Importance of Wnt-signaling during embryonic skeletogenesis

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INTRODUCTION: The canonical Wnt-signaling pathway has been implicated in playing various roles in skeletogenesis. The central component of this pathway is β-catenin, which is stabilized upon ligand/receptor interaction and translocated into the nucleus. Recent work by our lab showed that this pathway is absolutely required for skeletal lineage differentiation in mouse embryogenesis.

METHODS: Canonical Wnt-signaling was altered by conditional inactivation (fl/fl) or stabilization at the protein level (ex3fl/ex3fl) of β-catenin using the Prx1 Cre line and by genetically inactivation of Wnt9a and Wnt4. Primary cell cultures such as micromass and osteoblast cultures were established from E11.5 and E16.5 β-catenin fl/fl or β-catenin ex3fl/ex3fl embryos, respectively and infected with Adeno-Cre. Cultures were analyzed histologically, by in situ hybridizations and real-time PCR analysis.

RESULTS: Deletion of β-catenin in the early limb bud results in an expansion of Sox9, Runx2, Col2α1, Sox5 and Sox6, which are expressed in early skeletal precursors. In contrast stabilization of β-catenin leads to repression of the expression of Sox9 and the other markers. This suggests that active canonical Wnt-signaling is involved in limiting the differentiation of skeletal pre-cursor cells to the center of the limb bud.

Fig. 1: Sox9 expression in response to loss-of (lof) and gain-of -catenin activity.

While cartilage differentiation proceeds more or less normally in mice lacking β-catenin, no mature osteoblasts can be detected in the limb and head mesenchyme of those mice. Molecular analysis revealed that differentiation is blocked at the level of the osteoblast commitment factor Osterix. Absence of β-catenin leads to the transdifferentiation of osteoblast precursors into chondrocytes (Fig. 2) in a cell-autonomous manner.

Fig. 2: Chondrocytes form instead of osteoblasts in the skull lacking β-catenin (lof), as shown by van Kossa/alcian blue staining.

Within the joint lineage Wnt-signaling is required to maintain joint integrity as in the absence of the signal, Wnt9a, synovial chondroid metaplasia is observed in the humeral-radial joint (Fig. 3). Upon additional removal of the activity of Wnt4 this phenotype is also observed in the knee and ankle joints.

Fig. 3: Col2α1 in situ hybridization showing chondroid metaplasia (arrow).

DISCUSSION & CONCLUSIONS: Our work shows that canonical Wnt-signaling is important in cells of all skeletal lineages. Mesenchymal cells with low levels of β-catenin give rise to skeletal precursors. Later during development elevated levels of β-catenin are required in osteoblasts and joint cells to suppress their potential to differentiate into chondrocytes. Therefore chondrocyte differentiation might be the default pathway in the absence of elevated β-catenin levels. However, there is also evidence that β-catenin is also required in chondrocytes for further maturation.


ACKNOWLEDGEMENTS: This work was supported by Boehringer Ingelheim and EU NoE Cells into Organs.
The Isolation and Partial Characterisation of Progenitor Cells from Foetal and Aged Human Articular Cartilage.

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Previous work from our laboratory has demonstrated the existence of progenitor-like cells isolated from the surface of bovine articular cartilage. These cells can be expanded extensively in monolayer culture whilst maintaining chondrocytic potential unlike mature chondrocytes that lose this ability after 10 population doublings. Clearly, in terms of cartilage repair, the existence of equivalent populations within human cartilage would provide opportunities for devising strategies to heal large cartilage lesions which currently and not amenable to procedures such as autologous chondrocyte implantation (ACI).

I shall present data, describing the isolation and partial characterisation of cells from foetal, aged normal and osteoarthritic cartilage that have progenitor-like properties and will compare and contrast their characteristics (see abstracts by Dowthwaite, Haugthon and Redman). Central to the regulation of clonality is the Delta/Notch signalling pathway where the expression of Notch-1 is a pre-requisite for stemness although the same receptor is also expressed by non-progenitor cells.

I shall speculate on the potential clinical use of these cells as an alternative to other procedures of biological repair of joint tissues.
Integrin α10β1, a unique cell-surface marker for chondrogenic cells

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INTRODUCTION: Cartilage development and function depends on the interaction between the chondrocytes and the surrounding extracellular matrix (ECM). Integrins, present on chondrocytes, have a central role in mediating these interactions. Our work has focused on the collagen binding integrin α10β1, expressed mainly by chondrocytes. Knockout mice lacking α10β1 have defect growth plate and develop a growth retardation of the long bones. The length of the hypertrophic zone is reduced and the columns of the growth plate are disorganized with rounded chondrocytes. In addition, chondrocyte proliferation is reduced and the matrix contains less collagen. In healthy human articular cartilage α10β1 is expressed on the majority of the chondrocytes, however, in patients with osteoarthritis the chondrocytes loose expression of α10β1, starting in the upper layer.

Integrin α11β1, a collagen binding integrin mainly expressed by fibroblasts, is upregulated on cultured and dedifferentiated chondrocytes and appears to oppose the expression of α10β1.

METHODS: Human chondrocytes were cultured in monolayer for 4 weeks and MACS sorted using a monoclonal antibody specific for α10 (mAb365). mRNA expression of COL1A, COL2A, aggrecan and GAPDH was analyzed using real time PCR. C-28/I2 cells, stably transfected with α10, and wild-type C-28/I2 cells were differentiated in pellet-mass and mRNA expression of COL2A, Aggrecan, COL1A, integrin α11, MMP-13 and GAPDH was analyzed. Human mesenchymal stem cells (MSCs), were cultured with or without bFGF and subjected to chondrocyte differentiation in pellet-mass. On day 7, 14 and 21 mRNA expression of GAPDH, COL2A, aggrecan, integrin α10 (ITGA10), integrin α11 (ITGA11) and SOX9 was analysed. Supernatant was analyzed for synthesis of collagen type II (CPII pro-peptide ELISA) and proteoglycan (35-S incorporation).

RESULTS: Integrin α10β1 as well as collagen II expression is down-regulated during monolayer culture and up-regulated during re-differentiation of human chondrocytes. By sorting α10 expressing cultured chondrocytes we demonstrate that high expression of integrin α10 correlates with high expression of COL2A and aggrecan and low expression of COL1A. When we investigated the effect of growth factors on α10-expression we found that bFGF up-regulates while TGF-β down-regulates the expression in human chondrocytes. A chondrogenic role for α10 was also shown by transfecting the chondrocytic cell-line C-28/I2 with α10 which resulted in elevated expression of COL2A and aggrecan and reduced expression of COL1A, ITGA11 and MMP-13 after differentiation in pellet mass.

We also found that human MSCs express integrin α10 and that bFGF up-regulates the expression. Furthermore, after chondrogenic differentiation in pellet cultures, bFGF pre-treated cells, with high levels of α10 have an increased mRNA expression of COL2A, aggrecan and SOX9, compared to untreated cells. By analyzing the Col II pro-peptide, we could demonstrate that only the bFGF pre-treated MSCs synthesize and process collagen type II. The bFGF pre-treated MSCs also have increased proteoglycan synthesis compared to untreated cells.

DISCUSSION & CONCLUSIONS: We conclude that high expression of α10β1 on chondrocytes correlates with high expression COL2A and aggrecan and low expression of COL1A. We also conclude that MSCs cultured under conditions that favours expression of α10, has an enhanced capacity to synthesize cartilage-specific molecules. Taken together our results strongly suggest that integrin α10 is a unique cell surface marker for chondrocytes and MSCs with chondrogenic potential.

Our finding that over-expression of α10β1 in a chondrocytic cell-line resulted in increased expression of cartilage-specific markers suggests that α10β1 play an important role in modulating the homeostasis of cartilage.

Stages of murine articular cartilage development.

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INTRODUCTION: The structure of adult articular cartilage (AC) is intimately related to its function as a load bearing tissue. Determining the sequential development of this complex tissue will enhance the understanding of factors that may influence AC formation and expose possible mechanisms that may be used to aid tissue repair. Studying mouse tissue enabled a comprehensive examination of each stage of development due to the relatively condensed period of maturation.

METHODS: Mouse tibiae were dissected from pups aged 1-25 days old, raised either in natural litters or litters culled to 3 or 10 pups. The tibial plateaux were kept intact and fixed in formaldehyde in 0.1M Piperazine_1, 4- bis-2-ethanesulfopnic acid (PIPES) buffer (pH 8.5) for 24 hours, dehydrated in an ascending ethanol series, transferred to xylene, embedded in paraffin wax, sectioned at a thickness of 7.5µm and stained in picrosirius red. Sections were imaged using light and polarised light microscopy. Additional mouse tibiae were plunge frozen in propane cooled by liquid nitrogen, freeze substituted for 5 days in 58% acetone, 30% methanol, 10% acrolein an 2% tannic acid and a further 5 days in 100% acetone. Samples were then critical point dried, fractured and coated in platinum/palladium (80/20) for examination by scanning electron microscopy.

RESULTS: Several developmental stages were determined. At birth there was no birefringent collagen matrix or clear chondrocyte orientation. Subsequent development was characterised by an alignment of chondrocytes and collagen matrix parallel to the articular surface. This was apparent in tissue from mice up to 6 days old. An area lacking birefringence, containing chondrocytes obliquely orientated with respect to the articular surface, was apparent in the load-bearing regions of cartilage from mice 7 days and older. Progressive development of this region of reorganisation (ROR) was observed in the increasingly determinate appearance of collagen fibres and columns of chondrocytes aligned perpendicular to the articular surface. Extension of the ROR from the load-bearing region to cover the condyles was observed, finally resembling adult AC. Development of AC correlated with the initiation of weight bearing on the hindlimbs and anatomical maturation of the pups. Additionally, the rate of AC development was retarded in pups raised in large litters, which also affected the appearance of the tissue in skeletally mature adults.

DISCUSSION & CONCLUSIONS: The development of mouse AC appeared to be related to that of other species. Similar stages of development are observed in bovine tissue, and clearly affect the biomechanical properties of the cartilage. The formation of the ROR and realignment of the tissue to a structure that resembled adult AC occurred at the same time as the formation of the secondary centre of ossification. The subsequent expansion of the tissue to adult depths, which is observed in some species, appears to occur by a mechanism of appositional growth but was not observed in mouse tissue.


ACKNOWLEDGEMENTS: This work was funded by Smith and Nephew Research, York.
Indian hedgehog signaling in the embryonic growth plate

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INTRODUCTION: Endochondral ossification is a multistep process during which a cartilage template is successively replaced by bone tissue. Chondrocytes in the cartilage anlagen undergo several steps of differentiation until they become terminal hypertrophic and are subsequently replaced by bone. The secreted growth factor Indian hedgehog (Ihh) is expressed in a distinct population of chondrocytes that undergo hypertrophic differentiation. Ihh interacts with a second secreted molecule, Parathyroid Hormone related Protein (PTHrP), expressed in the distal ends of the cartilage elements in a negative feedback mechanism to regulate the onset of hypertrophic differentiation.

RESULTS & DISCUSSION: Analyzing a mouse line carrying a hypomorphic allele of Ext1, a glycosytransferase necessary for the synthesis of heparan sulfates (HS), we have recently shown that HS negatively regulates the propagation of the Ihh signal in a concentration dependent manner. Our data strongly indicate that Ihh acts as a long range morphogen directly inducing the expression of PTHrP.

To further investigate the interaction between Ihh and PTHrP, we have started to analyze the role of the zinc finger transcription factor Gli3, which acts downstream of hedgehog signals in other organs. Ihh;Gli3 double mutants indicate that Gli3 acts as a repressor downstream of Ihh in regulating chondrocyte proliferation and the expression of PTHrP, and, thus, the onset of hypertrophic differentiation. Furthermore, our studies revealed that Gli3 negatively regulates the differentiation of distal, low proliferating (zoneI) into central, high proliferating (zone II) chondrocytes. Whereas the domain of zone II chondrocytes is determined by the level of PTHrP, the transition of zone I into zone II chondrocytes is regulated by Gli3R independent of PTHrP. We have thus identified a new function for the Ihh/Gli3 system in regulating the differentiation of distal chondrocytes.
C-TYPE NATRIURETIC PEPTIDE REGULATES ENDOCHONDRAL BONE GROWTH IN A P38 MAP KINASE-DEPENDENT MANNER

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INTRODUCTION: Endochondral ossification, the process responsible for long bone formation, begins with the condensation of mesenchymal cells that is followed by successive stages of chondrocyte proliferation, differentiation, and hypertrophy. Studies in our lab and others have shown that C-type Natriuretic Peptide (CNP) and its effector cGMP are important regulators of chondrocyte proliferation and hypertrophy. In addition, p38 MAPK signaling has been shown to be important for cartilage development. The aim of this study is to investigate the mechanisms underlying the anabolic regulatory effects of CNP during endochondral bone growth and its potential interaction with p38 MAPK signaling.

METHODS: Tibiae from E15.5 day old embryonic mice were isolated and cultured in minimal media in the presence of control BSA/HCl (1mM)/DMSO, CNP (10^-6M), the p38-specific pharmacological inhibitor SB202190 (10µM), or SB202190 and CNP. Bone growth was determined by tibiae measurements on days 3, 6, and 8. The weights of six pooled bones were determined on day 6, along with Alcian Blue and Alizarin Red staining and histological analysis. We wanted to identify target genes of CNP in chondrocytes; therefore, on the sixth day of treatment cultured tibiae were micro-dissected into the resting/proliferating, hypertrophic, and mineralized areas. Distinct zones from approximately 24 bones were pooled together and RNA was isolated using the Qiagen RNeasy® Lipid Tissue Extraction Kit. Once the quality of total RNA from three independent trials was determined using the Agilent 2100 Bioanalyzer, microarray analyses were performed at the London Regional Genomics Centre using MOE430_2.0 Affymetrix arrays. Results were analyzed using GeneSpring 7.2 software.

RESULTS & DISCUSSION: We demonstrate that treatment of mouse tibiae with CNP significantly increases tibia growth, mainly through an enlarged hypertrophic zone of the growth plate. We show that inhibition of p38 activity with the pharmacological inhibitor SB202190 reduces both the bone growth and weight increases observed with CNP treatment. Bioinformatics analyses indicate the hypertrophic zone to be the major site of gene expression change after CNP treatment, with 309 probe sets undergoing at least two-fold change in expression in response to CNP. Our data also reveal that the genes encoding cGMP-dependent kinases I and II, which are major transducers of CNP signaling, are most strongly expressed in the hypertrophic zone, providing a potential explanation for the specific effects of CNP in this area. Microarray results were validated using real-time analyses for selected genes. For example, it was confirmed that expression of Ptgs2, the gene encoding cyclooxygenase-2 (Cox2), and Npr3, the gene encoding the clearance receptor for natriuretic peptides, are strongly up-regulated by CNP in the hypertrophic zone. However, the CNP-induced increase in Ptgs2 and Npr3 expression occurs through a largely p38-independent manner.

CONCLUSIONS: In summary, our data show that CNP stimulates endochondral bone growth through stimulation of chondrocyte hypertrophy through p38-dependent and –independent pathways. Further exploration of these pathways will increase our understanding of normal skeletal development and of potential therapeutic applications of CNP’s anabolic properties.

ACKNOWLEDGEMENTS: Work in the Beier laboratory is supported by the Canadian Institutes of Health Research (CIHR), The Canadian Arthritis Network, The Arthritis Society and the Natural Sciences and Engineering Research Council. H. A. is a recipient of an Ontario Graduate Scholarship, S. K. received a graduate scholarship from the Canadian Arthritis Network, and C. J. is a recipient of a CIHR Doctoral Award.
The PI3K/Akt pathway regulates endochondral bone growth

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INTRODUCTION: Most of our bones develop through the process of endochondral ossification which involves successive steps of chondrogenesis, chondrocyte proliferation, and hypertrophic differentiation [1]. A large number of growth factors and hormones have been implicated in the regulation of growth plate biology; however, not much is known about the signaling pathways involved [2]. PI3K has been identified as a major regulator of cellular proliferation, differentiation and death in multiple cell types, acting partially through the downstream kinase Akt1 [3].

We hypothesized that the PI3K/Akt pathway promotes endochondral bone growth by increasing proliferation and differentiation of chondrocytes and/or by suppressing apoptosis.

METHODS: Employing an organ culture system of embryonic mouse tibias and LY294002, a pharmacological inhibitor of PI3K we determined the length of different zones of the growth plate in H&E stained tibias, the number of BrdU labeled, p57 and cyclin D1 expressing cells. We performed similar experiments using long bones from Akt1 KO and control mice. Using micro CT analysis, skeletons of Akt1 KO and control mice were compared. Primary chondrocytes were isolated from long bones of E15.5 mouse embryos, cultured in the presence or absence LY294002 and analyzed by TUNEL staining for apoptosis and by cell counts.

RESULTS: PI3K inhibition results in smaller proliferative and hypertrophic zones of the growth plate, reduced number of BrdU labeled cells, reduced number of p57 stained cells and decreased cell numbers in primary chondrocyte cultures, indicating that this pathway is required for maximal proliferation and differentiation. TUNEL staining of primary chondrocytes also showed an increased number of apoptotic cells in LY294002 treated cultures compared to the control.

From birth until adulthood, Akt1 KO mice are smaller than their littermates [4]. Investigating the skeletal phenotype of Akt1 null mice, we have shown that most of their long bones are 10% shorter than in their wild-type littermates and show delayed growth plate development (as shown by micro-CT) as well as delayed formation of secondary ossification centers. Similar to LY29400-treated organ cultures, growth plates from Akt1-deficient mice display reduced chondrocyte proliferation.

DISCUSSION & CONCLUSIONS: Our results in organ culture system and primary cell cultures demonstrate that PI3K is required for normal chondrocyte proliferation and survival as well as endochondral bone growth in vitro. Targeted disruption of Akt1 gene results in reduced length of long bones decreased chondrocyte proliferation and delayed secondary ossification, demonstrating that this pathway is necessary for normal endochondral bone growth in vivo.


ACKNOWLEDGEMENTS: We thank Dr. Morris J. Birnbaum (University of Pennsylvania) for providing us the Akt1 deficient mice and Dr David Holdsworth for his help with microCT analysis.
Lack of Endochondral Ossification in Site-1 Protease Knockout Mice

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INTRODUCTION: Site-1 protease (S1P) is a member of the family of proprotein convertases and plays a role in the processing of membrane-bound transcription factors such as the sterol regulatory element-binding proteins (SREBPs) which is involved in cholesterol homeostasis. Defects in cholesterol metabolism during embryogenesis can affect growth plate function and can lead to skeletal abnormalities like Smith-Lemli-Opitz syndrome. The importance of S1P in cartilage development was demonstrated in literature by the study of the zebrafish gonzo mutant with knockdown in S1P and its subsequent effect on cartilage development [1].

METHODS: In order to follow these observations further we have created a cartilage-specific S1P knockout mouse (S1Pcko) to understand its role in cartilage development and the determination of cell fate. Stages of chondrogenesis were defined by expression of specific extracellular matrix and growth regulatory molecules.

RESULTS: S1Pcko mice die shortly after birth and have a severe chondrodysplasia with abnormally short and deformed limbs as compared to wild type mice (Fig. 1). Histological studies of forelimbs and hindlimbs show that these mice have an abnormal growth plate lacking the structured organization of chondrocytes that is typical of a normal growth plate, even though BrdU indices indicate that they proliferate normally and exit the cell cycle in a manner similar to wild type. Furthermore, endochondral ossification is totally lacking in S1Pcko mice and the diaphysis remains completely cartilaginous, even though mineralization of the hypertrophic cartilage, expression of Collagen type X, and VEGF seem to occur normally (Fig. 2). Lack of endochondral ossification is compensated by exuberant growth of the bone collar. The defect appears to lie in the inability of blood vessels, and subsequently the osteoclasts and endothelial cells to invade the mineralized matrix in the S1Pcko mice and deposit bone.

DISCUSSION and CONCLUSIONS: This is the first enzyme defect shown to yield a severe chondrodysplasia. The exact substrate of S1P with respect to cartilage development is not known as yet, but activation of SREBPs by S1P is known to coordinate expression of key enzymes of the cholesterol and fatty acid biosynthesis. Besides SREBPs, S1P is also known to process ATF6 which is involved in the ER stress response. Our studies in mice will help elucidate the importance of these regulatory pathways in cartilage and bone formation.

REFERENCES

ACKNOWLEDGEMENTS: NIH/NIAMS AR050847
Chondrocyte secreted CRTAC1: an extracellular matrix molecule of human articular cartilage facilitates cell adhesion.

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INTRODUCTION: Cartilage acidic protein 1 (CRTAC1) is a novel human marker which allowed discrimination of human chondrocytes from osteoblasts and mesenchymal stem cells in culture. It was so far studied only on the RNA-level [1-3]. Based on sequence analyses the following protein domains were predicted: a signal peptide for secretion, four FG-GAP repeat domains, one RGD integrin binding motif, and an EGF-like Calcium-binding domain. Two splice variants (CRTAC1-A and CRTAC1-B) resulting from alternate last exon usage were detected in human. The aim of this study was to characterize the expression and localization of CRTAC1 protein and to test our hypothesis that CRTAC1 represents a so far unrecognized extracellular matrix molecule of cartilage.

METHODS: Two different polyclonal CRTAC1 antisera were generated by immunising rabbits with two distinct CRTAC1 specific peptide epitopes. These antisera were used for Western blot analysis and for immunohistochemical staining of cartilage-bone-sections. Human chondrocytes were obtained by enzymatic digestion of cartilage slices, cultured in monolayer and cellular lysates as well as medium supernatants were subjected to Western blotting. Functional cell adhesion experiments were performed with a Multiple Substrate Array (MSA™) containing either different matrix molecules or different quantities of type II collagen. Adhesion assays were performed with SaOS cell lines stably transfected with CRTAC1-A, CRTAC1-B or the control vector. In addition CRTAC1 conditioned medium free medium was analyzed on its ability to influence cell adhesion on different substrates.

RESULTS: Western blot analyses showed CRTAC1 reactive bands in supernatants of cultured chondrocytes indicating an effective secretion of the molecule. In addition, polyclonal CRTAC1 antisera stained the extracellular matrix of deep zone cartilage close to the subchondral bone, a region where calcified cartilage matrix is located. Interestingly, consistent with this localization, some consensus domains of the molecule (repeat 3 and 4 of the FG-GAP domains and the EGF-like domain) were predicted to bind calcium, and the acidic nature of the molecule predisposes it for divalent cation binding. Of five putative O-glycosylation motifs in the last exon of splice variant CRTAC1-A, the most C-terminal one was modified according to exposure of serial C-terminal deletion mutants to the O-glycosylation inhibitor Benzyl-α-GalNAc. A functional assay on a Multiple Substrate Array showed that CRTAC1 supported cell adhesion of recombinant cells to several different matrix compounds. Its capacity to enhance cell-matrix interaction to type II collagen, the most prominent extracellular matrix compound of articular cartilage, was significant from 12.6 to 270 µg/ml of spotted substrate. In addition CRTAC1 conditioned medium enhanced adhesion of human cells to extracellular matrix molecules like fibronectin, vitronectin and type VI collagen.

DISCUSSION & CONCLUSIONS: In summary, human CRTAC1-A represents a chondrocyte-secreted new extracellular matrix molecule of deep zone articular cartilage that is glycosylated at Thr-626 with function in cell-matrix interaction.


ACKNOWLEDGEMENTS: This work was supported by a grant of the Deutsche Forschungsgemeinschaft (STE1131/1-1) and by a grant of the research fund of the Stiftung Orthopädische Universitätsklinik Heidelberg (F03.0030). The authors thank Stephanie Kadel, Katrin Götzke and Regina Föhr for excellent technical assistance.
Cartilage ECM molecules with roles in tissue homeostasis
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INTRODUCTION: The cartilage extracellular matrix contains collagen fibers contributing tensile properties and large proteoglycans, aggrecan, which contributes resilience and resistance to deformation. There are two networks of collagen, schematically illustrated in the figure, i.e. one containing a backbone of collagen VI forming beaded filaments. This is located to the territorial matrix in proximity to the cells. This network contains a number of non-collagenous proteins having roles in regulating its formation and providing networking to other tissue macromolecular assemblies.

The other major fibrillar network contains collagen II as the principle constituent with a minor component being collagen XI representing a few percent of the total collagen in the fiber.

The formation and organization of the networks depend on interactions of a number of molecules secreted by the cells.

METHODS: Interactions between matrix molecules were studied by solid phase assay, by plasmon resonance and by negative staining electron microscopy of molecular complexes. The effects of isolated molecules on the assembly of the major tissue macromolecular networks was monitored by light scattering and electron microscopic methods. Degradation of tissue macromolecules was studied by Western blots and by mass spectrometry.

RESULTS AND DISCUSSION: The many molecules of the family of leucine rich proteins bind to collagen and affects collagen fibril formation. Two such molecules are the small leucine rich proteoglycans, decorin and particularly biglycan, which regulate the assembly of the collagen VI secreted tetramer into a network and provide a linker from the completed network to other matrix structures. The proteoglycan thus binds at the same time to collagen VI and to a member of the matrilin family, a set of molecules with 3-4 usually identical subunits. These molecules in turn bind to other networks including those with aggrecan or collagen type II as major constituents as is schematically illustrated in the figure.

Additional proteins like COMP affect collagen fibrillogenesis, in this case by catalyzing formation of the collagen type II fibers.

In pathology, the molecules bound at the collagen fibers are degraded before the fibers themselves. This process involves proteinases not normally involved in tissue turnover and apparently specific to the pathological process. The process removes a polyanionic part of the molecule limiting its interactions.

All hyaline cartilages are characterized by their high content of the proteoglycan aggrecan, which exists in the form of proteoglycan aggregates in association with hyaluronan (HA) and link protein (LP). The proteoglycan aggregates provide the osmotic properties needed for tissue turgidity. Cartilages also contain a variety of small leucine-rich repeat proteoglycans (SLRPs), including decorin, fibromodulin and lumican, which help maintain the integrity of the tissue and modulate its metabolism.

