Advances in Cartilage Tissue Engineering *in vitro*

Erik Mandl

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The production of this thesis was supported by:

Nederlandse Orthopaedische Vereniging (NOV), Dutch Arthritis Association (Reumafonds), Stichting dr. A.A. van Puyvelde Fonds, Ortomed BV, Isotis OrthoBiologics, Stryker Nederland BV, Endo Plus Nederland, Smith & Nephew, DePuy / Johnson&Johnson, Mathys Orthopaedics, Bauerfeind, Somas Chirurgische Techniek, Penders Orthopedische Schoentechniek BV, Van Wijk Orthopedische Schoentechniek BV, Harlan Nederland BV, Jongenengel Orthopedisch Centrum.

ISBN: 90-8559-005-1

Printed by Optima Grafische Communicatie, Rotterdam

Advances in Cartilage Tissue Engineering *in vitro*

Vorderingen in het genereren van kraakbeen *in vitro*

Proefschrift
ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 1 december 2004 om 11.45 uur

door

Erik William Mandl
geboren te Groningen

Promotiecommissie

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Tissue Engineering 2002 Aug; 8(4):573-80

I

General Introduction

Cartilage structure and contents

Within the body three subtypes of cartilage can be distinguished: hyaline cartilage, elastic cartilage and fibrocartilage. Hyaline cartilage is the predominant subtype and is mainly located in articular joints and in less extent in the nasal septum and cricoid. Elastic cartilage can be found in the outer ear and in parts of the respiratory tract. The menisci and intervertebral discs are made from the third subtype: fibrocartilage.

Hyaline cartilage in joints, further refered to as articular cartilage, is a highly specialized tissue that ensures low-friction movement of articulating bones, whilst transmitting load. The only cell-type present in articular cartilage is the chondrocyte, which only composes less than 5% of the total tissue volume. Water (up to 80% wet weight) is the predominant molecule in cartilage, followed by different collagens (10-20% wet weight) and the group of GAG (GlycosAminoGlycan, 4-7% wet weight) [181]. From the latter two groups, collagen type II and aggrecan are the most prominent respectively. A simplified reflection of the cartilage structure and function would be as follows. The collagen molecules form a tightly woven network in which the GAG are contained (Figure I-1 next page). In solution, the GAG is negatively charged and creates an osmotic pressure via attraction of positive counter-ions. This osmotic pressure, together with repulsive forces of the negatively charged GAG, result in a swelling pressure within the matrix that accounts for the excellent capabilities of cartilage to absorb and transmit forces during joint movement and loading. Several smaller molecules also play a crucial role in the extracellular matrix of articular cartilage: decorin, anchorin, collagen type VI, IX and XI. Articular cartilage lacks blood vessels; chondrocytes receive oxygen and nutrients through diffusion from the synovium and the underlying bone.

Articular cartilage can be divided in four zones with respect to the architecture [68, 181, 213] (Figure I-2, next page). The superficial zone comprises approximately 10% of the cartilage and is characterized by a high cell density, flattened chondrocytes and thin collagen fibers that are oriented tangential to the articular surface. The middle zone or intermediate zone has randomly orientated collagen fibers and chondrocytes with a more spherical configuration.

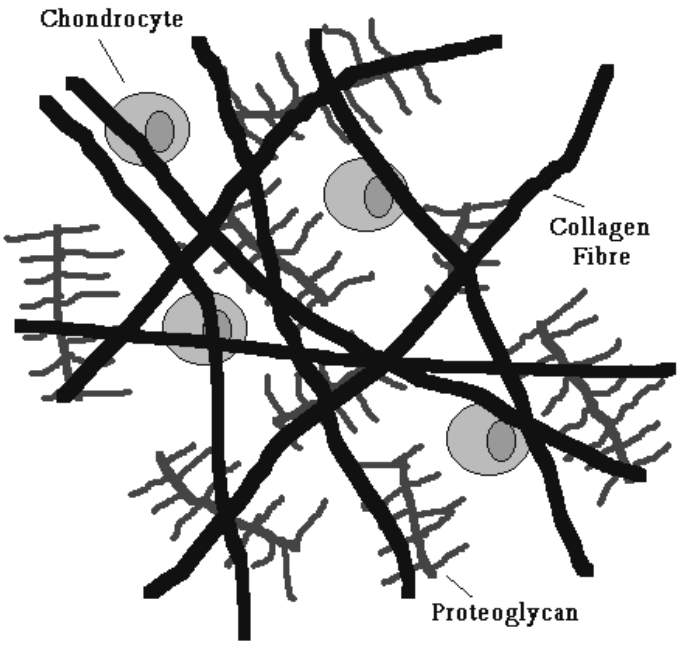

Figure I-1 A simplified overview of the articular cartilage matrix. Collagen fibres form a network in which the glycosaminoglycan are contained.

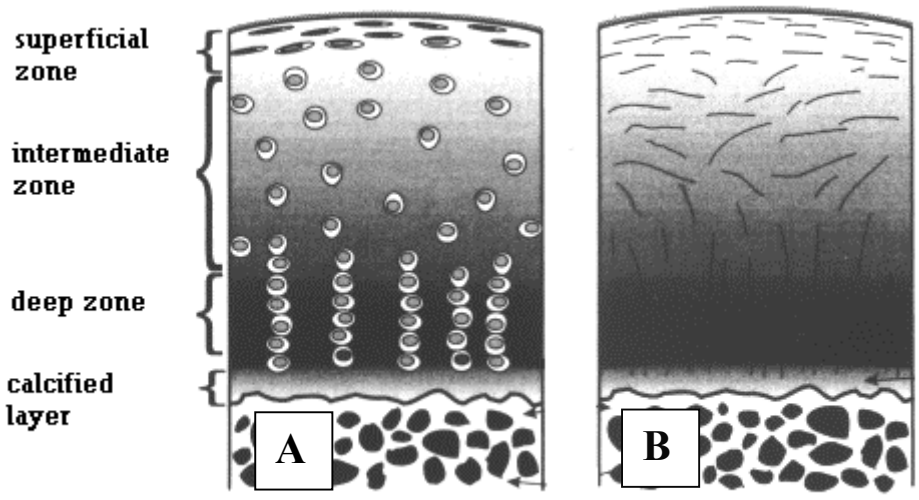

Figure I-2 Architecture of articular cartilage. Four zones can be distinguished with respect to cellular (A) and collagen fibre (B) orientation.

Furthermore, relatively more GAG is present than in the superficial zone. The deep zone has collagen fibers that are orientated perpendicular to the articular surface. The cell density is similar to the middle zone. However, the chondrocytes are orientated in a columnar fashion. The last zone is the calcified layer, rich in calcium salts and with a relatively low number of chondrocytes. The calcified layer connects the visco-elastic cartilage to the underlying bone.
Ear cartilage is of the elastic type and can be found in the outer ear, the inner ear and parts of the respiratory system where it has a supportive function. Similar to articular cartilage, it is of mesenchymal origin [137]. A major difference between articular cartilage and ear cartilage is that the latter contains an extracellular network of elastin, a long macromolecule that enhances the tensile properties of the tissue. Also, ear cartilage is structurally different. Surrounded by perichondrium, the ear cartilage has a more symmetrical architecture from top to bottom (Figure I-3, below). The chondrocytes are larger, contain more lipid vesicles and occasionally have two nuclei.
Fibrocartilage is an intermediate between dense regular connective tissue and hyaline cartilage. It is characterized by the presence of both collagen type I and II and can be found in the meniscus, intervertebral disc and pubic symphysis.

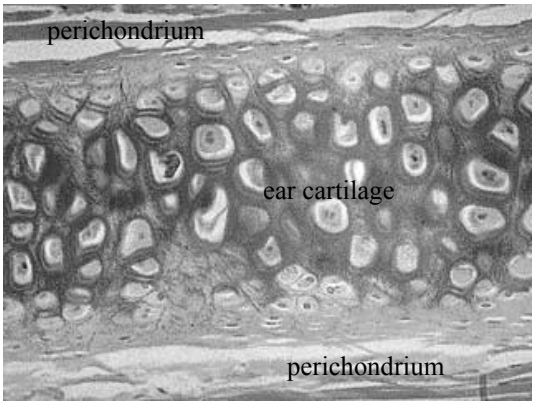

Figure I-3 Elastic cartilage (rabbit ear). Ear cartilage is covered by perichondrium on both sides and has a more symmetrical architecture from 'top to bottom' compared to articular cartilage.

Traumatic articular cartilage defects

Unfortunately, there are no reliable estimates of the prevalence of traumatic articular cartilage defects. However, it is clear that most cartilage defects arise during sport-related accidents. Around 16-20% of patients with a traumatic hemarthros undergoing arthroscopy have a cartilage defect [52, 146]. Furthermore, Hjelle et al. found some sort of cartilage defect in 60% of 1000 consecutive arthroscopies [58].

In case of a traumatic defect, articular cartilage itself has little capacity to repair or regenerate, as it lacks the prerequisite for classical wound healing: blood supply [18, 67-69]. Traumatic cartilage defects occur in two forms: chondral defects and osteochondral defects. Chondral defects are limited to the cartilage layer itself and occur either as partial or full thickness defects, which are both characterized by the absence of spontaneous repair. In osteochondral defects, full-thickness cartilage defects with a concurrent defect in the underlying subchondral bone, some sort of repair exists as blood and mesenchymal stem cells from the bone marrow cavity can enter the defect. Although this natural repair process is not well defined in humans, Shapiro et al. described this process in rabbits [67]. Briefly, from the blood a fibrin clot was formed in the cartilage defects. In subsequent days, mesenchymal cells began to invade the fibrin clot and differentiated into chondrocytes, which formed a proteoglycan-rich matrix. However, the repair tissue started to degenerate after 48 weeks. Also, the temporal sequence and the quality of the repair tissue have been shown to depend on the size of the initial cartilage defect [104]. Evenmore, Jackson et al. found no repair in 6 mm cartilage defects in a goat model [72]. Instead, the surrounding cartilage and underlying bone underwent progressive changes, leading to both bone and cartilage collapse. This, among other indirect evidence [18, 118, 132, 190], suggests that cartilage defects may lead to general symptomatic degeneration of a joint (i.e. secondary osteoarthrosis), when left untreated.

Current surgical treatments

The most widely accepted choice of initial surgical treatment of a cartilage defect is subchondral plate perforation, which is based on the natural repair observed in osteochondral defects. Pridie first described this concept in 1959 [71, 170]. Since, several other subchondral plate perforation techniques, i.e. microfracturing and abrasion arthroplasty, have been introduced [76, 184]. Clinical studies reported good symptomatic relief and improvement of joint function with these techniques. However, similar to the natural repair tissue observed in osteochondral defects, the resulting tissue represents fibrocartilage at best and with time the results deteriorate [68]. An alternative to subchondral plate perforation is osteochondral transplantation. This technique composes of transplantation of either allograft or autograft osteochondral plugs into the defect [51]. The autograft plugs are harvested from the less-load bearing surfaces of the joint. Short-term results are good to very good, like other current surgical treatments for cartilage defects [68].
Total knee arthroplasty or unicompartimental knee arthroplasty are successful treatment options for generalized osteoarthritis [7, 33, 54, 117, 121, 122]. However, as prostheses have a limited life-span and revision is difficult, knee arthroplasty is not a valid option for the treatment of cartilage defects in the often young patient with a cartilage defect. Therefore, knee arthroplasty should be reserved as final treatment option for generalized osteoarthritis or cartilage defects in the elderly.

A relatively new and promising technique to treat cartilage defects is ACT (Autologous Chondrcyte Transplantation). Although currently not widely employed in the Netherlands, it will be discussed in more detail, as it comprises several techniques employed in this thesis.

Tissue engineering and Autologous Chondrocyte Transplantation

Tissue engineering is a relatively new field and is being developed to overcome the general shortage of organs and tissue for transplantation purposes. The term 'tissue engineering' itself was only introduced in 1987 [43]. Since, many attempts have been made to give a proper definition; for instance: "Tissue engineering is the application of principles and methods of engineering and life sciences toward fundamental understanding and development of biological substitutes to restore, maintain and improve (human) tissue functions".[43] or "Tissue engineering is the art of reconstituting mammalian tissues, both structurally and functionally" [68]. Nowadays, for virtually every tissue or organ in the human body tissue engineering techniques are being developed [74, 147, 153, 161, 193, 216]. However, tissue engineering of musculoskeletal tissues is most widely investigated and at the forefront of this field.

As cartilage defects are not uncommon and present surgical treatments are not optimal, cartilage repair using tissue engineering techniques could offer wide potentials. In 1987 the first patient with cartilage lesions was treated with culture expanded autologous chondrocytes [15]. Since, thousands of patients have been treated. The procedure is known as ACT and can be regarded as first-generation cartilage tissue engineering. Briefly, autologous chondrocytes are harvested from a less-load bearing part of the involved knee and are culture expanded *in vitro* for several weeks. During a second procedure an arthrotomy is performed and the culture expanded chondrocytes are implanted in the defect behind a periostal flap, without any structural support. Several publications showed that ACT has good clinical results 2-9 years follow-up, especially for isolated cartilage defects on the femoral condyle [15, 62, 135, 156, 157]. However, most often the repair tissue represented fibrocartilage [62]. Interestingly, a relation was shown between the clinical outcome and the quality of repair tissue, assessed by taking a biopsy from the repair site [156]. As the first prospective randomized study comparing ACT with another surgical treatment for cartilage defects, osteochondral transplantation, Horas et al. found that both ACT and osteochondral transplantation decreased symptoms [62]. However, the improvement by ACT lagged behind that of osteochondral transplantation. Recently, Knutsen et al. compared ACT with the microfracture technique to repair articular defects in a randomized prospective study. Both techniques improved clinical signs and were comparable in macroscopic and histological outcome after 2 years [205]. Thus, these studies show that no current surgical technique is superior at present.

To date, cartilage repair using ACT remains the first and only cartilage tissue engineering technique that has been used clinically on a wide scale. Currently, new (second generation) cartilage repair techniques using tissue engineering are being developed, which aim at the implantation of a cell-seeded matrix scaffold [95, 106].

The tissue engineering process

Three constituents are involved in tissue engineering (Figure I-4, next page): Cells, Biomaterials and Bioactives.

Cells

The cartilage tissue engineering process with respect to c*ells* can be divided in several parts: Choice of donor source, expansion of donor cell-population (expansion culture) and tissue formation. Articular cartilage as donor tissue represents the most obvious choice. Many studies involving cartilage tissue engineering *in vitro* use articular cartilage from, often immature, bovine, porcine or human origin. Small amounts of articular cartilage can be harvested from less load-bearing areas of the affected knee, which is in fact the procedure of ACT [15, 157]. However, there are several theoretical disadvantages of using articular cartilage as donor tissue. In a clinical setting, tissue-engineering techniques will be initiated only after several weeks or months from the initial trauma, during which the presence of a cartilage defect influences joint homeostasis and can result in degeneration of surrounding cartilage [72, 118, 132, 171]. Therefore, the quality of donor cartilage from the compromised joint could be inferior. Furthermore, harvesting the donor cartilage itself is very likely to inflict additional joint morbidity, as it is merely creating an additional cartilage defect within the joint [203]. However, no reports can be found in literature on negative effects of harvesting cartilage from the joint for tissue engineering purposes like ACT. And although the cartilage is harvested from the 'less weight-bearing' parts of the joint, filling one cartilage defect by creating another cartilage defect is not an ideal solution. And, as the limited possibilities to use autologous articular cartilage for transplantation purposes is one of the main reason why cartilage tissue engineering techniques are being developed, it is obvious that other cell sources suitable for articular cartilage tissue engineering should be sought after.

