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Different Phenotype of Chondrocytes in Articular Cartilage: Mapping, Possible Mechanisms, and Impact to Implant Healing

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Abstract

A remodelling of articular cartilage due to damage or pathological state development is characterized by an alteration in cartilage homeostasis including distribution of the chondrocytes. Said alteration can be determined by cellular response changes during cartilage regeneration and remodelling. However, features of cellular distrubution in native cartilage is not yet clear. This study was undertaken to select representative set of regulatory molecules and their receptors related to the formation of mosaic structures in the native cartilage. We used bioinformatical approaches and mapping of the chondrocyte phenotype markers in different areas of the intact articular cartilage. Differences in the phenotype markers expression as like as in cellular density gradient from tidemark to articular surface were observed. Additionaly, we have found specific order of lateral cellular distribution in the intact cartilage. The database of molecular processes in

chondrocytes have been obtained, seems to be suitable for further investigation of the articular cartilage remodeling. The hypothetical model of scaffold to satisfy the conditions of mosaic structure formation in cartilage after substitution of full-layer defects and stimulation of colonization by cells from the bone marrow, have been proposed.

Keywords: articular cartilage; osteoarthritis; cartilage tissue engineering; scaffolds; molecular mapping.

Introduction

Articular cartilage has unique structural features and control mechanisms of its tertiary functions to provide noninvasive stress distribution due to joint movements. These features, as a minimum, include visco-elastic and, the same time, very sough characteristics of extracellular matrix (ECM), monomorphic cellular composition, full avascularity, and absence of innervations. The trophic and molecular control the chondrocyte phenotype are carried out by molecules diffused from the underlying bone and synovial fluid [1, 2]. These features are the biological foundations of extremely low ability of articular cartilage to full regeneration, therefore, different post-traumatic problems in large joints and chronic pathology of degenerative and inflammatory genesis, osteoarthritis (OA), are wide-spread in the human population [3-6].

Despite the chondrocytes are the only cell population composed of cartilage, the tissue forming the articular surface is not seems to be homogeneous in its volume. In addition to the classic subdivision into loaded and not loaded areas, as well as deep, middle, and superficial zones [3, 7], an articular cartilage, as shown recently in rats and rabbits [8, 9], has distinct structural heterogeneity. The revealed phenomenon concluded in a presence of mosaic plots (microclusters), different from the environment by the numerical density of chondrocytes, structure and optical density of ECM. Those microclusters took about 40% of cartilage volume and could be described as a polygon-based truncated pyramids with a diameter cross section about 60-120 microns near the tidemark and extending to 100-150 microns by superficial zone of the cartilage, and they were critically altered in experimental osteoarthritis [10].

Currently, there are over forty biologically active molecules being able to actively influence over the chondrocyte phenotype. Basically, they represent cytokines (TNF-α, interleukin 1), growth and/or differentiation factors (TGF-β, BMP-2, IIS-7, IGF-1 etc.), although the effects of a number of hormones (in particular, the number of thyroid, insulin and metabolites were described in [11-15]). Recently there have been published the evidence of involvement of some signaling molecules, as growth factor and differentiation 5 (GDF-5) [20] and connective tissue growth factor (CCN2) [21, 22] in the regulation of chondrocyte phenotype. The participation hypoxia-inducible factor HIF-1α [23], and integrin α1β1 in this process has been substantially revised [24, 25].

That is the reason to make significant changes in scaffold technology for cartilage tissue engineering. Based on the of biomimetic principle, such scaffolds should have such structure and properties that the maximum extent close to the one of intact cartilage [26]. Tissue regenerates in the scaffold replacement site should be remodel into native cartilage in natural manner within some reasonable period, including all the nuances of zonal and mosaic organization [27, 28]. In order to achieve the results it is necessary to construct the scaffold being able to form zonal and mosaic heterogeneity of cartilage after implantation, based on engineering principles and bioinformatics approach.

