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CLINICAL OVERVIEW OF CARTILAGE REPAIR

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The problem of managing patients with articular cartilage damage has tantalised surgeons from the earliest times and it has been recognised that intrinsic healing of cartilage is, at best, a limited occurrence. The subject was carried forward immensely by the work of Audrey Smith who first successfully isolated chondrocytes and made possible the early animal experiments to transplant freshly isolated and stored chondrocytes into joints to heal surface defects. This pioneer work laid the foundation for cell-engineering as it is now recognised and for the clinical applications in the last decade of various cell-based repair techniques.

Petersen and colleagues in Gothenburg have demonstrated that repair of articular cartilage defects with isolated cells which have been cultured and then re-implanted can be successful and this has been confirmed by a number of other individuals and centres throughout the world. Data is emerging which suggests that this method of cell implantation is superior to any method of transplanting intact cartilage with or without its subchondral bone.

However the results are not universally successful and the interaction of biomechanical with the biological factors which are necessary for success are crucial in achieving a high proportion of success in patients. Thus randomised controlled clinical trials are vital to carry the subject forward and to indicate much more precisely what constitutes long-lasting repair in the damaged joint as distinct from temporary repair and also the correct indications for particular techniques in different joints and different locations.

Many very exciting laboratory techniques are being developed which will enhance this work for clinical application. The prospect of having a matrix or membrane carrier for cells which can be inserted by minimally-invasive techniques would be a great advantage from the point of view of patient rehabilitation. Alternative methods such as the use of stem cells or allograft cells are interesting but carry possible risks which require careful evaluation by both animal experimentation and carefully designed clinical trials.

It now appears definitely established that cell-based cartilage repair is successful in appropriate cases but there are technical problems to be solved such as the presence of large bone defects and also there is uncertainty as to the longevity of such repair. These questions will be answered progressively by collaboration between clinicians and scientists and promise to reduce not only disability in young people following injury to joints, but also to prevent early onset osteoarthritis. In the longer term it may be possible to treat more advanced joint damage by such methods.

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THE AUTOLOGOUS OSTEOCHONDRAL TRANSPLANTATION (AOCT) FOR FOCAL CARTILAGE DEFECTS IN THE KNEE

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AOCT is an established form of treatment for focal cartilage defects of the knee, ankle and other joints of limited size. In the knee one agrees that the defect size should not exceed 25 by 25 mm. The ideal indication is the traumatic lesion; relative indications are a limited area of avascular necrosis of a femoral condyle or a case with predominantly femoral osteoarthritic involvement and unicompartmental disease where combined with generous unloading of the involved compartment by a femoral or tibial osteotomy. A useful indication is also the defect or wear situation of the femoropatellar joint when dislocation or subluxation is the origin or when there is a clear trauma as the start of the problem with a contusion followed by symptomatic cartilage necrosis some months later. Mosaicplasty of the patella and or trochlea with an alignment procedure and/or advancement of the tibial tuberosity performed at the same time can definitely improve the situation

The isolated trochlear defect however with no clear traumatic onset is unsuccessful in our hands.

In the talus the indication had been initially extended from the focal defect to the degenerative defects on both talus and tibial surface, has however been restricted due to unfavourable results to OD of the talus. An osteotomy of the medial or lateral malleolus is mandatory for the approach.

Our experience in the knee has convinced us of the necessity of an accurate placement of the grafts in regard to the intact neighbouring surface with which they should be flush. Parallel placement of the cylinders with partially non-perpendicular retrieval at the donor cartilage surface is required when one wants to avoid the "foot collision" between the plugs. This allows then to recreate the biconvex radius of the condyle that is congruent and that does not contain prominent or deep sunken plugs.

Insertion of the plugs should be done with great care to avoid traumatic cartilage necrosis.

Insecurity remains regarding the histomorphological behaviour of the cartilage of the transplanted cylinders. Animal work has shown the cartilage to become softer with time. There is also some worry about cyst formation in the base area of the grafts that could prevent a solid ingrowth of the grafts or maintain a passage of synovial fluid with gap formation at the surface and crack propagation and surface delamination.

Having performed this operation in 200 cases and despite of all of these worries there is an 80% success of subjective and objective evaluation of our results. We regularly practise the AOCT and like it because it is readily available and economic. There is however room for future methods that combine the mechanical advantage of a few osteochondral plugs with a tissue-engineered cartilage implant (De Novo) with a smoother surface mainly for defects that are mostly cartilagineous alone. This could help to decrease the number of plugs to be retrieved, allow at the same time to fill the defects created by the plug removal in the trochlea and to end up with a more homogenous surface and less osseous, subchondral changes. The key question to solve regards the histochemical properties of the cartilage implant and its properties on the long run.

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THE EFFECT OF THE STRUCTURE IN OSTEOCHONDRAL GRAFTS – A COMPARISON OF PHOTOOXIDIZED MUSHROOM STRUCTURED TO CYLINDRICAL GRAFTS IN AN EXPERIMENTAL STUDY IN SHEEP

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INTRODUCTION: Osteochondral grafting procedures for cartilage resurfacing have gained popularity in clinical orthopaedics over the last years. Auto-[1], pretreated allo- [2] and xenografts [3] were successfully implanted in clinical and experimental studies, where either single grafts or multiple grafts in form of mosaicplasty [4] were applied. Although in clinical cases cylindrical grafts were reported to be successful, experimental data demonstrated that central necrosis of the grafts and bone necrosis with subsequent collapse of the graft into the original defect proved to be a problem [3]. The structure of the cylindrical graft may be related to this problem and therefore a mushroom-structured osteochondral graft pretreated with photooxidation [3] was compared in an experimental animal study with sheep. It was hypothesized that in a mushroom-structured graft central necrosis and bone necrosis of the host bed would be avoided.

Materials and methods: Two main groups were made, with 8 Swiss Alpine sheep between 2-4 years of age in each group. In one group, photooxidized osteochondral grafts with a cylindrical structure, while in the other group a mushroom structured graft was used. For each main group, two subgroups with 4 animals each were formed. The first subgroups were sacrificed at 2 months and the second subgroup at 6 months after surgery. Both osteochondral grafts were implanted into the medial and lateral condyle of the distal femur at the weight bearing location using a pressfit technique. After sacrifice, the cartilage surface was assessed macroscopically, while the subchondral bone was evaluated with microradiography. Histology of nondecalcified bone sections embedded in methylmetacrylate based resin was performed, where ground sections (30-40µm) and thin sections (5µm) were stained with toluidine blue or von Kossa/McNeal.

Results: All grafts were still well positioned at the time of sacrifice. The bluish colour was slightly diminished and the interface between host and grafts was visible in all instances. Partial collapse of the grafts into the original defects was demonstrated with some cylindrical but not mushroom structured grafts. The cartilage surfaces were intact in the grafts and in the adjacent host matrix. Bone resorption and central necrosis was observed histologically and radiographically mainly in the cylindrical grafts. In the mushroom structured grafts, bone and cartilage remodelling as well as new extracellular matrix synthesis of the cartilage was further advanced, while the development of cyst-like lesions was more prominent in the cylindrical grafts, mainly at 6 months. Statistically significant differences in the percentage of fibrous tissue and bone matrix were found ($P<.05$), such that the mushroom-structured grafts demonstrated less fibrous tissue and more remodelled new bone matrix at 2 and 6 months.

Discussion and conclusion: Overall, mushroom-structured osteochondral grafts were superior to cylindrical grafts in regard of the remodelling of the subchondral bone as well as repopulation with living cells of the cartilage. This was attributed to the better mechanical stability of the mushroom structured grafts, assuming that the mechanical load would be distributed on two levels thereby reducing bone resorption at the base of the mushroom stem, while at the same time facilitating in-growth and bone remodelling at the level of the subchondral bone of the mushroom head. In addition, the better mechanical stability seemed to encourage faster ingrowth of cells from the subchondral bone area into the old cartilage matrix. Therefore, it was concluded that mechanical stability of osteochondral grafts is dependent on the structure of the grafts and is important for overall graft survival. In addition, repopulation of the old, nonviable photooxidized cartilage demonstrated that the extracellular cartilage matrix provided enough stability for tissue-guided regeneration of the cartilage surface.

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BERLIN CHARITÉ CONCEPT ON TREATMENT OF CARTILAGE DEFECTS

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Progressive degeneration of joints following the traumatic or non-traumatic destruction of the articular cartilage is one of the most common causes of musculo-sceletal disability worldwide. This occurs preferentially at the knee and hip joint and may be the source of long-term joint dysfunction. Defects in articular cartilage are classified as full or partial thickness depending on whether or not there is penetration down to the subchondral bone. Furthermore defect size, patient age and additional ligamentous injuries must be taken into consideration for treatment of cartilage defects.

For smaller grade III-IV defects (smaller than 2cm2) current methods for treatment mainly relies on induction of chondrogenesis by penetrating the subchondral bone. Induction of vascular mediated elements by bone drilling, abrasion or microfracturing is the basis behind these techniques. For this treatment arthroscopic techniques are standard. For lager defects (from 1-2 cm2) autologous osteochondral transplantation (OATS, Mosaicplasty) are proved methods. For defects which are larger than 4 cm2, osteochondral allografts are successfully used in many centres. Also osteotomies can lead to good clinical results. Autologous chondrocyte transplantation (ACI) has shown to be effective over many years. However this an expensive method of treatment and will not be covered by numerous ensurances in Europe.

Although the cartilage surface has been identified to play an important role in cartilage degeneration, not many successful attempts have been made clinically to treat the cartilage surface (and partial thickness defects) in order to prevent further cartilage damage. The problem associated with partial thickness defects is that they do not heal spontaneously because there is generally no contact to bone marrow and therefore no access to macrophages and endothelial cells. The most common tool to treat cartilage surface defects clinically is arthroscopic shaving. However this technique has various shortcomings. Radiofrequency treatment is gaining increasing popularity for treatment of partial thickness defects. However in numerous experimental studies it was shown that RF-treatment can lead to large cartilage defects.

Treatment of full and partial thickness cartilage defects by means of tissue engineered cartilage and use of chondrocyte seeded scaffolds for cartilage resurfacing and defect filling additionally enhanced by various growth factors is subject of current research or first clinical studies.

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CELLULAR RESPONSES OF ARTICULAR CARTILAGE TO SHARP AND BLUNT TRAUMA

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INTRODUCTION: Articular cartilage has a poor reparative response to injury, impeded by the lack of successful integration between the host and repair tissue. This is due in part, to an area of cell death present at the wound margin that is of an apoptotic and necrotic nature. The repair tissue integrates into effectively 'dead tissue' that will fatigue and fracture with time leading to the eventual disintegration of the wound site [1]. Behind this zone of cell death there is a limited repair response of chondrocyte proliferation and altered matrix metabolism [2]. We hypothesise that mechanical stress associated with injury contributes to this pattern of cell death. We have therefore, observed the response of articular cartilage to blunt trauma, caused by a trephine, and sharp trauma, caused by a scalpel, in terms of cell death, cell proliferation and matrix synthesis.

METHODS: Full depth immature (~7day) bovine articular cartilage explants were taken from the metacarpal-phalangeal joint. Blunt wounds were made with a 1.3mm internal diameter trephine [3]. The explant was sliced in half through the centre of the trephine wound with a sharp scalpel (#23 blade) so both blunt and sharp trauma were on the same explant. Wounded and unwounded explants were cultured for 10 days in DMEM/F12 supplemented with 10% serum. Prior to fixation at days 2, 5 and 10 medium was supplemented with 10µCi/ml ^{35}Sulphate, ^{3}H-proline or ^{3}H-thymidine for 24 hours. Explants were fixed and processed for routine histology and micro-autoradiography. Cell death was analysed by TUNEL labelling of wax sections and by live/dead labelling of unfixed tissue. Statistical analysis of ^{3}H-thymidine incorporation was carried out using a one-way analysis of variance.

RESULTS: In the case of sharp wounds, cell death was only seen within the first 20µm from the lesion edge (Fig.1A). Both ^{3}H-proline and ^{35}S-sulphate were detected up to the lesion edge at all time points (Fig.2A) whilst ^{3}H-thymidine incorporation was restricted to chondrocytes adjacent to the lesion edge. With blunt wounds histological analysis revealed a band of cell death extending ~150µm from the lesion edge at all time points, which was confirmed by TUNEL and live/dead labelling (Fig1B). Micro-autoradiography showed little incorporation in this region of cell death by either ^{35}S-sulphate or ^{3}H-proline (Fig.2B). ^{3}H-thymidine incorporation was located in the region behind the area of cell death.

DISCUSSION & CONCLUSIONS: Cell death at the lesion edge is a potential hindrance to successful integration [3]. After sharp injury we demonstrated that the majority of the observed cell death is inhibited and there is an up-regulation of matrix synthesis above normal levels adjacent to the wound edge. Cell proliferation is also promoted adjacent to the lesion edge where many animal studies have previously shown hypocellularity [4]. In terms of clinical relevance, the use of sharp precise instruments during the surgical management of cartilage defects may be necessary to reduce cell death and promote matrix synthesis at the lesion edge to aid successful integration.

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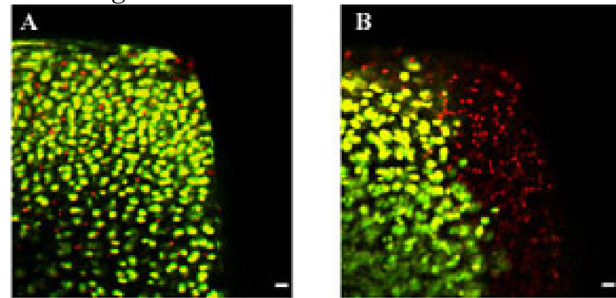

Figure 1: Live/Dead labelling of immature bovine articular cartilage wounded with a scalpel (A) or trephine (B) 2 days post-wounding. Membrane compromised cells are labelled red, viable cells green. Scale bars 20µm, the articular surface is to the top and wound edge is to the right of the image.

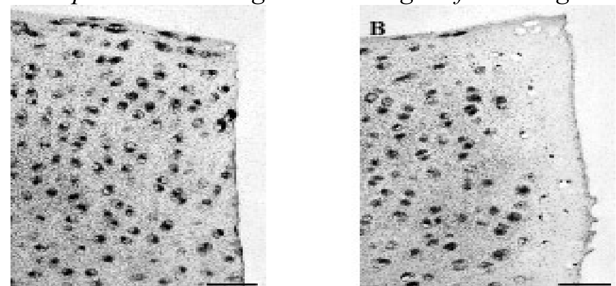

Figure 2: ^{3}H-proline labelling of tissue wounded with a scalpel (A) trephine (B) 2 days post-wounding. Tissue is counterstained with haematoxylin & eosin. Scale bars 50µm, the articular surface is to the top and wound edge is to the right of the image.

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THE REGULATION OF CARTILAGE MATRIX TURNOVER AND THE PATHOLOGY OF OSTEOARTHRITIS

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INTRODUCTION: Osteoarthritis (OA) is a degenerative joint disease involving the whole diarthrodial joint. Extensive remodelling of bone, changes in ligaments, menisci and synovia accompany the progressive degeneration of articular cartilage (AC). With the damage to AC there is a progressive loss of joint function.[1]

Idiopathic OA involves the formation of focal lesions that progressively enlarge, leading to the eburnation of subchondral bone and changes in the congruency of articulating surfaces. The development of these lesions involves excessive degradation of extracellular matrix, in particular the resident collagen fibrils (composed mainly of type II collagen), more remote from chondrocytes.[2, 3] In healthy cartilages only pericellular molecules are ordinarily degraded in young individuals (up to 35 years).[4] The degradation is generally seen in the same sites where the collagenases, matrix metalloproteinases (MMP)-1 and –13 are localized.[4] MMP-13 appears to be involved in the excessive cleavage of 'resident' collagen molecules: MMP-1 may often be more involved in the degradation of newly synthesized molecules.[5] Both aggrecanase and MMPs are involved in the excessive cleavage of aggrecan[6] There is also an increase in synthesis of these matrix molecules in OA. This involves onset of gene expression of the type IIA COL2A1 gene and especially upregulation of type IIB collagens.[1] Collagen synthesis is increased as revealed by radiochemical analyses and the increased generation of the c-propeptide of these type II molecules.[7] This frequently occurs in the same sites where cleavage is observed which, as discussed above, involves cleavage of newly made molecules.

The degenerative process is initiated at the articular surface, extending progressively into the deeper layers[4] – probably over a period of 10-20 years or more. It is normally very slow but can be accelerated in the presence of joint inflammation or following joint injury causing changes in joint loading. As the collagen network degenerates, so chondrocytes differentiate and become hypertrophic, expressing type X collagen, annexin V and other genes associated with hypertrophy. These also include MMP-13 and COL2A1, including type IIA collagen. There is partial calcification of cartilage matrix. Eventually this results in chondrocyte apoptosis as in the growth plate and fracture callous, as part of endochondral ossification.[1]

The excessive degeneration of type II collagen and chondrocyte hypertrophy can be induced by a single small peptide of this same collagen working via a specific cell surface receptor. This is dependent upon increased expression and activity of interleukin-1 or tumour necrosis factor α, both of which are often involved in the excessive degradation seen in OA cartilages in culture.[8] The chondrocyte is very sensitive to its environment and to the degradative changes that it creates through the excessive generation of MMPs. It is also very sensitive to changes in mechanical loading and there is evidence to indicate that mechanical loading combined with excessive proteolysis is required for the development of focal lesions.[9]

Through the work of many laboratories we now have a much clearer understanding of the pathobiology of this disease—one where the events seen in OA are likely also encountered in the engineering of new cartilage. Comparative studies of both OA and cartilage engineering may provide a better understanding of the problems we face in each field. Since the management of OA needs more effective cartilage repair and often cartilage repair results in a degenerative process..

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AGING & THE PATHOGENESIS OF OSTEOARTHRITIS – IMPLICATIONS FOR CARTILAGE REPAIR

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INTRODUCTION: After age 40 the incidence of osteoarthritis in humans increases progressively with increasing age. Age related declines have been demonstrated in articular cartilage chondrocyte synthetic activity and response to anabolic cytokines. Increasing age is also associated with alterations in extracellular matrix proteoglycans and collagens and deterioration of the mechanical properties of articular cartilage. Yet, the mechanisms responsible for these changes in articular cartilage and the age related increase in the incidence of osteoarthritis have not been defined. Furthermore, clinical experience suggests that methods of promoting articular cartilage repair or transplanting articular cartilage are less successful in patients 40 years of age and older. We hypothesized that in vivo articular cartilage chondrocyte senescence impairs the ability of the cells to maintain or restore articular cartilage and thereby increases the risk of osteoarthritis. Chondrocyte senescence may also decrease the effectiveness of methods of stimulating articular cartilage repair and cartilage transplantation.

METHODS: To test this hypothesis we measured senescence markers in human articular cartilage chondrocytes from 27 donors ranging in age from one to 87 years. The markers included expression of the senescence associated enzyme beta galactosidase, mitotic activity and telomere length. We also examined mitochondrial DNA and mitochondrial numerical density per cell. To determine if chondrocyte age changes are potentially reversible we transfected human articular cartilage chondrocytes from a 47 year old donor with human telomerase gene and human oncogenes.

RESULTS: Beta-galactosidase expression increased with age ($r = .84$, $p = .0001$) while mitotic activity and telomere length declined ($r = -.77$, $p = .001$ and $r = -.71$, $p = .0004$ respectively). Decreasing telomere length was strongly correlated with increasing expression of beta-galactosidase and decreasing mitotic activity. With increasing numbers of cell divisions mitochondrial DNA was degraded and the numerical density of mitochondria per cell declined. Transfection of human articular cartilage chondrocytes from a 47 year old donor with human telomerase and oncogenes created a cell line that has completed more than 300 population doublings as compared with an upper limit of 20 population doublings for normal cells. Telomere length increased in the transfected cells and then remained constant over more than 300 population doublings.

DISCUSSION & CONCLUSIONS: These findings help explain the previously reported age related declines in chondrocyte synthetic activity and response to anabolic cytokines, and indicate that in vivo chondrocyte senescence may contribute to the age related increase in the incidence of osteoarthritis. Degradation of chondrocyte mitochondrial DNA compromises synthesis of electron transport proteins. Deterioration of mitochondrial function and loss of mitochondria forces cells to increase reliance on glycolysis and exposes them to increased oxidative damage due to free radicals. The creation of an immortal line and increased telomere length by transfection of human chondrocytes suggests that it may be possible to alter the progression of human chondrocytes toward senescence. Future efforts to restore articular cartilage surfaces in middle age and older individuals might include either modification of autogenous chondrocytes or introduction of new cell populations.

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THE BIGLYCAN/FIBROMODULIN DOUBLE-DEFICIENT MOUSE: CHARACTERIZATION OF A NEW ANIMAL MODEL OF OSTEOARTHRITIS

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INTRODUCTION: Biglycan and fibromodulin are two extracellular small leucine-rich proteoglycans co-expressed in tendon, cartilage and bone [1]. Collagen fibrils in tendons from mice deficient in biglycan and fibromodulin are structurally and mechanically altered resulting in unstable joints. As a result, the mice develop successively and progressively: gait impairment, ectopic tendon ossification and severe premature knee osteoarthritis (OA) [2]. Forced use of the joints increases ectopic ossification and OA in the double-deficient mice further indicating that structurally weak tendons may cause the phenotype. In the knees, the articular cartilage lesions recapitulate the histological features of human OA. In order to further characterize and validate the use of the biglycan/fibromodulin double deficient mouse as an animal model of OA, other joints were processed for histology and an immunohistochemical characterization of the cartilage lesions was initiated.

METHODS: Knees, hands, feet, hips, shoulders, and elbows were fixed in Z-fix, decalcified in Immunocal and processed for histology. Immunostaining for cartilage oligomeric matrix protein (COMP), decorin and type II collagen was performed on wild type and double deficient knees. Two and 6 month-old knees, corresponding respectively to an early and an advanced stage of the disease in the double deficient mice, were analyzed.

RESULTS: All the observed joints develop OA although the severity of OA differs from joint to joint, with knees joints being affected first and most severely.

In two month-old wild–type knees, the expression of COMP was low and restricted to the extracellular matrix above the tidal mark. Comparatively, this area was more strongly stained in the two month-old double deficient knees. In addition, in the double deficient knees, the calcified matrix and its chondrocytes were also stained but at a lower level. At 6 months, the level of expression of COMP had increased in the wild-type mice but decreased in the double deficient mice.

Type II collagen stained the whole articular cartilage matrix and the chondrocytes in wild-type knees. In double deficient knees, type II collagen was not detected at the articular surface at 2 months, and by 6 months its absence had spread to the matrix surrounding the chondrocytes. Similar, but not identical, patterns of expression were observed for decorin.

DISCUSSION & CONCLUSIONS: Our data demonstrate that the biglycan/fibromodulin double deficient mouse develop polyarthritis. Because each joint develop OA at its own time and pace, the biglycan/fibromodulin double deficient mouse will provide a great deal of flexibility as an animal model to test the *in vivo* actions of molecules on OA. In addition, the immunohistochemical results reported here are similar to immunostainings performed on samples from natural OA and other animal models. The transient increase in COMP level observed here has also been reported in Del1 mice, a transgenic model of OA and in natural OA in horses. Superficial loss of decorin occurs in human OA and the collagen loss pattern reported here mimic the degradation pattern of type II collagen in human OA. Taken together, our data support the use of the biglycan fibromodulin deficient mouse as an animal model of OA.

