Chapter 1

Fibrochondrocytes and their Use in Tissue Engineering of the Meniscus

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Summary

The meniscus is a fibrocartilaginous tissue within the knee joint responsible for shock absorption, load transmission, lubrication, and stability. Damage to the meniscus can result in loss of some or all of the above functions. Unfortunately, current repair techniques do not adequately address the issue of meniscus regeneration. Tissue engineering is one possible solution to fix this difficult problem. Meniscal cells, also known as fibrochondrocytes, have the potential to play a central role in the tissue engineering approach. This article provides a description of various studies performed to-date, such as fibrochondrocyte characterization and their reaction to different culturing environments, peptides, and growth factors. The current tissue engineering attempts performed are also examined.

Keywords: Culture Conditions, Fibrochondrocytes, Growth Factors, Meniscus, Tissue Engineering

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Introduction

The meniscus is a fibrocartilaginous structure found within the knee joint responsible for shock absorption, load transmission, and stability (1-5). According to the National Center for Health Statistics in the United States, over 600,000 surgeries each year are the result of complications with the meniscus (6). Regions of the meniscus, namely those in the vascular zone, have an intrinsic healing capability, whereas the avascular zone does not heal (7). To repair damage in the avascular region and overcome tissue degeneration, methods that will assist the meniscus in healing itself need to be developed, and tissue engineering is a potential solution. Fibrochondrocytes constitute a major component of tissue engineering attempts. Fibrochondrocytes are responsible for filling a scaffold material with matrix, organizing the matrix in response to the mechanical stimuli present in the joint, and helping the tissue construct integrate with the surrounding tissue. A thorough understanding of the mechanics and anatomy of the tissue is necessary before attempting to tissue engineer the meniscus.

Meniscus Anatomy

The meniscus is a tissue consisting of two wedge-shaped semilunar sections of fibrocartilaginous tissue between the tibial and femoral bearing surfaces of the knee joint (Fig. 1). On gross inspection the meniscus is a white, glossy, and smooth tissue; this smoothness is also present at the microscopic level (8). The peripheral portion of the meniscus, also known as the red zone, is vascularized, whereas the inner portion, known as the white zone, is avascular (Fig. 2) (9). The meniscus is attached to the medial collateral ligament, the meniscofemoral ligaments, the transverse ligament, and the anterior and posterior horns. The anterior and posterior horns are where the meniscus joins with the tibial plate; these attachments are usually considered the most important (10).

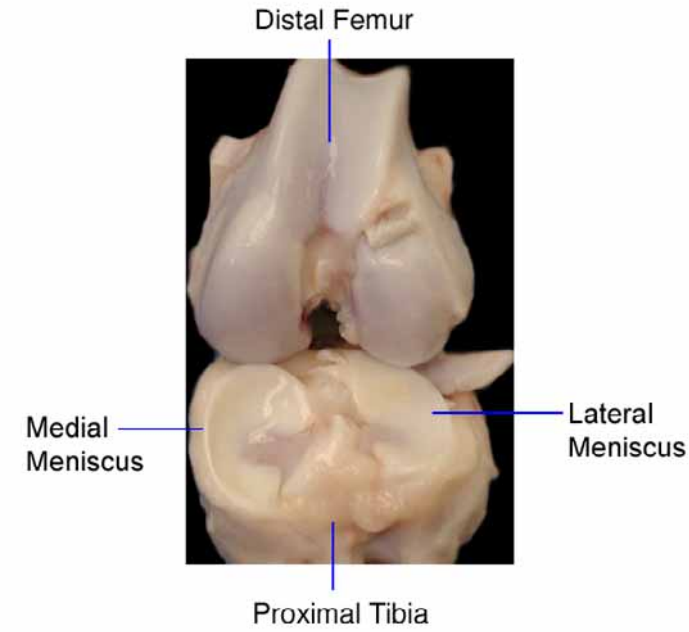

Fig. 1: Meniscus attached to the tibia.