The goal of this work, based on the above, is an attempt to develop principles for the fabrication of new scaffolds for cartilage tissue engineering.

Material and Methods

The first step in our study is an attempt to connect the imaginations about structural irregularity of cartilage with differences in chondrocyte phenotype. The samples of cartilage in the knee and elbow joints of six male Chinchilla rabbits weight of from 2.4 to 3.2 kg have been used for mapping the phenotype of the chondrocytes. Removing the animals from experiment was carried out in accordance within the context of Directive 2010/63/EU on the protection of animals used for scientific purposes.

Histological specimens were prepared after fixation in 10% solution of neutral buffered formalin (pH = 7.4) and decalcification in Cal-Ex® solution (Fisher Scientific). The sections were stained with hematoxylin, eosin, and with safranin O, to identify ECM. Primary phenotype

identification included such characteristics of chondrocytes, as the average size on the section (μm²) after visualization of their nuclei by fluorescent DAPI staining, and the average optical density of their territorial matrix (con. U/μm²), correlated to the distance between cell and articular surface (μm). The measurement was carried out in 4 or 5 slides from each specimen, 5-6 visual fields in each slide, so that the total number was of the order of 10^3.

Identification of the phenotype of chondrocytes was performed by sensitive immunohistochemical assays. Using the monoclonal antibodies against the proliferation marker Ki-67 (DakoCytomation, Denmark), TNF-related apoptosis-inducing ligand (TRAIL, Novocastra, UK) and the key enzyme of apoptosis cascade, caspase-3 (Novocastra, UK) we obtained the opportunity to reveal proliferative and apoptotic potential of chondrocytes. The antibodies against aggrecan (Santa Cruz Biotechonogy, USA) and lubricin (Santa Cruz Biotechonogy, USA), matrix metalloproteinases MMP-9 (Leica Mycrosystems, Germany) and MMP-13 (Santa Cruz Biotechonogy, USA), and their tissue inhibitors TIMP-1 (DakoCytomation, Denmark) and TIMP-3 (Santa Cruz Biotechonogy, USA) one used following the manufacturer's instructions to identify specific synthetic activity of cells. We also used temperature method of antigen demasking, secondary antibodies labeled with alkaline phosphatase with Fast Red visualization direct fluorescent label, negative control antigens and antibodies were used.

To examine visually and obtain digitized images we used research microscopes BIMAM R-13 (LOMO, Russia) with JCM camera (Japan) and AxioPlan 2 Imaging (Carl Zeiss, Germany) with visualization system AxioVision LE. We evaluate the expression immunopositive material in cells and/or territorial matrix as negative (-), very low, low, moderate and high. The most frequent variant of the conclusion was assigned to the specific site of the specimen.

Quantitative data were processed using Statistica 10.0 (StatSoft Inc., USA) with the calculation of the indices adopted to characterize the non-parametric samples in biomedical research: median [1st quartile ÷ 3rd quartile]. To prove the validity of differences the non-parametric Friedman criterion for multiple groups was applied. *P* values less than 0.05 were considered significant.

The next step was to build a scheme of genes encoding key proteins. It is required to determinate the chondrocyte phenotype. We used routine bioinformatics techniques, including the search free access NCBI resources (PubMed, PubMed Central, Gene, Protein, BioSystems), UniProtKB, PDB, KEGG, and SRS. We used comprehensive pool of synonymous constructs to success in data mining. As a result, genes from the operational database were associated with metabolism and the regulation of the functional activity of the chondrocyte (signaling molecules and their receptors, transcription factors). Representation of the signaling pathways controlling chondrocyte metabolism in the form of intellectual schema allowed to transform the information from the database, into a set of necessary properties of the chondrocyte environment in each compartment of articular cartilage: the surface area (1), the microclusters (2) and between them (3), and also near to the tidemark (4).

The final step was to establish a working model of scaffold for tissue engineering, taking into account the necessity of forming a cartilage with a three-dimensional heterogeneity of cellular setting and ECM density.