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CONTRAST-ENHANCED MRI AS A MEANS TO STUDY CARTILAGE STRUCTURE

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Introduction: A major issue in OA research has been the fact that cartilage changes develop over decades, however, our ability to identify such changes before severe joint cartilage disease occur has been limited. Since treatments attempted at restore the functional properties of the joint cartilage likely is more successful if initiated before such severe matrix changes develop, we must be able to identify the earliest phases of disease changes. Magnetic resonance imaging (MRI) has become the most accurate method to image articular cartilage structure as well as to provide information about surrounding soft tissues and subchondral bone non-invasively. However, in earlier stages of cartilage pathology, conventional MRI sequences have shown limitations in providing a detailed assessment of the matrix. In OA research, it would be more valuable to be able to image and quantify macromolecules in the matrix, particularly those that are altered early in the OA disease. A method to study cartilage GAG content is delayed contrast enhanced MRI of cartilage (dGEMRIC). This technique is based on the principle that the negatively charged contrast agent (Gd-DTPA^{2-}) distributes in the cartilage in an inverse relationship to the GAG content. In normal cartilage Gd-DTPA^{2-} is repelled by the abundant negatively charged GAG, whereas in conditions of GAG loss, more Gd-DTPA^{2-} will be distributed within the cartilage matrix. To address the issues of the *in vivo* dose-response distribution in cartilage as well as the time window for MR imaging after contrast injection we examined the temporal pattern of Gd-DTPA^{2-} distribution in knee cartilage at three different doses in healthy volunteers. It was shown that dGEMRIC has a linear dose-response distribution in femoral weight bearing cartilage with the highest concentration between two and three hours post-contrast (1,2,3).

To explore the potential of dGEMRIC, we compared Gd-DTPA^{2-} distribution in femoral knee joint cartilage in sedentary and physically active subjects after an intravenous injection of Gd-DTPA^{2-}. This to test the hypothesis that cartilage is an adaptive tissue, as suggested from animal studies. Moderately exercising dogs have shown an increased proteoglycan content in their knee cartilage (4).

Methods: 28 healthy volunteers (age 21-30 years) were included in this cross-sectional study. Obesity (BMI>30 kg/m^2) and previous knee injury were exclusion criteria. The subjects were divided into two groups according to their level of physical activity during the previous two years, no regular activity (n=12), and exercise 2-4 times a week (n=16). The most common activities were jogging and workout at a gym.

Gd-DTPA^{2-} was administered intravenously at the dose of 0.3mmol/kg.bw (triple dose). After the injection, subjects walked in stairs during approximately seven minutes in order to optimize the distribution of the contrast medium into the cartilage. MRI examinations were performed two hours post-contrast using a 1.5 T machine (Siemens Magnetom Vision). Quantitative relaxation rate (R1) measurements were made in regions of interest (ROI's) in the femoral cartilage using a single slice sagittal turbo inversion recovery sequence with different inversion times. All ROI's were drawn by one single investigator and positioned in the central weight-bearing medial femoral cartilages (ROI size: 150-250 pixels). Student's t-test was used for the statistical analyses.

Results: Subjects with no regular physical exercise had significantly higher distribution of the contrast medium in the weight-bearing medial femoral cartilage, approximately 15%, (p<0.01) (table). higher R1 than both groups of exercising individuals.

Exercise level	**1 (n=12)**	**2 (n=16)**
R1 medial (mean±SD)	2.8±0.3	2.4±0.2

Table. R1 (mean±SD) in medial femoral weight-bearing cartilage.

Discussion and Conclusions: Results suggest a higher proteoglycan content in the cartilage of regularly exercising individuals. To our knowledge this is the first human study to indicate that cartilage, in similarity to other skeletal tissues, may have the capacity to adapt to physical demand.

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The International Cartilage Repair Society (ICRS) -Histological Visual Scale. A Preliminary Report of the Histological End Point Committee. I. Human Biopsies, Toronto Consensus.

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*** Each author has equally contributed to the development of the present classification.**

Cartilage repair procedures have gained acceptance during the last few years. There is a growing interest in the cartilage community to develop proper assessment tools in order to evaluate current and future technologies enhancing/facilitating the repair/regeneration of this hyaline organized tissue.

Methods to evaluate repair procedures include arthroscopic imaging, probing (mechanical measurement), MRI, histological and biomarkers monitoring. All these outcome measures provide complementary information, have no real value as a "stand alone" indicator and must be correlated to the clinical data.

Based on the results of the discussion of the workshop that was organized in Murten in 1999 by the ICRS, The International Cartilage Repair Society has constituted a Histological End Point Committee whose mission is to suggest a simple method of evaluation and to provide a morphological catalogue which can be used for the validation of other techniques such as MRI, mechanical testing, biomarkers etc.

The committee has analysed hundreds of cartilage biopsies after repair procedures performed in humans. Only 60 % of the biopsies analysed had morphological criteria allowing a possible observation/diagnosis of the specimens. It has been decided that biopsies which can be considered for histological scoring are only biopsies that contain subchondral bone, that can be orientated and which are in one piece. All other samples should be excluded.

The classification relies on morphological criteria that can be observed on H&E and/or Toluidine Blue Stains. It is recommended to study histological specimens under polarized light.

<u>Morphological Criteria</u>

I) Surface
- Smooth, Continuous 1
- Discontinuities, irregular 0

II) Matrix
- Hyaline 4
- Mixture Hyaline/Fibrocartilage 3
- Fibrocartilage 1
- Fibrous Tissue 0

III) Cell Distribution
- Columnar 3
- Mixed Columnar/Clusters 2
- Clusters 1
- Individual Cells/ Disorganized 0

IV) Cell Population Viability
- Predominately Viable 2
- Partially Viable (population) 1
- < 10% Viable 0

V) Subchondral Bone
- Normal 2
- Active Remodeling 1
- Bone Necrosis/Granulation Tissue/ Detached/Fracture Callus at base 0

VI) Mineralization
- Normal 1
- Abnormal/Inappropriate Location 0

The individual scores must NOT be summed but reported separately. Method to report a biopsy score: i.e. (I/1, II/3, III/3;…)

REAL-TIME IMAGING OF SOLUTE TRANSPORT IN DYNAMICALLY COMPRESSED CARTILAGE

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INTRODUCTION: Chondrocyte metabolism is inhibited by static compression and stimulated by low-amplitude dynamic compression[1]. Since adult cartilage has no blood vessels or lymphatic transport system, physical regulation of diffusion and convection of bioreactive solute has been implicated as a mediator of cell metabolism[2]. To investigate effects of dynamic compression on solute transport in cartilage, our goal was to develop and apply a novel compression and real-time imaging technique.

METHODS: Cartilage discs (diameter=2mm, thickness=1mm) were obtained from adult bovine proximal humeri. Discs were bathed for 24 hrs in 5µM solutions of tetramethylrhodamine (TMR), 3-kDa dextran conjugated to TMR (Dx-TMR), or 10-kDa Dx-TMR. 3 modes of compression (10% static strain, 50% static strain, or 0.001 Hz dynamic ramp compression between 10%-50% strain) were applied for 1100s using a novel compression apparatus that enables real-time confocal imaging during static or dynamic compression (Figure 1).

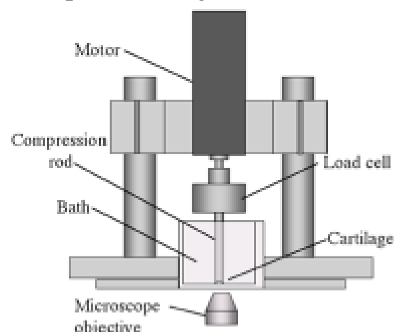

Fig 1: Compression apparatus on microscope stage.

The apparatus frame rests on a confocal microscope stage and supports an ultra-precision motorized actuator in series with a precision miniature load cell and a compression rod. An external pump system provides fluid flow and mixing within a plexiglass specimen bath. The fluorescent solute in the specimen was excited with a HeNe laser and data analysis of images recorded every 100s was performed using NIH Image. Average fluorescent intensity was measured within a circular region of interest that was centred on the disc and held constant for each sample throughout compression. The intensity was correlated with concentration using previously established standards[3] and normalized to initial values.

RESULTS: Concentration decreased with desorption time for all solutes and experimental conditions. The concentration decrease at 10% and 50% static compression was quasi-exponential for each solute (Fig.1a,b). Concentration in dynamically compressed samples appeared to additionally follow the changes in compression, with a time lag of ~100s (Figure 1c). The fraction of the initial concentration remaining at t=1100s (Figure 1d) was greatest under 50% static strain, with p<0.01 for TMR and 3kDa Dx. The greatest efflux of TMR and 3kDa Dx occurred under dynamic loading, though no significant difference was detected.

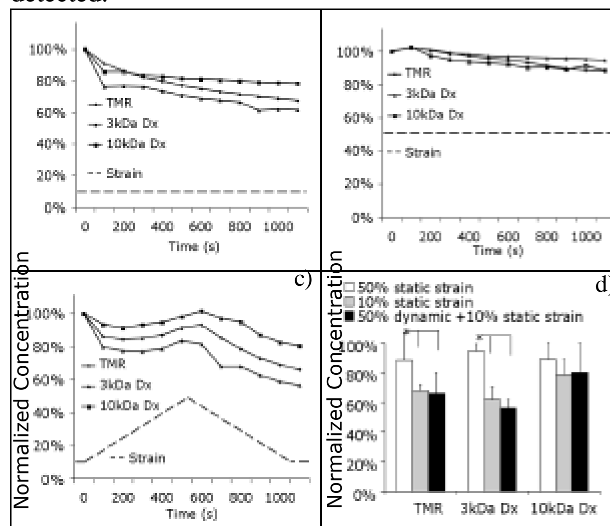

*Fig. II: Evolution of solute concentration within explant discs under a) 10% b) 50% static compression, c) 0.001 Hz dynamic ramp compression between 10% &50%, d) percentage of initial concentration at t=1100 (n=5, *p<0.01)*

DISCUSSION & CONCLUSIONS: Our compression and real-time imaging apparatus illustrated an inhibitory effect of increased static loading on transport, and suggested a stimulatory effect of dynamic loading. These results support the hypothesis that mechanical loading may influence chondrocyte metabolism via alterations to solute transport in the cartilage extracellular matrix. Concentration increases seen during dynamic compression may suggest attractive interactions between these solutes and the matrix that limit solute convection. Diffusion and convection coefficients, and fluid velocities associated with dynamic compression remain to be quantified in future work

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CONVENTIONAL AND CRYOTECHNICAL PREPARATION OF ARTICULAR CARTILAGE FOR TRANSMISSION ELECTRON MICROSCOPY

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INTRODUCTION: It is well recognized that rapid cryofixation preserves ultrastructural features in biological samples almost perfectly. Compared to chemical fixation, ultrastructural details are better preserved and structural features are easy to correlate with function. In the case of cartilage, the advantages of cryofixation compared to chemical fixation are well documented (1). Freezing quality determines structural preservation (2); follow–up procedures (e.g. freeze-substitution in different solvents) also influence structural preservation (e.g. 3). The only disadvantage of freezing samples is that their size is limited. Under high pressure (2000bar), samples about 200µm in thickness and with a diameter of 1.2 to 4mm are well frozen. Because of this restriction optimised chemical fixation is still important for the characterisation of larger samples.

METHODS: Samples of bovine articular cartilage were compressed to an extent of 50% of their initial thickness and either chemically fixed or high pressure frozen. Chemical fixation was done in isotonic cacodylate buffer containing glutaraldehyde. As a second step the samples were poststained in osmium tetroxide. They were dehydrated in ethanol and embedded in Epon-Araldite (for details see ref. 1). The cartilage samples, which were high pressure frozen, were cut with a vibratome into slices 400µm in thickness and punched to the right size to fit into the cavity of the platelets being 200µm in depth, resulting in a compression of the cartilage of 50% of its initial thickness. The samples were then high pressure frozen (Leica HPF or EMPACT, Leica Microsystems, Vienna), freeze-substituted in acetone containing 2% osmium tetroxide at –90°C. After the temperature was raised to 4°C, the samples were washed in pure acetone and embedded in Epon-Araldite (for details see ref. 2)

RESULTS: High-pressure frozen samples show an optimal structural preservation (Fig. 1A, 1B). Nucleus, mitochondria, smooth and rough endoplasmatic reticulum, golgi apparatus, cell membrane and extracellular matrix are shown in detail. In chemically fixed sample (Fig. 2A, 2B) most of the organelles are recognizable, however, details are by far not as well preserved. Membranes are not clearly depicted and the extracellular matrix is much less densely stained. There is loss of matter.

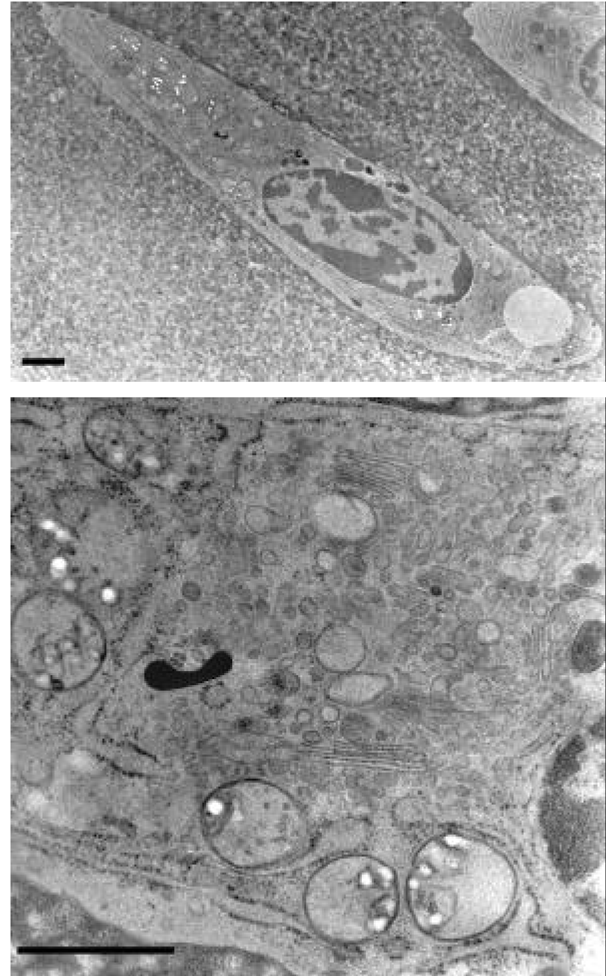

Fig. 1A, 1B: High pressure frozen chondrocyte compressed to 50% of its initial thickness. Cell and extracellular matrix are well preserved. The blow up shows all details of the organelles completely clearly. The membranes are well depicted. Bar represents 1µm.

DISCUSSION & CONCLUSIONS: Optimal ultrastructural preservation of cartilage is important to understand structure and function of this unique tissue. We have shown that best preservation is achieved when the samples are high pressure frozen. High pressure freezing allows to increase

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the size of a well frozen sample by a factor of ten when compared to cryofixation protocols at ambient pressure. However sample thickness is limited to about 200µm (2) for inherent physical reasons. In chemically fixed samples molecular details are not preserved, however, for large samples the approach of chemical fixation is still the only possible one.

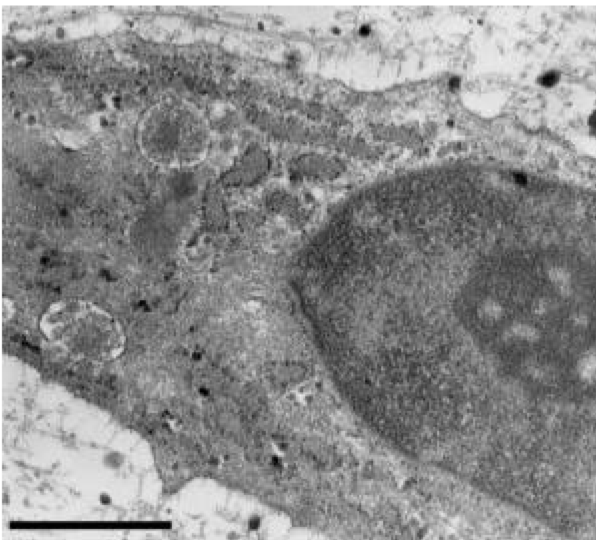

Fig. 2A, 2B: Chemically fixed chondrocyte compressed to 50% of its initial thickness. The blow up shows organelles not really well preserved. Bar represents 1µm.

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PREPARATION OF ARTICULAR CARTILAGE FOR SCANNING ELECTRON MICROSCOPY

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The preparation of articular cartilage, because of its inherent properties, presents a challenge to the microscopist. The collagen fibril matrix, containing osmotically active proteoglycans, along with a large proportion of water makes up the bulk of the tissue. If the proteoglycans become exposed to an aqueous solution, they will take up any available water very rapidly. This process can modify the structure of the tissue.

Ultrastructural studies of this tissue, using a scanning electron microscope (SEM), require that the sample be cut open in some way to reveal its internal structure. The most direct method involves freezing the specimen, fracturing and etching it before sputter coating and then placing it on the specimen stage of the microscope while keeping it frozen. This is known as cryo-SEM.

When tissue is frozen, then any water that is available for freezing will form ice crystals when the tissue is above the recrystallisation point of water. This means it should be taken down to below minus 80°C very rapidly, and kept below this temperature[1]. How rapidly this can, and needs to be achieved is a matter of discussion. High-pressure freezing, the most rapid method, requires that the sample size is considerably smaller than the total depth of articular cartilage tissue. If the integrity of the tissue needs to be retained, as seems possible, then this is not an option. Reasonably fast rates of cooling can be achieved by plunging the tissue samples into liquid propane at boiling nitrogen temperature. Any ice crystals that form following this procedure are comparatively small.

Following freezing, freeze substitution can also carried out. This approach allows frozen tissue to be studied without the need for a special cryo stage on the SEM and involves the dissolving of the frozen water out of the specimen by means of suitable organic solvents. Once all the water has been removed, the specimen can be brought up to room temperature and critical point dried. Mounted samples can then be sputter coated before viewing in the SEM.

The most commonly practiced approach to preparing articular cartilage for SEM study has been to apply a chemical fixation protocol to the tissue, dehydrating it in organic solvents and critical point drying before placing the dried tissue in liquid nitrogen for fracturing. The inherent problem with this approach is the effects of the chemical fixation media used.

Aldehyde based fixing agents (formaldehyde, glutaraldehyde and acrolein) have long been established as the most suitable chemical agents for cross-linking and stabilizing biological macromolecules within cells and tissues. These agents are usually dissolved in a buffer solution, often with added ions etc. Most of the images of the ultrastructure of cells and tissues are based upon tissue prepared in this way. Others have added osmium tetroxide, with thiocarbohydrazide treatment, as an additional stage in the process[2]. There is no doubt that severe changes occur to tissues as a result of such treatments, and some have argued for many years that the freeze substitution route provides a much more accurate representation of the *in vivo* arrangement of tissue components than does any form of chemical fixation[3].

One of the reasons for the disruptive effects of aldehydes upon tissues derives from their tendency to remove the basic groups of macromolecules[4]. Clearly, this property has the potential to create severe electrostatic imbalances on and between these molecules, and the structures that they compose. There is little doubt that this is one of the reasons for the differences observed between chemically fixed and cryo preserved ultrastructure.

The preservation obtained as a result of the cross-linking properties of aldehydes is obviously preferable to treatments based on the use of traditional histological fixatives that are merely coagulants. This explains their long-standing popularity. However, it must be realized that changes do occur to the ultrastructure as a result of applying chemical fixation protocols to tissues. When that tissue is articular cartilage such changes can be considerable, but were minimized by a suitable choice of fixation medium pH and osmolarity, the use of acrolein as an aldehyde as well as the application of microwave energy to the

tissue. Freeze-substitution, however appears to provide an even better preservation[5].

Fracturing of blocks of critical point dried tissue, however prepared, presents some practical problems to overcome. In order to view the internal structure of the tissue, by SEM, it is necessary to fracture the block in a controlled direction, keeping the tissue under liquid nitrogen. To this end we designed a small fracturing jig to hold the tissue, as well as the fracturing blade, in position (Fig.1). Once the fracture had been performed it was necessary to bring the sample back to room temperature, while preventing moisture in the surrounding atmosphere from condensing onto its delicate fractured surfaces. This was achieved by using a specially designed warming-up device (Fig. 2).

The fracturing jig, with tissue and razor blade mounted in position, was lowered gently into the small plastic lunchbox (microwave and freezer proof). Liquid nitrogen was poured into the box, until the fracturing jig was covered completely. The fracturing was performed by striking the razor blade with a small hammer. Immediately following the fracturing the lid was placed on the plastic box. This lid had been modified by having a 150mm long piece of 5mm diameter polyethylene tubing attached to it. The closed box was then left on the bench, until it reached room temperature. The principle of this device was that the boiling nitrogen drove all of the, moisture containing, air out of the box – through the polyethylene tube. As the fractured specimen emerged from the liquid it was then only exposed to an atmosphere of dry nitrogen that remained until the box was opened at room temperature. The fractured samples could then be removed and mounted on specimen holders for sputter coating.

High-resolution field emission scanning electron microscopy provided images that suggested a high degree of three-dimensional organization to the collagen matrix of articular cartilage tissue.

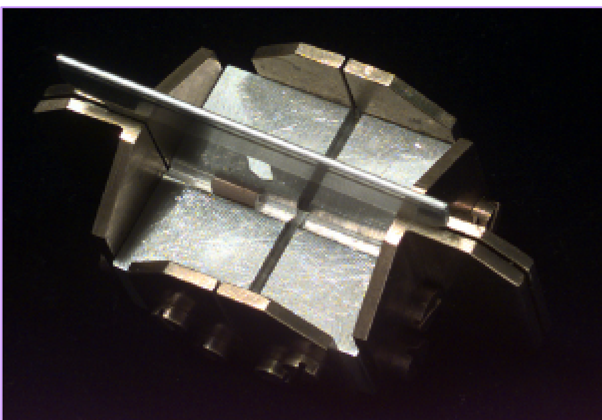

Fig. 1: Fracturing jig used for the preparation of articular cartilage samples (built by P.C.Lloyd).

Fig. 2: Warming-up device used to bring cold fractured articular cartilage samples back to room temperature.

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RECOMBINANT HUMAN COLLAGENS FOR BONE & CARTILAGE REPAIR

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INTRODUCTION: The development and commercialisation of recombinant forms of human collagens will have a significant impact on clinical approaches to bone and cartilage repair. Collagens represent the most abundant extracellular structural proteins in man and play an essential role in the development, maintenance, repair and regeneration of almost every type of tissue. The physical and functional differences of tissues that are apparent at a macroscopic level underscore the diversity in form and function of the collagen family of proteins. There are now well over 20 different types of collagen that have been identified within the human genome, each with a unique but related structure and function. The clinical utility of collagen has been limited, for the most part, to the use of Type I collagen isolated from animal sources such as bovine, porcine and equine hides, bones or tendons. The abundance and relative ease of isolation of Type I collagen from these tissues has made them an attractive and economical source of bulk material. The application of genetic engineering and recombinant protein expression technology for the commercial production of recombinant human collagens now opens up the possibility to utilize additional collagen family members in the design of new tissue engineering scaffolds. This presentation will preview the use of recombinant collagen-based biomaterials in scaffolds for bone and cartilage repair.