Aggrecan is a modular proteoglycan with multiple functional domains. The amino terminal G1 domain is responsible for the interaction of aggrecan with HA, whereas the adjacent structurally related G2 domain presently has no known function. The carboxy terminal G3 domain is essential for normal posttranslational processing of the aggrecan core protein and subsequent aggrecan secretion. The intervening keratan sulfate (KS) and chondroitin sulfate (CS)-attachment domains provide the high anionic charge density needed for the unique osmotic properties of aggrecan. In the human, the first of the two CS-attachment domains (CS1) exhibits size polymorphism due to a variable number of 19 amino acid repeats. This results in different individuals possessing aggrecan core proteins of different length and different CS content. Those individuals with the shortest core protein length and lowest CS substitution may possess functionally inferior aggrecan and be at risk for cartilage degeneration.

Link proteins are structurally related to the G1 domain of aggrecan and also possess the ability to interact with HA. Mammals possess four link protein genes of which one is predominantly expressed in cartilage. The cartilage LP serves several functions in the proteoglycan aggregate. First, by virtue of its ability to interact with both HA and the G1 domain of aggrecan it stabilizes the proteoglycan aggregate and prevents its dissociation under physiological conditions. Second, it participates in a phenomenon termed delayed aggregation, in which it interacts with newly secreted aggrecan and promotes subsequent interaction with HA. Third, together with the G1 domain of aggrecan, LP forms a protein coat covering the surface of HA, which helps protect the HA from undesirable degradation by both hyaluronidases and free radicals.

HA is synthesized at the plasma membrane of the cell via a hyaluronan synthase (HAS). Mammals possess three HAS genes, with HAS2 expression predominating in cartilage. HA is present as a coat surrounding all chondrocytes and it is likely that proteoglycan aggregate formation occurs in this pericellular location. The mechanism whereby the proteoglycan aggregates are released from this environment and move to the more remote parts of the extracellular matrix is not clear. Studies on knockout mice have demonstrated that the absence of HAS2 in cartilage is catastrophic; resulting in a severely abnormal skeleton and perinatal death, confirming the essential role of the HA and proteoglycan aggregates in normal cartilage function.

SLRPs are characterized by multiple adjacent domains bearing a common leucine-rich motif. In the case of decorin, fibromodulin and lumican there are ten leucine-rich repeats. Decorin possesses a CS chain in the amino terminal region of its core protein, whereas fibromodulin and lumican possess KS chains within their central leucine-rich repeat region. The CS and KS can interact with a variety of growth factors and provide a sink for growth factor accumulation within the extracellular matrix, so modulating chondrocyte metabolism. The core proteins allow the SLRPs to interact with the collagen fibrils, and in so doing they help regulate fibril diameter and fibril-fibril interaction. They also limit the access of the collagenases to their unique cleavage site on each collagen molecule, and help protect the fibrils from proteolytic damage.

All cartilage proteoglycans are essential for normal tissue function, and perturbation in their abundance or structure can have dire consequences.

ACKNOWLEDGEMENTS: The author wishes to thank the Shriners of North America and the Canadian Institutes of Health Research for financial support.
Chondrocyte Gene Expression in A Rat Model Of Osteoarthritis

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INTRODUCTION: Osteoarthritis (OA) is a complex degenerative disease that results in architectural changes to both the articular cartilage and subchondral bone of synovial joints. A common characteristic of OA is cartilage degradation. Studies have shown that much of the degradation is due to the misexpression of proteases, cytokines, chemokines, growth factors, and other secreted factors1,2. Accordingly, we hypothesized that altered expression of growth factors in articular cartilage causes cartilage degradation in OA.

METHODS: Total RNA was harvested directly from the articular knee cartilage of rats that had undergone anterior cruciate ligament transection (ACL-T) and partial medial meniscectomy (PM) (to induce OA), or Sham surgery (control), followed by 28 days of 30-minute forced mobilization sessions 3 times per week on a rota-rod apparatus. Affymetrix microarrays (RAE230_2.0 GeneChips®) were used to assess changes in articular chondrocyte gene expression due to OA stimuli (n = 5). Data were subsequently categorized using Gene Ontology classifications. The expression patterns of genes known to play a role in OA pathogenesis (e.g. Mmp13)2 and genes novel to the study of OA were also confirmed using real-time PCR. Functional studies are being carried out with identified growth factors to determine their effects on primary chondrocyte gene expression, morphology, and phenotype.

RESULTS: After data normalization and statistical analysis, 1,619 gene probe sets showed at least 1.5-fold changes in expression between operated knees and sham controls. Several growth factors were among these, and many of the regulated genes are implicated in metabolism, cell signal transduction, cell differentiation, and transcriptional regulation as indicated by GeneOntology annotations (Figure 1). Consistent changes in gene expression were also observed in the contralateral knee. Novel factors identified included endothelin type A receptor (Ednra), kit-ligand (Kitl), and several members of the cathepsin family of proteases including cathepsin S (Ctss). Further, groups of related genes were also dynamically regulated including many ECM genes, members of the TGF-β superfamily and the insulin-like growth factor (IFG) axis.

Fig. 1: Distribution of genes across metabolic (52%), cell communication (25%), cell differentiation (12%), and transcription (11%) functional classifications.

DISCUSSION & CONCLUSIONS: Overall, these results implicate dysregulated expression of growth factors (and other types of genes) in cartilage degradation and support the contention that multifactorial influences contribute to OA pathogenesis. Several avenues of research into the causes and potential therapeutic targets of OA will stem from this exciting pool of candidate genes.


ACKNOWLEDGEMENTS: This project was funded by the Canadian Institutes of Health Research and the Canadian Arthritis Network.
Role of Osteogenic Protein-1 in the Regulation of Catabolic and Anabolic Pathways

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INTRODUCTION: Osteogenic Protein-1 (OP-1) is one of the most potent growth factors for cartilage repair due to its pro-anabolic and anti-catabolic activities [1,2]. The goal of the current project was to evaluate the role of OP-1 in human articular cartilage homeostasis by gene array approach under conditions of lack (inhibition of endogenous OP-1 expression by antisense oligos; [3]) or excess of OP-1 (stimulation with recombinant OP-1 [rhOP-1]). This report is limited to the following selected genes: IGF-1 and the members of its pathway (anabolic) and chemokines and cytokines, interleukin-6 (IL-6), IL-8, IL-11, and leukemia inhibitory factor (LIF).

METHODS: Human normal adult articular cartilage was obtained from the ankle joints of 12 tissue donors, age 55-70 y/o. After enzymatic digestion chondrocytes were plated at high-density monolayers and either transfected with OP-1 antisense oligos in the presence of lipofectin [3] or treated with rhOP-1 for 48 hours followed by RNA isolation. Gene expression profile was analyzed by HG-U133A gene chips from Affimetrix. RNA within each treatment group was pooled from all donors. Statistical analysis consisted of 1) analysis of differentially expressed genes under single experimental condition in comparison to control (up- or down-regulated in the presence of OP-1 antisense or rhOP-1); 2) analysis of differentially regulated genes by both treatments (OP-1 antisense and rhOP-1); and 3) gene ontology. Selected gene array data were verified in vitro by real-time PCR.

RESULTS: Gene array results showed that the inhibition of endogenous OP-1 led to down-regulation of a number of anabolic genes among which were the members of the IGF-1 pathway: IGF-1 receptor (1.73-fold decrease), IGFBP-5 (1.9 fold) and IGFBP-7 (1.58-fold, P<0.01). In vitro validation experiments confirmed and extended these findings: in chondrocytes treated with OP-1 antisense expression of IGF-1 and its receptor was down-regulated, while stimulation with rhOP-1 up-regulated their expressions. This in turn restored the response to IGF-1 that is reduced with aging of human cartilage [4]. Among catabolic genes the most highly regulated by OP-1 were LIF, IL-6, IL-8, IL-11, and NF-kB. Inhibition of OP-1 led to about 2-fold stimulation in their expression (p<0.001), while treatment with rhOP-1 dramatically inhibited these genes. LIF was down-regulated by 6.2-fold; IL-6 by 2-fold; IL-8 by 2.6-fold; and IL-11 by 4.7-fold (p<0.001). These changes were confirmed by real-time PCR with corresponding primers. Additional experiments showed that OP-1 was not only able to inhibit gene expression of autocrine catabolic mediators, but also counteracted their inhibitory effect on proteoglycan synthesis.

DISCUSSION & CONCLUSIONS: The initial analysis of gene array data strongly suggests a critical role of endogenous and exogenous OP-1 in cartilage homeostasis. OP-1 regulates numerous metabolic pathways that are not only limited to its anabolic function, but also to its anti-catabolic activity. Understanding of OP-1 function in cartilage will provide strong justification for the application of OP-1 protein as therapeutic treatment for cartilage regeneration and repair.


ACKNOWLEDGEMENTS: The authors would like to acknowledge the Gift of Hope Organ & Tissue Donor Network and donors’ families. The work was supported by the NIH grant AR 47654 and Stryker Biotech grant SC-001.
ARTICULAR CARTILAGE COLLAGEN: AN IRREPLACEABLE FRAMEWORK?

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INTRODUCTION: Collagen provides the tensile fabric and two-thirds of the dry weight of mature articular cartilage [1]. The collagen is highly cross-linked and its turnover is at best extremely slow and the articular chondrocytes appear incapable of repairing any overt damage. It is still unclear, however, to what extent molecular remodeling, fibril growth and repair or other metabolic changes to the collagen framework are essential features of joint cartilage biology and functional longevity.

METHODS: By dissecting the collagen fabric proteolytically, determining the structure of cross-linked peptides and identifying the sites of interaction of the various gene products, steps in the molecular assembly and inherent heteropolymeric nature and tissue-specific differences in the collagen polymer can be defined. Using the latest methods in protein mass spectrometry, this type of work has been greatly facilitated and is allowing us to define in more detail the post-translational phenotype that characterizes articular cartilage collagen.

RESULTS: The basic polymeric fabric begins as a cross-linked copolymer of types II, IX and XI collagens [2]. Other types of collagen including collagens VI, XII and XIV are present in the pericellular space (type VI) or associated with fibril surfaces (types XII and XIV), but these molecules are extractable by protein denaturants not part of the covalently bonded polymer [1]. The placement of covalent intermolecular bonds between collagens II, IX and XI suggests a hierarchical assembly, with a collagen XI filament providing a scaffold for collagen II and collagen IX covalently bonding to the surface of the nascent fibril network [3,4]. As the tissue matures, much of the collagen IX disappears, presumably by proteolysis, to allow the collagen II fibrils to grow laterally but the mechanism, whether by fusion of thin fibrils or incremental addition of more collagen monomers, is unclear. With maturity, collagen III appears as a significant component of adult articular cartilage. Our recent findings reveal that collagen III molecules are extensively cross-linked to type II collagen, primarily in the form of pN-type III molecules attached to the surface of collagen II fibrils (Fig. 1). This appears to be an addition imposed metabolically on the existing collagenous matrix by the articular chondrocytes, and can be considered a form of matrix repair response.

DISCUSSION & CONCLUSIONS: In summary, as we continue to dissect the molecular details of collagen hierarchical assembly, cross-linking and chain isoform usage, for example in the collagen V/XI subfamily, it becomes clear that articular cartilage collagen has acquired distinctive molecular properties that presumably have evolved with the tissue’s adaption for a load-bearing function and longevity. The results also indicate that new monomers (in this case collagen III) can become covalently added to the surface of an existing fibrillar network (collagen II). This has implications for collagen fibril growth and remodeling in tissues in general.

A Model of Synovial Fluid Lubricant Composition in Normal and Inflamed Joints

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INTRODUCTION: The synovial fluid (SF) of human joints functions as a biological lubricant, providing low-friction, low-wear properties through the putative contributions of proteoglycan 4 (PRG4) and hyaluronic acid (HA). These lubricants are secreted by articular chondrocytes (AC) in cartilage and synovial cells (SC) in synovium, and concentrated in the synovial space by the semi-permeable synovial lining (Fig 1). Several chemical and mechanical regulatory mechanisms exist to modulate secretion of lubricants by the cells into SF. The objective of this study was to develop a mathematical model of lubricant composition in SF, considering the joint as a system of specific cell types, a semi-permeable fluid compartment, and regulatory factors.

METHODS: The joint was modeled as SF and interstitial fluid (IF) compartments. The rate of change in concentration of lubricant $i$ ($c_i$) in SF is assumed to depend on the rate of secretion of $i$ ($r_i$) by AC and SC, the permeability ($p_i$) of the membrane to $i$ and the concentration gradient between the SF and IF compartments. Secretion rates depend on the chemical and mechanical regulatory factors in the system. Model variables and membrane characteristics are outlined in Fig 2.

RESULTS: The form of the solution for $c_{iSF}$ at steady state conditions is shown in Equation 1.

$$c_{iSF} = \frac{r_{iAC}^SC + r_{iSC}^AC}{p_i A} + c_{iIF}$$  \hspace{1cm} (1)

As $p_i$ and $A$ increase, $c_i$ decreases, while increases in $r_i$ cause increases in $c_i$. The ranges of model predictions for $c_{iHA}^{SF}$ and $c_{iPRG4}^{SF}$ with and without chemical regulatory factors present are shown in Fig 3.

DISCUSSION & CONCLUSIONS: Model predictions are within the observed physiological range. Enhancements to the model could include interactions between different compartments, such as binding of lubricants to tissues, and mechanical regulation on model parameters.


ACKNOWLEDGMENTS: NIH, NSF, UC BREP GREAT training grant (MEB).
Effects of relative sliding stimulus on the friction properties of regenerated cartilage

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INTRODUCTION: Objective of this study was to evaluate the effect of mechanical stimuli on properties of regenerated cartilage tissue and to develop a culture system for cartilage regeneration. A simple magnetic stirring system to culture a tissue under sliding condition and Fibroin sponge were used to form cartilage tissue in vitro, and the tribological function was measured.

METHODS:
[Cell culture] Chondrocytes were harvested from humerus, femur and tibia of 4-weeks-old Japanese white rabbits, and were inoculated into the fibroin sponge at a cell concentration of 1x10^7 cells/ml. Fibroin sponge (diameter: 8 mm, thickness: 1±0.2 mm) made of fibroin hydro gel was used as a scaffold for cartilage regeneration.

[Magnetic stirring culture] Magnetic stirring system is consisted of a discoidal magnetic stirrer manufactured by Teflon and a glass petri dish. Each specimen was placed into the holes that were made along the circumference to discoidal magnetic stirrer. The surface of a specimen was set to confront the bottom of the petri dish. The specimens were cultivated under the mechanical stimuli by setting stirring frequency 5 rpm (stirring group). Some specimens were cultivated in same system without mechanical stimuli (control).

[Evaluation] Frictional properties of the regenerated cartilage were evaluated by using a reciprocating friction-testing apparatus. Frictional experiments were performed at 7 and 14 days after the seeding (sliding velocity: 0.8 mm/s, loading time prior to sliding: 1 minute, applied load: 0.029N). The counterface for regenerated cartilage was a flat stainless steel (Ra=0.06µm). All experiments were performed in the saline solution and at room temperature (25°C). Specimens before and after the frictional tests were evaluated by histological observations using safranin-O staining and collagen type II immunostaining.

RESULTS: The friction coefficient of regenerated cartilage against stainless steel tended to fall with cultivation period and to increase with sliding distance. However, that at 14 days cultivation in stirring group showed no increasing with sliding distance (Fig.1). The thickness of tissues at 14 days in stirring group was almost as three times as that in control group (Fig.2).

DISCUSSION & CONCLUSIONS: ECM formation and frictional function of the regenerated cartilage were improved by the magnetic stirring system. Our previous work suggested that the durability of low-friction performance of the regenerated cartilage was related to the collagen-proteoglycan network on the surface [1], and this result implies that the mechanical stimuli with magnetic stirring affects setting to the collagen-proteoglycan network on the surface.

Regulation of Lubricin Expression and Metabolism in Synovial Joints

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INTRODUCTION: Lubricin, also denoted as superficial zone protein (SZP) and proteoglycan 4 (PRG4), is a ~345 kDa mucinous sialoglycoprotein that is present on the surface of articular cartilage and other joint tissues such as synovium, tendon and meniscus. Functionally, lubricin contributes to the critical boundary lubrication mechanisms that enable remarkably low friction levels between interfacing surfaces of joint tissues. Genetic disruption of lubricin synthesis leads to joint pathology and dysfunction, and lubricin dysregulation has been linked to early stages of cartilage pathology. The aim of this study was to examine the effect of a variety of arthritis-related cytokines on lubricin mRNA and protein expression by bovine cartilage, and to examine the stability of exogenous lubricin in synovial fluid (SF) aspirates from patients with osteoarthritis (OA).

METHODS: Calf articular cartilage explants (discs harvested from full-depth cores) were cultured ex vivo in the absence or presence of various cytoregulatory factors. These included IL-1β, TGF-β, TNF-α, retinoic acid (RA), oncostatin M (OSM), BMP-2, IL-6 and LIF. Analysis of cartilage-associated lubricin removed under non-denaturing conditions and soluble lubricin levels in conditioned media was performed by Western blotting with monoclonal antibody (MAb) 6-A-1, raised against bovine lubricin. For quantitative RT-PCR analysis, RNA was extracted from bovine chondrocyte monolayer cultures maintained in a similar fashion. Full-length recombinant human lubricin expressed in CHO cells was purified under non-denaturing conditions and soluble lubricin levels in conditioned media was performed by Western blotting with monoclonal antibody (MAb) 6-A-1, raised against bovine lubricin. For quantitative RT-PCR analysis, RNA was extracted from bovine chondrocyte monolayer cultures maintained in a similar fashion. Full-length recombinant human lubricin expressed in CHO cells was purified and labeled by biotinylation of sialic acid residues following periodate oxidation. Biotinylated lubricin (bLub) was added to OA SFs (New England Baptist Hospital, Boston, MA) or PBS, ± proteinase inhibitors to a final concentration of 50µg/ml and incubated for 24 hours at 37°C. Samples were then treated with chondroitinase ABC for 1h, and after SDS-PAGE separation bLub was visualized by affinity blotting with streptavidin-HRP.

RESULTS: Stimulation of calf articular cartilage explant cultures with IL-1β, TNF-α, IL-6, or retinoic acid for 48h resulted in a reduction in the levels of cartilage-associated and soluble lubricin. Anabolic growth factors such as BMP-2 and TGF-β increased levels of lubricin, as did OSM. Preliminary qPCR data indicates that these changes in protein levels are effected at the mRNA level, whereas IL-1 conditioned media does not appear to degrade exogenously added recombinant lubricin. Incubation of bLub with OA SFs, however, resulted in limited proteolysis of lubricin. Incubation of the proteinase inhibitors PMSF, 6-AHA or benzamidine-HCl did not prevent the degradation of lubricin, but inclusion of EDTA in the incubation mixtures prevented bLub fragmentation, suggesting metalloproteinase-mediated catabolism.

DISCUSSION & CONCLUSIONS: Lubricin at the boundaries of joint surfaces forms an integral part of the synovial ‘tribosome’, a term proposed for the macromolecular components that provide cartilage (and other interfacing tissues) with low-friction (wear-minimizing) attributes. The diminution of lubricin levels mediated by a number of proinflammatory cytokines, as observed in this study, could result in increased friction levels and decreased resistance of the articular surface to cellular adhesion. In addition, catabolic factors (i.e. proteases) expressed in pathological joints may also disrupt joint proficiency by exacerbating lubricin dysfunction.


ACKNOWLEDGMENTS: MAb 6-A-1 was generously provided by Prof. B. Caterson and Dr. C.E. Hughes, Cardiff University, UK.
Experimental and Finite Element Analysis of Strain Fields in Chondrocytes and the Extracellular Matrix of Cartilage Under Physiological Loading

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INTRODUCTION: The goal of this study was to investigate the deformation behavior of the chondrocyte and its microenvironment under transient loading, in order to address the relationship between the applied dynamic deformation and cellular strain. In-situ strain measurements were performed on cells in the middle (MZ) zone at early time points during ramp loading. Furthermore, the cellular deformation results were interpreted in the context of a finite element analysis of chondrocyte deformation.

METHODS: 2-mm cartilage samples from bovine calf carpometacarpal joints were cut diametrically to create semi-cylindrical specimens. The deformation at the zonal and cellular levels was characterized on the cross-section, under compressive loading using a custom motorized loading device mounted on an inverted microscope. The specimen was then loaded with a ramp displacement to 2% platen-to-platen strain, applied at 1 µm/sec. Deformation and strain were assessed using digital image correlation in four regions of interest within a field of view consisting of a cell with its pericellular matrix (PCM, Fig. 1). A total of 10 cells in two tissue samples were analyzed.

To investigate the local deformation behavior of chondrocytes in-situ, a finite element analysis (FEA) was also performed, using a multiscale approach1. The chondrocyte, modeled as a homogeneous protoplasm surrounded by a semi-permeable membrane, was embedded in the center of a cylindrical cartilage disk, modeled with the biphasic theory2. The FEA model was loaded as in the experimental study.

RESULTS: Representative principal normal strain contours, measured experimentally under transient ramp loading, are presented in Fig. 1. In general, the minimum (compressive) principal normal strain was observed to be highest inside the cell and lowest at the axial poles. The minimum strain ($e_1$) averaged -0.47±0.11% in the ECM, but was significantly higher in the intracellular region ($e_1$ = 3.24±0.43%, $p < 0.05$). The PCM was exposed to a compressive strain of $e_1$ =-2.05±0.69%, significantly smaller than measured intracellularly ($p < 0.05$).

For 2% ECM strain, the FEA produced $e_1$ =-9.93±0.5% intra-cellularly. Strain contours showed the highest strain inside the cell and significantly lower $e_1$ strains at the axial poles (Fig. 2).

DISCUSSION & CONCLUSION: Experimental and FEA results demonstrate that intracellular strain magnitudes are considerably larger than the applied strain on the ECM, with a strain amplification factor of ~6. The patterns of strain distribution in the finite element results show a reasonable qualitative agreement with experimental results. This localized response under dynamic loading represents a novel finding relative to earlier studies of chondrocyte height and volume change under static equilibrium3. They suggest that mechanotransduction may be significantly mediated by this amplification mechanism.


ACKNOWLEDGEMENTS: NIH AR46532.
Multiscale Finite Element Modeling in Tissue Engineering of Articular Cartilage

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INTRODUCTION: Mechanical loading can impact the process of extracellular matrix (ECM) formation in tissue engineering (TE) of cartilaginous constructs for articular cartilage repair. The determination of the most effective type of loading, the appropriate load history, and the mechanical field variables responsible for the stimulation of cell activity is still subject of contrary discussions and motivation of numerous investigations. Numerical approaches like the finite element method (FEM) may become a powerful tool, and provide useful hints for more efficient TE processes.

METHODS: To analyze the hydrated and complex composite structure of hyaline cartilage we propose a biphasic, poroelastic model considering the interactions between solid and fluid phase of the tissue. Further, we assume an anisotropic, elastic solid matrix with tension-compression nonlinearities and rate dependent stiffening due to viscoelastic effects of the solid phase.

Following an idea of Guilak and Mow [1] to determine the mechanical loading conditions for a single cell, and deducing from the cell response the prediction of local cell stimulation, a multiscale FE approach has been chosen. Starting with a macro-scale FE model of the native tissue or the TE construct the local load history of a selected element of this FE mesh provides the boundary conditions for a micro-scale FE model with a single cell and its neighborhood. The material model mentioned above has been implemented into a commercial FE-code. Effects of variations in boundary conditions, loading regime and material properties on the evolution of mechanical field variables in the tissue and the mechanical loading of a single cell have been obtained [2]. Those were compared against the spatial distribution of cell messages of TE constructs [3].

RESULTS: It was shown that the fluid flow and the distribution of the pore pressure as well as its gradient essentially influenced mRNA expression. Thereby, the permeability of the construct plays a major role. The dependency on load velocity in the range of frequencies under consideration is less distinct.

DISCUSSION & CONCLUSIONS: In conclusion, the results suggest that the mechanical properties of the scaffold need to function in concert with the experimental concept of the bioreactor to successfully generate cartilaginous tissue. A multi-scale finite element model has been presented as the essential part of a tissue engineering strategy. A macro-scale model is designed to investigate mechanical conditions of native and tissue engineered cartilage as well as tissue property changes in a bioreactor, while a micro-scale model will be used to determine the stress and strain fields in a chondrocyte and in its local mechanical environment. Modeling will be continuously adapted to biochemical and biomechanical data.


ACKNOWLEDGEMENTS: This work was supported by AO Research Grant 02-W66.
Mechanical and Cellular Response of Impaction Cycles on Osteochondral Grafts

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INTRODUCTION: Osteochondral grafting procedures are routinely used to replace articular cartilage lesions with true hyaline tissue. However, the physical manipulation of articular cartilage during transplantation can be traumatic, leading to cell death and subsequently tissue degeneration [1]. The present study focused on cell viability of cartilage after impaction in a controlled laboratory setting. Further, the dynamic behavior of the osteochondral graft during impaction was studied in detail.

METHODS: Fresh osteochondral grafts were obtained from bovine stifles joints. The 8 mm diameter plugs were removed using the Arthrex OATS system. Next, a pneumatic impaction device delivered consistent loads at a rate of 100 kN/s to the plugs that were held in unconfined compression, similar to the clinical situation [1]. The applied loads ranged from 37 to 300 N, while the number of impaction cycles ranged from 74 to 11 and was adjusted to keep the overall impulse constant at 7.0 Ns. Each loading scenario was repeated with n=10 randomizing animal and retrieval site. Using a confocal microscope and Live/Dead cell assays, tissue samples were investigated at the day of impaction, as well as after 4 and 8 days in culture. Not impacted plugs served as a control. High speed videos were utilized to study the deformation of the osteochondral plugs during impaction. Every plug was impacted multiple times, while the load was recorded. Cartilage deformation was then calculated from the digitally stored grayscale images. The local in-plane displacements between two consecutive frames were found using a two-dimensional correlation analysis [2].

RESULTS: The highest local deformation was observed within the top layer of the cartilage. Tissue deformation was up to 0.4 mm in z-direction and involved the subchondral bone. Radially it was highest at the edges of the cartilage reaching values up to 0.2 mm (Fig.1).

