TISSUE ENGINEERING AND CARTILAGE REPAIR: LIMITS AND PERSPECTIVES.

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Abstract

The repair of damaged tissues represents a major challenge in our aging society. The destruction of articular cartilage represents the outcome of most inflammatory and degenerative rheumatic diseases and leads to severe disability. Articular cartilage is unable to repair spontaneously, and damage of the joint surface often results in end-stage osteoarthritis, requiring surgical intervention and total joint replacement.

Cartilage harbors only one cell type, the chondrocyte, which synthesizes and secretes specific proteins such as type II collagen and high molecular weight proteoglycans. These matrix proteins are responsible for the conservation of the chondrocyte phenotype and are essential for the maintenance of the mechanical function of cartilage tissue. Thus, development of therapeutic strategies for cartilage repair should comprise not only the replacement of lost cartilage cells but also that of an abundant extracellular matrix with cartilage-like properties.

Different protocols are under investigation. The most commonly employed materials include transplantation of autologous osteochondral tissue. More recently, cell-based therapies using autologous mature chondrocytes or pre-chondrogenic stem cells have drawn particular attention. Tissue-engineering procedures represent the actual trend in cartilage repair. This approach involves the use of biodegradable polymeric three-dimensional matrixes and isolated chondrocytes from tissue biopsies. The cells are seeded inside the biocompatible matrix and then implanted into the joint. Numerous non-degradable and degradable polymers have been studied. Among natural polymers, chitosan constitutes a very interesting family of glycosaminoglycans which efficiently "mimic" the natural surroundings of cartilage cells.

Introduction.

The repair of damaged tissues represents a major challenge for our aging society. Heart, liver, kidneys, lungs or skin are more frequently involved in organs or tissues failure. However, the locomotor system is of equal importance for the preservation of quality of life.

The destruction of articular cartilage represents the outcome of most inflammatory and degenerative rheumatic diseases and leads to severe disability. Articular cartilage is unable to repair spontaneously, and damage of the joint surface often results in end-stage osteoarthritis, requiring surgical intervention and total joint replacement (1, 2)

This paper will focus on the physical and mechanical properties of articular cartilage tissue, in close relationship with the nature and the architecture of extra-cellular proteins of the tissue. Thus the development of bio-engineering material for cartilage repair should take in account these very special properties (3).

Normal human cartilage tissue.

Structure
Articular cartilage is a remarkable tissue, critical for adult skeletal function. It protects the articulating surfaces of bones with a composite material that resists to compressive skeletal loads during normal activities. Cartilage is practically avascular and mainly contains 70% water with specific matrix proteins. It harbors only one cell type, the chondrocyte, which undergoes a low process of differentiation and secretes specific proteins. These matrix proteins are responsible for the conservation of the chondrocyte phenotype and are essential for the maintenance of the mechanical function of cartilage tissue .