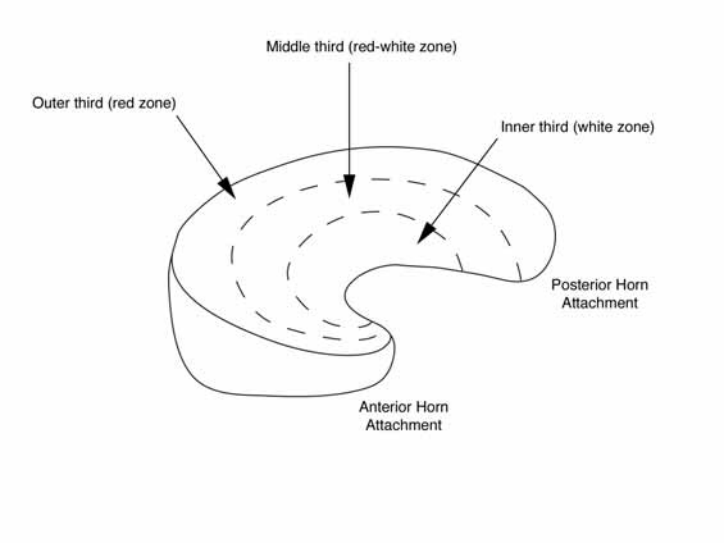

Fig. 2: Schematic drawing of the meniscus showing the vascular profile and tibial attachment locations. The white zone is avascular, the red-white zone is a transitional area, and the red zone is vascularized.

Cells in the Meniscus

Most meniscal cell characterization has been carried out in humans and the rabbits (8, 9, 11-13). Meniscal cells are generally considered to be a cross between chondrocytes and fibroblasts. The cells have a rounded morphology and are protected by a territorial matrix, as are chondrocytes, yet the cells produce type I collagen, like fibroblasts. In 1985, Webber and coworkers (13) coined the term 'fibrochondrocytes' to describe these unique cells. There are two relatively distinct fibrochondrocyte populations found in different locations within the human meniscus. Oval or fusiform fibrochondrocytes with a few small processes are found in the superficial layers (Fig. 3). Fibrochondrocytes from the deeper zone are rounded or polygonal and contain more processes (9). In-depth studies have also been carried out on the rabbit meniscus. A study by Moon *et al.* (12) found that the periphery of the meniscus contains cells that are fibroblast-like, whereas chondrocyte-like cells were found in the inner rim of the meniscus. This correlates with the functional aspects of the tissue. The inner rim of the meniscus is a bearing surface for the knee joint, is subjected to compressive forces, and contains a combination of type I and II collagen. The periphery is responsible for shock absorption, is subjected mainly to tensile forces, and contains large quantities of type I collagen. A recent study on rabbit fibrochondrocytes (11) provides an in-depth look at the different locations of the meniscus. This study found four morphologically distinct classes of fibrochondrocytes within the meniscus. Cells in the superficial layer were found to be fusiform in shape, as shown in other studies (8, 9). Cells from the inner rim, also known as the white zone, contained rounded cells that lacked projections. The last two cell types, found in the red zone and red-white zone, had long cellular processes (larger quantities in the red zone) and a large number of gap junctions. Cells within these two regions were also organized into rows, something not seen in the white zone. This row-like organization of cells along the collagen fibers is also noted in other fibrous tissues, such as tendon. It is believed that the large number of processes helps the tissue organize the extracellular matrix so that it can withstand the tensile hoop-stress. It should also be noted that the processes observed in fibrochondrocytes *in vivo* are not found in these cells if grown in culture, indicating that culture conditions result in morphological changes.