Next to articular cartilage, the nasal septum cartilage is also of the hyaline type. Several *in vitro* studies have been performed with nasal septum chondrocytes, which showed the possibility of this cell-type to multiply *in vitro* and form hyaline cartilage-like tissue during subsequent culture [19, 20, 79, 93, 167, 196]. In fact, Kafienah showed that chondrocytes from the nasal septum had superior qualities over articular chondrocytes in the ability to multiply and form extracellular matrix *in vitro* [79].

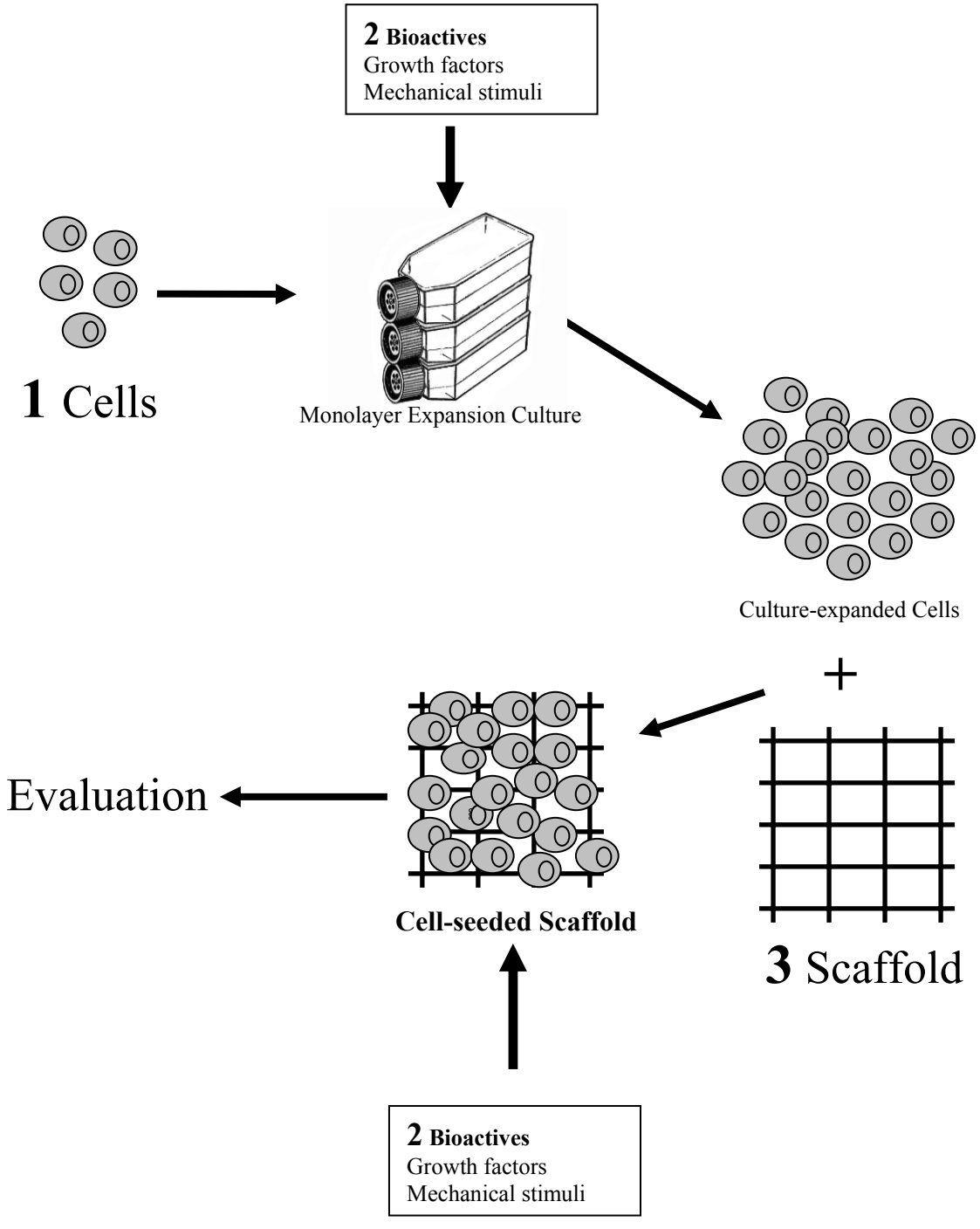

Figure I-4 The cartilage tissue engineering process as it was used during the experiments in this thesis. The three essential constituents are: Cells, Bioactives and Scaffold.

Another cartilage subtype that could be a very relevant donor tissue is elastic cartilage, which is mainly located in the outer ear. Cartilage from the outer ear has the advantage that a relatively large amount can be harvested without cosmetic changes and without major donor-site morbidity [46, 50, 103, 144]. Furthermore, chondrocytes from the outer ear, called ear chondrocytes, have proven to multiply and to form extracellular matrix *in vitro* [75, 99, 110, 138, 159, 201].

A cell source which has gained much interest in recent years are BMSC (Bone Marrow Stromal Cells), in which mesenchymal progenitor cell can be found [26, 44, 82, 136, 164, 172]. These cells are characterized by their ability to differentiate into the different mesenchymal tissues, i.e. cartilage, given the right stimuli [78]. Harvesting BMSC is done via a bone marrow aspiration. However, a complicating factor is that the frequency of BMSC in bone marrow is $1:10^4$ to $1:10^6$ and decreases with age, which makes identification of BMSC in bone marrow aspirates difficult [164]. Various studies using culture expanded BMSC as donor cells for cartilage tissue engineering *in vitro* have been performed [34, 111, 125, 214], showing collagen type II and GAG production by proper use of chondrogenic medium. However, the repair of cartilage defects with BMSC in animal studies showed variable results [68, 70, 173]. Next to bone marrow, other sources of mesenchymal progenitor cells, i.e. the periosteum [91, 148] and fat tissue [39], have recently been explored.

For cartilage tissue engineering to be successful it is vital that the limited amount of harvested donor cells is expanded in number. Classically, this is performed *in vitro* using monolayer culture (2D culture). The donor cells are seeded at a certain density in plastic culture flasks. Monolayer culture of chondrocytes has been performed this way as early as the 1960's [59, 102, 113, 183]. However, these early experiments also showed a phenomenon that nowadays, 40 years later, is still one of the major limiting factors in successful cartilage tissue engineering: loss of the differentiated phenotype during multiplication *in vitro*. Isolated chondrocytes in suspension have a spherical configuration. When introduced in monolayer culture they attach to the culture plastic and start to spread, assuming a more flattened (monolayer, 2D) configuration. During this process the chondrocytes gradually shift the expression of the differentiated phenotype towards a more fibroblast-like phenotype (dedifferentiation) [59, 209, 210]. On molecular level this is represented by a shift in expression of collagen type II and aggrecan, both typical molecules of cartilage, towards collagen type I and versican. Ongoing multiplication, passaging and low seeding densities enhance dedifferentiation during monolayer culture [112, 211]. Unfortunately, to go from a low number of cells after biopsy to a sufficient number of cells to repair a cartilage defect, monolayer cultures of several weeks using several passages are usually needed.

After sufficient numbers of cells are obtained during expansion culture a second part of the engineering process is initiated, which is aimed at restoring the typical chondrogenic phenotype from the dedifferentiated stage (redifferentiation) and, more importantly, at the actual tissue formation. This can be done *in vitro*, *in vivo* or *in situ*, in the actual defect. Regardless which system is chosen, Benya [10] and

Bonaventure [12] showed in the mid 1980's that a prerequisite for restoration of the chondrogenic phenotype and production of the proper extracellular matrix is allowing the dedifferentiated chondrocytes to regain a spherical configuration (3D), like in cartilage. For this purpose many matrix scaffolds, both biological and artificial have been designed and will be discussed below. For experimental purposes, redifferentiation and tissue formation is usually assessed during *in vitro* cultures or *in vivo* using different animal species.

Biomaterials

In general, the biomaterials used in the cartilage tissue engineering process are scaffold matrices. Scaffold matrices are essential in cartilage tissue engineering for several reasons. During a significant part of the tissue engineering process, *in vitro* and *in situ*, the chondrocytes lack structural support of a mature and functional extracellular matrix; a scaffold matrix can substitute for this [13]. Furthermore, scaffold matrices can provide a structural template in which the chondrocytes are contained and can deposit their extracellular matrix. Within time the produced extracellular matrix within a cell-scaffold construct should substitute for the functions of the scaffold. Furthermore, the waste products of the degrading scaffold should be eliminated from the body and should be non-toxic, like the scaffold itself (biodegradable and biocompatible respectively). Also, the material should allow easy cell seeding and medium or nutrient diffusion. Next generation scaffold design and production are the so-called 'smart scaffolds', which are scaffolds loaded with bioactive factors (see below) that can direct and stimulate extracellular matrix production by the cells [27, 65, 93, 182, 213].

Scaffold matrices can be divided in two categories: natural or synthetic. Examples of natural scaffolds include the hydrogels alginate and agarose. Both hydrogels are polysaccharides derived from brown algae and red algae respectively and have an inert nature. Therefore, they are popular scaffold matrices for *in vitro* culture of chondrocytes [36, 90, 101, 105, 120, 130, 141, 160, 201, 212]. Other natural scaffolds are composed of collagen i.e. DBM. (Demineralized Bone Matrix), fibrin or hyaluronic acid [213]. Synthetic scaffolds include the copolymers PGLA (PolyGlycolic Lactid Acid) and PEGT/PBT (Polyethylene Glycol-Therephthalate/PolyButylene Therephthalate).

Advantage of using synthetic scaffolds is the possibility of tailor-made design and absence of animal or human substances, thus reducing the risk of disease transmission [213].

Scaffold matrices are essential in cartilage tissue engineering for several reasons. During a significant part of the tissue engineering process, *in vitro* and *in situ*, the chondrocytes lack structural support of a mature and functional extracellular matrix; a scaffold matrix can substitute for this [13]. Furthermore, scaffold matrices can provide a structural template in which the chondrocytes are contained and can deposit their extracellular matrix. Within time the produced extracellular

matrix within a cell/scaffold construct will substitute for the functions of the scaffold. Ideally, the scaffold should thus degrade and lose function in linear function with the elaborate extracellular matrix. Furthermore, the waste products of the degrading scaffold should be eliminated from the body and should be non-toxic, like the scaffold itself (biodegradable and biocompatible respectively). Also, the material should allow easy cell seeding and medium/nutrient diffusion. Next generation scaffold design and production are the so-called 'smart scaffolds', which are scaffolds loaded with bioactive factors (next paragraph) that can direct and stimulate extracellular matrix production by the cells [27, 65, 93, 182, 213].

Bioactives

Bioactives comprise all molecules, signals or stimuli that can improve the quality of the tissue engineering process. One of the most powerful bioactives is the group of growth factors. FGF2 (Fibroblast Growth Factor 2) is the most widely studied growth factor with respect to cartilage tissue engineering. Formally known as cartilage derived growth factor or basic FGF2, it is a molecule of 18 kDa and larger. It is a member of the large family of FGF2 and has a ubiquitous distribution in the human body. FGF2 is known to be one of the key regulators during embryonic limb formation where it acts as a mitogen [123]. For cartilage tissue engineering purposes FGF2 has been shown to enhance cell multiplication during monolayer culture as well as in suspension culture (3D environment) [6, 20, 29, 73, 99, 126, 127, 129]. Other relevant growth factors in cartilage tissue engineering are IGF1 (Insulin-like Growth Factor 1) and TGFß-2 (Transforming Growth Factor ß-2) [42, 65, 73, 145, 154, 196, 197, 199, 201, 215]. IGF1, a protein of 7.5 kDa, is structurally homologous to pro-insulin and is known as an anabolic factor of proteoglycan synthesis. TGFß-2 is a 25 kDa protein, known for enhancing proliferation and proteoglycan synthesis. Also, addition of TGFß-2 is thought to enhance autocrine TGFß-2 production. For tissue engineering purposes the combination of IGF1 and TGFß-2 significantly increases GAG production and collagen type II expression of dedifferentiated chondrocytes during suspension culture [201, 215].

Another important bioactive might be mechanical loading (Figure I-5, next page). Animal experiments have shown that the absence of loading can have negative effects on cartilage [204]. The chondrocyte, as the only cell type present in cartilage, plays a central role in this process. Within a joint the mechanical loading on cartilage is a combination of factors like compression, shear stress, hydrostatic pressure and osmotic stress. However, *in vitro* experiments with cartilage explants or chondrocytes have shown that these factors separately can elicit effects on the cell's metabolism. The most frequently used form of mechanical loading in *in vitro* experiments is compression. In general, cyclic compression

Figure I-5 An *in vitro* bioreactor-loading device for controlled mechanical loading of cell-seeded scaffolds. This actual device was developed in our laboratory and used in the experiments discussed in chapter VIII.

with specific regimes stimulates GAG and collagen synthesis [23, 25, 92, 97, 130, 150, 185-187], whereas static compression inhibits extracellular matrix synthesis [22-24, 30, 48, 66, 92, 97, 169]. For the cartilage tissue engineering process, mechanical loading *in vitro* could offer several advantages. Mechanical loading could be useful to stimulate (re)differentiation towards the chondrocyte phenotype. Furthermore, the production and organization of extracellular matrix could be enhanced. However, few studies have been published with positive results of mechanical loading on longer-term basis, which is a prerequisite for implemantation of mechanical compression as a bioactive during the tissue engineering process. Mauck et al. showed an increase of the compressive modulus of a chondrocyte-seeded agarose gel after several weeks of cyclic loading, indicating an increase in extracellular matrix [130]. Only recently, Lee et al. performed experiments in which cyclic mechanical loading was found to increase the accumulation of radioactive molecules in the extracellular matrix, synthesized by dedifferentiated chondrocytes [96]. This scarcity of studies with either long-term loading experiments and/or with dedifferentiated cells, which are both relevant with respect to cartilage tissue engineering, indicates that the use of mechanical loading as a bioactive is difficult and yet to be established.

Summary

Summarizing, no solid therapy to treat articular cartilage defects, which occur mainly in young and active adults, exists. Tissue engineering techniques do form a new and promising treatment for such defects. However, many problems have to be addressed. In this thesis we focus on the chondrocyte phenotype and extracellular matrix production during the tissue engineering process *in vitro* and on using ear cartilage as an alternative cell source for articular cartilage repair.

II

Aims of this thesis

Introduction

As stated in the General Introduction (Chapter I), the expansion culture is a necessary step in cartilage tissue engineering since the low number of cells after biopsy should be multiplied to a sufficient number of cells for the repair of a defect. First generation cartilage tissue engineering techniques, the ACT (Autologous Chondrcyte Transplantation), have shown promising clinical results [15, 156, 157]. However, as yet, there have been no reports that ACT is superior to other surgical techniques for articular cartilage repair, like subchondral plate drilling or mosaic plasty. Histology from repair sites in both animal and human joints most often show inferior repair tissue, resembling fibrocartilage, after ACT [14, 62, 156]. Although not entirely consistent [89], a better long-term functional outcome has been shown to correlate to a more hyaline cartilage-like repair tissue at biopsy during a second-look arthroscopy [156]. Furthermore, animal studies have shown that cartilage repair with differentiated chondrocytes results in a longer durability of the repair tissue than with culture-expanded, and hence dedifferentiated, chondrocytes [81]. Thus, to further elucidate the potentials of tissue engineering for cartilage repair the process should be improved in such ways that dedifferentiation during monolayer expansion culture is minimized and redifferentiation during subsequent *in vitro* culture or after *in situ* implantation is maximal. This thesis focuses on this for the *in vitro* part of the cartilage tissue engineering process.