Results

Morphological analysis confirmed that the chondrocytes in the articular cartilage had quite heterogeneous phenotype; this heterogeneity cannot be reduced to the differences caused by different loads on specific areas of joint or different zonal location. Typical chondrocyte columns formed by chondrocyte isogroups in the deep and middle zones of cartilage are additionally aggregated into specific microclusters the size of about 100 μm (fig. 1a).

In the horizontal scanning the cartilage, we found distinct areas of more dense settlement of cells, usually about 5-8 in cross section (fig. 1b). When you restore the volume structure of microclusters they consisted generally up to a couple hundred of chondrocytes. As a detailed morphological description had been given in previous publications [8, 9], we specify only key features of the chondrocyte phenotype in the description of the cartilage under this perspective.

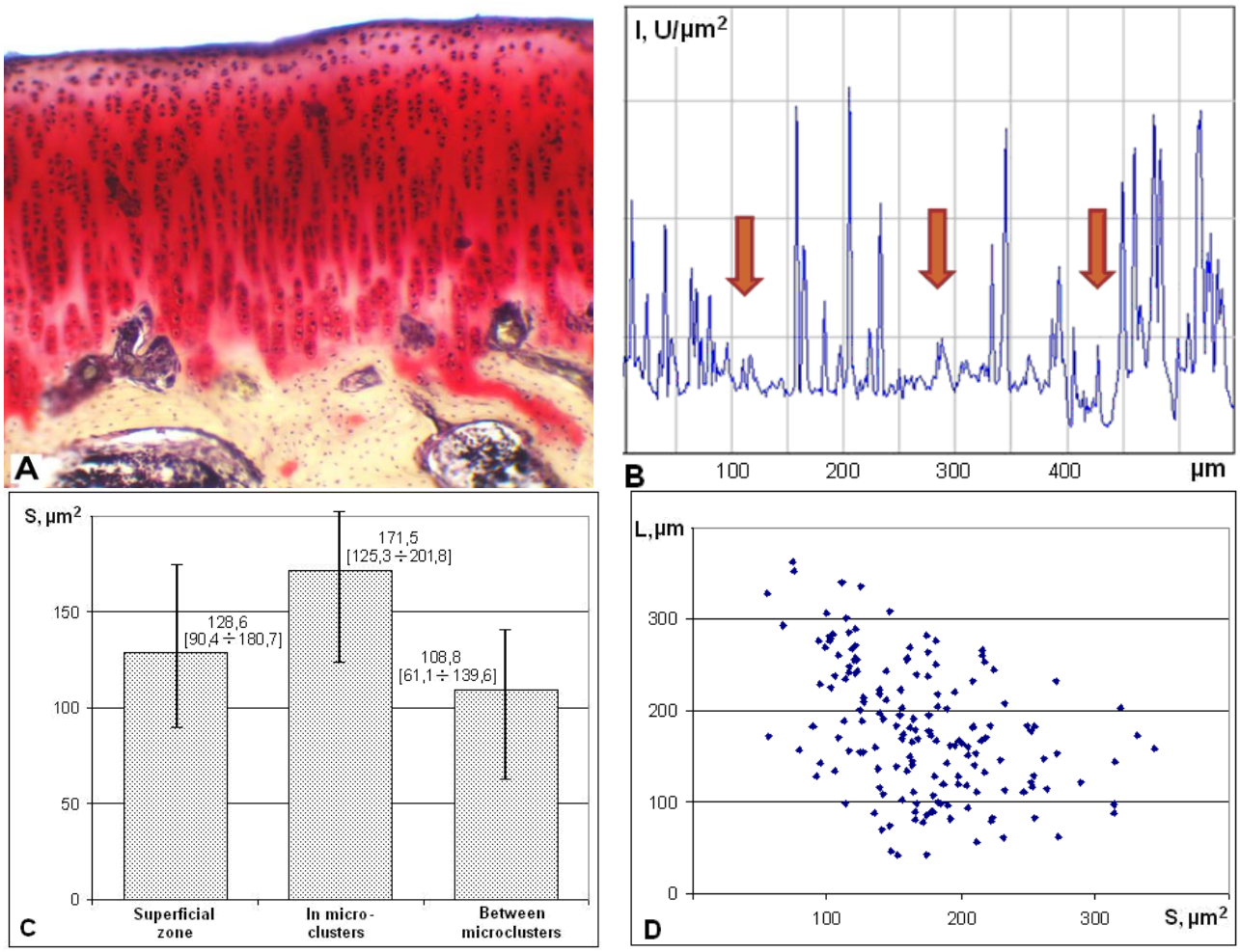

Figure 1. Chondrocytes in the cartilage of rabbit knee joint particularly group into microclusters different from surrounding areas of cartilage by cellular phenotype and denser ECM. A. Chondrocyte microclusters are presented by groups of several columns, spreading and widening from deep to superficial zone of cartilage. Safranin O staining, x 240. B. The characteristic distribution of DAPI fluorescence intensity in the articular cartilage reflects the presence of microclusters and intermediate areas (shown as arrows). C. Average sizes of chondrocytes, presented as Median [25% ÷ 75%], are different in superficial zone of cartilage, in microclusters of middle zone, and between them. D. Average size of chondrocytes in microclusters depends on the distance to articular surface in inversely proportional manner. The sample image contains about 200 cells in sight (estimation using Image J program).

First, the size and shape of the chondrocytes differed not only in the comparative study the superficial, middle and deep zones of cartilage. The size of chondrocytes in microclusters is much larger than the similar one between microclusters. The chondrocytes of the superficial zone, as well known, characterizing by a more elongated shape, had intermediate size in this list (fig. 1c). Then, the size of chondrocytes in microclusters was largely depended on their distance from the articular surface, this dependence was close to inversely proportional (fig. 1d). Finally, the density of the territorial matrix of the chondrocytes in the structure of microclusters was significantly higher than the similar one between microclusters.