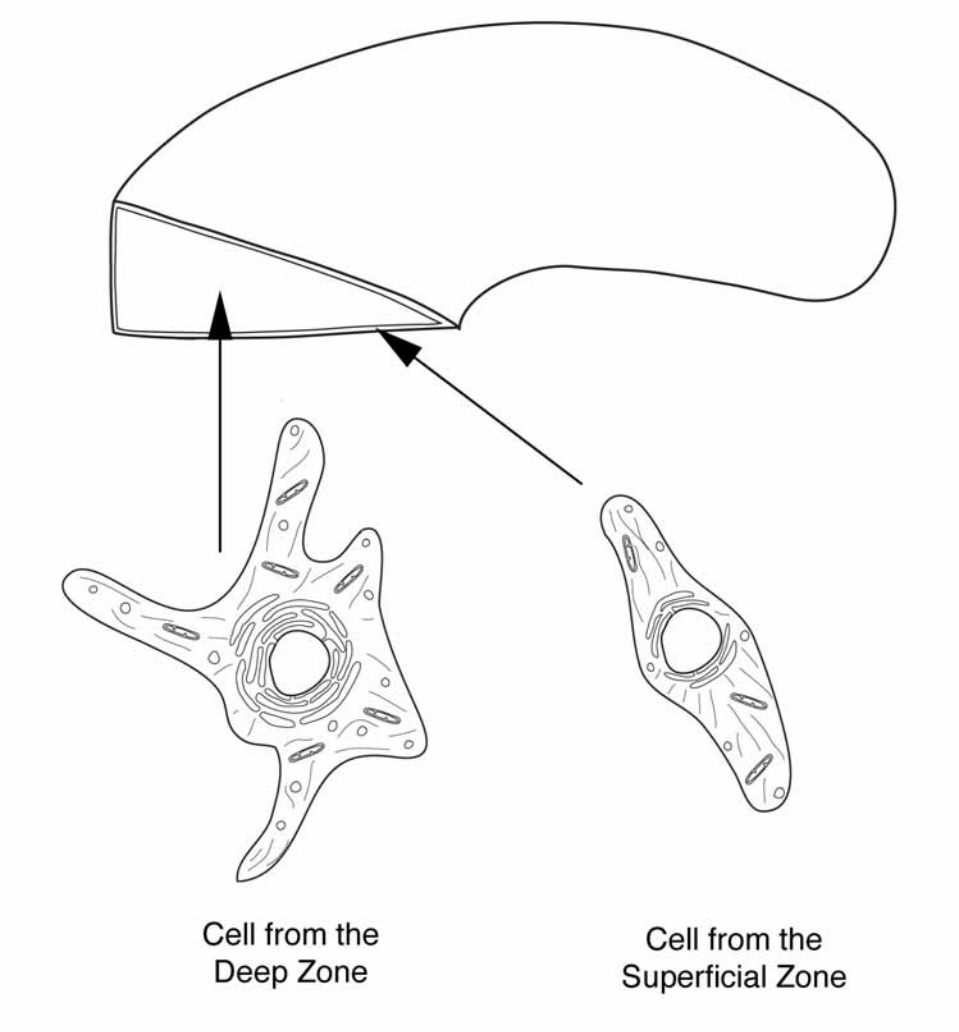

Fig. 3: Morphological differences of fibrochondrocytes at the superficial zone and deep zone.

Extracellular Matrix of the Meniscus

The extracellular matrix is made of four different components: water, fibrillar proteins, proteoglycans, and adhesion glycoproteins. Biochemical analysis has shown that human meniscal tissue contains 72% water, 22% collagen, 0.8% glycosaminoglycans; the rest is made up of DNA and adhesion molecules (14). These numbers can vary depending on age, species, and location within the tissue (15, 16).

Fibrillar Components

The main type of fibrillar component found in the meniscus is collagen. Collagen types I, II, III, V, and VI have been found within meniscal tissue, and account for 60-70% of the dry weight (15). Type I collagen is by far the most predominant, accounting for more than 90% of the collagen within the tissue (17). For example, in bovine menisci the outer 2/3 of the tissue collagen is predominantly type I collagen, whereas the inner 1/3 is 60% type II collagen and 40% type I collagen (18). The meniscus has a unique collagen fiber orientation that is related to its function and consists of three different layers. The superficial layer consists of a thin layer of randomly orientated fibers (19). The lamellar layer, situated just inside the superficial layer, also consists of randomly orientated fibers, with the exception of the peripheral portions at the anterior and posterior sections; here the fibers are orientated radially (19). The deep zone consists of circumferentially orientated fibers with a small amount of radial fibers, also referred to as tie fibers (19). The combination of the different mechanical stimuli most likely cause the cells to organize the collagen fibers in such a fashion.