Besides phenotype modulation the choice of donor tissue used for the cartilage tissue engineering process is also adressed. As briefly discussed in the general introduction, new cell sources should be sought after to bypass shortage of donor articular cartilage and donor-site morbidity. In this thesis ear chondrocytes were used in a substantial part of the experiments to assess the feasibility of ear cartilage as donor tissue for cartilage tissue engineering in general and articular repair in particular.

The specific aims of this thesis

Chondrocyte phenotype and extracellular matrix production during the tissue engineering process

To achieve minimal dedifferentiation during monolayer culture, without losing the high cell yield needed, experiments were performed with various seeding densities and growth factor supplemented culture media. Chapter III, V and VI describe the effects of FGF2 (Fibroblast Growth Factor 2) on cell yield, dedifferentiation during monolayer and redifferentiation during 3D culture. Chapter IV describes the effect of different seeding densities in monolayer culture on these outcome measures.

To increase redifferentiation after monolayer expansion culture chemical and mechanical stimuli can be used. Chemical stimuli are described in Chapter III, IV, V and VI, where experiments with a SFM (Serum-Free Medium) with IGF1 (Insulin-like Growth Factor 1) and TGFß-2 (Transforming Growth Factor β-2) on dedifferentiated chondrocytes were performed. To test whether mechanical loading can have effects on matrix production of isolated chondrocytes in 3D culture a custom-designed loading device was built. Eventually, mechanical loading might be used to direct dedifferentiated chondrocytes seeded onto a scaffold towards the differentiated phenotype. In Chapter VIII (paragraph: The loading device, page 17) the loading device is introduced and loading experiments on isolated bovine articular chondrocytes are discussed.

The feasibility of ear cartilage as donor tissue for cartilage tissue engineering

Harvesting articular cartilage as donor tissue for cartilage tissue engineering has several disadvantages. Firstly, because articular cartilage has the important function of transferring loads and stresses through a joint, only a limited amount can be harvested without compromising its function. Secondly, the articular cartilage is usually harvested from the injured and/or arthritic joint. This is likely to inflict additional morbidity to an already diseased joint [94, 203]. In turn, the only function of ear cartilage is offering structural support to the outer ear. Transplantation of ear cartilage has been used for many years to repair nasal septum defects [28, 77, 83, 155, 189]. Harvesting ear cartilage for this purpose has proven to be relatively easy and is without major donor-site morbidity [50, 77, 103, 133, 144]. In Chapter III, IV and V either immature or mature human ear chondrocytes were used for the experiments. Furthermore, in Chapter VII the considerations of using ear chondrocytes as donor cells for cartilage tissue engineering in general and for articular cartilage tissue engineering in particular are discussed.

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III

Serum-free medium supplemented with high concentration FGF2 for cell expansion culture of human ear chondrocytes promotes redifferentiation capacity

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Tissue Engineering 2002; Aug 8(4): 573-580

Abstract

For tissue engineering of autologous cartilage, cell expansion is needed to obtain the number of cells required. Standard expansion media contain bovine serum. This has several disadvantages, i.e. risk of transmitting diseases and serum-batch variations. The aim of this study was to find a SFM (Serum-Free Medium) with at least the same potentials to expand the number of cells as serum-containing media. Ear chondrocytes of three young children were expanded in either SCM (Serum-Containing Medium; DMEM with 10% fetal calf serum) or SFM (Serum-Free Medium; DMEM with ITS+) supplemented with 5 ng/ml or 100 ng/ml FGF2. To promote cell adherence onto the culture flask the serum-free conditions were cultured with 10% serum for one day after each trypsinisation. After the fourth passage the chondrocytes were encapsuled in alginate beads and redifferentiated in a SFM (DMEM with ITS+, hydrocortisone and L-ascorbic acid) supplemented with 10 ng/ml IGF1 and 10 ng/ml TGFß-2. Results showed that expansion in SFM with 100 ng/ml FGF2 was comparable to expansion in SCM. Redifferentiation with SFM with IGF1 and TGFß-2 showed high collagen type II expression and high GAG/DNA production regardless which expansion medium had been used. However, chondrocytes expanded in SFM with 100 ng/ml FGF2 resulted in less positive cells for collagen type I and 11-fibrau (a fibroblast membrane marker). The present study shows that it is possible to use SFM for tissue engineering of cartilage. Expansion of immature ear chondrocytes in SFM supplemented with high concentration FGF2 resulted in a high number of cells which in addition had a better redifferentiation capacity than cells expanded in medium with 10% serum.

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Introduction

Surgical repair and reconstruction of cartilage defects in articular joints as well as the head and neck area require extra cartilage. Often the amount of donor site cartilage is limited. The expansion of donor site cartilage using tissue-engineering techniques could be a possibility to solve this problem. Cells are generally isolated from the tissue and expanded using monolayer culture. Standard culture medium consists of 10% serum. A culture medium without serum is preferable because of the risk of transmitting disease. Furthermore, a SFM (Serum-Free Medium) would exclude the variation between serum batches. Cell expansion in SFM should at least be comparable to expansion in medium supplemented with 10% FCS (Fetal Calf Serum).
During expansion the cells lose their phenotype and become more fibroblast-like, producing less GAG (GlycosAminoGlycan)and they change collagen type II production to collagen type I [4, 38, 210] . After sufficient multiplication the dedifferentiated cells should be stimulated to regain their specific phenotype. Besides obtaining sufficient chondrocyte proliferation, this is essential in order to engineer a functional piece of cartilage.
In earlier publications FGF2 (Fibroblast Growth Factor 2) was added to medium with serum to stimulate chondrocyte proliferation [2, 32, 166]. Sometimes, very high concentrations (up to 100 ng/ml) were used. Furthermore, recent publications showed that supplemention of 5 ng/ml FGF2 to SCM (Serum-Containing Medium) promoted redifferentiation capacity [127]. In the present study we composed a SFM supplemented with FGF2 for expansion of human auricular chondrocytes. To evaluate redifferentiation capacity after expansion in monolayer, the chondrocytes were cultured in a three dimensional environment, i.e. alginate beads in SFM supplemented with IGF1 (Insulin-like Growth Factor 1) and TGFß-2 (Transforming Growth Factor β-2) [201, 215].

Materials and methods

Cartilage
Human ear cartilage was obtained from three children (aged 5-10) during protruding ear correction surgery after informed consent. No 'extra' ear cartilage was harvested for this study. The perichondrium was carefully dissected from the cartilage and the cartilage was sliced into small pieces and washed several times in physiological saline. A two-step digestion protocol was used. First the cartilage was pre-incubated in pronase E (2 mg/ml; Sigma, St Louis, Mo.) for two hours, followed by overnight digestion in collagenase B (1.5 mg/ml; Boehringer Mannheim, Germany). The chondrocytes were filtered using a 100 μm filter to remove small parts of undigested cartilage. Cell viability was tested using trypan blue staining. No significant cell death was noted.

Expansion culture

Chondrocytes were seeded onto culture flasks at a density of $4 \cdot 10^4$ cells/cm^2. Four different types of expansion media were prepared. Dulbecco's modified eagle medium (Life Technologies, Breda, the Netherlands; Glutamax-I) was supplemented with either 10% FCS, ITS+ (1:100; Becton Dickinson, Insulin, Transferrin, selenious acid, bovine serum albumin (1.25 mg/ml final concentration in medium), linoleic acid), ITS+ with 5 ng/ml FGF2 (recombinant human, Instruchemie, Hilversum, the Netherlands) or ITS+ with 100 ng/ml FGF2. The SCM will be referred to as SCM and the SFM as SFM + supplementations. Bacterial and fungal growth was inhibited by using gentamycine (50 µg/ml; Life Technologies) and fungizone (1.5 µg/ml; Life Technologies). At subconfluency chondrocytes were trypsinized using trypsin-EDTA (Life Technologies). At the start of each new passage, chondrocytes in the serum-free conditions were cultured in 10% FCS for one day to promote cell adhesion onto the culture flask. Medium was changed every other day. Cells were expanded for four passages.

Redifferentiation culture

After the fourth passage the chondrocytes from the four conditions were encapsulated separately in alginate beads to enhance cell redifferentiation [12]. The cells were suspended in sterile 1.2% alginate in saline (Keltone LV, Kelco, Chicago, Ill.) at a density of $4 \cdot 10^6$ cells/ml. This suspension was slowly dropped in a 102 mM $CaCl_2$ solution using a 23-gauge needle, creating gelled alginate beads. After 10 minutes in the $CaCl_2$ solution the beads were washed three times with physiological saline.

DMEM was supplemented with ITS+, 10 ng/ml IGF1 (recombinant human, Boehringer Mannheim, Germany), 10 ng/ml TGFß-2 (recombinant human, R&D systems, Abingdon, UK) and 0.1 µg/ml hydrocortisone (Becton Dickinson Labware); reffered to as SFM+IGF1/TGFß-2. This condition is described before to induce redifffferentiation [201, 215]. As control, alginate beads were cultured in DMEM with 10% FCS, shown before to induce little redifferentiation of chondrocytes [32, 201]. L-ascorbic acid (25 µg/ml) was freshly added. The beads were cultured for 21 days in a 24-wells plate. Each well contained 10 beads and 50 µl medium per bead was added. The medium was changed every other day.

Biochemical analysis

Alginate beads were dissolved by 150 µl of 55 mM sodium citrate in 150 mM sodium chloride per bead. The samples were divided in two parts to determine DNA content and GAG amount.

To determine the amount of DNA Papain (Sigma, St Louis, MO) was added to the samples (final concentration 125 µg/ml) and the samples were incubated overnight at 60 °C. Using Hoechst 33258 dye the amount of DNA per sample was measured with a fluorometer (Perkin-Elmer LS-2B) at 440 nm. The emission

of the samples was compared to the emission of a standard (calf thymus DNA; Sigma).
GAG content was measured using the dimethylmethylene blue assay [40]. To extract the GAG GuHCl was added to the samples at a final concentration of 4 M and incubated at 4 °C for 48 hours on a shaker. Using a microplate reader the ratio of absorption at 540 nm and absorption at 595 nm was measured. The amount of GAG was calculated using a standard of chondroitin sulphate C (shark; Sigma).
For each sample the amount of GAG was divided by the amount of DNA.

Immunohistochemistry
Alginate beads were dissolved in sodium citrate. Cytospins were prepared and stored at -80 °C. The cytospins were stained for collagen type I, collagen type II, elastin and 11-fibrau.

Collagen staining
The cytospins were fixed in acetone and treated by 1% hyaluronidase (Sigma) for 20 minutes. The next steps included pre-incubation with normal goat serum followed by incubation with monoclonal antibodies against procollagen type I and collagen type II (1:100 M38 and 1:100 II-II6B3 respectively; Developmental Studies Hybridoma Bank). Fab-fragments against mouse conjugated with alkaline phosphatase (GAMAP; Immunotech, Marseille, France, 1:100) were added. Next, mouse monoclonal alkaline phosphate anti-alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark; 1:100) was added. A red signal in positive cells was obtained by incubation with a new fuchsin substrate (Chroma, Kongen, Germany), demonstrating alkaline phosphate activity.

Elasin staining
The cytospins were fixed in formaline at 4 °C overnight. The next day the cytospins were treated with 0.1% trypsin for 15 minutes at 37 °C.
After pre-incubation with 10% normal goat serum the cytospins were incubated with monoclonal antibodies against elastin (BA4 1:50, Sigma). Next, goat Fab-fragments against mouse conjugated with boitine (1:50, Vector Laboratories, Burlingame, CA) were used, followed by mouse monoclonal biotine anti-alkaline phosphatase (1:50, Vector Laboratories, Burlingame, CA). New fuchsin substrate was used to demonstrate positive cells.

11-fibrau
Monoclonal antibody 11-fibrau binds a 112 kDa protein on the cell surface of fibroblasts. This protein is absent on differentiated chondrocytes, but is expressed by dedifferentiated chondrocytes[200].
The cytospins were fixed in acetone. Next, the cytospins were washed and pre-incubated with 10% normal goat serum. This was followed by incubation with

the 11-fibrau antibody (1:400, Imgen, distributed by ITK diagnostics, Uithoorn the Netherlands) for two hours. Fab-fragments against mouse conjugated with alkaline phosphatase (1:100) were added for 30 minutes. Next, mouse monoclonal alkaline phosphate anti-alkaline phosphatase (1:100) was added for 30 minutes. Incubation with a new fuchsin substrate demonstrated alkaline phosphate activity.
Controls for the four staining procedures were made by following the same staining routine but by omitting the first antibody.
The percentage of positive cells for each cytospin was determined by randomly selecting and counting 300 cells.

Data analysis
For each donor, three samples of each experimental group are used for analysis of GAG/DNA and one sample is used to prepare cytospins. Each sample contained three beads. Mean and standard deviations of three donors were presented. When data of less than three donors were used this is indicated. Differences among experimental groups were analysed using the Mann-Whitney test. A p-value < 0.05 is considered to indicate a statistically significant difference.

Results

Cell expansion
Cells cultured in SFM with no growth factors, did not expand. The addition of 5 ng/ml FGF2 to this SFM increased the expansion to 1.7 times (SD = 0.67), but this was still less than expansion in SCM. Expansion in SFM supplemented with 100 ng/ml FGF2 appeared always higher than expansion in medium with 10% FCS. In average 2.5 times as many cells were obtained by replacement of 10% FCS by ITS+ and 100 ng/ml FGF2 . The time to reach passage four was not significantly different for these conditions. Cells cultured with 100 ng/ml FGF2 remained a polygonal shape for longer period of time in monolayer culture.

Chondrocyte Redifferentiation
Alginate culture in SCM showed poor chondrocyte redifferentiation of expanded chondrocytes, independent of the expansion medium used. Collagen type I expression was high and both GAG/DNA production and collagen type II expression were low (Figure III-1 and Figure III-2 next page). Alginate culture in SFM+IGF1/TGFß-2 showed redifferentiation for chondrocytes expanded in either SCM or SFM supplemented with FGF2 (both 5 and 100 ng/ml); Production of GAG/DNA showed a 10-fold increase ($p=0.01$) and collagen type II expression increased from virtually 0% to 80-90%.