As remembered by V Hascall in a Professional Health Information note of the Cleveland Clinic (SpineUniverse.com), the functional properties of cartilage result from two macromolecules around the chondrocytes. Collagen (mostly type II collagen) forms fibrillar networks and defines the shape and tensile properties of the cartilage, but cannot bear weight on its own. The other macromolecule is aggrecan. This complex macromolecule has a large core protein divided into subdomains characterized by the presence of covalently attached sulfated glycosaminoglycan (GAG) side chains. The GAG chains are linear polymers of repeating disaccharides containing either hexosamine and hexuronic acid (chondroitin sulfates) or hexosamine and galactose (keratan sulfate). The central region of the aggregan macromolecule contains approximately 200 polysaccharide chains of chondroitin sulfate and keratan sulfate, each with a sugar unit motif repeated up to 100 times. When secreted by chondrocytes, neosynthesised aggrecans form large molecular size aggregates (MW $\geq 10.10^6$ Daltons) by interaction between a binding site in the core protein and hyaluronan, a very long polysaccharide with several thousand sugar units. GAG chains (with the exception of hyaluronic acid) contain negatively charged sulfates. This polyanionic structure attracts cations and binds water (Figure 1).
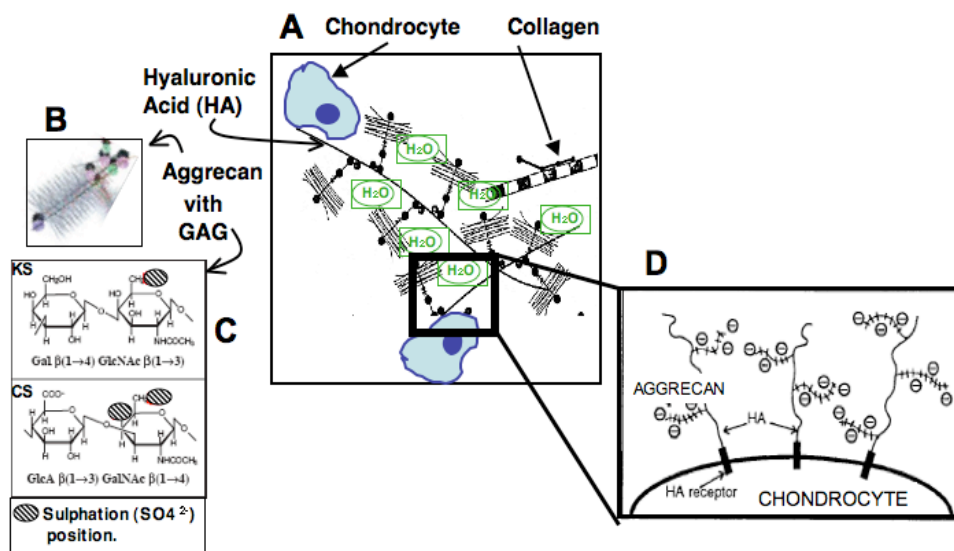

Figure 1 :   Shematic representation of cartilage extracellular matrix proteins. A. The main proteins synthesized and secreted by Chondrocytes are type II collagen and aggrecans. When secreted by chondrocytes, aggrecans form large molecular size aggregates by interaction with hyaluronan. GAG chains of aggrecans contain negatively charged sulfates and this polyanionic structure attracts cations and binds water. B. Representation of an aggrecan with a central core proteins and . C. Glycosaminoglycans of cartilage aggrecans are mainly composed of keratan sulfate and chondroitin sulfate. C. Brush-like configuration of hyaluronan-aggrecan complexes on the cell surface with the negative charge repulsion of the GAG chains. The result is that the complexes straighten and extend outward from the surface. Such a peri-cellular coat has important functional effects on cartilage cells.

Aggrecan molecules are shock absorbers in cartilage.
The main function of aggrecans is to protect the cell from compressive forces exerted by joint movements. This resistance to compression is due to the osmotic pressure produced by hydrated aggrecans (4). Compression of polymers increases osmotic pressure by packing the chains closer and expelling water. This increases the density of negative charges and hence the repellant forces inside the macromolecule (Figure 1). When the load is released, aggrecan re-expands to the extent allowed by the collagen network.

What happens in cartilage defects ?
Many factors lead to compromised function in an articular joint (5, 6). Injurious and excessive mechanical stress can result in depletion of proteoglycans and damage the collagen network. Mechanical factors, leading to joint damage, comprises factors increasing joint vulnerability, including muscle weakness, genetic predisposition, and aging. Other factors, such as obesity, certain physical activities, and acute trauma can also lead to joint damage by excessively loading the joint. The lost of extra-cellular matrix leads to a chondrocytic reaction. Cells start to divide and to synthesize new protein material. However, the dividing cells rapidly dedifferentiate into fibroblastic-like cells, which are unable to synthesize cartilage-type proteins. The regenerating tissue is thus a fibrillar tissue without any mechanical properties.