These differences already suggest the existence of metabolic phenotype heterogeneity for chondrocytes, including their relation to synthesis, proliferation, and apoptosis.

Table 1 summarizes our findings in respect of these phenotypic traits. For ease of comparisons, we subdivided the chondrocytes in accordance with their position in the cartilage into 4 groups: surface area (1), in microclusters (2), between them (3) and in areas of deep zone near the tidemark (4).

Table 1: The distribution of phenotypic markers in chondrocytes and territorial matrix of the rabbit articular cartilage in the concept of its mosaic structure

Marker	Location of chondrocytes			
	Superficial zone	In microclusters	Between microclusters	Near tidemark
Ki-67	-	Very low	Very low	Moderate
TRAIL	Moderate	Low	Low	High
Caspase-3	Low	Low	Low	Moderate
Aggrecan	Low	High	Moderate	Low
Lubricin	High	Very low	-	-
MMP-9	Moderate	Low	Moderate	Very low
MMP-13	Moderate	Low	Moderate	-
TIMP-1	Low	Moderate	Low	Low
TIMP-3	Very low	Moderate	Low	Low

Besides the obvious marker findings, such as high lubricin expression of almost exclusively in the superficial zone of the cartilage, and the presence of proliferative potential in the deep zone, we discover several interesting findings. Pre-apoptotic and apoptotic potentials, estimated balance TRAIL and caspase-3 have been quite high in the deep zone of cartilage and moderate in the surface zone. These markers almost never appears in the middle zone's chondrocytes of native cartilage. The aggrecan expression was higher in chondrocytes of microclusters, less in chondrocytes localized between clusters, and it was tending to zero as it approached the surface. The chondrocytes in microclusters were characterized by a higher expression of TIMP-1 and TIMP-3, whereas chondrocytes between the microclusters and in surface area were characterized by the prevalence of MMP-9 and MMP-13 expression and low expression of TIMPs.

