Chapter 2

Differentiation Factors and Articular Cartilage Regeneration

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Summary

Numerous differentiation factors have been examined for effects on chondrocytes in culture with the goal of sustaining these cells in culture for tissue engineering. This review examines several of these factors and evaluates their successes and shortcomings. As the quality of an engineered tissue is based upon comparisons to the native tissue, discussion of articular cartilage structure and function is also provided. Current therapeutic use of differentiation factors is presented in addition to the latest technologies incorporating differentiation factors in the tissue engineering paradigm.

Keywords: Cartilage, Chondrocytes, Differentiation Factors, Growth Factors, Tissue Engineering.

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Introduction

Articular cartilage is a necessary evil in musculoskeletal pathophysiology. Necessary because it provides the lubricated surface necessary for the smooth functioning of the articulating joints. Evil because once damaged, the tissue initiates a downward spiral into a degenerative state. Few tissues can withstand the punishing biomechanical environment that articular cartilage is exposed to on a daily basis. This tissue has evolved specifically to function in this location, providing the cushion for our every step and the smooth surfaces for joints to articulate.

There is a clear relationship between the structure of cartilage and the function that it performs *in vivo*. Articular cartilage allows the many joints in the body to move smoothly as they are used on a daily basis, and also dissipates the large biomechanical energy generated by simple activities such as jogging, dancing, climbing stairs or driving a manual car. The forces are absorbed and redistributed, allowing the activity to continue without undue discomfort.

Articular Cartilage Structure and Function

There is a wide variety of components that make up articular cartilage. Aside from the chondrocytes, which maintain the healthy tissue, the predominant species are the extracellular matrix molecules, including collagens and proteoglycans (1), and water (2). Mature chondrocytes are semi-quiescent. As most of the supportive structures have been synthesized during development, they maintain the environment and interact with it through integrin mediated attachments. This surrounding milieu of proteins serves a variety of functions. It maintains the cells in their differentiated state, sequesters growth factors and other useful molecules, and protects the joint from the various forces experienced by everyday actions. The matrix is composed of collagen fibrils, primarily type II, which form rope-like cords running throughout the tissue to maintain the structural integrity; proteoglycans, bottle-brush looking moieties that provide resistance to

compressive forces and also harbor a vast amount of water within their glycosaminoglycan chains; link proteins, forming connections between the various matrix components (3), and a variety of other, smaller contributors. Within the articular cartilage covering at the end of a bone, there are several zones, each with a particular function in the process alluded to above. Each zone has its own distinct combination and orientation of matrix proteins and morphological characteristics (4). These zones also vary with age. With aging and wear, the zones become substantially altered, rendering the cartilage layer susceptible to its environment (1, 5-7).

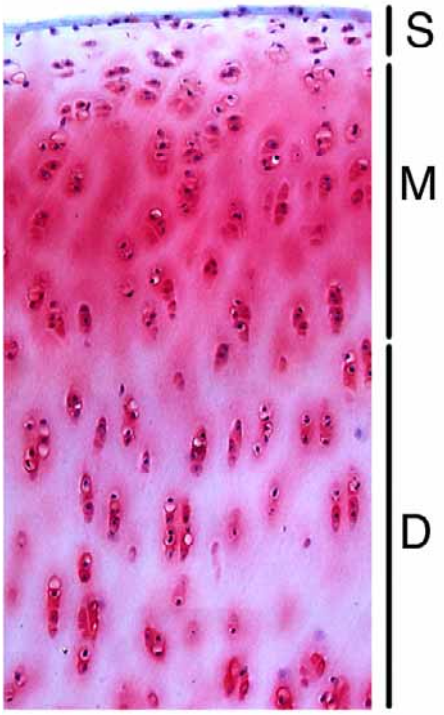

Fig. 1: Zonal arrangement of articular cartilage. Articular cartilage stained with Safranin O to indicate proteoglycans. S = superficial zone, M = middle zone and D = deep zone.