Proteoglycans

Proteoglycans are responsible for hydration within the meniscus and the compressive properties of the tissue (20, 21). The concentration of proteoglycans in meniscal tissue is 8 fold lower than the concentration found in articular cartilage (22, 23). Various studies have been performed on meniscal proteoglycans, which are responsible for tissue hydration (21). The inner 2/3 of the meniscus produces more proteoglycans than the outer 1/3. More proteoglycans are produced laterally than medially, though the glycosaminoglycan makeup of the proteoglycans stays the same at all of these locations (24, 25). This higher proteoglycan content in the inner third correlates with the bearing

surface nature in the avascular portion of the tissue.

Adhesion Glycoproteins

As the name suggests, adhesion molecules are partly responsible for binding with other matrix molecules and cells. There are three of these molecules that have been identified within the meniscus: type VI collagen, fibronectin, and thrombospondin (15). While the exact nature of these three glycoproteins has not yet been described, the RGD peptide, which plays a central role in cell adhesion, has been found in type VI collagen, fibronectin, and thrombospondin (15).

Meniscal Biomechanics

Functionally, the meniscus acts as a shock absorber, helps with load bearing and transmission in the knee joint, improves stability in the knee, and helps with lubrication (1-5). Because all of these different functions and the geometry of the tissue, the meniscus is subjected to compressive, tensile, and shear stress. Whenever a load is applied to the knee joint, the meniscus is compressed, but due to its wedge-shape it is also displaced away from the center of the femoral condyles, resulting in tensile stress because of the anterior and posterior attachment to the tibial plate (3, 26, 27). In terms of shear properties of the meniscus, it is known that they depend heavily on the collagen orientation of the meniscus and the low circumferential shear strength is thought to be partly responsible for the occurrence of longitudinal tears (28, 29). The menisci partially cover the articular cartilage on the tibial plate and are responsible for absorbing some of the load transmitted through the knee and protecting the articular cartilage within the joint (3, 5, 26, 27, 30, 31). In general, the lateral meniscus is displaced more than the medial meniscus during compression, showing that while both menisci have the same general function they do react differently (27). These differences can also be noted in the biomechanical and biochemical properties of the meniscus among various animal models (30, 32).

Tissue Engineering of the Meniscus

The amount of work that has been performed in the attempt to tissue engineer the meniscus has been limited when compared to other musculoskeletal tissues, such as bone or articular cartilage. Many components, such as the fibrochondrocytes, the animal model, scaffold material, and evaluation techniques, are of importance. Several aspects of the cellular components have been studied, such as cell isolation, cell culture, the effect of peptides, and the effect of growth factors. Much of this information has been used in the few attempts to engineer the meniscus carried out to-date.

Fibrochondrocyte Isolation Methods

A variety of different methods have been used to isolate the fibrochondrocytes from the surrounding matrix for tissue culture applications (24, 33-38). Webber *et al.* (34) used a method adopted from Green (33) for articular chondrocytes. This isolation method consisted of sequential treatment of minced tissue with 0.05% hyaluronidase, 0.2% trypsin, and 0.2% clostridial collagenase, in conjunction with mechanical stirring, to release the cells, followed by filtering and washing the fibrochondrocytes several times to remove the debris (33, 34). Tanaka *et al.* (24) treated meniscal tissue with 0.8% pronase for 25 minutes, followed by digestion with 0.4% collagenase for 40-60 minutes to isolate fibrochondrocytes from the meniscus. Mueller *et al.* (36) used a simple collagenase digestion (0.2%), in conjunction with mechanical stirring, for a period of six hours to release the fibrochondrocytes from the meniscal tissue. Nakata *et al.* (35) looked at each of the three above methods and determined that a simple collagenase digestion provided the most consistent results in terms of cell number and phenotype. In their studies a 0.4% collagenase solution was used for a period of 2-3 hours. Ibarra *et al.* (37, 38) also used a simple collagenase digestion method.

In Vitro Cell Culture

The majority of *in vitro* studies have been performed in monolayers. Fibrochondrocytes from several different species have been used, such as the human, rabbit, dog, and pig, but the majority of studies have been performed on the human and rabbit models (13, 34, 35, 39, 40). Human fibrochondrocytes grown in monolayer culture have shown three distinguishable types: elongated fibroblast-like cells, polygonal cells, and round chondrocyte like cells (35). There are many different factors which can have an effect on the cells, such as the media type, the age and sex of the animal, and the amount of CO_2 in the culture environment.

