

Joints: Regeneration of Bone, Cartilage, Meniscus, Ligament, and Tendon

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BONE

Calcium Phosphate Materials as Matrices for Bone Regeneration: Bone Graft Substitute Materials

The terms "permanent" and "resorbable" have generally been used to describe the two broad classifications of bone substitute materials. In fact, all of the substances referred to as being permanent undergo some degree of biodegradation. This might take the form of physicochemical dissolution and/or fragmentation. The term resorbable has been used somewhat indiscriminately to describe the loss of implant substance over time. Strictly speaking, this term implies that the mechanism of breakdown of the synthetic material is the same as that occurring with osteoclast-mediated bone resorption. However, mechanisms governing the degradation of synthetic calcium phosphate substances have not yet been determined.

While it is generally been considered a nonresorbable material, synthetic HA has also been found to undergo physicochemical dissolution, albeit at a very slow rate. Because it is only slightly soluble in biological fluid, synthetic HA substances can functionally be considered as long-lasting implants, especially when they are incorporated into bone. This behavior is in contrast to that of anorganic bone, that appears to undergo a cell-mediated resorption. In an animal (rabbit) study, histology revealed the presence of greater numbers of osteoclast-like cells on the surface of the implants of natural bone mineral than on the surface of synthetic HA samples (111) after 40 days of implantation. Investigation of the remodeling of implanted matrices can be challenging because the period for normal remodeling of bone in the adult can be from months to years.

Initially, bone substitute materials were developed to serve as a temporary scaffolds or templates to facilitate osteogenesis. While some of the more recent investigations have explored the use of polymers as matrices to be used as implants in defects in bone, most efforts have implemented calcium-containing ceramics. Calcium sulfate and tricalcium phosphate (TCP) were two of the calcium-containing substances first studied. Potential problems with these materials related to the fact that they often underwent physicochemical dissolution too soon after implantation for many applications. However, there is still some question as to the rate at which these materials become absorbed by the body. Differences in experimental findings might be related to variations in the composition and structure of the substance referred to as TCP, and the different physiological characteristics of the implant sites and animal models. Uncertainty about the bonding of bone to TCP may be due to the fact that in many situations the TCP undergoes physicochemical dissolution at a rate which precludes the precipitation of biological apatite and subsequent bone formation on its surface; the dissolving surface does not allow for protein adsorption and cell attachment.

Much of the early work with HA began in the mid 1970s. Mechanisms underlying the bone-bonding associated with incorporation of these substances in osseous tissue are beginning to be revealed. However, despite these advances, limitations in the ability of synthetic bioceramics to duplicate the behavior of natural bone remain. These limitations relate to the fact that, as noted above, the synthetic products do not replicate the chemical composition and structure of natural bone mineral, which is a calcium deficient carbonate apatite. This prompted implementation of methods to treat bone in order to prepare natural bone mineral (*i.e.*, anorganic bone) to be used as an implant material. Removing the organic phase of bone without altering the chemical composition, morphology, and microstructure of the mineral crystallites is challenging, but has been achieved.

Biological Response

Some of the earliest studies employing calcium phosphate ceramics in the mineral form of HA were reported by Jarcho (56). His animal investigations yielded histology suggestive of bone-bonding to the material. Jarcho interpreted the results of electron microscopy studies as showing the precipitation of bone apatite on the surface of implants (56). It was postulated that collagen fibrils synthesized by nearby osteoblasts initially formed a zone between the calcified layer on the implant and the bone-forming cells. The continuum of HA-like crystallites was then generated between the calcium phosphate specimen and the matrix of the newly forming bone. Jarcho also suggested that there was evidence that the zone immediately adjacent to the implant material included glycosaminoglycans similar to those comprising the "ground" or "cementing" substance of natural bone. Later electron microscopy studies revealed other ultrastructural features of the interface between HA-like implants and tissue (103).

Heughebaert, *et al.*, (47) employed methods to detail the composition and structure of synthetic HA implants before and after implantation in soft tissue sites in animals. They found that the mineral phase that deposited on the implants *in vivo* had characteristics consistent with bone apatite. The precipitating substance was found to be a carbonate-apatite with an electron diffraction pattern and infrared spectrum similar to that obtained from bone mineral. This mineral phase was significantly different from the carbonate-free synthetic HA ceramic implant.

Recent investigators in France (24, 25) and Japan (63, 64) have employed *in vitro* and *in vivo* models to evidence the deposition of biological apatite onto certain calcium phosphates and bioactive glasses in the course of bone-bonding. One recent study proposed that the time required for deposition of apatite *in vitro* from a "simulated body fluid" could be correlated with the relative strength of bone-bonding to various bioactive glass and HA substances (64).

In vitro studies and animal investigations indicate that biological apatite deposits on the surface of calcium-containing implants soon after insertion into the body. This biological apatite layer presumably serves as a substrate for subsequent protein adsorption and bone cell attachment. The fact that biological apatite deposition is an obligatory precursor phase for bone bonding would indicate that implants comprising anorganic bone might be more rapidly incorporated into host bone because their surface already comprises biological apatite. Recent investigations have suggested that such may be the case (57).

Calcium Phosphate Coatings for Permanent Prostheses

A major problem associated with the use of internal skeletal prostheses is the fixation of the device to bone. Deficiencies in current methods of fixation limit the function and longevity of total joint replacement prostheses. Recent studies have indicated that bone will bond (chemically) to a variety of

calcium-containing substances. Current work is focused on the implementation of calcium phosphate coatings that have been plasma-sprayed onto metallic hip replacement prostheses. Histological evaluation of specimens retrieved from animals and human subjects has revealed that bone forms directly on the surface of these calcium phosphate coatings within a few weeks of implantation. However, investigations also indicate that the strength of attachment of the calcium phosphate coating to the metallic substrate degrades with time, and thereby leads to detachment of the coating.