As seen in Figure 1, the uppermost zone is the outer surface, termed the superficial zone, that makes up approximately 10% of the cartilage thickness. To provide for the incessant movement of the joint, this layer is extremely smooth on the external surface. This smooth outer layer also serves as a barrier, maintaining fluid within the cartilage, which provides nutrient transport during compression as well as resistance to compression (8). Fibrils of collagen type II, run parallel to the surface of the articulating surface (9). The orientation of the fibers provides the mechanical support

to withstand the shear forces generated by joint movement (10). As the purpose of this zone is not to withstand compressive forces, the superficial zone contains little proteoglycan (11).

Underneath the superficial zone is the middle zone, rich in proteoglycans. This zone functions as the shock absorptive layer due to the high concentration of proteoglycans, as indicated by the deep red staining with Safranin O seen in Figure 1. The major proteoglycan of cartilage, aggrecan, is found in abundance within this zone. With the bottlebrush appearance, aggrecan, or other proteoglycans, can sequester water as well as charged ions within the glycosaminoglycan chains (3). When the tissue is subjected to compression, hydrostatic pressure in the interstitial water provides the bulk of the resistance to the applied stress. Since the middle zone experiences primarily direct compressive forces rather than shear forces, there are fewer collagen fibrils in this zone and they do not display the same degree of organization as seen in the superficial or deep layer. The chondrocytes in this layer appear more randomly dispersed, neither aligned with the surface as in the superficial zone or in the columnar arrangement seen in the deep zone.

The third layer is the deep zone, which is connected to the final layer of calcified cartilage, overlying the subchondral bone. Similar to the superficial zone, the deep zone has lower levels of proteoglycans than the middle layer and greater expression and organization of collagen type II. In this region, the collagen fibers are aligned to best anchor the cartilage to the underlying bone. Thus, they are perpendicular to the surface. The columns of chondrocytes also share this orientation.

One can see from the diversity of functions provided by this tissue, how critical it is to maintain the integrity of the articulating layer. Natural processes have developed a multi-zonal, functional tissue better than any engineer could ever create.

Unhealthy Cartilage

When the articular cartilage structure is intact, the joint functions smoothly. However, as the average life span increases, and the variety of abuses we subject our bodies to increases, articular cartilage suffers significant accumulated insult. This induces damage that in turn leads to further disease and degradation of the articular layer. The main pathology resulting from wear is osteoarthritis, which can occur in younger people, although it is typically diagnosed in older age. Osteoarthritis begins with a minor defect in the superficial surface. With repeated motion, this defect becomes larger as more cells are exposed to aberrant mechanical forces, similar to the development of potholes in a road surface. As the fault expands, the affected cells begin releasing inflammatory proteins into the environment, which starts the tissue in a downward spiral towards an advanced disease state (12). The advanced state results in the progressive destruction of the articular cartilage, releasing degraded matrix proteins into the synovial fluid, eventually exposing the underlying bone (13,14). The degradation occurs both through physical stress on the tissue and through an enzymatic process initiated by the release of cytokines into the environment (15).

In addition to disease brought on by normal wear of the tissue, osteoarthritis can also be initiated at any stage of life by acute damage to the joint. This is typically seen more frequently in athletes, but the occasional skier who ventures out and suffers a joint-twisting fall can also set the process of cartilage degradation into motion. In cases of injury, cartilage can make some attempt to heal the initial defect. However, as chondrocytes are not highly proliferative, nor do they respond to damage stimuli well *in vivo* to initiate a repair process, the subsequent patch is not true cartilage, but fibrocartilage, which cannot withstand the mechanical forces experienced over time. This patch, similar to the initial repair to a pothole, wears away and becomes the initiation site for further damage.

What can be done about this damage? Currently, cartilage is unsuccessful at self-regeneration. It falls, therefore, in the hands of the physicians and scientists to come up with a functional solution that can guarantee more years of active, high quality life for the athlete or the average person who has already started down the road to osteoarthritis.