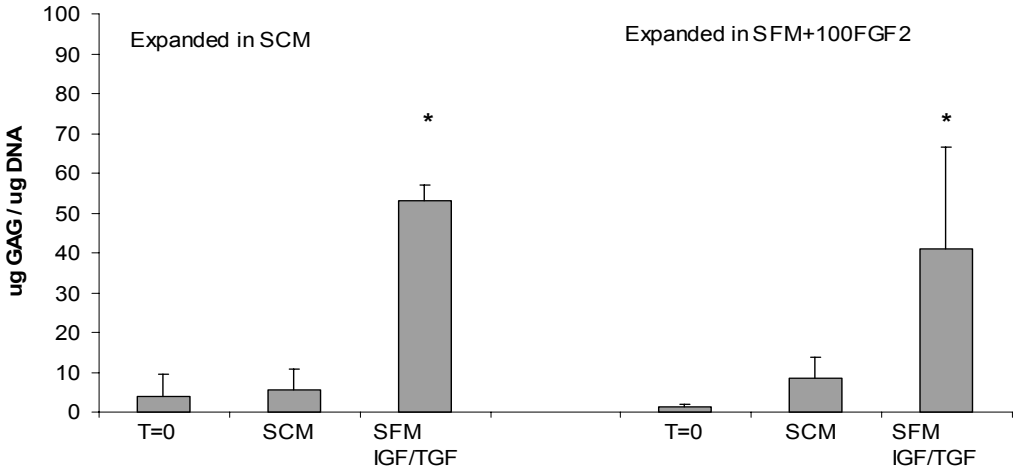

Figure III-1 The amount of glycosaminoglycan per DNA (GAG/DNA) produced by chondrocytes after redifferentiation culture in alginate. The three bars on the left side of the figure represent chondrocytes expanded in SCM and the three bars on the right side represent chondrocytes expanded in SFM + 100 ng/ml FGF2. The star represents statistical significant difference of GAG/DNA to T=0 and SCM.

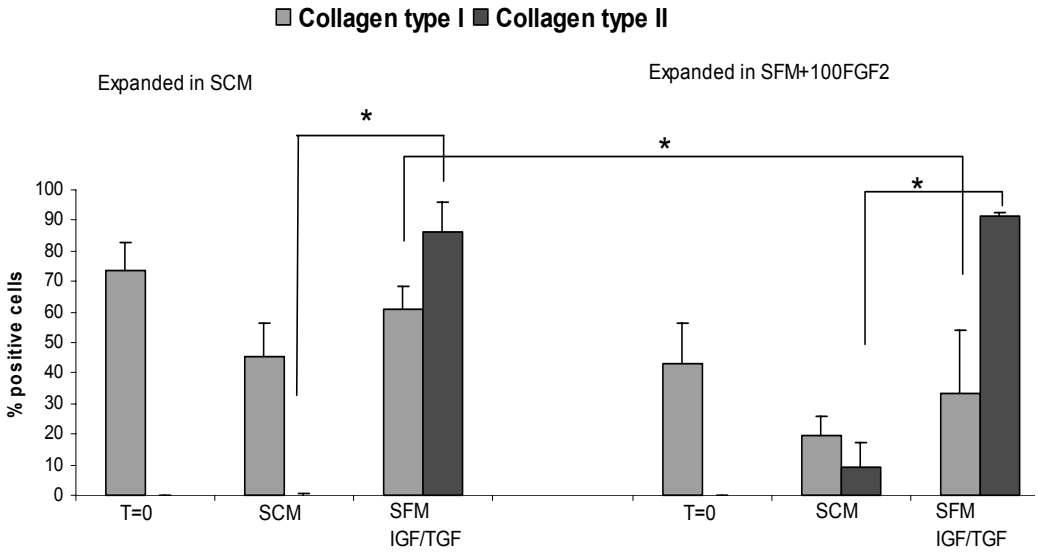

Figure III-2 The number of cells positive for collagen type I and II afterredifferentition culture in alginate. The three bars on the left side of the figure represent chondrocytes expanded in SCM and the three bars on the right side represent chondrocytes expanded in SFM + 100 ng/ml FGF2. A star indicates a statistical significant difference between the two conditions compared. Only comparisons important for this study were submitted to statistical analyses in this figure.

Chondrocytes expanded in SFM alone were not redifferentiated, showing high collagen type I expression and both low collagen type II expression and GAG/DNA production.
Elastin expression increased from minimal amounts after alginate culture in SCM upto 22% when SFM+IGF1/TGFß-2 had been used. No differences were found between cells expanded in different types of medium.
Because expansion in both SFM alone and SFM+5 ng/ml FGF2 resulted in a very low number of cells, these conditions are considered non-relevant for clinical application. Furthermore, insufficient amount of cells were obtained to perform redifferentiation cultures of all experiments. Therefore only the results for expansion in SCM and expansion in SFM+100 ng/ml FGF2 are further evaluated using 11-fibrau.
After expansion in medium with serum, most cells were positive for 11-fibrau (Figure III-3, opposite page).
Redifferentiation in SFM+IGF1/TGFß-2 slightly but significantly reduced the amount of 11-fibrau positive cells. When cells after expansion in SFM with 100 ng/ml FGF2 were cultured in alginate with SCM, the number of 11-fibrau positive cells was lower. Moreover, culture in alginate in SFM+IGF1/TGFß-2 almost completely removed the epitope for 11-fibrau, indicating these cells expressed chondrocyte phenotype.

Discussion

Expansion cultures should be safe, reproducible and fast in order to introduce tissue-engineered cartilage in clinical practice. Using SFM supplemented with specific growth factors these three conditions are met. Basic FGF2 in a concentration of 100 ng/ml added to expansion culture has been shown to stimulate chondrocyte expansion in monolayer and to enhance the capacity to express the cartilage phenotype. Both high expansion numbers and good redifferentiation are essential for cartilage tissue engineering. Expansion in SFM+100 ng/ml FGF2 showed highest multiplication.
After three weeks of redifferentiation in alginate culture, immunohistochemical staining showed chondrocytes expressing mainly collagen type I when SCM had been used for redifferentiation culture. Evenmore, when expansion had been performed in the presence of serum, collagen type II and the ratio collagen type II / collagen type I was overall lowest. This is indicative for poor redifferentiation. Yaeger et al. and van Osch et al. showed a positive synergistic effect of IGF1 and TGFß-2 in SFM on collagen type II expression and GAG production during redifferentiation culture of articular and auricular chondrocytes respectively. Based on these results we supplemented our SFM with 10 ng/ml IGF1 and 10 ng/ml TGFß-2. Subsequently, collagen type II expression and GAG/DNA production increased dramatically for chondrocytes expanded in both SCM and SFM+100 ng/ml FGF2 to the same amount.

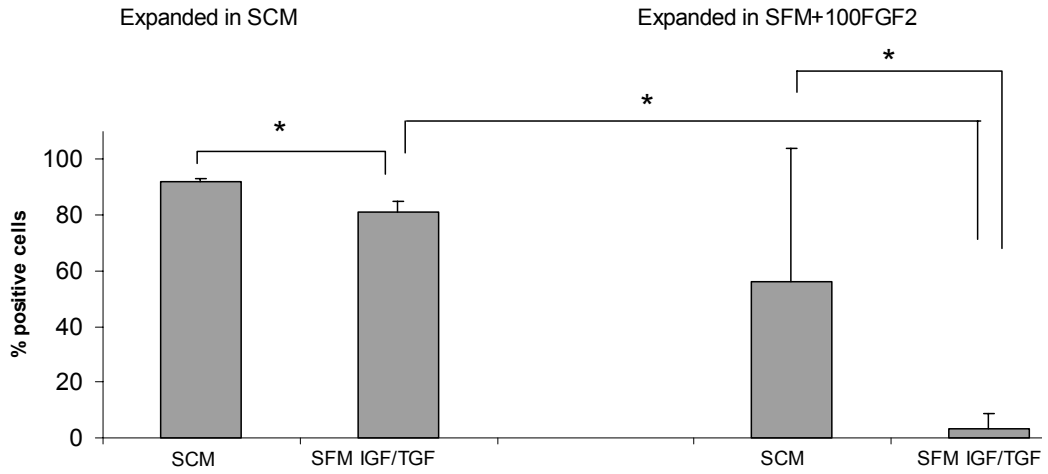

Figure III-3 The results of the immunohistochemical staining with 11-fibrau (a fibroblast membrane marker) are presented of cells redifferentiated in alginate. The three bars on the left side of the figure represent chondrocytes expanded in SCM and the three bars on the right side represent chondrocytes expanded in SFM+100 ng/ml FGF2. A star indicates a statistical significant difference between the two condition compared. When chondrocytes had been expanded in SFM+100 ng/ml FGF2 and redifferentiated in SFM+IGF1/TGFß-2 almost 95% of the chondrocytes were negative for 11-fibrau, indicating superior redifferentiation compared to the other conditions.

Van Osch et al. showed that 11-fibrau is a useful antibody to mark the state of dedifferentiation for *in vitro* expanded chondrocytes [200] . Fibroblast-like chondrocytes stain positive when 11-fibrau antibody is used, confirming a dedifferentiated phenotype. Immunohistochemical staining with 11-fibrau on chondrocytes expanded in SCM and 'redifferentiated' in in SFM+IGF1/TGFß-2 still showed many positive cells, indicating cells were not completely redifferentiated in spite of the high number of collagen type II positive cells. However, cells expanded in SFM+100 ng/ml FGF2 showed an almost complete absence of 11-fibrau staining when cultured in alginate in SFM+IGF1/TGFß-2, indicating better re-expression of the chondrocyte phenotype. These data implicate the usefulness of assessing more parameters than collagen type I and II expression when evaluating chondrocyte differentiation stage. The use of antibody 11-fibrau provides additional information on chondrocyte differentiation stage.

What mechanism(s) is(are) responsible for the effect of FGF2 on expression of chondrocyte phenotype is not fully clear yet. Recently, a growing number of studies report a relation between the group of FGF2, Sox9 and chondrocyte matrix production [9, 31, 143]. Sox9 is a transcription factor, which is responsible for

the differentiation of mesenchymal stem cells to chondrocytes. Also, Sox9 promotes upregulation of several chondrocyte-specific marker genes including the genes for collagen type II and aggrecan. FGF2, including FGF2**,** can enhance the expression of Sox9. Addition of FGF2 to expansion cultures might slow down the dedifferentiation process or enhance preservation of redifferentiation capacity.
Next to collagen type II, elastin is an important extracellular matrix component of elastic cartilage. Elastin production during the primary differentiation of mesenchymal stem cells to ear chondrocytes is temporally linked to the expression of collagen type II and proteoglycans production. Elastin expression during redifferentiation culture might therefore be an indicator of redifferentiation of auricular chondrocytes. We could demonstrate a low percentage of cells positive for elastin/tropoelastin when cells were redifferentiated in SFM+IGF1/TGFß-2. This low percentage could be due to the fact that elastin is produced slowly by chondrocytes in culture [110]. Moreover, we can not exclude the possibility that our redifferentiation medium is not optimal for re-expression of elastin.

Tissue engineered cartilage without the presence of bovine serum is safer in clinical use.
However, it should be noted again that after each passage during cell expansion chondrocytes had to be cultured with serum containing media for one day to promote cell adhesion onto the culture flask. Also, the ITS+ used for the SFM contains BSA (Bovine Serum Albumin), mainly to serve as a carrier for linoleic acid and to bind growth factors. In future studies autologous serum could be used for cell adhesion because only a relatively small amount of serum is needed and HSA (Human Serum Albumin) could be supplemented to ITS+ to replace BSA.

In this study ear chondrocytes from children were used. Cartilage tissue engineering of immature donors could be used to treat microtia or traumatic defects in children. However, cartilage tissue engineering will also be used for mature patients. Several publications on culturing young versus adult chondrocytes have shown that donor age can be an important factor in redifferentiation capacity and responds to growth factors [107, 201]. Therefore, chondrocytes derived from adult tissue should be tested to detemine whether similar results can be obtained as with immature chondrocytes.

Finally, we can say that we have made significant progression in excluding bovine serum from the cartilage tissue engineering culture system. Futhermore, expansion in SFM with 100 ng/ml FGF2 promotes multiplication and re-expression of chondrocyte phenotype compared to expansion in medium with 10% serum. This has several advantages. First, a higher number of cells gives larger pieces of tissue engineered cartilage. Second, superior redifferentiation

should give better quality extracellular matrix and hence a more functional piece of engineered cartilage.

Acknowledgements

The authors would like to thank Dr. Leon van Adrichem (Plastic and Reconstructive Surgeon) as well as the patients for providing human ear cartilage.
The monoclonal antibodies, II-II6B3 and M38 were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. This work was funded by the Dutch Ministry of Economic Affairs in a grant of Senter (nr. BTS00021).

IV

Multiplication of human chondrocytes with low seeding densities accelerates cell yield without losing redifferentiation capacity

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Tissue Engineering 2004; Jan-Feb 10(1-2): 109-118

Abstract

To treat a cartilage defect with tissue engineering techniques, multiplication of donor cells is essential. However, during this multiplication in monolayer expansion culture chondrocytes will lose their phenotype and produce matrix of inferior quality (dedifferentiation). Dedifferentiation occurs more extensively by low seeding densities and passaging. To obtain cartilage of good quality it is important that the multiplicated cells regain their cartilaginous phenotype (redifferentiation capacity). A gold standard for the multiplication of chondrocytes in monolayer, with respect to seeding density and passaging is lacking. In numerous studies available, various cell densities have been used making comparison of the results of these studies difficult. Therefore, we performed a comparative study to gain insight in the effect of seeding density and passaging on the capacity of the cells to redifferentiate. From the resulting data we will deduce the seeding density in monolayer culture for which cell expansion is both sufficient and fast, whereas the cells retain a capacity to redifferentiate. As a guideline we calculated that a minimum of 20 times multiplication is needed to fill an average cartilage defect of 4 cm^2 with the amount of donor chondrocytes we obtained. For this study we used isolated ear chondrocytes from five children. Four different seeding densities in monolayer culture were used, ranging from 3500 cells/cm^2 to 30,000 cells/cm^2. The cells were cultured for 4 passages. The capacity of the expanded chondrocytes to redifferentiate (redifferentiation capacity) was studied after an additional three weeks culture in alginate beads and was assessed by GAG production and immunohistochemical stainings for collagen type I, collagen type II, elastin and a fibroblast marker (11-fibrau). In general we found that both passaging and decreasing seeding density yielded an increase of expanded chondrocytes, but at the same time decreased the redifferentiation capacity. Further analyzing our data with the proposed guideline we found that with lower seeding densities sufficient multiplication (20 times) was reached in less time and with less passaging than at higher seeding densities. Importantly, the redifferentiation capacity of these chondrocytes was preserved. It was equal or might even be better than chondrocytes multiplicated 20 times at higher seeding densities, which used more time and more passages in monolayer culture. Thus, for cartilage tissue engineering purposes we propose that expansion culture with low seeding densities is preferable.