The received data specify the number of mechanisms associated with relatively higher ECM density in microclusters of articular cartilage. They, at least, are in the presence of high synthetic potential of chondrocytes, their resistance to apoptosis, and in MMP/TIMP balance preventing any intensive destruction of the matrix.

Bioinformatics approach allows particularly explain structural irregularity of cartilage using a metabolic map creation. Key bonds between signal molecules and specific synthesis in chondrocyte is considered a control network for chondrocyte phenotype.

A first control circuit for the control chondrocyte phenotype is a set of signaling molecules coming from the bloodstream and bone marrow through the underlying bone. In accordance with the diffusion mechanism of receipt such molecules increasingly act to young cells near the tidemark and, in a much lesser extent, to the middle zone chondrocytes. Necessary information about intake of almost all known cytokines and growth factors, as well as a number of hormones and other regulatory molecules to the cartilage has been found in open access databases.

The second control circuit is in a pool of molecules coming from the synovial fluid. Similar to the first one, as is clear from the revealed cartilage structure, this pool is able to influence the chondrocytes of the superficial zone and cells between microclusters in middle zone. Many cytokines were found in the synovial fluid: more than ten interleukins (IL-1β, IL-6, IL-8, IL-10, IL-17 etc.), TNF-α, interferon γ, interferon-inducible protein 10, cell-derived factor-1, oncostatin M, monocyte chemoattractant proteins 1 and 2, and RANTES. Also growth factors such as transforming growth factors (TGF-α and TGF-β1-3), granulocyte colony-stimulating factor, insulin-like growth factors (IGF-1 and IGF-2), platelet-derived growth factor (PDGF-BB), vascular endothelial growth factor (VEGF), connective tissue growth factor (also known as CCN2) were detected in the synovial fluid. A number of hormones, including leptin, were also identified there. In addition, synovial fluid contains many products of disintegrating cartilage, and therefore it is possible to discover in it a variety of glycosaminoglycans, their sulfated fragments, glycoconjugates, polygalacturonase acid, fibronectin-aggrecan complexes, fractalkine, cartilage oligomeric matrix protein, osteopontin, and matrix metalloproteases 1, 3, 9, and 13. Collectively, they represent an additional pool of substances seems to regulate composition and properties of the superficial zone in articular cartilage.

Directly in the middle zone the paracrine and autocrine regulation mechanisms are probably prevalent particularly stimulating by mechanical loads on articular surface. Such factors can

include hyaluronic acid and its derivatives, matrix metalloproteinases and their inhibitors, a number of transcription factors capable of intercellular transfer (for example, Nupr1, NF-kB), etc.

The most important of all these regulators and their possible receptor-dependent involvement in the formation of the chondrocyte phenotype we group in table 2.

Table 2: Proved presence of regulatory molecules in the environments supplying articular cartilage and their influence on the chondrocyte phenotype

Regulatory molecule	Evidence of presence		Impact to processes in chondrocytes			
	Synovial fluid	Bone and bone marrow	Proliferation and differentiation	Apoptosis	ECM synthesis	ECM degradation
Cytokines						
Growth regulated oncogene	+++	+	+++			+
Interferon γ	+++	+++		+		+++
Interferon-inducible protein 10	+++	+++	+	+		+++
Interleukin 1α, 1β	+	+++		+++	-	+++
Interleukin 6	+++	+++	+++	+	+	+
Interleukin 8	+	+++		+	-	+++
Interleukin 10	+++	+++	+	-	+	-
Interleukin 17	+	+++		+++	-	+++
Monocyte chemoattractant protein 1, 2	+++	+++	+++	+		+
Oncostatin M	+++	+++	-			-
RANTES	+	+++		+++		+++
Tumor necrosis factor α	+++	+++		+++		+
Growth factors						
Connective tissue growth factor	+++	+++	+++		+++	
Bone morphogenetic protein 2	+	+++	+++	-	+++	+
Bone morphogenetic protein 4	-	+++	+		+++	+
Bone morphogenetic protein 6	-	+++	+		+++	+
Bone morphogenetic protein 7	-	+	+		+++	+
Granulocyte colony-stimulating factor	+++	+++	+++			
Growth differentiation factor 5	-	+++	+++		+	
Insulin-like growth factor 1, 2	+++	+++	+++		+++	
Platelet-derived growth factor	+++	+++	+++		+	
Transforming growth factor β1-3	+++	+++	+++	-	+++	+++
Vascular endothelial growth factor	-	+++	+++			+
Hormones and other regulatory molecules						
Angiotensin 1, 2	-	+++	+++			
Hyaluronic acid	+++	-	+++		+++	
Leptin	+	+++	+++		+++	
Lipoplysaccharide	+	+		+++	+	
Osteopontin	+	+++	+		+	+
Prostaglandin E-2	+	+++	+			+++