Current Treatment

Currently, there are limited options available to orthopedic physicians treating the osteoarthritic patient or the patient suffering from a focal injury. If the patient is older and suffers from overall degeneration, they may be best served with total joint replacement. This option is best suited for older patients, as the life of the implanted joint is not great enough for use in younger people. For those patients, especially for treatment of focal lesions, the options are to remove cartilage from a less load-bearing region and place it in the area of the defect, now removed. While this does put more healthy cartilage in the affected region, it leaves regions surrounding the implant, as well as the donor site, to fill with the less mechanically sound fibrous tissue. The other option with focal defects is to harvest cartilage cells from the individual, culture them for a limited amount of time and then inject them into a partial thickness defect, created at the affected site, covering the area with a periosteal flap. Although this approach is widely used, no conclusive data exist regarding the quality of the new tissue resulting from the transplanted cells. It may be that neither joint replacement nor autologous cell transplantations set the stage for a suitable regeneration mechanism, but there are limited options to choose from.

Today, tissue engineers are pursuing a variety of approaches that show some promise in the effort to regenerate cartilage. By engineering replacement tissue for those damaged, there is potential for restoration and the return to a healthy condition. There are numerous scaffold materials (16) under study in coordination with differentiated chondrocytes, stem cells or other types of cells to determine which combination may produce a cartilage-like tissue *in vitro*. Most current approaches for tissue engineered cartilage involve culturing cells on either natural or synthetic scaffolds, treating with growth factors or mechanical stimulation and placing these constructs in the defective joint. Scaffold materials include coral, chitosan, collagen type II, collagen type I as well as synthetic polymers such as polylactic acid (PLA), polyglycolic acid (PGA), combinations of the two, as well as polyfumarate. Agarose and alginate are also commonly used scaffold materials. Cells, typically primary or cultured chondrocytes are either seeded onto the scaffold material, or enmeshed in the material as it is formed. This construct is either maintained in static culture for a period of time, or can be subjected to a variety of mechanical forces, including direct compression, hydrostatic

pressure and shear forces. A potentially pivotal action in the tissue engineering approach for articular cartilage is the inclusion of growth or differentiation factors.

Tissue Engineering and Differentiation Factors

Overview

When discussing growth and differentiation factors, there are many molecules that could be considered to function in this manner. For the purposes of this article, growth factors are defined as proteins that cause cell proliferation while differentiation factors are proteins that result in a change in the phenotype or behavior of a cell. Thus, if cells are cultured with basic fibroblast growth factor (bFGF) and they maintain their current phenotype but fill a scaffold at a much shorter time interval, then the bFGF is acting as a growth factor, or mitogen. If cells are cultured with bone morphogenetic protein 2 (BMP-2) and the cells begin to express a mineralized matrix, then BMP-2 has acted as a differentiation factor by causing the cells to change their normal behavior and become cells that express bone matrix.

There are numerous substances that can be added alone, or in combination to cell cultures that can induce differentiation of cells. While their effects may be worthwhile, factors such as insulin and dexamethasone will not be considered in this review. Instead, the focus will be on the more traditional factors such as the transforming growth factor β (TGF-β) superfamily, insulin-like growth factor type I (IGF-I), FGFs and others.

Treatment with TGF-β

When culturing chondrocytes, de-differentiation of primary cells is always a concern. The cells will spread in culture, losing their rounded phenotype, and after a few passages, will also cease to express chondrocytic proteins such as collagen type II and high levels of proteoglycans such as aggrecan, and decorin. Thus, the identification of factors that can maintain the phenotype of these cells in culture will increase the numbers of cells available for cartilage engineering. Some of the