Introduction

The treatment of traumatic or congenital cartilage defects is limited by several factors. First, cartilage has no or little intrinsic repair mechanisms. Furthermore, the amount of cartilage available for autologous transplantation is limited. However, during the last 15 years cartilage tissue engineering techniques have been studied to overcome these problems. With these techniques a relatively small amount of donor cells, either chondrocytes or progenitor cells, are expanded *in vitro* until sufficient cells are obtained. Currently, expansion using monolayer culture is most widely used. However, it is well known that *in vitro* multiplication of chondrocytes in monolayer results in dedifferentiation of these cells [59, 209, 210]. They lose their specific phenotype and become more fibroblast-like. The production of the specific cartilage matrix components collagen type II and aggrecan is gradually shifted toward collagen type I and small proteoglycan [112, 211]. To re-express the differentiated phenotype (redifferentiation) a three dimensional environment, like a gel [10, 12], a pellet [73] or a 3D scaffold [45] in which the expanded chondrocytes can obtain a spherical configuration, is essential. However, studies showed that many of these redifferentiated chondrocytes still produce collagen type I and small proteoglycans to some extent indicating a not fully restored cartilage phenotype [73, 116]. To enhance chondrocyte redifferentiation changes can be made in either the redifferentiation culture itself or in the expansion culture. In this study we focussed on the expansion culture. Since the early 1960's it has been known that dedifferentiation in monolayer is enhanced by low seeding densities, passaging and ongoing multiplication. Therefore, high initial seeding densities (30,000 cells/cm^2 and higher) are being used to reduce dedifferentiation. However, expansion in high seeding density cultures often fail to produce sufficient chondrocytes, even after several passages. Lower seeding densities may increase cell yield but bear the risk of decreased redifferentiation capacity. Although much research is performed, there is no gold standard for cell density in monolayer culture to expand cell number for cartilage tissue engineering purposes. Various seeding densities have been used, making comparison of the numerous results impossible. Therefore, we performed several experiments to study the relations between the seeding density in monolayer, the number of passages in monolayer and subsequent chondrocyte redifferentiation capacity in more detail. From the results we hoped to find a seeding density for which cell expansion is both sufficient and fast whereas the cells retain the capacity to redifferentiate. As a guideline we presumed a cartilage defect of the human nasal septum or an articular joint of 5 mm thickness and with an area of 4 cm^2. We also presumed the possibility to harvest $2 \cdot 10^6$ donor chondrocytes from the biopsy [116] and that the minimal concentration to generate new cartilage *in vivo* is $20 \cdot 10^6$ cells/cm^3 [158]. To treat our presumed cartilage defect the donor cells have to be multiplied at least 20 times. Isolated ear chondrocytes from children were expanded in monolayer using four different seeding densities ranging from 3500 cells/cm^2 to 30,000 cells/cm^2. At different passages the

chondrocytes were encapsulated in alginate to evaluate redifferentiation capacity. By correlating seeding density and number of passages to the resulting expansion and redifferentiation capacity we provide a scientific base for the choice of seeding density of human ear chondrocytes in monolayer for cartilage tissue engineering.

Materials and Methods

Two experiments were performed with five separate donors. In each experiment chondrocytes were expanded using monolayer culture. After expansion the chondrocytes were encapsulated in alginate to promote redifferentiation. A brief overview of the separate experiments is given below.

Experiment 1: seeding densities
Cells from two donors (aged 6 and 12, respectively donor I and donor II) were used. Freshly isolated chondrocytes were seeded in monolayer culture in four different densities; 3500 cells/cm^2, 7500 cells/cm^2, 15,000 cells/cm^2 and 30,000 cells/cm^2. The chondrocytes were expanded until passage 4. A culture of three weeks in alginate was performed with P4 chondrocytes from donor I and with both P2 and P4 chondrocytes from donor II.

Experiment 2: passaging
Cells from donor II and three additional donors (aged 7,11 and 7, respectively donor III, IV and V) were seeded in monolayer in a density of 7500 cells/cm^2. At each passage (1,2,3 and 4) part of the expanded cells were encapsulated in alginate and a redifferentiation culture was performed. The remaining cells were seeded at the initial density in monolayer again. After passage four the expansion culture was stopped.

Chondrocyte isolation
The pieces of cartilage were resected from five children (aged 6-12) during protruding ear corrections and were used by informed consent. Perichondrium was removed in order to prevent any contamination with chondrogenic cells and the cartilage was digested using 2 mg/ml pronase (Sigma, St Louis, Mo) with saline for 1.5 hours, followed by overnight digestion in 2 mg/ml collagenase B (Boehringer Mannheim, Germany) in Dulbecco's modified eagle medium (Life Technologies, Breda, the Netherlands; Glutamax-I) with 10% FCS (Fetal Calf Serum). The next day the chondrocytes were washed three times in physiological saline and cell viability was tested using trypan blue staining. Cell yield was 9000-40,000 cells/mg prepared cartilage. Next, the chondrocytes were seeded in monolayer at different densities.

Expansion culture

Four different seeding densities were used: 3500 cells/cm^2, 7500 cells/cm^2, 15,000 cells/cm^2 and 30,000 cells/cm^2. The chondrocytes were expanded in culture flasks (either 25 cm^2 (3 ml medium) or 75 cm^2 (10 ml medium)) using DMEM with

10% FCS,gentamycine (50 µg/ml; Life Technologies) and fungizone (1.5 µg/ml; Life Technologies). At confluency the cells were detached from the culture flask using trypsin-EDTA (Life Technologies). Next, the cells were counted using a haemacytometer to determine the expansion. The remaining cells were either seeded in new culture flasks at the same density as before and/or encapsulated in alginate. At passage four the cell expansion culture was stopped.

Alginate culture

Alginate culture was performed to promote chondrocyte redifferentiation [12]. Briefly, the expanded chondrocytes were suspended in 1.2% alginate in saline ($4 \cdot 10^6$ cells/ml). Using a 23-gauge needle the suspension was carefully dropped in $CaCl_2$ (102 mM), creating gelled beads. After 10 minutes in $CaCl_2$ the beads were washed three times with physiological saline. The alginate beads were cultured in a 24-wells plate with twelve beads per well. A defined SFM (Serum-Free Medium) was used to stimulate redifferentiation [116, 201, 215]: DMEM supplemented with ITS+ (1:100; Becton Dickinson, Insulin, transferrin, selenious acid, bovine serum albumin {1.25 mg/ml final concentration in medium}, linoleic acid), 10 ng/ml IGF1 (Insulin-like Growth Factor 1, recombinant human, Boehringer Mannheim, Germany), 10 ng/ml TGFß-2 (Transforming Growth Factor β-2, recombinant human, R&D systems, Abington, UK) and L-ascorbic acid 2-phosphate (25 µg/ml, freshly added). The culture medium was changed every other day and 50 µl medium was used per bead. After three weeks the beads were harvested and stored at –18 °C until further biochemical and immunohistochemical analyses.

Biochemical analysis

Each sample contained three alginate beads and for each condition three samples were analyzed. Alginate beads were dissolved by 150 µl of 55 mM sodium citrate in 150 mM sodium chloride per bead. After the samples were dissolved they were divided in two parts to determine DNA content and GAG (GlycosAminoGlycan) amount.

To determine the amount of DNA Papain (Sigma, St Louis, MO) was added to the samples (final concentration 125 µg/ml) and the samples were incubated overnight at 60 ^oC. The amount of DNA per sample was measured with Hoechst 33258 dye using a fluorometer (Perkin-Elmer LS-2B) at 440 nm Calf thymus DNA(Sigma) was used as a standard.

GAG content was measured using the dimethylmethylene blue assay [41]. To extract the GAG, Guanidine HCl was added to the samples at a final

concentration of 4 M and incubated at 4 °C for 48 hours on a shaker. Using a microplate reader the ratio of absorption at 540 nm and absorption at 595 nm was measured. The amount of GAG was calculated using a standard of chondroitin sulfate C (shark; Sigma).
For each sample the amount of GAG was related to the amount of DNA.

Immunohistochemistry
A sample of 3 alginate beads were dissolved in sodium citrate. Cytospins were prepared and stored at -80 °C. The cytospins were stained for collagen type I, collagen type II, elastin and 11-fibrau.

Collagen staining
The cytospins were fixed in acetone and treated by 1% hyaluronidase (Sigma) for 20 minutes. The next steps included pre-incubation with normal goat serum followed by incubation with monoclonal antibodies against procollagen type I and collagen type II (1:100 M38 and 1:100 II-II6B3 respectively; Developmental Studies Hybridoma Bank) for 2 hours. Fab-fragments against mouse conjugated with alkaline phosphatase (GAMAP; Immunotech, Marseille, France, 1:100) were added. Next, mouse monoclonal alkaline phosphate anti-alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark; 1:100) was added. A red signal in positive cells was obtained by incubation with a new fuchsin substrate (Chroma, Kongen, Germany).

Elastin staining
The cytospins were fixed in formalin at 4 °C overnight. The next day the cytospins were treated with 0.1% trypsin for 15 minutes at 37 °C. After pre-incubation with 10% normal goat serum the cytospins were incubated with monoclonal antibodies against elastin (BA4 1:50, Sigma) for 2 hours. Next, goat Fab-fragments against mouse conjugated with biotin (1:50, Vector Laboratories, Burlingame, CA) were used, followed by mouse monoclonal anti-biotin alkaline phosphatase (1:50, Vector Laboratories, Burlingame, CA). New fuchsin substrate was used to demonstrate positive cells.

11-fibrau
Monoclonal antibody 11-fibrau binds a 112 kDa protein on the cell surface of fibroblasts. This protein is absent on differentiated chondrocytes, but is expressed by dedifferentiated chondrocytes [200].
The cytospins were fixed in acetone. Next, the cytospins were washed and pre-incubated with 10% normal goat serum. This was followed by incubation with the 11-fibrau antibody (1:400, 11-fibrau = SM1399, Imgen, distributed by ITK diagnostics, Uithoorn, the Netherlands) for two hours. Fab-fragments against mouse conjugated with alkaline phosphatase (1:100) were followed by mouse

monoclonal alkaline phosphate anti-alkaline phosphatase (1:100) and a new fuchsin substrate.
Controls for the staining procedures were made by following the same staining routine but by omitting the first antibody. The percentage of positive cells for each cytospin was determined by randomly selecting and counting 3 times 100 cells.

Data Analysis
For each donor, three samples of three alginate beads were used for each experimental group to analyze GAG/DNA, and one sample was used to prepare cytospins. Mean and standard deviations of five donors are presented. Differences among experimental groups were analyzed using the Wilcoxon's rank test and the Spearman's correlation coefficient (r_s) for non-parametric analysis. A p-value < 0.05 was considered to indicate a statistically significant difference.
The average saturation density of 4 passages was calculated by taking fourth root of the total expansion after four passages of a certain seeding density in monolayer and multiplying this with the seeding density.

Results

Experiment 1: seeding density
For both donors from experiment 1 total expansion after four passages increased when the seeding density was decreased from 30,000 cells/cm^2 to 3500 cells/cm^2 (Table IV-1 page 41, correlation coefficient = 0.97, $p < 0.001$). In the introduction we hypothesized that minimal 20 times multiplication is needed for cartilage tissue engineering. For the lower seeding densities (3500 cells/cm^2 and 7500 cells/cm^2) the required 20 times multiplication was reached after passage 2 or 3 within 3-4 weeks. With higher seeding densities multiplication was insufficient after four passages. A remarkable observation was that donor I had a much higher cell yield after multiplication than donor II. For donor I the number of chondrocytes harvested from a culture flask at confluency (saturation density) was much higher (Table IV-2 page 42). The saturation density was fairly constant for each donor regardless the amount of chondrocytes seeded initially (Table IV-2 page 42).
With decreasing seeding density and thus increasing total expansion, both GAG/DNA production and collagen type II expression after three weeks redifferentiation culture were decreased (Table IV-1 page 41). Collagen type I, a marker of chondrocyte dedifferentiation, was always expressed after redifferentiation culture. However, it was variable and did not show any correlation with the seeding density used during expansion. The 11-fibrau staining, another dedifferentiation marker, also showed that chondrocytes after redifferentiation culture were still not fully redifferentiated. No correlation

between seeding density and the amount of 11-fibrau positive cells was present. The third important matrix component of ear cartilage is elastin. However, elastin expression after redifferentiation culture was low, ranging from 0.6 % to 6 % positive cells (data not shown).

Experiment 2: passaging

In experiment 2, chondrocytes from four donors were expanded at 7500 cells/cm^2 and after each passage a redifferentiation culture in alginate was performed. Passaging and the resulting increased expansion were correlated with a decrease in GAG/DNA production during subsequent redifferentiation culture ($p = 0.040$, Table IV-3 page 42 and Figure IV-1 page 43). For all donors except donor V, the collagen type II expression after redifferentiation decreased with higher passage number. Comparable to experiment I, collagen type I expression was variable and not correlated with passaging and resulting expansion. The amount of 11-fibrau positive chondrocytes after redifferentiation culture tend to increase with higher passage number. Cells of donor I and III were dividing rapidly and had a high saturation density in monolayer (the amount of cells at confluency). These cells reached sufficient multiplication for cartilage tissue engineering (20 times) in the second passage. Except for donor IV, sufficient multiplication for cartilage tissue engineering (20 times) was reached before passage three. Remarkably, the subsequent redifferentiation culture after 20 times multiplication showed that GAG production and collagen type II expression were comparable to chondrocytes expanded at high seeding density for 4 passages, that had not multiplied enough yet (experiment I).

Discussion

The expansion of isolated donor chondrocytes is an important step in cartilage tissue engineering. Without a sufficient number of cells an engineered construct will definitely fail. To obtain a clinical application, expansion culture should yield large amounts of cells in a short period of time. Secondly, these expanded chondrocytes should preferably express or at least be able to re-express their chondrocyte phenotype (redifferentiation capacity) in order to produce a functional extracellular matrix. Thus, the number of cells and the redifferentiation capacity are important outcome parameters in cartilage tissue engineering. The seeding density used in monolayer culture has been known to be a powerful factor that can influence both outcome parameters. In the present study we used different seeding densities in monolayer to multiplicate human ear chondrocytes and study the effects on total expansion and subsequent redifferentiation capacity. Our results are consistent with previous publications and the generally accepted opinion that both low (text continues on page 43)

Passage	Multiplication (cumulative)		Days from seeding to confluency		µgGAG/µgDNA (value ± SD)		%Collagen type II positive cells		% Collagen type I positive cells		% 11-Fibrau positive cells	
	Donor I	Donor II	Donor I	Donor II	Donor I	Donor II	Donor I	Donor II	Donor I	Donor II	Donor I	Donor II
3500 cells/cm²												
P1	11.4	5.7	13	12								
P2	9.7 (110)	5.6 (32)	14 (27)	9 (21)		39.2 ± 2		18		38		100
P3	13.1 (1,448)	13.2 (421)	9 (36)	10 (31)								
P4	15.8 (22,800)	10.0(4,213)	12 (48)	7 (38)	22.8 ± 5.1	11.0 ± 2.7	13	2	53	42	54	100
7500 cells/cm²												
P1	6.3	3.2	10	12								
P2	4.3 (27)	2.3 (7.4)	7 (17)	7 (19)		30.9 ± 5.2		34		23		95
P3	8.0 (217)	3.3 (24)	7 (24)	5 (24)								
P4	6.6 (1,417)	4.7 (111)	7 (31)	7 (31)	29.1 ± 9.5	19.6 ± 0.9	30	25	81	16	78	100
15,000 cells/cm²												
P1	3.0	1.7	10	10								
P2	2.4 (7.2)	1.7 (2.9)	7 (17)	4 (14)		38.0 ± 5.9		55		39		75
P3	4.0 (29)	1.5 (4.4)	7 (24)	5 (19)								
P4	2.9 (84)	2.5 (10)	7 (31)	5 (24)	34.1 ± 20	23.2 ± 4.6	16	27	57	35	85	89
30,000 cells/cm²												
P1	1.7	1.1	10	10								
P2	1.6 (2.7)	1.4 (1.5)	7 (17)	4 (14)		32.3 ± 4.2		49		35		NA
P3	1.4 (3.8)	1.4 (2.2)	7 (24)	5 (19)								
P4	1.5 (5.7)	0.8 (1.7)	7 (31)	5 (24)	55.5 ± 8.8	28.7 ± 2.1	37	34	75	NA	56	97
r_s	- 0.970	-0.959			0.648	0.876						
p-value	<0.001	<0.001			0.023	<0.001						

NA = no data available

Table IV-1 Expansion and redifferentiation capacity of ear chondrocytes in relation to seeding density in monolayer culture. Chondrocytes from two donors were seeded in monolayer at different densities and after passage 2 (donor II) and passage 4 (donor I and II) a culture in alginate was performed. Spearman's coefficients (r_s) and p-values were determined for correlation between increasing seeding density from 3500 cells/cm² to 30,000 cells/cm² and expansion or µgGAG/µgDNA produced (for chondrocytes from passage 4).