Media Type

The type of media used in monolayer culture can have a profound effect on morphology, proliferation, and protein synthesis capabilities of fibrochondrocytes (13, 35, 39, 40). A study performed by Nakata *et al.* (35) compared morphology, proliferation ability, and mRNA synthesis of fibrochondrocytes in three different culture mediums: Ham's F-12 with 10% fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, and a 1:1 mixture of Ham's F-12 and DMEM supplemented with 10% FBS. Fibrochondrocytes proliferated the fastest in DMEM and the slowest in Ham's F-12. Morphology was maintained in the Ham's F-12 media and the Ham's F-12:DMEM media, but the elongated fibroblast like cells became predominant in the DMEM media within one week. Fibrochondrocytes grown in the Ham's F-12:DMEM mixture also showed expression of mRNA for types I, II, III, and IX collagen, and aggrecan. Additional studies looking into the effect of the different media types on fibrochondrocytes have been carried out by Webber *et al.* (13, 39, 40) In a study from 1985 (13) fibrochondrocytes were grown in either a DMEM/10% FBS media or a Ham's F-12/10% FBS media. The cells were then passaged into secondary culture and analyzed for morphology, growth, and proteoglycan synthesis. The morphology of the fibrochondrocytes varied depending on the initial media type. The cells grown in the DMEM had a polygonal shape and resembled chondrocytes, whereas the cells from the F-12 media were fusiform, with a fibroblast-like morphology. Cells grown in the F-12 media had a slightly faster growth rate in primary culture, but in secondary culture the growth rate of the cells from the DMEM was more rapid. The addition of ascorbate to the media had a drastic effect on the population doubling time of the fibrochondrocytes. In secondary culture the addition of ascorbate to the F-12 media increased the

population doubling time (PDT) from 20 hours to greater than 24 hours, whereas the PDT of the cells grown in the DMEM decreased from 15 hours to 8.8 hours with the addition of ascorbate. The addition of ascorbate also decreased the uptake of $^{35}SO_4$, a marker used for estimating proteoglycan synthesis, though it should also be noted that the uptake was higher in the F-12 media than the DMEM media. In another study, performed by Webber *et al.* (39), a serum-free culture media was developed that gives similar results to 10% FBS. The serum-free media developed consists of a 1:1 ratio of DMEM:Ham's F-12, transferrin (1 µg/ml), selenium (1 pg/ml), trace metal mix (1:100), dexamethasone (100 ng/ml), insulin-like growth factors I and II (50 ng/ml each), pituitary fibroblastic growth factor (100ng/ml), and lactalbumin hydrolysate (2 µg/ml). The one major difference between the serum-free media and the media with 10% FBS was the morphology. Fibrochondrocytes from the serum-free media had a more polygonal morphology. This trait continued for the length of the experiment (20 days).

Age and Gender

Webber *et al.* (34, 41) have also performed two studies examining the effect of age and gender on fibrochondrocyte quantity, proliferation, and proteoglycan synthesis. A 1986 study (34) looked into these characteristics in rabbit fibrochondrocytes. Results showed that female menisci contain more fibrochondrocytes than male menisci, regardless of age. Gender and age did not have a significant effect on the PDT, but an effect was noted on proteoglycan synthesis. Fibrochondrocytes from six-month-old male rabbits incorporated four times as much $^{35}SO_4$ as the female fibrochondrocytes, though this trend was reversed in older rabbits (24 months), with the fibrochondrocytes from the females exhibiting twice as much uptake as the fibrochondrocytes from the males. A later study (41), performed using human explants, showed that ingrowth of fibrochondrocytes into a fibrin clot was significantly quicker in skeletally immature individuals (14 and 16 years old) when compared to skeletally mature individuals (>22 years old).

CO_2 Levels

Different CO_2 levels have been used in fibrochondrocyte culture both for fibrochondrocyte characterization studies and tissue engineering attempts. Ten percent CO_2 was used by Webber *et al.* (13, 34, 39, 40) for all of their cell characterization work, whereas Nakata *et al.* (35) and Bhargava *et al.*

(42), Ibarra *et al.* (43, 44), and Mueller *et al.* (36) used 5% CO^2 for their tissue engineering efforts. No direct comparison has been performed to determine the effect of these two culture environments on fibrochondrocytes.