Note: Note: the sign '+++' indicates the presence of information in multiple sources and reflects the established view this molecule, '+' denotes a single source on this fact, '-' indicates evidence of a negative effect. Blank cells indicate absence of information.

Based on these data, a list of genes encoding key proteins for the chondrocyte phenotype was compiled. Factors connected to strong pro-apoptotic stimuli are not included. With respect to

future plans to research the possible effectiveness of new scaffolds for tissue engineering in rats, data about genes of human and rat have been included into table 3.

Table 3: Common characteristics of the genes encoding key proteins involved in the differentiation and phenotypic expression of articular chondrocytes

Coding protein	Gene	Gene ID		Function of coding protein
		Human	Rat	
Encoding chondrocyte receptors				
TGF-βR1	*tgfbr2*	7046	29591	Reception of relevant molecules from transforming growth factor β super family.
TGF-βR2	*tgfbr2*	7048	81810	
BMPR-1	*bmpr1a*	657	81507	Reception of bone morphogenetic proteins 2, 4, 6, 7, and growth differentiation factor 5 (BMP-14)
BMPR-2	*bmpr2*	659	140590	
IGFR-1	*igfr1*	3480	25718	Reception of insulin-like growth factor
PDGFR-α	*pdgfra*	5156	25267	Reception of platelet-derived growth factor
PDGFR-β	*pdgfrb*	5159	24629	
FGFR2	*fgfr2*	2263	25022	Reception of connective tissue growth factor (CCN2)
FGFR3	*fgfr3*	2261	84489	
IL-6R	*il6r*	3570	24499	Reception of interleukin 6
CXCR1	*cxcr1*	3577	54258	Reception of interleukin 8
CXCR2	*cxcr2*	3579	29385	
CD44	*cd44*	960	25406	Reception of hyaluronic acid
LEPR	*lepr*	3953	24536	Reception of leptin
Encoding common ECM components				
Aggrecan	*acan*	176	58968	Base proteoglycan of cartilage ECM
Collagen type II	*col2a1*	1280	25412	Base protein of cartilage ECM
MMP-3	*mmp3*	4314	171045	Common matrix metalloproteinases of cartilage involving to its remodeling and ECM degradation
MMP-13	*mmp13*	4322	171052	
TIMP1	*timp1*	7076	116510	The specific inhibitors of matrix metalloproteinases involving to ECM protection
TIMP3	*timp3*	7078	25358	
Encoding autocrine and paracrine regulators				
Intergin β1	*itgb1*	3688	24511	Key protein of cell adhesion in skeletal tissues
Hypoxia inducible factor 1α	*hif1a*	3091	29560	The inducible regulator of cellular metabolism by activating transcription
Nuclear protein 1	*nupr1*	26471	100912108	Stress-inducible protein involved in gene transcription including MMP-13 expression
Tenascin C	*tnc*	3371	116640	Extracellular matrix protein, controlling the spatial and temporal distribution of ECM

The data presented in table, in our opinion, could be used to design DNA probes for mapping the gene expression in native and altered cartilage, to detail the mechanisms of its formation and remodeling under the physiological and pathogenic conditions. Secondly, this information is useful to connect elements of gene therapy to replacement technology of cartilage defects in clinical practice.