most utilized soluble factors added to culture media are the members of the TGF- β superfamily. In this realm, the most notable members of the family are TGF- β itself and the multifaceted bone morphogenetic proteins (BMPs). TGF-β is well known for the ability to stimulate production of proteoglycans and other components of cartilage matrix (17, 18). Recent reports identify TGF-β functioning in the differentiation, or inhibition of differentiation in the development of cartilage. It appears that *in vivo*, TGF-β prevents terminal hypertrophic differentiation in the developing growth plate (19, 20). With such an *in vivo* response, the ability to maintain primary chondrocytes in culture follows closely. TGF-β has been used successfully on primary cultures from many species: rabbit, pig, bovine and human (21-26). Cell lines can also be used with TGF-β, but as these cells are farther from the native phenotype, their use in tissue engineering is more difficult. TGF-β is often used in combination with other factors in stimulating differentiation and matrix synthesis. One example of this combinatorial effect is in sequential treatment of bovine cells grown on a PGA scaffold (24). Chondrocytes were treated with TGF-β and bFGF, increasing cell numbers, prior to treatment with IGF-I or bFGF alone. The initial treatment followed by IGF-I resulted in a large increase in matrix production as well as a stronger construct than with bFGF or no treatment (24). TGF-β expression can be upregulated by mechanical forces, as demonstrated using fluid shear (27).

Treatment with IGF-I

Another factor commonly used alone, or in combination with TGF-β, to promote or maintain differentiation of cartilage cells is IGF-I. While IGF-I has been shown to be an effective upregulator of matrix synthesis, namely proteoglycans, it also stimulates the production of IGF binding proteins (IGFBPs) which bind to the protein and inactivate it. IGF-I has been shown to increase the ability of the joint to undergo self-repair, but the newly synthesized tissue still lacks equivalent structural components and compressive ability as compared to the undamaged native tissue (28, 29). When used to re-differentiate chondrocytes seeded in alginate, IGF-I, in combination with TGF-β, works most effectively on cells initially isolated from younger animals, stimulating the production of proteoglycans and increasing the number of cells producing collagen type II from 10% of the population to 97% of the population. In the adult cells, IGF-I was able to increase the collagen type II positive cells to only 33% of the population (30). When repeated with human cells, the age of the donors made no difference. Proteoglycan synthesis was stimulated, but collagen type II production

was only increased above 10% by the addition of media suplement insulin-transferin-selenium (ITS+) and cortisol (30). IGF-I has also been shown to have a protective effect, preventing the damaging action of interleukin one (IL-1) and tumor necrosis factor type alpha (TNF-α)/matrix metalloproteinases (MMPs) on cultured chondrocytes (31). Recently, more use has been made of IGF-I in engineered constructs, either with or without mechanical stimulation.

Treatment with TGF-β and IGF-I in combination

In studies utilizing mechanical stimulation, simple forces such as centrifugal stimulation of rabbit chondrocyte cultures maintained in centrifuge tubes stimulate the native expression of IGF-I. The initiation of IGF expression is followed by collagen type II expression, increased cell number and total protein synthesis (32). Using insulin to bind to the IGF-I receptor can also stimulate matrix production, as seen using bovine chondrocytes on PGA scaffolds (33). Similar studies using IGF-I itself with bovine chondrocytes under a variety of conditions found that mechanical stimulation alone produces cartilage-like tissue as indicated by biochemical composition, mechanical properties and morphology; the combination of mechanical stimulation and IGF-I produced a construct more like articular cartilage than with mechanical stimulation alone (29). Similarly, in one of the most exciting reports combining mechanical stimulation and growth factors, explants from bovine cartilage were cultured with IGF-I, mechanical stimulation or a combination of both. While IGF-I was able to increase protein and proteoglycan synthesis 90% and 120%, respectively, and mechanical stimulation (dynamic compression) was able to increase protein and proteoglycan 40% and 90%, respectively, the combination of both resulted in a 180% increase in protein and 290% increase in proteoglycan synthesis (34). While the addition of growth factors can result in outstanding results such as these, it is always necessary to consider the form the chondrocytes are presented in. For example, the synergistic results using dynamic compression made use of cartilage explants. These are pieces of cartilage tissue harvested in one piece, not subjected to further digestion or processing once out of the joint. A study using isolated bovine chondrocytes seeded on PGA scaffolds was also carried out (29). These cells were isolated from the joint and shortly after, seeded onto the scaffolds. The rapid seeding minimizes any dedifferentiation that the cells might undergo during prolonged culture periods. Also noteworthy is the type of scaffold used. PGA mesh, collagen type I gels, alginate and fibrin are some of the many scaffold types used in these studies. The scaffold type can have an effect on how the cells respond to the various factors. When bovine chondrocytes are

