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Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering

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Summary

The abundant extracellular matrix of articular cartilage has to be maintained by a limited number of chondrocytes. *Vice versa*, the extracellular matrix has an important role in the regulation of chondrocyte function.

Objective: In this review we discuss the role of the extracellular matrix in the regulation of chondrocyte function and the relevance for cartilage tissue engineering. To reach this goal the international literature on this subject has been searched with a major focus on the last 5 years.

Results: Structural matrix macromolecules (e.g. collagen, hyaluronate), but also growth factors (e.g. IGF-I, TGF β) entrapped in the matrix and released under specific conditions affect chondrocyte behavior. These factors communicate with the chondrocyte via specific membrane receptors. In this way there is a close interaction between the extracellular and intracellular milieu. Articular cartilage has a limited capacity of intrinsic repair, which has resulted in the development of tissue engineering approaches to repair damaged cartilage. Successful application of scaffolds has to take into account the important role of both soluble and insoluble matrix-derived factors in cartilage homeostasis.

Conclusion: Functional tissue engineering will only be realized when the scaffolds used will provide cartilage cells with the correct extracellular signals. © 2002 Published by Elsevier Science Ltd on behalf of OsteoArthritis Research Society International

Key words: Chondrocytes, Cartilage, Tissue engineering, Extracellular matrix, Growth factors.

Introduction

Articular cartilage is a tissue with a remarkable durability. However, trauma and abnormal joint loading may induce localized cartilage damage. At short term this will lead to pain, joint dysfunction and effusions. Later on localized lesions may result in progressive, more generalized cartilage destruction and to gross deformation of the joint. To induce cartilage healing the use biological scaffolds with or without cells has gained great attention. However, to be able to rebuild the articular cartilage matrix one has to take into account the subtle interaction between the chondrocytes and their surroundings. Therefore the focus of this paper will be on the interaction between chondrocytes, extracellular matrix and growth factors and the relevance of these interactions for cartilage tissue engineering. Articular cartilage consists of sparsely distributed chondrocytes surrounded by extracellular matrix. The chondrocytes are responsible for the maintenance of the articular cartilage as a functioning entity. The extracellular matrix can be divided in the pericellular, territorial and interterritorial compartments, going from near to further removed from the cells. Based on dry weight, the main constituents of articular cartilage are type II collagen and the proteoglycan aggrecan. Other components, however, are equally important on a molar base. Moreover, the various compartments of the extracellular matrix differ in their composition of matrix molecules. To maintain cartilage homeostasis, the chondrocytes have to sense changes in matrix composition to compensate for those changes. The feedback of the matrix to the chondrocytes can be carried out by a direct interaction of the matrix component with the chondrocyt, or by soluble factors released after matrix damage. To reach the goal of repairing damaged articular cartilage using (semi)artificial scaffolds, those scaffolds have to provide the necessary signals, both soluble and insoluble, for rebuilding functional articular cartilage by the chondrocytes.

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Articular cartilage ECM receptors

Chondrocyte proliferation, differentiation and homeostasis is not only governed by soluble mediators in interaction with the genetic make up of these cells but also the ECM provides important signals for chondrocyte behavior. The interaction between chondrocytes and ECM is mediated in part by a family of ECM molecule receptors called integrins. The integrins are transmembrane receptors composed of a α subunit and β subunit. Until now 15α and 8β subunits have been identified which pair in a multitude of variations resulting in large number of dissimilar integrins.