Fig. 1: Radial tissue displacement at 300 N. Bars indicate 95% confidence intervals

Higher loads caused a decrease in cell viability (Tab.1). The pattern of cell death was consistent with the deformation pattern.

Table 1. Relative amount of viable cells in control and loaded plugs (means). * controls were more viable than loaded samples(p<0.05) # indicate group specific differences (p<0.05)

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<th>37.5N</th>
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<td>0.591</td>
<td>0.50</td>
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<tr>
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<tr>
<td>day 8</td>
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<td>0.20</td>
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DISCUSSION & CONCLUSIONS: Cartilage impaction has a detrimental effect on cell viability. There is a dose response relationship with respect to the magnitude of force, which needs to be taken into account. The dynamic behavior of cartilage plugs during impaction grafting is far from equilibrium, which has implications for future theoretical modeling.


ACKNOWLEDGEMENTS: This study was financed in part by Arthrex, Inc.
PROTEASES AND CARTILAGE DEGRADATION

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Cartilage degradation is fundamental to both joint development and joint pathology. The major degradative agents mediating this process are thought to be proteolytic enzymes, in particular members of the metalloprotease class. When articular cartilage is maintained in organ culture in the presence of catabolic cytokines members of the ADAMTS family of proteases ("aggrecanases") are produced and their activity in the tissue is evident by the generation of characteristic degradation products of aggrecan. These fragments can be detected and quantitated using anti-neoepitope antibodies specific for the different cleaved aggrecan products. However, analysis of adult human cartilage extracts demonstrates that additional proteases are operating in vivo. In particular matrix metalloproteinases such as MMP-13 (collagenase-3) appear to be involved in the turnover of aggrecan in addition to their action on triple helical collagen.

The contribution of metalloproteases, in vivo, was demonstrated in mice null for tissue inhibitor of metalloproteinases-3 (TIMP-3) which inactivates MMPs such as stromelysin-1 (MMP-3) and collagenase-3. Importantly, it also inhibits the aggrecanases (ADAMTS-4 and -5) and members of the ADAM family such as ADAM-17 (tumor necrosis factor-α converting enzyme [TACE]). The animals appear normal and show no irregularities in skeleton development. However, histochemical analysis of the articular cartilage of adult animals demonstrated increased degradation of aggrecan by both aggrecanases and MMPs and of collagen by collagenase, relative to wild-type mice.

While the role of metalloproteases in cartilage degradation is evident, this is by no means the whole story. It has recently become clear that cysteine proteases, in particular cathepsin K, are expressed in osteoarthritic cartilage. The activity of this enzyme in cartilage has been demonstrated using anti-neoepitope antibodies specific for cathepsin K cleavage products of link protein and collagen. This protease is active at neutral pH and thus may be responsible for collagen degradation in this tissue.

In addition to the role of proteolytic enzymes, increasing evidence points to the action of glycohydrolases in cartilage turnover. It is generally assumed that following cleavage of the aggrecan core protein, the G1-link protein-hyaluronan complex remains localized in the tissue due to its large size. Cartilage explants cultured in the presence of catabolic cytokines have been shown to release the G1 aggrecan domain and hyaluronan into the culture medium. In addition, it was shown that the hyaluronan released has a lower molecular size than that isolated from the tissue, suggesting its degradation. Two candidate mechanisms can be proposed for hyaluronan cleavage. While the molecule is very susceptible to free radical attack there is little evidence to support this mechanism under non-inflammatory conditions and no effect of free radical scavengers has been observed. In contrast, hyaluronan release was dramatically reduced in the presence of the hyaluronidase inhibitor, apigenin, suggesting that one or more of these glycohydrolases is responsible for the extracellular cleavage of hyaluronan and the concomitant release of G1 and link protein. At least five hyaluronidases are coded for by the human and bovine genomes, and mRNA for of most of them have been detected in chondrocytes.

Thus while metalloproteases are certainly important mediators of cartilage degradation, there is clearly a role for proteases from other classes and for glycohydrolases in the turnover of this tissue.

ACKNOWLEDGEMENTS: This work was supported by the Canadian Institutes of Health Research and the Shriners of North America.
INTRODUCTION: Increased nitric oxide (NO) production is associated clinically with osteoarthritis (OA), and the severity of disease is significantly decreased in vivo using nitric oxide synthase (NOS) inhibitors. Mechanical stress has strong influences on cartilage metabolism and plays an important role in the maintenance of articular cartilage in both health and disease. Pro-inflammatory cytokines and mechanical stress can increase the production of inflammatory mediators such as NO by articular cartilage. Articular cartilage, however, is avascular and exists at a reduced oxygen tension, the superficial zone at approx 6% O2 and the deep zone at 1% O2. As the production of NO is an oxygen dependent process, our studies have investigated the effects of oxygen tension on mechanically and cytokine induced NO production.

METHODS: Full thickness explants of articular cartilage were harvested from the femoral condyles of 2-3 y old female pigs and cultured in DMEM with 10% FBS, non-essential amino acids, HEPES, penicillin and streptomycin. Treated and control explants were paired from adjacent sites in the joint. After 72 hrs in culture at 37°C, 5% CO2, 95% air, explants were incubated in culture media supplemented with 37.5 µg/ml ascorbate-phosphate at either 5% CO2, 95% air, or 5% CO2, 5% O2, 90% N2 or 5% CO2, 1% O2 and 94% N2.

The effects of recombinant porcine IL-1α (rpIL-1α, R&D Systems) and oxygen were tested on site-matched paired explants cultured in control or experimental culture media and incubated at different oxygen tensions for 72 hrs.

The effects of mechanical compression were tested by applying intermittent compressive loads, at 0.5 Hz (1 sec on, 1 sec off) for 24 hrs, using a modified version of the Biopress system™ (Flexcell International), at 20%, 5% or 1% O2.

The role of NOS2 was determined using the selective NOS2 inhibitor 1400W (2 mM, Alexis). NO production was measured via the Griess reaction and cell viability determined using a fluorescent live/dead assay (Molecular Probes).

RESULTS:

Figure 1: Nitric oxide production from articular cartilage explants in 1% vs 20% O2 in the presence or absence of rpIL-1α and 1400W. Mean ± SEM, n=12 ***p<0.001.

Figure 2: Nitric oxide production from articular cartilage explants compressed at 0.05 MPa, 0.5 Hz for 24 hrs at either 20% O2, 5% O2 or 1% O2, Mean ± SEM, n = 18, ***p<0.001.

DISCUSSION & CONCLUSIONS: Oxygen tension significantly alters endogenous NO production in articular cartilage, as well as the stimulation of NO in response to both mechanical loading and pro-inflammatory cytokines. In other studies, we have shown that oxygen tension also influences the production of peroxynitrite, the production of ATP, and the mechanical properties of articular cartilage. These findings suggest that the relatively low levels of oxygen within the joint may have significant influences on the metabolic activity and inflammatory response of cartilage as compared to ambient levels.


ACKNOWLEDGEMENTS: NIH AR49790 and VA Research Services.
Interleukin-1 induction of DNA damage in articular cartilage: roles of nitric oxide and superoxide.

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INTRODUCTION: Increased nitric oxide (NO) is associated with both osteoarthritis (OA) and rheumatoid arthritis (RA)¹. Cartilage destruction associated with these diseases is partially mediated by cytokines such as interleukin-1 (IL-1). IL-1 causes an increase in the pro-inflammatory mediator NO. NO has pleiotrophic effects and in some cell types can cause DNA damage. The aim of this study was to determine if IL-1 can cause DNA damage in articular chondrocytes through an NO dependent mechanism.

METHODS: Articular chondrocytes were enzymatically isolated from porcine distal femoral cartilage and embedded in 1.2% alginate (4 x 10⁶ cells/ml). The alginate beads were maintained at 37°C, 5% CO₂, 95% air, in DMEM supplemented with 10% FBS, HEPES, NEAA, pen/strep. 24h after harvest, and incubated with 0, 0.1, 1, 10, or 100 ng/ml IL1-α (R & D Systems), for 24 hrs. The effects of the NOS2 selective inhibitor (1400W, 2mM Cayman Chemical Co), and the free radical scavenger superoxide dismutase (SOD, 50 µM, Sigma Chemical Co.) were also tested. Chondrocytes were released from the alginate beads and subjected to single cell gel electrophoresis¹ using the “comet” assay (Trevigen). Briefly, cells were combined in molten LMAgarose (1x10⁵ cells/ml agarose) on microscope slides, lysed, and alkali treated². Electrophoresis was performed at 1V/cm for 30 min. DNA was stained with SYBR® green and DNA damage was identified by migration of nuclear material from the nuclei (head) to give a comet-like appearance (tail).

![Comet showing DNA damage](image)

**Fig1:** Comet showing DNA damage. ¹Olive tail moment = (Tail Mean – Head Mean) X Tail % DNA/100

Images were captured using LSM scanning confocal microscopy and analysed using CASP™ software. DNA damage was quantified by combining tail length and the distribution of DNA in the tail to give a "tail moment" (Fig.1).

NO production was measured as NO₂⁻ and NO₃⁻ (NOx) using the Griess assay. Cell viability was determined with the live/dead assay (Molecular Probes).

RESULTS: IL-1 caused significant DNA damage in a concentration-dependent manner, with significant DNA damage occurring as low as 1 ng/ml IL-1. The DNA damage observed by the comet assay was not associated with cell death. IL-1 induced DNA damage was inhibited by the NOS2 selective inhibitor or SOD (Fig.2). Incubation with IL-1 was associated with increased NO production.

![IL-1 (100ng/ml) causes significant DNA damage which can be inhibited by 2 mM 1400W or 50 µM SOD. Mean ± SEM, n = 2 pigs, 50 cells analyzed per group. *** = p < 0.001 (ANOVA).](image)

**Fig.2:** IL-1 (100ng/ml) causes significant DNA damage which can be inhibited by 2 mM 1400W or 50 µM SOD. Mean ± SEM, n = 2 pigs, 50 cells analyzed per group. *** = p < 0.001 (ANOVA).

DISCUSSION & CONCLUSIONS: Incubation of articular cartilage with IL1-α caused DNA damage via NO production. We are currently identifying the type of DNA damage that occurs and investigating DNA damage response proteins. Some levels of IL-1 used in this study are hyper-physiological and further studies will be carried out using lower concentrations of IL-1 for longer periods of time.


ACKNOWLEDGEMENTS: Jessica Son for excellent technical assistance. NIH AR49790 and VA Research Services.
Is ADAMTS-5 the only aggrecanase in mouse cartilage?

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Introduction: Aggrecan provides cartilage with its weight-bearing properties. In arthritic cartilage, aggrecan is degraded by one or more "aggrecanases" that are members of the ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin motifs) family of zinc-dependent enzymes. ADAMTS-4 and -5 are thought to be the primary aggrecanases, although ADAMTS-1, -8 and -9 have weak aggrecan degrading activity in vitro. Using targetted gene mutations we identified ADAMTS-5 as the major aggrecanase in mouse articular cartilage. The ADAMTS-5 deficient mouse is protected against aggrecan loss and cartilage erosion in models of inflammatory arthritis [1] and osteoarthritis [2], whereas the ADAMTS-4 deficient mouse is not [1,3]. In this study we examine the expression and modulation of aggrecanase activity by IL-1α and retinoic acid in ADAMTS-4 and ADAMTS-5 deficient mice.

Materials and Methods: Catalytically-deficient mice were created by targetted exon deletions in either the adamts-4 (TS-4Δ-cat mice) or adamts-5 (TS-5Δ-cat mice) genes. Aggrecan catabolism was examined in explant cultures of femoral head cartilage harvested from 3 week old mice and stimulated with either IL-1α (10 ng/mL) or retinoic acid (10 µM) for 3 days. Aggrecan release into the medium or remaining in the cartilage was measured on each of days 1, 2, and 3 by the dimethylmethylene blue dye binding assay. Aggrecanase activity was examined by Western blot analysis of both medium and cartilage extracts, using neoepitope antibodies that recognise the EGE373↓374ARG, SELE1279 and FREEE1467 neoepitopes in mouse aggrecan.

Results: The TS-4- and TS-5Δ-cat mice are viable and fertile, with no obvious abnormalities in any tissues, including the joints and skeleton. Treatment of cultured femoral head cartilage with IL-1α or retinoic acid promoted loss of aggrecan from wildtype cartilage. Loss of aggrecan from TS-4Δ-cat cartilage was slightly reduced, and aggrecan loss from TS-5Δ-cat was markedly reduced. Western blot analysis showed that proteolysis at the E373↓374A site in the aggrecan interglobular domain was blocked in the TS-5Δ-cat mouse. However, ADAMTS-5 deficiency was not sufficient to block cleavage in the CS-2 domain at either the E1279↓1280G site or the E1467↓1468G site, since SELE1279 and FREEE1467 epitopes were generated in TS-5Δ-cat cartilage following stimulation with IL-1α or with retinoic acid. The SELE1279 and FREEE1467 fragments were detected as single bands in cartilage extracts and as doublets in the culture medium.

Discussion: These results show that aggrecan loss stimulated by either IL-1α or retinoic acid, is driven predominantly by ADAMTS-5 in mouse cartilage. The data suggest that whereas ADAMTS-5 is responsible for cleavage in the interglobular domain, ADAMTS-5 is not essential for cleavage in the aggrecan CS-2 domain. ADAMTS-4 may cleave in the CS-2 domain and ADAMTS-5 may compensate for loss of catalytic activity in the TS-4Δ-cat mouse. Alternatively, an aggrecanase other than ADAMTS-4 or -5 may be responsible for cleavage in the aggrecan CS-2 domain.

References:
INTRODUCTION: Catabolic cytokines and anabolic growth factors are thought to be important for maintaining the tissue homeostasis of articular cartilage in the adult. This relates mainly to their important role in regulating the expression and synthesis of matrix constituents (anabolism) as well as the expression of matrix-degrading proteases (catabolism), namely matrix metalloproteinases (MMPs) and molecules of the ADAMTS-family of proteinases. In this respect, over the last years and decades in particular interleukin 1 and members of the BMP/TGFß-superfamily gained a lot of scientific interest. Additionally, the balance of anabolic-catabolic stimulation is likely to be very important also in cartilage destruction occurring in rheumatoid arthritis and osteoarthritis. Of note, work by Sandell and colleagues showed additionally a close influence of interleukin 1 activity also on BMP gene expression itself suggesting that both mediators are directly interwoven also on the gene expression levels.

METHODS: In vitro experiments were performed using freshly isolated adult human articular chondrocytes from normal knee joints (femoral condyles). (Non-passaged) High-density monolayer cultures were stimulated with and without IL-1ß (1 and 10ng/ml) and BMP-7 (50ng/ml; kindly provided by Stryker Biotech). Gene expression levels (collagen type II, aggrecan, MMP-1, MMP-2, MMP-3, MMP-13, ADAMTS-4) were detected by real-time PCR.

RESULTS: After OP-1 stimulation, the anabolic genes collagen type II and aggrecan were significantly up-regulated in articular chondrocytes. No significant changes were observed for the matrix degrading enzymes. Interleukin 1 showed a significant down-regulation of aggrecan and collagen type II genes. In contrast, the matrix degrading proteases - except MMP-2 - were induced by IL-1ß dose-dependently.

DISCUSSION & CONCLUSIONS: Clearly, interleukin 1 and BMP-7 (but according to results by Sandell et al. also e.g. BMP-2) exert opposite effects on anabolic and catabolic expression on the effector level. This interplay appears to play an important role in physiological cartilage tissue homeostasis. Thus, the knockout of BMP stimulation by antisense technology showed a catabolic imbalance in cartilage tissue culture.


ACKNOWLEDGEMENTS: This work was supported by the BMBF (Leitprojekt osteoarthritis grant 01GG9824) and the DFG (grant Ai20/7-1).
AMIC technique for cartilage repair, a single-step surgical intervention as compared to other methods

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The past ten years have brought plenty of research and technical innovations and also preliminary clinical success in cartilage repair. The common target of all methods utilised is to produce a sufficiently stable quality of cartilage repair or regenerate. However, yet today clinical, radiological and histological results analysing the different techniques are somewhat contradictory. The different lines of clinically applied and basic research have focused on:

1. a. Spontaneous natural filling of the defect with fibrocartilage of variable solidity.
   b. Abrasion chondroplasty, drilling or microfracturing to allow for mobilisation of progenitor cells and mesenchymal stem cells from the cancellous bone into the defect and develop to a hyaline like cartilage.
   c. Stem cell treatment (in vivo or ex vivo theory of potential technique by which stem cells could be brought to a defect to create cartilage; so far no directly linked product available)

2. Transplantation of osteochondral auto grafts (Mosaicplasty, OATS, SDS, patellar graft) or allograft.

3. a. Autologous chondrocyte transplantation and periosteal coverage (ACT) to cover bigger surfaces.
   b. Implantation of second and third generation ex vivo products and create less morbidity but without knowing whether the results are as long-lasting as for the originally described technique (chondrocytes cultured on membranes, MACI, in gels, implantation of a stable three-dimensional de novo cartilage disk or even engineered osteochondral grafts, AMIC: autologous membrane induced chondrogenesis).

A fair amount of today’s laboratory research is focusing on the culture of the patients own chondrocytes or his own stem cells.

Clinically, some methods can be applied in all indications regardless of size, localisation, depth of the lesion up to the age of fifty years and this is valid for lesions in the knee, the shoulder, the talus, the elbow etc. Other methods like AOCT should not be used for lesions over 2 cm in diameter because of donor side morbidity. All methods claim to have an 85% outcome success rate. Regarding the histological content of the successful implants or the reformed cartilage, microfracturing produces a cartilage implant containing a fibrocartilage that looks similar to the hyaline like cartilage of ACI at two years. Mosaicplasty plugs provided great care is applied during insertion avoiding damage of the cylinders and cartilage death-a special instrumentation has been developed with ZIMMER, the Soft Delivery System, SDS to avoid force during impaction. They remain hyaline provided they are inserted without being prone or deep sunken and the surface convexity of the femoral condyle is restored and provided they are inserted tightly next to each other. There is agreement that this is more difficult in arthroscopic techniques. One agrees also that results are dependent on the alignment of the limb. If the compartment treated is overloaded, there is less chance for integration. Osteotomy has therefore a solid position in the armamentarium of the cartilage surgeon- up to 50% of our cases get an osteotomy as part of their treatment regardless of which technique is utilised.

As complications in autologous osteochondral grafting we may observe destruction of the hyaline cartilage cap, non integration and pseudarthrosis or fractures of the cylinders (of special risk are smokers), especially when grafts are not inserted tightly to each other and there is lack of stability with fluid leakage out of the cartilage caps. Rarely ossification of the cartilage is observed when a thin capped cylinder retrieved in the peripheral zone of the femoral trochlea is implanted in an area of thick cartilage as in the centre of the patella where the cartilage is 5 mm thick. Donor site pathology in mosaicplasty is an issue of concern mainly if more than six plugs are removed from the femoropatellar joint. This alone can create clinical symptoms.
Nicotine abuse, probably for all techniques decreases the rate of success of cartilage repair or regeneration and osteotomy healing. Roughly 300 cases have been treated during the last 10 years in our institution. The results were reported in 2002 (Jakob et al).

As an alternate single surgery technique to microfracturing and mosaicplasty we adopted the “Autologous membrane induced chondrogenesis” (AMIC) technique proposed by Behrens. Microfracture of subchondral bone results in intrinsic repair of cartilage defects. Stem or progenitor cells from bone marrow have been proposed to be involved in this regenerative process. In a study Kramer et al (2006) demonstrate for the first time that mesenchymal stem (MS) cells can be recovered from matrix material saturated with cells from bone marrow after microfracture. This introduces a new instant, single time technique for automatic MS cell isolation during arthroscopic treatment. This study demonstrates that MS cells can be attracted to a cartilage defect by guidance of a collagenous matrix after perforating subchondral bone. Protocols for application of MS cells in restoration of cartilage tissue include an initial invasive biopsy to obtain the MS cells and time-wasting in vitro proliferation and possibly differentiation of the cells before implantation. The new technique already includes attraction of MS cells to sites of cartilage defects and therefore may overcome the necessity of in vitro proliferation and differentiation of MS cells prior to transplantation.

Furthermore Breinan et al (2000) have shown that the fibrocartilaginous filling of a microfracture treated defect is superior when covered with a collagen membrane than when left uncovered.

We find this technique especially useful in OCD. In this relatively young technique we curette the defect and apply microfractures to the basis of the osseous defect. Then we gain cancellous bone from the tibial plateau and mix it with fibrin glue, of which 50% of the thrombin portion is replaced by the serum of the patient as a source of growth factor. This paste of bone and enriched fibrin glue is filled in the defect which is then covered by the porcine Chondrogide membrane (Geistlich) that is glued on and which we can as well suture to the defect. The AMIC technique in combination with microfractures can be utilised for the coverage of pure cartilage defects alone, where the membrane is glued alone or fixed on the defect in combination with 5-0 resorbable sutures. In the first two weeks following surgery, aftertreatment is very defensive to avoid loss of the membrane. After two months of crutch walking with 15 kg of weight we observe a nice osseous integration of the graft and a covering layer that looks promising. After 4-6 months activity can be increased depending on the size of the defect. This is a young technique that we adopted in mid 2003 with 40 cases treated so far. Femoropatellar joint with OA due to chronic subluxation, 21 cases; femoralcondylar pure cartilage lesions, 6 cases; OCD lesions, 7 cases; talar lesions, 6 cases; total 40 cases.

Strict observation is required over the upcoming years regarding clinical results and durability and also the composition of this neocartilage, mainly also to see whether it is superior to Microfracturing alone.

So far it seems to be an interesting alternative to Mosaicplasty or Microfracturing alone since it combines principles of cell therapy with an artificial and instant biological containment that acts against the loss of cells thus acting as a internal bioreactor with the patients own growth factor support.

Literature

Breinan H.A., Hu-Ping Hsu, Martin S, and Spector M, Healing of canine articular cartilage defects treated with microfracture, a type II collagen matrix, or cultured autologous chondrocytes; J Orthop Research 2000, 18, 781

Is ACI a success? Current evidence and trials in progress

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In Google you will get over 100,000 web pages from a search on ‘autologous chondrocyte’. The paper in 1995 by Petersen, Lindahl and Brittberg began a revolution in orthopaedics with many consequences. It is not that large numbers of surgeons or patients were affected, but orthopaedics was brought to the forefront of tissue engineering. New possibilities of treatment were opened up.

Another aspect of AC has been the way it challenged accepted dictum of the inability of cartilage to heal. This has fed the emotional debates that surround the histology of ACI. Is true hyaline cartilage formed?

Many new treatments arrive in orthopaedics, become fashionable, and pass – as John Hunter said, ‘nothing is as vicarious as surgical fashion’. Most new treatments are started by a champion, often with commercial support, and progress on the basis of a personal series, eventually a gathering of other individual series and occasionally fade as reports of complications increase. Orthopaedic surgeons appear more concerned than many specialties about the cost of procedures. Most of our procedures are highly cost-efficient as measured by QUAYS, but criticism of ACI was also partly of the apparent high cost. This has not proved to be significant when assessed by cost benefit, but studies are very limited.

Many treatments have become popularized since the advent of ACI, including mosaicplasty and microfracture. An excellent randomized trial by Hubbard on simple debridement has never been challenged in a trial, but there is a natural instinct to ‘try and do something’, and therefore soething more, with the assumption it will be better than simple debridement. Is there good evidence?

Only one randomized multicentre trial has been reported and this is the study by Gnutsen and the dedicated group of Norwegian surgeons with 80 patients who must all be congratulated on showing us the way. The problems with clinical trials in orthopaedics are numerous.

Vague end-points, the need for long term studies of at least 10 years, differences in technique, and learning curves all make trials very difficult. Results so far from Norway are inconclusive. Larger trials are probably needed. The MRC in the UK is funding a 660 patient study where independent observers will hopefully provide sufficiently accurate measures of outcome to come up with an answer. European funding of ‘Myjoint’, a collaboration between Keele in the UK and Kiel in Germany, is to build on the science of ACI to investigate the possibility of growing a living joint in the Latissimus dorsi of the patient. The aim is to allow a joint to grow in the security of the patient with Autologous cells and over a sufficient time to become mechanically strong and suitable for transplantation into the arthritic joint.

So although the clinical trial evidence is not strong for ACI, it has been successful in drawing a lot of resource to improve treatments of localized cartilage loss, and in the long term I believe advances the possibility of a biological joint replacement.
MR Imaging and Early Cartilage Degeneration Detection

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INTRODUCTION: Interactions between all the major joint tissues, including the articular cartilage, synovium, bone marrow, subchondral bone, trabecular bone and muscle, have been implicated in osteoarthritis (OA) (1). Magnetic resonance (MR) images have been used to quantify the cartilage morphology, volume and thickness (2) and focal defects (3), and may reflect changes in biochemical composition of articular cartilage (4-6). Cartilage loss in OA is preceded by damage to the collagen-proteoglycan matrix and elevation of cartilage water content. These changes are associated with changes in cartilage relaxation times T2 and T1 (7), as well as in the uptake of contrast agents such as Gd-DTPA in the cartilage matrix (dGEMRIC) (4). Furthermore, injury and OA-related changes in bone marrow manifested by an increase in the signal intensity in bone marrow on fat-saturated T2-weighted images (bone marrow edema, BME) have been associated with severity and progression of OA (8). Such marrow changes are also associated with acute injuries such as anterior cruciate ligament injuries (9), that sometimes progress to OA and joint degeneration. The purpose of this paper is to review the interrelationships of T2, T1, cartilage volume and BME in patients with OA versus those with acute knee injury, and markers of early cartilage degeneration.

METHODS: In controls and subjects with OA and ACL injury studies relating cartilage volume, thickness or degeneration to relaxation time T2, T1 and bone marrow edema changes were conducted. High-resolution, fat-suppressed, sagittal images were acquired for assessing cartilage structure, using a 3-D spoiled gradient echo (SPGR) sequence. The cartilage was segmented using a spline-based, semi-automatic technique and was defined in four distinct regions: medial and lateral tibia, and medial and lateral femur. Total cartilage volume and average thickness were calculated for each region. Sagittal images were acquired for measuring T2 relaxation time, using a dual echo spin echo sequence. A map of T1 values was calculated. Using a T1 relaxation time mapping technique T1 maps were reconstructed. In patients with OA and with ACL (anterior cruciate ligament) tears, who showed BME T1-weighted and fat-saturated T2-weighted fast spin-echo images were acquired. Point RESolved Spectroscopy (PRESS) volume selection was used to acquire spectroscopic fat/water quantitative data in the edema. BME was semi-automatically segmented using a threshold method based on T2-weighted images and volume of BME was calculated.