encapsulated in hydrogels with microspheres containing IGF-I and TGF-β, the growth factors were able to increase the cell number and synthesis of proteoglycans, but collagen type II production was decreased after culture with both factors (35). While these gels were not subjected to mechanical stimulation, the decrease in collagen expression remains different from that seen in monolayer cultures or other culture systems.

Treatment with BMPs

The effects of the BMP family are numerous, and vary with cell type and treatment conditions. The predominant effect of BMP proteins is differentiation or maintenance of differentiation. Extensive studies have been performed using BMP proteins *in vivo* to determine their effect on cartilage and bone development. While it is clear that the BMPs are critical for cartilage and skeletal development in animals, it is not always possible to apply information from the *in vivo* state to cells in culture. Also, the BMPs are often commonly used to differentiate other cell types such as mesenchymal stem cells (36), or cell lines such as ATDC5 (37) or C3H10T1/2 (38). The most commonly used BMP for cartilage studies is BMP-2 (39-44). Similar to its family member TGF-β, BMP-2 is able to increase proteoglycan synthesis and increase production of collagen type II. This response is seen not only in monolayer cultures, but also when cells are cultured in PGA scaffolds. In a comparison between BMP-2, BMP-12 and BMP-13, BMP-2 increased the total weight of the construct the greatest percentage, with the highest increase in both proteoglycans (45). In this instance however, the bovine chondrocytes also initiated hypertrophy. As the BMPs are also used in the differentiation of bone, it may be that in this case BMP-2 would have driven the chondrocytes to form bone, rather than remain as articular chondrocytes. Such findings are useful for applications directed towards repairing growth plate cartilage as in studies of dwarfism, or chondroplasia, where BMPs are seen acting to increase the size of both the proliferative and hypertrophic zones of the developing limb (46). Other studies utilizing BMPs have shown that BMP-7 is perhaps a stronger candidate for cartilage differentiation than BMP-2. When bovine chondrocytes were transfected with cDNA for various BMPs, embedded in scaffolds and then cultured subcutaneously in nude mice, only the cells transfected with BMP-7 prevented the invasion of surrounding host fibroblasts while increasing the expression of cartilage matrix (47). Studies *in vivo* also identify BMP-4 as participating in cartilage development (36, 48, 49). Few mechanical studies make use of BMPs for formation of cartilage, rather most are focused on bone (50-52). However, those employing scaffolds without mechanical

stimulation do find that addition of BMPs, either in the media or through transfection, enhance the chondrocytic phenotype (45, 47, 53, 54).

Treatment with bFGF

Another well-characterized differentiation factor often used in combination with TGF-β is bFGF. A plethora of studies have been performed using bFGF in differentiation of non-chondrocytic cells and report several functions, occasionally contradictory, for the protein. Most importantly, bFGF has been recognized as a factor capable of maintaining chondrocytes in their dedifferentiated state such that they are able to return to a full chondrocytic phenotype, expressing a complete array of cartilage proteins. Adult human chondrocytes have been maintained for up to six months (24 population doublings) in the presence of bFGF while maintaining phenotype (55). Bovine chondrocytes can also be carried for extended periods of time in the presence of bFGF (56). Apparently, bFGF prevents the complete dedifferentiation of the chondrocytes into fibroblasts by preventing the change in actin expression. There does not appear to be a direct stimulatory effect of bFGF on the synthesis of cartilage matrix as seen with TGF-β, IGF-I or BMPs. While bFGF can stimulate the expression of these other differentiation factors, this can have varied results on the culture. In studies of dwarfism, or achondroplasia, bFGF serves to hasten hypertrophic differentiation in the limb (46). In normal pig chondrocytes, bFGF interferes with TGF-β and IGF-I, preventing the synthesis of matrix proteins (57).