METHODS: Previous attempts to express recombinant forms of human collagens have been hampered by thermal instability problems that are due to the lack of post-translational modifications, such as the hydroxylation of proline residues, that are important for stabilization of the collagen triple helical structure. The multigene technology approach to be described here expresses both the structural chains of the collagen molecules and the enzyme machinery (prolyl-4-hydroxylase) necessary to stabilize the protein structure in a manner similar to native material. This approach has resulted in the successful expression of nine of the over twenty known collagen family members in thermally stable formats using host systems (yeast) that are well adapted for commercial scale processes. The resulting recombinant collagen is well suited for fabrication into 2D films/membranes and 3D porous matrices that are common in bone and cartilage tissue engineering applications. Recombinant human collagen Types I, II and III are currently being evaluated in a series of *in vitro* and *in vivo* analyses designed to demonstrate their potential utility in bone and cartilage repair.

RESULTS:

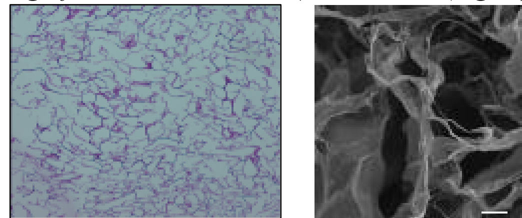

Fig. 1: Histologic (left panel, cross-section, 40X magnification, H&E stain) and SEM (right panel, 500X magnification, bar = 10 μm) of a recombinant human Type I collagen 3D matrix.

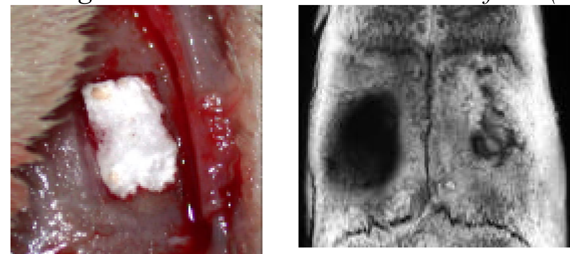

Fig. 2: Implantation of a recombinant human Type I collagen matrix into rat cranial defect (left panel) and contact radiograph of defect at 28 days post-implantation (right panel). Right side defect treated with matrix, left side defect is the untreated control.

DISCUSSION & CONCLUSIONS: Recombinant versions of human collagen have been produced in thermally stable forms that can be further manipulated into matrix formats suitable for tissue engineering applications such as bone and cartilage repair. The availability of pure, non-animal-derived forms of human collagen family members now allows the custom fabrication and application of matrices that match recipient tissues at the levels of primary protein sequence and tissue-specific matrix composition and architecture.

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Polymeric matrices in the treatment of articular cartilage defects

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INTRODUCTION: Permanent pain, disability and joint dysfunction are the typical consequences of articular cartilage injuries. The surgical treatment of articular cartilage defects almost never results in the formation of hyaline cartilage. The defects are filled with fibrocartilage, which does not take on the functions of a normal articular surface. These problems call for new procedures that might allow the regeneration of functional hyaline cartilage tissue. One of such procedures involves transplantation of cultured human autogenous chondrocytes in combination with a periosteal flap [1]. Tissue engineering approach exploits the possibility of using constructs consisting of autogenic chondrocytes cultured on suitable matrices - scaffolds. When implanted into a cartilage defect the construct may potentially induce the regeneration of functional hyaline cartilage.

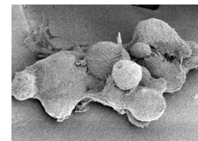
Materials for scaffolds could be medical ceramics or polymers of natural or synthetic origin. The use of bioresorbable polymers for scaffolds is especially attractive as the cartilaginous tissue may replace the space occupied by the scaffold.

Natural bioresorbable polymers used in scaffolds are collagen, gelatine, fibrin, and alginates. Synthetic bioresorbable polymers are primarily polyhydroxyacids, including polylactides, polyglycolide, and copolymers the lactide or glycolide units and other monomers.

Optimally, scaffolds for tissue repair, regeneration and engineering should be: biocompatible, bioresorbable or biodegradable; have adequate degradation profile; and micro- and/or macroporous to allow for the ingrowth of blood vessels, tissues and the flux of nutrients. Scaffolds should support attachment, activity and proliferation of cells and allow for the formation and maintenance within the porous structure of extracellular matrix.

The success of tissue repair and regeneration when using polymeric scaffolds will to a great extend depend on the interactions of implants with cells and tissues. The biological quality of the polymer, the biocompatibility of the released by-products, the chemistry and texture of the scaffold surface and its mechanical compatibility with tissues influence these interactions. Additional factors include the technique of the scaffold preparation, the post-treatment, i.e. cleaning and sterilization.

During the last two decades scaffolds from various polymers have been extensively tested for their capability to support the growth of chondrocytes harvested from various animal species [2-8]. Gels from collagen type I supported growth of bovine, rabbit and canine chondrocytes [2-4]. Collagen type II was better scaffold material than collagen type I [4]. Collagen scaffold stimulated the synthesis of collagen and was better for bovine chondrocytes than polyglycolide or poly(lactide-co-glycolide) The latter, however, enhanced the synthesis of proteoglycans [5]. Porous polylactide matrix promoted growth of neocartilage at the articular surface of the rabbit knee [6]. Bovine and human chondrocytes seeded on polyglycolide nonwoven mesh or porous poly(L-lactide) scaffold produced neocartilage *in vitro* and *in vivo* [7]. Porous scaffold from poly(L/DL-lactide) supported the attachments and growth of sheep articular chondrocytes [8].



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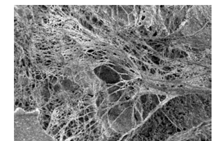

CONCLUSIONS: Tissue engineering approach using scaffolds seeded with autogenic chondrocytes can be a means to repair defects, and ultimately to regenerate a whole hyaline articular cartilage. Results of cartilage repair in experimental animals are promising, the road to successful clinical procedures in human may still be long.

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CONTROL OF CHONDROCYTE PHENOTYPE IN A CHITOSAN / CHONDROCYTE CONSTRUCT

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INTRODUCTION: Articular cartilage repair remains a clinical and scientific challenge. Cell delivery to cartilage defect area appears as a promising therapy. However, isolated chondrocytes loose very rapidly their phenotypic expression in the absence of their extracellular matrix environment. Cartilage engineering offers new possibilities to prepare chondrocyte/matrix constructs that can reproduce a cartilage-like biomaterial, suitable to be injected into the defect area.

The aim of the present work was to elaborate such a cartilage-biomaterial by combining a chitosan-based physical hydrogel with isolated chondrocytes and to analyse the effects of different physical properties of the biogel and different culture conditions, on the chondrogenic properties of the construct.

METHODS: Articular chondrocytes were isolated from adult rabbits and either grown alone or mixed with various formulations of chitosan hydrogels with various degrees of acetylation (DA) (see abstract by Morfin et al). Different ways of combining the cells with the chitosan gel were used: cells in suspension or in micromass; large or small biogel fragments; different ratio of cell/fragment number. The culture was performed during one, two or three weeks in DMEM containing 5% fetal calf serum. Chitosan biogels were maintained in similar culture conditions without chondrocytes.

The resulting constructs were then analysed for cartilage properties. In each experimental condition, three sets of samples similarly treated were performed. One of them was treated for histochemical proteoglycan observation by using Alcian blue coloration; the other two samples were used for biochemical analysis: Aggrecan and type I and II collagen genes expressions were studied by RT-PCR; the amount of superaggregated proteoglycans accumulated into the matrix was studied after radiolabelled sulphate incorporation and analysis of newly synthesized sulphated macromolecules by chromatography on sepharose 2B column.

RESULTS: The primary goal of this study was to assess whether or not the cells will penetrate the biogel. Whatever the DA of chitosan used to prepare the biogel and the selected experimental conditions used, cells were always laying outside of the biogel. When observed in situ under phase contrast microscopy, chondrocytes cultured alone were rapidly dropping to the bottom of the flask where they stuck and grew as a monolayer. Biogels incubated without cells in similar culture medium were visualized as translucid and birefringent masses floating into culture medium, more or less well detectable depending on the DA. When chondrocytes and the biogels were mixed and cultured in similar conditions, one could observe numerous groups of aggregated cells floating into culture medium in between biorefringent material. These aggregated cells were still floating after 8, 15 or 21 days in culture. Only spared cells stuck to the bottom of the plate. After 8 days in culture, the histochemical analysis of the cultured samples showed that the constructs were composed of aggregated rounded cells surrounded with cartilage-type matrix proteins with a strong affinity to Alcian blue coloration in the pericellular area. Each cell aggregate is in contact with several fragments of non-alcyanophil biogel. Serial sections of the constructs observed at high magnification show that biogel fragments were in close contact with the alcyanophil matrix area localised around the cells. The size of the pericellular alcyanophil area increased with time (15 to 21 days). Otherwise, biogel fragments become heterogenous with increased alcyanophil coloration. When gels were prepared with chitosan

whose DA was higher (up to 60%), a strongest alcyanophil coloration of the biogel was observed. RT-PCR products from chondrocytes mixed with biogel expressed type II collagen and aggrecan but not type I collagen genes, whatever the DA of the biogel. At the protein level, sulfated proteoglycans were shown to be newly synthesized and accumulated as high molecular weight superaggregates.

Finally, matrix components morphological changes of the of the constructs were associated with modifications of the cell arrangement but not of their chondrocytic phenotype. Thus, cells never looked fibroblastic-like but increased in size from the periphery (close to the biogel surface) to the center of the aggregate, suggesting that biogel acted as an inductor of the cellular maturation process.

CONCLUSION : Our data provide evidences that chitosan biogels cannot be used as scaffold for chondrocytes. In contrast the present initial results show that chondrocytes have high affinity to chitosan hydrogels, which, in turn, may play a role of “biological decoy”, mimicking the presence of cartilage-like macromolecules being presented to the cells. The resulting effect of mixing the chondrocytes with chitosan biogels consists of a bio-construction in which chondrocytes maintain their phenotype and are surrounded by functional cartilage-type matrix proteins. Further studies will be necessary, using defined culture medium with combinations of growth factors, which may control the maturation process of the chondrocytes inside the construct.

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BIODEGRADABLE MICROPARTICLES FOR ARTICULAR CARTILAGE TISSUE ENGINEERING

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INTRODUCTION: Articular cartilage has a limited capability for self-repair. The consequence of injuries to articular cartilage tissue is typically degeneration until surgery becomes necessary. However, surgical procedures available today for the treatment of articular cartilage degeneration often lead to a mechanically inferior fibrocartilage tissue. Results obtained from tissue engineering procedures using autologous chondrocyte transfer suggest that this technique is promising but may lead to a mixture of hyaline and fibrocartilage. Here, we present a chondrocyte culturing and delivery system which is based on the use of biodegradable microparticles. Advantages of this system include the control over the chondrocyte phenotype and a reduction in volume contraction during tissue formation.

METHODS: Fresh sheep articular cartilage chondrocytes were isolated from tissue samples by digestion with 10% w/v trypsin, bacterial collagenase and hyaluronidase. Chondrocytes ($1x10^5$ cells) were cultured in monolayers on TCPS substrates in DMEM/10% FBS containing 100µg/ml penicillin and streptomycin, at 37ºC in 5% CO_2. In addition, chondrocytes were cultured in spinner bottles on various biodegradable microparticles including poly(lactide-co-glycolide) (PLGA) microspheres (prepared by an emulsion method as seen in Figure 1A), crushed PLGA particles, gelatin, bone or demineralised bone particles. Cytodex beads (Pharmacia Biotech), were used as control particles. The diameter of particles ranged from 50µm to 250µm. Surface modifications carried out particularly on PLGA particles included protein pre-adsorption, ammonia plasma treatment and the covalent immobilization of dendrimers.

For *in vitro* tissue growth experiments, chondrocytes ($3x10^6$ cells) on beads were mixed with an equal volume of 20 mg/ml type I collagen or a mixture of type II/I collagen at neutral pH in DMEM. After collagen gel formation at 37°C, the samples were incubated in cell culture medium at 37°C in 5% CO_2 in the presence of ascorbate.

All samples were evaluated in regard to the proliferation rate and the resulting phenotype. The phenotype was monitored using a variety of histochemical and immunohistochemical markers, including specific antibodies against type I and type II collagens (Southern Biotech). The Glycosaminoglycan (GAG) content was assessed using a dimethyl methylene blue assay.

RESULTS: Evaluation of chondrocyte monolayer cultures showed rapid proliferation and significant de-differentiation within 3 to 7 days, observed by a change in cell morphology (rounded vs elongated) and the secretion of collagen type I. In comparison, culturing on control Cytodex beads, coated with type I or type II collagen, in spinner flasks resulted in proliferation rates comparable to monolayer cultures and maintenance of the phenotype, observed by the rounded cell morphology and the maintained secretion of collagen type II.

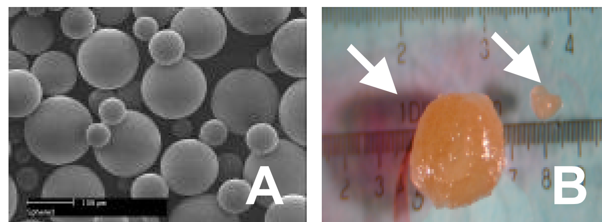

Figure 1: (A) The morphology of PLGA microspheres used in this study and (B) the effect of volume contraction seen in tissue growth experiments with and without particles, respectively

Good proliferation rates were observed on all protein-based particles, and the secretion of type II collagen and GAG demonstrated the maintenance of the phenotype. PLGA beads required modification to allow good cell attachment and proliferation. Ammonia plasma modification, followed by treatment with type I collagen, enhanced the proliferation rate 8-fold and resulted in the correct phenotype. Gelatin and gelatin/collagen particles were excellent carriers producing high proliferation rates and phenotypic control. Demineralised bone particles produced the highest proliferation rates.

Tissue growth experiments demonstrated the ability of chondrocytes to migrate into collagen gels and to synthesize new matrix. In all instances where particles were used in combination with cells, GAG synthesis was maintained over a 3-week culture period. Without particles, cells alone produced GAG at 1 week, but this decreased substantially over the 3-week culture period. In addition, the presence of particles significantly reduced the extent of gel shrinkage (as seen in Figure 1B) and increased the wet weight of tissue formation.

DISCUSSION & CONCLUSIONS: Chondrocytes for transplantation into articular cartilage defects can be proliferated on a variety of biodegradable particles with biological or synthetic origin. The use of these particles has shown excellent proliferation rates while maintaining the cell phenotype. In addition, the combination of cells and particles has been shown to reduce the extent of volume contraction when used with a gel delivery system.

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BIOMECHANICS IN CARTILAGE TISSUE ENGINEERING

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INTRODUCTION: The growth in basic research and clinical trials for cartilage tissue engineered materials has generated a need for assessment of material properties and functional performance of these constructs before, during development and after implantation. Applications require detailed assessment of the biomechanical properties of neo-tissue constructs *in vitro* and *in vivo*. Based on the known properties of normal tissues, investigators have identified a range of biological, biochemical, and biophysical end-point parameters that must be quantified to determine the potential for success of a given methodology. It is important to determine if constructs are capable of withstanding the forces associated with locomotion *in vivo*, and whether construct properties compare to the corresponding native tissue. For tissue engineering approaches in which cells must re-synthesize a functional ECM within a scaffold, the mechanical properties of the construct will indicate whether the native structure is being replicated. There is still debate as to whether neocartilage constructs must attain biological and biomechanical properties similar to native tissue before or after implantation.

METHODS: We have focused on the development of a novel methodology for encapsulating chondrocytes in a three-dimensional peptide nanofiber network[1]. Certain peptides are able to self-assemble into stable hydrogels at low (0.1-1%) peptide concentrations. We hypothesized that a self-assembling peptide hydrogel would provide an appropriate environment for the retention of chondrocyte phenotype and the synthesis of a mechanically functional cartilage ECM. In addition, the self-assembling nature of the peptide hydrogel and the flexibility of molecular design may offer advantages in controlling scaffold degradation, cell attachment, and the delivery of tethered stimulatory growth factors to the microenvironment of encapsulated cells. Together, these features offer a flexible approach to optimising scaffold-cell interactions for cartilage repair.

RESULTS: Chondrocytes were harvested from the femoropatellar grooves of 1-2 week-old calves and resuspended in a solution of KLD-12 peptide (Fig.1a). The suspension was injected into a stainless steel casting frame to produce gel slabs that were cultured in serum-free or serum supplemented medium. During 4 weeks of culture *in vitro*, chondrocytes retained their morphology and developed a cartilage-like mechanically functional ECM rich in proteoglycans and type II collagen. Time dependent accumulation of this ECM was paralleled by increases in equilibrium modulus and dynamic stiffness (confined compression, Fig. 2) up to about ¼ of the values for native calf cartilage. Explants. Further studies demonstrated chondrocyte biosynthesis was increased significantly by dynamic compressive loading relative to free-swelling controls.

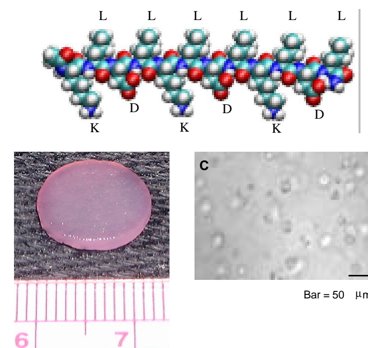

Fig. 1 A. KLD-12 peptide structure **B.** disk specimen for biomechanical tests and loading-induced biosynthesis **C.** Encapsulated chondrocytes

DISCUSSION and CONCLUSIONS: Taken together, these results demonstrate the potential of a self-assembling peptide hydrogel as a scaffold for the synthesis and accumulation of a cartilage-like ECM in a 3-D cell culture for cartilage tissue repair. The peptide KLD12 used in this study represents one of a class of specially designed self-assembling peptides made through molecular engineering that can be tailored to the specific cell and tissue application of interest.

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KNEE CARTILAGE OF SPINAL CORD INJURED PATIENTS DISPLAYS PROGRESSIVE THINNING IN THE ABSENCE OF NORMAL JOINT LOADING AND MOVEMENT

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INTRODUCTION: Alterations of cartilage morphology, biochemical and mechanical properties occur after unloading and immobilization in animals. However, findings have been inconsistent, and it is unclear whether such changes also occur in humans (1). Due to a lack of accurate noninvasive imaging methods, there has, so far been no report on morphological changes of cartilage in humans following immobilization. This knowledge is important to anticipate cartilage changes in patients immobilized after surgical procedures, accidents, or spinal cord injury. In this in vivo study, we tested the hypothesis that progressive thinning of knee joint cartilage is observed after spinal cord injury.

METHODS: We evaluated the right knee cartilage of patients with complete, traumatic spinal cord injury (after receiving oral and written information). Magnetic Resonance images from patella and tibia were taken within the first month, three, six and 12 months post-injury. The patients were examined with a 1.5 T scanner (Magnetom Symphony, Siemens), and a circular polarized transmit-receive extremity coil. A previously validated fat-suppressed gradient echo sequence (FLASH = fast low angle shot; repetition time = 53 ms, echo time = 10.3 ms, flip angle = 30 deg) (2) was used to acquire one transverse data-set of the patellar cartilage, and one coronal data-set of the tibial cartilages. Images were obtained at an in plane resolution of 0.31 x 0.31 mm2 and a slice thickness of 1.5 mm (matrix =512x512 pixels, field of view = 160 cm. Morphologic parameters for the knee cartilage (mean and maximum thickness, surface area) were computed from magnetic resonance imaging data.

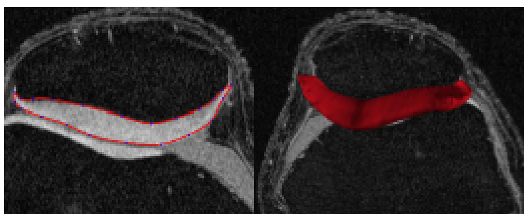
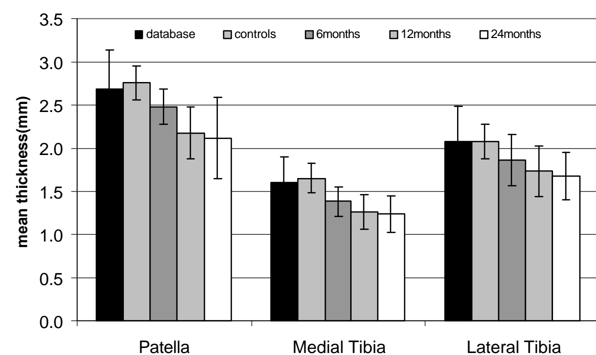


Fig. 1: Transverse MR image (top) and coronal image of the knee joint at 6 months post spinal cord injury. Fat suppressed FLASH sequence (TR/TE 53/10 msec, flip angle 30 deg) with a resolution of 0.31 mm x 0.31 mm x 1.5 mm (matrix = 512 x 512 pixels, field of view = 160 cm).

RESULTS: After 6 months of injury, the mean articular cartilage was significantly less in the patella and medial tibia (-10 and -16%, respectively, $p < 0.05$), but not in the lateral tibia (-10%) compared with healthy volunteers. After 12 and 24 months, the differences amounted to -21%/-23% for the patella, -24%/-25% for the medial tibia, and -16%/-19% for the lateral tibia. The changes were significant in all three surfaces ($p < 0.05$ to 0.01).

Figure 2. Mean cartilage thickness of the patella, medial tibia, & lateral tibia of normal healthy men



(n=49), control group (n=9) & spinal cord subjects 6 months (n=9), 12 months (n=11) & 24 months (n=6) post injury. The bar graph shows the mean values & standard deviation in each group

DISCUSSION & CONCLUSIONS: Our data show, for the first time, that progressive thinning (atrophy) of human cartilage occurs in the absence of normal joint loading and movement. Thinning of the cartilage may render the joint unstable, and these joints may encounter abnormal stresses during passive standing training in spinal cord injured patients. This may have important implications for patient management, for spinal cord injured patient and patients immobilized after surgery.

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CARTILAGE MECHANICS: LOW-ENERGY WATER JET ALLOWS TO DIFFERENTIATE CARTILAGE QUALITY

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INTRODUCTION: In order to assess the efficacy of articular cartilage repair procedures for trauma, osteoarthritis or other diseases, quantitative information about cartilage quality can be obtained from compressive stiffness measurements. Small handheld devices have been developed to determine arthroscopically cartilage mechanical properties by indentation [1]. Cartilage appears to be highly sensitive to mechanical deformation. Therefore a device, which determines the stiffness of soft materials without compromising the healing tissue, will have advantages compared to common indentation instruments. The aim of the study was to validate the measuring of different cartilage qualities in vitro using a new low-energy water jet system.

METHODS: A method was developed to measure mechanical stiffness of soft biological materials arthroscopically (e.g. joint cartilage) without direct contact between the tip of the measurement device and the measured object. Deformation is produced by a defined flow of liquid (NaCl) and measured optically (mean error < 5 µm). The technical solution is applicable to measure mechanical stiffness of native, degenerated and regenerated joint cartilage and other soft biological materials. Eight non-paired knees (sheep, two years) were retrieved and stored at -20°C until testing. The medial femoral condyle and the lateral tibia plateau were potted in PMMA for mechanical testing (native group). The lateral femoral condyle and medial tibia plateau were placed in 0.1%-trypsin solution (Merck KG, Germany) at 37°C for 48h to simulate cartilage degeneration [2]. After Trypsin treatment, the degenerated group was potted. During mechanical testing, the cartilage samples were rinsed with 0.9%-NaCl-solution. Cartilage stiffness was non-destructively determined using the new water jet system during indentation testing in a materials testing device (Zwick 1455, Germany) according to Mow et al. [3]. For each sample, 6 consecutive measurements were performed.