Some integrins recognize specific ECM molecules while others bind with several different ECM proteins. The cytoplasmatic domain of the integrins is connected with the cytoskeleton. The signaling of integrins is transmitted in two directions, 'inside-out' such that the extracellular domain of the integrins becomes more adhesive for their ligands and 'outside-in', whereby binding of integrins to their ligand results in cellular activation[1]. Since cellular behavior is strongly influenced by signals from the ECM it is crucial that artificial scaffolds provide the adequate signals to embedded or invading cells to induce or maintain these cells in their desired differentiation stage.Chondrocytes have been reported to express several integrins. Receptors have been detected for type II and type VI collagen ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha11\beta1$), vitronectin and osteopontin ($\alpha V\beta3$), laminin ($\alpha6\beta1$) and most abundantly for fibronectin ($\alpha5\beta1$)[2–4]. Remarkably, in cartilage integrins have not only been found in the cell membrane but also in the ECM itself[5]. In the cartilage matrix $\beta1$, $\alpha1$ and $\alpha5\beta1$ are detected by fluorescence, electron microscopy and immunoprecipitation. These integrins could play a role in the development and structural organization of the cartilage matrix.The most abundant integrin detected on chondrocytes is the fibronectin receptor $\alpha5\beta1$. Isolated chondrocytes cultured in monolayer undergo dedifferentiation to a fibroblast-like phenotype and show an increased $\alpha5\beta1$ expression[6,7]. This might be related to the cell attachment on plastic or serum proteins present in the culture medium. Binding of chondrocytes to collagen, amongst others by $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha10\beta1$ integrins, has been shown to be essential for chondrocyte survival[9–11]. Deprivation of chondrocytes from collagen derived signals induces apoptosis[10]. Moreover, the interaction of chondrocytes with osteopontin via $\alpha V\beta3$ protects articular cartilage against interleukin-1-mediated damage[11].

Besides the integrins, chondrocytes express other transmembrane molecules that function as receptors for ECM molecules. Annexins, mainly chondrocyte annexin V (anchorin II), is a type II collagen-binding molecule mainly expressed by superficial chondrocytes[12,13]. Antibodies against annexin V inhibited binding of chondrocytes to type II collagen[13]. However, it is unclear if chondrocyte annexin V has a function in the regulation of chondrocyte homeostasis. Another well-studied membrane receptor for ECM molecules is the hyaluronan receptor CD44[14]. CD44 has a domain which is structurally similar to the hyaluronan binding site of aggrecan and cartilage link protein. Binding of chondrocytes to hyaluronan by the CD44 receptor affects chondrocyte functioning and is essential for cartilage homeostasis[15]. Blocking of CD44 hyaluronan binding on chondrocytes results in degradation of the cartilage matrix[15].

Articular cartilage ECM and growth factors

Molecules from the extracellular matrix not only regulate cellular behavior by providing signals to the embedded cells through binding of integrins or other ECM receptors, but also by binding, storage, release and presentation of soluble mediators. Transforming growth factor β is very abundant in articular cartilage[16]. In most cell types TGF β is produced in a latent form in which the mature peptide is complexed to the so-called Latency Associated Peptide (LAP[17]). Chondrocytes, and several other cell types, produce TGF β as a high molecular weight macromulecule in association of the latent homodimer with Latent TGF β Binding Protein (LTBP[18,19]). In growth plate chondrocytes it has been shown that storage of TGF β by LTBP is cell maturation-dependent[18,19]. LTBP has a function in directing to and storage of TGF β in the extracellular matrix and appears to be co-localized in the extracellular matrix with fibrillin[20].