The elusive goal of investigations of a wide range of implantable devices is the production of a biomaterial surface to which tissue will chemically bond. The focus of the majority of these studies is the surface immobilization of amino acid sequences (*viz.*, R-G-D) that have been shown to be the ligand for integrins of several types of cells. Studies of this type have yet to prove the idea. In this light, it is remarkable that, not only has the bonding of osseous tissue been shown to occur with a wide range of inorganic substrates, but certain substrates have already been implemented in large numbers clinically as prosthesis coatings. In the bone-bonding to calcium phosphate surfaces, we have found materials to which certain tissue (osseous) will bond, but have not yet revealed the mechanisms. What is also remarkable is that there is already discussion to discard bone-bonding substances because of their inability to be permanently attached to metallic substrates. There is a consensus developing that calcium phosphate substances should be implemented as resorbable coatings on porous metal only, with the role being early, temporary bone-bonding. It has become clear and compelling that to judiciously implement calcium phosphate surfaces as permanent attachment vehicles for orthopedic prostheses we must elucidate the chemical and crystalline features of the surface that are determinants of the bone-bonding process .

Studies conducted during the last few years have not yielded definitive findings regarding the mechanism by which bone bonds to the surface of calcium-containing implants. There continue to be two working hypotheses, one that emphasizes the initial deposition of biological apatites on the implant surface and the other that proposes that the adhesion of certain proteins and/or proteoglycans is the determining factor in the bone-bonding process. The following critically reviews recent investigations performed *in vivo* and *in vitro*.

***In Vivo* Studies**

A recent investigation employed transmission electron microscopy to investigate apatite formation on dense sintered HA, tricalcium phosphate, and a "bioactive glass ceramic."⁽⁸⁴⁾ Deposition of biological apatite differed on these three specimens reflecting their "bioactivity," and suggesting differences in their bone-bonding behavior. A thin collagen-free apatite layer was identified on the surface of the implants. The apatite layer was observed before the mineralization of surrounding bone matrix, and was also evident on surfaces bordering bone marrow.

Another investigation (8) examined by scanning electron microscopy (SEM) HA specimens obtained from laboratory dissolution experiments and animal implantation to determine the role of reprecipitation of apatite in the bone-bonding process. It was concluded that "the formation of the bone/HA bond seems to involve dissolution/reprecipitation phenomena." The findings were interpreted as being the "first morphological evidence of epitaxial growth involvement in the formation of this bond."

Other studies have correlated bone-bonding behavior with the biological mineralization process involving matrix vesicles. Z. Schwartz, *et al.*, found that the ability to support primary mineral formation, involving matrix vesicle production and maturation, may contribute to the bonding of bone to HA (93).

***In Vitro* Investigations**

Several investigations have recognized the value of tissue culture systems for study of the response of osteoblast-like cells to biomaterial surfaces. One series of investigations employed cells obtained by enzymatic digestion of neonatal rat calvaria (89). The focal contacts of these cells with HA and other substrates was investigated by epi-fluorescence microscopy with staining for F-actin and vinculin. There were no conclusions drawn relative to the correlation of focal contact formation by the osteoblast-like cells and the chemical characteristics of the biomaterial surface. Subsequent investigations employed SEM to investigate qualitatively the spreading of these cells on HA and other biomaterial surfaces within two hours in culture (73). After 0.5 hr, cells on the HA surface were flat, with the edge of the cytoplasm difficult to discern. This was in contrast to the rounded morphology of cells on alumina, tissue culture polystyrene, and bone. The authors concluded that "the cellular morphological response may be related to the bioreactivity of hydroxyapatite." The expression of certain non-collagenous bone proteins by osteoblasts on biomaterials surfaces and culture was also studied (90). Qualitative findings suggested that there was a greater level of message for osteopontin in cells on the HA surface than in cells on tissue culture plastic or titanium alloy. The authors emphasized that these findings required follow up.

Bone marrow derived cells from rats have also been used in tissue culture studies investigating the response of osteoblast-like cells to HA and other biomaterials surfaces. Results of one study were interpreted as supporting the hypothesis that differentiating bone cells synthesize an organic matrix that deposits on the surface of the bone-bonding material before the mineralization phase. Shen, *et al.*, (95) demonstrated that a proteoglycan (CS-56 antibody positive) and osteopontin deposited initially on surfaces that subsequently became bone-bonded. These authors concluded that these proteins "could be implicated in providing seeding sites for calcium phosphate crystal and cell attachment." Other investigators (48) studied the role of the adhesion proteins, fibronectin and vitronectin, on the attachment of human bone-derived cells on HA substrates *in vitro*. They found that removal of fibronectin from the medium did not significantly reduce the number of cells spread on HA. Removal of vitronectin, however, did influence cell spreading, leading to the conclusion that this adhesion protein might be critical for bone-bonding. The presence of receptors for vitronectin on the surface of the cultured bone-derived cells was detected by reaction with polyclonal antibodies raised against vitronectin subunits.

ARTICULAR CARTILAGE

Defects - fissures and larger gaps - in adult articular cartilage, that do not penetrate the underlying vascularized tissues, generally do not heal (16). This reflects in some part the inability of adult articular chondrocytes to contribute to a reparative process (14). While cloning of chondrocytes may be found in the cartilage near the site of injury (14, 51), a hypercellular zone, as seen around defects in other connective tissues, is not seen bordering articular cartilage lesions. The absence of a hypercellular response may be related to the low number density and mitotic activity of the chondrocytes (15) and the absence of a fibrin clot (14). Another factor, however, may be the inability of the cells to extricate themselves from their extracellular matrix and freely migrate through the matrix to the wound edge (14).

While several approaches have been employed in the laboratory and in the clinic toward the goal of engineering articular cartilage, none have yet been successful. Recent clinical studies, however, have suggested that significant symptomatic relief can be provided by treatments resulting in the formation of tissue with the cellular and matrix composition and architecture of fibrocartilage and non-articular hyaline cartilage. The principal question is how long these tissues will provide pain-free

articular function. The answer will come only after longer term prospective clinical investigations employing novel non-invasive imaging modalities or histological assessment of biopsies to correlate clinical outcome with reparative tissue make-up. One result of these studies may be to demonstrate the clinical value of tissue engineering approaches that, while falling short of the goal of producing articular cartilage, yield a reparative tissue with a clinically meaningful longevity.