RESULTS: Cartilage stiffness was significantly reduced following Trypsin treatment, confirmed by both measurement methods (water jet device $p < 0.002$; material testing $p < 0.001$).

The results of the newly developed testing method correlated with those from a standard testing protocol (r=0.659). After 50s of fixed deformation using the materials testing device, native cartilage carried 50% of the initial load while degenerated cartilage carried less than 5% of the initial load.

	Water Jet System	Material Testing	Cartilage Thickness
Femur medial, native	24.9±5.2	35.7±4.4	1.09±0.24
Tibia, lateral, native	23.7±2.4	34.9±5.2	0.97±0.28
Femur, lateral, degenerated	11.0±2.0	23.3±7.2	0.84±0.14
Tibia, medial, degenerated	8.5±2.0	23.9±6.1	0.92±0.35

Table 1. Comparison of intact and degenerated cartilage stiffness measured by water jet system and material testing system in N/mm (mean±sd), cartilage thickness in mm (mean±sd).

DISCUSSION & CONCLUSIONS: The study reports that the water jet system clearly allows differentiation between native and degenerated cartilage samples. The lower stiffness measurements using the water jet system may be attributed to the lower applied forces and depth of penetration that measures only the superficial tissue layer. The stiffness values were well within the range of those previously reported using the arthroscopic system [4].

Compared to mechanical indentation testing, this new technique appears to minimize the risk of surface damage of the cartilage during mechanical loading. The system is extremely sensitive to testing of soft tissues. It may therefore be considered a valuable tool in the arthroscopic diagnosis of cartilage lesions. The water jet system provides an alternative to the existing indentation techniques with their inherent invasiveness.

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MECHANICAL REGULATION OF CARTILAGE MATRIX PROTEINS AND ANGIOGENIC FACTORS BY MECHANICAL STRESS

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INTRODUCTION: Endochondral ossification involves a complex series of events including chondrocyte differentiation towards hypertrophy, calcification of the matrix, vascular invasion and the deposition of bone. The rate has been suggested to be influenced by intermittent patterns of shear/tension and hydrostatic stress[1.] Single-phase continuum models of skeletal development show areas of high shear stress in secondary ossific nuclei of epiphyses and suggests that vascular invasion is inhibited in areas of high hydrostatic pressure[2]. The goal of this study is to examine the effect of hydrostatic pressure and tension on the expression of cartilage matrix proteins and angiogenic factors.

METHODS: Primary chondrocytes were isolated from 6 calf bovine humeral heads cartilage using a sequential pronase/collagenase digestion. Cartilage from each shoulder was digested separately. The chondrocytes were suspended in a 2% alginate solution containing $4x10^6$cells/ml and were polymerised in cylinders for hydrostatic pressure and rectangular beams for tensile experiments. Struts of polyethylene were embedded in both ends of the rectangular beam for attachment to the tensile apparatus. The gels were cultured for five days and subjected to 3 additional days of tension, compression or pressure. Labview software was used to control movement of the 3 microtesting systems. All 3 loading modes were performed at 0.5 Hz, 3 hrs/day for 3 days. Alginate specimens were solubilised after the third day of loading and RNA was isolated using an RNeasy kit (Quiagen). RNA was reversed transcribed using the Gene Amp RNA kit (Perkin-Elmer). TAQman probe and primer sets were designed using the Primer Express software. Cartilage matrix proteins examined included collagen 1, 2, and 10, aggrecan, COMP, and superficial zone protein. Factors associated with angiogenesis during endochondral ossification include connective tissue growth factor (CTGF), MMP13, TIMP-1, and vascular endothelial growth factor (VEGF). PCR reactions were run on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using 10ng cDNA/reaction and a primer and probe concentration of 900 nM and 300 nM, respectively.

RESULTS: The largest change in gene expression resulted from uniaxial tension and compression. These loading modes caused an upregulation in the expression of collagen 10, COMP, and superficial zone protein. Uniaxial tension also significantly upregulated the expression of CTGF, MMP-13 and down-regulated the expression of TIMP-1. Hydrostatic pressure had the chondroprotective effect of down regulating MMP-13 expression and collagen 1 expression.

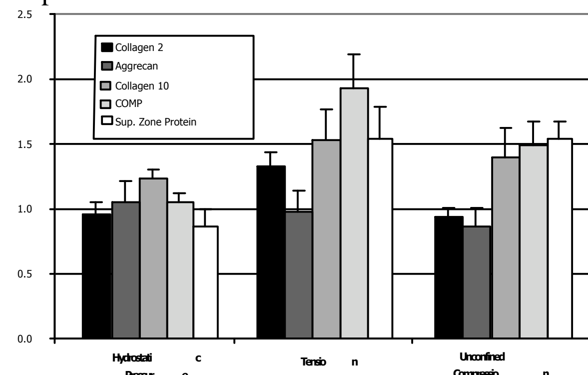

Fig. 1: Effect of mechanical loading on synthesis of cartilage matrix proteins (n=6).

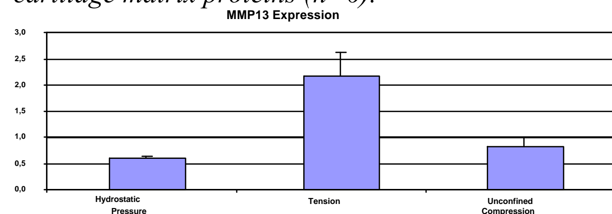

Fig.2: Tension up-regulates MMP-13 expression while hydrostatic pressure down-regulates its expression.

DISCUSSION & CONCLUSIONS: Hydrostatic pressure and uniaxial tension have opposing effects on the expression of cartilage gene expression. Uniaxial tension upregulated expression of matrix proteins involved in hypertrophy (type X collagen) and vascular invasion (MMP-13, CTGF) and down-regulated expression of chondroprotective genes such as TIMP-1. The effect of hydrostatic pressure was to inhibit the expression of MMP-13 and to maintain the chondrogenic phenotype by down-regulating expression of type I collagen. Interestingly, the gene expression pattern of the two large-strain loading modes, uniaxial tension and compression, were quite similar. The expression of superficial zone protein was upregulated by both high strain modes, a finding which is consistent with its in vivo pattern of expression in the highly strained superficial layers.

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A TRIBOLOGY APPROACH TOWARDS CARTILAGE ENGINEERING

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INTRODUCTION: There are estimates that 900,000 Americans suffer articular cartilage injuries each year. Cartilage damage is irreversible, mainly because cartilage has an inherent limited capacity for self-repair. Thus, assisted articular cartilage repair remains a clinical and scientific challenge. It is the general scientific view that in the future mesenchymal stem cells or chondrocytes will be seeded into three-dimensional matrices ex vivo, and then surgically implanted into the cartilage defect. However, these cartilage-like constructs obtained in vitro, at present, do not match the properties of in vivo cartilage tissues, both at the biochemical and biomechanical level.

BACKGROUND: Scientific investigation over the last decade has shown that chondrocyte activity is stimulated by the mechanical demands made on their environment, i.e. by the stress and/or deformation in and around the cells and by the flow rate of the interstitial fluid resulting from mechanical loading. Hence, it has become evident that the regulation of the biomechanical environment is a powerful modulator for tissue engineering and is nowadays employed in a variety of bioreactors [e.g. 1]. Our research group believes that synovial joints are designed to move and are meant to do so. Thus, in addition to forces, interface slip may be important. Indeed, it has been clinically shown that continuous motion brings about the best healing results for full thickness cartilage defects [2]. Therefore, the following concept is based on the hypothesis that biomechanical loading, which closely reproduces in vivo joint dynamics, including interface motion, will produce cartilage with properties of well-functioning hyaline cartilage.

BASIC THEORY & CONCEPT: An analysis of displacement trajectories on the bearing surfaces of natural joints revealed that the wear tracks form quasi-elliptical to rectangular paths during human locomotion [3]. Those paths vary widely in shape as well as in length over the contact area. Based on these premises, a bi-axial "pin-on-ball" (PoB) concept has been developed (Figure 1a). The interface is comprised by a cylindrically shaped scaffold, which is pressed onto commercially available alumina balls and static or cyclic loads *L(t)* may be applied. By applying rotationary motion of ball and pin in phase difference, elliptic trajectories can be generated on the scaffold's surface. If Φ is the angular amplitude and ω_b the frequency of oscillation of the ball, further Ψ and ω_p are angular amplitude re. frequency of the pin (scaffold), the motion trajectory s_A (t) of any single point *A* on the scaffold's surface can be described:

$$\vec{s}_A(t) = \begin{pmatrix} x_A(t) \\ y_A(t) \end{pmatrix} = \begin{pmatrix} \Phi \cdot \sqrt{R^2 - y^2}\,\sin(w_b t) + \sqrt{x_0^2 + y_0^2}\,\cos\left[\Psi \cdot \sin(w_p t + j_p) + \arctan\left(x_0/y_0\right)\right] \\ \sqrt{x_0^2 + y_0^2}\,\sin\left[\Psi \cdot \sin(w_p t + j_p) + \arctan\left(x_0/y_0\right)\right] \end{pmatrix}$$

with t as the time and φ_p as the phase shift between pin and ball oscillation and the initial conditions for the location $(x_0; y_0)$ of *A* at the time t = 0: $-\pi/2 < x_0/y_0 < \pi/2$ and y_0 0. Since the trajectories cross each other at any location (Fig. 1b), multiple shear is introduced to the surface.

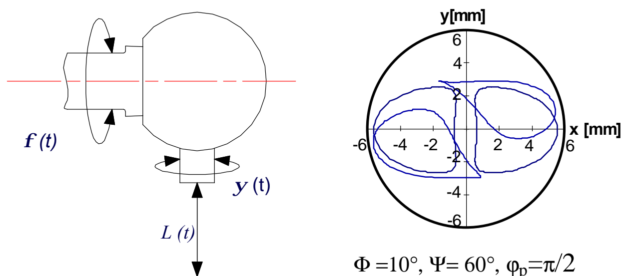

Fig. 1: (a) PoB concept *(b) X-ing trajectories*

EXPERIMENTAL DESIGN: The design of the pin-on-ball device is based in principal on the concept outlined above. All three motions are generated using step motors. The linear movement, which applies static and dynamic compression, is produced with a connected gearbox. The whole apparatus is placed in an incubator, while its control station is located outside. Nutrition of the cartilage can be backed-up with an internal nutrition system, which pumps fluid through the scaffold. The final design will include four individual stations in line.

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EFFECTS OF CYCLIC COMPRESSION AND SURFACE MOTION ON GENE EXPRESSION OF CHONDROCYTES SEEDED ONTO 3D SCAFFOLDS

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INTRODUCTION: A surgical treatment of articular cartilage defects almost never results in the formation of functional hyaline cartilage. The defects are filled with fibrocartilage, which does not take on the functions of a normal articular surface. A biological approach to cartilage injuries exploits the possibility of using constructs of chondrocytes cultured on suitable scaffolds. The use of resorbable polymers is especially attractive as the inflammatory response to the implant recedes after the scaffold is resorbed. In recent years it has become evident that the regulation of the biomechanical environment is a powerful modulator of chondrocyte activity. In the present study the effects of a simultaneous application of rotation and dynamic compression on the gene expression of chondrocytes seeded onto resorbable polyurethane scaffolds was evaluated.

METHODS: Polyurethane scaffolds of 8 mm in diameter and 4 mm thickness were attached to cylindrical holders and seeded with primary bovine articular chondrocytes. The cell-scaffold constructs were cultured for six days and subsequently subjected to cyclic compressive loading, with or without articulating against a commercially available ceramic hip ball. The interface between ceramic ball and cell-scaffold construct was intended to reproduce similar forces and surface motion characteristics as the natural joint. Loading was performed at 0.1 Hz, with 5%, 10%, or 20% compressive strain, superimposed on an equal preload. Ball rotation was bi-directional with ±60° amplitude at 20 cycles per minute. Two cycles of 1 hour were performed daily over a period of three days. After loading, total RNA was extracted from the cells, and the gene expression of different anabolic and catabolic molecules was analysed by TaqMan real-time RT-PCR using 18S ribosomal RNA as endogenous control. mRNA levels were expressed relative to levels of unloaded control constructs.

RESULTS: Depending on the strain amplitude, cyclic unconfined compression affected the expression of distinct genes. Especially, the expression of type II collagen mRNA was significantly downregulated, independently of the strain amplitude and surface motion. Collagen I mRNA was increased by compression at strain amplitudes of 5% and 10%, but not at 20%, where additional surface motion even decreased its expression. Neither the transcription factor Sox-9 nor aggrecan core protein was affected by the applied loading regimes. Type X collagen and MMP-9 were markedly down regulated in response to cyclic compression, indicating suppression of the hypertrophic phenotype. The effect on MMP-3 was dependent on the strain amplitude; no effect was observed at 5%, a slight up-regulation at 10% and a stronger increase at 20% compression. MMP-13 showed a tendency to decrease following compressive loading. Surface motion by ball rotation affected two of the genes tested. It namely caused a slight up-regulation of cartilage oligomeric matrix protein (COMP) and, most interestingly, a strong induction of superficial zone protein (SZP).

DISCUSSION & CONCLUSIONS: These results demonstrate that the phenotype expression of articular chondrocytes may be significantly altered by application of defined compressive forces and ball rotation movements. Especially, the marked induction of SZP expression suggests that in our system, which resemble the forces and motion characteristics of natural joints might be an efficient tool to generate constructs with properties similar to natural articular cartilage. SZP is a lubricating proteoglycan that is known to be specifically synthesized and secreted by chondrocytes of the superficial zone. Our findings suggest that surface motion may be an important modulator of its expression. Further biochemical, structural and biomechanical analyses and long term studies will be required to evaluate the most appropriate conditions to generate cartilage-like tissue in vitro.

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MESENCHYMAL STEM CELLS AND CHONDROGENESIS

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Mesenchymal progenitor or stem cells can be isolated from many tissues of the body. These cells were first described by Friedenstein *et al* (1). In recent years, their potential utility in musculoskeletal tissue repair and regeneration has been explored by many groups. We have been interested in the ability of these cells to undergo chondrogenesis. In 1998, we developed a method that facilitated the *in vitro* differentiation of these cells into chondrocytes (2). Our first studies were with bone marrow-derived stem cells from rabbits. Since then, we have used the *in vitro* chondrogenesis culture system to examine stem cells from many tissues and species, including human (3).

The culture system is based on the pellet culture system first described by Holtzer and co-workers (4) for use in studying embryonic chick vertebral chondrocytes, and later human articular cartilage chondrocytes (5), in conditions that maintained their differentiated state. In other studies, the system was used for culturing growth plate chondrocytes (6, 7) and producing further chondrogenesis to hypertrophy. It was the last variant of the system that we adapted in order to promote chondrogenesis of the bone marrow-derived cells. The conditions that induced bone marrow stem cell differentiation required a defined medium, based on that used by Ballock and Reddi (7), with further supplementation.

The method is relatively simple and has been used by several groups since we first developed it. Stem cell-containing populations of cells are isolated and expanded from the tissue of choice in monolayer. These cells are then pelleted by centrifugation in a defined medium. The cells form a free-floating aggregate and, in the presence of the defined medium containing TGF-β1 and dexamethasone, undergo chondrogenic differentiation (Figure 1) with consequent hypertrophy and apoptotic death. We have used the system to study many aspects of chondrogenesis, including the condensation-related events, cartilage production, chondrocyte hypertrophy and apoptosis. We have adapted the system to study other aspects of chondrogenesis, such as the influence of mechanical forces on chondrocyte differentiation.

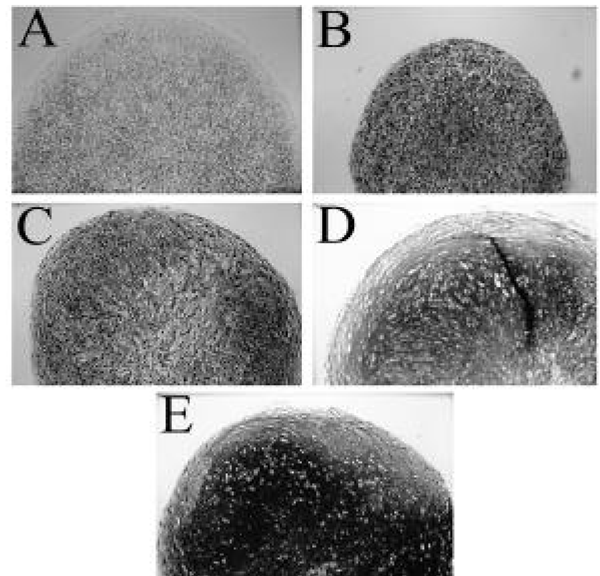

Figure 1. Toluidine blue stained sections of human mesenchymal stem cell aggregates at (A) 1, (B) 3, (C) 6, (D) 9 and (E) 14 days of culture.

We use what we learn from these basic science studies for our attempts to create cartilaginous tissue for implantation *in vivo* in musculoskeletal repair and regeneration models. We have developed matrices for use with these cells. In one study, we have produced cartilage tissue *in vitro* that has been implanted in meniscus defects in a rabbit model with good success (8). Other work with a range of materials continues as we apply lessons learned from our *in vitro* basic science studies to the field of tissue engineering.

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BONE MARROW STROMAL CELLS: CELL BIOLOGY & CLINICAL APPLICATIONS

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INTRODUCTION: Bone marrow is the tissue where hemopoiesis occurs in close contact with the stromal microenvironment, which support haemopoietic stem cell growth and differentiation. The bone marrow stroma is composed of a variety of different cell types providing structural and functional support for hemopoiesis: endothelial cells, adipocytes, smooth muscle cells, reticular cells, osteoblasts and stromal fibroblasts. Among these cell types, stromal fibroblasts have a peculiar biologic relevance. They are in fact able to support hemopoiesis, to differentiate towards osteogenic, chondrogenic and adipogenic lineage and to form a bone structure complete of hemopoietic marrow in in vivo assays. Their in vitro clonogenic counterpart is represented by Colony Forming Units-fibroblasts (CFU-f), which in turn give rise to Bone Marrow Stromal Cells (BMSC) and Mesenchymal Stem Cells (MSC), possibly corresponding to a single cell population. Hemopoietic stem cells commitment, differentiation and proliferation need complex interactions with the marrow environment which is mostly cellular, with relatively little extracellular matrix compared to the collagenous scaffolds in most other organs. BMSC in particular provide the absolutely essential support for hemopoiesis through both direct contact with cell surfaces and stromal cell derived soluble mediators. In vivo bone formation by CFU-f derived fibroblasts has been strikingly demonstrated and therefore these cells are considered a progenitor compartment for endosteal osteoblasts, responsible for the maintenance of bone turnover throughout life.

BMSC can be easily isolated from iliac crest bone marrow aspirates. Nevertheless, a step of extensive in vitro expansion is required to obtain a consistent number of cells available for both reconstruction and repair of mesodermally derived tissues, given the low frequency of BMSC in a marrow sample. Moreover, their use for gene and cell therapy of skeletal diseases requires the long-lasting engraftment of BMSC endowed with a residual proliferation potential sufficient to sustain the low, but continuous, bone turnover in adulthood. The maintenance of their stem properties and the possibility to reprogram their commitment is therefore a field of primary interest given their potential use in regenerative medicine.

Cell therapy of bone lesions by ex vivo expanded osteogenic progenitor is passing from the phase of experimental animal model to the phase of clinical trials. Bone is repaired via local delivery of cells within a scaffold. Extremely appealing is the possibility of using mesenchymal progenitors in the therapy of genetic bone diseases via systemic infusion. There are experimental evidences that mesenchymal progenitors delivered by this route engraft with very low efficiency and do not produce relevant and durable clinical effects. Under some conditions where the local microenvironment is either altered (i.e. injury) or under important remodelling processes (i.e. fetal growth) engraftment of stem and progenitor cells seems to be enhanced.

DISCUSSION & CONCLUSIONS: Although multilineage differentiation potential of this cell population is supported by an substantial amount of experimental evidences, a better understanding of the mechanisms, which control differentiation, is required for their exploitation in therapy of human diseases. Furthermore, a better understanding of their engraftment mechanisms will hopefully extend the field of therapeutic applications of mesenchymal progenitors.

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FUNCTION OF THE RETINOID SIGNALING PATHWAY IN SKELETAL DEVELOPMENT

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INTRODUCTION: Development of the appendicular skeleton relies on a complex interplay between multiple signalling pathways to coordinate condensation and differentiation of chondroprogenitors. Although the phenotypic changes associated with chondroblast differentiation have been well characterised, much less is known about the mechanisms underlying these changes.

Vitamin-A and its metabolites, including retinoic acid (RA), are potent teratogens, which, in excess, adversely affect formation of the limb cartilages. Retinoic acid is the natural ligand for two classes of nuclear hormone receptors, the RA receptors (RARs) and the retinoid-X-receptors (RXRs). In the presence of ligand, these receptors recruit a co-activator complex, which augments gene transcription, while in the absence of ligand the receptors form a complex with nuclear co-repressors and histone deacetylase(s) to repress gene transcription. Thus, retinoid receptors have an active role in regulating gene transcription both in the presence and absence of ligand.

RESULTS and DISCUSSION: Previously, we demonstrated that mice ectopically expressing a weak constitutively active form of RARα in the developing limb bud present with severe skeletal malformations. These defects were subsequently shown to be the result of an inhibition of chondroblast differentiation[1,2]. Consistent with this, antagonism of RAR-mediated signalling in limb mesenchymal cultures causes an early increase in collagen type II (*col2a1*) expression that is preceded by a transient early increase in *Sox9* expression. Moreover, the activity of a reporter construct containing four repeats of a Sox9 binding sequence from the *col2a1* gene is increased several fold in mesenchymal cultures treated with an RAR-selective antagonist or co-transfected with a dominant-negative RARα (dnRAR). These results reveal a close association between RAR activity and the transcriptional activity of Sox9. Specifically, inhibition of RAR-mediated signalling in primary cultures of mouse limb mesenchyme, results in increased Sox9 expression and activity. This induction is attenuated by the histone deacetylase inhibitor, TSA indicating a requirement for RAR-mediated repression in skeletal progenitor differentiation.

Using the Sox9 reporter assay, we have further delineated the pathways downstream of retinoid signaling and identified additional modulators of chondrogenesis. Of particular interest, the p38 mitogen-activated protein kinase (MAPK) signaling pathway is activated in response to expression of a dnRAR, whereas inhibition of p38 MAPKs attenuates the chondrogenic stimulation by RAR-selective antagonists. These results will be presented along with additional findings that provide a framework for understanding the role of retinoid signalling in chondrogenesis.