The ECM proteoglycans decorin, biglycan and fibromodulin regulate TGF β activity by sequestering of TGF β within the ECM[21]. The interactions of these small proteoglycans with TGF β are not primarily mediated by the proteoglycan glycosaminoglycan chains but depend on the core protein. The decorin-like proteoglycans function as physiological regulators of TGF β. Both inhibition and stimulation of TGF β activity after binding to these small proteoglycans has been reported[22–25]. For example, binding of TGF β to TGF β receptors on osteoblastic MC3T3-E1 cells was enhanced by decorin[23]. However, in osteosarcoma cells addition of TGF β prevented TGF β-mediated up-regulation of biglycan synthesis[24]. Adenoviral overexpression of decorin has been shown to prevent TGF $\beta1$ induced inhibition of lung morphogenesis[26]. Decorin is bound to collagen in the ECM. Also type II collagen itself has been shown to affect TGF β effects on cells. The response of chondrocytes to TGF β was enhanced by non-denatured type II collagen while heat-inactivated type II collagen had no effects[27,28]. Moreover, TGF β expression was increased by incorporation of type II collagen into the cell culture[29]. These data show that typical ECM molecules, like the small interstitial proteoglycans and type II collagen, can affect the effect of TGF β on chondrocyte behavior. Insulin-like growth Factor-I (IGF-I) is considered to be the main anabolic growth factor of normal cartilage[30,31]. The biological function of IGF-I is modulated by the interaction with the IGF-binding proteins (IGFBPs). Six IGFBPs, designated IGFBP-1 to IGFBP-6, are known today. In cartilage IGFBP-2 and -3 and -6 are detected but the role of these binding proteins in this tissue is not clear[32,33]. However, since IGF analogs without affinity for the binding proteins are more potent than the natural peptide, an inhibitory role of the IGFBPs in cartilage seems likely[33]. IGFBP-3 accumulates with increasing age in the territorial matrix of articular chondrocytes where it co-localizes with fibronectin[34]. Co-presentation of IGFBP-3 with fibronectin increased the inhibitory activity of IGFBP-3[34]. IGF-I appears to be stored in the chondrocyte territorial matrix through binding with IGFBP-3. Binding of IGF to cartilage IGFBPs regulates the transport and availability of IGF-I to the chondrocytes[35]. Other growth factors known to be important in cartilage homeostasis and pathology such as the Bone Morphogenetic Proteins (BMPs) are also sequestered within the ECM. BMP-2 contains a heparin-binding site in the N-terminal region[36]. Binding of BMP-2 to immobilized heparin modulates the biological activity of BMP-2. Important for articular cartilage is the binding of BMP-2 to type IIA procollagen. The cartilage specific type II collagen is expressed in two splice forms, IIA and IIB. Type IIA contains an additional 69 amino acids and is synthesized by chondrocyte precursors[37]. BMP-2 binds with type IIA procollagen but not with IIB procollagen. This points out to a role for type IIA procollagen during chondrogenesis by modulation of BMP availability. Fibroblast Growth Factors (FGFs) are small polypeptide factors most of which bind to heparan proteoglycans of the ECM. The FGFs bind specific receptors in the context of the heparan proteoglycans, which results in cellular activation[38–40]. Perlecan, a heparan sulfate proteoglycan, is highly expressed in cartilage and augments the FGF-mediated receptor activation

on cells expressing this proteoglycan by augmentation of FGF receptor binding[41]. Targeted disruption of the gene encoding perlecan in mice results in severe disorganization of the cartilage matrix[42].

Interaction of integrins and growth factors

The ECM itself, as well as free and ECM-bound mediators, regulates cartilage homeostasis. However, these factors do not operate alone but there also appears to be an important interplay between ECM molecules and soluble mediators on the intracellular level. Binding of chondrocyte integrins to ECM molecules, such as fibronectin, results in the intracellular formation of focal adhesion plaques. The formation of these focal adhesion plaques is a prerequisite for articular chondrocytes to be responsive to growth factors such as FGF and IGF-I[43,44]. Cell adhesion via integrin ligation regulates activation of growth factor receptors[45]. These observations show a mutual dependence between chondrocyte adhesion to the ECM and the regulation of chondrocyte behavior by growth factors. This indicates that in the development of new scaffolds for tissue engineering, cells should not only be provided with the correct three-dimensional architecture but also with the adequate signals from both the ECM and growth factors. If these signals are not initially provided to the cells by the scaffolds, cartilage regeneration will not be obtained. During the course of the restoration process it can be expected that the chondrocytes themselves will generate the neccesary signals for further cartilage regeneration and homeostasis.

Development of tissue engineered cartilage

Since articular cartilage is a relatively uncomplicated tissue judged on the limited cell diversity, absence of vascularity and innervation, the development of *in vitro* engineered cartilage has been attempted in the early days of tissue engineering. However, although cartilage has a relatively simple structure it is a dynamic tissue with characteristics that contradict this tissue as an ideal model for tissue engineering. The extracellular matrix of this tissue has to be maintained by the scarcely distributed chondrocytes, asking for a great demand on the anabolic capacity of these cells. The poor intrinsic repair capacity of articular cartilage restricts the process of cartilage tissue formation compared with tissue with a strong inherent repair response, such as liver. Also the high biomechanical demands on this tissue when placed *in situ* is a great challenge for cartilage tissue engineering. Ideally, tissue engineered cartilage has a similar structure, biochemical composition, mechanical properties and capacity of self-maintenance as healthy native articular cartilage.