Matrices

The matrix can have several roles in the process of tissue engineering. These roles include: (1) structural support to the defect site, (2) barrier to the in-growth of undesirable cell and tissue types, (3) scaffold for cell migration and proliferation, and (4) carrier or reservoir of cells and/or regulators. A variety of matrices have been studied as potential carriers and scaffolds specifically for articular cartilage regeneration.

Recent *in vivo* studies have used collagen sponges (38) and gels (61), devitalized articular cartilage, natural coral, agarose gels, PLA sponges (21), and PLA/PGA co-polymers as matrix materials (7). Many of these investigations also involved cells and regulators, but none were able to achieve complete regeneration of articular cartilage in the surgically created defects. Implanted matrices alone have been shown to increase the rate and completeness of subchondral bone formation in osteochondral defects. The scaffolds increased the rate and total amount of filling as well as the quality of the tissue filling the defect. Unseeded devitalized osteochondral allografts and unseeded bilayer collagen matrices used by Toolan, *et al.* and Frenkel, *et al.*, respectively, both increased the amount of hyaline cartilage filling the defect. Defects treated with chondrocyte-seeded collagen gels were filled as early as one day post-operatively. The rapid filling of the defect was likely due to the implanted chondrocytes with the implanted gel providing a mechanism for cell delivery and mechanical support for the defect.

Although type I collagen matrices have generally been researched, type II collagen matrices may better support the chondrocyte phenotype and matrix molecule synthesis. Nehrer, *et al.* found that cells seeded in type II collagen sponges exhibited a more chondrocytic morphology (spherical) and synthesized matrix components at a higher rate compared to cells seeded in type I collagen sponges (82, 83).

Cells

Chondrocytes exhibit a very specific phenotype in that they synthesize type II collagen and proteoglycans that make up the articular cartilage matrix. As a result of this demand, most of the research involving cells as a component in articular cartilage regeneration in recent years has focused on the use of articular chondrocytes. However, other cell types have also shown promise.

The first human trial of cultured articular chondrocytes for treatment of cartilage defects was reported in 1994 (11). The clinical findings were more favorable for defects on the femoral condyle than on the patella. Histological evaluation of biopsies revealed hyaline-like and fibrous tissue comprising the reparative tissue of asymptomatic patients. An associated rabbit study (12) revealed filling of chondrocyte implanted defects with hyaline cartilage 52 weeks post-operatively. However, a more recent canine investigation questions the longevity of the procedure as no difference in the types of reparative tissue filling treated and untreated defects was detected after 12 and 18 months (10).

The use of articular chondrocytes, while advantageous in that the cells already possess the desired phenotype, presents practical problems for tissue engineering. Articular chondrocytes have a low capacity for proliferation – one of the reasons why natural healing of cartilage defects does not

occur. Thus, cells must be grown up in culture. When chondrocytes are brought out of their natural three-dimensional environment onto two-dimensional culture plates, they switch to a fibroblastic phenotype. Studies have shown that these cells will regain the chondrocytic phenotype when returned to the proper environment. However, recent research has aimed to eliminate de-differentiation.

The second problem in using articular chondrocytes is obtaining the cells. It may be preferable to use autologous cells in tissue engineering so as to obviate any immunological reaction or transmission of disease. In order to obtain autologous chondrocytes, healthy cartilage must be harvested from uninvolved regions of the joint. However, the harvesting of healthy cartilage for a source of autologous cells introduces problems related to donor site morbidity and the possibility that the harvest procedure might result in joint responses resulting in changes of the uninvolved cartilage.

The difficulty in obtaining chondrocytes has led to research on the potential use of other cell types for articular cartilage engineering. Non-chondrocytic cells have been induced *in vitro* to exhibit the chondrocyte phenotype given the appropriate culture conditions. A recent overview of cartilage repair investigated the healing procedure of articular cartilage lesions in the rabbit knee treated with cultured autologous mesenchymal stem cells from marrow (19). The authors propose a method of articular cartilage regeneration that employs pretreatment of the defect with trypsin and subsequent implantation of a high density of cultured mesenchymal stem cells delivered in type I collagen gel.

Non-articular cartilage cells have also been studied as a potential source of chondrocytes. Isolated cells from ear perichondrium cultured *in vitro* with BMP-7 (rhOP-1) were reported to have differentiated into articular chondrocytes by the third week and neo-natal foreskin fibroblasts cultured at high-densities and under hypoxic conditions took on the chondrocytic phenotype. *In vivo*, perichondrial cells seeded in PLA and implanted into surgically defined defects in the rabbit knee yielded variable results, including an apparent decrease in filling from week 6 to week 12, post-implantation.

Regulators

Because of the inherent difficulties in trying to implement cell-based treatments, a number of researchers have investigated the roles that regulators may play in articular cartilage tissue engineering.

BMP-7 (rhOP-1) can be useful in triggering expression of the chondrocytic phenotype in non-chondrocytic cells *in vitro*. Hepatocyte growth factor (HGF) is also believed to play a role in stimulating progenitor stem cells to differentiate into chondrocytes *in vivo*. Using regulators to turn non-chondrocytic cells into functional chondrocytes could eliminate the problem of obtaining and culturing articular cartilage chondrocytes.

Enzymes may play a role in articular cartilage tissue engineering in enhancing the attachment of cells or implants to the defect site. Hunziker and Kapfinger treated superficial lesions of mature rabbit cartilage with chondroitinase ABC and noted increased cell attachment up to one month after enzyme treatment (50). They suggest that extraction of surface proteoglycans results in improved cell adhesion, but that the cells are able to replace the lost proteoglycans.