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REGULATION OF CHONDROCYTE PROLIFERATION IN ENDOCHONDRAL BONE GROWTH

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INTRODUCTION: Most of pour bones are formed through the process of endochondral ossification, in which bones are first formed as cartilage precursor. The majority of the cartilage is then replaced by bone and bone marrow. Longitudinal growth of these bones is regulated through the coordinated proliferation and differentiation of chondrocytes in the growth plates at the cartilage/bone interface. Many skeletal disorders are caused by deregulation of these processes. For example, different chondrodysplasias (genetic diseases generally characterized by growth retardation and skeletal deformities) are caused by mutations in genes controlling growth plate function, such as the FGF receptor 3 and the PTH/PTHrP receptor genes. Furthermore, disturbance of chondrocyte proliferation and differentiation likely also contributes to other diseases such as osteoarthritis and skeletal tumourigenesis. The intracellular pathways regulating chondrocyte biology are not completely understood. Deeper knowledge of these pathways might not only lead to a better understanding, diagnosis, prevention, and treatment of skeletal disorders, but may also be used for the manipulation of chondrocyte behaviour during cartilage regeneration in patients or in tissue engineering approaches.

RESULTS: Many extracellular factors such as TGFβ, Indian hedgehog, PTHrP, fibroblast growth factors, insulin-like growth factors and growth hormone have been implicated in the regulation of chondrocyte proliferation. In addition, signals from the extracellular matrix also control proliferation and differentiation of growth plate chondrocytes. However, the intracellular signalling molecules mediating these responses remain largely unknown. We have identified the transcription factors ATF-2 (Activating Transcription Factor 2) and CREB (cAMP response element-binding protein) as key modulators of chondrocyte proliferation and cell cycle progression (Beier et al., 1999, 2000). Chondrocytes from ATF-2-deficient mice display slower proliferation and severely reduced levels of cyclin D1 and cyclin A proteins, compared to wild type cells. We have also show that ATF-2 and CREB transmit mitogenic signals from TGFβ and PTHrP to the cyclin D1 gene, which is necessary for normal proliferation of chondrocytes *in vivo* and *in vitro* (Beier et al., 2002).

DISCUSSION & CONCLUSIONS: While our results clearly suggest a role of ATF-2 and CREB, as well as their downstream targets cyclin D1 and cyclin A, in the control of chondrocyte proliferation and differentiation, it is likely that a large number of additional factors and pathways contribute to these processes. We are currently studying the role of several of these pathways in skeletal development. Results from these studies will enhance our understanding of skeletal biology and contribute to a more efficient manipulation of chondrocyte behaviour in skeletal disorders.

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MODULATION OF CHONDROCYTE PHENOTYPE TO GENERATE IMPLANTABLE GRAFTS

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INTRODUCTION: Large osteochondral defects are associated with mechanical instability of the joint and usually need surgical intervention to prevent the development of degenerative joint disease. We recently demonstrated that engineered cartilage could provide a tissue-like template allowing the orderly repair of very large osteochondral defects in adult rabbits [1]. In order to extend the procedure to a clinical setting, several challenges have to be faced, related to the control of the phenotype of adult human chondrocytes.

METHODS: Cells were isolated from human articular cartilage and expanded in monolayers in medium containing 10% foetal bovine serum, without or with additional growth factors. The chondrogenic capacity of the expanded cells was first tested in high cell density pellets using different culture media, and subsequently in 3D polymer scaffolds (5 mm diameter x 2 mm thick discs) based on either synthetic polymers (i.e., Polyactive, IsoTis, NL) or naturally occurring polymers (i.e., Hyalograft-C, Fidia Advanced Biopolymers, IT). Bioreactors were developed and built to apply either perfusion through or deformation [2] of tissue specimens at defined and controlled regimes. Generated tissues were assessed histologically, biochemically, and for the expression of cartilage-related genes at the mRNA level using real-time quantitative RT-PCR.

RESULTS: During expansion in monolayers, human articular chondrocytes lost their differentiated phenotype and displayed a reduced ability to re-differentiate. Specific growth factors supplemented to the culture medium during cell expansion (namely FGF-2, TGF-β1 and PDGF-bb) enhanced the proliferation rate of human chondrocytes and their ability to redifferentiate and deposit cartilaginous matrix when transferred into a 3D environment [3]. Supplementation of culture medium with molecules contained in the synovial fluid, particularly prostaglandin F2α, further improved the production of extracellular matrix by articular chondrocytes and thus the quality of the generated tissues [4].

Human articular chondrocytes were loaded into biodegradable polymer scaffolds and the cell-polymer constructs cultured in the previously selected culture media. Chondrocytes in Polyactive scaffolds differentiated with time in culture and produced increasing amounts of cartilage-specific extracellular matrix molecules. In contrast, chondrocytes cultured in hyaluronic acid-based scaffolds remained at a pre-chondrogenic stage and developed cartilaginous matrix only upon implantation in vivo in nude mice.

In order to investigate how the phenotype of chondrocytes in the constructs would change in response to loading upon implantation in the joint, engineered tissues based on human articular chondrocytes and Polyactive scaffolds were exposed to dynamic deformation (10% $\pm$ 7.5%) at 0.1Hz for 2 hours every 12 hours for 3 days. The applied regime of physical stress induced loss of the accumulated glycosaminoglycans and upregulation of catabolic chemokines, including IL-8 and MCP-1.

DISCUSSION & CONCLUSIONS: In order to generate durable 3D cartilage grafts for the successful repair of large osteochondral defects, it is necessary to control and appropriately modulate the phenotype of articular chondrocytes. Our results provide evidence that human articular chondrocyte behaviour in vitro is highly sensitive to biochemical (e.g., soluble bioactive molecules), structural (e.g., 3D scaffolds) and physical (e.g., cyclic compression) factors.

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TISSUE ENGINEERING OF CARTILAGE BASED ON HUMAN MESENCHYMAL STEM CELLS FROM BONE MARROW AND ADIPOSE TISSUE

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INTRODUCTION: The regenerating response of cartilage to injury is very limited. To prevent early degenerative arthritis "healing" of cartilage defects with in vitro generated bioartificial cartilage is desired. Tissue engineering approaches aimed at the regeneration of articular cartilage would profit from an easy availability of large numbers of cartilage-forming cells. If possible, such cells should be obtained without the need to harvest cartilage from patients and avoid a two-fold intervention at the same joint. Bone marrow- and adipose tissue-derived mesenchymal stem cells are capable of differentiating into various cell types including chondrocytes [1-3]. In this project the suitability of these stem cells as a source for autologous cell based tissue engineering of human cartilage was evaluated.

METHODS: Mesenchymal stem cells were isolated from bone marrow (BMSC) from patients undergoing hip surgery, or from adipose tissue (ATSC) from individuals undergoing liposuction. Following expansion, differentiation into chondrocytes, osteoblasts and adipoblasts was induced. In addition to histochemical analyses, the multilineage potential of the cells was compared by gene expression profiling using a cDNA array. Chondrogenesis was optimised by modulating cell density, the culture conditions and media supplements to obtain a cartilage-like gene expression pattern.

RESULTS: Both BMSC and ATSC were able to differentiate into adipoblasts, osteoblast- and chondroblast-like cells as assessed by histochemistry and expression of characteristic genes. Chondrogenic differentiation in monolayer culture resulted in strong up-regulation of several cartilage-specific molecules like cartilage oligomeric matrix protein (COMP), lumican, biglycan, and osteonectin. To induce a whole panel of cartilage-expressed molecules including collagen type 2, aggrecan, cartilage link protein, COMP, cartilage intermediate layer protein, and fibromodulin, a switch to 3D culture conditions was necessary. By optimising the culture conditions we were able to produce cartilage-like spheroids derived from BMSC expressing collagen type 2 protein throughout their matrix 4 weeks after induction. The gene expression profile of these cells was similar to that of human cartilage samples obtained from osteoarthritic joints and thus of cartilage attempting matrix reconstruction and differentiation in vivo.

DISCUSSION & CONCLUSIONS: BMSC and ATSC possessed a similar multilineage potential when differentiated in monolayer culture. 3D-spheroids with a cartilage-like gene expression profile could be generated from BMSC, but less successfully from ATSC. Mesenchymal stem cells thus represent a very attractive cell source for autologous cell therapy and tissue engineering approaches for articular cartilage lesions. Moreover, BMSC spheroids could be used as an in vitro system for drug screening on human OA cartilage-like tissue outside the human body.

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ISOLATION AND EXPANSION OF HUMAN ARTICULAR CHONDROCYTES IN MONOLAYER CULTURE INDUCES A PROINFLAMMATORY CYTOKINE PROFILE

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INTRODUCTION: For autologous chondrocyte transplantation (ACT), chondrocytes are expanded in monolayer culture. During this expansion chondrocytes may dedifferentiate into fibroblast-like cells, characterised by e.g. decreasing type II collagen expression. However, little is known about changes in the expression of cytokines involved in cartilage anabolism and catabolism during this process. Since the production of a good quality cartilage might be enhanced through the transplantation of chondrocytes with an anabolic cytokine profile, we analysed the cytokine expression patterns of chondrocytes, which were expanded in monolayer culture and subsequently recultivated in alginate beads.

METHODS: Human articular chondrocytes were obtained from cartilage biopsies (n=6). mRNAs encoding type I and II collagen, IL-1, -4, -10, -17, -18 and BMP-2 and -4 were evaluated by quantitative RT-PCR in one aliquot. Expression patterns were established for freshly isolated chondrocytes, chondrocytes after primary expansion (~2-4 population doublings) in monolayer culture, and for expanded chondrocytes recultivated in alginate beads.

RESULTS: After primary expansion in monolayer cultures, increased IL-4, IL-18 (Fig 1), type I collagen and BMP-4 transcription in comparison with freshly isolated chondrocytes was observed. At the same time, type II collagen, BMP-2, IL-10 and IL-17 mRNA transcription decreased. Recultivation of the monolayer expanded cells in alginate resulted in the reexpression of type II collagen, BMP-2 and IL-10 whereas IL-18 and BMP-4 expression was reduced.

DISCUSSION & CONCLUSIONS: This study shows that in chondrocytes cultivated in monolayer culture IL-10 expression is strongly reduced, while IL-18 expression is increased. IL-10, is known for its IL-1 antagonizing properties; it protects chondrocytes from destructive and catabolic IL-1 effects. IL-18 induces proinflammatory and catabolic responses in chondrocytes and contributes to cartilage degeneration. These results suggest that monolayer culture of chondrocytes not only results in the dedifferentiation of these cells (as shown by the shift from type II towards type I collagen production), but also leads to the establishment of a proinflammatory phenotype. Combined this may lead to a suboptimal formation of cartilage upon transplantation of these cells. Therefore, it is of great interest that the 3D-recultivation of monolayer-expanded chondrocytes not only results in the restoration of the chondrogenic phenotype, but also results in a reduced expression of proinflammatory cytokines.

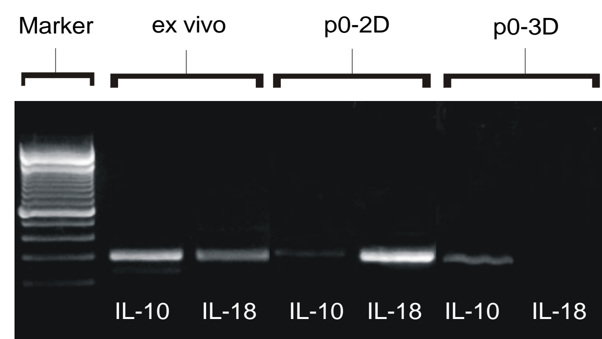

Fig 1. IL-10 and IL-18 expression of human articular chondrocytes seeded in monolayer culture and alginate beads. Ex vivo = freshly isolated human articular chondrocytes. p0-2D = chondrocytes after expansion in primary monolayer culture. p0-3D = expanded chondrocytes from p0-2D recultivated in alginate.

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PLASTICITY OF HUMAN DE-DIFFERENTIATED CHONDROCYTES

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INTRODUCTION: Mesenchymal progenitor cells with the capacity to differentiate along different connective tissue lineages have been identified from an increasing number of tissues, including bone marrow stroma[1], periosteum[2], synovial membrane[3], and blood vessels[4,5]. Chondrocytes from articular cartilage are known to de-differentiate during *in vitro* culture in monolayers, and several biochemical and structural factors have been reported to support their redifferentiation into the chondrocytic lineage. In this work, we addressed: (i) Can de-differentiated human adult articular chondrocytes (HAAC) redifferentiate into other tissue lineages, similar to mesenchymal progenitor cells? (ii) Can single clonal subpopulation of HAAC differentiate into multiple lineages after monolayer expansion?

METHODS: Human articular cartilage biopsies were collected from the knee joint of 3 individuals (age range 25-43). Isolated cells were expanded in medium containing 10% fetal bovine serum (CTR) or supplemented with 1 ng/ml TGFb1, 5 ng/ml FGF-2 and 10 ng/ml PDGFbb (TFP) (factors shown to enhance HAAC proliferation and capacity to redifferentiate[6]). After 2 passages, cells were cultivated (i) as 3D pellets in serum-free medium containing 10ng/ml TGFb1 and 100 nM dexamethasone (chondrogenic medium); (ii) in monolayers in medium containing 10% FBS, 10 nM dexamethasone and 10 mM b-glycerophosphate (osteogenic medium), with or without 100 ng/ml BMP-2; (iii) in monolayers in medium containing 10% FBS, 1 µM insulin, 100 nM dexamethasone, 1 µM indomethacin & 0.5 µM methylisobutylxanthine (adipogenic medium). Single colony-derived strains of articular chondrocytes were established by limiting dilution of freshly digested cells. Clones were expanded in CTR and TFP media and differentiated using above culture conditions. Cells differentiation was assested by histological analyses (safranin-O & oil red O stains) and by expression of collagen types II, X, bone sialoprotein (BSP), osteocalcin (OC) and adipsin at the mRNA level using real-time quantitative PCR.

RESULTS: TFP expanded cells in pellet culture upregulated collagen type II expression at higher level and formed cartilaginous structures more stained for Safranin-O than CTR expanded cells. Culture of CTR and TFP expanded chondrocytes for 3 weeks in osteogenic medium without BMP-2 induced expression of BSP and OC, but not of collagen II and X. Further supplementation of the osteogenic medium with BMP-2 induced the formation of mineralized structures and upregulation of collagen types II and X more efficiently in TFP than in CTR expanded chondrocytes. High and low numbers of respectively CTR and TFP expanded cells accumulated lipid droplets stained with oil red O when exposed to adipogenic medium for 3 weeks. A total of 27 clones were isolated from one primary culture and expanded with (20 clones) and without (7 clones) TFP. Clones proliferated at different rates and had different morphologies. The frequency of clones able to differentiate into chondrocytic (C), hypertrophic (H), osteocytic (O) and adipocytic (A) lineages are summarized in *Table 1*.

Phenotype			CTR		TFP	
O	C or H	A	No clon	% of total	No clon	% of total
+	+	+	1	14	2	10
+	+	-	1	14	2	10
-	+	+	1	14	1	5
+	-	+	0	0	0	0
+	-	-	0	0	1	5
-	+	-	1	14	7	35
-	-	+	2	29	1	5
-	-	-	1	14	6	30
Total			7	100	20	100

Table 1. Frequency & differentiation potential of clonal HAAC

DISCUSSION & CONCLUSIONS: De-differentiate HAAC could be re-differentiated into chondrocytes (hyper or non-hypertrophic), osteoblast and adipocytes, indicating (*in vitro)* extensive plasticity of the chondrocyte phenotype. Based on the results of the clonal analysis, the ability of a population of HAAC to differentiate into different lineages may mostly be due to coexistence of different subpopulations with restricted differentiation capacities. Studies are in progress to identify features or markers to characterize different clonal subpopulations of HAAC.

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CHARACTERISATION OF ARTICULAR CARTILAGE PROGENITOR CELLS

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INTRODUCTION: There are two major problems that afflict current strategies in cartilage repair. One problem is tissue integration between host and reparative tissue. The second problem is the generation of a repair tissue with the structural characteristics of articular cartilage. We have shown that articular cartilage grows by apposition from the articular surface towards the subchondral bone and that this growth is driven by the proliferation of surface zone cells [1,2], Additionally, a population of cells with an increased cell cycle time was identified within the surface zone; a property typical of many progenitor cell populations [2]. The aim of our research is to identify and characterise a chondroprogenitor population from articular cartilage to enable the rapid culture of undifferentiated chondrocytes in vitro for future clinical use. Here we describe the isolation and partial characterisation of a cell population from the articular surface which exhibits differential adhesion to fibronectin, differential integrin and Notch family expression and the ability to form large numbers of colonies from an initially small seeding density; properties that are common to known progenitor cell populations of other tissues.

METHODS: *Tissue culture and differential adhesion assay:* Cartilage slices were isolated from the surface (SZ), middle (MZ) and deep (DZ) zones of 7 day old bovine metacarpal-phalangeal joints by fine dissection and sequential pronase/collagenase digestion (7U ml^{-1} pronase in DMEM/5%FCS for 3 hours at 37°C followed by 0.16U ml^{-1} collagenase in DMEM/5%FCS for 16 hours at 37°C). Chondrocytes were seeded onto fibronectin (10µg ml^{-1})-coated or PBS/1% BSA-coated 35 mm dishes at 4,000 cells ml^{-1} in serum free DMEM (DMEM-) for 20 minutes. After 20 minutes, media and non-adherent cells were removed and placed in similarly treated dishes for a further 40 minutes before this media and nonadherent cells were placed in a third dish. After removal of media at 20 and 40 minutes, fresh DMEM- was added to the remaining cells that were cultured for up to 10 days. In all experiments 6 fibronectin and 6 uncoated dishes were used for each zone of cartilage. Fibronectin was used as a ligand in the experiments since it is known to be differentially expressed at the articular surface during mammalian development [3]. Within three hours of plating, chondrocyte adhesion was assayed by counting the total number of cells per dish using phase contrast microscopy and expressed as a percentage of the initial seeding density. Additionally, colonies of chondrocytes consisting of more than 32 cells were counted at 0, 3, 6 and 10 days after differential adhesion (n = 6 experiments). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n = 3) the numbers of cells per colony was counted to determine the average number of cells per colony. Results were analysed using the Students *t* test.

Flow cytometry: Four hours after differential adhesion, chondrocytes were removed from dishes non-enzymatically and 2 x 10^{5} cells were incubated for 3 hours with antibodies to α5 (AB1928) and β1 (MAB1951) integrin subunits and anti-N1 (SC 6014) at room temperature. Cells were centrifuged at 3,000rpm, supernatants removed and cells washed three times in PBS with centrifugation between each wash. Cells were then incubated with relevant FITC conjugated secondary antibodies for 1 hour at room temperature and washed three times in PBS as described above. Finally, labelled cells were resuspended in 500µl PBS and subjected to flow cytometry. *Notch 1 Immunolabelling and Immunomagnetic Isolation:* Frozen sections of 7 day bovine full depth articular cartilage were immunolabelled with anti-N1 antibody and localised with the appropriate secondary FITC conjugated secondary antibody. Chondrocytes were isolated by sequential pronase/collagenase digestion from surface middle and deep zone articular cartilage and incubated with M450 tosyl-activated Dynal beads conjugated to goat anti-human N1 antibody for 4 hours at 4°C. N1 selected cells were counted and 4,000 cells ml^{-1} subjected to differential adhesion to fibronectin for 20 minutes. Initial adhesion and CFE were assessed as described above.

RESULTS: Initial adhesion ranged between 3.5% and 14.5% of the original cell number. Significant differences in adhesion were evident between surface zone chondrocytes seeded on fibronectin for 20 minutes (9.05% +/- 0.44) and those seeded on

PBS coated dishes for 20 minutes (3.83% +/- 0.27; $p < 0.001$) and also with those seeded on fibronectin for 40 minutes (4.89% +/- 0.43; $p < 0.001$). Middle zone chondrocytes were significantly more adherent at 20 minutes (14.53% +/- 0.86) than at 40 minutes (10.58% +/- 0.51) when seeded onto fibronectin-coated dishes ($p < 0.01$). Additionally, middle zone chondrocytes were more adhesive to fibronectin at both time points compared with PBS-coated dishes ($p < 0.001$). No differences in adhesion were observed between deep zone chondrocytes regardless of substrate or time point ($p > 0.05$ in all cases).

At days 0 and 3, no colonies containing 32 or more cells were present in any sample. At 6 and 10 days (Fig 1), the CFE of surface zone chondrocytes initially cultured on fibronectin for 20 minutes was greater than that of the other samples ($p < 0.01$ at 6 days, $p < 0.001$ at 10 days). In addition, the CFE of surface zone cells initially cultured for 20 minutes on fibronectin was greater at 10 days compared with that at 6 days ($p < 0.05$). No change in CFE was evident between 6 and 10 days for any other sample ($p > 0.05$ in all cases). The average number of cells per colony was greater in surface zone cells initially grown on fibronectin for 20 minutes at both 6 ($p < 0.05$) and 10 ($p < 0.01$) days compared with all other samples. FACS analysis showed elevated levels of both $\alpha 5$ and $\beta 1$ integrin subunits in surface zone cells compared with middle and deep zone cells ($p > 0.05$).

N1 immunolabelling revealed occasional N1 positive cells within uppermost 2-3 cell layers of the articular cartilage. When surface zone cells were isolated and analysed for N1 using FACS over 84% of the surface zone population were N1 positive and this result was reflected in the cell counts obtained after N1 selection. Adhesion assays performed using N1 selected chondrocytes revealed that the N1 positive cells were more adherent than either negative cells or unselecetd cells ($p > 0.05$) and that the CFE of N1 selected cells was increased 4 fold relative to negative cells and unselected cells ($p > 0.001$).

DISCUSSION & CONCLUSIONS: The ability of a population of cells to form large numbers of chondrocyte colonies from a low seeding density, differences in $\alpha 5\beta 1$ integrin subunit expression, differential N1 expression and when taken together with previous results demonstrating the prolonged cell cycle time at the articular surface (2), strongly suggest that a subpopulation of progenitor chondrocytes resides in the articular surface. Additionally, the prolonged adhesiveness of mid zone cells and their restricted ability to form large numbers of colonies and their relatively short cell cycle (2) strongly indicates the presence of transit amplifying cells within this zone. The further purification and in vitro expansion of this chondroprogenitor population will prove to be vital in advancing strategies for cartilage repair.

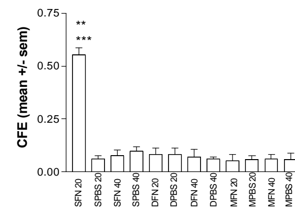

Fig 1: Surface zone CFE is significantly enhanced 10 days after differential adhesion to fibronectin.

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CHONDROPROGENITOR REPAIR OF ARTICULAR CARTILAGE *IN VITRO*

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INTRODUCTION: There are two major problems which afflict current strategies in cartilage repair. One problem is tissue integration between host and reparative tissue. The second problem is the generation of a repair tissue with the structural characteristics of articular cartilage. We have shown that articular cartilage grows by apposition from the articular surface towards the subchondral bone and that this growth is driven by the proliferation of surface zone cells [1,2], Additionally, a population of cells with an increased cell cycle time was identified within the surface zone; a property typical of many progenitor cell populations [2]. The aim of our research is to identify and characterise a chondroprogenitor population from articular cartilage to enable the rapid culture of undifferentiated chondrocytes in vitro for future clinical use. Here we describe the isolation and partial characterisation of a cell population from the articular surface which exhibits differential adhesion to fibronectin, differential integrin and Notch family expression and the ability to form large numbers of colonies from an initially small seeding density; properties that are common to known progenitor cell populations of other tissues. Additionally we report that this population of chondroprogenitor cells can prevent cell death and matrix loss in an in vitro model of articular cartilage wounding.