Replacement of damaged cartilage with healthy cartilage tissue or cells.
Since local chondrocytes, situated within or around a cartilage lesion, cannot restore aggrecan successfully, it has been proposed to fill the lesion either with pieces of autologous normal cartilage (mosaicplasty), or with healthy cartilage cells (autologous chondrocyte transplantation) (7, 8). In the latter case, mature autologous chondrocytes are extracted from a biopsy of healthy cartilage, amplified *in vitro* and re-introduced as a suspension into the defect, under a periosteal flap. In the past 10 years, reports of transplantations of isolated chondrocytes have described clinical improvement of the patients but did not demonstrate a direct link between the transplantation process and actual regeneration of hyaline-like cartilage tissue (9).
Several questions remain to be answered (10, 11). Chondrocytes culture conditions have been described many years ago with successive improvements (12, 13, 14), but it remains a critical phase. The phenotype instability of autologous chondrocytes during amplification process *in vitro* still represents a major challenge (15, 16). Other important points are the maintenance of newly synthesized and secreted matrix proteins around the cells when transplanted into the lesion (17) and the recovery of mechanical properties in the graft (18, 19). In addition, adult autologous chondrocytes being well-differentiated mature cells, can rapidly reach a terminal differentiation phase after implantation. This process may reproduce endochondral ossification, including uncontrolled neo-calcifications of the graft and subsequent loss.

Perspectives.
Growth factors are currently required to activate re-differentiation pathways of de-differentiated chondrocytes (20, 21, 22) or to induce the chondrogenic differentiation of stem (23). The best candidates that could provide appropriate signaling are molecules involved in embryonic chondrogenesis, namely members of TFGβ family, BMP-2 and BMP-7, and IGF.

Mesenchymal stem cells seem to be a very promising tool to counteract the ectopic endochondral ossification and chondrocyte apoptosis depicted above as they mimic partially the plasticity of embryonic stem cells. They also have supplementary advantages in terms of availability, expandability, transplantability, and ethical implications (24, 25). Stem cells may be recovered from bone marrow (26, 27), but also from other adult tissues such as adipose tissue (28), periosteum (29) or synovial membrane (30). As the recent literature indicates that stem cell preparations are heterogeneous, even when they are enriched with a specific subpopulation of cells, the major problem is the standardization and the reproducibility of this biological material.

Cartilage bio-engineering is also rapidly developing (3, 31, 32). This approach involves the implantation in the joint of biodegradable polymeric three-dimensional matrixes mixed with amplified cartilage cells from tissue biopsies (33). Addition of a three-dimensional natural or synthetic material has at least two purposes. First, it serves as a cell carrier allowing either chondrogenic re-differentiation of dedifferentiated mature chondrocytes or chondrogenic differentiation from precursors stem cells. Second, this 3D-material will retain the accumulating extracellular cartilage matrix secreted by the adjacent chondrogenic cells, favoring the acquisition of optimal mechanical strength.

Numerous non-degradable and degradable polymer materials have been studied. Natural and artificial three-dimensional matrixes have been extensively used in order to maintain the chondrocytic characteristics of the cells *in vitro*. Beneficial effects have been reported with either synthetic polymers such as polyesters, polyglycolic acid, or polyethylene oxide (33, 34) or natural polymers such as hyaluronan, alone (35, 36) or combined with fibrin (37), alginate (38), heparin, collagen (39), or chitosan (40, 41, 42, 43, 44, 45). Among natural polymers, chitosan constitutes a very interesting family of glycosaminoglycans. Chitosan belongs to the family of the linear copolymers of (1→4)-2-amino-2-deoxy-β-D-glucan (GlcN) and (1→4)-2-acetamido-2-deoxy-β-D-glucan (GlcNAc).

Chitosan-based matrixes have been shown to be biocompatible with bone and cartilage (46, 47). Recent studies have presented an initial attempt to use chitosan and to assess its potency to provide a substrate for *in vitro* neo-chondrogenesis (43, 44). In these experimental conditions, chitosan scaffolds supported cell attachment and maintenance of a rounded cell morphology. A chitosan-based hyaluronic acid hybrid biomaterial (45) and a lactose derivative of a highly deacetylated chitosan (41) showed very interesting biological effects when added to rabbit or pig chondrocytes in primary culture. In addition to the stimulation of chondro-specific aggrecan and type II collagen, these authors report an induction of chondrocyte aggregation leading to the formation of nodules, that might be used as a new cell-delivery system for chondrocyte implantation in defective cartilage. Similar findings have been recently observed using a pure chitosan physical hydrogel and human chondrocytes (42).