In order to choose the main directions in modification of existing scaffolds for cartilage tissue engineering, it is necessary to map them to current structure with general organization of the surrounding cartilage. Ideally, the cartilage, formed at the site of implantation after scaffold remodeling, should have a similar structure. Figure 2A presents a schematic structure of articular cartilage, taking into account the presence of zones, distinguished by the morphology of chondrocytes and ECM, as well as microclusters presence.

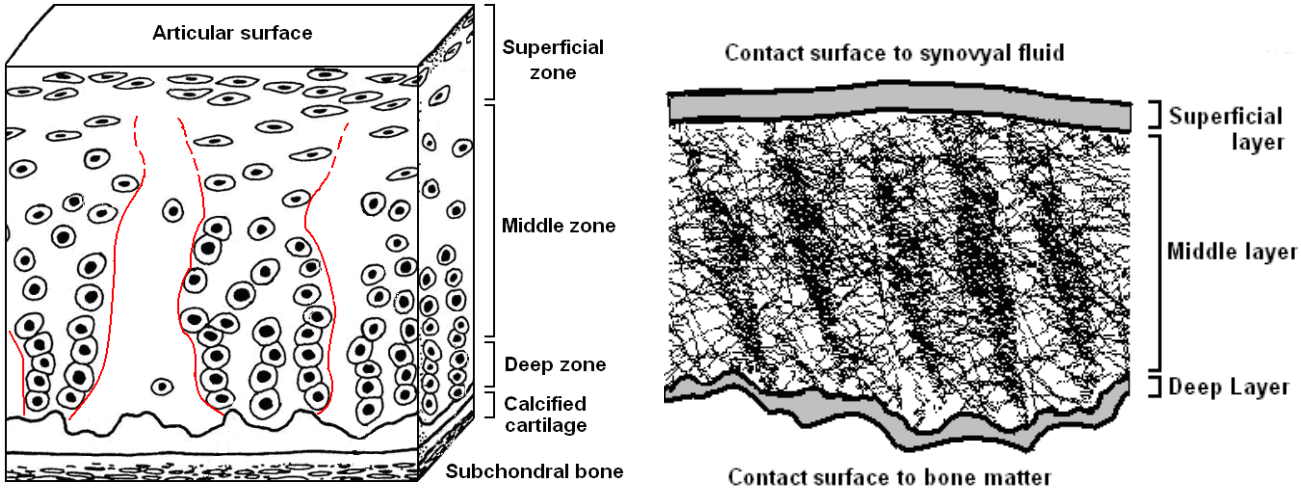

Figure 2. A. Scheme illustrates zonal organization of the articular cartilage with the selection of microclusters (bordered by red lines). B. Hypothetic model of scaffold for cartilage tissue engineering based on principles of zonal and mosaic organization of natural articular cartilage. Explanations are in the text.

Based on these representations, it is not sufficient for bulk of scaffold to have the three-dimensional porous structure for cell seeding (1), and certain strength to ensure early load on the articular surface after implantation (2). This part of scaffold should initially contain certain irregularities look like seal columns the size of about 100-150 microns. They should become the basis for microcluster formation in the remodeling matter scaffold into cartilage ECM (fig. 2b).

To ensure the specific properties of scaffold surface layer ought to be an important aspect of scaffold building. In most existing tissue engineering constructions, this task is left to nature, so that surface lubricants were formed by the active function of the synovial fluid. It seems appropriate to provide neo-formed articular surface of the lubricating layer with a thickness of about 50 μm in the first few days after implantation. For example, it can be done by applying hyaluronate gel on the outer scaffold surface.