RESULTS: The studies revealed that higher medial T2 results in greater loss of medial cartilage volume at twelve months. Specifically, the correlation between baseline medial femoral T2 and change in medial femoral cartilage volume was r = -0.38 (p < 0.05). A significant correlation was found between average T1, and T2 values within the cartilage, with a correlation coefficient R=0.69 and p=0.017. The increase of average T1, in cartilage from the controls to patients was 19.1% (43.90 ms for controls and 52.28 ms for patients), while the increase was 9.6% for the average T2 (34.94 ms for controls and 38.31 ms for patients). The difference in average T1, in cartilage between controls and patients was significant (p = 0.003) while it was not significant for average T2 ( p = 0.202). Patients with similar average T2 may have different T1, or vice versa. The average T1, values in BME-overlying cartilage were significantly higher than that in surrounding cartilage (51.8 ± 10.8 ms vs. 43.0 ± 8.3 ms, p=0.032). Volume of BME correlated significantly with volume of elevated water (R=84.4%, p=0.004) but not with volume of elevated unsaturated lipids.

DISCUSSION & CONCLUSIONS: Quantitative imaging appears promising and may potentially provide information beyond morphological changes in articular cartilage, with regards to early cartilage degeneration and biochemistry and further studies are clearly warranted.

REFERENCES:
INTRODUCTION: The successful application of tissue engineered cartilage constructs depends on the redifferentiation of chondrocytes after the in vitro cultivation expressed by the synthesis of cartilage specific ECM components. Several approaches are investigated to enhance the cell response and cell differentiation including the choice of appropriate scaffold materials as well as biochemical and biomechanical stimulations. The valuation of preimplantative in vitro cultivated scaffolds including cell morphology, cell distribution, cell number and metabolic activity by non-invasive techniques enables a sample specific controlling of the required cultivation time and the type of stimulation. We introduce methodical aspects of the visualization of chondrocytes on collagen I/III scaffolds by two photon laser scanning microscopy (TPLSM).

METHODS: Bovine chondrocytes were isolated from knee biopsies, expanded in monolayer cultures and cultivated on collagen I/III scaffolds with two different structures (fleece and sponge-like). The TPLSM analysis was performed using a TriMScope of LaVision BioTec (Bielefeld, Germany). The excitation wavelength was in the range of 790-820 nm (Ti:Sa). The autofluorescence of samples (Z-stacks) was detected by CCD camera and/or photomultiplier. The selective visualization of autofluorescent signals of cells and scaffolds was achieved by a) analysis of Second Harmonic Generation (SHG), b) the spectral analysis of autofluorescence and c) Fluorescence Lifetime Imaging (FLIM).

RESULTS: The results demonstrate that cells and collagen scaffolds can be visualized selectively using autofluorescent signals. Spectral differences of collagen signals and cells provide a powerful tool to distinguish cells from scaffold materials. SHG signals of collagen fibres in fleece structures as well as FLIM analysis increase the possibilities of a selective visualization. Furthermore the results indicate the stimulation of cell differentiation processes of chondrocytes growing on sponge-like collagen scaffolds.

DISCUSSION & CONCLUSIONS: The results provide the basis for targeted online investigations on the influence of biochemical and biomechanical stimulations on the redifferentiation of in vitro cultivated chondrocytes. The quantitative analysis of cell number, distribution and morphology depending on the scaffold structure and cell stimulating cultivation conditions can be achieved by powerful 3D rendering software tools.

ACKNOWLEDGEMENTS: The authors would like to thank the Federal Ministry of Education and Research (BMBF) Az. 13N8432, 13N8434 and 13N8435 for the financial support.
Biophysical diagnostics of normal, degenerated and repaired articular cartilage

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INTRODUCTION: We have developed and/or applied several clinically potential biophysical methods for the quantitative diagnostics of articular cartilage [1-6]. In this study, we compare the diagnostic performance of these techniques to detect early degenerative cartilage changes.

METHODS: In situ ultrasound (attenuation and speed), ultrasound-indentation (dynamic modulus (Edyn) and ultrasound surface reflection coefficient (R)), MRI (T₂ relaxation time, T₁ relaxation time in presence of Gd-DTPA²⁻ (T₁GD)) and reference biomechanical and microscopic analyses were conducted for the same bovine articular cartilage samples (n=32). Mankin score of the samples (0=normal (n=11), 1-3=early degeneration (n=11),>3=advanced degeneration (n=10)) was evaluated from safranin-O stained sections.

RESULTS: A n example of the high-frequency ultrasound images of the native and repaired porcine cartilage (3 months after autologous chondrocyte transplantation (ACT)) is presented in Fig. 1. The mean values for measured parameters in each group are presented in Table 1. With the cutoff value R=2.3% the specificity and sensitivity values to differentiate normal and degenerated groups were 0.89 and 0.91, respectively. For US attenuation (cut-off 2.47 dB/mm) the specificity and sensitivity values were 0.82 and 0.95, respectively. For US speed (cut-off 1576 m/s) the same values were 0.82 and 0.91, respectively. The specificity and sensitivity values for MRI T₂ (cutoff 44 ms) and for T₁GD (cutoff 386 ms) were typically slightly lower, 0.73 and 0.81, respectively.

DISCUSSION & CONCLUSIONS: The non-destructive methods of the present study showed promising potential for the early OA diagnosis as well as for monitoring cartilage repair. Especially, US indentation results corresponded highly with the histological and biomechanical integrity of the tissue as well as sensitively and specifically classified samples into normal and degenerated ones. US speed was also an effective parameter for evaluation of cartilage integrity. Also, diagnostic performance of MRI was relatively good. In summary, the further development of novel biophysical methods provides potential tools for detecting signs of early OA in clinical practice.

**Fig. 1: Example of the in situ high frequency ultrasound image (below) of ACT cartilage repair, along with the corresponding histological sections (above).**

**Table 1. Mean values of diagnostic parameters in different degenerative groups. n=27-32 *p<0.01**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Cartilage</th>
<th>Early Degeneration</th>
<th>Advanced Degeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edyn (MPa) *</td>
<td>9.12 ± 6.06</td>
<td>3.69 ± 3.62</td>
<td>1.48 ± 0.47</td>
</tr>
<tr>
<td>R (%) *</td>
<td>3.65 ± 1.20</td>
<td>1.91 ± 1.13</td>
<td>0.55 ± 0.26</td>
</tr>
<tr>
<td>Thickness (μm) *</td>
<td>1715 ± 264</td>
<td>2032 ± 311</td>
<td>2098 ± 360</td>
</tr>
<tr>
<td>MRI parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attenuation (dB/mm) *</td>
<td>2.65 ± 0.58</td>
<td>2.01 ± 0.45</td>
<td>17.62 ± 0.44</td>
</tr>
<tr>
<td>Speed (m/s) *</td>
<td>1603 ± 27</td>
<td>1572 ± 15</td>
<td>1548 ± 14</td>
</tr>
<tr>
<td>T₂ (ms) *</td>
<td>42.8 ± 14.0</td>
<td>69.4 ± 58.6</td>
<td>120.0 ± 105.2</td>
</tr>
<tr>
<td>T₁GD (ms) *</td>
<td>401 ± 40</td>
<td>373 ± 35.0</td>
<td>327 ± 32.0</td>
</tr>
</tbody>
</table>

The Pros and Cons of Studies of Animal Joints to Improve Articular Cartilage Regeneration

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Animal models of articular cartilage repair/regeneration have greatly contributed to the understanding of the biology of the repair mechanisms and our capacity to manipulate or enhance these events. Many factors need to be considered when choosing an animal model for novel cartilage repair strategies. The ideal model should be similar to humans in respect to anatomy, joint mechanics and cell biology. However, in reality, this ideal is impossible to achieve because of the great heterogeneity which already exists in the target human population (genetic profile, age, weight, joint geometry…). Adult animals should preferably be used to mimic the adult human joint environment, when attempting to predict the response of the intervention in humans. However they may not be easily available and it is costly to house them until they attain an adult age. The animals should at least be skeletally mature (growth plate closure on radiographs). It is possible to obtain adult horses, but the majority of studies include 2-3 year old (post – pubertal) horses and are consequently models of acute injury in young athletes.

An advantage of all the animal models is that the anatomy of the knee joint is similar to that of humans. On the other hand, all, except primates, are quadrupeds and consequently joint mechanics and loading are very different from humans. It should be borne in mind that differences also exist at the cartilage structural and cellular level that could affect outcome. It is interesting that the size of the chondrocytes do not differ between species, but differences in cell volume density exist (1.7% human v 12.2% rabbit femoral condyles) which could impact cell dosing. There is also considerable variation in cartilage thickness between species and the thickness of mature equine femoral cartilage most closely approximates that of humans.

The characteristics of the cartilage defect in the animal model (partial or full thickness/area/depth/shape,) should also mimic that encountered in humans. The larger animals, because of the increased cartilage thickness, are preferable for studies of partial thickness defects confined to the hyaline cartilage.

Fig. 1: Differences in cartilage structure between species x25 (rabbit left, horse right)

One of the most valuable contributions of animal models is that they allow the determination of the kinetics of repair mechanisms and efficacy of cartilage repair strategies temporally. The ability to assess the treatment strategy non-destructively in vivo is an important consideration. Repeated imaging with MRI & CT is possible in all species but resolution is problematic in small animals such as rabbits where the cartilage is thin and powerful magnets are required to accurately quantify cartilage changes. Large animals (goat, sheep, pig, horse) are required when studying efficacy and functionality of the intervention as the size of the joints more closely approximate the human knee. The large joints facilitate minimally invasive surgical procedures such as arthroscopy to visualize, biopsy and assess the mechanical properties of the repair site. They also permit harvest of undiluted synovial fluid and yield abundant joint tissues for assessment. Joint function analyses (force plate and kinematics) also make the larger animals more appealing. The ultimate test however for any novel repair strategy is a long term clinical trial in the target species-homo sapiens.

REFERENCES

Stem Cell Strategies for Cartilage Repair

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INTRODUCTION: There are several characteristics of stem cells that make them unique in comparison to other mammalian cells. Firstly, they exist as unspecialized cells lacking tissuespecific characteristics and they maintain this undifferentiated phenotype until exposed to appropriate signals. Secondly, they have the capacity for extensive self-renewal. Thirdly, under the influence of specific biological signals they can differentiate into specialized cells with a phenotype fully distinct from that of the precursor. Mesenchymal stem cells (MSCs) in the bone marrow conform to this definition. These cells, as their name implies, are the precursors of cells of mesenchymal lineage, including cartilage, bone, fat, muscle and tendon. Despite a lack of understanding of these cells and their natural history, it is very likely that they have therapeutic potential in a variety of clinical applications.

There is now strong interest in the evaluation of stem cells in cardiac repair, treatment of neurological diseases and bone and cartilage repair, among others. Recent data have suggested that stem cells may also play a role in the suppression of the host immune system, tolerance of allogeneic transplants and in the suppression of graft versus host disease (1). This work describes the evaluation of MSCs in cartilage and joint repair following transplantation of cells from a mismatched donor to an immunocompetent recipient.

METHODS:
MSCs were isolated from bone marrow aspirations from male donor goats and expanded in culture (4). A total of 17 male goats, unrelated to the cell donors, received intra-articular MSC injections to the knee following partial meniscectomy surgery.

MLR assays were performed on the group of animals on study for a period of 12 months. Whole blood was processed on Ficoll-Hypaque to enrich peripheral blood mononuclear cells (PBMCs). One-way MLR was performed using recipient PBMCs as the responder population and irradiated recipient (autologous) PBMCs or donor (allogeneic) PBMCs as the stimulator populations. Allo-antibody assays were also performed on all animals.

RESULTS: ³H-thymidine incorporation of PBMCs obtained prior to MSC-treatment was similar to that of PBMCs obtained after MSC treatment (1 – 12 months). Primary response kinetics of the MLR in day 7 proliferation were higher than day 3 proliferation (day 3 data not shown), indicating that injection of allogeneic MSCs did not result in T cell priming to donor allo-antigens, even after 3 injections of cells from the same donor. None of the animals produced allo-antibody levels that were above the positive cutoff, and no consistent peak in allo-antibody response was observed at the 4-week time point, 1 week after the final injection (Fig. 1).

DISCUSSION AND CONCLUSIONS:
Delivery of allogeneic MSCs to the joint does not generate an immune response in immunocompetent recipients. Allo-antibody production is not elevated after multiple exposures, indicating that allogeneic therapy is clinically useful.

REFERENCES:
A comparison of the different growth factor repertoire and the factor requirement for successful chondrogenesis of MSC’s from bone marrow and adipose tissue


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Introduction: Mesenchymal stem cells (MSCs) can differentiate into various cell types including chondrocytes and they may represent an alternative cell source for the treatment of cartilage defects. Under standard chondrogenic in vitro differentiation protocols MSCs derived from adipose tissue (ATSCs) have a lower chondrogenic differentiation potential compared to MSCs from bone marrow (BMSCs). The aim of this study was to analyze limiting factors in chondrogenic differentiation of ATSCs with respect to endogenous production of growth factors and the requirement of exogenous factors for successful chondrogenesis.

Methods: In vitro expanded BMSCs and ATSCs were expanded in vitro and differentiated under standard chondrogenic conditions (dexamethasone, ascorbic acid) for 6 weeks in micromass culture in the presence of TGF-beta3 and/or other growth factors and inhibitors. Differentiation was assessed by alcian-blue and collagen type II-staining and gene expression was quantified by Real Time-PCR.

Results: Undifferentiated BMSCs differed from ATSCs by expression of mRNA for BMP2, 4, 6, osterix, osteocalcin and PDGF-BB while no differences were obvious for BMP 7, TGF-beta 1, 3, FGF1, FGF2, IGF, CTGF, MATN1, Cbfa1, Sox9, MMP3, MMP13. In addition, BMSC but not ATSC were positive for osterix and osteocalcin indicating that osteoprogenitor cells may be present in BMSC cultures. In contrast to BMSCs, ATSCs required BMP6 besides TGF-beta for successful differentiation during which Col2A1, Col10A1, BMP4 and Indian hedgehog mRNA and alkaline phosphatase enzyme activity were up-regulated. All other tested growth factors could not replace BMP6 in this function.

No difference was evident in the expression of growth factor receptor genes BMPRIA, BMPRIB, and BMPRII which are crucial for BMP signaling. However, in contrast, the TGFbeta receptor subunit Alk-5 was not detected in ATSC while consistent signals were obtained from BMSCs.

Both cultures were positive for ALK-2 and negative for Alk-1 indicating that only one type of TGFbeta receptor, ALK-2/TBRII exists in ATSC while BMSC could form both ALK-2/TBRII and ALK-5/TBRII receptors.

While enhanced concentrations of TGFbeta did not improve chondrogenesis of ATSC, the exogenous addition of BMP2, BMP4 or BMP6 besides TGF-beta improved chondrogenesis of ATSC indicating that signaling via BMP receptors could compensate for a potentially reduced capacity of ATSC to sense TGFbeta.

Conclusions: Discrepant expression of BMP2, BMP4 and BMP6 between ATSCs and BMSCs indicates that these factors may be crucial for chondrogenic differentiation of MSCs and supplementation experiments demonstrate that BMP6 + TGFbeta addition is sufficient and most effective for chondrogenic differentiation of ATSCs. Pilot experiments indicate that BMPs are functionally relevant for in vitro chondrogenesis since the BMP inhibitor noggin is capable to fully block TGFbeta-driven chondrogenesis.

References

Acknowledgements
We thank K. Götzke and R. Föhr for technical assistance.
This work was supported by a grant of the research fund of the Division of Experimental Orthopaedics of Heidelberg.
Suppression of Hypertrophy and Osteogenesis in Committed Human Mesenchymal Stem Cells Cultured on Novel Nitrogen-Rich Plasma Polymer Coatings

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INTRODUCTION: Mesenchymal stem cells (MSCs) are pluripotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament and fat. However, recent evidence indicates that a major drawback of current cartilage- and intervertebral disc-tissue engineering is that human MSCs isolated from some arthritic patients (a clinically relevant source of stem cells) express type X collagen (a marker of chondrocyte hypertrophy associated with endochondral ossification) and osteogenic markers [1]. Some studies have attempted to use growth factors to inhibit type X collagen expression, but none has addressed the possible effect of the chemical composition of the substratum on chondrocyte hypertrophy and osteogenesis. Here, we examine the growth and differentiation potential of human MSCs cultured on extremely N-rich plasma polymer layers, which we call "PPE:N" (N-doped plasma-polymerised ethylene, containing up to 36% [N]).

METHODS: Deposition of PPE:N - PPE:N films were deposited on biaxially oriented polypropylene (BOPP 50 μm thick isotactic polymer film, graciously provided by 3M Company. Films containing 29.5% nitrogen, [N], were deposited from the precursor gas mixture composed of nitrogen (N₂, 10 standard liters per minute, slm) and ethylene (C₂H₄, 10 standard cubic centimeters per minute, sccm). Other PPE:N films with different nitrogen concentrations, [N], were also studied, for comparison. Changing the flow rate of the ethylene gas precursor, F C₂H₄, from the lowest value of 5 sccm to higher values gradually reduces [N]. Thus, for F C₂H₄ = 5 sccm, [N] ≈ 36%, while for the highest F C₂H₄ used here, 20 sccm, [N] ≈ 25%.

Source and preparation of mesenchymal stem cells (MSCs). MSCs were obtained from aspirates from the intramedullary canal of donors (60-80 years of age) undergoing total hip replacement for osteoarthritis, using a protocol approved by the Research Ethics Committee of the Jewish General Hospital. Bone marrow aspirates were processed essentially as previously described [1].

Cell culture

One million of 3 or 4 passage MSCs were cultured on each of the three different PPE:N coatings on BOPP, in DMEM + 10%FBS. Commercial polystyrene (PS) tissue culture dishes (Sarstedt, Inc. Canada) were used as controls. The medium was changed every 2 days for up to 14 days, after which cells were harvested at different times or at the endpoint for gene expression studies.

Polymerase Chain Reaction (PCR)

PCR was performed as previously described [1].

RESULTS: We show that PPE:N almost completely suppresses the expression not only of type X collagen but also of osteogenic marker genes such as alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OC). In contrast neither aggrecan nor type I collagen expression were significantly affected.

DISCUSSION & CONCLUSIONS: These results indicate that PPE:N coatings may be suitable surfaces for inducing MSCs to a chondrocyte or disc-like phenotype for tissue engineering of cartilage or intervertebral disc in which hypertrophy and osteogenesis are suppressed.

REFERENCES:


ACKNOWLEDGEMENTS: This work is supported by the Canadian Institutes for Health Research (CIHR), AO Foundation Switzerland, and the Natural Sciences and Engineering Research Council of Canada (NSERC).
Cells with chondrogenic potential have been isolated from many postnatal tissues including bone marrow, synovial tissue, fat and periosteum. Methods for their isolation, expansion and differentiation have been developed in recent years, with the growing interest in using these cells in tissue engineering applications for skeletal tissue repair and regeneration. In vitro systems for their differentiation have been developed that work well for the examination of the process of chondrogenesis. However, there are many challenges to their use in creating cartilaginous tissues on the scale and of the quality required for in vivo implantation in skeletal pathologies. These challenges include controlling the differentiation to create chondrocytes that do not go on to hypertrophy, as would be found in permanent cartilage. The elaboration of the correct extracellular matrix, both in content and organization is also difficult to control. Furthermore, the size of implants required to fix human skeletal tissue defects means that scaffolds to hold cells and shape the tissue regenerate are needed.

In recent years, we have developed tissue culture systems that allow for expansion of large numbers of cells with reparative potential. We have also experimented with scaffolds of various types, examining cell behavior and matrix production. Many scaffolds have been explored for their potential usefulness in tissue engineering of cartilage. We have reported on the use of hyaluronan/collagen composite scaffolds and their usefulness in conjunction with adult human bone marrow-derived stem cells in vitro, and with these cells of several animal species, both in vitro and in vivo. These biocompatible and biodegradable scaffolds are an example of the sponge and mesh-like scaffolds that have potential clinical use. There are also scaffolds that are solidified from an initial aqueous solution that allows cell mixing before creation of the scaffold. Derivatives of poly(ethylene oxide) (PEO) are widely utilized in these hydrogels due to their hydrophilicity, biocompatibility, and intrinsic resistance to cellular adhesion and protein adsorption. Although there are reports of chondrogenesis of mesenchymal progenitor cells within this type of scaffold, the production of clinically useful load-bearing cartilage with sufficient matrix production remains an issue. We have developed a photopolymerizable semi-interpenetrating network composed of PEO and PEODA in which the extensive chondrogenic differentiation of bone marrow-derived mesenchymal progenitor cells can be achieved in a hydrogel of clinically significant size (Figure 1). Many factors can influence cell viability, differentiation and matrix elaboration in these gels. We have explored the relationship of crosslink size, density, gelation time, interpenetrating network size and also cell-related factors to the extent of chondrogenic differentiation that occurs in this type of gel. In the optimized gels, chondrogenic differentiation occurred in all areas of the cell-seeded constructs with extensive elaboration of a cartilaginous matrix throughout the construct. This photopolymerizing system may have applications in the repair of cartilaginous tissues.

![Figure 1](image_url)

*Figure 1* Sections of cultured cell-seeded constructs stained with toluidine blue (A-D), after 3 (A,B) and 6 (C,D) weeks in culture. Immunohistochemistry for type II collagen (E,F), under permissive (E) or non-permissive conditions (F).
Chondrogenic Differentiation in Adipose Tissue Derived Mesenchymal Stem Cells: Effects of Growth Factors and PTHrP

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INTRODUCTION: Due to their remarkable abilities in proliferation and differentiation mesenchymal stem cells are an attractive cell source for application in tissue engineering and regenerative therapy. Special interest has been paid on mesenchymal stem cells originating from adipose tissue (ATSC) as they are easily available in great amounts. Though many similarities to bone marrow derived mesenchymal stem cells (BMSC) have been reported, the chondrogenic differentiation potential of ATSC is inferior to that of BMSC using common TGF-ß driven protocols (Winter et al. 2003). Further, chondrogenic induction of BMSC with standard induction medium results in a hypertrophic phenotype. The aim of this study was to improve the chondrogenic differentiation of ATSC without inducing hypertrophy by applying different growth factors and growth factor combinations.

METHODS: ATSC were derived by liposuction surgery after informed consent. After expanding the cells for up to 8 passages in a standard expansion medium, differentiation was induced by incubation in high density pellet culture in serum-free medium containing ascorbate and 10ng/ml of TGF-ß, BMP2, 4, 6, 7, FGFa, FGFb, IGF-1 or PThrP, respectively. The same growth factors were also applied in combination with TGF-ß (10ng/ml). Success of chondrogenic differentiation was analyzed by immunohistological staining and gene expression profiles were determined by cDNA-array analysis and real-time RT-PCR.

RESULTS: The combination of TGF-ß and BMP6 was most effective for chondrogenic induction of ATSC. Though the combination of TGF-ß with BMP2 or BMP4 also resulted in proteoglycan deposition and collagen type II immunostaining of ATSC spheroids, it did not lead to a complete differentiation. Both, TGF-ß and BMP6 had to be administered simultaneously for successful chondrogenesis. The gene expression profile of ATSC induced with TGF-ß and BMP6 was similar that of BMSC induced with TGF-ß showing expression of cartilage relevant molecules like Col2, aggrecan, CRTL-1, COMP, PRELP and fibromodulin. Like BMSC, ATSC showed an early upregulation of hypertrophic markers like Col10A1 and enhanced alkaline phosphatase (AP) activity from day 14 on. When PThrP, a known inhibitor of chondrocyte hypertrophy, was added to the TGF-ß and BMP6 supplemented chondrogenic induction medium, there was no immunostaining for collagen type X and no upregulation of AP enzyme activity over 6 weeks. There was, however, also no staining for collagen type II, indicating an inhibition of chondrogenic differentiation. Interestingly, mRNA of Col2 and Col10 was still detected after addition of PThrP.

DISCUSSION & CONCLUSIONS: Application of TGF-ß and BMP6 completely eliminated the reduced chondrogenic potential of ATSC. Like BMSC they can be differentiated into chondrocyte like cells, which, however, express hypertrophic markers. Attempts to suppress the hypertrophic phenotype by PThrP prevented the differentiation of ATSC and BMSC to chondrocyte like cells. Addition of PThrP to standard chondrogenic induction media, thus, is no means to obtain phenotypically stable chondrocytes known from articular cartilage.


ACKNOWLEDGEMENTS: We thank K. Goetzke and R. Föhr for technical assistance. This work was supported by a grant of the research fund of the Division of Experimental Orthopaedics of Heidelberg.
Biochemical Markers of the Mechanical Quality of Engineered Hyaline Cartilage

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INTRODUCTION: The mechanical properties of articular cartilage are dependent on the composition of the extracellular matrix. However, it is still unclear which extracellular matrix proteins are the most appropriate indicators of the mechanical properties of tissue-engineered cartilage. The aim of this study was to determine which biochemical markers can be used as surrogate measures of the mechanical quality of engineered cartilage. We modified the matrix composition of engineered cartilage by forming constructs from scaffolds seeded with varying numbers of chondrocytes or by seeding with a single cell concentration/scaffold and varying the length of time constructs were cultured. We then compared changes in GAG, total collagen (by measurement of hydroxyproline), collagen I and II concentrations and collagen cross-links with the mechanical properties of the constructs.