METHODS: *Tissue culture and differential adhesion assay:* Cartilage slices were isolated from the surface (SZ), middle (MZ) and deep (DZ) zones of 7 day old bovine metacarpal-phalangeal joints by fine dissection and sequential pronase/collagenase digestion (7U ml^{-1} pronase in DMEM/5%FCS for 3 hours at 37°C followed by 0.16U ml^{-1} collagenase in DMEM/5%FCS for 16 hours at 37°C). Chondrocytes were seeded onto fibronectin (10µg ml^{-1}) -coated or PBS/1% BSA-coated 35 mm dishes at 4,000 cells ml^{-1} in serum free DMEM (DMEM-) for 20 minutes. After 20 minutes, media and non-adherent cells were removed and placed in similarly treated dishes for a further 40 minutes before this media and nonadherent cells were placed in a third dish. After removal of media at 20 and 40 minutes, fresh DMEM- was added to the remaining cells which were cultured for up to 10 days. In all experiments 6 fibronectin and 6 uncoated dishes were used for each zone of cartilage. Fibronectin was used as a ligand in the experiments since it is known to be differentially expressed at the articular surface during mammalian development [3]. Within three hours of plating, chondrocyte adhesion was assayed by counting the total number of cells per dish using phase contrast microscopy and expressed as a percentage of the initial seeding density. Additionally, colonies of chondrocytes consisting of more than 32 cells were counted at 0, 3, 6 and 10 days after differential adhesion (n = 6 experiments). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n = 3) the number of cells per colony were counted to determine the average number of cells per colony. Results were analysed using the Students *t* test.

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resuspended at 2 X 10^6 cells ml^{-1} and 500µl aliquots placed in 1.5ml microtubes. Cells were pelleted at 2000 rpm for 5 minutes and resultant pellets maintained in culture (DMEM/F12, 10%FCS 1% HEPES buffer) for up to 14 days. Pellets were fixed in 10% NBFS and processed for routine histology and immunocytochemistry. Additionally, pellets cultured for 3 days were transplanted into previously wounded bovine explants and the explants maintained in culture for 7 days. Cell death was assesssed using ethidium homodimer (90 minute incubation at 2mM) prior to fixing in 10% NBFS and wax embedding.

RESULTS: Initial adhesion ranged between 3.5% and 14.5% of the original cell number. Significant differences in adhesion were evident between surface zone chondrocytes seeded on fibronectin for 20 minutes (9.05% +/- 0.44) and those seeded on PBS coated dishes for 20 minutes (3.83% +/- 0.27; $p < 0.001$) and also with those seeded on fibronectin for 40 minutes (4.89% +/- 0.43; $p < 0.001$). Middle zone chondrocytes were significantly more adherent at 20 minutes (14.53% +/- 0.86) than at 40 minutes (10.58% +/- 0.51) when seeded onto fibronectin-coated dishes ($p < 0.01$). Additionally, middle zone chondrocytes were more adhesive to fibronectin at both time points compared with PBS-coated dishes ($p < 0.001$). No differences in adhesion were observed between deep zone chondrocytes regardless of substrate or time point ($p > 0.05$ in all cases).

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Zonal derived pellets were homogenous at day 3 of culture but displayed increasingly distinct morphologies after this time. By 14 days, surface zone pellets were highly cellular and separated by a sparse yet intensely safranin O stained matrix. Mid-zone derived pellets fewer cells separated by a more extensive and intensely stained matrix whilst deep zone pellets contained large vacuolated cells in an extensively stained matrix.

Wounded explants displayed a distinct loss of safranin O staining at the articular surface and around the wound margin as did explants containing middle and deep zone derived pellets. This loss of safranin O staining was abbrogated in explants containing surface zone derived pellets. Additionally, cell death was prominent around control wound margins and wounds containing mid and deep zone derived pellets consistent with the zone of matrix loss identified with saffranin O staining. Wounds containing a surface derived pellet exhibited minimal cell death around the wound margin.

DISCUSSION & CONCLUSIONS: The ability of a population of cells to form large numbers of chondrocyte colonies from a low seeding density, differences in α5β1 integrin subunit expression, and differential N1 expression when taken together with previous results demonstrating the prolonged cell cycle time at the articular surface (2), strongly suggest that a subpopulation of progenitor chondrocytes resides in the articular surface. Additionally, the reductions in cell death and matrix loss in wounded explants treated with surface zone derived pellets emphasises the future use of this chondroprogenitor in articular cartilage repair.

REFERENCES: [1]Archer, CW (1994), *Ann Rheum Dis* **53:** 624-630. [2]Hayes et al (2001) *Anat Embryol.* **203:** *469-279.* [3]Salter et al (1995); *J Histochem. Cytochem.* **43:** 447-457.

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CELL BIOLOGY OF TENDONS

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INTRODUCTION: Tendons and ligaments have a highly ordered cellular organisation within their tissue. Of key importance to attempting to replicate such structures *in vitro* is understanding (i) the purpose of that organisation and (ii) how it arises in development. The aim of the work reported here is to accurately describe cellular architecture and to report major roles of cell-cell interaction in tendon physiology, and to give a preliminary indication as to how such understanding can be applied to a tissue-engineering environment.

METHODS: The approach taken has been to use a range of labelling techniques allied to confocal microscopy to examine the 3 dimensional organisation of cells in tendon, and to describe the arrangement of cell-cell junctions and the cytoskeleton. We have then used in vitro cell loading equipment (Flexcell FX3000; Flexercell International, USA) to examine the effects of tensile strain on cytoskeletal architecture and to determine the roles of direct cell-cell communication on collagen synthesis under cyclic tensile load. Finally, we have developed cell culture procedures that prioritise cell-cell interaction in the formation of tendon material in cell culture

CELL AND MATRIX ORGANISATION: Tendon cells are arranged in longitudinal rows between collagen fibre bundles. Within rows, they are in longitudinal contact end-to-end; between rows they are in contact via sheet-like lateral cell processes that extend around collagen fibre bundles and meet up with processes from adjacent cells. An individual cell is associated with 6-9 collagen fibre bundles, and fibre bundles run from cell to cell longitudinally along the cell row.are thus enclosed in cell bounded compartments *(Figure 1)*.

CELL-CELL COMMUNICATION: Where the tendon cell membranes meet, they contain gap junctions, which in other tissues are associated with coordination of cell function, often by calcium signalling. Different types of gap junction occur in lateral and longitudinal planes. Thus, longitudinally within a row cells have the gap

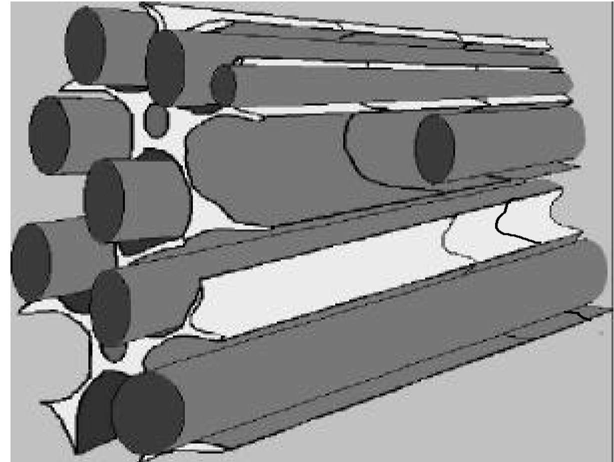

Figure 1. Diagram to illustrate interaction of cells and association with collagen fibre bundles (cylinders); 3 cells are shown longitudinally, and 2 laterally. Collagen bundles are enclosed by lateral cell processes and passed from cell to cell longitudinally.

junction proteins connexin 32 and connexin 43 at points of contact, whereas laterally, cell processes only have connexin 43 on their cell processes. Gap junctions with these connexins have different communication characteristics, and thus there are different abilities of tendon cells to directly pass messages longitudinally, i.e. along the line of principal strain, and laterally. Cell cultures of tendon cells contain both connexin types. Normally, cultures exposed to cyclic tensile load increase their collagen synthesis by about 50%. In the presence of general blockers of gap junction communication, this response is abolished. If the specific connexins are downregulated using antisense techniques, we can show that communication via connexin 32 is *stimulatory* to the load response, whereas via connexin 43 is *inhibitory*. Thus there must be a balance of inhibitory and stimulatory signals that determines what the response of a population of cells will actually be.

CYTOSKELETAL ORGANISATION: Gap junctions cannot hold cells together, and nor can they originate the load signals – they just pass signals from cell to cell. Cytoskeletal associated anchoring junctions, in particular adherens junctions linking actin filaments cell to cell, are

associated with cell-cell adhesion via cadherins and vinculin, and possibly with generation of load signals. Labelling techniques and confocal microscopy show that tendon cells in vivo contain longitudinal actin-myosin stress fibres; these are aligned in longitudinally adjacent cells, and linked via adherens junctions to form long "transcellular" stress fibres extending along the cell rows. Significantly, these are not seen laterally. The stress fibres and their junctions are in ideal position aligned to the line of principal strain in the tendon to (a) hold cell rows together when tendons stretch under load and (b) to monitor strain within the tendon, generating signals that are then integrated via the gap junction signalling system. In cell cultures exposed to strain, tendon cells synthesise more adherens junction components, and assemble more of their available actin into stress fibres, suggesting that they enhance cell-cell adhesion under load.

DISCUSSION & CONCLUSIONS: Tendon cells, and indeed ligament cells and cells of the annulus fibrosus, have a highly ordered architecture within their tissue, and an intimate association with one another and with their extracellular matrix. The cell-cell associations are of clear importance in load sensing and modification of the extracellular matrix. Cell-cell interactions are also of crucial importance in the early development of connective tissues. Both tendon and cartilage initially develop as a dense condensation of cells, with much direct cell-cell contact. In cartilage, cell differentiate, secrete matrix and push one another apart, losing their contact. In tendon, cells retain their contacts and form the highly ordered structure as described. Given the importance of cell-cell contacts in tendon biology, we have developed a high cell density suspension culture system to study roles of cell-cell interactions in tendon development. In this system we allow large numbers of tendon cells (2-3x10^7 cells/ml) to interact. The cells aggregate together within 6 hours, and over the next 14 days form elongate structures 1-2mm in diameter and up to 2 cm long with evidence of cell and matrix organization, at least in parts. This system will be developed further as both a scientific tool and as a potential means of developing substantial amounts of organized fibrous tissue in vitro, without the use of exogenous scaffolds.

ACKNOWLEDGEMENTS: This work has been funded by the Wellcome Trust and the EPSRC. I also thank a number of people for their important contributions: Drs Andrew Waggett, Ceinwen McNeilly and Mike Benjamin; Prof Al Banes; Rhiannon Fish; and Ed Brydon for assistance with microscopy.

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NON-COLLAGENOUS MATRIX COMPONENTS INFLUENCE THE MICRO-MECHANICAL ENVIRONMENT OF TENOCYTES WITHIN TENDON FASCICLES SUBJECTED TO TENSILE STRAIN

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INTRODUCTION: Tendon is a fibrous connective tissue, composed predominantly of type I collagen fibrils arranged in a hierarchical manner, comprising fibrils, fibres and fascicles. The collagen fibrils are interspersed by non-collagenous matrix, composed primarily of the proteoglycan decorin, which influences the structural integrity of tendon, by binding to collagen and creating a hydrated microenvironment[1].

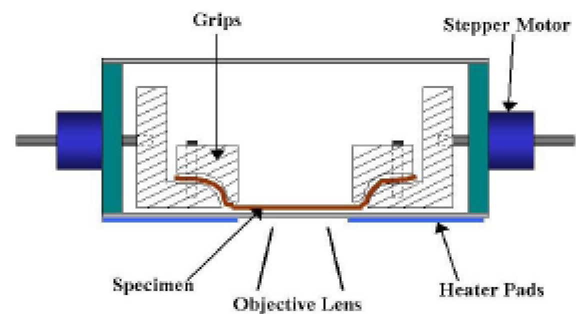
Collagen fibres possess a natural periodic waveform, along which cells, known as tenocytes, are positioned in short rows. During physiological loading tendon responds initially with the straightening and realignment of crimped collagen fibres. Once fibres have straightened, further tendon elongation is achieved through the relative sliding of the collagen components at the fibre and fibril levels. These mechanisms may be regulated by the proteoglycan matrix[2].

The mechanism of tendon elongation will necessarily affect the local strains experienced by the tenocytes, which respond by the initiation of mechanotransduction pathways, thereby stimulating tissue remodelling [3]. This study tests the hypothesis that proteoglycan-rich matrix regulates tendon elongation mechanisms. Specifically the relative contribution of crimp straightening, fibre sliding and fibre extension were determined during the elongation of isolated rat-tail tendon fascicles. Chondroitinase ABC digestion was used to selectively remove dermatan sulphate and chondroitin sulphate glycosaminoglycan (GAG) side chains on proteoglycans.

METHODS: Tendon fascicles, approximately 60 mm in length, were prepared from the proximal region of the tails of male Wistar rats, aged 19 to 22 weeks. Control samples were immediately stained in the vital fluorescent nuclei stain acridine orange (5µl.ml^{-1} in EBSS) for 1 hour at room temperature, and briefly rinsed in EBSS before testing. Further samples were incubated for 24 hours at 37 °C in 1 ml of PBS to permit hydration, or PBS + 1U.ml^{-1} chondroitinase ABC to remove GAG. These samples were subsequently stained with acridine orange as above.

The stained samples were placed in a custom designed test rig (Fig. 1), for use on the stage of an inverted confocal microscope (UltraView, Perkin Elmer, UK). The test rig enabled accurate visualisation of cell nuclei during tensile loading of tendon fascicles[4].

Fig. 1: Schematic of the test rig



Groups of nuclei, equidistant from the grips and within the body of the fascicle were identified for imaging. Samples were strained in 1% increments and the nuclei positions recorded at each increment, by taking a z-series of images through the sample.

The relative nuclei co-ordinates within the matrix were recorded at each strain increment, taking the intersection of the nucleus long axis and its perpendicular width as its centre point. Relative displacements between groups of nuclei along the same collagen fibre were expressed as within-group strains. Displacements between the nuclei of different fibres, expressed as a percentage of the applied displacement, were also recorded and termed between-group displacements. Hydration and chondroitinase treatments were characterised by assessment of wet and dry weight and GAG and collagen content.

RESULTS: Biochemical analysis of samples indicated that GAG constituted 0.3±0.1% of the dry weight of tendon. However no GAG could be detected after chondroitinase ABC digestion. Negligible levels of collagen were releasaed during hydration or chondroitinase treatment. Hydrated samples showed 246±54% increase in wet mass,

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compared to a 170±53% increase in chondroitinase treated fascicles.

Both cell nuclei and collagen fibres were clearly visible using confocal analysis, as shown in figure 2. In all fascicles, crimp straightening and fibre alignment were noted during the first 4% of applied strain, and rotation of the fascicle was recorded throughout testing.

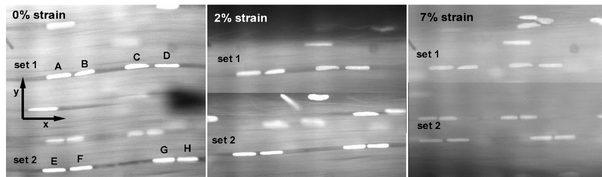
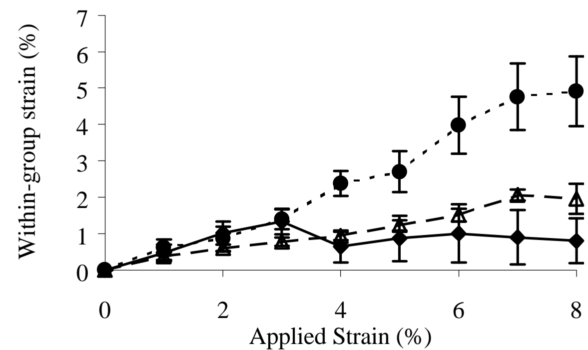


Fig. 2: Relative motion of tenocyte nuclei in a rat-tail fascicle at 0, 2 and 7% applied strain:

After 4% applied strain, control samples exhibited little further increase in within-group strains, but a continual increase in between-group displacements. Figure 3 compares data for hydrated, chondroitinase treated and control samples. Hydrated samples exhibited a continual increase in within-group strains after 4% applied strain, and little alteration of between-group displacements. By contrast, between-group displacement was enhanced in chondroitinase-treated samples when compared to control and hydrated specimens. Using unpaired student t-tests, to compare chondroitinase treated and hydrated samples, within-group strains were significantly different beyond 4% applied strain and between-group displacements were significantly different beyond 6% applied strain (n=0.05).

DISCUSSION & CONCLUSIONS: The results from this study highlight the role of non-collagenous matrix components in regulating tendon elongation mechanics. Within-group strains are indicative of sliding between fibrils, whereas between-group displacements record the relative sliding of fibres. Compared to control samples, hydrated samples displayed an increase in sliding at the fibril level and consequent decrease between fibres.

The increase in fibril sliding post-swelling may therefore be a result of weakened fibril interactions in hydrated fascicles. Chondroitinase treated samples exhibited increased fibre and reduced fibril sliding, relative to control samples, suggesting that fibril binding strength is increased. Collagen binds to the core protein of decorin, hence digestion of GAG chains does not directly affect this linkage. By removing the GAG chains however, fibrils are able to move into closer contact, potentially increasing the number of strongly binding collagen crosslinks present between the fibrils.



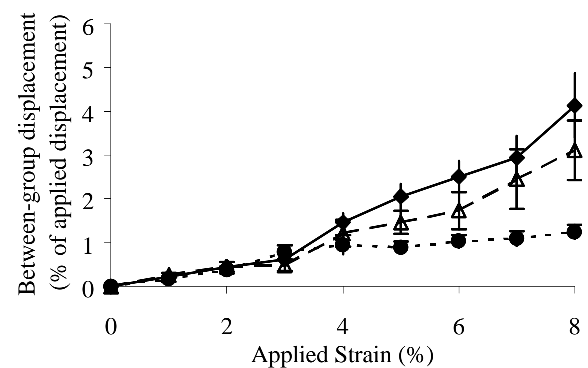


Fig. 3: Within-group strains (a) and between-group displacements (b) in (⋯•⋯) hydrated, (—♦—) chondroitinase digested and (—△—) control fascicles. Mean ± sd.

This study demonstrates the importance of proteoglycan matrix in regulating tendon elongation mechanics, and consequently cell mechanotransduction processes.

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TISSUE-ENGINEERED LIGAMENTS: A NEW TECHNOLOGY.

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INTRODUCTION: Tissue-engineering belongs to the latest technologies developed for the reconstruction of tissues and organs in vitro. This approach was used for the reconstruction of anterior cruciate ligament (ACL) of the knee joint. Rupture of the ACL often happens during intensive sport practice. Unfortunately, current options for its reconstruction are associated with many potential complications[1]. We have developed a tissue-engineered ACL for in vitro studies of connective tissue repair and for clinical applications[2]. Implantation in goats has shown very promising results. Eventually, the technology will be adapted to produce other ligaments and tendon substitutes.

METHODS: Autologous ACL cells were isolated from each of the 6 goats (45 kg) of the experimental group. Tissue-engineered ACL (te-ACL) substitutes were constructed using autologous living cells, seeded in a bovine Type I collagen matrix anchored by 2 bone plugs (Fig.1A)[2]. Some additional procedures were performed to increase the strength of the tissues prior to implantation. The surgical implantation was performed according to the method initially described by Dunn et al. [4]. The te-ACLs were grafted (Fig.1B) for periods ranging from 1-12 months.

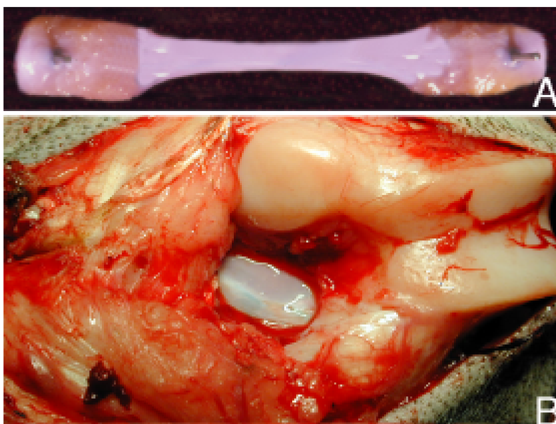

Fig.1: A: te-ACL ready to be grafted and B: the same te-ACL immediately after its implantation in a goat's knee joint.

The histological analyses of the grafts post-mortem ex-vivo were performed using Masson's trichrome staining method. Ultrastructural analyses were also done on some grafts biopsies under transmission electron microscopy.

To evaluate the weight applied on each leg of the 3 goats, two force plates were used and connected to a computer recording the data in real time. One goat was grafted on only one knee while the two others were grafted on both knees, all weighing about 70 kg. Therefore, the data must be considered as preliminary, since a sham knee on each animal would have provided more accurate information. Nevertheless, the results of these tests are reported.

RESULTS: Our technological strategy to produce te-ACL is rather simple. We use the autologous cells of the host to seed them into a bovine Type I collagen matrix. The diameter of the te-ACL is slowly reduced as a result from the contraction of the matrix by the cells. The te-ACL is cultured under static elongation, which promotes collagen fibres alignment in its long axis. Collagen fibres are neosythesized and organized by the living cells in culture prior to the implantation of the autologous te-ACL.

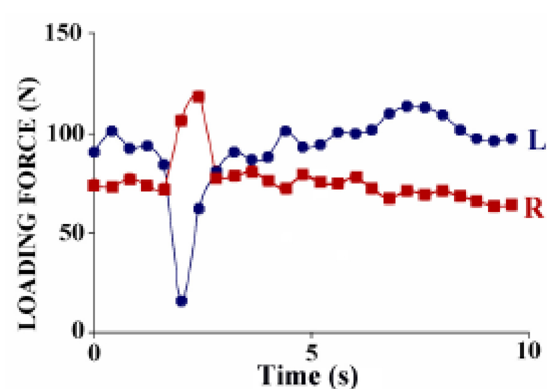

Fig. 2: Load bearing on the goat left (L) and right (R) hind limbs.

Once implanted, the te-ACLs get integrated in the knee joint of the animals.

The experiments performed after 2 months with the force plates showed equal balance of weight bearing on both legs, the grafted and the control contralateral leg of one goat (Fig. 2). Similar results were obtained with 2 other goats grafted on their two legs (data not shown). The animal experimentation is currently pursued to see the results after a year of implantation.

DISCUSSION & CONCLUSIONS:

Our results suggest that the grafting of our te-ACL are promising. The animals are walking and jumping normally at two months post-grafting.

The histological, ultrastructural and biomechanical analyses of the grafts ex-vivo post-mortem will give precious information about the potential of our te-ACL as an eventual ACL prosthesis for fundamental and clinical applications.

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COLLAGEN MATRIX STRUCTURE IN THE DEEP ZONE OF HUMAN ARTICULAR CARTILAGE

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INTRODUCTION: The collagen matrix of articular cartilage deep zone has been described as a spongy network, although suggestions of the existence of a more organised arrangement are made[3]. The nature of the images obtained is dependent upon the specimen preparation protocols applied, the age of the tissue and area studied. If rapid freezing of the tissue, followed by freeze substitution, then an organised structure is seen[1]. The collagen fibrils of rabbit deep zone articular cartilage, arranged perpendicular to the surface of the tissue, are packed together, forming fine tubular structures within the matrix. This arrangement is absent from the tissue of very young animals[2]. Human articular cartilage was found to have a similar structure.