Previous studies investigating the migration of isolated chondrocytes *in vitro* have identified agents that affect the motility of the cell (37, 102). Others employing articular cartilage explants have reported the migration of cells to the edge of the tissue (72, 94). Malesud, *et al.*, (72) found that the times to chondrocyte outgrowth from explants of adult human articular cartilage from one patient were 7 and 10 days. In another investigation using calf cartilage explants (70), cell proliferation was seen at the margins of the tissue after 6 weeks. In a later study also employing calf articular cartilage explants, Scully, *et al.*, (94) found that chondrocytes repopulated a laceration site in the explant between 21 and

42 days in culture, and displayed a proliferative response to bFGF but not TGF- β 1. Importantly, they showed that the cells that repopulated the defect maintained the chondrocyte phenotype. In a recent study employing adult bovine articular cartilage explants, Reindel, *et al.*, (92) found some indication of migration of chondrocytes to the surface of the explants after 3 weeks; moreover, these cells appeared to be contributing to the integrative repair of the experimental constructs. While collectively the authors of these previous explant studies considered that the cartilage matrix restricted the chondrocytes from migration, none previously employed enzymatic treatment prior to culture to enhance outgrowth.

The effect of chondroitinase on the adhesion of reparative cells to a wound surface was investigated in a recent study performed in a rabbit model (50). Treatment of the surface of superficial defects with 1U/ml of the enzyme for 4 minutes almost doubled the extent of the surface covered by reparative cells at 1 month but demonstrated no effect after 6 months. Of particular importance was the source of the reparative cells. While this issue was not expressly addressed in the aforementioned *in vivo* study, based on their results from prior work (51), the authors suggested that the adhering cells originated from the synovium.

Current Status

While a variety of matrices, cells, and regulators, alone and in combination, have shown promise as components for tissue engineering of articular cartilage, the current methods have not yet successfully resulted in the production of articular cartilage. *In vitro* attempts at engineering articular cartilage have produced tissue that is cartilage-like and has some similar mechanical properties. However, this tissue still lacks the full composition of natural cartilage and it remains to be seen how it will integrate with host tissue and perform *in vivo*. The best *in vivo* attempts at regenerating articular cartilage have produced tissue that histologically matches normal cartilage. However, the reparative tissue does not fully integrate with the surrounding host tissue. Failure to integrate with the host tissue results in implant instability and micromotion that may lead to the eventual degradation and failure of many implants.

Despite the great amount of progress made in recent years towards the development of tissue engineering articular cartilage, scientists have yet to achieve the goal of engineering fully functional articular cartilage for the resurfacing of joints. However, non-articular cartilage repair tissue can restore pain-free joint function. The benefits of such treatment, however, may be short-lived if the functional demands on the articular surface lead to breakdown and degeneration of the newly formed tissue. Further studies are still needed to determine the longevity of various reparative tissues and to match various treatment modalities with the subpopulation of patients that would most likely benefit from a given procedure.

MENISCUS

The biomechanical importance of the knee meniscus, a wedge-shaped fibrous structure interposed between the femoral condyles and the tibial plateau, is well recognized. The mechanical properties of the tissue are largely the result of a unique structural arrangement of the predominantly type I collagen fibers. The cells that maintain this matrix comprise two cell populations: an elongated, fibroblast-like cell type in the superficial zone of the meniscus, and, throughout the meniscus, a spherical or chondrocyte-like cell type, which resembles the chondrocytes in articular cartilage (42). Many studies (41, 43, 62, 74) dating back to the 1930s have indicated that there is little capacity for intrinsic healing of injuries to the meniscus by the proliferation of meniscal cells. The roles of these cells in remodeling, degradation, and healing of the meniscus are incompletely understood.

Given the incomplete healing of meniscal injuries, the value of certain surgical treatments remains in question. King described an ingrowth of fibrous tissue hardly distinguishable from normal fibrocartilaginous tissue after partial or total meniscectomy (62). However, despite the fact that this new “meniscus-like” tissue had formed, the observed roughening and degradation of the opposing articular cartilage⁶ suggested that this reparative tissue was not normal. While other follow-ups of partial and full meniscectomies were promising (98, 99), later work suggested that the degenerative changes of the knee joint were due to the inadequate weight-bearing capability of the reparative meniscus (20, 31). Other authors have since described the adverse long-term effects of total meniscectomy (5, 49, 55, 58). Despite the initial optimistic reports of partial arthroscopic meniscectomy (79, 86, 87, 107), follow-up revealed only fair or poor outcomes (32), and the long-term results of meniscectomy appear to be even worse (1, 75, 100, 108).

A recent trial of a porous collagen matrix (101) employed as an implant to fill meniscal lesions has yielded encouraging clinical results in nine patients followed for at least 3 years. A 3-month biopsy displayed newly formed collagen, but it appeared very immature and without organization. Six-months biopsy specimens revealed more collagen, however the newly formed tissue still displayed a chondroid appearance indicating its immaturity. Nevertheless the finding of some dense fibrocartilage in some sections was promising. In this light, pre-seeding matrices with meniscus cells, previously harvested arthroscopically from parts of the injured meniscus and grown in culture, might accelerate the tissue regeneration process.

Previous studies have demonstrated the capability of fibrochondrocytes, isolated from the menisci of rabbits, to proliferate in culture and to synthesize matrix molecules (*viz.*, proteoglycans) (104). Significant effects of age and gender on a) yield of cells from digested tissue, b) growth rate in culture, and c) proteoglycan synthesis, were noted (106). That the proteoglycans synthesized by the fibrochondrocytes in culture resembled those synthesized in organ culture, based on size and GAG content, indicated that meniscal cells were “capable of expressing their differentiated phenotype in short-term monolayer cell culture.” (105) Moreover, these findings suggest that, while there is little evidence of a healing response of this tissue to injury *in vivo*, meniscus may have some potential for intrinsic repair.

In a recently published study (52), polyglycolic acid scaffolds seeded with newborn calf meniscus fibrochondrocytes were implanted subcutaneously into nude mice for up to 16 weeks. The resulting tissue appeared to display a pattern of collagen fiber orientation resembling normal meniscus. The promising findings of the previous and current studies warrant continued investigation of meniscal cell-seeded matrices for regeneration of the meniscus.