METHODS: Chondrocytes were isolated as described previously [1]. Non-woven HYAFF¹¹ scaffolds (2mm depth, 5mm diameter, Fidia Advanced Polymers, Italy) were seeded dynamically with 2, 4, 8, or 16 x 10⁶ chondrocytes and cultured for 42 days as described previously [1]. 3mm cores were taken and tested under confined compression. A ramp displacement corresponding to 10% strain at a ramp speed of 0.001 mm/sec was applied. Two subsequent ramp displacements of 5% were then applied to give a total strain of 20%. The cores were analyzed [2] to determine proteoglycan content (measured as glycosaminoglycan (GAG), by calorimetric assay using dimethylmethylene blue) total collagen (measured as hydroxyproline by amino acid analysis), collagens I and II (measured by inhibition ELISA) and collagen cross-links (measured by amino acid analysis). For experiments varying the time in culture, the HYAFF¹¹ scaffolds (2mm depth, 8mm diameter) were seeded with 16 x 10⁶ chondrocytes and cultured for 20, 30, 40 or 80 days [1]. The constructs were tested under non-confined compression using the described loading regimen.

RESULTS: All the engineered constructs formed an extensive extracellular matrix with hyaline characteristics. In experiments changing the number of chondrocytes seeded, the aggregate modulus correlated positively with the percentage matrix composition of both GAG (P<0.0001, r=0.5737 to 0.9147) and collagen II (P<0.0001, r=0.6215 to 0.9261) but not with collagen I content. Varying the length of culture showed that Young’s modulus increased over the culture period and correlated positively with GAG (P<0.0001, r=0.3926 to 0.7375), collagen II (P<0.0001, r=0.48268 to 0.7557), ratio of mature to immature collagen cross-links (P=0.0001, r=0.2802 to 0.6739). No correlation was found between Young’s modulus and matrix hydroxyproline.

CONCLUSIONS: The results suggested that measurement of collagen II and GAG are good predictive markers of the mechanical quality of tissue-engineered hyaline cartilage. The lack of correlation of Young’s modulus with hydroxyproline concentrations suggested that collagens other than collagen II were a significant matrix component of these relatively immature engineered cartilage constructs.


ACKNOWLEDGEMENTS: This project was supported by the European Commission under the 5th Framework Programme (SCAF CART project GRDR1-1999-00050.)
Cell-based approaches to joint surface repair: from bench to bedside
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INTRODUCTION: Biological repair of skeletal tissues such as articular cartilage and bone is intensely pursued. Mesenchymal stem cells (MSCs) are very attractive as they can be extensively expanded in culture while maintaining their multilineage potential towards mesenchymal tissues.

We have identified and characterized multipotent MSCs from the adult human synovial membrane (SM), an easily accessible and rapidly self-renewing tissue. Regardless of donor age, culture-expanded SM-MSCs can differentiate at the single cell level to cartilage, bone, adipocytes, and skeletal muscle (1-3).

Another source of adult human MSCs is the periosteum (Pe). We have recently reported that human periosteal cells, commonly regarded as chondro-osteogenic (4), display mesenchymal multipotency that is inherent at the single cell level, regardless of donor age (5). To investigate chondrogenic potential in vivo, we implanted PKH26-labeled expanded autologous periosteal cells in an experimental joint surface defect under a periosteal flap in the goat. At 3 weeks after implantation, there was no repair, with delamination of the periosteal flap. However, a cartilage tissue was histologically evident at the margins of the defect. In this area we detected clusters of cells displaying double fluorescence for PKH26 and type II collagen, thus providing proof of concept that a proportion of the implanted periosteal cells can undergo chondrogenesis in vivo.

Our recent findings also indicate that MSCs from SM and Pe display a strikingly different propensity to form either cartilage or bone. This biological variability of MSC populations is likely to affect the outcome of clinical applications. Pe-MSCs differentiate more rapidly and efficiently towards bone than SM-MSCs, a property that is inherent at the single cell level. Following a molecular screening of single-cell-derived clonal populations, we have developed an algorithm that predicts amount of bone formation, regardless of donor age or MSC source. This algorithm could be used to quality control MSC populations in pre-clinical and clinical studies of bone repair. Our study constitutes an additional step towards establishing MSC populations with consistent and reproducible biological behaviours, quality-controlled for specific therapeutic applications (6).

We are currently investigating the niche(s) of MSCs in vivo within the joint environment using animal models. The elucidation of the molecular regulation of “joint stem cell niches” may lead to the development of technologies to activate and direct resident stem cells in vivo, without ex vivo manipulations, to achieve joint surface repair and restore joint homeostasis. This would address a growing concern in cell-therapies that ex vivo manipulations, such as culture expansion, are associated with phenotypic instability, difficult upscaling, high costs, and variability (6).

REFERENCES:
Cell Sheet Technology for Cartilage Repair

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Introduction
The repair of the partial thickness defect of articular cartilage fills the one of the requirements for the future treatment of Osteoarthritis. We used the temperature-responsive culture dishes, which show both hydrophilic and hydrophobic property alterations in response to temperature change, because of coating with N-isopropyl-acrylamid polymers. Cultured cells reach to confluent at 37°C and only reducing the temperature from 37°C to 20°C, and they are detached from the surface without any enzymatic treatment because the surface property becomes more hydrophilic (Fig.1). We already reported chondrocyte sheet, synovial cell sheet, and anterior cruciate ligament sheet were successfully collected. Chondrocyte sheets have good adhesion to the partial thickness defect of rabbit articular cartilage. The purpose of this study was to clarify the property of layered chondrocyte sheet compared to conventional monolayer culture, and to examine the potential of cell sheet engineering as a new treatment for the repair of partial thickness defect of articular cartilage, as a means to directly care for the osteoarthritis.

Materials and Methods
Consent was obtained from 10 patients who were to undergo reconstruction of the anterior cruciate ligament of the knee. During the surgery, synovial membrane and cartilage were obtained and enzymatically treated to isolate the cells. Temperature-responsive culture dishes were then seeded with a high cell concentration (more than 10000 cells/cm²), and cell sheets were prepared (Fig.2). The cell sheets were then layered. To analyze the phenotypic expression of layered cell sheets and adhesive factors, single-layered cultured cells prepared according to the conventional methods and multi-layered cell sheets were examined using microarrays and real time PCR.

Results & Discussions
Compared with conventional single-layer culture methods, greater expression of proteins such as fibronectin1, collagen type II, aggrecan, and TIMP 1, which are related to adhesion and to synthesis of extracellular matrix., was seen with layered cartilage cell sheets. In addition, activation was confirmed of genes such as SOX9 and HAS, which are important in maintaining articular cartilage characteristics.

Fig. 1. Cell Sheet Technology

Good adhesiveness was also seen ex vivo with layered cell sheets, which adhered and grafted to the damaged portion of the joint. From the above we considered that layered cell sheets function as a barrier that protects the surface of damaged cartilage from enzymes existing within the joint and prevents defluxion of proteoglycans from the damaged area, thereby contributing to repair and regeneration of partial thickness defect of articular cartilage (Fig.3). We are now studying to develop the focal gene delivery system using this adhesiveness of the cartilage cell sheet.

- Easy to culture and proliferate
- Good adhesion
- Barrier function:
  - Protection of intra-articular enzyme
  - Prevention of proteoglycan escape
- Cell & cell sheet manipulation:
  - Layered sheets of chondrocytes, synovial cells, etc.
  - Focal gene delivery

Fig. 3. Advantages of Cell Sheet

Conclusions
1. The cell sheet adheres well to the surface of articular cartilage.
2. The cell sheet technology and focal gene delivery might be a useful tool for the treatment of partial thickness defect of articular cartilage.
INTRODUCTION: Formation of multicellular aggregates is of particular interest, since aggregates show not only morphological but also functional similarities to tissues and organs; unlike conventional monolayer cell cultures [1-2]. In this study, a new method of making (histologically) homogeneous cell aggregates [3] is introduced. In this method, single cells are cultured on micro-patterned non-adhesive substrate. The purpose of this study is to evaluate the performances of this method using chondrocytes and pitted substrate as an example.

METHODS: [Micro-Folding Culture] Micro-patterned polydimethylsiloxane (PDMS) substrates with two-dimensional arrays of pits were prepared by using simple soft-lithography processes. Depth of the pit was approximately 100µm. Two types of substrates with different pit diameter were prepared: Large; D =200µm, S=80µm, Small; D=100µm, S=40µm, D represents diameter of pit, S represents spacing between pits. Chondrocytes were harvested from humerus, femur and tibia of 4-weeks-old Japanese white rabbits. These chondrocytes were seeded into pits and cultured for 48 hours. [Cartilage regeneration] Aggregates were inoculated into the fibroin sponge at a cell concentration of 1.25x10⁷ cells/ml. Fibroin sponge (diameter: 8 mm, thickness: 1±0.2 mm) made of fibroin hydro gel was used as a scaffold for cartilage regeneration. [Chondroitin sulfate assay] Chondroitin sulfate was measured by using DMB method.

RESULTS: Chondrocytes seeded into the pits of micro-patterned substrate formed multicellular aggregates (Fig. 1). Mean diameter of aggregate was 56.3µm (Small) and 84.8µm (Large). Amount of ECM synthesis was shown in Fig. 2, where the small-aggregates group tends to show higher ECM synthesis. This advantage become clearer after serial subcultivation (when passage-2 cells were used).

DISCUSSION & CONCLUSIONS: Diameter of aggregate could be controlled by using substrates having pits of different diameter. The aggregates made by the Micro-Folding Culture showed higher performance of ECM synthesis. This tendency seems to be more prominently seen when proliferated cells were used. We are now evaluating the tribological functions of the Micro-Folding Culture derived cartilage tissue.


ACKNOWLEDGEMENTS: We are grateful to Prof. H. Kotera and Mr. H. Yamamoto for their assistance and helpful suggestions to make micro-patterned substrates.
INTRODUCTION: Recent animal experiments have demonstrated that engineered cartilage tissues generated by culturing chondrocytes into 3D scaffolds provide functional templates for the orderly repair of critically sized osteochondral lesions. In order to reproducibly generate functional cartilage tissues starting from adult human cells, efforts have to be directed not only to the identification of stimulatory biochemical factors, but also to the development and use of controlled bioreactor systems, applying defined regimes of physical forces. In this work, we present some examples on the use of bioreactors for processes that are key for engineering of 3D cartilage tissues based on cells and scaffolds, namely the chondrocyte seeding into porous scaffolds, their efficient nutrition, and the physical conditioning of the developing tissues.

CHONDROCYTE SEEDING AND CULTURE UNDER PERFUSION: In the cell seeding process, cells must be utilized with maximum efficiency to minimize the biopsy size needed and/or the extent of cell expansion, and must be dispersed uniformly throughout the scaffold volume to form the basis for uniform tissue formation. To overcome limitations associated with the most commonly employed seeding techniques, we developed a bioreactor for the automated cell seeding of three-dimensional scaffolds by continuous perfusion of a cell suspension through the scaffold pores in oscillating directions. Perfusion seeding of chondrocytes into Polyactive foams (IsoTis OrthoBiologics, NL) or Hyaff®-11 non-woven meshes (Fidia Advanced Biopolymers, IT) resulted in the highest fraction of viable cells within the foam pores, the greatest efficiency of seeding and the highest uniformity of cell distribution in comparison to the typically used static and spinner flask methods.

Constructs uniformly seeded by perfusion and then cultured statically for 2 weeks were highly heterogeneous in structure, consisting of a layer of cells and matrix at the periphery and an essentially void interior region. Instead, constructs cultured under prolonged perfusion were remarkably homogeneous, containing a uniform distribution of both cells and matrix throughout the cross-section.

PHYSICAL CONDITIONING OF CARTILAGE CONSTRUCTS: Application of dynamic compression to cell-polymer constructs could potentially improve the development of cartilaginous tissue in vitro. We exposed human articular chondrocytes-based cartilaginous constructs at different stages of maturation, as defined by the glycosaminoglycan (GAG) content, to intermittent compressive deformation for 3 days. Compression-induced changes in GAG synthesis and accumulation were positively correlated to the GAG content prior to loading, such that compression was stimulatory only for the most developed constructs. Therefore, under our experimental conditions, cyclic loading appears to be applicable for the enhancement of cartilaginous tissue development only in the late phases of tissue regeneration. Our results also point out the possible use of bioreactors applying defined regimes of physical forces as a quality control tool for engineered cartilage, with the goal of defining when the tissues are sufficiently developed for immediate load bearing after implantation.

CONCLUSION: The reviewed studies indicate that bioreactors enable generation of cartilaginous tissue constructs and may contribute to understand the function of specific chemico-physical culture parameters on cartilage tissue development. In the future, bioreactors are expected to efficiently translate laboratory- to industrial-scale cartilage tissue engineering, possibly providing an economically viable approach to the automated manufacture of functional cartilage grafts for broad clinical use.

Transplantation of nasal chondrocytes with an innovative self-setting hydrogel for cartilage repair
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INTRODUCTION: Cartilage is a specialized connective tissue that consists of relatively few cells embedded in an extracellular matrix. It is well known that the intrinsic repair capacity of articular cartilage is limited. To promote the repair of this tissue, amplification and transfer of autologous chondrocytes using a three-dimensional matrix appear promising. In this attempt, we developed a self-setting and three-dimensional matrix consisting of a silanized cellulose derivative (Si-HPMC). In previous works, we showed that Si-HPMC enabled the proliferation of articular chondrocytes and the expression of the main chondrocytic markers during a three-dimensional culture in vitro. The aim of the present work was to determine whether this hydrogel could be a suitable scaffold for nasal chondrocytes-based cartilage engineering. We therefore tested the ability of nasal chondrocytes associated with Si-HPMC to allow the formation of a cartilaginous tissue in nude mice subcutaneous pockets and rabbit articular cartilage.

MATERIALS AND METHODS: The synthetic scaffold consisted of a reticulated hydrogel of silanized hydroxypropylmethyl cellulose (Si-HPMC). Primary chondrocytes isolated from human nasal cartilage (HNC) were investigated for cellular viability (MTS assay) and proliferation assay in contact with the hydrogel. To assess cell viability within hydrogel, cells were stained with cell tracker green (CTG) and observed in confocal microscopy. To assess the influence of Si-HPMC on chondrocytic phenotype, specific hallmarks of chondrocytes (type II Collagen and aggrecan) as well as type I collagen were investigated at the level of mRNA using RT-PCR. The sulfated glycosaminoglycans (GAG) synthesis in three-dimensional culture was investigated by Alcian blue staining. The ability of Si-HPMC to form a cartilaginous tissue was evaluated by in vivo transplantation of human nasal chondrocytes associated with Si-HPMC in subcutaneous site of nude mice for three weeks. The preclinical interest of nasal chondrocytes transplantation was thereafter investigated in rabbit. Rabbit nasal autologous chondrocytes (RNAC) were amplified in vitro during 4 weeks before transplantation with Si-HPMC in defects created in rabbit articular cartilage for 6 weeks. Implants were histologically characterized for the presence of GAG (Alcian blue staining) and collagen (Masson's trichrome staining). Cell morphology was observed with haematoxylin/eosin staining. The presence of type II collagen was investigated by immunostaining.

RESULTS: Results showed that our hydrogel enabled the proliferation of HNC, the production of sulphated GAG and the expression of the main chondrocytic markers during a three-dimensional culture in vitro. Histological analysis of human nasal chondrocytes transplanted in nude mice during three weeks revealed the production of a cartilage-like extracellular matrix containing GAGs and collagen. The presence of chondroid nodules positively stained for type II collagen confirmed the formation of a hyaline cartilaginous tissue.


ACKNOWLEDGEMENTS: This study was supported by grants from “Association de Recherche pour la Polyarthrite Rhumatoïde”, “Société Française de Rhumatologie”, “ANR-young researcher”. CV received a fellowship from the French Ministry of Research and Technology. Authors also thank Guy Daculsi and Olivier Malard for their fruitful contribution.
Nasal Chondrocytes And Fibrin Sealant For Cartilage Tissue Engineering

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**INTRODUCTION:** Articular cartilage is a specialized connective tissue with highly limited self-renewal capacity. To promote cartilage repair, the transfer of autologous articular chondrocytes using biomaterials has been proposed. Fibrin sealant has been considered as a three dimensional matrix for delivering articular chondrocytes in hyaline cartilage repair procedures. With respect to the donor site morbidity occurring after harvesting articular cartilage, the use of alternative chondrocyte sources are also currently considered. The aim of the present work was to determine whether nasal chondrocytes associated with fibrin sealant could be a promising strategy for cartilage tissue engineering. In a first set of experiments, we questioned whether human nasal chondrocytes associated with fibrin sealant could allow the formation of a cartilaginous tissue in subcutaneous pockets in nude mice. Thereafter, we sought to evaluate the preclinical interest of nasal autologous chondrocyte transplantation using fibrin sealant in rabbit articular cartilage defects.

**MATERIALS AND METHODS:** Fibrin sealant (Tisseel\(^\circledR\)) was from Baxter and consisted of fibrinogen/aprotinin and thrombin. The ability of human nasal chondrocytes associated with fibrin sealant to form a cartilaginous tissue was first evaluated by transplantation in subcutaneous pockets of nude mice for three weeks. Implants were histologically characterized for the presence of GAG (Alcian blue staining) and collagen (Masson's trichrome staining). Cell morphology was observed with haematoxylin/eosin staining. The presence of type II collagen was investigated by immunostaining. The preclinical interest of nasal autologous chondrocytes transplantation with fibrin sealant was thereafter investigated in rabbit. Primary rabbit nasal chondrocytes (RNC) were isolated from nasal septa. For cellular viability and proliferation assay, RNC were cultured in contact with fibrin sealant and a Methyl Tetrazolium Salt (MTS) assay was performed. To assess the influence of fibrin sealant on chondrocytic phenotype, specific hallmarks of chondrocytes (type II Collagen and aggrecan) were investigated at the level of mRNA using RT-PCR. Rabbit nasal autologous chondrocytes were finally implanted using fibrin sealant in defects created in rabbit articular cartilage. After 6 weeks, implants were histologically analysed as described above.

**RESULTS:** Histological analysis of human nasal chondrocytes transplanted in nude mice during three weeks revealed the production of a cartilage-like extracellular matrix containing GAGs and collagen. The presence of a positive staining for type II collagen confirmed the formation of a hyaline cartilaginous tissue. Our results also showed that cellular viability of rabbit nasal chondrocytes was maintained in contact with fibrin sealant. In addition, the expression of type II collagen and aggrecan were up-regulated after a three-dimensional culture in fibrin sealant as compared to a monolayer-type culture. Histological analysis of autologous rabbit nasal chondrocytes transplanted in an articular cartilage defect revealed the formation of a repair tissue presenting an extracellular matrix containing sulfated GAG and collagen. Immunohistological analysis of type II collagen showed that the repair tissue was a hyaline-like cartilage.

**DISCUSSION & CONCLUSION:** Taken together, our results indicate that nasal chondrocytes could be an alternate cell source for cellular therapy of articular cartilage. Our data also strongly suggest that fibrin sealant is a promising scaffold for the transfer of chondrocytes. Long-term in vivo experiments and biomechanical assays are now required to further consider the association of nasal chondrocytes with fibrin sealant as a potential strategy for cartilage repair.

**ACKNOWLEDGEMENTS:** This study was supported by grants from Baxter Biosciences Biosurgery and INSERM U 791. C. Vinatier received a fellowship from the French Ministry of Research and Technology.
Gene transfer offers the possibility of delivering chondrogenic factors to sites of cartilage damage in a sustained, cost-effective and efficacious manner. Depending upon the sophistication of the construct, more than one transgene may be delivered and levels of transgene expression may be independently regulated. The strategies employed will differ depending on the size of the lesion and whether it is an isolated injury in an otherwise healthy joint or part of the degenerative process associated with osteoarthritis.

Gene transfer to synovium is technically quite straightforward and has already been achieved in human clinical trials of arthritis. In the context of cartilage repair, it is a convenient intra-articular depot from which to synthesize secreted growth factors that can influence the metabolism of articular cartilage by diffusion. This has been demonstrated for IGF-1 and BMP-2 in rabbits’ knees. However, delivery of TGF-β1 in this fashion has serious adverse effects.

Disadvantages of gene delivery to synovium include is its failure to increase the cellularity of the cartilaginous lesion and its limitation to secreted, diffusible gene products. Ex vivo protocols permit the implantation of genetically modified chondrocytes or chondroprogenitor cells that can, if required, be engineered to express increased levels of intracellular proteins, such as transcription factors. Although successful in animal models, this strategy involves cell culture and scaffolding issues that render it tedious and uneconomical in clinical practice. Two approaches are being developed to obviate these limitations. Madry and colleagues have pioneered the direct application of recombinant adeno-associated virus vectors to cartilaginous lesions at the time of repair by subchondral drilling. By this means, genes are transferred to chondroprogenitor cells as they enter the defect via the subchondral bone. Using a rabbit model, they have reported success with various transgenes, including IGF-1 and FGF-2. In a related approach, we have removed bone marrow from rabbits, allowed it to clot in the presence of viral vectors carrying chondrogenic genes, and inserted the resulting autologous “gene plug” into full-thickness lesions in articular cartilage. Encouraging preliminary data have been obtained.

For lesions in osteoarthritic joints, where repair is attempted in the presence of a disease process, it may be advantageous to combine the synovial delivery of a cDNA encoding IL-1Ra with intra-lesional delivery of a chondrogenic cDNA.
PRG4/LUBRICIN/SZP AND THE SLIPPERY SIDE OF CARTILAGE

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INTRODUCTION: Proteoglycan-4 (PRG4), lubricin, superficial zone protein (SZP), and megakaryocyte stimulating factor (MSF) are synonyms for a proteoglycan which provides lubricating properties within synovial joints. It is synthesized by cells lining synovial joints including synovial cells, superficial zone chondrocytes and tendon cells. The lubricating properties of the molecule have been studied by several laboratories including those of Swann¹, Jay², Sah³, Bonasser⁴ and my own. Here we report that a custom tribometer using a cartilage-plastic interface is able to detect dramatic changes in the coefficient of friction when microgram quantities of PRG4 are coated on the plastic surface and tested in a PBS solution.

METHODS: PRG4 was purified from human synovial fluid by a combination of ion exchange, gel filtration and affinity chromatography. PRG4 was coated on Falcon tissue culture dishes (35 mm diameter) in 2 ml of 50 mM sodium carbonate buffer pH 9.0 for 18 h at room temperature. Unbound PRG4 was removed by washing in PBS and the plates were tested in PBS. Additional plates were coated with bovine serum albumin (BSA) or hyaluronan. In other experiments the PRG4 was reduced with various concentrations of dithiothreitol and alkylated with an excess of iodoacetamide before coating the plates. The ability of PRG4-coated plates to lower the coefficient of friction was tested in a custom pin-on-disc tribometer using an adult rabbit phalangeal bone with its articular cartilage opposed to the coated plastic surface. All tests were performed in PBS. The normal force was 0.4 MPa and the frictional force measured with a load cell. Tests were performed at several sliding velocities for 5 or 10 minutes each.

RESULTS: Falcon tissue culture dishes were coated with purified PRG4 at different concentrations and the coefficient of friction measured between rabbit articular cartilage and the coated plastic surface in PBS. Figure 1 shows low concentrations of PRG4 in the coating solutions were unable to lubricate the cartilage-plastic interface. However a threshold coating concentration is reached between 3-4 ug per plate which caused a dramatic reduction in the coefficient of friction. When petri dishes or Primaria plates were coated, more PRG4 bound to the plates and lower coefficients of friction were obtained. This lubrication assay was used to test if reduction and alkylation affected the lubricating activity of PRG4. Titration experiments showed 20 mM dithiothreitol treatment almost completely eliminated the lubricating activity of PRG4 in these assays.

DISCUSSION & CONCLUSIONS: PRG4 has been shown to reduce the coefficient of friction in cartilage-glass¹,⁴, rubber-glass² and cartilage-cartilage³ test systems. Usually the [PRG4] needed to produce this effect is relatively high (50-450 ug/ml)¹⁴. We found very low concentrations (~5 ug/ml) were necessary to coat plastic surfaces and generate similar effects. When PRG4 was reduced and alkylated it lost its ability to lubricate the cartilage-plastic interface. This lubrication assay should be useful to dissect the molecular features of PRG4 which support its lubricating function.


ACKNOWLEDGEMENTS: This work was supported by NIH grant AR050457.
Articular Motion Induces SZP in Chondrocytes-Seeded 3-D Scaffolds: Link between Joint Motion and Joint Lubrication?

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INTRODUCTION: Mechanical loading has widely been used as a stimulus to support the development of cartilage-like constructs in tissue engineering applications in vitro. In particular, dynamic compressive loading, shear stresses and hydrostatic pressure can modulate the metabolic activity of chondrocytes and have been employed in various systems. However, the kinematics of natural joints in vivo are complex as is the composition and structure of articular cartilage, and therefore one simple loading regime may not sufficiently stimulate the generation of functional tissue from cartilage cells in vitro.

To achieve its range of functionality, articular cartilage is organized with zone differences in matrix composition, matrix architecture, and cellular metabolism. It is likely that these different regions may require distinct types of mechanical stimulation, depending on their functions. In particular, superficial zone protein (SZP) plays essential roles in maintaining proper joint lubrication and in providing a protective barrier for the cells in the deeper zones of the cartilage. SZP, also known as megakaryocyte stimulating factor (MSF), or proteoglycan 4 (PRG4), is a glycoprotein that is synthesized and secreted by superficial zone chondrocytes and synovial fibroblasts and has been shown to be homologous to lubricin. Despite the perceived importance of SZP on proper joint function, the effects of physical forces on its synthesis are not known. Therefore, a novel bioreactor has been designed to approximate the movements and surface motion characteristics of natural joints. In particular, this bioreactor creates interface motion with crossing motion trajectories by oscillation of a ceramic ball on the surface of the specimen or of ball and specimen in phase difference. This causes a complex shear force pattern on the specimen’s surface and is intended to mimic the characteristics of the natural joint. Using this novel bioreactor, the specific effect of applied articular motion on the gene expression and on the release of SZP by chondrocytes seeded onto polyurethane scaffolds was investigated.

METHODS: Cylindrical porous polyurethane scaffolds were seeded with bovine articular chondrocytes and subjected to static or dynamic compression, with or without articulation against a ceramic hip ball and/or rotation around the perpendicular axis. After loading, the mRNA expression of SZP and its immunoreactivity of conditioned media was also determined.