METHODS: Samples of healthy articular cartilage from human tibial plateau and proximal femoral joints were stored in cool phosphate buffered saline for transportation to the laboratory.

2mm diameter cores, complete from the cartilage surface to the bone, were removed. The samples were then frozen by plunging into liquid propane at -197°C. The frozen samples were fractured under liquid nitrogen, before being transferred into dry acetone at -80°C to freeze substitute for 7 days, with a change of acetone every 24h. All samples were then critical point dried, using liquid CO_2. Where necessary, further fracturing of the dried samples was performed under liquid nitrogen, bringing the samples back to room temperature, while ensuring that no condensation of water from the atmosphere occurred on the specimen surface. All samples were sputter coated in a *Baltec Med020* coating unit with gold/palladium and studied using a Field emission scanning electron microscope.

RESULTS: Collagen fibrils in the 2mm thick deep zone were arranged in the radial direction. Essentially; two patterns of arrangement were seen in the areas removed from the immediate vicinity of the chondrons. The bulk of the fibrils were arranged in a spiral fashion, forming the lining of broadly tubular structures. In some regions, much straighter fibrils were seen in tightly packed arrangements. The two types combined, in load bearing areas, to form sheet-like structures lining the tubules. An orthogonal arrangement of finer fibrils appeared on the surfaces of these structures. These were also observed in the superficial zone. This arrangement was similar to that discovered in tissue from other species. We have also shown that, in rabbit tissue, the application of conventional chemical fixation techniques result in a reorganisation of the extra cellular matrix components of this tissue. Considerable volume changes result from such treatments, not apparent after cryo fixation[4]. An appropriate modification of the chemical fixation protocol can result in the preservation of at least some of the tubular and orthogonal fibril structure.

CONCLUSIONS: The arrangement of collagen fibrils in the loaded area of human tibial plateau tissue was essentially similar to that of the tubular matrix like structure found in other species, with a strong helical component to the 30nm fibrils and orthogonally arranged fine fibrils apparently binding them together

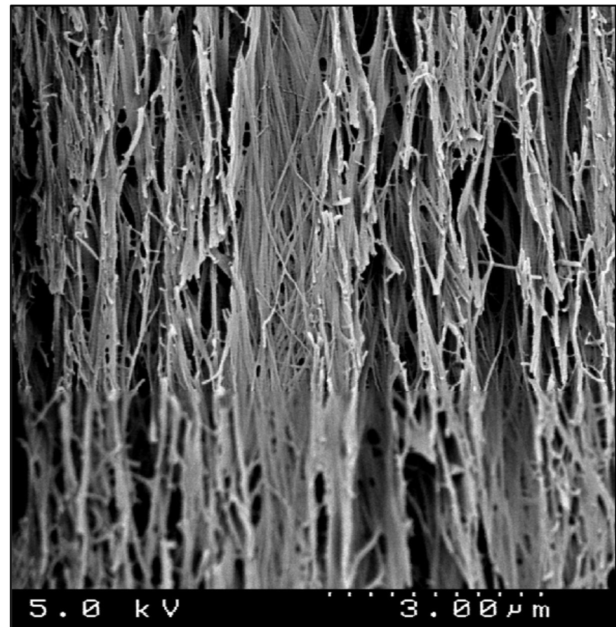

Fig. 1: Scanning electron micrograph of tubular arrangement of the collagen matrix in human tibial plateau articular cartilage, showing spirally arranged fibrils.

REFERENCES: [1]ap Gwynn, I., *et al* (2000). *J.Microscopy* 197(2), 159-172. [2]ap Gwynn, I., *et al* (2002) Collagen arrangement in the Radial Zone of Articular Cartilage. (Submitted). [3]Eyre D. (2002). *Arthritis Res*. 4, 30-35. [4]Kääb, M.J., *et al* (1999) *Scanning Microscopy* 13(1) 61-70.

ACKNOWLEDGEMENTS: The specimens were supplied by Dr M. J. Kääb of Charité, Humboldt University, Berlin & Dr. P. Holzach, Orthopaedics & Surgery, SportClinic, Zürich, Switzerland. The work was supported by grants from the AO Research Fund (AORF), Switzerland (grant numbers 98G36 & 2000G50).

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ORTHOGONALLY ARRANGED FINE FIBRILS BINDING TO COLLAGEN IN ARTICULAR CARTILAGE.

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INTRODUCTION:

The structure of articular cartilage is continuously being evaluated in an attempt to determine how it functions as a load bearing tissue. Recent studies [1,2] utilising a variety of different preparation methods show a tubular formation of collagen fibres in the radial zone of articular cartilage. It is shown here that within the collagen tubules, of several different mammalian species, are arrays of orthogonally arranged fibrils.

METHODS:

Human, sheep, rabbit and mouse tibias were obtained as fresh as possible and cryofixed using rapid freezing and freeze-substitution techniques. Standard chemical fixation techniques for electron microscopy were also used for some samples.

The samples for scanning electron microscopy (SEM) were critical point dried and fractured under liquid nitrogen. The tibias were brought up to room temperature in a special container that released the liquid nitrogen gas and prevented condensation onto the samples. The fractured tibias were then mounted onto stubs and coated with platinum/palladium before being viewed in a Hitachi S-4700 field emission SEM

The samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) while exposed to bursts of microwave energy. The tibias were embedded in resin, sectioned and stained using standard preparation techniques. The sections were imaged using a JEOL CX-100.

RESULTS & DISCUSSION:

The collagen matrix in the radial zone of the articular cartilage was arranged into structures resembling a tubular formation in all the different species examined. High-resolution images of the matrix (both SEM and TEM) revealed fine fibrils arranged orthogonally to the main radial collagen fibrils. These fibrils were observed raised on the surface of the collagen fibres and were present in all the species examined. The spacing between the orthogonal fibirils appeared to coincide with the distance expected for the characteristric 'D' banding of collagen. This arrangement may form a barrier to lateral fluid movement in the radial zone of the cartilage.

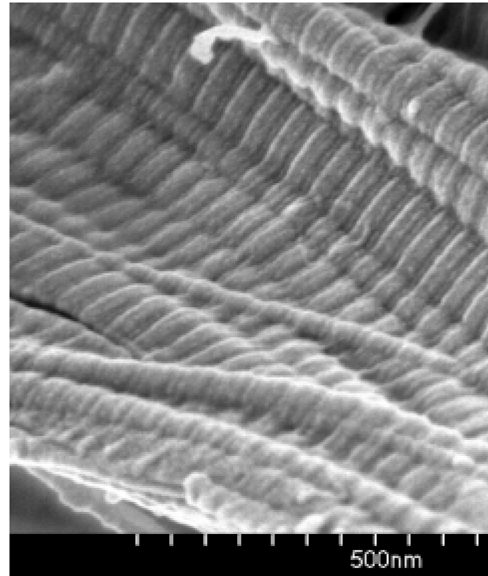

Fig. 1: Orthogonally arranged fine fibrils attached to collagen fibrils in the radial zone of adult rabbit articular cartilage.

REFERENCES: [1] ap Gwynn et al (2000) J. Microsc. 197 (2): 159-172.[2.] ap Gwynn et al (20002) Collagen arrangement in the radial zone of articular cartilage (submitted).

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CELL PROLIFERATION DURING SECONDARY OSSIFICATION OF RAT TIBIA

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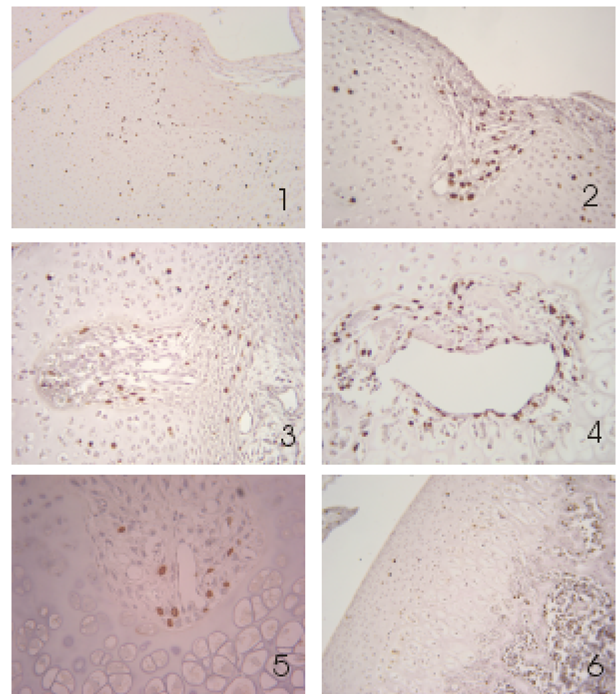
INTRODUCTION: Development of long bones takes place via formation of cartilaginous templates, which are subsequently replaced by osseous tissue in a well-defined temporal and spatial pattern. A primary centre of ossification is formed at the diaphysis whereas secondary centres develop latter at the template epiphyses. Activity of the primary ossification centre results in longitudinal growth whereas secondary ossification leads to the formation of two roughly spherical epiphyses at the ends of the bone [1,2]. Epiphyseal shape and size is critical for connection between adjacent bones to form a functional skeleton. Since cell proliferation is a key process during development, the present study was focused to study the relationship between changes in cell proliferation and the progression of the secondary ossification in rat tibiae.

METHODS: Male Sprague-Dawley rats were sacrified on days 3, 5, 7, 10, 12, 15, 18, 21, 28 and 35 after birth (n=4). All animals were injected intraperitoneally with 5-bromo-2'deoxy-uridine (BrdU) (1mg/10 g body weight) (Sigma, St Louis, MO, USA) one hour before sacrifice. Tibiae were immmediately isolated, cut through the sagittal plane, fixed by immersion in 4% paraformaldehyde, decalcified in EDTA and embedded in paraffin. Proliferating cells (S phase) were immunocyto-chemically identificated by using an anti-BrdU monoclonal antibody (Sigma).

RESULTS: In 3-day-old rats the proximal tibial epiphysis was completely cartilaginous and a number of scattered BrdU-positive chondrocytes were found especially at peripheral portions (Fig.1). There was a marked uptake of BrdU associated with the beginning of intrachondral canal formation at the 5th postanatal day (Fig.2). Canals entered the chondroepiphysis from the perichondrium and contained many BrdU-positive cells. Positive cells included both endothelial and mesenchymal type inside the canals and surrounding chondrocytes (Figs.2,3). About the 7th postanatal day, canals reached the middle of the epiphysis and a central vascularized region appeared. At this stage, BrdU-labeling was abundant in vascular and perivascular cells of the ossification centre located in proximity to the osseochondral invasion edge (Figs.4,5). From 12 days onwards the secondary growth plate is

formed and proliferating cells are mainly located in the proximal and lateral portions of the epiphyseal cartilage (Fig.6).



Immunocytochemical demonstration of S-phase cells in rat epiphyses. Fig.1, 3-day-old; Fig.2, 5-day-old; Fig.3, 7-day-old; Fig.4, 10-day-old; Fig5, 12-day-old; Fig.6,21-day-old.

DISCUSSION & CONCLUSIONS: Results obtained show that the pattern of cell proliferation varies in the diverse cell types at different stages of the development of the secondary ossification centre. Regional changes in cell proliferation are necessary to induce growth according to specific directions of cartilage canals, bone invading cells and epiphyseal cartilage. Thus, control of cell proliferation during secondary ossification could be a critical link in the sequence of tightly regulated events that lead to epiphyseal morphogenesis.

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DEVELOPMENT OF A METHOD FOR MEASUREMENT OF ANISOTROPIC AND INHOMOGENEOUS SOLUTE DIFFUSION IN COMPRESSED CARTILAGE

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INTRODUCTION: Adult articular cartilage is avascular; chondrocyte metabolism therefore relies heavily upon solute transport through the extracellular matrix. Previous studies have explored solute effective diffusivities (D_{eff}) [1] and effects of compression on transport [2] as mediators of the cell biological response to compression. We hypothesize that the inhomogeneous structure of cartilage may give rise to inhomogeneous and anisotropic transport phenomena in compressed cartilage [3]. We are therefore developing experimental and theoretical methods for evaluation of these phenomena.

METHODS: We plan two-directional (2D) diffusion measurements that are an extension of an existing technique for measuring unidirectional (1D) diffusion with a confocal microscope [2]. The dimensions of the cartilage sample are 3mm x 1mm x 2mm (Fig. 1a). The cartilage sample is equilibrated overnight in a solution of fluorescent molecules. Once compressed, the bottom face of the sample is set flush against a microscope slide and the assembly put into a bath container on a confocal microscope stage. A flow of PBS on the free edges and through the porous inserts starts the diffusion process and is designed to make the solute concentration at these boundaries zero (Fig. 1b).

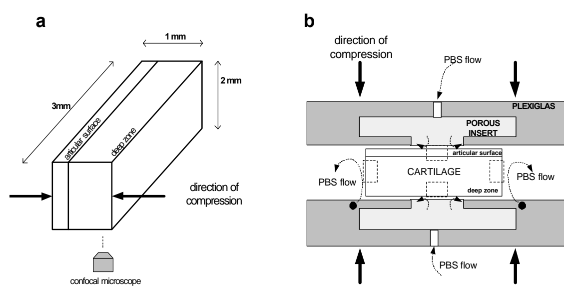

Fig. 1. (a) Orientation of sample. (b) Schematic of 2D diffusion apparatus. Dashed boxes indicate where images will be recorded.

Optimising the length of the face of the porous insert to ensure that the measured image of fluorescent solute concentration could be analysed as a locally 1D diffusion process was an important design issue. Simulations of this 2D experiment were done in Mathematica using the Crank-Nicolson differencing scheme to solve the partial differential equation of diffusion. Each iteration was solved by direct matrix inversion methods. For initial simulations unit size was 33.3µm, time step was 0.5s and D_{eff} was 50µm^2/s in both directions. The model can be adapted so that D_{eff} can vary with position and direction.

RESULTS: Comparisons of the theoretical 1D concentration profile with the simulated 2D concentration profiles for 200µm wide image windows at the articular surface and at the free edge of the sample were done (Fig. 2). With the face of the porous insert being 1000µm long, results indicated that each of the imaging locations should represent locally 1D diffusion processes. Agreement between the 1D diffusion theory and the 2D simulations indicates that measurements can reliably be made for elapsed times of less than 600 s.

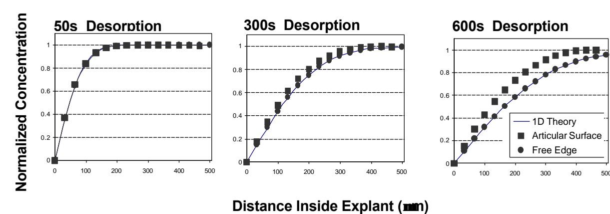

Fig. 2. Concentration profiles for theoretical 1D diffusion and simulated 2D diffusion with a 1000µm wide porous insert.

Images from preliminary 2D diffusion experiments have been acquired (Fig. 3). The sample (2mm x 750µm x 2mm) was equilibrated in a solution of 1µM tetramethylrhodamine (TMR) and then compressed in the 2D apparatus to 2mm x 650µm x 2mm. The intensity of the fluorescence corresponds to the concentration of solute in the cartilage and decreases with time after initiating flow of PBS.

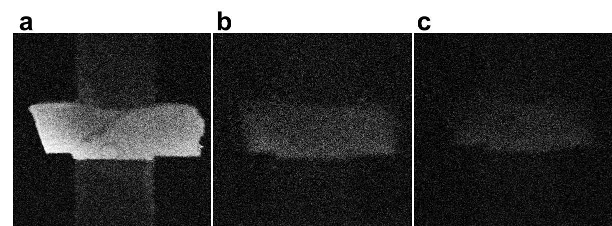

Fig. 3. Confocal images of TMR in a cartilage sample at (a) 0s (b) 60s and (c) 480s into the diffusion process.

DISCUSSION & CONCLUSIONS: The simulations of the 2D diffusion experiment suggest that the confocal images obtained can be analysed as separate 1D diffusion processes. Measuring D_{eff} in the directions parallel and perpendicular to the articular surface will allow us to look for anisotropies in diffusion. Measuring D_{eff} at both the articular surface and at the deep zone will allow us to look for inhomogeneities in diffusion. Controlling the degree of sample compression will allow us to measure the effects of compression on diffusion processes.

Analysis of preliminary images suggests that diffusion occurs in all four locations (along the free edges and against the porous inserts). To confirm that the diffusion information acquired with this 2D method is independent of the location of the image, experiments will be done with isotropic, homogenous gels.

The feasibility of using fluorescently labelled globular proteins (bovine serum albumin (66kD), ovalbumin (45kD) and parvalbumin (12.3kD)) as solutes in these diffusion experiments is being tested. Using approximately spherical solutes will allow us to build a model of 1D and 2D diffusion in cartilage using current macroscopic transport theories.

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PHENOTYPIC MODULATION OF HUMAN ARTICULAR CHONDROCYTES BY BISTRATENE A

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INTRODUCTION: Articular chondrocytes undergo phenotypic alterations following extended periods in monolayer culture, i.e. they become "fibroblast-like" cells, proliferate, and synthesise type I as opposed to type II collagen. This process has been termed chondrocyte dedifferentiation. Such expansion of monolayered chondrocyte cultures is utilized by clinicians for autologous transplantation procedures in the repair of damaged cartilage[1]. However, for such a tissue repair strategy to work, it is necessary for dedifferentiated chondrocytes to "redifferentiate" and again adopt a mature chondrocyte phenotype. Bistratene A is a macrolide polyether that induces morphological changes and differentiation of a number of cell types (including cell rounding of fibroblasts and maturation of melanocytes) through activation of the delta isoform of protein kinase C (PKCδ). Here, we report the response of dedifferentiated human articular chondrocytes to treatment with bistratene A.

METHODS: Human articular chondrocytes were obtained from macroscopically normal cartilage by collagenase digestion and placed into monolayer culture (for 2-4 passages) to promote their dedifferentiation. Cultures were then treated with 100ng/ml bistratene A or with carrier alone and chondrocyte morphology, cell viability and proliferation assessed over a 5-day time course. Parallel cultures were treated with 100ng/ml bistratene A in combination with the PKCδ-specific inhibitor, rottlerin. Activation of PKCδ is associated with translocation of the enzyme to the nuclear membrane[2]. Immunolocalisation for PKCδ at early time points post-treatment, and for collagen (types I and II) in harvested cells at experimental end-points, were performed. The presence of F-actin stress fibres was determined using FITC-labelled phalloidin.

RESULTS: Dedifferentiated human articular chondrocytes became rounded and underwent cell growth arrest after treatment with bistratene A. Bistratene A-treated chondrocytes also became more immunopositive for type II collagen, but less immunopositive for type I collagen (Figure 1). These phenotypic changes were associated with a prior and extensive disruption of actin microfilaments and translocation of PKCδ to the nuclear membrane. Concurrent treatments of chondrocytes with rottlerin partially blocked the morphological effects of bistratene A.

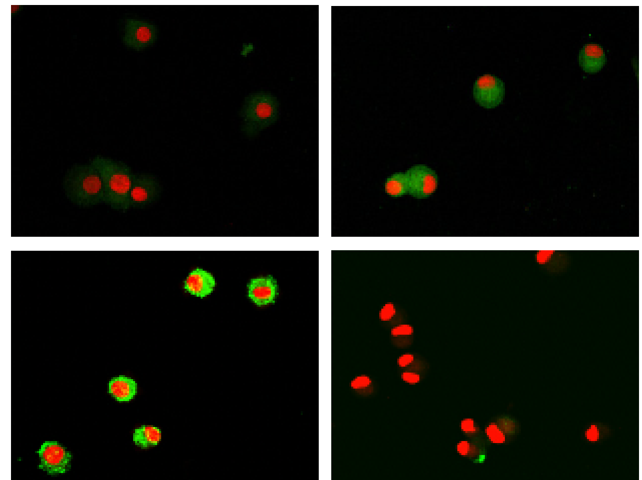

Fig. 1: Human articular chondrocytes become more immunopositive for collagen type II, and less immunopositive for collagen type I, following treatment with bistratene A. (Top panels=collagen II, bottom panels=collagen I: left panels =control cells, right panels=bistratene A-treated cells).

DISCUSSION & CONCLUSIONS: Signalling mechanisms that regulate articular chondrocyte phenotype are likely to be of great importance to tissue engineering strategies for cartilage repair, but are largely unknown. The bistratene A-induced alterations in chondrocyte behaviour reported here may provide a new model system to study an as yet unresolved pathway, potentially involving cytoskeletal and signalling components, that regulates chondrocyte differentiation in vitro.

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THE USE OF BIODEGRADABLE POLYURETHANE SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING: A PRELIMINARY STUDY

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INTRODUCTION: To improve treatment options for articular cartilage injuries, current research has increasingly focused on the in vitro generation of cell-polymer constructs for implantation. Biomaterial scaffolds should provide a three-dimensional structure, support growth and activity of the cells and allow for the maintenance of their differentiated phenotype. Various biocompatible materials have been tested for their potential as suitable matrices for generating cartilaginous tissue from seeded cells. The use of synthetic resorbable polymers offers interesting possibilities, because their biochemical and biomechanical properties can be varied and they can be manufactured with a high degree of reproducibility. Stable polyurethanes have widely been used in implantable devices for various biomedical applications. Since recently, experimental biodegradable polyurethanes have gained an increasing interest. The aim of the present study was to evaluate the capability of novel biodegradable polyurethane scaffolds to support attachment, growth and phenotype of chondrocytes in vitro.

METHODS: Three-dimensional polyurethane scaffolds with an average pore size of 150 µm and a pore-to-volume ratio of 85 % were cut into discs of 8 mm in diameter and 4 mm in thickness. After sterilization with ethylene oxide, discs were seeded with bovine articular chondrocytes by active cell seeding in spinner flasks. 8×10^6 cells were seeded per scaffold, and cell-polymer constructs were cultured in tissue culture plates for up to 6 weeks. After 2, 14, 28, and 42 days, they were analysed biochemically, biomechanically and histologically. Cell viability was assessed by fluorescence staining. DNA contents of the constructs were measured, and amounts of glycosaminoglycans and total collagen were determined in constructs and in the culture media. Collagen type I and type II were specifically identified by western blot analysis. Gene expression of aggrecan, procollagen type I and type II was quantified by real-time RT-PCR using TaqMan PCR analysis. Compressive stiffness of the constructs was assessed by stress-strain behaviour. For histological evaluation constructs were embedded in methyl methacrylate and sections were stained with toluidine blue.

RESULTS: Fluorescence staining showed a high degree of viability and a uniform distribution of the cells throughout the scaffold two days after seeding. After an initial decrease, the DNA content remained nearly stationary during the culture period. Amounts of glycosaminoglycans and total collagen steadily increased with time in culture, indicating the formation of a new extracellular matrix within the construct. However, up to 80% of the totally synthesized matrix molecules were found in the culture medium. On the transcriptional level, a decrease in aggrecan and procollagen II mRNA expression was noticeable, whereas procollagen I expression was increased, indicating dedifferentiation of the seeded chondrocytes. Western blot analysis also demonstrated that, in addition to the cartilage specific type II collagen, type I collagen was present in the constructs and in the culture media. Toluidine blue staining confirmed the homogeneous distribution of the cells in the scaffolds two days after seeding and the formation of a rich proteoglycan containing extracellular matrix by day 42 of culture. An increase in compressive stiffness was also observed, although the compressive modulus was still ten times lower than that of native articular cartilage.