	Average Saturation Density at seeding densities of			
	3500 cells/cm^2	**7500 cells/cm^2**	**15,000 cells/cm^2**	**30,000cells/cm^2**
Donor I	43,000	46,015	45,343	46,347
Donor II	28,176	24,344	26,872	33,581
Donor III		41,920		
Donor IV		20,697		
Donor V		28,752		

Table IV-2 The average saturation densities from passage 1 to passage 4 are shown for all experiments. Note that the average saturation density was donor-dependent and independent of seeding density or passage number.

Passage	**Multiplication factor (cumulative)**	**µgGAG/µgDNA** (value ± SD)	**%Collagen type II positive cells**	**%Collagen type I positive cells**	**% 11-Fibrau positive cells**
Donor I					
P1	6.3	NA	NA	NA	NA
P2	4.3 (27)	NA	NA	NA	NA
P3	8.0 (217)	NA	NA	NA	NA
P4	6.6 (1417)	29.1 ± 9.5	30	81	78
Donor II					
P1	3.2	NA	NA	NA	NA
P2	2.3 (7.4)	30.9 ± 5.2	34	23	95
P3	3.3 (24)	33.7 ± 2.9	39	14	84
P4	4.7 (111)	19.6 ± 0.9	25	16	100
Donor III					
P1	7.3	45.3 ± 6.4	65	34	44
P2	5.4 (39)	33.5 ± 2.5	48	15	83
P3	4.5 (177)	28.5 ± 5.2	20	43	49
P4	5.5 (976)	12.4 ± 0.8	7	37	84
Donor IV					
P1	2.3	45.8 ± 1.4	42	20	70
P2	2.0 (4.6)	10.8 ± 0.1	22	32	90
P3	3.5 (16)	12.0 ± 0.3	26	29	95
P4	3.6 (58)	12.2 ± 0.6	14	19	100
Donor V					
P1	2.8	8.0 ± 0.5	26	11	67
P2	4.0 (11)	15.8 ± 1.3	49	25	100
P3	4.2 (47)	9.4 ± 1.4	46	38	NA
P4	4.6 (216)	13.2 ± 3.2	47	18	100
Statistics					
r_s	0.884	-0.311	-0.488	0.066	0.612
p-value	<0.001	0.040	0.065	0.814	0.020

NA = no data available

Table IV-3 Expansion and redifferentiation capacity of chondrocytes seeded at 7500 cells/cm^2 from passage and p-values are determined for correlation between passaging and subsequent expansion or redifferentiation capacity parameters.

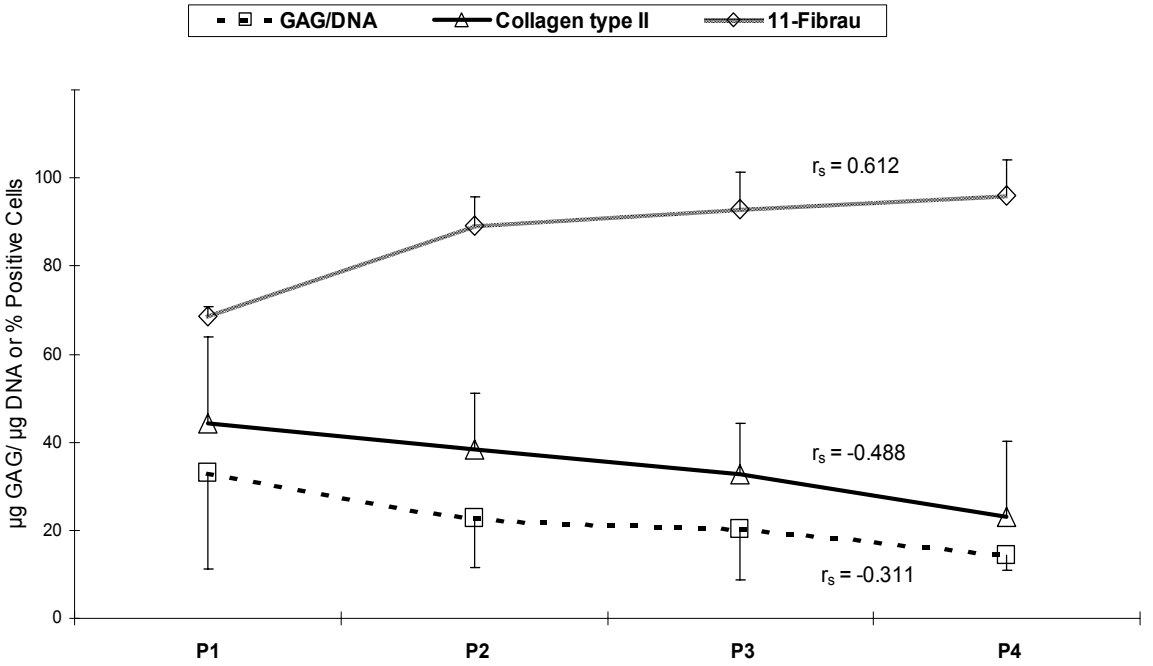

Figure IV-1 The relation between cell passage number and cell redifferentiation parameters. The average amount of µgGAG/µgDNA, the average percentage of cells positive for collagen type II and 11-fibrau after redifferentiation culture are shown for P1 to P4 chondrocytes expanded at a seeding density of 7500 cells/cm^2 (average calculated from donor II, III, IV and V). Both the Spearman's correlation coefficients (r_s) and a line drawn between the averages indicate a relation between passaging from P1 to P4 and a subsequent redifferentiation capacity.

seeding densities and ongoing passaging promote expansion but also decrease redifferentiation capacity [55, 59, 112, 211]. Total cell expansion increased dramatically with lower seeding densities, with up to 2500-4000 times more multiplication with a seeding density of 3500 cells/cm^2 compared to 30,000 cells/cm^2. However, with this increase in total expansion the redifferentiation capacity after alginate culture, indicated by GAG production and collagen type II expression, decreased significantly. Several mechanisms have been proposed by which lower seeding densities result in more dedifferentiation. Firstly, cells seeded at lower density have to multiply more until confluency is reached. Furthermore, with lower seeding density the cell-cell contact inhibition will be less. This has been shown to inhibit chondrogenic differentiation in chick mesendermal cells [209]. Moreover, secreted factors by the chondrocytes that condition the medium can have a role in stabilizing phenotype [59, 175]. These factors will have lower concentrations in low seeding density cultures. Another proposed mechanism of dedifferentiation is the changing cell shape during monolayer culture. Changes in cell shape are thought to be a (morphologically visible) result of changes in microfilament organization

that have been suggested to have a direct effect on chondrogenic phenotype *in vitro* [10, 16, 63, 108, 114].

With the correlation's we found between seeding density, total multiplication and subsequent redifferentiation capacity we tried to deduce a pathway to multiplicate chondrocytes in monolayer culture for which the resulting total expansion, culture time and redifferentiation capacity is adequate for clinical purposes. In theory two different pathways can be chosen to obtain an adequate number of cells. Expansion culture with high seeding densities (30,000 cells/cm^2 and 15,000 cells/cm^2) could have the advantage of a better-retained phenotype, whereas more time in culture and more passages would be needed. On the contrary, expansion with low seeding densities (3500 cells/cm^2 and 7500 cells/cm^2) would need less time and less passages to reach an adequate number of cells. However, loss of phenotype would be more prevalent. As a guideline to find the optimal pathway we presumed a cartilage defect of 4 cm^2 area and 5 mm thickness. To treat such a cartilage defect with the required minimal of $20 \cdot 10^6$ cells/ml [158], we need $40 \cdot 10^6$ cells. The donor cartilage we used yielded a minimum of $2 \cdot 10^6$ chondrocytes after digestion. Thus, with $2 \cdot 10^6$ donor cells from a biopsy a minimum of 20 times multiplication is needed. By using low seeding densities of 3500 cells/cm^2 or 7500 cells/cm^2 20 times multiplication was reached between passage 2 and passage 3 for most donors (< 28 days). Chondrocytes seeded at a density of 15,000 cells/cm^2 and 30,000 cells/cm^2 only rarely reached 20 times multiplication after 4 passages. Therefore, the redifferentiation capacity of both groups cannot be compared directly. However, when for example the results from donor I are extrapolated, chondrocytes seeded at 30,000 cells/cm^2 would have reached a sufficient number of cells at passage 7 taking 49 days. After such prolonged expansion culture the redifferentiation capacity is equal or even lower than chondrocytes expanded at lower seeding densities. Thus, for a clinically relevant amount of multiplication the advantages of using low seeding densities, less time in culture and fewer passages needed are in balance with or will even outweigh the negative effects of low seeding densities on differentiation level. The advantage of using fewer passages could have several grounds. Holtzer et al. showed by using labeled globulin that up to 20% of chondrocytes can be injured after trypsinisation and are probably lost [59]. Also, the whole purpose of passaging is seeding the chondrocytes in a lower density again, with the previously mentioned negative effects on phenotype.

Further analyzing the data shows that the correlation between higher multiplication (by decreasing seeding density or continued passaging) and decreased capacity applies for each donor individually, but it does not apply when results between donors are compared. The number of cells per unit of area at confluency was donor dependent and independent of initial seeding density (Table IV-2 page 42). The number of cells per unit of area at confluency was previously reported by Kolettas to be approximately 10,000 cell/cm^2 for human articular chondrocytes and was referred to as the saturation density [90]. In our

experiment with human ear chondrocytes the saturation density ranged from approx. 21,000 cell/cm^2 to 46,000 cells/cm^2 for the different donors. Higher 67saturation density resulted in higher total expansion. However, higher saturation density and hence higher total expansion did not result in a lower redifferentiation capacity. On the contrary, expansion culture with a chondrocyte population with high saturation density took less time, less passaging and showed a better preserved redifferentiation capacity. Again, less time in culture, less passaging, or a combination of these, seems beneficial for cellular performance in redifferentiation culture. For example, comparing donor III with donor IV. The mean saturation density for donor III was 41,920 cell/cm^2 and for donor IV 20,697 cells/cm^2. When chondrocytes from both donors had to be expanded 20 times, the chondrocytes from donor III reached this number at passage 2, while chondrocytes from donor IV reached 20 times multiplication between passage 3 and 4. Furthermore, chondrocytes of donor III showed a better redifferentiation capacity, GAG/DNA production was higher ($p = 0.05$) and more cells were positive for collagen type II (48% versus 26%). We assume that the relatively large variation between the saturation densities of the different donors could be due to differences in internal biological activity (other than donor age, which did not correlate with the results) or due to the presence of subpopulations of cells in monolayer. Although the perichondrium was dissected from the donor cartilage before digestion, we can not exclude that a small amount of perichondrium cells was included in expansion culture. The perichondrium has been known to contain cells that have chondrogenic progenitor potentials [191, 199]. Expansion of these cells could result in a relative overgrowth. Furthermore, cartilage itself has different subpopulations. Several reports have stated the possibility that chondrocytes from the superficial layer also show progenitor potentials [5, 194] . The progenitor cells might expand better and retain better capacity to express the cartilage phenotype. Other factors that influence saturation density total expansion and redifferentiation were excluded as much as possible. Interobserver variability was ruled out as one person performed culturing and decided when cultures were confluent. Furthermore, for all expansion cultures one serum-batch was used. Finally, correcting protruding ears is an elective operation; therefore all the obtained cartilage for the experiments was of patients in normal health.

Ear cartilage could prove to be a useful source of chondrocytes for articular cartilage tissue engineering. It has the advantage of a high cell yield and it is metabolically very active, although elastin production *in vitro* has been demonstrated to be poor [57, 116, 159, 165]. Furthermore, ear cartilage is easier to harvest than autologous articular cartilage and relatively large pieces can be harvested without cosmetic problems. Finally, harvesting ear cartilage seldomly causes large donor site morbidity [46].

Although we have used young ear chondrocytes for our experiments we do not anticipate that using adult ear chondrocytes will show a completely different

relationship between seeding density, passaging and redifferentiation capacity. We do hypothesize that using lower seeding densities in monolayer for adult chondrocytes also would be advantageous. Details of growth rate, saturation densities of adult ear chondrocytes in monolayer, as well as a possible worse redifferentiation capacity, have to be investigated. Also, for our experiments we have used DMEM with 10% FCS. Recently, a growing number of reports show that using a (serum-free) medium supplemented with growth factors enhances cell yield during expansion cultures [116, 127]. Concurrently, these defined expansion media promote redifferentiation capacity [73, 127]. We previously published data in which SFM with high concentration FGF2 (Fibroblast Growth Factor 2) was used for expansion of young ear chondrocytes seeded at 40,000 cells/cm^2 in monolayer [116]. Although total multiplication was only slightly increased, redifferentiation capacity increased significantly compared to chondrocytes expanded in 10% FCS. Future experiments will indicate whether FGF2 also enhances the lower redifferentiation capacity of chondrocytes expanded at low seeding densities.

In summary, we show a relationship between initial seeding density, passaging and the subsequent redifferentiation capacity of human ear chondrocytes in monolayer culture. For cartilage tissue engineering purposes we propose that expansion culture with low seeding densities is preferable. This will result in an adequate number of cells, using less time and fewer passages than expansion culture with high seeding densities. Importantly, the redifferentiation capacity of these cells is preserved.

Acknowledgements

The authors would like to thank the Department of Plastic and Reconstructive Surgery for providing human ear cartilage.
The monoclonal antibodies, II-II6B3 and M38 were obtained from the Developmental Studies Hybridoma Bank (DSHB) maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. This work was funded by the Dutch Ministry of Economic Affairs in a grant of Senter (nr. BTS00021).

V

Fibroblast Growth Factor 2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture

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Matrix Biology 2004 Jul; 23(4): 231-241

Abstract

The loss of the differentiated phenotype (dedifferentiation) during the expansion culture of donor chondrocytes remains a large problem in cartilage tissue engineering. Dedifferentiated chondrocytes produce other matrix components and therefore the tissue produced will be of less suitable quality. Previously, the addition of FGF2 to a SCM (Serum-Containing Medium) during expansion culture was shown to have positive effects on the phenotype of articular chondrocytes. In the present study we focused on a more defined, SFM (Serum-Free Medium), to expand chondrocytes in monolayer culture for the purpose of cartilage tissue engineering. Adult human ear chondrocytes were expanded in SFM supplemented with 100 ng/ml FGF2. Expansion culture in a conventional SCM (10% fetal calf serum) served as control. The cell yield during expansion culture in SFM with FGF2 was significantly higher compared to SCM. In addition, chondrocytes expanded in the SFM with FGF2 expressed a more differentiated phenotype at the end of monolayer culture, as indicated by higher gene expression ratios of collagen type II to collagen type I and aggrecan to versican. Also, a higher gene expression of Sox9 was found. Next, suspension in alginate and subsequent culture *in vitro* or subcutaneous implantation in nude mice was used to evaluate the capacity of the chondrocytes, expanded in either medium, to re-express the differentiated phenotype (redifferentiation) and to form cartilage. The observed beneficial effects of the SFM with FGF2 on the chondrocyte phenotype at the end of monolayer culture were sustained on both transcriptional and extracellular level throughout both redifferentiation methods.