ANTERIOR CRUCIATE LIGAMENT

The human anterior cruciate ligament (ACL) is a complex structure of extracellular matrix proteins maintained by a diverse population of cells (3, 4, 36). The extracellular matrix chemical composition (3, 36) and hierarchical structure (6, 22) have been previously defined; however, the cellular distribution within the ligament itself has not been described. The ligament is comprised of longitudinally oriented fascicles, 20 to 400 μm in diameter, that course from femoral to tibial insertion sites. The fascicles consist of densely organized type I collagen fiber bundles that are approximately 20 μm wide and display a sinusoidal, organized waveform referred to as crimp, which has been shown to be a critical determinant of the load-deformation behavior of ligament (29). References to the cells which maintain this highly organized structure have identified different fibroblast morphologies, but a

histologic evaluation of the distribution of cell density, cell morphology and cell phenotype within the ligament has yet to be reported.

The cells that maintain the ACL have been distinguished histologically on the basis of nuclear shape and the presence of a lacunar space. Three principal cell types have been described: fusiform, ovoid and spheroid. Fusiform, or spindle shaped, cells have been noted to be intimately related to the crimped collagen fibers, and distributed throughout the midsubstance of the ACL. These cells have also been found in the "outer zones" of human ACLs (85). Ovoid cells have been identified in columns (4, 71, 109) and in lacunae (22, 109). They have also been noted in "central areas" of the human ACL (85). Periodic acid-Schiff staining has been positive in the ovoid cell columns, suggesting the presence of glycosaminoglycans (22). Transmission electron microscopy (TEM) of the area surrounding the ovoid cells has shown amorphous extracellular matrix in the peri-cellular space (71). Spherical, or cuboid, cells have been described in the rabbit ACL (22). While morphologic criteria have distinguished certain cells in the ligament, little is yet known about the distribution and function of these cells in the ligament.

The human anterior cruciate ligament (ACL) is one tissue which appears to lack the ability to repair itself after rupture [Arnold, 1979 #90; Cabaud, 1979 #91]. Successful healing of other ligaments, such as the medial collateral ligament (MCL), follows a known pattern. Initially, a provisional matrix of clotted blood fills the space between the ruptured ends of the ligament. This provisional matrix is then infiltrated by fibroblasts, the source of which may be intrinsic or extrinsic to the injured ligament (91). In tendon and meniscus, as in dermis, the presence of a contractile fibroblasts phenotype, the myofibroblast, has been associated with the process of wound closure in this stage of healing. While myofibroblasts have also been found in the healing lapine MCL, they have not yet been directly related to the contracture process that re-establishes the normal in situ strain. Little is known about the response of the human ACL to injury, particularly with respect to the migration and contractile behavior of the constituent cells.

Previous work has demonstrated outgrowth from human and animal ACL explants onto 2-D surfaces [Geiger, 1994 #10; Deie, 1995 #74]. Outgrowth from explants likely has two components - cells which migrate from the explant and cells which have migrated and begin to proliferate on the 2-D surface. Previous work has assumed minimal contribution from the proliferation component and reported outgrowth rates as migration rates [Geiger, 1994 #10]. This method has yielded a migration rate from rabbit ACL explants of 0.48 mm/day. The previously reported migration rate of fibroblasts is 0.20 mm/day [Stearns, 1940 #87].

TENDON

Tendon is a specialized dense connective tissue that links muscle to bone and allows for the transmission of muscle contraction forces to the bone for skeletal locomotion; for example, the Achilles tendon, one of the largest in the body, links the triceps surae muscle, the grouping of the gastrocnemius, the soleus, and the plantaris muscles to the calcaneous bone. As organs, the tendons of the body vary greatly in their anatomy and structural mechanical properties. An approach that might result in the regeneration of one tendon may require modification to facilitate the regeneration of another. Tendons consist of three parts: the muscle attachment region, the substance of the tendon itself, and the bone attachment region. These three parts of tendon vary in their cellular make-up, histology, and function, and may require different tissue engineering approaches. This chapter will focus on the midsubstance of tendon.

The extracellular matrix of tendon is composed predominantly of collagen fibers highly aligned along the long axis of the tendon. Spindle-shaped cells - fibroblasts - aligned in columns along the direction of the collagen fibers, are sparsely dispersed through the tissue. When viewed transversely, the cells appear as star-shaped figures among bundles of collagen. The mid-substance of the tendon is generally avascular and absent innervation.

That tendon is arguably the least complex of the connective tissues with respect to composition and architecture might reasonably lead to the expectation that it would be more amenable to tissue engineering approaches than other biological structures. However, decades of experience have shown how refractory tendon is to treatment. This limited capability of tendon injuries to regenerate is a better indication of the challenge it poses to being engineered, and evidences the importance of developing a procedure to do so.

Extracellular Matrix

Tendons have a crimped, waveform appearance when seen under polarized light microscope. The periodicity of the alternating light and dark bands comprising the zig-zag pattern of crimped fibers can be used to determine the crimp angle (28 degrees), crimp length (65 μm), and crimp wavelength (120 μm). The crimp pattern of tendon has been shown to play an important role in its mechanical properties (23, 26); Diamant *et al* (26, 28) demonstrated that the crimp pattern unfolds during initial loading of tendon.

Tendons that generally move uniaxially, such as Achilles tendon, have a loose areolar connective tissue, the paratenon, which is continuous with the tendon. The paratenon stretches several millimeters and recoils without tearing or disrupting tendon blood supply. An interlacing mesh work of thin collagen fibrils, elastic fibers and glycosaminoglycans gives the paratenon this elasticity and extensibility. On the other hand, tendons which bend sharply, such as the flexor tendons of the hand, are enclosed by a synovial sheath. The sheath helps to direct the path of tendon movement by acting like a pulley and allows low friction movement between tendon and the adjacent bones and joints. The sliding of these tendons through the sheath is assisted by the presence of synovial fluid between the outer wall of the tendon and the inner wall of the tendon sheath.

