</td>
<td>All increased sulfate uptake, All except bFGF increased proline uptake</td>
</tr>
</tbody>
</table>
Table 2a. $^3$H-proline and $^{35}$S-sulfate incorporation data for cells treated with growth factors at low concentration levels. All results are mean ± standard error. All units are cpm. * indicates a significant difference compared to the control (p<0.05).

<table>
<thead>
<tr>
<th>Growth Factor (ng/ml)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
<td>$^3$H-proline</td>
</tr>
<tr>
<td>TGF-β1 (1)</td>
<td>24841.75±3621.05</td>
<td>6054.67±459.37</td>
<td>3890.33±546.50</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1 (5)</td>
<td>20083.07±1963.40</td>
<td>7761.00±425.05</td>
<td>7820.50±2671.78</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF-AB (10)</td>
<td>17977.16±1100.05</td>
<td>4712.67±508.41</td>
<td>4265.33±115.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bFGF (10)</td>
<td>12194.07±3066.70</td>
<td>4756.00±411.84</td>
<td>5685.33±517.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (0)</td>
<td>15326.01±513.01</td>
<td>5023.33±66.52</td>
<td>7076.56±3291.65</td>
</tr>
</tbody>
</table>
Table 2b. $^3$H-proline and $^{35}$S-sulfate incorporation data for cells treated with growth factors at medium concentration levels. All results are mean ± standard error. All units are cpm. * indicates a significant difference compared to the control (p<0.05).

<table>
<thead>
<tr>
<th>Growth Factor (ng/ml)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
<td>$^3$H-proline</td>
</tr>
<tr>
<td>TGF-β1 (10)</td>
<td>38231.11±870.76</td>
<td>7803.67±335.89</td>
<td>7118.30±1975.93</td>
</tr>
<tr>
<td>IGF-I (12.5)</td>
<td>20177.70±394.88</td>
<td>6276.33±139.22</td>
<td>6183.67±1243.24</td>
</tr>
<tr>
<td>PDGF-AB (25)</td>
<td>20297.19±2634.39</td>
<td>6948.67±250.17</td>
<td>5292.00±142.92</td>
</tr>
<tr>
<td>bFGF (25)</td>
<td>21209.77±1380.70</td>
<td>6760.33±104.24</td>
<td>7970.70±1812.13</td>
</tr>
<tr>
<td>Control (0)</td>
<td>15326.01±513.01</td>
<td>5023.33±66.52</td>
<td>7076.56±3291.65</td>
</tr>
</tbody>
</table>
Table 2c. $^3$H-proline and $^{35}$S-sulfate incorporation data for cells treated with growth factors at high concentration levels. All results are mean ± standard error. All units are cpm. * indicates a significant difference compared to the control (p<0.05).

<table>
<thead>
<tr>
<th>Growth Factor (ng/ml)</th>
<th>Week 1</th>
<th></th>
<th>Week 2</th>
<th></th>
<th>Week 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
</tr>
<tr>
<td>TGF-β1 (100)</td>
<td>43986.67±</td>
<td>918.79</td>
<td>10671.33±</td>
<td>812.65</td>
<td>9382.33±</td>
<td>504.24</td>
</tr>
<tr>
<td></td>
<td>*</td>
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<td>*</td>
<td>*</td>
</tr>
<tr>
<td>IGF-I (50)</td>
<td>21205.66±</td>
<td>1341.16</td>
<td>6061.33±</td>
<td>1175.55</td>
<td>4655.00±</td>
<td>249.76</td>
</tr>
<tr>
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<td>*</td>
</tr>
<tr>
<td>PDGF-AB (100)</td>
<td>15524.32±</td>
<td>2648.43</td>
<td>6890.67±</td>
<td>563.88</td>
<td>4588.67±</td>
<td>23.13</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>bFGF (100)</td>
<td>14806.27±</td>
<td>2754.56</td>
<td>9697.33±</td>
<td>494.08</td>
<td>12902.15±</td>
<td>4717.95</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Control (0)</td>
<td>15326.01±</td>
<td>513.01</td>
<td>5023.33±</td>
<td>66.52</td>
<td>7076.56±</td>
<td>3291.65</td>
</tr>
</tbody>
</table>
Table 3a. $^3$H-proline and $^{35}$S-sulfate incorporation data for scaffolds treated with growth factors at low concentration levels. All results are mean ± standard error. All units are cpm. * indicates a significant difference compared to the control (p<0.05).