CHONDROCYTE AVAILABILITY

The development of tissue engineered cartilage *in vitro*, based on (semi)artificial matrices in combination with chondrocytes or chondrocyte precursors, is hampered by the limited availability of cells and the absence of biomaterials, which can adequately substitute for the natural cartilage matrix. Articular cartilage chondrocytes can be harvested from cartilage but the limited accessibility of this tissue, mainly because of its inherent poor repair capacity makes (autologous) cartilage harvesting problematic. A large number of chondrocytes is needed for the construction of clinically significant implants. This has resulted in the search for alternative sources for articular chondrocytes and the application of (mesenchymal) stem cells appears to be a logical alternative.

Autologous bone marrow can be a suitable source for mesenchymal stem cells. Mesenchymal stem cells are present in bone marrow in a quantity of about 1 out of every 105 cells[46]. These cells can be used as chondrocyte precursors, which will develop in fully differentiated chondrocytes when supplied with the correct differentiation signals from the surrounding matrix and soluble mediators. Many years ago it was shown that mesenchymal stem cells could be directed to the chondrocyte lineage under the influence of bone-derived growth factors[47,48]. Following this line it can be expected that (genetically modified) embryonic stem cells will be a source of chondrocytes in the near future.

SCAFFOLDS

In addition to the search for an optimal cell source the quest for the optimal matrix has resulted in the development and testing of both (semi) artificial and natural materials (scaffolds). Numerous matrices have been used as culturing systems for articular chondrocytes. Matrices based amongst others on polyglycolic acid, polylactic acid, hyaluronate esters, polymerized or non-polymerized fibrin, collagen, alginate, chitosan, calcium carbonate and combinations of those compounds have been used as substrates for *in vitro* cartilage tissue engineering.

Polyglycolic acid, polylactic acid and a combination of both polymers can be used as substrate for chondrocytes resulting in cartilage tissue after 20 weeks with a relatively high resistance to loading[49]. However, toxic monomers are shown to be derived from these resorbable polymers after degradation[50]. Probably more natural substrates are hyaluronan-based polymers. These polymers have been evaluated to support chondrocyte and mesenchymal stem cell-derived cartilage formation. The hyaluronan-based scaffolds are biocompatible and seeded cells easily adhere and proliferate on this material[51,52]. Another widely used biomaterial is fibrin. Polymerized fibrin can be used as a moldable gel and is used for the growth of chondrocytes *in vitro* before implantation *in vivo*[53]. As we have experienced in our own experiments with fibrin, the stability of the material is limited and it can easily be degraded by the embedded chondrocytes. Culturing of rabbit articular chondrocytes resulted in disintegration of the fibrin matrix after 3 days[54]. These results have led to the development of methods to stabilize the fibrin matrix by increasing fibrinolytic inhibition using aprotinin and tranexamic acid[55]. An alternative method is mixing of fibrin with other matrix materials such as alginate[56]. Both methods stabilize the fibrin-chondrocyte constructs and enhance the synthesis of cartilage-specific molecules by the embedded chondrocytes[55,56]. A more recently used biomaterial is chitosan, a copolymer of glucosamine and N-acetylglucosamine, a derivative of chitin[57,58]. Chitosan is used alone and in combination with chondroitin sulfate. Chondrocytes cultured on chitosan were spherical, in contrast to spindle shaped cells cultured on plastic, and maintained the synthesis of type II collagen and aggrecan[57,58].

Scaffolds based on type I collagen have been used by our own group. The sources of commercial type I collagen

influenced chondrocyte behavior, probably due to contaminating molecules in the matrices[59]. Compared to chondrocytes cultured in alginate, chondrocytes grown in type I collagen have a higher proliferation capacity but lose their differentiated phenotype[60]. Studies in our and other groups are continuing, using the cartilage specific type II collagen as a substrate[61]. Highly purified type II collagen may be a better matrix for chondrocyte culture than type I collagen[62,63]. However, to provide the chondrocytes with a matrix that provides similar signals to cells as natural cartilage, other matrix molecules have to be incorporated in the type II collagen matrix.