The organization of the extracellular matrix molecules of tendon at the nanometer and micrometer levels is the principal determinant of the physiological function of this tissue, its mechanical behavior, as will be discussed below. The degree to which tissue engineering approaches are successful will be reflected in the degree to which the normal composition and architecture of the extracellular matrix has been restored.

Cells

Fibroblasts (also called tenocytes) are the predominant cell type in tendon. Endothelial cells and nerve processes form only a small part of the of the cell population. Ippolito *et al* (53) has shown that there is a subpopulation of myofibroblast-like, contractile cells present in normal tendon and has noted their presence in healing tissue (9, 39, 46, 53, 97) This subpopulation of cells has been speculated to be involved in the "tensioning" of the tendon and in the modulation contraction-relaxation of the muscle-tendon complex (53).

As the parenchymal cell of tendon, the tenocyte has the role of maintaining matrix structure through the degradative and formative processes comprising remodeling, and to some extent can contribute to healing. However, that tendon has a relatively low density of cells that display a low mitotic activity, explains the low turnover rate of this tissue and questions the degree to which these

parenchymal cells can promote intrinsic healing. Moreover, this fact prepares for the likelihood that exogenous cells may be an important component of tissue engineering strategies for tendon.

Tendon Healing

The extent to which injuries to tendon heal depends on many factors such as the anatomical location, vascularity, skeletal maturity as well as the amount of tissue loss. Spontaneous healing of tendon has been studied extensively in both the Achilles tendon and the flexor tendons of the hand. Tendon healing normally results in the formation of scar, which is different morphologically, biochemically, and biomechanically from tendon. With time, the scar tissue may assume some of the characteristics of tendon; however, complete regeneration does not appear to occur. Although both Achilles and flexor tendons respond to injury by forming scar tissue, scarring in the flexor tendon appears to have a more detrimental effect on the function of the tissue. Flexor tendons need the ability to glide within their synovial sheath to function properly and formation of adhesions to the sheath during healing interferes with this gliding function.

Following tendon injury by full transection, there is a spontaneous retraction of the cut tendon ends. This retraction has also been reported to also occur in a full-transection rabbit animal model for the medial collateral ligament. In the medial collateral ligament, the retraction of the cut ligament ends produced a gap as large as 2-4 mm (34). In the Achilles tendon of both a rat and rabbit animal model, the gap formed by the retraction of ends, with the joints held in neutral position, was observed to be approximately 9-12 mm (13). Additional retraction of the ends can occur with movement of calcaneus and knee joints.

The response of Achilles tendon to injury, reviewed in several articles (2, 17, 68), follows a sequence similar to that found in other connective tissues such as ligament and skin. This sequence is generally considered to consist of three overlapping phases: inflammation, repair, and remodeling. Following is a summary of the response of tendon to a lesion produced by a full transection of tendon as reported by several authors. Within the first few hours of injury, the collagenous matrix is disrupted and tendon and blood cells die. A hemorrhagic exudate fills the lesion site and within minutes, a fibrin clot forms and seals the wound (13). The clot contains inflammatory products (fibrin, platelets, red cells, and nuclear and matrix debris) (13, 88). This clot has little tensile strength.

The inflammatory stage generally starts within hours of injury and can take from 3 to 10 days to complete. This stage is associated with "clean-up" of the lesion site. Polymorphonuclear neutrophils (PMNs) and lymphocytes, and other acute inflammatory cells, invade and populate the wound site within hours of injury. Monocytes and macrophages appear soon after to continue the phagocytosis of cell and matrix debris begun by the PMNs.

The period of dramatic fibroblast migration and proliferation and matrix synthesis, the repair phase, starts as early as 10 days post-injury and can take up to 2 to 5 weeks to complete (13, 45). Undifferentiated and disorganized fibroblasts containing well-developed endoplasmic reticulum, infiltrating from the wound edge and paratenon, begin to proliferate in wound site within the fibrin mesh of the clot. Simultaneously, endothelial cells of surrounding vessels enlarge and proliferate forming capillary buds that follow the migrating fibroblasts. Together the fibroblasts, macrophages and capillaries form the granulation tissue in the wound site (30). This early stage of the repair phase is characterized by increased cellularity. The fibrin clot is gradually replaced by a collagen bridge, initially comprised predominantly of collagen type III collagen. The type III collagen fibers, which are smaller in diameter than the collagen type I to be deposited in the next stage of healing, are referred to as reticular fibers because of the network-like pattern of their deposition; the collagen type III fibers do not

aggregate in a preferential direction the collagen type I fibers. Both collagen production and fibroblast proliferation peak during this phase (characterized as loosely organized fibrous tissue), and subsequently decrease over the next several months (33).

The remodeling phase, which can begin as early as 3 weeks and last for over 1 to 2 years, is marked by a reduction in the production of type III collagen and reorganization of the type I collagen fibers (33). During the remodeling stage, the matrix fibers reorient themselves along the long direction of the tendon. This direction coincides with the direction of tensile stress in the tendon. The remodeling stage is also marked by a decrease in the number of fibroblasts present in the tissue and a decrease in the overall volume of the scar tissue. The tensile strength of the tendon increases through this period of remodeling even though the total volume is decreasing. This increase has been explained by the reorganization of the collagen fibers, which has been observed to occur during the same period. In the analogous case of ligament healing, however, it has shown that the tensile strength of the reparative tissue did not return to normal ligament levels even after a year (35). These data can probably be extended to the case of the tendon.

In summary, the response of mature Achilles tendon to an injury involving a full transection of the tendon, results in reparative fibrous tissue that lacks the structure of normal tendon. During the process of remodeling, collagen fibers initially arranged in random directions become reoriented in the longitudinal direction of the tendon. However, there is never a return to normal composition and architecture (13, 88), thus demonstrating the need for tissue engineering approaches.