Introduction

Cartilage has limited intrinsic repair mechanisms. Therefore, cartilage defects beyond a certain magnitude will not heal spontaneously and can lead to significant joint morbidity [64, 180]. During the last 15 years new techniques, grouped under the name of tissue engineering, are being developed to treat cartilage defects using culture- expanded donor cells. Classically, 2D-monolayer culture on a plastic surface with serum-supplemented medium is used for expansion culture. A serious side effect of monolayer expansion culture is loss of chondrocyte phenotype (dedifferentiation) [1, 59, 208]. During monolayer culture chondrocytes spread onto the culture flask and expression gradually shifts from collagen type II and aggrecan to collagen type I and versican, molecules characteristic for a more fibroblast-like phenotype [208]. In an attempt to improve the quality of tissue engineered cartilage by culture-expanded chondrocytes, various expansion media supplemented with growth factors were recently developed [32, 73, 116, 126, 127, 154, 159, 178, 195]. The most widely used growth factor for this purpose is FGF2 (Fibroblast Growth Factor 2). Previous studies showed that addition of FGF2 to the conventional SCM (Serum-Containing Medium) increased both cell yield during monolayer expansion culture and the capacity of expanded chondrocytes to re-express cartilage specific molecules during subsequent 3D culture *in vitro* [126, 127]. Controversially, it was shown in these studies that FGF2 increased dedifferentiation during monolayer culture [73, 127].
Most studies with growth factors were performed in the presence of serum. Addition of growth factors to serum-supplemented media could influence the actions of the growth factors due to possible interacting factors in the serum [202]. Also, large variations in bioactivity can exist between different serum batches, making the reproducibility of results difficult [215]. In a previous study we were able to multiply immature ear chondrocytes in a defined SFM (Serum-Free Medium) with FGF2 and thereby enhancing the quality of the matrix generated lateron in 3D tissue formation, compared to chondrocytes multiplied in medium with 10% serum [116]. However, in that study we used high cell densities in monolayer (40,000 cells/cm^2), which limited cell multiplication. The aim of the present study is to evaluate and further elucidate the effects of SFM with FGF2 on cell expansion and redifferentiation capacity of human adult chondrocytes. Therefore, adult human ear chondrocytes were expanded in monolayer culture in either defined SFM with 100 ng/ml FGF2 (SFM+FGF2) or conventional SCM without additional growth factors. Next, the ability of expanded chondrocytes to re-express the differentiated phenotype (redifferentiation) and to form cartilage was assessed via suspension in an alginate gel. The alginate-cell suspension was either cultured *in vitro* or implanted subcutaneously in nude mice after seeding into a biomaterial. Quantitative RT-PCR technique was used in combination with biochemical assays and histology to analyze the effects of our experimental medium with FGF2 on cellular phenotype at different stages of the process and the quality of extracellular matrix produced.

Materials and Methods

Chondrocyte expansion

Pieces of ear cartilage from three adults (resp. 18, 43 and 46 years old) , obtained as leftover material from patients undergoing reconstructive surgery of the nose using ear cartilage, was used. The perichondrium was removed and cartilage was digested for 1.5 hours with pronase (Sigma, St Louis, Mo) followed by overnight digestion with collagenase B (Boehringer Mannheim, Germany). Chondrocytes were seeded in monolayer using two different densities: 7500 cells/cm^2 and 15,000 cells/cm^2. Expansion culture was performed using either a SFM (SFM) or serum-containing control medium, thus creating four different conditions (Figure V-1 next page). SFM was composed of DMEM (Dulbecco's modified eagle medium, Life Technologies, Breda, the Netherlands; Glutamax-I) supplemented with ITS+ (1:100; Becton Dickinson, insulin, transferrin, selenious acid, bovine serum albumin, linoleic acid) and 100 ng/ml FGF2 (recombinant human, Instruchemie, Hilversum, The Netherlands). Control medium consisted of DMEM with 10% heat-inactivated FCS (Fetal Calf Serum). Gentamycine (50 µg/ml; Life Technologies) and fungizone (1.5 µg/ml; Life Technologies) were added to prevent microbial growth. Chondrocytes in the serum-free conditions were cultured in 10% FCS for one day to promote cell adhesion in the culture flasks and to ensure that cell adhesion was not influenced by the experimental medium. At subconfluency the cells were detached from culture flasks using trypsin-EDTA (Life Technologies) and cell multiplication from seeding till trypsin treatment is determined. At the start of each new passage, chondrocytes were again seeded at their original seeding density of either 7500 cells/cm^2 or 15,000 cells/cm^2 and the serum-free conditions were cultured for one day in 10% FCS. Medium was changed two times a week. Expansion culture was stopped after the third passage (25 days for SCM and 28 days for SFM+FGF2) and cells were suspended in alginate for redifferentiation culture.

In vitro redifferentiation culture

To promote redifferentiation, expanded chondrocytes were suspended in 1.2% alginate in physiological saline ($4 \cdot 10^6$ cells/ml) [12]. Beads were prepared and cultured in a 24-wells plate with twelve beads per well in a defined SFM to stimulate redifferentiation [116, 201, 215] : DMEM supplemented with ITS+, 10 ng/ml IGF1 (Insulin-like Growth Factor 1, recombinant human, Boehringer Mannheim,Germany), 10 ng/ml TGFß-2 (Transforming Growth Factor β-2, recombinant human, R&D systems, Abington, UK) and L-ascorbic acid 2−phosphate (25 µg/ml, freshly added). Culture medium (50 µl was used per bead) was changed every other day. After three weeks beads were harvested. Part was used immediately for RNA extration, the rest was stored at –20 °C until further biochemical and immunohistochemical analyses (Figure V-1 next page).

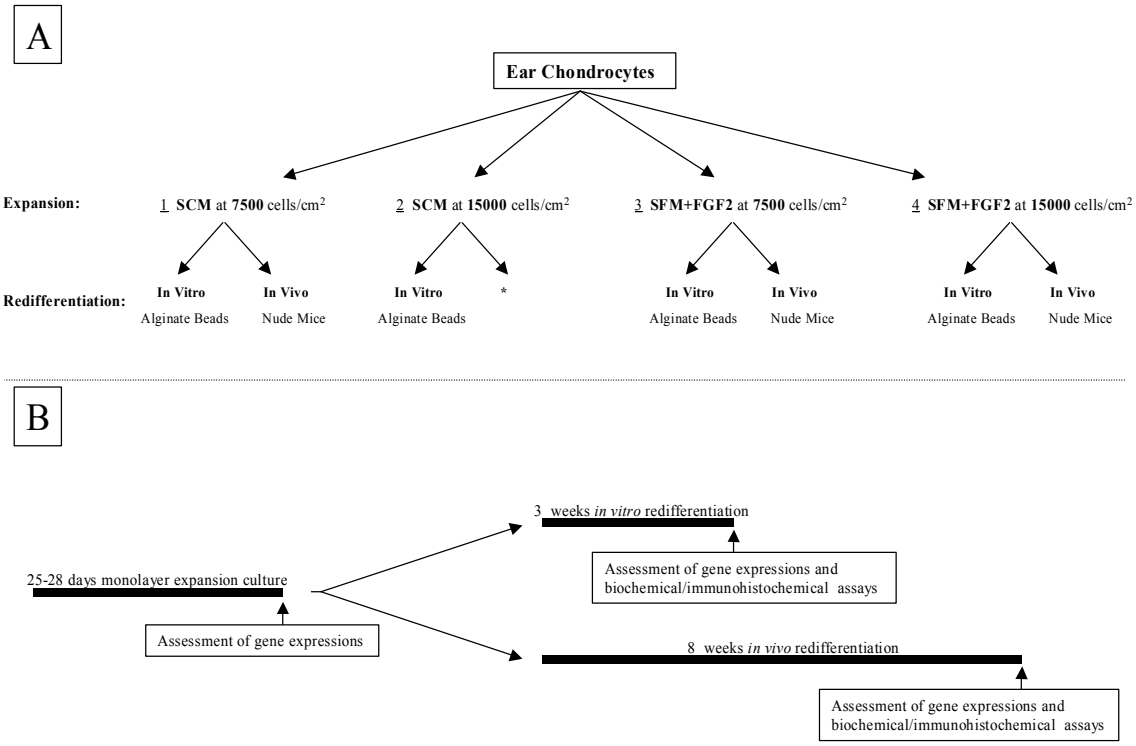


Figure V-1 A schematic overview of the experiments:

A= Chondrocytes from each donor were culture-expanded in monolayer using four different conditions**. (1)** Serum-containing medium (SCM) at a seeding density of 7500 cells/cm^2, **(2)** Serum-containing medium at 15,000 cells/cm^2, **(3)** serum-free medium with FGF2 (SFM+FGF2) at 7500 cells/cm^2 and (**4)** serum-free medium with FGF2 at 15000 cells/cm^2. After expansion culture the chondrocytes were suspended in alginate and cultured *in vitro* (alginate beads) or *in vivo* (subcutaneous implantation in nude mice).

***** Condition seeded at 15,000 cells/cm^2 in SCM failed to generate sufficient cells to perform experiments in nude mice.

B= An overview to underscore significant time points during the experiments. Gene expression profiles were assessed at the end of monolayer culture, the end of three weeks *in vitro* redifferentiation culture and after 8 weeks subcutaneous implantation in nude mice. Alongside, the amount and quality of the extracellular matrix produced during either redifferentiation method was also assessed at these end points.

In vivo redifferentiation

Expanded chondrocytes were suspended in 1.8% alginate at a concentration of $20 \cdot 10^6$ cells/ml [149]. Demineralized trabecular bone matrix (DBM, bovine collagen type I, Osteovit, Braun, Oss, The Netherlands), cut into cores of 3 mm height and 8 mm diameter, were impregnated with alginate-cell suspension for 15 minutes and put into 102 mM $CaCl_2$ for 20 minutes allowing the alginate to gel.

Subsequently, the constructs were washed two times in plain DMEM. As an in-vivo redifferentiation model the constructs were implanted subcutaneously in six nude mice (male, SWISS Nu/Nu, Charles River). Approval for this animal experiment was obtained from the local ethical committee (116-02-01). Four pockets were created subcutaneously; one pocket near each limb. For each condition two construct were implanted, one in the pocket close to the forelimb and one close to the hindlimb. In total four constructs were implanted per mouse. The six mice were housed in two filter-top cages. Mice were sacrificed using cervical dislocation eight weeks after implantation and the constructs were harvested (Figure V-1 previous page). Each construct was divided into four equal parts; one part for paraffin embedding was fixed in formaline, the other three parts, one for biochemical assays, one for cryo-sectioning and one part for RNA isolation were stored at –80 °C. Due to insufficient cell yield during expansion culture, the condition 15,000 cells/cm^2 in combination with SCM was not used for *in vivo* alginate experiments.

Biochemical analysis

Alginate Beads

Each sample contained three alginate beads and for each condition three samples were analyzed. Alginate beads were dissolved in 150 µl of 55 mM sodium citrate in 150 mM sodium chloride per bead and divided into two parts to determine DNA content and GAG (GlycosAminoGlycan) amount.

The amount of DNA was determined fluorometrically with Hoechst 33258 dye, after papain digestion (final concentration 125 µg/ml; Sigma, St Louis, MO) using calf thymus DNA (Sigma) as a standard. GAG was determined after extraction in 4 M Guanidine HCl using dimethylmethylene blue assay [41]. Absorption ratio's at 540 nm and at 595 nm were measured and the amount of GAG was calculated using a standard of chondroitin sulfate C (shark; Sigma).

Alginate/DBM contructs

Samples were weighted and a Proteinase K digestion was used to digest the samples overnight at 56 °C. The reaction buffer contained TRIS EDTA (pH = 7.6) with 0.19 mg/ml iodoacetamide (ICN Biomedical, Ohio, USA), 10 µg/ml Pepstatin A (ICN Biomedicals, Ohio, USA) and 1 mg/ml Proteinase K (Boehringer Mannheim, Germany). The next day the samples were heated to 100 ^{o}C for 10 minutes in order to inactivate proteinase K. Next, the amount of GAG and DNA was measured as described above under *Alginate beads*.

Immunohistochemistry

Alginate Beads

Three alginate beads per condition were dissolved in sodium citrate and cytospins were made which were stained for collagen type I, collagen type II and

elastin. The percentage of positive cells for each cytospin was determined by randomly selecting and counting three times 100 cells. Controls for the staining procedures were made by following the same staining routine but by omitting the first antibody. For collagen staining the cytospins were fixed in acetone and treated with 1% hyaluronidase (Sigma). For elastin staining the cytospins were fixed in formalin and treated with 0.1% trypsin. Sections were incubated with monoclonal antibodies against procollagen type I, collagen type II (1:100 M38 and 1:100 II-II6B3 respectively; Developmental Studies Hybridoma Bank) or elastin (BA4 1:50, Sigma) for two hours. Finally, alkaline phosphatase conjugated antibodies in combination with a new fuchsin substrate (Chroma, Kongen, Germany) were used to obtain a red signal in positive cells.

Alginate/DBM constructs

Alkaline phosphatase conjugated anti-mouse F_{ab}-fragments (GAMAP, 1:400) were coupled overnight with monoclonal anti-collagen type II (1:100 II-II6B3) or anti-elastin (1:1000 BA-4) antibodies. Two hours before staining 0.1% normal mouse serum was added. Paraffin embedded histological slices were deparaffinised using xyleen and alcohol. For collagen staining, the sections were incubated with 1 mg/ml pronase and subsequently with hyaluronidase. For elastin staining the sections were incubated with trypsin. Next, the sectons were incubated with the conjugated antibody for two hours. Subsequent steps were identical to the immunohistochemical staining on cytospins.

Quantification of gene expression

Isolation of total RNA

Cells in monolayer were rinsed with phosphate-buffered saline, homogenized in RNA-Bee™ (TEL-TEST, Inc; Friendswood, TX, USA) by scraping and stored at –80 °C. Alginate beads were dissolved in ice-cold sodium citrate/EDTA buffer. Cell pellets were suspended in RNA-Bee™ and stored at –80 °C. Alginate/DBM constructs were snap frozen in Liquid nitrogen, disrupted for one minute at 47 Hz in a Micro-Dismembrator-S (B. Braun Biotech International GmbH, Melsungen, Germany), then homogenized in RNA-Bee™ and frozen at –80 °C.

RNA was precipitated with 2-propanol and purified with lithium chloride (cells) or RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) (alginate/DBM constructs).

Reverse transcriptase

Quantities and purity of extracted total RNA were determined spectrophotometrically at 260 and 280 nm. Ratios between 1.7 and 2.0 indicated reasonable purity. Total RNA was quantified using Ribogreen™ reagent (R-11490, Molecular Probes Europe BV, Leiden, The Netherlands) prior to cDNA

synthesis using RevertAid^{TM} First Strand cDNA Synthesis Kit (#1622, MBI Fermentas, Germany).