One of the less studied differentiation factors is platelet derived growth factor (PDGF), which also has variable effects. For example, PDGF stimulates rat chondrocytes to increase proteoglycan synthesis in culture (58) but if injected directly into the knee joint in rats, it has little to no effect (59). In studies using cells cultured on scaffolds, PDGF again has varied responses. Depending on the amount of fetal bovine serum (FBS) included in the cultures, PDGF either increased proteoglycans synthesis (1% FBS, 10-100 ng/ml PDGF) or decreased production (10% FBS, PDGF) on a per cell basis (60). This variability in effects is not unusual for these different factors.

The current experimental techniques, as seen briefly above, employ a variety of scaffold types to harbor cells while stimulating them in various ways to generate the most cartilage-like tissue possible. Of the various mechanical stimuli available, direct compression and rotating bioreactors

are most commonly used. Not all scaffold types can be used with every form of stimulation. For instance, compression of fibrous scaffolds at high frequencies would result in a permanently compressed scaffold, as it takes some time for the material to recover from the compression. In direct compression, typically a platen (a movable surface made to the shape desired) is brought into contact with the scaffold to compress it a specific amount. After the compression, the platen is raised and the cycle repeats. A wide range of cycle times have been reported, although frequencies close to 1 Hz appear to have the most beneficial effects on matrix synthesis and differentiation (61). As mentioned above, the combination of differentiation factors and compression can have a synergistic effect on the behavior of chondrocytes. Rotating bioreactors are beginning to come to the forefront of tissue engineering. These vessels enhance nutrient diffusion while maintaining the constructs in a relatively low shear environment. Excellent results have been generated using rotating bioreactors (62, 63). Another form of potentially stimulatory mechanical forces is hydrostatic pressure. In this instance, cells are surrounded by fluid, often sealed into a pouch filled with media, and placed in a chamber in which the fluid is subjected to pressure, directing hydrostatic pressure on the chondrocyte constructs (64, 65). The best measure of success in generating cartilage *in vitro* is to compare it to native cartilage. While many of the studies using differentiation factors alone are able to generate large amounts of matrix, the constructs either lack the mechanical strength of native tissue, or this parameter was not examined. In several studies culturing scaffolds *in vivo*, the constructs fail to reach equivalence with the native tissue (21, 53). However, with the addition of mechanical forces, the constructs gain both matrix and mechanical strength, approaching the composition of native tissue (62). As yet, no one has been able to replicate the structure entirely. Collagen type II production trails that seen with proteoglycans, and the organization of the synthesized tissue is not yet comparable to native, but progress is being made.

Conclusion

Healthy articular cartilage is a strong, resilient tissue that is central to a comfortable, active lifestyle. When articular cartilage is damaged, tissue deterioration ensues. Current treatment options are limited, with natural repair of the tissue insufficient. These conditions make cartilage an optimal candidate for repair by engineering. While chondrocytes alone are difficult to culture and maintain in the differentiated state, differentiation factors added to the media, transfected into cells or encapsulated into a scaffold can both maintain the differentiated state as well as boost production of chondrogenic proteins. Although these factors are beneficial to the chondrocytes in culture, to better recreate cartilaginous structures, mechanical forces such as direct compression, hydrostatic pressure or culture in a rotating bioreactor can be added to the culture regimen. The combinations of these differentiation factors and mechanical stimuli are bringing the field of cartilage regeneration to the forefront of the tissue engineering field. The next challenge facing the cartilage engineers is that of cell source for use in the engineered tissue.

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