Source of Reparative Cells

There is controversy surrounding the identity and location of the cells responsible for collagen synthesis during tendon repair. On one side of the controversy is the concept that tendon has the necessary cells to produce collagenous tissue (the intrinsic mechanism)(40, 76, 77), while on the other side, there is the belief that the source of collagen-producing cells is outside of the tendon (*i.e.*, an extrinsic source such as the surrounding tissues or from the tendon sheath). Some believe that both intrinsic and extrinsic sources of collagen-producing cells contribute to the healing process.

In vitro studies have shown that in response to tendon injury, cells within the tendon had the ability to migrate to and proliferate in the wound site. In these studies, by six weeks, the injury site appeared to be filled with collagen. *In vivo* data appear to parallel these *in vitro* studies. Matthews and Richards (78) showed that cells within the rabbit flexor tendon participated in the wound healing process when the synovial sheath was both not violated, and the tendon was mobilized with early controlled passive motion. However, if the tendon sheath was compromised and the tendon was immobilized, cells from external sources (*e.g.*, tendon sheath, blood vessels, and other neighboring tissue) migrated and proliferated into the wound site. Tendon repair, in that case, involved the participation of all surrounding tissue in the healing of the entire wound.

Questions remain about whether there is a sufficient pool of parenchymal fibroblasts in tendon to adequately populate a defect. This uncertainty is rationale for the use of exogenous cultured cells in tissue engineering modalities.

Normal Tendon versus "Scar"

Regeneration of tissue results in a tissue that is indistinguishable from the original tissue, *i.e.*, the newly formed tissue is morphologically, ultrastructurally, biochemically, biomechanically, and functionally indistinguishable from the original tissue. Repair, in the classical use of the term, results in a fibrocollagenous tissue that is distinguishable from the original tissue, and is generally referred to as

"scar". Many studies have claimed regeneration of tendon (59, 88), but close examination of the studies shows that the tissue in question may appear to fulfill the criteria of regeneration in one area but not in another. In the field of tendon healing, regeneration and repair have, in general, not been clearly distinguished in the literature. There is accordingly, a lack of consensus on the degree of functional recovery which can be considered acceptable to restore function.

There are many similarities between normal adult Achilles tendon and tissue formed in a tendon wound site. Both tissues tend to have highly aligned matrix fibers and a relatively low density of fibroblast cells present in the tissue. Morphologically, differences between these two tissue appear to be the crimp pattern and the average fibril diameter and distribution of the tissue. For example, one year post-injury, Kato *et al* (59) found the crimp length of the healing tendon in their animal model, was smaller than that of normal tendon. Collagen fibril diameters were also significantly smaller than that of normal tendon (54, 88). Biomechanically, mechanical properties of tissue formed in a tendon wound site appear to be 40-60% of normal tendon levels (13, 80).

These considerations can serve as the basis for criteria to assess the success of certain tissue engineering approaches in regenerating tendon.

Tendon Tissue Engineering

Tissue engineering approaches applied to tendon, as with other tissues, are founded on the use of matrices, cells, and soluble regulators, alone or in combination. Also, as with other applications, one of two goals might be set: to engineer tendon *in vitro* for subsequent implantation or to develop an implant to facilitate regeneration *in vivo*. A problematic issue associated with the former approach is the need to have the tendon tissue, engineered *in vitro*, incorporated in the host tissue when it is implanted. Work to date has focused on the implementation of absorbable synthetic and natural matrices, alone, as implants for the engineering of the tissue by the host. While the results of this approach have been promising, there are indications that exogenous cells will be necessary for more complete regeneration. This has led to a preliminary studies of methods for seeding certain matrices with tendon and mesenchymal stem cells *in vitro*.

While there have been reports of the use of soluble regulators alone, injected into healing ligaments to attempt to facilitate regeneration (65), no such approach has yet been reported as a modality to promote the engineering of tendon.

Recently, an animal model was developed to isolate a 1-cm gap in the rabbit Achilles tendon by "entubulating" the tendon stumps in a silicone tube (67) as was done in an early study of flexor tendon healing (45) and prior studies of bone (81) and peripheral nerve regeneration (69). In this model, the Achilles tendon was transected at its mid-point, after which the tendon stumps retracted about 8-10 mm. The stumps were subsequently inserted into a silicone tube. A 10-mm gap in the tendon was maintained by stitches through each stump and the tube, that allowed any mechanical stresses imparted to the system to be taken up by the sutures. In this model, the tendons of the peroneus long, brevis and tertius were cut and a portion of each tendon dissected away to immobilize the tendon gap site. Moreover, the plantaris tendon was cut with a "Z" plasty, the ends separated, and then sutured under minimum tension. The knee joint was immobilized by external fixation to reduce loading to the tendon. The spontaneous healing in the entubulated 1-cm gap after 6 weeks comprised a thin continuous cable of the fibrous tissue, less than 1 cm in diameter. There was no significant increase in the diameter of the reparative tissue after 12 weeks. The tissue comprised dense aggregates of crimped collagen fibers, with a wavelength (12 μm) and fiber bundle thickness that were both significantly shorter than those in

normal tendon. Future studies need be directed toward answering the question: Is this crimped tissue end-stage scar?.

Findings with the entubulated tendon gap model in the rabbit demonstrate that a tendon wound site can be isolated from the effects of extrinsic factors during healing, and that there is only limited capability for spontaneous intrinsic healing, with no indication of regeneration of tendon. An *in vivo* model of this type could thus be valuable for the evaluation of constructs developed for engineering tendon. Future studies need consider how certain characteristics of the tube affect the healing process. One potentially important feature is permeability. Because of the limited blood supply to the mid-substance of many tendons, regenerating tissue in the tube need derive nutrients from the surrounding milieu. While the permeability of the tube may facilitate this nutrition of the tissue forming in the entubulated gap, it might allow for the loss of intrinsic regulators of growth and remodeling from the lesion. These considerations have been addressed in entubulated peripheral nerve regeneration, and may be as relevant for tendon healing.