RESULTS: Uni- or multidirectional (bi-axial) articular motion significantly up-regulated the mRNA expression of SZP, whereas axial compression alone had no effect on SZP mRNA levels. MSF (SZP) immunoreactivity of conditioned media of constructs exposed to articular motion was markedly enhanced compared to media of unloaded controls.

DISCUSSION: These results indicate that specific stimuli that mimic the kinematics of natural joints, may promote the development of a functional articular surface - synovial interface. Complex motion trajectories, as they occur in vivo in the joint, may provide beneficial stimuli for chondrocyte-seeded scaffolds. Although the character of the motion trajectories is greatly simplified and no attempt was made to model any specific joint, our study demonstrates that applied surface motion is an important mediator for maintaining the homeostasis of the cartilage-synovial interface, in particular for the synthesis of molecules involved in joint lubrication. Loading systems that approximate natural joint kinematics more closely may help in identifying mechanical stimuli for in vitro tissue engineering or for in vivo application for repair and regeneration of joint surfaces during rehabilitation protocols.

ACKNOWLEDGEMENTS: This work was in part supported by the SNSF (grant # 3200B0-104083).
FRICCTIONAL PROPERTIES OF ENGINEERED CARTILAGINOUS TISSUES IN BOUNDARY LUBRICATION

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INTRODUCTION: Frictional properties of cartilaginous engineered tissues have not been thoroughly investigated despite being a major function of cartilage. Lubricin is a component of synovial fluid shown to lubricate cartilage in boundary lubrication in a dose dependant manner similar to that of synovial fluid (SF)\(^1,2\). Removal of lubricin from cartilage with salt increases the equilibrium friction coefficient (\(\mu_{eq}\))\(^3\). The objectives of this study were to determine 1) the inherent friction coefficient of engineered tissues in boundary lubrication 2) the ability of these tissues to be lubricated by SF and 3) the reversibility of SF lubrication by removal of lubricin with a high salt solution.

METHODS: Equine sternal mesenchymal stem cells (MSC), primary bovine chondrocytes (CON) and meniscal fibrochondrocytes (MEN) were encapsulated in alginate disks (25x10\(^6\) cells/ml) utilizing standard protocols\(^4\). The constructs were placed in culture with standard culture media supplemented with 10% FBS, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. MSC media was supplemented with 5 ng/ml TGF-\(\beta\)\(^1\) to enhance chondrogenic differentiation. Engineered constructs were cultured for 0, 2, 4, 6 weeks and frozen for later friction testing. Friction testing: Engineered tissue constructs were tested in a custom friction apparatus utilizing PBS as a lubricant in boundary lubrication. The instrument linearly oscillated the sample at 0.32 mm/sec against glass with an imposed 20% normal strain and \(\mu_{eq}\) was calculated\(^1\). Samples were thawed in PBS and friction coefficients were measured (PBS). Each sample was then incubated in equine synovial fluid for 1 hour and rinsed with PBS followed with friction testing in PBS (ESF Soak). Following the 2\(^{nd}\) friction testing, the samples were then incubated in 1.5M NaCl in PBS for 5 min and then equilibrated in standard PBS for 1 hour. Exposure to 1.5M NaCl has been shown to extract lubricin from the surface of cartilage\(^3\). Friction coefficients were then measured for a third time (ESF + 1.5M Extract).

RESULTS: The equilibrium friction coefficient was the same for all engineered constructs from all cell types over 6 weeks in culture. Incubating of the engineered tissues in ESF (testing in PBS) decreased friction coefficient over culture time by approximately 20% in MSC generated cartilage and 50% in both CON and MEN generated tissue. The friction coefficient increased and returned to values similar to unincubated tissue following the extraction protocol with 1.5M PBS.

DISCUSSION & CONCLUSIONS: Changes in \(\mu_{eq}\) are minimal with time in culture, suggesting insufficient production and localization of lubricin by three cell types. Constructs from all cell types are lubricated by equine synovial fluid at later culture times and lubrication is reversed with 1.5M NaCl, suggesting production of ECM components that can localize lubricin. The ability to localize lubricin seen by a decrease in \(\mu_{eq}\) is cell type dependant with CON and MEN similar but greater than MSC generated tissues.

REFERENCES:  

ACKNOWLEDGEMENTS: NASA GSRP NNG-04GN57H (Gleghorn), Wyeth Research.
FRICITION REDUCING PROPERTIES OF LUBRICIN (PRG4): A NANOSCALE STUDY OF CHONDROPROTECTION

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INTRODUCTION: Lubricin (PRG4) is a classical mucinous glycoprotein [1] providing boundary lubrication of apposed synthetic and cartilaginous surfaces in the absence of viscosity. Cartilage bearings lubricated by synovial fluid possess a very low coefficient of friction \( \mu \approx 0.001 \) [2] which is attributable to lubricin [3] and interstitial fluid pressurization [4]. Lubricin works in concert with hyaluronate where it regulates the mesh size of the polymer network and also synergistically lowers \( \mu \). Although the tribological properties of deposited synovial fluid and lubricin films have been studied on the macroscale, the details of the boundary lubrication mechanism are poorly understood.

METHODS: Friction measurements were conducted by lateral force microscopy between \( \omega \)-substituted alkane thiol modified surfaces in the presence of physisorbed lubricin.[5] Specifically, friction was measured between colloidal probes (Figure 1) and surfaces functionalized with self assembled monolayers (SAMs) of hydroxyl or methyl terminated thiols. The objective here was to study the effect of lubricin on friction in absence of fluid pressurization on model surfaces that recapitulate the salient chemical features of the superficial surface of cartilage.

RESULTS: Surface plasmon resonance studies showed that on the OH-terminated thiol surface a monolayer formed at a lubricin concentration of \( \approx 200 \mu g/ml \) (measured between OH-terminated thiol surfaces) (Figure 2). The addition of hyaluronate to lubricin lowered \( \mu \) (~0.020) compared to lubricin alone on the methyl-terminated thiol surface.

DISCUSSION & CONCLUSIONS: These values of \( \mu \) are much higher than \( \approx 0.001 \) which occurs between cartilage surfaces in vivo. In the functioning diarthroidal joint, the large majority of the load is carried by interstitial fluid pressurization. This implies that friction at contacting asperities is low, and that there is not necessarily a need to lower friction. The role of “boundary lubricants” may thus be one to protect cartilage asperities by strong steric repulsion, in absence of interstitial fluid pressurization.


ACKNOWLEDGEMENTS: NIH AR050180 (G.D.J), NSF DMR-0239769 CAREER (S.Z.)
Therapeutic Perspectives for Lubricin and Joint Function

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INTRODUCTION
Articular cartilage is a biomechanical bearing with specialized surface properties which repel friction and provide critical chondroprotective attributes. Apposing and sliding interfaces of this thin and compliant, regeneratively refractive tissue are able to dispel the biomechanical challenges and potential tribological consequences engendered by contact pressures several-fold the magnitude of human body-weight load, thereby enabling fluid joint motion and ambulation1-4.

Both historically, as well as more recently, considerable attention has been focused on lubricin (aka superficial zone protein/SZP, PRG4) as a critical cartilage boundary lubricant and cytoprotectant5-9. In a review article from 2001 highlighting ‘Modern Concepts of Osteoarthritis’, Dr. Leon Sokoloff considers: “Whether there may be a future for therapeutic application of lubricin now must be regarded only as a beautiful dream”2.

More recent insights provide cues toward strategies for restitution of lubricin loss-of-function, with the realizable potential to help promote joint function via therapeutic modulation of lubrication and/or adhesion.

OVERVIEW & DISCUSSION
The synovial “tribosome”, comprising macromolecules such as lubricin and aggrecan, facilitates lubrication and fluid pressurization of articular cartilage, thereby contributing to the maintenance of proficient joint functionality3-9.

Dysregulated metabolism of articular cartilage constituents, including aggrecan and type II collagen, is a hallmark of arthritic joint diseases such as osteoarthritis (OA), and degenerative changes at cartilage surfaces, including lubricin depletion, emblemize early OA pathophysiology10,11. Furthermore, deficits in secreted and cartilage surface-associated lubricin can be elicited by chondrodisruptive cytokines such as IL-1 and TNF-α6,12,13.

Cartilage endurance might therefore be enhanced by the induction of lubricin synthesis in response to appropriate cytokiregulatory growth factors and/or suitable biophysical stimuli6,12-16.

In addition, recombinant human lubricin can effectively augment articular cartilage lubrication17, suggesting evident therapeutic implications for delivery of applicable biolubricant formulations.

REFERENCES

ACKNOWLEDGMENTS
The financial and infrastructural support provided by Wyeth, and collaborations with co-workers and colleagues, are gratefully acknowledged.
The adaptation of human pisiform entheses to compressive forces.

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INTRODUCTION: According to the literature two types of entheses (i.e. tendon / ligament attachment sites), fibrous and fibrocartilaginous can be distinguished. Fibrous entheses transmit tensile stress to bone, while fibrocartilaginous entheses in addition are subject to local compressive stress. Local compressive stress is closely related to the function of the enthesis which is to dissipate shear forces away from the hard/soft tissue interface. Shear forces at the attachment site are caused by the insertional angle change of the tendon / ligament during limb movement. The fibrocartilaginous layer resembles a functional adaptation to the special local mechanical situation. The aim of our study was to test, if there is a correlation between the size of the fibrocartilaginous parts of the entheses and the insertional angle changes of the tendon and ligaments of the human pisiforme complex. The pisiforme as a bony pulley distributes the force of the flexor carpi ulnaris muscle between two ligaments. One of these (pisometacarpal ligament) is running in line with the tendon of flexor carpi ulnaris muscle, while the other (pisohamate ligament) is branching in an angle of 45°, which means that both ligamentous entheses are exposed to more or less the same forces, but experience different types of insertional angle change during hand movement.

METHODS: Five human pisiforme complexes were removed within 48h post mortem. The specimens were fixed in methanol, decalcified and cryosectioned at the level of the entheses. Sections were labeled with monoclonal antibodies directed against collagens I and II. The varying sizes of the collagen II positive zones at the entheses were assessed using Axiovision image analysis software (Zeiss).

RESULTS: All attachment sites showed fibrocartilaginous (i.e. collagen II positive) entheses. The fibrocartilaginous zones showed the largest collagen II positive layer at the attachment site of the flexor carpi ulnaris muscle and the smallest at the distal entheses of the pisometacarpal ligament. Furthermore there was a difference between both distal entheses of the pisohamate and the pisometacarpal ligaments.

DISCUSSION & CONCLUSIONS: It is well documented that during hand movement (i.e. during carpal flexion and extension) the major changes of insertional angles occur at the attachment site of M. flexor carpi ulnaris. During radial and ulnar deviation the insertional angle change at the distal entheses of the pisohamate ligament is greater than at the distal entheses of the pisometacarpal ligament. This is due to the specific movement of the pisiforme. Our results on the different sizes of the fibrocartilaginous layer are apparently reflecting the adaptation to the mechanical environment that is created by the average insertional angle changes occurring at either enthesis.


Fig.1 Schematic drawing of a right pisiforme complex.
Adverse cell and tissue reactions during delayed degradation of PLDLA-anchors for shoulder joint repair

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INTRODUCTION: Torn ligaments or tendons can be reattached to the bone by so-called suture anchors during reconstructive joint surgery. Particularly for minimal invasive arthroscopic surgery, anchors made of biodegradable polymers, mainly poly-glycolic and/or poly-lactic acid (PGA, PLA) were introduced [1]. However, these anchors seem not to be readily resorbed, and not bone healing, but osteolysis observed 8 months to years at radiologic follow-ups. In this prospective study, the bone reactions to biodegradable anchors were followed by radiologic imaging, and augmented by histopathologic evaluation of two illustrative cases.

METHODS: So far, 43 patients (males> females, age range 54-83 yrs) from a single institution, with 9 to 24 months follow-up after arthroscopic rotator cuff repair, using 1 to 3 PLDLA-anchors (Arthrex, Inc., Naples FL, USA), were evaluated on plain radiographs and arthroMRIs. Bone healing, or residual bone defects and/or cystic lesions in the respective anchor positions were assessed. At endoprosthetic replacement after clinical failure, for biopsy 1, one anchor bone bed was retrieved from a male patient (66a) 9 months p.implantation. For biopsy 2, two segments of the humeral head with anchor bone beds were retrieved from a female patient (74a) 24 months p.implantation. All specimens were plastic embedded, and undecalcified microtome and ground sections were evaluated by light microscopy.

RESULTS: Except in one case, in 35 cases the anchors were detectable up to 24 months p.impl., the anchor sites appeared as osteolytic defects, containing foreign body giant cells and macrophages with PLDLA remnants(?) and abundant hemosiderin. In between, lymphocytes and plasma cells were found, and lymph follicles in the surrounding edematous fatty marrow.

DISCUSSION & CONCLUSIONS: In the bone bed, “bioabsorbable” implants seem not to degrade in the same amount of time as e.g. suture materials. During delayed degradation, cases with osteolytic or cystic lesions and joint effusions have been observed [3]. In the present study, bone healing of the anchor sites seemed not only hindered by the presence of the not totally degraded implant, but also impaired by demineralization due to low pH at acid release, and by chronic inflammation with an immune cell reaction. One explanation for this immune response could be denaturation of proteins adsorbed to these semi crystalline degradable polymers [4].

REFERENCES:
INTRODUCTION: Osteoarthritis (OA), or degenerative joint disease, is the most prevalent of the musculoskeletal diseases with characteristic, progressive destruction of cartilage. Adult mesenchymal stem cells (MSCs) contribute to the maintenance of connective tissues and local delivery of MSCs to the injured joint in a goat model of OA retarded the progressive destruction of cartilage (1). However, MSCs did not engraft to either intact or fibrillated cartilage in this model. We are evaluating strategies to promote homing of MSCs to fibrillated cartilage to promote tissue repair using a human osteoarthritic cartilage explant model.

METHODS: Full thickness cartilage explants (1-2 mm thick and 2mm diameter) were taken from the medial tibial plateau or femoral head of tissue obtained after total knee and hip arthroplasty. Human MSCs were obtained from normal donors and all procedures were performed with informed consent and approved by the Clinical Research Ethical Committee at University College Hospital, Galway. Explants were placed in 10% FCS containing media for 48 h at 37°C to recover and subsequently cultured in serum-free chondrogenic media without TGF-β3 (ICM) for 24 h at 37°C. MSCs were labelled with Cell Tracker™ Red and DAPI and added to the explants at 0.5-2 x 10⁶/ml for 1-4 h at 37°C. After stringent washing to remove unattached cells, explants were processed immediately. Cartilage disks incubated without cells were used as controls. Some explants were pre-treated with 0.25% trypsin/EDTA and collagenase (1mg/ml). Formalin-fixed, paraffin-embedded sections were stained with Toluidine blue or processed for fluorescence microscopy. Proteoglycan (GAG) release into the media was determined by DMMB assay over 14 days for explants cultured in ICM and chondrogenic media containing TGF-β3 (CCM).

RESULTS: The culture model was validated using fibrillated goat cartilage explants. Mean accumulated GAG/DNA was not different in explants cultured in serum-containing media, ICM or CCM over 14 days. Sulphate incorporation was also similar in serum-containing media and CCM. Sulphate incorporation was significantly lower in explants cultured in ICM. Proteoglycan release by human cartilage explants was constant over a 14 day culture period.

Initial MSC attachment experiments were performed with explants from both the femoral head and tibial plateau embedded in a fibrin gel. Cell attachment to the OA tissue in the fibrin-implanted explants was insignificant at all cell concentrations and times used. Maximum binding of MSCs to the OA cartilage was observed in a free floating system (without fibrin) with cartilage from the medial aspect of the tibial plateau (Figure 1). Binding increased with time, cell concentration and exposure to collagenase and trypsin. Collagenase treatment alone resulted in less cell binding than PBS treatment.

DISCUSSION & CONCLUSIONS: Strategies for stem cell-mediated repair of damaged cartilage as a result of OA will require targeting stem cells to damaged cartilage. We have established a functional culture system to evaluate in vitro MSC binding to OA cartilage to allow development of specific targeting strategies. The explants are maintained in culture for up to 14 days in serum-free media and will enable testing of the ability of the targeted stem cells to repair the surface.


ACKNOWLEDGEMENTS: Research funding was from Science Foundation Ireland.
MAINTENANCE OF CHONDROCYTE PHENOTYPE BY THE VIMENTIN INTERMEDIATE FILAMENTS

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INTRODUCTION: The vimentin cytoskeleton is believed to facilitate extracellular signal transduction inducing transcriptional responses within the chondrocyte. The spatial organisation of vimentin differs in human osteoarthritic chondrocytes, and a reduction in vimentin protein was observed in a rat model of osteoarthritis. The aim of this study was to assess the role of the vimentin filaments on cartilage chondrocyte homeostasis.

METHODS: Chondrocytes from 7-day-old bovine articular cartilage were plated at 1x10^6 cells/24 well plate, and vimentin filaments disrupted with 1-5mM acrylamide for 24, 48 and 72hrs. Disruption of the vimentin filaments was confirmed using a FITC-conjugated vimentin antibody visualized using confocal microscopy. The cytotoxic effects of acrylamide were assessed using the CytoTox® 96 assay. Changes in phenotypic markers of chondrocytes i.e. type II collagen and sGAG were measured by immunoblotting and DMMB assay, respectively. The amount of phosphorylated versus total MAP kinase proteins (ERK 1/2, p38 and JNK) was also conducted by immunoblotting. Specific MAP kinase inhibitors (U0126, SB203580 and SP600125 respectively) were added to chondrocyte cultures in the presence of acrylamide to determine the involvement of each in vimentin-mediated signal transduction.

RESULTS: In the presence of acrylamide, the vimentin filaments were observed to collapse around the nuclei, whereas the filaments in control cells were organized into a dense network throughout the cytoplasm. Acrylamide was cytotoxic in a dose-dependent manner, which was significant after 72 hours in culture. Hence, all other data are normalized to cell number. Levels of sGAG in the media decreased with increasing acrylamide concentrations. This was also evident with pro type II collagen expression; interestingly, disruption of vimentin inhibited the processing of the pro-collagen into the II (α1) chains. An increase in phosphorylated ERK 1/2 was observed with increasing acrylamide concentration. Preliminary data indicates that increased phosphorylation of ERK 1/2 correlates with a change in chondrocyte phenotype. There were minimal effects on phosphorylated p38 kinase and JNK in acrylamide treated chondrocytes. We are currently analyzing the effect of blocking ERK phosphorylation using specific inhibitors to determine whether this prevents the change in phenotype.

DISCUSSION & CONCLUSIONS: We have shown that cytoskeletal vimentin element modulation by disruption with acrylamide alters the phenotype of the chondrocyte. ERK phosphorylation is widely reported to inhibit chondrogenesis\(^1\),\(^2\), therefore we believe that disassembly of the vimentin architecture, by an as yet unknown mechanism, results in phosphorylation of ERK 1/2 which may alter the phenotype of the chondrocyte i.e. inhibits sGAG and type II collagen production. We are currently determining the mechanism(s) involved in mediating these matrix changes, and the role of the chondrocyte cytoskeleton in signal transduction, changes in which may contribute to joint pathologies such as osteoarthritis.


ACKNOWLEDGEMENTS: Arthritis Research Campaign for funding (EJB)
COMPARING THE RESPONSIVENESS OF CHONDROCYTES ISOLATED FROM YOUNG AND MATURE ARTICULAR CARTILAGE TO IGF-1 AND TGFβ1

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INTRODUCTION: Injury to articular cartilage does not result in a spontaneous repair process due to the avascular and aneural properties of the tissue. Surgical strategies to enhance this repair process result in filling of the defect but with poor integration between the endogenous and repair tissue. This study investigates whether exposure to IGF-1 and TGFβ1 may enhance the repair process by increasing chondrocyte migration and matrix biosynthesis in chondrocytes isolated from young and mature articular cartilage.

METHODS: Cell Migration/Chemotaxis – Isolated chondrocytes were allowed to adhere to Boyden chambers coated with fibronectin, aggregan, type II collagen or BSA as a control. After 48 hours IGF-1 and/or TGFβ1 (both at 10ng/ml) or BSA were added to the bottom of the wells. After 24 hours, cells remaining on the top of the chamber were removed and the migrated cells stained with crystal violet and counted.

Matrix Biosynthesis – Chondrocytes were plated at 5 x 10^5 cells/well and allowed to adhere to the plate. After 24 hours the cells were supplemented with IGF-1 and/or TGFβ1 (both at 10ng/ml). Media served as control. All treatment groups were supplemented with 10µCi/ml 35Sulphate and 20µCi/ml 3H-proline and incubated for 1, 3 or 7 days. Radiolabel was counted in both the media and cell lysates (and associated material) giving biosynthesis values for both sulphated glycosaminoglycans (sGAGs) and collagen. Additional 'cold' experiments were set up as described above for protein and RNA extraction. PCR was used to confirm the chondrocytic phenotype using Sox 9 and Col 2 as markers. Western blotting using the monoclonal antibody AVT-6E3 was performed to confirm that the collagen synthesised was the characteristic type II collagen expressed in articular cartilage.

RESULTS: Cell Migration/Chemotaxis. Chondrocytes from both age groups were seen to migrate on all the substrates however aggregan significantly inhibited the migration of chondrocytes isolated from young cartilage when compared to BSA controls (P=0.012). The addition of growth factors only enhanced migration of the young chondrocytes if coated onto fibronectin, with TGFβ1 having an additive effect on IGF-1 (P=0.028). Only combined growth factor treatment enhanced the migration of the mature chondrocytes (P=0.025).

Matrix Biosynthesis. Increases in both sGAG and collagen biosynthesis were seen in both age groups of chondrocytes with growth factor treatment. Treatment with TGFβ1 increased biosynthesis at 24 hours and then within the cell associated material by 3 days, suggesting deposition of the newly synthesised matrix. No significant differences in biosynthesis levels were seen in either the conditioned media or cell associated material by day 7, suggesting a possible up-regulation of matrix degradation. PCR and Western blotting confirmed maintenance of the chondrocytic phenotype through time in culture with Sox 9 and Col 2 present within RNA samples and type II pro-collagen found in protein extracts from the treated cells.

DISCUSSION & CONCLUSIONS: Both IGF-1 and TGFβ1 can enhance chondrocyte migration in a substrate dependent manner, only occurring on fibronectin. IGF-1 and TGFβ1 also exert an anabolic effect on both age groups of cells up-regulating their sGAG and collagen biosynthesis whilst maintaining the chondrocytic phenotype. These results suggest that IGF-1 and TGFβ1 may have an application in cartilage repair where a combination of increased chondrocyte migration and matrix biosynthesis are required for the generation and integration of a biomechanically stable repair tissue.

ACKNOWLEDGEMENTS: Thanks to the EPSRC and Cardiff University for funding this research.
Identification of Human Chondroprogenitor cell populations

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INTRODUCTION: We have previously identified a population of chondroprogenitor cells from the surface zone of bovine articular cartilage using differential adhesion to fibronectin (1). This population of cells can form large numbers of colonies from a low seeding density and is capable of extended culture without losing the chondrogenic phenotype. Here we show that human foetal and adult articular cartilage contain a population of cells with similar clonality to the bovine chondroprogenitor population and describe the effects of Notch signal modulation on Chondroprogenitor behaviour.

METHODS: Human foetal and adult cartilage was obtained with institutional ethical approval. Foetal femoral condyle cartilage was removed from 4 foetuses (71-86 days) and chondrocytes obtained by sequential pronase/collagenase digestion. Adult human femoral chondrocytes were obtained from patients undergoing hemiarthrotyomy (54-64 yrs; normal cartilage obtained from non-diseased compartment) and isolated using sequential pronase/collagenase digestion. Isolated chondrocytes were subjected to differential adhesion to fibronectin and the initial adhesion and colony forming efficiency calculated as previously described (1).

RESULTS: Foetal chondrocytes plated onto fibronectin for 20 minutes were more adherent than foetal chondrocytes plated onto fibronectin for 40 minutes or foetal chondrocytes plated onto PBS for 20 or 40 minutes (Fig 1A, * p < 0.01) and showed increased colony forming efficiency at 6 and 10 days compared with controls (Fig 1B, * p < 0.05).

Adult chondrocytes plated onto fibronectin for 20 minutes were more adherent than chondrocytes plated onto PBS for 20 minutes (Fig.2A). Colonies first became apparent in human samples at 8 days and the CFE was greater at 12, 14 and 17 days compared with cells adhered to PBS (Fig 2B).

DISCUSSION & CONCLUSIONS: Using differential adhesion to fibronectin, we have shown that a subpopulation of both foetal and adult articular chondrocytes adheres to fibronectin in a short time span and that this adhered population is capable of forming large colonies. These results closely mimic those of the well defined bovine chondroprogenitor population (1).


ACKNOWLEDGEMENTS: Department of Trade and Industry, NovaThera, and The Wellcome Trust for funding this work.
Identification of Hyperelastic Parameters of Porous Polyurethane Sponges

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INTRODUCTION: Porous polyurethane sponges have recently been used as a scaffold for cartilage tissue engineering [1]. A poroviscoelastic overlay model [2] can be used to describe the constitutive behavior of this material. In this study a method to evaluate elastic material parameters of the solid phase of the scaffold is described.

METHODS: Displacement controlled compression tests were performed on a cylindrical sample of 4 mm in height and 8 mm in diameter. To eliminate friction effects, radial displacements were inhibited at the top and the bottom surface of the sample (Fig. 1a). The displacement was applied in five steps with a relaxation time of 15 minutes after each step (Fig. 1b). At the end of this relaxation phases, where an equilibrium state of the sample was assumed, images of the sample were taken and crosshead displacement as well as force response was recorded. The radial displacements at the edge of the sample were determined from the images using grey scale correlation software (VEDDAC, Chemnitzer Werkstoffmechanik GmbH).