Fibrochondrocytes and Peptides

It has been shown that the RGD peptide enhances the attachment of fibrochondrocytes (40). Canine fibrochondrocytes were harvested and seeded on surfaces either coated with chondroitin sulfate alone, or with chondroitin sulfate conjugated to a peptide containing the RGD sequence. A large quantity of cells attached to the surface containing the RGD peptide, but not on the surface with chondroitin sulfate alone (40). Overall, the RGD sequence shows great potential for supporting fibrochondrocytes attachment to a scaffold for tissue engineering.

Fibrochondrocytes and Growth Factors

Numerous growth factors have been used on meniscal fibrochondrocytes to test their effects on the healing of tears or defects, and on protein synthesis in tissue and cell culture. Table I summarizes the different growth factors that have been used. All of these have the potential to help in tissue engineering of the meniscus.

Growth Factor	***Cells***	***In Vitro or In Vivo (Animal)***	***Results***	***Reference***
FGF	Lapine	*In vitro*	Proliferation was stimulated	[13]
Human PL	Lapine	*In vitro*	Proliferation was stimulated	[13]
ECGF	No cells	Dog	Improved healing in cylindrical defect	[50]
ECGF	No cells	Dog	Increased short term healing in meniscal tears	[49]
PDGF-AB	Ovine	*In vitro*	Affected mitogenic response from the outer 1/3 of meniscus	[45]
PDGF-AB	Bovine	*In vitro*	Stimulated cell migration and increased DNA synthesis	[42]
TGF-β	Ovine	*In vitro*	Increased proteoglycan synthesis	[24]
TGF-β	Human	*In vitro*	Increased proteoglycan synthesis	[25]
Hyaluronic Acid	No cells	Rabbit	Increased rate of healing in a cylindrical meniscal defect	[47]
HGF	Bovine	*In vitro*	Stimulated cell migration, increased DNA synthesis	[42]
BMP-2	Bovine	*In vitro*	Some cell migration and increased DNA synthesis	[42]
IGF-1	Bovine	*In vitro*	Some cell migration	[42]
IGF-1	Bovine	*In vitro*	Increased proteoglycan synthesis	[46]
Epidermal GF	Bovine	*In vitro*	Some cell migration	[42]
Interleukin-1	Bovine	*In vitro*	Some cell migration	[42]
Hyaluronan	No cells	Rabbit	Stimulated collagen remodeling in peripheral zone	[48]

Table 1: Growth Factors that were studied for meniscal tissue engineering

Most experiments have used cultures of fibrochondrocytes or small tissue explants to examine the proliferative response of the cells or protein synthesis. One growth factor that has a potential application is transforming growth factor-β (TGF-β). Studies by Tanaka et al. (24) and Collier *et al.* (25) showed that TGF-β increases proteoglycan synthesis in fibrochondrocytes from all different regions of the meniscus in a dose-dependent manner. In these studies Collier and Ghosh used ovine fibrochondrocytes and Tanaka *et al.* used human fibrochondrocytes (24, 25). Studies by Spindler *et al*. (45) and Bhargava *et al*. (42) tested the effect of human platelet-derived growth factor-AB (PDGF-AB) on ovine and bovine cells. The ovine study showed that PDGF-AB only affected the mitogenic response from the peripheral third of the meniscus; there was no effect on the inner 2/3 of the tissue (45). Bhargava *et al.* (42) bovine test showed that PDGF-AB stimulated the migration of fibrochondrocytes from the inner, middle and outer 1/3 of the meniscus; PDGF-AB was also shown to increase DNA synthesis by the cells from all three sections. Bhargava *et al.* (42) also found increased DNA synthesis when hepatocyte growth factor (HGF) or bone-morphogenic protein-2 (BMP-2) was used. HGF also increased the cell migration rate similar to PDGF-AB. BMP-2 and IFG-I (insulin-like growth factor-1) stimulated the migration of fibrochondrocytes from the middle zone by 40-50%. This study also tested the effects of two other growth factors: interleukin-1, which stimulated migration of cells taken from the peripheral 1/3 of the tissue, and epidermal growth factor (EFG), which stimulated migration of cells from the inner and outer zones by 40-50% (42). A study by Imler *et al.* (46) found that in bovine explants the addition of IGF-1 increased proteoglycan synthesis in a dose dependent manner. An earlier study by Webber *et al*. (13) tested the effect of fibroblast growth factor (FGF) and human platelet lysate (PL) on proliferation of fibrochondrocytes and both were found to stimulate their growth.