Structural features of the scaffold deep layer depend on the depth of the defect, they should provide strong fixation to the underlying bone and, at the same time, the possibility of cell seeding and diffusion of necessary substances in remodeling area. It is here, in our opinion, should be placed microspheres containing the required number of growth factors for the successful trigger of cellular differentiation and providing the necessary chondrocyte phenotype.

Discussion

Thinking about the need to provide adaptability to environmental changes for a certain area through the formation of irregular structures with conditional repetitive elements is a consequence of the cellular principle of Life organization and general biological principle of mosaicism. In this sense, our findings are not inconsistent with recent detailed studies of the articular cartilage, including the use of computer-conversion and modeling [2, 7, 29].

Microcluster described in our study, should be distinguished from pathological clusters of chondrocytes in osteoarthritis. These structures are formed with long-term progression of the disease, they are mainly located in the deep zone of the cartilage, and not surrounded by a dense ECM [30].

Possible mechanisms for the formation of such clusters from cellular columns, most likely conclude in the spatial characteristics of molecular signal distribution near tidemark in connection to the heterogeneity of underlying bone surface. One of the mechanisms leading to the advanced differentiation of some cellular groups may be earlier deprivation of oxygenation. As the formation of dense territorial and extraterritorial ECM, the mechanical stimuli, as a result of total redistribution of mechanical loads from the articular surface, are connected to these mechanisms.

Such switching and mechanisms of mechanic signal reception have been recently described directly in the cartilage [29].

Range markers of chondrocyte phenotype, formed in this research, meet the tasks used to improve and monitor the results of the cartilage tissue engineering. They could change significantly if, for example, we are talking about the cultivation of chondrocytes on artificial media. The results of mapping the lubricin, metalloproteinases, their inhibitors, the characteristics of the chondrocyte receptors, as well as the role of these differences in functioning and remodeling of ECM appeared very recently [13, 31-33]. In relation to apoptosis of chondrocytes the discussion is still ongoing due to the ambiguity of the propagation mechanisms of molecular signals in the thickness of the cartilage [15, 34].

The elaboration of new principles for the scaffold fabrication in cartilage tissue engineering was practical objective of this research. Articles, where the authors focused on creating scaffolds with zonal structure for these tasks, only appear in the open access [26, 28, 35], but attempts to create structures reproducing the horizontal irregularity of ECM, has not yet been undertaken. This study proves the relevance of this approach in cartilage tissue engineering.

Conclusion

The study suggests that native articular cartilage had a mosaic structure, which was provided by differences in chondrocyte phenotype. Based on bioinformatics approach and mapping the actual chondrocyte phenotype in different areas of articular cartilage it is possible to explain this structural mosaicism. As a result, the intellectual map and database of molecular processes in chondrocytes have been obtained, seems to be suitable for further investigation of the articular cartilage remodeling. The hypothetical model of scaffold to satisfy the conditions of mosaic structure formation in cartilage after substitution of full-layer defects and stimulation of colonization by cells from the bone marrow, may be a basis for future elaboration of scaffold technologies.

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Различия фенотипа хондроцитов в составе суставного хряща: картирование, возможные механизмы и влияние на приживление имплантатов

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Аннотация

На основании биоинформационного поиска и сопоставления с реальным фенотипом хондроцитов выбран спектр биологически активных молекул-регуляторов и их рецепторов, имеющих отношение к формированию мозаичных структур в неизмененном хряще и их ремоделированию при развитии экспериментального остеоартроза. Как итог, получена интеллектуальная карта молекулярных процессов в хондроците, сопряженная с базой знаний об этих молекулах, пригодная для дальнейшего исследования процессов ремоделирования суставного хряща. Предложена гипотетическая модель скаффолда, удовлетворяющая условиям формирования мозаичной структуры хряща после имплантации на место полнослойных дефектов хряща в условиях стимуляции заселения клетками из костного мозга.

Ключевые слова: суставной хрящ; тканевая инженерия; скаффолды; остеоартроз; молекулярное картирование.