Besides the chemical properties of the matrix, other parameters also determine the final outcome of cartilage formation. The microtopography of the material resulting in differences in porosity and surface roughness contributes to the chondrocyte behavior[63]. Moreover, the *in vitro* culture conditions itself strongly modulate cartilage formation. Incubation of chondrocytes on polymer scaffolds in rotating vessels showed higher synthesis of cartilage specific molecules and better mechanical properties compared with chondrocytes cultured in mixed or static flasks[64,65]. These results show that the hydrodynamic culture conditions influence the formation of functional tissue engineered cartilage.

In vitro tissue engineering is the application of the principles of developmental biology and tissue regeneration to the *in vitro* situation. Since soluble factors significantly contribute to the morphogenesis of newly formed tissues, these factors can be used to stimulate *in vitro* cartilage formation. Many studies have been performed with cartilage explants and chondrocytes cultured in monolayer but the number of studies applying these growth factors to cartilage tissue engineering *in vitro* is limited so far.

GROWTH FACTORS

Members of the TGF β superfamily are known to play an important role in cartilage formation during embryonic stages and can stimulate cartilage repair *in vivo*[66–68]. Equine chondrocytes cultured in a three-dimensional fibrin matrix showed an increased cell proliferation and proteoglycan synthesis in the presence of TGF β. The effect of TGF β was most pronounced in the absence of serum in the culture medium[69]. TGF β stimulated proteoglycan synthesis in bovine articular chondrocytes cultured in alginate and appeared to be more potent under these conditions than BMP-2[70,71]. Others have shown that BMP-7 not only stimulates proteoglycan synthesis but also synthesis of matrix molecules known to be important in retention of the major cartilage proteoglycan aggrecan, namely hyaluronan and CD44[72].

Besides the members of the TGF β superfamily other growth factors have been tested. IGF-I stimulates proteoglycan synthesis of articular chondrocytes cultured in alginate[70,71]. Platelet-derived growth factor (PDGF-BB) increased cell proliferation of bovine chondrocytes cultured on collagen gels but production of proteoglycan was significantly decreased under the influence of PDGF, indicating an harmful effect of PDGF on chondrocyte differentiation[73]. During expansion in monolayer, chondrocytes loose their differentiated phenotype. Culturing of articular chondrocytes in monolayer in the presence of FGF-2 followed by transfer of these cells to a three-dimensional polymer carrier induced redifferentiation to mature chondrocytes[74]. This suggests that FGF can be used to increase chondrocyte cell number if this treatment is followed by the culturing of the cells in a three-dimensional scaffold.

Differentiation of mesenchymal stem cells to a particular lineage, osteoblast, adipocytes, muscle cells and chondrocytes, can be regulated by specific growth factors. Myogenic differentiation of mesenchymal stem cells is inhibited by BMP-2 and TGF β[75]. Chondrogenic differentiation of marrow-derived mesenchymal stem cells is strongly stimulated by TGF β in rabbit, equine and human systems[76–78]. Embryonic stem cells of murine origin can be directed to chondrocytic differentiation by application of BMP-2 or BMP-4[79].

An alternative to the addition of growth factors to the culture medium is the incorporation of genes coding for these growth factors in the cells itself. Introduction of IGF-1, BMP-2 or TGF β by adenoviral transfection of articular chondrocytes resulted in increased synthesis of matrix molecules by these cells[80]. Similar findings were reported after retroviral and non-viral transfection of, respectively, meniscal cells or chondrocytes with TGF β[81,82]. The application of gene-enhanced tissue engineering will result in the development of cartilage grafts with the potential for prolonged synthesis of growth factors after transfer to the *in situ* lesion site. The latter might stimulate the integration of the scaffold into the cartilage lesion when implanted *in vivo*. Optimization of the regeneration of articular cartilage should be based on knowledge of the normal function and development of this tissue. Functional tissue engineered cartilage can only be achieved when those matrices are used for cartilage regeneration which provide invading or embedded cells with the correct signals, both soluble and insoluble, Results from other organs and tissues show that the application of the correct differentiation signals in combination with cells able to respond to these signals (e.g embryonic stem cells) can achieve remarkable results in the field of tissue generation and tissue engineering.

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