Bioresorbable Fibrous Scaffolds and Sponge-like Matrices

Bioresorbable prostheses currently being investigated to facilitate tendon healing include: collagen fibers tows, resorbable fibers tows of dimethyltrimethylene carbonate - trimethylene carbonate copolymer (96), and a composite artificial tendon of poly (2-hydroxyethylmethacrylate)/ poly (caprolactone) blend hydrogel matrix and poly (lactic acid) fibers (27).

Kato, *et al.*, (44, 59, 60) reported the use of a carbodiimide-cross-linked and a glutaraldehyde-cross-linked collagen-fiber prosthesis for the Achilles tendon of rabbits. They found that the healing in gaps in the tendon, bridged by the devices, was affected by the rate of implant degradation. The carbodiimide-cross-linked implant was resorbed by 10 weeks and was replaced with "neotendon". This reparative tissue was characterized by "aligned, crimped collagen fiber bundles" as early as at 20 weeks. The slower degrading glutaraldehyde-cross-linked implant was surrounded by a "capsule of collagenous connective tissue" at twenty weeks and both capsule and implant were still present at one year. Repair tissue infiltrated into the glutaraldehyde-cross-linked implant but the tissue was "not as developed" (was not as aligned and was not crimped) as the carbodiimide-cross-linked implant. While these results are promising, it should be noted that Kato, *et al.*, stated that the neotendon was "similar, but not identical, to normal tendon", one year after implantation of the prosthesis. The tissue in Kato's tendon lesion site was described to have a crimp wavelength of 10 μm . In Kato's study, it was also observed that this crimp pattern was present from 3 weeks to 52 weeks with minimal change in the crimp characteristics. That fibrous tissue with this crimp pattern did not appear to remodel significantly by 52 weeks may suggest that it is a terminal "scar." This raises the question of whether complete regeneration is necessary for the reparative tissue to be of functional value.

Cell-Seeded Matrices for Engineering Tendon

That the mid-substance of tendon is poorly vascular with a low density of parenchymal cells, suggests that matrices seeded with exogenous cells may be necessary to facilitate regeneration. Recent preliminary investigations have seeded fibers of a synthetic polymer (18) and a collagen-GAG sponge (66) with tenocytes *in vitro* in order to ultimately develop cell-seeded implants for tendon engineering. Another approach recently taken was to seed mesenchymal stem cells into a collagen gel for encapsulation of collagen sutures to bridge tendon gaps (110).

Tenocyte-Seeded Polyglycolic Acid Fiber Mesh

In a recent investigation (18), samples of nonwoven meshes of polyglycolic acid fibers, with interstitial spaces from 75 to 100 μm in diameter, were seeded with tendon cells isolated from newborn calves by collagenase treatment. After 1 week in culture, the cell-seeded specimens were implanted subcutaneously in nude mice for up to 10 weeks. Histological evaluation of the 10-week samples showed "parallel linear organization of collagen bundles throughout the specimens." Mechanical testing revealed that the tissue engineered neo-tendon structures had approximately one-third the tensile strength of normal tendon (11 versus 32 MPa), 8 weeks postimplantation. These promising findings are serving as the basis for efforts to further improve the engineered tendon. Additional studies will be required to determine if comparable results can be achieved with adult cells.

Seeding of Cultured Tenocytes into Porous Collagen-GAG Matrices

Tenocytes, recovered by collagenase digestion of the Achilles and plantaris tendon of adult New Zealand white rabbits, were grown to confluence for 2-3 days and subsequently passaged to increase cell number. In a preliminary experiment (66), collagen-GAG matrices (described in a previous section) were seeded with 0.75 to 1.5 million cells.

The number of cells and their distribution in collagen-GAG matrices varied from few cells uniformly distributed throughout the matrix to cells concentrated on the surface of the matrix. Cells were either spread out along the surface of the collagen-GAG material or aggregated. A cell count of the medium and trypsinized contents of the wells, in which the samples were seeded, revealed that from 2% to 50% of the seeded tendon cells were not incorporated into the collagen-GAG matrices after one day. In general, the tenocytes appeared to infiltrate to a depth of 0.35 mm into the 1 mm thick samples. In many of the matrices, the interior of the matrix was devoid of cells. The majority of cells were near or on the surface of the cells. The degree of infiltration appeared to be dependent on the pore diameter of the collagen-GAG matrices. Matrices with a larger pore diameter (120 μm) allowed for the infiltration of cells into the interior of the matrices and produced, in general, matrices with evenly distributed cells throughout the device. While matrices with larger pore diameters would be desirable as cell transplantation devices because of the deeper cell infiltration and the uniformity of cell distribution, there might be drawbacks to using too large of a pore diameter. For the matrices with pore sizes averaging 120 μm , the cells attached and spread out along the surface of collagen-GAG fibers. The surface area of collagen-GAG sponges is inversely proportional to the matrix pore diameter; therefore, with the larger pore diameters, there is less surface area to which the cells can attach. Future studies need systematically investigate the matrix characteristics that yield optimal cell seeding.

Mesenchymal Stem Cell-Seeded Gel

In a recently reported study (110), autologous marrow-derived mesenchymal stem cells seeded in a collagen gel were implanted into 1-cm long defects in the lateral gastrocnemius tendons of rabbits for 3 months. The mesenchymal stem cells (MSCs) were mixed with the collagen solution and incubated for 36-40 hours in the presence of biodegradable sutures, such that the gel contracted around the suture to form an "integrated implant." During the culture period tensile loading was applied to the sutures in order to align the cells seeded in the incorporated gel. The implant was then sutured into the gap in the rabbit tendon.

The reparative tissue after 3 months comprised "dense bands of matrix organized in the axial direction of the tendon." Controls with the sutures alone (but absent the unseeded gel) "demonstrated similar attributes but with less volume and less organization than those seen in MSC-treated repair

tissue." While the mechanical properties of the cell-treated defects were improved relative to the suture-alone group, they were still significantly below normal levels. Further investigation of this novel approach will be required to determine how the system can be modified to achieve a more physiological neotendon.

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