Other studies have been performed to check the effects of growth factors on healing of open defects and tears in the meniscus. Hyaluronic acid, hyaluronan and endothelial cell growth factor (ECGF) have been studied in both tear healing and defect repair (47-50). Suzuki *et al.* (47) created a cylindrical defect in a rabbit anterior lateral horn and then made weekly injections of hyaluronic acid, demonstrating increased rate of healing. A study by Sonoda *et al.* (48) tested hyaluronan's effect on the healing of tears in the peripheral and avascular region of the rabbit meniscus. They found that hyaluronan stimulated collagen remodeling in the peripheral zone and inhibited swelling in the avascular zone (48). Another study tested the effect of ECGF, a member of the acidic FGF

family (51), on the healing of an allograft to the joint capsule, found that ECGF increases short-term healing, but over the long term no difference was found (49). A study by Hashimoto (50) tested the effect of ECGF on assisting the healing of a cylindrical full-thickness defect placed in the meniscus of a dog. The defect was filled with a fibrin sealant (some containing ECGF) and then allowed to heal over a 24-weeks period. The defects that contained both the fibrin sealant and ECGF showed the best results. Roughly, 90% of the defect was filled at the end of the study (50).

Tissue Engineering Attempts

Only a limited amount of work has been carried out to attempt to tissue engineer the meniscus with fibrochondrocyte seeded scaffolds (35-37, 52). Ibarra *et al.* (37) used polyglycolic acid (PGA) scaffolds seeded with bovine fibrochondrocytes in an attempt to engineer the meniscus. The cells were seeded onto the PGA scaffold, implanted subcutaneously in nude mice, and evaluated after 12 weeks. Grossly, the construct resembled meniscal tissue, and histology indicated meniscal repair tissue. Unconfined compression on the constructs demonstrated compressive properties that were 40% of the native tissue levels. These studies were also performed using ovine, canine, and human cells. In a later study, ovine cells were seeded onto a copolymer of PGA and polylactic acid (PLA), implanted subcutaneously in a sheep, and later implanted within the knee joint. Histological evaluation showed the presence of fibroblastic and chondrocytic cells within the repair tissue, along with collagen and proteoglycans. In a study by Ertl *et al.* (52) a PGA mesh was seeded with rabbit fibrochondrocytes, grown in culture for one month, and implanted into a full-thickness defect for a period of one year. Histological results showed healing at one year, but no biochemical or biomechanical testing was performed. Collagen scaffolds have also been examined for tissue engineering of the meniscus. A study by Nakata *et al.* (35) showed that human fibrochondrocytes can attach to a collagen sponge and infiltrate some of the scaffold. Mueller *et al.* (36) examined the effect of collagen-glycosaminoglycan (GAG) scaffolds made from either type I collagen or type II collagen. The scaffolds were seeded with canine fibrochondrocytes and evaluated over a period of 21 days. Results showed that in the type I collagen matrix the cells remained near to the periphery of the scaffold, whereas they were evenly distributed throughout the type II scaffold. The type II

scaffold contained 50% more GAGs than the type I scaffold, and did not contract, whereas the type I scaffold shrunk to half of its original size. To the authors' knowledge, no seeded collagen scaffold has yet been implanted in an animal model.

Conclusion

Successful tissue engineering of the meniscus would provide great help for the treatment of meniscal defects, but much work needs to be carried out before this can be achieved. More characterization of the cells is needed, particularly with respect to their culture environment and growth factors. The use of bioreactors for small and large scale cell production needs to be examined, and more animal studies need to be performed. Hopefully, fibrochondrocytes can be used to tissue engineer constructs that will successfully address the difficult problem of meniscal regeneration.

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