DISCUSSION & CONCLUSIONS: These preliminary experiments demonstrate that polyurethane based biodegradable scaffolds are able to support attachment and extracellular matrix production of bovine articular chondrocytes. The limitations of the system are the diffusion of large amounts of matrix molecules into the culture medium and the accumulation of type I collagen. Possible strategies to improve and maintain the chondrocytic phenotype of the cells include the modification of the polymer scaffold (pore size, active groups) and the modulation of the chondrocytic activity by specific growth factors. In addition, due to the favourable mechanical properties of the polyurethane matrix, stimulation of chondrocytes by mechanical loading can be considered in order to improve the formation of a functional cartilage-like extracellular matrix.

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CHANGES IN CELL MORPHOLOGY AND CYTOSKELETAL ORGANISATION IN OSTEOARTHRIC CARTILAGE

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INTRODUCTION: The cytoskeleton plays a fundamental role in the control of numerous cell processes including cell division, control of cell morphology, phenotypic modulation, transport and signalling. Within articular cartilage the cytoskeleton, and in particularly the vimentin intermediate filament network, is believed to be involved in chondrocyte mechanotransduction and the pathophysiology of osteoarthritis. This study utilises confocal microscopy to visualise vimentin intermediate filaments within intact human articular cartilage and to investigate changes in cytoskeletal organisation and cell morphology associated with osteoarthritis.

METHODS: Full depth cartilage explants were excised with subchondral bone from various locations of the femoral heads of 20 age-matched patients in two groups. The first group underwent total hip replacement for osteoarthritis; the second group underwent hemiarthroplasty for femoral neck fracture but had no macroscopic osteoarthritic features. Cartilage explants were fixed, sectioned and enzymatically predigested to enable antibody penetration. Cells were permeabilised and labelled with anti-vimentin (clone 9) primary antibody and FITC-conjugated secondary antibody (OA n=6, control n=5). Samples were visualised using a confocal microscope (Ultra View, Perkin Elmer).

RESULTS: Light microscopy confirmed the appearance of pannus over the surface of some specimens of osteoarthric cartilage. Pannus was not observed in the normal, non osteoarthric group. Vimentin staining revealed the characteristic intracellular fibrous network of intermediate filaments as previously reported [1,2] (Fig 1). The presence of cells with 'abnormal' elongated cell processes was characterised at different depths below the articular surface (Fig 2). Less than 10% of cells within normal, non-osteoarthritic cartilage showed elongated processes and these were predominantly located in the surface and middle zone cartilage from the superior parafovial site. This anatomical site often showed macroscopic signs of fibrillation even in otherwise normal joints.

At all depths, osteoarthritic cartilage contained a significantly greater percentages of cells with elongated process compared to normal cartilage. The differences were greater towards the articular surface with osteoarthritic pannus containing the highest percentage of cells with process (Fig 2).

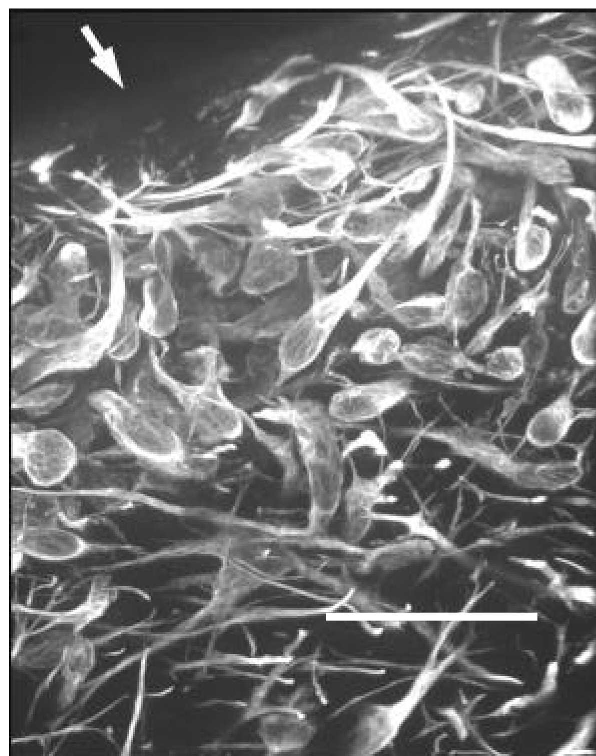

Fig. 1: Confocal image of vimentin intermediate filaments in the chondrocytes within the surface zone of human OA cartilage. Arrow indicates the articular surface. Scale bar = 50µm

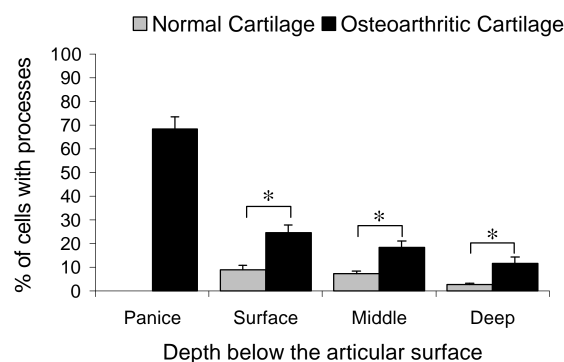

Fig. 2:Prevalence of cells with elongated cell processes in normal and OA cartilage. Values

represent means ± SEM. Statistically significant differences have been indicated ($p<0.05$)*

DISCUSSION & CONCLUSIONS: OA was found to be associated with profound changes in chondrocyte morphology, characterised by the appearance of elongated vimentin-rich cell processes. These changes were most evident in the surface zone and, where present, the pannus layer. The appearance of cells with elongated processes may be associated with early degradation of the extracellular matrix and a change in the cellular biomechanical environment. Indeed previous studies have shown that isolated chondrocytes also exhibit similar elongated processes when cultured in very low modulus agarose gels [3] or exposed to tensile strain [4].

The influence of these changes on chondrocyte behaviour is not yet known. However it is likely that the changes in morphology and associated cytoskeletal organisation will considerably alter the cellular mechanotransduction pathways which are essential for the health and homeostasis of the tissue. This study represents an important step in understanding the development of osteoarthritis.

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OSTEOARTHRITE-ACTIVATED CHONDROCYTES PR0DUCE SUPER PATTERN OF MATRIX METALLOPROTEINASES IN OSTEOARTHRITE EQUINE CARTILAGE

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INTRODUCTION: Trigger of a cascade of molecular events after the development of the initial osteoarthritic (OA) steps in joint cartilage involves a large number of enzymes, including the family of matrix metalloproteinases (MMPs), important in remodelling of its structure [l] The aim is to study the effects of OA diseases on the expression of matrix metalloproteinases in both equine cartilage extracts and chondrocyte cultivated cells derived from normal (N) and OA tissues.

METHODS: Horses with particular OA diseases (17 joints with the chips and traumatic OA) were used. The cell cultures from cartilage chondrocytes were obtained by enzymatic digestion of the pieces of articular cartilage and were used as the primary cultures. The standard cell cultures were washed exhaustively, i) homogenates were prepared using Laemmli sample buffer or ii) cultures were exposed for 3.5 hrs with serum-free Häms F-12 media to produce N/OA chondrocyte secretion patterns of MMPs. The pieces of N or OA cartilage were washed and homogeniesed with Laemmli sample buffer. The suspension supernatants (10 000 g, 3x3 min) containing 2-3 µg of a general protein were used per well. A high sensitive modification of reverse zymography [2] was developed in order to detect MMP activity in extracts.

RESULTS: With these procedures, it has been shown that the extracts from normal adult equine cartilage and those of N chondrocyte cultivated cells of the Po–P2 generations contain only MMP-64 kDa (K), (MMP-2,[3]). MMP-2, and 2 additional MMPs, namely MMP-53K & MMP-46K, characterise MMP pattern of N junior equine cartilage ("juvenile motiv"). It has been also observed that MMP secretion patterns of N chondrocyte cultivated cells contain usually MMP-2 and MMP-94 K (MMP-9, [3]). Surprisingly, both OA equine cartilage extracts and those of OA cultivated chondrocytes of Po generations express, principally, two distinct MMP patterns: *TYPE I*: MMP-2, MMP-53 K, MMP-46 K (2 or 3 of the MMPs together, with "juvenile motiv" , *Fig.1*); *TYPE II* : MMP-2, MMP-53 K, MMP-46 K, MMP-85 K, MMP-9 etc (11 – 14 of the MMPs together). Their Po secretion patterns of MMPs reflect also *TYPE I* or *TYPE II*, with small modifications. OA synovial fluids have demonstrated approximately *TYPE II* patterns, but relative concentrations of the MMP-9 were often 1.5 – 2.5 times higher compared to the normal ones.

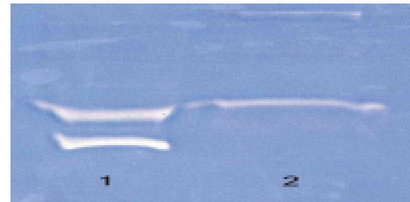

Fig. 1: MMP pattern of OA equine cartilage extract, chip with "juvenile motiv"plus MMP-2 (left, 1); normal chondrocyte secretion MMP pattern with MMP-2 plus MMP-9 (right, 2).

DISCUSSION & CONCLUSIONS: The investigation showed the existence of constant MMP patterns in both normal cartilage extracts derived from different joints and normal cultivated chondrocytes, but also the existence of different MMP patterns in both the OA cartilage extracts and the OA chondrocyte cultivated cells. The MMP fractions observed are the enzymes with gelatinolytic activities which catalyse the hydrolysis of gelatin Type A, and it was possible to inhibit their activity with EDTA or to reactivate it by followed incubation of gels with the Ca-containing media. MMP-2 was observed in both N chondrocyte cultivated cell extracts and N joint cartilage ones, but the secretion pattern of N cultivated chondrocytes contain MMP-2 and MMP-9. We concluded that proenzyme forms of MMP-9 cannot be activated in cytosol extracts, it is important for the activation to cleave signal peptydes by the transport process through the membranes. TYPE I OA MMP pattern includes MMP-2 and the "juvenile motiv", these MMP fractions characterise mainly the chips. Traumatic OA MMP patterns (TYPE II OA MMP pattern) are more complicated, the number of enzyme fractions increases up to 11-14 in both OA cartilage extracts and in OA chondrocyte cultivated cells. The data obtained permit to conclude that TYPE I OA MMP pattern is the obligate phenotype of osteoarthrite diseases and also activities of the MMP-85 K, MMP-9 and 7-9 additional minor MMP fractions contribute to OA diseases.

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PERCOLATING HYDROGELS FOR TISSUE ENGINEERING

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INTRODUCTION: Chitosan is a linear copolymer of linked $\beta(1\rightarrow 4)$ glucosamine and N-acetyl glucosamine. Although present in biomass, it is produced from chitin, a glycosaminoglycan, extensively widespread on earth. Indeed, the latter constitutes the structure polymer of the cuticles of all arthropods and endoskeletons of all cephalopods, etc. It is noteworthy to observe that both chitin and chitosan are completely absent in mammals.

Numerous results reported in the literature reveal that chitosan exhibits very interesting biological properties, which can be summarized by the fact that this polysaccharide is a bioactive biopolymer useful for tissue regeneration[1]. In the field of cartilage some results on chitosan films have demonstrated its capability to favor the chondrocyte proliferation, but with an elicitation of the production of both collagens 1 and 2[2].

Our work consists in the introduction of a new concept of biomaterials for tissue engineering, based on the processing of chitosan physical hydrogels with a well defined and appropriate chemical structure and morphology. These materials constitute "decoys" for biological media. Our work also consists to show that this concept completely disagrees with the old theory of the "scaffold". Thus, the formation of an appropriate interface between the surface of the material and the living media is sufficient to allow the induction of a tidy neo-tissue.

METHODS: All the materials were produced from a unique batch of chitosan obtained from squid pens, kindly provided by France Chitine. Its degree of acetylation (DA) was 5.2% and the weight-average molecular weight close to 420.000 g/mol. In order to study the role of DA, the sample was reacetylated as described previously[3]. Then, we disposed of a series of polymers of same molecular dimensions but with DA's varying within 5.2-60%.

Gelation of chitosan was made from solutions in an hydroalcoholic media subjected to an evaporation at 40°C up to observe the gelation. The gels were then transferred into an alkaline solution to convert the ammonium groups of glucosamine residues into free amines. After a thorough washing in distilled water, a true physical hydrogel only constituted of a hydrated polymer network at less than 5% was obtained. This gel was then sterilized by wet heating at 120°C for 20 min. The morphology of the gels was studied both by atomic force- and scanning electron-microscopies.

These gels were then subjected to a cell culture media and thus were ready for experiments of chondrocyte cultivation (see M. Corvol et Al.).

RESULTS AND DISCUSSION: Gelation of chitosan solutions is observed when two conditions are verified. 1- the polymer concentration must be initially over the critical concentration of chain entanglement C*; 2- a critical value of the balance between attractive and repulsive interactions must be achieved. This situation occurs when a percolating condition of gelation takes place in the media. Two examples leading to this situation have been produced in our laboratory. The role of various parameters related to the chemical structure, especially the DA of the polymer and/or to its environment were studied.

The gel morphology has been observed both by atomic force- and scanning electron- microscopies. We show that as in the case of most living tissues, chitosan physical gels are three-dimensional networks quite different from sponge-like systems. The dimension of the pores whether on the surface or in the bulk of the material preclude any penetration by living cells.

These gels can be easily sterilized by wet drying at 120°C. This treatment, contrary to γ irradiation, has no significant influence on the properties of the material, especially as concerns the preservation of the chemical structure, molecular weight and

morphology of the gel.

In order to favour the interaction between this gel and living media, in particular with the surrounding material of chondrocytes, we also prepared microgels thus increasing the surface potentially in contact with this material. Chitosan microgels were then prepared by crushing of macrogels before to be subjected to chondrocyte cell cultures (See M. Corvol et Al.). This kind of material bears various characteristics allowing a behaviour of decoy of living media by its structure and morphology.

CONCLUSION: It is possible to process micro-hydrogels of chitosan which surface in contact with living cells constitutes a decoy on which an appropriate bioinductive interface can be generated. This interface, by the nature of both the structures and the kind of interaction involved in its formation generates favourable responses from the cells leading to a continuous remodelling of the interface allowing then the production and deposition of an extracellular matrix and/or cell proliferation.

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ENHANCED EXPRESSION OF THE HUMAN CHITINASE 3-LIKE 2 GENE (CHI3L2, YKL-39) IN OSTEOARTHRITIS.

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INTRODUCTION: Although there has been significant effort to identify biochemical markers to diagnose and monitor OA progression, a clearly validated marker is still unknown. Analysis of differential gene expression between non-diseased and osteoarthritic cartilage is one approach to detect genes that may be novel diagnostic or prognostic tools. The aim of this study was to compare the gene expression profile of human osteoarthritic versus normal cartilage using a method that allows the identification of differentially expressed genes.

METHODS: The gene expression profile from normal and osteoarthritic cartilage biopsies was compared by cDNA representational difference analysis (RDA) [1] and cDNA array hybridisation. Results were verified in the original samples and additional osteoarthritic and normal cartilage biopsies by quantitative real-time PCR using the housekeeping gene GAPDH as the reference control.

RESULTS: Among three fragments selectively amplified in OA cartilage one belonged to the oesophageal cancer related gene 4 (ECRG4) that was not described to be expressed in cartilage before. It encodes a 148 amino acid protein harbouring a transmembrane motive at the N-terminus. ECRG4 was expressed in all cartilage samples analysed but an enhanced expression in OA could not be verified in a larger collective. Two fragments belonged to YKL-39, the chitinase 3-like 2 (CHI3L2) gene, which was significantly upregulated in OA according to cDNA array analysis and real-time PCR (19-fold, p=0.009). We included YKL-40, also known as chitinase-3 like 1 (CHI3L1) or human cartilage glycoprotein (gp-39), in our analysis since YKL-40 is closely related to YKL-39 and was described to be enriched in serum and synovial fluid of patients with OA or rheumatoid arthritis [2,3]. However, no significant upregulation of gene expression in OA cartilage was evident for YKL-40.

DISCUSSION & CONCLUSIONS: Overexpression of YKL-39 mRNA but not of YKL-40 characterizes cartilage degeneration in OA while YKL-40 peptide detected in serum and synovial fluid may be produced by the synovium.

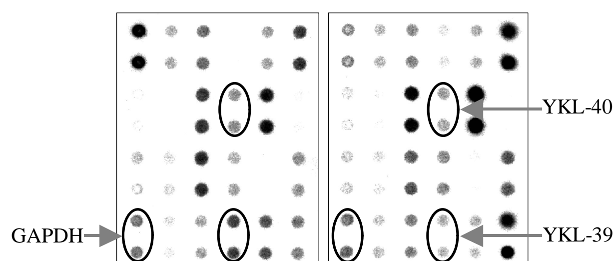

Fig. 1: Representative hybridization results of cDNA probes generated from OA (left) and normal (right) cartilage samples on two identical cDNA-arrays.

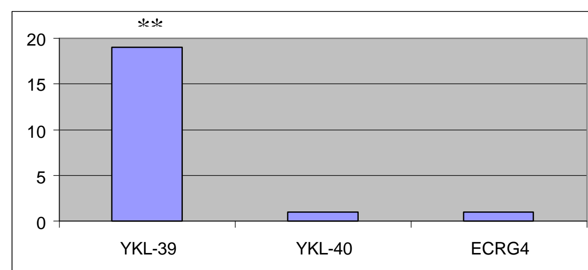

Fig. 2: x-fold increase of YKL-39, YKL-40 and ECRG4 in OA versus normal cartilage. Only for YKL-39 a significant difference with p=0.009 was obtained. The data are based on the mean values of 6 OA and 6 normal samples by light cycler analysis.

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HISTAMINE STIMULATES PROLIFERATION Of HUMAN CHONDROCYTES IN VITRO

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Introduction.

Increased levels of histamine have been reported in synovial fluids of osteoarthritic joints [1] and both H_1 and H_2 histamine receptors have been demonstrated on human articular chondrocytes (HAC) [2]. In addition to degeneration and loss of articular cartilage, abnormal clusters of chondrocytes are a common observation in OA cartilage. This study has examined whether histamine may play a role in the abnormal proliferation of HAC in vitro.

Methods.

Isolated HAC in culture (n = 5) were treated with either culture medium alone or with histamine (5 - 100µM), then incubated at 37°C. The medium was replaced every second day and after 6 days cell numbers were assayed using the napthol Blue-black method [3]. To determine whether any response was H_1 or H_2 receptor-mediated, experiments incorporated the specific H_1 and H_2 receptor antagonists, mepyramine and ranitidine respectively, along with the histamine treatment. In addition alphafluoromethyl histidine (αFMH, an inhibitor of histamine synthesis) was also used into some experiments to determine whether HAC proliferation is under autocrine control by histamine.

Results.

A modest but significant stimulation (15-40% above control) of proliferation was found when HAC were treated with histamine (Figure 1). This stimulation reached a maximum at 20µM histamine and was inhibited by the H_1 histamine-receptor antagonist, mepyramine, but not by the H_2 histamine-receptor antagonist, ranitidine. Addition of αFMH reduced the proliferation of HAC to below that of control cells, suggesting that HAC may synthesize histamine, which could contribute to the proliferative activity of these cells.

Discussion.

Exposure of HAC to histamine in vitro produced a significant stimulation of cell proliferation, probably mediated via the H_1-histamine receptor. Preliminary data suggest that HAC may also produce their own histamine. These findings, together with our earlier published data which demonstrates that histamine stimulates MMP-13 and MMP-3 production by HAC [4], suggest that histamine:chondrocyte interactions may contribute to the degenerative changes associated with OA cartilage.

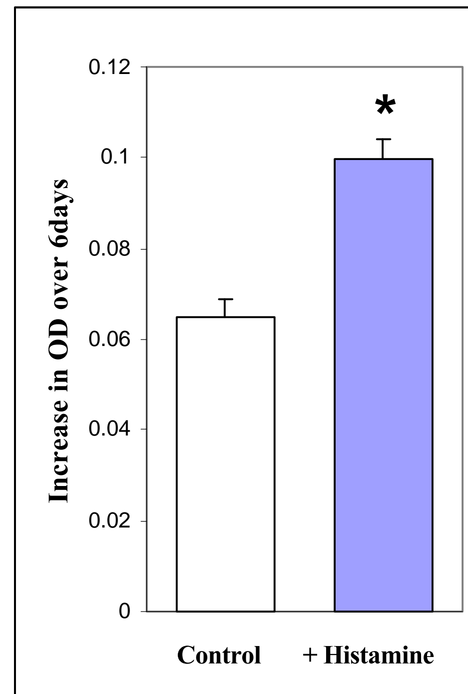

Figure 1. *Effect of histamine on proliferation of HAC in vitro (t-test *p= 0.000012, n=5)*

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CYTOKINE AND MATRIX METALLOPROTEINASE EXPRESSION BY SUBCHONDRAL MULTINUCLEATED CELLS OF THE RHEUMATOID LESION.

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Introduction.

Osteoclasts and chondroclasts have been implicated in bone and cartilage degradation of subchondral sites of the rheumatoid lesion [1]. Specific cytokines and proteinases are reportedly associated with osteoclast differentiation and function. This study has examined the expression of three proinflammatory cytokines and four matrix metalloproteinases (MMPs) by multinucleated cells in situ using immunohistochemistry.

Methods.

Cartilage/pannus junctions with underlying bone were sampled form rheumatoid knee specimens obtained from joint replacement surgery. Tissues were fixed in Carnoy's, processed to paraffin wax and tissue sections cut at 5µm. Twenty three specimens containing multinucleated cells were used for an immunohistochemical (IHC) study of cytokine (IL-1β, TNFα and IL-15) and MMP (MMP-1, -8, -9, -13) distributions. Sheep polyclonal antibodies to MMP-1, -9 –13, a monoclonal antibody to MMP-8 and goat polyclonal antibodies to the cytokines were used with alkaline phosphatase-conjugated avidin-biotin complex for IHC [2]. Osteoclastic cells were also identified by acid phosphatase (AP) staining as described [1]. Photomicrographs were taken using a Zeiss Photomicroscope III and Ektachrome 160T film.

Results.

A proportion of multinucleated cells were positively stained for interleukin-1β (IL-1β), interleukin-15 (IL-15) and tumour necrosis factor-α (TNFα), this being more prominent than that for neighbouring cells. AP-stained osteoclasts were also shown to contain MMP-1, MMP-8 , MMP-13 and MMP-9, but this was variable with some multinucleates appearing negative for these MMPs. Thus evidence is presented to show that multinucleated osteoclasts have the ability to produce these seven proteins in situ, but unlike AP staining which appeared to be a consistent feature, the cytokine and MMP expression was variable within the multinucleated cells of the same specimen.

Discussion.

The resorption cycle of osteoclasts is considered a multistep process. The variable expression of cytokines and MMPs may relate to stages of osteoclast differentiation/maturation, or to locally induced phenotypic changes in function [3]. The intracellular staining of TNFα, a potent factor for the induction of differentiation and bone resorption by osteoclasts [4], suggests an autocrine function. However, as yet it remains uncertain whether any of the visualised proteins have an extracellular function.

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