The hyperelastic model

\[ T = 2\frac{\partial \psi}{\partial C} \]

(1)

establishes a relation between the 2nd PIOLA-KIRCHHOFF stress tensor \( T \) and the right CAUCHY-GREEN tensor \( C \). The polyconvex energy density function

\[ \psi = \frac{(C_1/\alpha)}{[e^{(I-\lnIII-3)}-1]} + D_2 (\lnIII)^2 \]

(2)

defined in terms of the first and third invariant of \( C \) (I and III) and containing the parameters \( C_1, D_2 \) and \( \alpha \) characterizes the actual material behavior. A semianalytical algorithm assuming a homogeneous state of strain in a cylindrical layer in the middle of the sample was employed to obtain a first estimation for suitable material parameters.

The finite element code SPC-PM2AdNl (SFB 393, TU Chemnitz) was then utilized to identify the values of \( C_1 \) and \( D_2 \) which lead to the best possible fit of the hyperelastic model to the measured data. The parameter \( \alpha \) was kept constant at \( \alpha=-9.0 \). The commercial finite element code MSC MARC with a custom made material subroutine was used to simulate the measured data with the obtained material parameters.

RESULTS: Parameter identification with a fixed \( \alpha = -9.0 \) led to \( C_1 = 1.51 \times 10^{-2} \) and \( D_2 = 4.46 \times 10^{-4} \) (equivalent to \( E = 0.063 \text{MPa} \) and \( v = 0.053 \) at small deformation) as best fit. In Fig. 2 the curves computed with these parameters are shown in comparison to the measured ones.

Fig. 1: a) diagram of the undeformed and the deformed state of the sample. b) applied displacement (* marks points of equilibrium)

Fig. 2: measured (solid) & computed (dashed) force-displacement-curve (a) and radial edge displacements (b) in the points of equilibrium (marked by *)

DISCUSSION & CONCLUSIONS: The force-displacement-curve observed in the experiment could be fit quite well with the hyperelastic model. However, the less good curve fit for the radial edge displacements suggests that the model does not represent too well the porous structure of the scaffold.


ACKNOWLEDGEMENTS: B. Gueorguiev, C. Sprecher & D. Wahl (AO Research Institute) for help with mechanical testing.
BMP-2 transgene-stimulated adult mesenchymal cells for articular cartilage repair

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INTRODUCTION: Adult primary mesenchymal cells of different origin, which are obtainable with minor donor site morbidity, may be considered as an alternative for autologous chondrocytes for articular cartilage repair approaches¹. This study compares the chondrogenic potential of adult primary bone marrow-, perichondrium/periosteum- and fat-derived cells following stimulation by bone morphogenetic protein-2 gene transfer.

METHODS: Mesenchymal cells were obtained from perichondrium/periosteum, bone marrow or fat of adult rats or miniature pigs and characterized using a panel of cell surface markers. Differentiation was induced by application of BMP-2, either in form of recombinant protein or via viral gene transfer, followed by micromass culture². The expression of cartilage-specific genes (aggrecan, collagen types II and IX), collagen type I and alkaline phosphatase was analyzed by RT-PCR, immunofluorescence and immunohistochemistry in comparison with unstimulated control cells. The chondrogenic potential of transgene-activated and unstimulated cells was then studied in vivo following cell transplantation into small partial-thickness lesions in the knee joints of rats³ or extensive chondral defects at the patella of miniature pigs.

RESULTS: In micromass-cultures, stimulation with BMP-2 led to an up-regulation of cartilage-specific gene expression in all three cell populations studied. The most prominent and rapid increase of type II and IX collagen mRNA levels was observed in perichondrial/periosteal cells after stimulation. Similar results were obtained for bone marrow stromal cells in vitro, while fat stromal cells showed only a moderate increase of the respective transcript levels with a subsequent decline. Without BMP-2 stimulation, a moderate expression of cartilage-specific genes (aggrecan, type IX collagen) could only be observed in perichondrial/periosteal cells, while the other two cell types did not express relevant levels.

Following transplantation in vivo, BMP-2 transgene-activated periosteal/perichondrial cells produced a proteoglycan-rich, type II collagen-positive repair tissue with only faint staining for type I collagen. The matrix originating from bone-marrow stromal cells was also rich in proteoglycans but showed a weaker staining for type II collagen. Transgene-activated fat stromal cells formed rather fibrous tissue mainly composed of type I collagen. The transplantation of unstimulated cells of any origin led only to the formation of fibrous repair tissue.

In the large chondral defects in the minipig model, BMP-2 transgene-activated autologous periosteal cells were capable of generating hyaline-like repair tissue, which was well integrated into the preexisting cartilage.

DISCUSSION & CONCLUSIONS: Perichondrium/periosteum-derived cells seem superior to cells from bone marrow or fat with respect to forming hyaline cartilaginous tissue. However, a chondrogenic stimulus appears to be required for initiation and support their chondrogenic differentiation.

REFERENCES:

ACKNOWLEDGEMENTS: This work was supported by the Interdisciplinary Centre of Clinical Research Erlangen, Germany.
Spatial Pattern of the Delta-Notch Signalling Pathway in Osteoarthritic Cartilage

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INTRODUCTION: Notch family members (Notch 1, 2, 3 and 4) are single-pass transmembrane proteins, consisting of a cytoplasmic and an extracellular domain. The extracellular domain binds its ligands, Delta and Jagged (Serrate in Drosophila). The Notch signalling pathway involves three proteolytic cleavages and functions in cell fate decisions including apoptosis, proliferation and terminal differentiation during development¹. Notch is also involved in homeostasis of adult self renewing stem cells and is implicated in disease e.g. osteoarthritis, rheumatoid arthritis and alzheimer's¹,²,³

Previous work has shown the presence of Notch family members within articular cartilage during mouse development². Localisation of Notch 1 in 7-day-old bovine articular cartilage corresponds with a chondrogenitor population present at the articular surface ⁵. The aim of this study was to map expression of Notch family members and its ligands in articular cartilage from human osteoarthritic tibial plateaus in order to determine the role of Notch signalling in disease progression.

METHODS: Osteochondral plugs were removed from tibial plateaus obtained from consented patients undergoing TKR. Plugs were fixed in NBFS, decalcified (10% EDTA), wax embedded and sectioned. Sections were stained with toluidine blue and safranin O and immunolabelled for Notch 1-4 and Notch ligands Jagged and Delta and visualized using Vector Nova Red peroxidase substrate.

RESULTS: Localisation of various Notch receptors differed throughout the depth of the osteochondral plug from the surface zone to the underlying bone. Notch 1 was localized to the surface zone and the deep zone. Intense label for Notch 1 was also present within chondrocyte clusters. Notch 2 was absent throughout the depth of articular cartilage but both fibrocartilage and areas of vascular invasion within the underlying subchondral bone labelled positively for Notch 2. Notch 3 and 4 showed similar patterns of Notch expression in that chondrocyte clusters and hypertrophic chondrocytes labelled positively. Expression of Notch 3 and 4 also occurred in areas of vascular invasion and fibrous repair. Delta and Jagged labelled positively within chondrocyte clusters and also areas associated with vascular invasion within the underlying subchondral bone.

DISCUSSION & CONCLUSIONS: Notch 1 labelling in the surface zone of osteoarthritic cartilage mimics the expression pattern seen during murine development and in 7-day-old bovine tissue⁴,⁵. Positive labelling of Notch 1 within chondrocyte clusters suggests Notch may play a role in cluster formation and, therefore, the pathogenesis of osteoarthritis. The absence of Notch 2 in osteoarthritic cartilage suggests that although Notch 2 plays a role during development, it may not play a role in reparative responses thus not recapitulating developmental processes. Expression of Notch 3 within chondrocyte clusters appears to be a novel pattern. Previous work suggested a role for Notch 3 retarding the cell cycle which is contrary to its presence within rapidly proliferating osteoarthritic chondrocyte clusters⁴. The presence of Notch 4 within chondrocyte clusters contradicts previous work which suggested its role in preventing vascular invasion as invasion from the underlying subchondral bone is a feature of OA and, therefore, may play an alternative role in the pathogenesis of osteoarthritis⁴. The localisation of the ligands Delta and Jagged, further supports evidence for a role of the Notch signalling pathway in osteoarthritic cartilage. Therefore, further research of the Notch signalling pathway is necessary in order to investigate cluster formation through uncontrolled proliferation.

REFERENCES:
INTRODUCTION: Micromass cultures of human adipose-derived stem cells (ASC) show the ability to express chondrocyte specific genes and to form cartilage-like matrix [1-2]. Enhancing effects can be gained by addition of different growth factors to the medium.

METHODS: Liposuction material was digested with collagenase and ASC were expanded up to passage 4. For differentiation cells were pelleted and cultured in chondrogenic differentiation medium (Cambrex) for 4 weeks. Fibroblast growth factor-2 (FGF-2), bone morphogenetic protein-6 (BMP-6) or a combination of these two were added to the medium at a concentration of 10 ng/mL. Cartilage specific matrix formation was investigated by measuring the sulfated glycosaminoglycan (S-GAG) content using the 1,9-dimethyl-methylene blue (DMMB) assay. Gene expression was examined by quantitative RT-PCR and the pellet size was evaluated.

RESULTS: FGF-2 and a combination of FGF-2 and BMP-6 show inducing effects on the synthesis of S-GAG in 3D micromass cultures. Chondrospecific gene expression could be confirmed by quantitative RT-PCR. FGF-2 and BMP-6 in combination yielded a larger pellet diameter compared to single treated cultures.

DISCUSSION & CONCLUSIONS: Fat tissue represents a promising source for mesenchymal stem cells. Treatment with BMP-6 and FGF-2 enhances chondrogenic differentiation compared to control cultures. Differentiation could be demonstrated by the detection of S-GAG and quantitative RT-PCR.


ACKNOWLEDGEMENTS: This work was partially supported by the European STREP Project HIPPOCRATES (NMP3-CT-2003-505758) and the Lorenz Boehler Fonds and was carried out under the scope of the European NoE EXPERTISSUES (NMP3-CT-2004-500283).
INTRODUCTION: There are several different knockout mice that carry mutations in genes encoding key articular cartilage (AC) molecules. Some of these mutations are lethal and the structure of adult articular cartilage cannot be examined. There are several mutations, however, that do not appear to affect the normal development of the mice but which may reveal information regarding the importance of those proteins in the normal function and development of the tissue. It is shown that collagen IX null mice develop osteoarthritis at a relatively young age and skin fragility is noted in the decorin mutants1, 2, while matrilin-1 deficiency shows no obvious physiological changes. In each of these knockouts the development and ultrastructure of the AC appears unaffected1, 2, and 3. Optimisation of preparation protocols for light (LM), polarised light (PLM) and high resolution scanning electron (HRSEM) microscopies establish novel ultrastructural features and distinct stages of tissue formation in adult and developing normal (CD1 strain) mouse AC4. The investigation into the development and ultrastructure of the knockout strains AC revealed distinct differences between the mutants and CD1 mice.

METHODS: Mouse tibiae were dissected from adult (2-9 month old) and developing mice (1-18 days old) from each of the three knockout strains. The tibial plateaux were kept intact and fixed in formaldehyde in 0.1M Piperazine_1, 4-bis-2-ethanesulfonic acid (PIPES) buffer (pH 8.5) for 24 hours, dehydrated in an ascending ethanol series, transferred to xylene, embedded in paraffin wax, sectioned at a thickness of 7.5µm and stained in picrosirius red5. Sections were imaged using light and polarised light microscopy. Additional mouse tibiae were plunge frozen in propane cooled by liquid nitrogen, freeze substituted for 5 days in 58% acetone, 30% methanol, 10% acrolein an 2% tannic acid and a further 5 days in 100% acetone. Samples were then critical point dried, fractured and coated in platinum/palladium (80/20) for examination by scanning electron microscopy.

RESULTS: There was no discernible difference between normal and matrilin-1 mouse AC. Decorin and collagen IX null mutants showed, respectively, more rapid or retarded AC development than normal mice, although the final adult AC appeared normal. The rate of development varied from the normal by a few days only, but the results support suggestions describing the involvement of collagen IX and decorin in fibrillogenesis6, 7. In addition, altered physicochemical properties of the collagen matrix affected the chemical fixation of the collagen matrix ultrastructure in decorin and collagen IX adult tissue. The structural integrity of the collagen IX null AC also degraded with age and changes in the tissue ultrastructure were observed at 9 months, correlating with studies suggesting an early onset of osteoarthritis1.


ACKNOWLEDGEMENTS: P. Bruckner for supplying the knockout mice and R. Fässler, A. Aszódi & R. Iozzo for permission to use their mutants. Smith & Nephew for financing the study.
INTRODUCTION: Postoperative pain pumps are increasingly used to deliver a continuous infusion of local anesthetic into the surgical wound or the joint. Recently, there have been concerns that the use of such devices may be associated with chondral toxicity and even cases of chondrolysis in the shoulder [1]. An experimental model is presented that investigates potential chondrotoxic effects of a continuous intra-articular infusion of Bupivacaine in the rabbit shoulder.

METHODS: 30 rabbits were divided into three groups that received continuous infusions of either saline, Bupivacaine, or Bupivacaine with Epinephrine over 48 hours into the glenohumeral joint. Animals were sacrificed after one week and tissue samples underwent analyses with confocal microscopy for live/dead cell assay, metabolic sulfate uptake assessment, and conventional histology.

RESULTS: Infusion of Bupivacaine with and without Epinephrine decreased sulfate uptake 58% and 49%, respectively, when compared with saline; cell viability decreased by 20% and 32%. Histology demonstrated significantly worse scores for Bupivacaine infusion. The results for Bupivacaine with or without Epinephrine were not significantly different.

DISCUSSION & CONCLUSIONS: Continuous intra-articular infusion of Bupivacaine with and without Epinephrine led to significant histopathologic and functional changes in articular cartilage.


ACKNOWLEDGEMENTS: Supported (in part) by an Alpha Omega Alpha Carolyn L. Kuckein Student Research Fellowship
Prospective Evaluation of Prolonged Fresh Osteochondral Allograft Transplantation of the Femoral Condyle: Minimum 2 Year Follow-Up

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INTRODUCTION: Focal osteochondral lesions of the knee in young patients present a therapeutic problem [1,2]. One potential treatment is fresh osteochondral allograft (OCA) transplantation. This study presents the results of 25 consecutive patients whom underwent prolonged fresh OCA transplantation for defects in the femoral condyle.

METHODS: The average age of the patients was 35 (range, 17-49). All of the defects were on the femoral condyles. They included degenerative, traumatic, osteonecrotic and osteochondritis dissecans lesions. Five patients (20%) had more than one defect. The average size of the primary lesion was 5.24 cm² and for the secondary lesion was 2.31 cm². Twenty-four patients (96%) had a previous procedure to address the lesion, and the average number of previous procedures was 2.28 (range, 0-6). The average interval between injury and surgery was 25 months (range, 3-70 months). There were 14 patients (56%) in whom comorbidities required either a concurrent high tibial osteotomy or meniscal transplantation. There were 13 patients (52%) on worker’s compensation at the time of surgery.

RESULTS: The average age of the prolonged fresh grafts at implantation was 24 days (range, 15-43 days). The average length of follow-up was 35 months (range, 24-67 months). Prospective data was collected using several subjective scoring systems, as well as objective and radiographic assessments. Statistically significant improvements were seen for Lysholm (39 to 67), IKDC (29-58), all five components of the KOOS scores (Pain 43 to 73, Symptom 46 to 64, ADL 56 to 83, Sports 18 to 46, Quality of life 22 to 50), and the SF-12 physical component score (36 to 40). Overall, patients reported 84% satisfaction with their results and felt the knee functioned at 79% of their unaffected knee. The objective measures of knee range of motion and quad size were not significantly different from the unaffected knee. Radiographically, 22 of the grafts (88%) were incorporated into host bone. There were 2 (8%) complications; one patient had graft fragmentation, and one patient had pain greater than 6 months.

DISCUSSION & CONCLUSIONS: Fresh osteochondral allograft transplantation is an acceptable intermediate procedure for treatment of localized osteochondral defects of the femur. At two year follow-up, it is well-incorporated and offered consistent improvements in pain and function.


ACKNOWLEDGEMENTS: N/A
Oxidative stress induces expression of markers of early osteoarthritis and chondrocyte terminal differentiation in cultured bovine explants

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INTRODUCTION: Articular cartilage is susceptible to many forms of injury, some of which may lead to secondary arthritis at a much later time. Free radicals are formed by articular cartilage in response to injury, by chondrocytes themselves or through inflammatory pathways. We have examined the behavior of chondrocytes embedded within their native matrix to oxidant stress following exposure to hydrogen peroxide (H₂O₂). In addition to observing degradative effects of oxidant stress we have attempted to examine the synthetic reactions that occur as a result of repair and remodelling.

METHODS: Bovine articular cartilage explants (obtained from the metatarsalphalangeal joint of 18 month-old steers) were cultured in DMEM, 10 mM Hepes, insulin-transferin-selenium and gentamycin. Explants were treated with a single dose of 0.1, 0.5 and 1.0 mM H₂O₂, the culture medium was changed after 24 hours and subsequently after every third day. Immunohistochemical and immunofluorescence labelling of control and treated explants was performed using mouse anti-3B3(-), rabbit anti-procollagen type IIA, mouse anti-PCNA and mouse anti-nitrotyrosine antibodies.

RESULTS: Bovine explants were treated with a single application of 0.1, 0.5 and 1.0 mM for 7 to 21 days. Presence of the oxidant was undetectable in the culture medium within 6 hours of its addition. Cell death occurred in a statistically significant dose-responsive manner with increasing concentrations of H₂O₂ in serum-free medium. Addition of ITS to the culture medium also led to a dose-dependent increase in cell death but significantly less than was observed in serum-free cultures. There was evidence of cellular proliferation in explant cultures. PCNA positive nuclei were detected in treated explants and treated explanted were also positive for gene expression of cyclin b2 part of the mitosis-promoting factor in dividing cells. In contrast control explants were negative for PCNA labelling and cyclin b2 expression.

3B3(-) is an antibody that recognises an atypical change in the biochemical structure of chondroitin sulphate chains covalently attached to aggrecan. Reactivity for 3B3(-) occurs in the normal fetal growth plate and articular cartilage undergoing osteoarthritic changes. We found that treatment of explants with H₂O₂ induced expression of the 3B3(-) epitope in the superficial and upper middle zones, Figure 1. Moreover we discovered that expression of the 3B3(-) epitope progressively increased over a period of 3 weeks (with no further addition of H₂O₂), at which time labeling was intense and throughout the territorial matrix in the surface and middle zones of the explants.

Figure 1. Progressive increase in 3B3(-) labelling over time following 0.5mM H₂O₂ treatment.

Procollagen IIA that is expressed during limb development and re-expressed in OA was observed in treated explants. Procollagen IIA antibody labelling correlated with increased collagen type II gene expression. Our work has also shown that late stage OA in age matched animals is accompanied by reduced expression of 3B3(-) epitopes and a reduction in procollagen IIA labelling, indicating that failure of repair processes either through cell loss or dedifferentiation may presage irreversible decline in joint function.

DISCUSSION & CONCLUSIONS: Oxidatively-stressed cartilage explants recapitulate many of the earliest molecular signatures of early OA, whether this represents a transient repair process or a sustained degenerative process remains to be seen. Oxidant injury therefore represents a useful, reproducible in vitro experimental model to study molecules critical in repair and progression of cartilage degeneration.

ACKNOWLEDGEMENTS: The authors would like to acknowledge support given by the Department of Health, UK.
INTRODUCTION: Articular cartilage is a complex tissue comprising phenotypically distinct zones with a low capacity for repair. Recent research has identified the presence of a progenitor cell population found in the surface zone of articular cartilage based on differential adhesion to fibronectin. These cells were shown to possess an extended cell cycle time, form colonies from a single cell and shared many of the properties of mesenchymal stem cells.

The aim of the present study was to determine the in vivo plasticity of articular cartilage progenitor cells isolated from the surface of 7 day old bovine metacarpal-phalangeal joints.

METHODS: Chondrocytes were collected from the bovine metacarpal-phalangeal joints by fine dissection and progenitor cells were isolated by differential adhesion to fibronectin and expanded in supplemented DMEM-F12 with 10% fetal calf serum. To determine progenitor cell plasticity in vivo, 2 x 10^7 cells ml^-1 were labelled with PKH26 and injected into the rear thigh muscle of 8-week-old, skeletally mature severe-combined immunodeficient (SCID) mice. After 2 weeks, the muscles were dissected to determine the presence of a cartilage pellet. If no pellet was present, the muscle fibres from each sample were pooled and frozen for cryosectioning. Sections were then examined under a fluorescent microscope and stained with safranin O. Immunohistochemical analysis was carried out looking for sox9 and collagen type II.

RESULTS: Fluorescent PKH26 labelled cells were detected after two weeks in all the samples analysed. A cartilage pellet was present after intramuscular injection of freshly isolated full depth chondrocytes. After injection with a clonal population of chondroprogenitors, a distinct pellet was not present although regions of intense safranin O stain and sox9 expression corresponded to areas containing fluorescently labelled cells. Low levels of collagen type II were also detected in these areas. The intensity of the staining was weaker than the staining seen after injection of full depth chondrocytes.

DISCUSSION & CONCLUSIONS: The plasticity of bovine chondroprogenitors isolated from the surface zone of articular cartilage has already been demonstrated in vitro and in ovo. Here we demonstrate that full depth chondrocytes maintain the chondrocytic phenotype when injected intramuscularly into the SCID mouse as shown by the presence of an intensely stained safranin O pellet and the expression of sox9 after 2 weeks in vivo. The injection of pure chondroprogenitor populations resulted in the absence of a distinct pellet but the presence of sox9 and PKH26 positive cells distributed throughout the muscle fibres. These results suggest that these progenitor cells can express and maintain a chondrogenic-like phenotype within a foreign tissue albeit at a low level. Current work is concentrated on analysing the expression of homotypic genes such as MyoD.


ACKNOWLEDGEMENTS: This project is funded by the BBSRC and Smith & Nephew.
Two year long-term study of human MACT-grafts in vitro

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INTRODUCTION: Matrix-associated autologous chondrocyte transplantation (MACT) has already been broadly investigated but remains poorly understood concerning the biological reasons for the variability of clinical results. Due to the restricted availability of repair tissue of patients, long-term cultures were performed. The aim of this study was to investigate tissue formation, differentiation of human articular chondrocytes under artificial conditions and degradation of the scaffold material.

METHODS: Grafts of human articular chondrocytes cultivated on the collagen I/III scaffold Chondrogide® (MACI®) were taken 1) at the time of transplantation (three days after seeding) 2) three weeks, 3) three months and 4) two years after in vitro culture. Investigations were performed using light, scanning and transmission electron microscope and in situ hybridization.

RESULTS: At the time of transplantation, grafts of the patients were very different concerning cell number and cell morphology. During the following cultivation time cell-scaffold constructs with little infiltration but a differentiating tissue at the scaffold surface developed. The differentiation proceeded in a cell multi-layer underneath an outer fibroblast layer simultaneously with the secretion of extracellular matrix (ECM). First signs of differentiation, such as spherical cells containing round or lobed nuclei, few cytoskeletal elements, glycogen accumulations, lipid droplets and further dense ECM consisting of flocculent and fibrous elements (Fig.1), appeared after three weeks of cultivation. In situ hybridisation confirmed the differentiated stage of the cells by the presence of collagen type II mRNA. After two years of in vitro cultivation a hyaline-like tissue has been formed (Fig.1). Chondrocytes infiltrated the whole scaffold but without the formation of larger cell populations. During the first month of cultivation, some of the transplants formed roulades with the cell population in the middle (Fig.1). The biodegradable fleece showed obvious dissociation of the large, loose fibres; the small fibres remained morphologically unaffected during the whole cultivation time.

Fig. 1: Chondrocyte grafts after long-term cultivation: ‘Roulade’ of transplant (upper left); differentiating chondrocyte (upper, right), hyaline-like tissue (lower left), spherical chondrocyte in hyaline-like tissue (lower right).

DISCUSSION & CONCLUSIONS: During the long term cultivation of the MACI®-grafts a hyaline-like tissue developed without supplementary stimuli, such as mechanical stimulation or additional factors (e.g.: growth factors). The differentiating cell populations were located on the fleece surface suggesting only few or hardly any influence of the scaffold material. Differentiation proceeded simultaneously with an increase of autogen ECM, which is probably responsible for the phenotype switch. Contractile properties of the superficial cells of the cell layer are thought to affect the curving of the transplant and the formation of ‘roulades’.

ACKNOWLEDGEMENTS: This study was supported in part by the Lorenz-Boehler-Society (10/04). We thank the institution ‘Cell Imaging and Ultrastructural Research’ for the possibility to use of the electron microscopic equipment.
Mesenchymal Stem Cell Senescence In Osteoarthritis


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INTRODUCTION: Osteoarthritis (OA) is the most common cause of musculoskeletal disability in elderly. There are few effective treatments and most therapeutic approaches have little impact on the progressive degeneration of joint tissues in OA. Mesenchymal Stem Cells (MSCs) may function to maintain normal homeostasis of articular cartilage, bone and other connective tissues. However, this ability is diminished or altered in osteoarthritis as the capacity of endogenous stem cells to proliferate and differentiate into articular cartilage is significantly reduced1. This study tests the hypothesis that senescence contributes to a decreased capacity of MSCs in OA to self-renew leading to normal tissue homeostasis and to study the mechanism by which this may occur. To this end, we examined factors which contribute to cellular senescence, such as the expression of cell cycle inhibitors, telomerase activity and telomere length.

METHODS: MSCs were isolated from the bone marrow of OA patients undergoing hip and knee replacement surgery and normal control volunteers. All procedures were performed with informed consent and approved by the Clinical Research Ethical Committee at University College Hospital, Galway. MSCs were expanded and subjected to osteogenic, chondrogenic and adipogenic assays. Doubling times for OA and control MSCs were measured. Genomic DNA and RNA was isolated from MSCs at different passages, and used to examine telomere length using the Telomere Length Assay (Roche), with preliminary results suggesting no difference in telomere length. Total RNA was extracted from both groups, converted to cDNA and analysed for relative p27 gene expression using SYBR green real-time quantitative PCR chemistry and the 2^ΔΔCT method. p27 mRNA expression was reduced in OA relative to control samples (Fig. 2).