<table>
<thead>
<tr>
<th>Growth Factor (ng/ml)</th>
<th>Week 1</th>
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<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
<td>$^3$H-proline</td>
</tr>
<tr>
<td>TGF-β1 (1)</td>
<td>9887.63±665.52</td>
<td>14544.00±972.21</td>
<td>24567.78±1067.52</td>
</tr>
<tr>
<td>IGF-I (5)</td>
<td>11153.24±1092.20</td>
<td>13139.67±892.17</td>
<td>28304.76±374.31</td>
</tr>
<tr>
<td>PDGF-AB (10)</td>
<td>11364.33±954.83</td>
<td>12282.67±90.46</td>
<td>23452.00±1717.18</td>
</tr>
<tr>
<td>bFGF (10)</td>
<td>11923.23±644.36</td>
<td>13888.33±2105.48</td>
<td>27884.17±1079.47</td>
</tr>
<tr>
<td>Control (0)</td>
<td>8690.07±1259.96</td>
<td>9538.00±2157.64</td>
<td>31661.21±5998.79</td>
</tr>
</tbody>
</table>
Table 3b. \(^3\)H-proline and \(^35\)S-sulfate incorporation data for scaffolds treated with growth factors at medium concentration levels. All results are mean ± standard error. All units are cpm. * indicates a significant difference compared to the control (p<0.05).

<table>
<thead>
<tr>
<th>Growth Factor (ng/ml)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^3)H-proline</td>
<td>(^35)S-sulfate</td>
<td>(^3)H-proline</td>
</tr>
<tr>
<td>TGF-β1 (10)</td>
<td>13797.22± 2138.80</td>
<td>15368.60± 2971.55</td>
<td>31573.85± 3459.25</td>
</tr>
<tr>
<td>IGF-I (12.5)</td>
<td>14076.75± 2907.62</td>
<td>15956.33± 1533.02</td>
<td>35858.70± 2604.22</td>
</tr>
<tr>
<td>PDGF-AB (25)</td>
<td>9761.07± 502.67</td>
<td>9675.00± 932.740</td>
<td>22942.17± 1426.00</td>
</tr>
<tr>
<td>bFGF (25)</td>
<td>10529.39± 1753.89</td>
<td>11998.67± 1732.11</td>
<td>26329.75± 3993.14</td>
</tr>
<tr>
<td>Control (0)</td>
<td>8690.07± 1259.96</td>
<td>9538.00± 2157.64</td>
<td>31661.21± 5998.79</td>
</tr>
</tbody>
</table>
Table 3c. $^3$H-proline and $^{35}$S-sulfate incorporation data for scaffolds treated with growth factors at high concentration levels. All results are mean ± standard error. All units are cpm. * indicates a significant difference compared to the control (p<0.05).

<table>
<thead>
<tr>
<th>Growth Factor (ng/ml)</th>
<th>Week 1</th>
<th></th>
<th>Week 2</th>
<th></th>
<th>Week 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
<td>$^3$H-proline</td>
<td>$^{35}$S-sulfate</td>
</tr>
<tr>
<td>TGF-β1 (100)</td>
<td>13063.12± 878.75</td>
<td>14499.86± 5020.23</td>
<td>28304.76± 374.31</td>
<td>36168.27± 4918.02</td>
<td>*</td>
<td>56098.67± 4346.07</td>
</tr>
<tr>
<td>IGF-I (50)</td>
<td>11201.54± 929.56</td>
<td>19752.00± 6105.12</td>
<td>24907.59± 1631.98</td>
<td>17993.03± 4068.10</td>
<td>34504.92± 2563.67</td>
<td>18642.76± 2539.045</td>
</tr>
<tr>
<td>PDGF-AB (100)</td>
<td>9542.12± 920.43</td>
<td>11748.67± 1230.95</td>
<td>31279.36± 4379.65</td>
<td>11537.33± 889.64</td>
<td>30496.03± 4454.86</td>
<td>15822.67± 1717.88</td>
</tr>
<tr>
<td>bFGF (100)</td>
<td>11941.83± 859.81</td>
<td>11120.00± 746.65</td>
<td>35624.44± 4219.92</td>
<td>21310.45± 534.53</td>
<td>36710.22± 2434.48</td>
<td>*</td>
</tr>
<tr>
<td>Control (0)</td>
<td>8690.07± 1259.96</td>
<td>9538.00± 2157.64</td>
<td>31661.21± 5998.79</td>
<td>13775.33± 1632.83</td>
<td>22985.56± 2846.56</td>
<td>19045.67± 7303.62</td>
</tr>
</tbody>
</table>
Table 4. Biochemical data for scaffolds. All data are mean ± std dev. * indicates significance compared to control (p<0.05).

<table>
<thead>
<tr>
<th>PDGF-AB</th>
<th>TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (ng/ml)</td>
<td>GAG (µg/10^6 cells)</td>
</tr>
<tr>
<td>10</td>
<td>0.20±0.13</td>
</tr>
<tr>
<td>25</td>
<td>0.23±0.14</td>
</tr>
<tr>
<td>100</td>
<td>0.11±0.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IGF-1</th>
<th>bFGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (ng/ml)</td>
<td>GAG (µg/10^6 cells)</td>
</tr>
<tr>
<td>5</td>
<td>0.22±0.10</td>
</tr>
<tr>
<td>12.5</td>
<td>0.22±0.05</td>
</tr>
<tr>
<td>50</td>
<td>0.22±0.02</td>
</tr>
</tbody>
</table>

**Control Data:**

GAG = 0.13 ± 0.16µg/10000 cells  Collagen =0.05 ± 0.07µg/10000 cells  Cell/Scaffold (/10000) = 16.39±.94
WORKS CITED