As shown on figure 2, a significant amount of neo-formed extra-cellular matrix (aggrecan and type II collagen) began to accumulate in-between cells and hydrogel fragments and was later widely distributed within the neo-construct. This paper points out that chondrocytes never penetrated the chitosan material used, but bound tightly to the surface of the hydrogel. These data allowed the authors to suggest that such a chitosan hydrogel does not work as a scaffold, but could be considered as mimicking cartilage extra-cellular matrix components, thus favoring the binding of chondrocytes to chitosan.
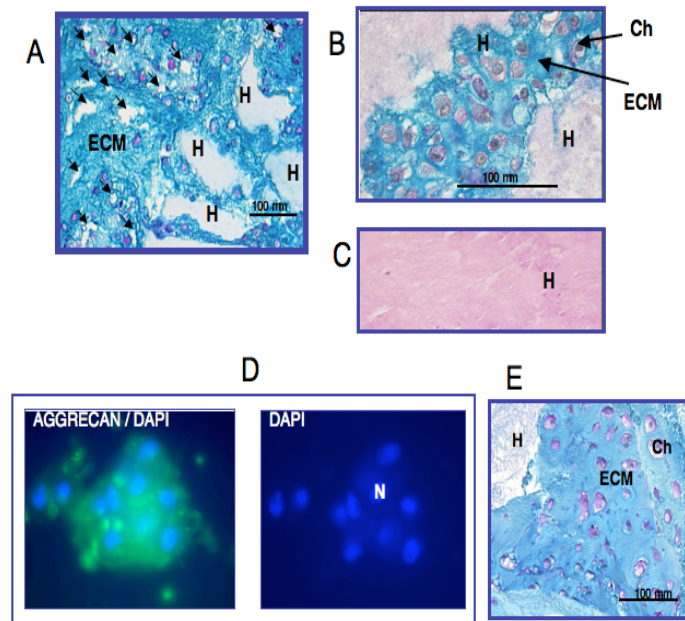
Figure 2 : . Morphological aspect of constructs prepared with human chondrocytes and chitosan hydrogel ($C_{polymer}$ = 1.5%, DA of chitosan = 40.4%). A and B. Histological morphology of the constructs after 8 days in culture. C. Histological aspect of a fragment of chitosan hydrogel incubated alone in similar conditions. A strong Alcian blue coloration, specific of aggrecans (ECM), was observed in between cells (purple nuclei) and hydrogel fragments (↘ and H). D. In situ Immunocytochemistry and E. Histological aspect of a construct cultured for 45 days. The ICC was performed with antibodies directed against Aggrecan (green fluorescenceAcknowledgements) with nuclei colored in Blue by DAPI, and the histological colorations were as in A and B. Symbols : Ch chondrocyte ; H chitosan hydrogel ; ECM: extracellular matrix; N nucleus.

The choice of the competent matrix as well as the elaboration of the cellular construct, have to take in account the high degree or the architectural organization and compartmentalization of articular cartilage in vivo, with a strictly delineated stratification from the surface of the non vascularized cartilage tissue to the vascularized subchondral bone.

In conclusion, although cell-based-engineering therapy appears as a potentially effective strategy for cartilage repair, a number of issues remain to be addressed before these therapies may be used in clinic. The major challenge is the characterization, the standardization and the reproducibility of the bio-construct. The use of so-called "bioreactors" where specific physicochemical culture parameters can be reproducibly maintained at defined levels, has the potential to improve the quality of engineered cartilage tissues (48, 49). A more sophisticated approach, including the impact of mechanical loading (18), is probably necessary for long term success of the repair of human cartilage defects.

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