RESULTS: OA and control MSCs differentiated along the adipo-, osteo- and chondrogenic pathways under appropriate stimulation. Population doublings of OA (6.4 ± 2.5 days) and control MSCs (3.85 ± 2.12 days) were measured. Protein (10µg) was extracted from MSC samples and subjected to the PCR-based TRAP assay (Roche) to measure telomerase activity. Both OA and control MSCs were negative for telomerase relative to identical amount of protein extracted from the Jurkat T cell line (Fig.1). One µg genomic DNA isolated from both groups was analysed for telomere length using the TeloTAGGG Telomere Length Assay (Roche), with preliminary results suggesting no difference in telomere length.

DISCUSSION & CONCLUSIONS: Our initial results do not suggest an association between cellular senescence and OA. The finding that p27 levels are reduced in OA relative to controls is interesting and warrants further investigation. However, expression levels of p16, p21 and hTERT and further analysis of telomerase activity must be carried out in order to confirm our findings.


ACKNOWLEDGEMENTS: The authors wish to acknowledge the funding of Science Foundation Ireland.
Porous bioactive glass scaffold with high compression strength

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INTRODUCTION: Tissue engineering poses new challenges in the area of biomaterials. For bone and cartilage tissue engineering applications, in particular, there is a need for the development of better scaffold materials. Porous bioactive glass scaffolds have been manufactured by sintering bioactive glass fibres. The relative porosity of the scaffolds, size of the pores and compression strength can be controlled by altering the manufacturing parameters like fibre dimensions and sintering temperature and time.

The aim of this study was to manufacture porous structures with high compression strength by sintering bioactive glass fibres.

METHODS: The composition of the bioactive glass used in this study was as follows; 12% Na2O; 15% K2O; 3% MgO; 13% CaO; 1% B2O3; 2% P2O5 and 54% SiO2 (all in weight %). Glass fibres, with mean diameter of 100 µm, were manufactured from the glass by melt spinning. The fibres were further chopped either to a length of 1.3 mm or 2.0 mm. Porous scaffolds (height = 6 mm) were manufactured by sintering the fibre segments in a mould (with inside diameter of 15 mm) at 630 °C for time periods of 15, 20 or 30 minutes. Porosity and compression strength and compressive shear strength (as a maximum load) of the manufactured specimen were analyzed. The cross head speed of 5 mm/min was used in both testing methods.

RESULTS:

As seen in Table 1 the increasing sintering time does increase the compression strength of scaffolds, though the brittle nature of the scaffolds introduce uncertainty on the results (high standard deviations).

DISCUSSION & CONCLUSIONS:

Porous bioactive glass is proposed as a good materials choice to achieve repair in articular osteochondral defects. Bioglass® has also been shown to have the ability to support the growth of chondrocytes. By using sintering technique porous bioactive glass scaffold with high compression strength are obtained. This type of networks may therefore prove useful as scaffold in applications, in which load bearing materials are required.

REFERENCES:

Scaffold-free chondrocyte transplantation as alternative to matrix associated chondrocyte transplantation

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INTRODUCTION: Tissue engineering (TE) is a modern discipline to repair destructed joint cartilage. One of the most favorized methods of TE is the MACT (matrix associated chondrocyte transplantation). This method combines the use of different types of artificial scaffolds to support a tri-dimensional structure of in monolayer proliferated autologous chondrocytes. Contrary to common used procedures a new technique of scaffold free chondrocyte transplantation (SFCT) was developed by the fzmb. As a model of neo cartilage production joint cartilage from horse was used.

METHODS: Chondrocytes were isolated from joint cartilage by enzymatic hydrolysis and propagated using autologous serum. After cultivation and proliferation in monolayer cultures cells were transferred into a three-dimensional structure without any artificial matrix or biocompatible material. An undifferentiated mechanical pressure (manual) was used for producing a scaffold free product and with development of extracellular matrix. These scaffold-free constructs were compared with native cartilage of the same animals. Cartilage development was documented by biochemical, histological and immuno-histological analysis.

RESULTS: Within three weeks of cultivation applying a mechanical stimulation (ten minutes a day) chondrogenesis was induced without additional growth factors. With mechanical stimulation the SFCT can reach a 20 mm diameter and a height of 2-3 mm (Fig.1). Data obtained clearly demonstrate that three-dimensional scaffold-free cartilage constructs derived by mechanical stimulation have a high proteoglycan (35%) and collagen (40%) content in comparison to native cartilage (Tab.1). Histological results revealed a distinct proteoglycan and collagen staining. Immuno-histological staining showed collagen type II.

DISCUSSION & CONCLUSIONS: Mechanical stimulation can be used as a stimulation factor for cartilage development. In comparison with native cartilage the collagen content of the SFCT was about 40% and the proteoglycan content was about 35%. These scaffold free chondrocyte transplantats can be used for repair of defect horse joint cartilage.


ACKNOWLEDGEMENTS: This study was supported by Freistaat Thüringer Grant 2004 WF 0163.
Evidence of a Chondroprogenitor Population in Human Osteoarthritic Cartilage

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INTRODUCTION: We have previously reported the presence of a chondroprogenitor population that resides in the superficial zone of immature bovine articular cartilage (1). There is much interest surrounding the therapeutic potential of this cell in terms of generating hyaline-like tissue in articular cartilage repair procedures such as autologous chondrocyte implantation. The question remains, however, as to whether this cell is present in mature human articular cartilage or during a disease state such as osteoarthritis. Alsalameh et al (2) have reported the presence of a mesenchymal progenitor cell population in normal and osteoarthritic articular cartilage, based upon the co-expression of cell surface markers CD105 and CD166. Here, we describe the isolation and expansion of clonally derived chondroprogenitor cells from human osteoarthritic articular cartilage.

METHODS: Articular cartilage was excised under sterile conditions from tibial plateaus obtained from patients undergoing total knee replacement for osteoarthritis. The articular cartilage was either subjected to a sequential digestion with pronase and collagenase or cryopreserved for cryosections. The isolated cells were then subjected to a differential adhesion assay to fibronectin or PBS (control) coated 6-well plates for 20 minutes after which non-adherent cells were removed (1). The adherent cells were cultured for up to 10 days in DMEM/F12 containing 10% FCS. Distinct colonies >32 cells were then cloned and cultured in DMEM/F12 containing 10% FCS in the presence or absence of 5ng/ml bFGF. Colonies of more than 32 cells were also fixed in 95% ethanol or pre-treated with monensin and then fixed in 95% ethanol for immunohistochemistry. Cartilage cryosections and fixed colonies were immunolabelled for Notch-1, CD166 and CD105. Colonies pre-treated with monensin were immunolabelled for type II, type IIA and type I collagen.

RESULTS: Following differential adhesion to fibronectin, colonies of greater than 32 cells formed. Clonally derived chondrocytes cultured in the presence bFGF were expanded through 10 population doublings. Immunolabelling of the tissue cryosections showed strong Notch-1 (fig1b) and CD166 labelling in the chondrocyte clusters. Immunolabelling of the colonies demonstrated that all cells in the colonies were Notch-1 positive (fig 1a). The colonies were also positive for CD166. Monensin blocked colonies demonstrated the presence of type I and type IIA collagen, however, they were negative for type IIB collagen.

DISCUSSION & CONCLUSIONS: We have used a previously published method (1) to isolate a chondroprogenitor population from osteoarthritic articular cartilage that can be expanded successfully under the influence of specific growth factors. Immunohistochemical analysis of colonies formed from these cells demonstrates they may represent an immature chondrogenic phenotype. Notch-1 is involved in cell fate selection and in combination with CD166 and CD105 immunolabelling may represent a marker of the chondroprogenitor cells in situ. The implication that a chondroprogenitor population is present within adult diseased articular cartilage presents many possibilities for the use of autologous tissue in the repair of articular cartilage.

IDENTIFYING PROGENITOR CELLS WITHIN ARTICULAR CARTILAGE

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INTRODUCTION: Previous studies have shown that articular cartilage grows by apposition from the articular surface driven by proliferation of a progenitor cell sub-population that resides in the surface zone. This study concentrates on identifying markers for this progenitor cell population. Cell surface receptors CD105 and CD166 are known markers of progenitor/stem cells in endothelial cells, bone marrow constituents as well as other tissues and have recently been reported within normal and osteoarthritic human articular cartilage. Notch 1, Delta and Jagged 1 and 2 are known to be expressed by stem/progenitor cells during various stages of limb development. Localisation of these markers to progenitor cells in articular cartilage would enable selective isolation facilitating further characterisation of the chondroprogenitor.

METHODS: Full depth cartilage explants were taken from 7-day-old bovine metacarpophalangeal joints. The tissue was then snap frozen and cryosectioned. Immunofluorescence was carried out using antibodies for the cell surface markers CD105, CD166 (Ancell, USA), Notch 1, Delta, Jagged 1 and Jagged 2 (Santa Cruz, USA). Surface zone explants were also enzymatically digested and the resulting cell suspension, filtered and counted. Cells were plated in monolayer at 30,000 cells/cm² and cultured for 4 days. Cells were lifted using accutase (Sigma, UK), then immunolabelled using directly conjugated CD105RPE and CD166FITC (Ancell). Cells were analysed using flow cytometry (BD FACSCanto).

RESULTS: Positive immunolabelling was observed for all markers. Jagged 2 occurred throughout the thickness of the cartilage, Delta was also present throughout the tissue at low levels but highly expressed at the articular surface. CD105, CD166 and Jagged 1 were localised to the superficial zone cells. Notch 1 labelling was found within the superficial layer and also in the deep zone. Labelling of CD105, CD166, Jagged 1 and Notch 1 were predominately present as clusters within the superficial layer (Figure 1).

Using flow cytometry surface zone cells were labelled for all the previous mentioned markers. CD105 labelled 10% of the surface population and CD166 labelled 45% of the surface population.

Fig.1: Immunofluorescence showing superficial zone labelling of CD105 (A), CD166 (B) Notch 1(C), Delta (D) and Jagged 1 (E) and uniform labeling throughout the tissue for Jagged 2 (F)

DISCUSSION & CONCLUSIONS: The above results demonstrate that CD105, CD166, Jagged 1, Delta and Notch 1 are promising markers for chondroprogenitor cells. At present, studies are concentrating on sorting labelled cells using flow cytometry (BD FACS Aria) to select the progenitor cells that can be expanded for use in monolayer and pellet systems.


ACKNOWLEDGEMENTS: With thanks to The Department of Health for funding this research.
MRI OF IRON OXIDE LABELED STEM CELLS: APPLICATIONS TO TISSUE REGENERATION

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INTRODUCTION: The ability to monitor stem cell therapies in vivo is rapidly becoming a significant consideration. Recently, it has been shown that stem cells can be labeled with superparamagnetic iron oxide (SPIO) particles and detected using MRI¹. The SPIO contrast agent is metabolized by the cell, increasing the cell’s magnetic susceptibility. As negative contrast agents, labeled cell populations appear as hypointense regions, making them distinguishable on MR images.

METHODS: Human mesenchymal stem cells (hMSCs) obtained from the VA Medical Center (San Francisco, CA) were labeled using Feridex IV (Fe) (Berlex Laboratories, Wayne, NJ). Protamine Sulfate (Pro) (APP, Schaumberg, IL) was used as a transfection agent. Cells were labeled using the Fe-Pro complex as described in Arbab et al². Following labeling, a Trypan Blue Assay was performed to assess cell viability.

For in vitro imaging, cells were placed in tubes (10⁶-10⁷ cells/mL) containing Ficoll (Amersham Biosciences, Piscataway, NJ) to maintain osmolarity. Tubes were placed in a water bath and imaged at room temperature (20°C). Imaging was done on a 3T scanner (GE Medical Systems, Waukesha, WI), and Mayo wrist coil (Mayo Clinic, Rochester, MN). A spin-echo sequence was used to determine T₁ (TE/TR = 12/60-1000 ms) and T₂ (TE/TR = 12-48/4000 ms) times. A gradient-echo (GRE) sequence was used to measure T₂* times using flip = 90°, and TE/TR = 4-20/34 ms. Dedicated software was used to quantify relaxation times. For ex vivo imaging, two cylindrical osteochondral defects (R = 2 mm, H = 4 mm) were created in the trochlea of the rabbit distal femur. Gelfoam Size 100 (Pharmacia and Upjohn, Kalamazoo, MI) cut to the size of the defect was used to load the labeled hMSCs. The two defects were used as follows: (1) labeled cells on Gelfoam (3x10⁶ cells/mL); (2) empty Gelfoam. The excised knee was imaged using a T₂*-weighted 3D GRE sequence.

RESULTS: The results of the in vitro experiments indicate efficient cellular uptake of the Fe-Pro complex and resulting MR signal intensity (SI) loss (Fig 1). This can be quantitatively characterized in plots of SI versus TR (T₁ values) and SI versus TE (T₂ and T₂* values) (not shown). In addition, labeling does not appear to affect cell viability. While 10⁶ cells/mL results in strong SI loss, 10⁵ cells/mL loss of SI can also be detected in vitro relative to unlabeled cells.

In the rabbit model, cells labeled with Fe-Pro appear as a signal void on the MR image (Fig 2). In contrast, the defect loaded with empty Gelfoam (no cells) appears brighter with a signal resulting from the scaffold.

DISCUSSION AND CONCLUSIONS: Stem cell labeling with Fe-Pro results in a significant loss of SI in MRI. The results suggest that this method of cell tracking could be applied to in vivo detection of stem cell therapy. Following initial detection of transplanted cell populations, this minimally invasive technique could allow for in vivo longitudinal tracking of tissue regeneration.


ACKNOWLEDGEMENTS: This research was supported by NIHRo1-AG17762.
Effect of mechanical load on matrix synthesis in scaffold free, de novo cartilage –like tissue

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INTRODUCTION: The development of autologous de novo cartilage in vitro requires chondrocyte isolation and a period of pre-culture before reimplantation. Implantation of an immature three dimensional implant may lead to premature failure. With this in mind we investigated whether the application of cyclical load and shear would increase the matrix synthesis within scaffold free, de novo cartilage-like constructs.

METHODS: Chondrocytes were isolated from calf knee articular cartilage and cultured in DMEM:F12 with 10% Foetal calf serum. Chondrocytes were encapsulated in alginate [1]. Implants were produced by liberating the chondrocytes and seeding them into a rectangular mould containing space for 20 implants, each 5 mm in diameter and 1 mm deep. 0.5N of load was applied by way of a roller that is able to apply both load and shear. For mRNA analysis of Collagen II and Aggrecan, the mRNA was isolated by the procedure of Chomczynski and Sacchi [2] and quantification was performed by competitive PCR using a known quantity of plasmid DNA as standard. The resultant values were normalized to GAPDH expression. Glycosaminoglycan content was quantified by alcian blue precipitation and DNA content measured by Hoechst 33258 fluorescence.

RESULTS: RNA samples were taken hourly from implants subjected to load applied at a frequency of 0.276Hz for 4 hours. The expression of collagen II was increased within one hour of load (171% of control) as was aggrecan (202% of control). The increased expression of both genes peaked at 2 hours (collagen II 239% control, aggrecan 253% control) before returning to almost basal levels by four hours. The peak at two hours indicated a desensitization to the load being applied. Thus, further experiments consisting of 2 hours interspersed with a 2 hour pause. To investigate longer term loading on matrix production, the implants were subjected to a loading regime consisting of 0.276Hz, 2 hours each morning and afternoon. After 4 days of load the samples were harvested and the RNA expression and GAG/DNA content was determined. Loaded samples demonstrated an increased expression of both matrix genes investigated (221 % collagen II and 292% aggrecan). There was also an increase of GAG/ DNA to 167% of the unloaded controls.

DISCUSSION & CONCLUSIONS: The increase in incorporated matrix noted within 4 days of loading indicates the cyclic dynamic load, along with shear, may indeed be a viable method for increasing the matrix content of 3 dimensional implants prior to implantation.

REFERENCES:

ACKNOWLEDGEMENTS: This work was supported by the Swiss National Science Foundation (Grant number- 32-52945.97), and the Hirslanden Group, Zürich, Switzerland.
Dexamethasone releasing chitosan scaffold for cartilage development
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INTRODUCTION: In articular cartilage defects, penetration of the subchondral bone allows rapid migration of inflammatory cells and blood cells which initiate a wound healing response [1]. Subcutaneous, intravenous, or intra-articular delivery of growth factors or non-steroidal anti-inflammatory drugs (such as dexamethasone), appear to overcome the joint pain associated with inflammation. However they have numerous side effects such as requirement of frequent injections in high drug concentrations as due to drug loss with molecular diffusion and/or deactivation. One possible method to overcome this problem is the development of sustained drug release systems [2]. We have recently prepared dexamethasone-loaded chitosan scaffold which is releasing constant amount of drug for long-term control of inflammatory responses.

METHODS: Porous chitosan scaffolds were prepared by freeze-drying method. Chitosan (Sigma, >85% deacetylated) solutions (2% and 3%(w/v) in acetic acid) were frozen at -20°C for 24 h and lyophilized at -80°C for 4 days. Incorporation of dexamethasone to chitosan scaffolds was performed by embedding technique through different concentrations of dexamethasone solutions, i.e. 5, 10 and 15 µg/ml. In vitro release studies was performed by incubating scaffolds in polypropylene tubes containing Dulbecco’s PBS at 37°C with 15 rpm agitation. Release media were changed at preset intervals and supernatants were analyzed by UV spectroscopy at 239 nm to calculate cumulative amounts of released dexamethasone.

RESULTS: The release behaviour of dexamethasone from chitosan scaffolds, which have 2% and 3% (w/v) compositions, showed a long period of sustained release after an initial burst of 8 h and 6.5 h, respectively (Figure 1). For both chitosan scaffolds a complete release of dexamethasone was observed and release periods were changed according to incorporated doses of drug into scaffolds. The kinetic data obtained for in vitro release studies were evaluated using the equation given below [3].

\[
\frac{M_t}{M_\infty} = k t^n
\]  

DISCUSSION & CONCLUSION: Porous chitosan scaffolds were prepared in order to achieve a sustained release of anti-inflammatory drug dexamethasone for at least a week in vitro. Not only a successful complete release of dexamethasone from chitosan scaffolds with different compositions was maintained but also a sustained release was achieved with a diffusion controlled release mechanism. Consequently, dexamethasone releasing porous chitosan scaffolds could preclude side-effects associated with systemic delivery in cartilage development.

Notch family signalling and notch signalling modulation during chondrogenesis

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INTRODUCTION: It has been shown that expression of the Notch signalling pathway regulates many aspects of limb development and plays a vital role in chondrocyte maturation¹. The Notch family of transmembrane signalling receptors comprises the Notchs 1-4 and their corresponding ligands Delta (D) and Jagged (J). In this study, we first demonstrate the differential distribution of the Notch family receptors during development and secondly, we investigate the effects of inhibiting the Notch pathway using the γ-secretase inhibitor DAPT² demonstrating the importance of Notch’s role in chondrogenesis.

METHODS: Chick hind limbs (HH st26-st44) and micromass cultures were immunolabelled with antibodies to Notch 1-4, D1 and J1 (10µg/ml, Santa Cruz Biotech Inc, USA). Micromasses were established from wing mesenchyme of st23 chick embryos at a density of 2 x10⁵ cells per 10ul as described³. Micromasses were treated with the γ-secretase inhibitor DAPT (Calbiochem,UK) to inhibit S3 cleavage of Notch preventing Notch signal activation. Controls consisted of the DAPT vehicle, DMSO. Media samples and micromasses were analysed for GAG content using the DMMB assay⁴. Results were expressed as total GAG content normalised to DNA. Micromasses were fixed in NBFS and stained with alcian blue pH1.0 overnight. Labelled nodules were counted. One way ANOVA was used for all statistical analysis.

RESULTS: The 4 Notch receptors and their ligands D1 and J1 showed distinct distribution throughout development of the chick limb. Notch 1 (N1) was seen extensively in the interzone region of the developing limb where D1 and J1 expression was also evident (Fig1). Post cavitation D1 and J1 were present in the cartilage surface but to a lesser extent than N1.

In vitro studies showed that on addition of the Notch inhibitor DAPT significant (P<0.05) increases in the total amount of GAG occurred. No change in DNA content was observed. A 3 fold increase in chondrogenic nodule number was revealed by alcian blue staining in DAPT treated micromasses (Fig2).

Immunolabelling of DAPT treated micromasses showed a higher proportion of N1 label within the chondrogenic regions. D1 was seen to be expressed in the DAPT treated micromasses whereas J1 expression was lost.

DISCUSSION & CONCLUSIONS: Mapping Notch family member expression in development of the avian limb shows a differential distribution pattern of expression suggesting Notch signalling is a necessary factor in the development and homeostasis of the cartilage anlagen. Inhibition of the Notch pathway using the γ-secretase inhibitor DAPT in mesenchyme micromass cultures demonstrates enhanced chondrogenesis that may reflect precocious cartilage differentiation of normally non-chondrogenic cells. This could be the result of a loss of lateral inhibition, suggesting that the Notch signalling pathway plays a vital role in chondrogenesis.


ACKNOWLEDGEMENTS: Work is funded by Arthritis Research Council.
BioDynamic Test Instrument for Characterization of Tissues and Biomaterials

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INTRODUCTION: The objective of this work was to employ the novel design of the BioDynamic testing platform to evaluate the mechanical properties of hydrogels and other composite biomaterials. The testing platform allows for continuous test and stimulation in a fully integrated and instrumented configuration by providing material characterization (viscoelastic properties, strength, creep and stress relaxation) within a physiological environment (nutrient flow, pressure loading, pH, dissolved oxygen, and temperature).

METHODS: An advanced BioDynamic testing platform has been designed that can be used to evaluate the mechanical properties of tissue-engineered constructs for both cardiovascular and musculoskeletal applications. The BioDynamic instrument was used to test a variety of specimens to demonstrate its versatility and advanced features with two examples described here. The dynamic mechanical properties of polyvinyl alcohol hydrogels (Cambridge Polymer Group, Boston, MA) were evaluated with our unique computer-controlled moving magnet linear motor that provides load, displacement, strain or pressure profiles. The hydrogel samples were 3-4 mm in diameter and 3-4 mm in height, and testing was performed in compression with a 5 mm displacement transducer and a 250 gram force transducer.

Vascular graft distension with increasing pressure was also evaluated in a BioDynamic instrument using a laser micrometer. The graft material used (Gel-Del Technologies, Inc., St. Paul, MN) is composed of proteins and polymers fabricated to mimic the viscoelastic properties of native blood vessels. The inner diameter of the grafts used was 3 mm and total length was approximately 30 mm. A laser micrometer was placed over the chamber with the laser beam penetrating the transparent chamber doors and measuring outer diameter (OD) changes with pressure and time.

RESULTS: The ability to perform very low force applications was tested using hydrogel specimens. The peak-to-peak loading on the hydrogel was approximately 2 mN with a corresponding peak-to-peak displacement of 28 µm. A distinct linear region was not observed with a displacement ramp as the specimen made a very gradual change in stiffness as a function of % strain. Upon completion of data acquisition, the software calculated the modulus and tan delta for the specimen, which appeared to exhibit resonance between 20 and 100 Hz.

When two cycles of a sinusoidal pressure waveform from 0 to 25 mmHg were followed by a cycle of pressure increase to 250 mmHg, the diameter response of the composite vascular graft biomaterial followed the pressure changes very closely throughout the test. After each cycle, OD did not return to its initial value within the test’s time frame, indicating potential creep behavior. A cycle of pressure increase from 0 to 295 mmHg is also shown. The specimen is again exhibiting creep by not returning to its initial diameter over the time frame studied.

CONCLUSIONS: The development goal was to create a fully integrated computer-controlled data acquisition and analysis testing system to characterize biomaterials, scaffolds and tissues in a biological environment.

The data obtained with the hydrogels show that the BioDynamic instrument is suitable for evaluating the dynamic properties of soft biomaterials. The results obtained with the vascular graft biomaterial confirmed the biphasic composition of the specimen that is composed of a protein-rich material and synthetic polymers and can serve as an indicator of vascular wall elasticity and compliance. Preliminary results with hydrogel disks for orthopaedic applications and vascular grafts show that the BioDynamic test instrument is a powerful tool for the integration of biochemical and mechanical stimulation and properties characterization in one system.
WATER-JET CUTTING OF ARTICULAR CARTILAGE TO IMPROVE CELL VIABILITY IN CARTILAGE REPAIR

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INTRODUCTION: Osteochondral grafting is a standard procedure in the surgery of cartilage defects. It involves the retrieval of osteochondral plugs from the donor site, typically with a punch device and the similar preparation of a recipient bed. Conventional punch sets cause chondrocyte death at the edge of either the transplant or the recipient bed 1-2. Water Jets (WJ) are a group of “cold” cutting methods, where the loads applied to the target material are minimized when compared to conventional tools. The cut is performed using water under high pressure (potential energy) by transforming it into water with high velocity (kinetic energy) using a nozzle, as seen in Fig. 1. Aim of the study was to compare cell viability of a conventionally prepared cartilage to the WJ.

METHODS: 24 full-thickness patellar articular cartilage slices (20mm x14mm) were obtained from 22 month-old cattle under sterile conditions. The recipient tool of an 8 mm osteo-articular transfer system (OATS, Arthrex) was used to remove a cartilage cylinder from the center of each sample. Then the WJ was used to cut the sample in half. The nozzle (Ø=0.2mm) was positioned with a stand off distance of 5mm and cutting was performed at a traverse speed of 1mm/s using saline solution and a pressure of 50MPa. The samples were cultured up to 8 days with live dead staining and confocal microscopy competed at days zero, four and eight. One-way analyses of variance were computed with the depth of cell death as dependent variable.

RESULTS: The resulting area of dead cells was significantly smaller for the WJ cut samples if compared to the OATS- punched samples (Fig.2) at any time point of the tissue cultivation (p<0.001, Fig.3).

DISCUSSION & CONCLUSIONS: The results suggest that WJ have great potential for cartilage surgery. The tremendous reduced cell death could hypothetically be traced back to minor mechanical stress applied to the chondrocytes during cutting. An additional advantage is the potential to cut varying geometrical shapes and sizes as need to machine the individual defect fitting geometry as per the surgeon’s requirements.