Primer and probe design and TaqMan® assay
All Taqman assays were performed in triplicates in 96-well optical plates using qPCR^{TM} Core Kit (#RT-QP73-05; Eurogentec Nederland B.V., Maastricht, Nederland B.V.). Oligonucleotide sequences and assay concentrations for *COLI, COLII, COL10, CSPG2 (versican)*, and *GAPDH* were adopted from [124]. The other primers and probes were designed using PrimerExpress1.5 software (Applied Biosystems, Foster City, CA, USA) the way that the probe binds to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. Specificity of primer sets was checked by BLASTN search and gel electrophoretic analysis of amplicons after reverse transcription PCR. Gene expression was quantified relatively using the $2^{-\Delta\Delta CT}$ formula from ABI Prism 7700 Sequence Detection System user bulletin #2 . The amount of target was normalized to endogenous GAPDH expression, with the efficiencies amplification of the targets and that of the reference being approximately equal. Primer and probe nucleotide sequences for gene amplification were as follows:

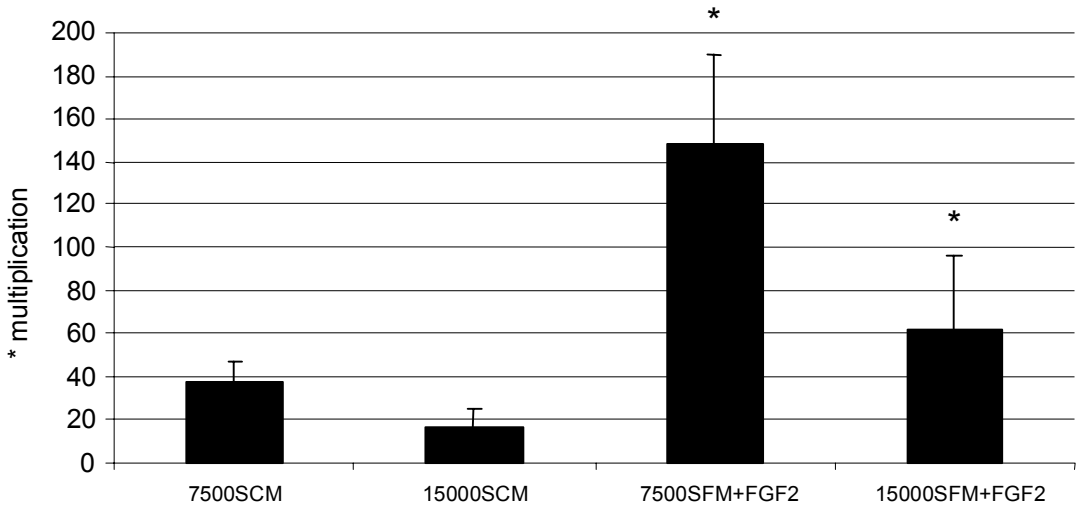
	Access. No.	Primers and position (5'-3')	Probe (FAM) (5'-3')
AGC1	NM_001135	*Forward 1066* GGCTACCCCGACCCCTC *Reverse* TCCACAAAGTCTTCACCTCTGTGT *1127*	TCCCGCTACGACGCCATCTGC
ALPL	NM_000478	*Forward 1074* GACCCTTGACCCCCACAAT *Reverse* GCTCGTACTGCATGTCCCCT *1122*	TGGACTACCTATTGGGTCTCTTCGAGCCA
ELN	NM_000501	*Forward 927* AGCCGCTAAGGCAGCCA *Reverse* ACCAACTACTCCCGGGCC *988*	TATGGAGCTGCTGCAGGCTTAGTGCC
Sox9	NM_000346	*Forward 765* CAACGCCGAGCTCAGCA *Reverse* TCCACGAAGGGCCGC *825*	TGGGCAAGCTCTGGAGACTTCTGAACG

Data Analysis
Differences in multiplication, GAG production, DNA content, the percentages of positive cells on cytospins and relative gene expression, among experimental groups were analyzed using the Mann-Whitney U test for non-parametric nalysis. A p-value < 0.05 was considered to indicate a statistically significant difference.

Results

Monolayer expansion culture

On average four times more proliferation was observed with SFM with FGF2 (SFM+FGF2) compared to SCM (Figure V-2 next page). The highest number of cells was found with 7500 cells/cm^2 in SFM+FGF2, where at least 7 doublings had occurred after 3 passages. In all conditions, Q-RT-PCR data obtained from chondrocytes at the end of monolayer culture showed a very low expression of aggrecan and collagen type II, which is typical for chondrocyte dedifferentiation (Figure V-3A page 58). The degree of dedifferentiation can be assessed by the ratios of expressed collagen type II to collagen type I and aggrecan to versican (COL2/COL1 and AGC/VERS). At the end monolayer culture, for chondrocytes expanded in SFM+FGF2 both ratios were higher than for chondrocytes expanded in SCM, indicating less dedifferentiation. For collagen this was the result of significant lower collagen type I expression in chondrocytes expanded in SFM+FGF2 compared to SCM. On the proteoglycan level however, the higher ratio of differentiation was caused by a combination of higher aggrecan expression and lower versican expression. Expression of *Sox9* was 3-5 times higher in chondrocytes expanded in SFM+FGF2, whereas elastin was significantly lower expressed compared to chondrocytes expanded in SCM (Table V-1 next page).



* = a statistical significant increase in total multiplication compared to SCM at similar seeding density.
7500SCM = expansion in serum-containing medium at 7500 cells/cm^2.
15000SCM = expansion in serum-containing medium at 15,000 cells/cm^2.
7500SFM+FGF2 = expansion in serum-free medium with FGF2 at 7500 cells/cm^2.
15000SFM+FGF2 = expansion in serum-free medium with FGF2 at 15,000 cells/cm^2.

Figure V-2 Total chondrocyte multiplication after three passages in monolayer for each of the four conditions used. Averages ± SD of three donors are shown

Monolayer	*Sox9*	*Elastin*	*Alkaline Phosphatase*	*Collagen type X*
7500 SCM	**6.1*10^{-3}**±1.4*10^{-3}	**0.09**±0.03 * #	**270*10^{-6}**±24*10^{-6} * #	**3.3*10^{-7}**±2.1*10^{-7}
15000 SCM	**8.6*10^{-3}**±3.7*10^{-3}	**0.06**±0.03 *	**340*10^{-6}**±20*10^{-6} *	**1.6*10^{-7}**±0.59*10^{-7}
7500 SFM+FGF2	**0.04**±2.6*10^{-3} *	**0.01**±3.5*10^{-3}	**0.57*10^{-6}**±0.39*10^{-6}	**2.3*10^{-7}**±0.92*10^{-7}
15000 SFM+FGF2	**0.03**±0.01 * #	**0.01**±3.7*10^{-3}	**2.9*10^{-6}**±2.9*10^{-6}	**9.0*10^{-7}**±13*10^{-7}
In Vitro				
7500 SCM	**0.14**±0.02	**1.17**±1.02	**220*10^{-4}**±240*10^{-4} * #	**0.07**±0.06 * #
15000 SCM	**0.10**±0.03	**0.50**±0.19	**130*10^{-4}**±68*10^{-4} *	**0.04**±0.02
7500 SFM+FGF2	**0.11**±0.04	**0.96**±0.48	**4.1*10^{-4}**±4.3*10^{-4}	**0.01**±2.1*10^{-3}
15000 SFM+FGF2	**0.08**±0.04	**0.85**±0.20	**6.5*10^{-4}**±2.2*10^{-4}	**0.02**±0.01
In Vivo				
7500 SCM	**0.55**±0.31	**10.5**±4.9	**15*10^{-4}**±6.1*10^{-4}	**2.1*10^{-4}**±1.5*10^{-4}
7500 SFM+FGF2	**0.78**±0.21	**6.3**±3.4	**8.2*10^{-4}**±6.1*10^{-4}	**5.4*10^{-4}**±3.1*10^{-4}
15000 SFM+FGF2	**0.76**±0.15	**8.4**±2.3	**7.9*10^{-4}**±3.1*10^{-3}	**5.4*10^{-4}**±3.9*10^{-4}

* = a significant higher expression compared to either SCM or SFM+FGF2 at the same seeding density.
# = a significant difference in gene expression between 7500 SCM and 15,000 SFM+FGF2.
7500 SCM = expansion in serum-containing medium at 7500 cells/cm^2.
15000 SCM = expansion in serum-containing medium at 15,000 cells/cm^2.
7500 SFM+FGF2 = expansion in serum-free medium with FGF2 at 7500 cells/cm^2.
15000SFM+FGF2 = expansion in serum-free medium with FGF2 at 15,000 cells/cm^2.

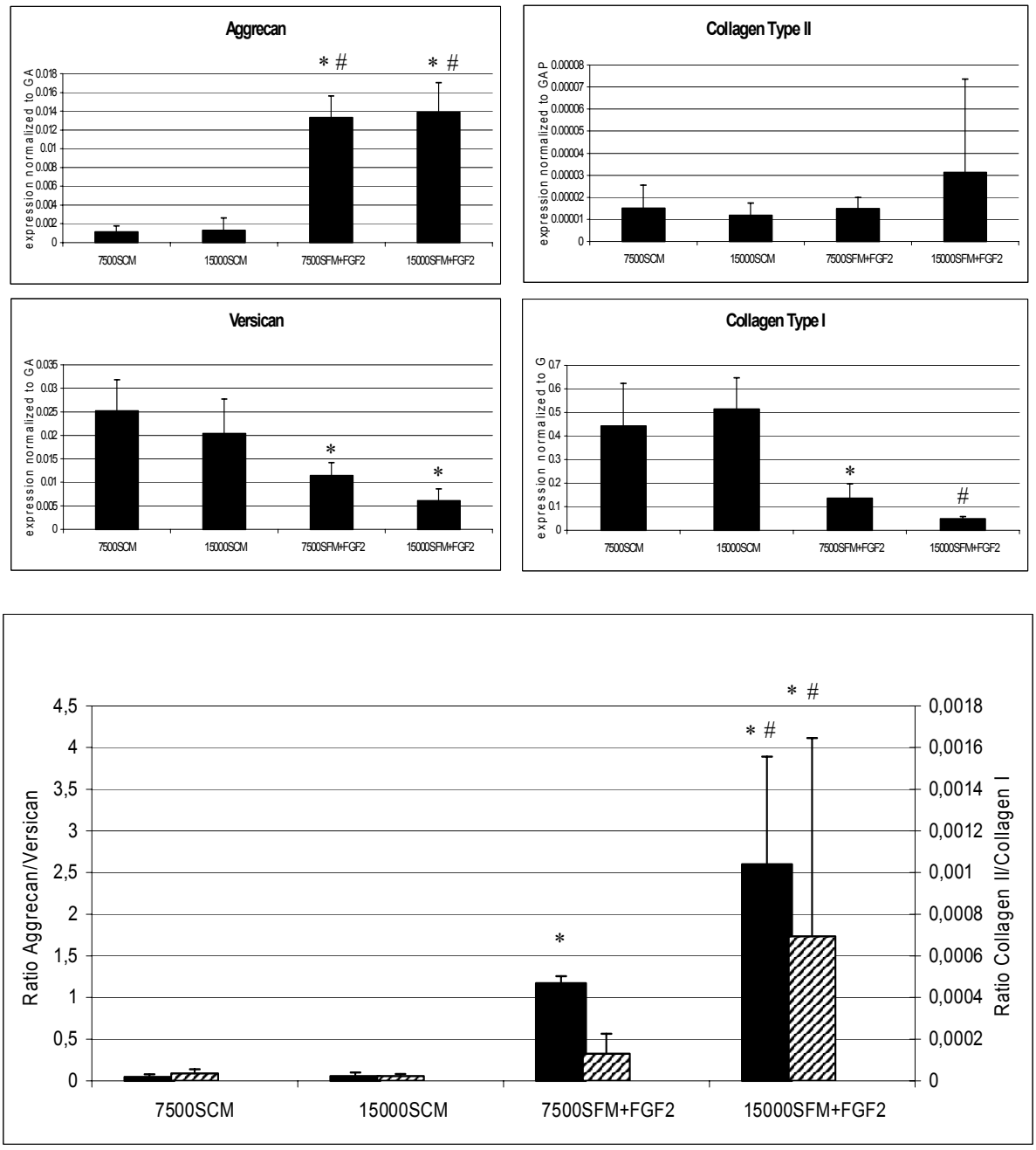
Table V-1 .For the four conditions used during expansion culture the expression of typical genes is shown at harvesting from the monolayer culture, *in vitro* redifferentiation culture and the *in vivo* implantation in nude mice (*in vivo* alginate). Averages ± SD of three donors are shown with expression levels normalized to GADPH.

In vitro redifferentiation culture

At the end of three weeks redifferentiation culture in 1.2 % alginate beads gene expressions were upregulated compared to the monolayer culture, except for versican (Figure V-3B page 59). The higher expression ratio of COL2/COL1 observed at the end of monolayer culture in chondrocytes expanded in SFM+FGF2 compared to SCM was sustained, again due to a lower collagen type I expression. This was confirmed by immunohistochemical staining for collagen type I on cytospins, which showed a similar pattern (Figure V-4 page 61). However, in contrast to gene expression, collagen type II was also significant higher expressed on cytospins when SFM+FGF2 had been used during expansion culture. The gene expression ratio of AGC/VERS at the end of the *in vitro* redifferentiation culture was not significantly different between either expansion medium used (Figure V-3B page 59). Also, on the extracellular matrix level no significant differences were found in total sulfated GAG (Figure V-5 page 61). *Sox9* gene expression levels were similar in the four conditions (Table V-1 page 56). Although elastin gene expression was considerate (Table V-1 page 56), only a maximum of 20% of the chondrocytes were positive for elastin on cytospins, showing no significant difference between expansion medium or seeding density used (data not shown)**.**

In vivo redifferentiation

At harvesting after eight weeks the constructs had a cartilage-like macroscopical appearance. The biochemical assays on GAG and DNA showed that chondrocytes which had been expanded in SFM+FGF2 produced more GAG per milligram tissue than constructs with cells expanded in SCM (Figure V-6 page 62). Histological methods revealed cartilage-like tissue, which was more positive for collagen type II when SFM+FGF2 had been used during expansion culture (Figure V-7 page 62). Higher expression ratios of COL2/COL1 were found for chondrocytes expanded in SFM+FGF2 with a similar AGC/VERS ratio in all conditions (Figure V-3C page 60). Elastin expression levels were similar in all conditions. However, immunohistochemical staining for elastin was only faintly positive, with highest staining in constructs initially seeded with chondrocytes expanded in SCM. Expression of chondrocyte hypertrophy markers, such as alkaline phosphatase and collagen type X, was low with no significant differences between the conditions (Table V-1 page 56).



* = statistically significant higher gene expression compared to expansion culture in serum-containing medium (SCM) at the same seeding density.
\# = significant difference in gene expression between 7500SCM and 15000SFM+FGF2.
7500SCM = expansion in serum-containing medium at 7500 cells/cm^2.
15000SCM = expansion in serum-containing medium at 15,000 cells/cm^2.
7500SFM+FGF2 = expansion in serum-free medium with FGF2 at 7500 cells/cm^2.
15000SFM+FGF2 = expansion in serum-free medium with FGF2 at 15,000 cells/cm^2.

Figure V-3A. Gene expression after monolayer only. In the upper four frames, the expressions of four typical genes (aggrecan, versican, collagen type II and collagen type I) is shown, normalized to *GAPDH*. In the lower frame, the ratios of differentiation, i.e. the expression of the 'differentiated' gene divided by the expression of the 'dedifferentiated' gene are plotted. The left y-axis represent the ratios of differentiation on the proteoglycan level (filled bars), whereas the right y-axis represent the ratios of differentiation on the collagen level (shaded bars). Averages ± SD of 3 donors are presented.

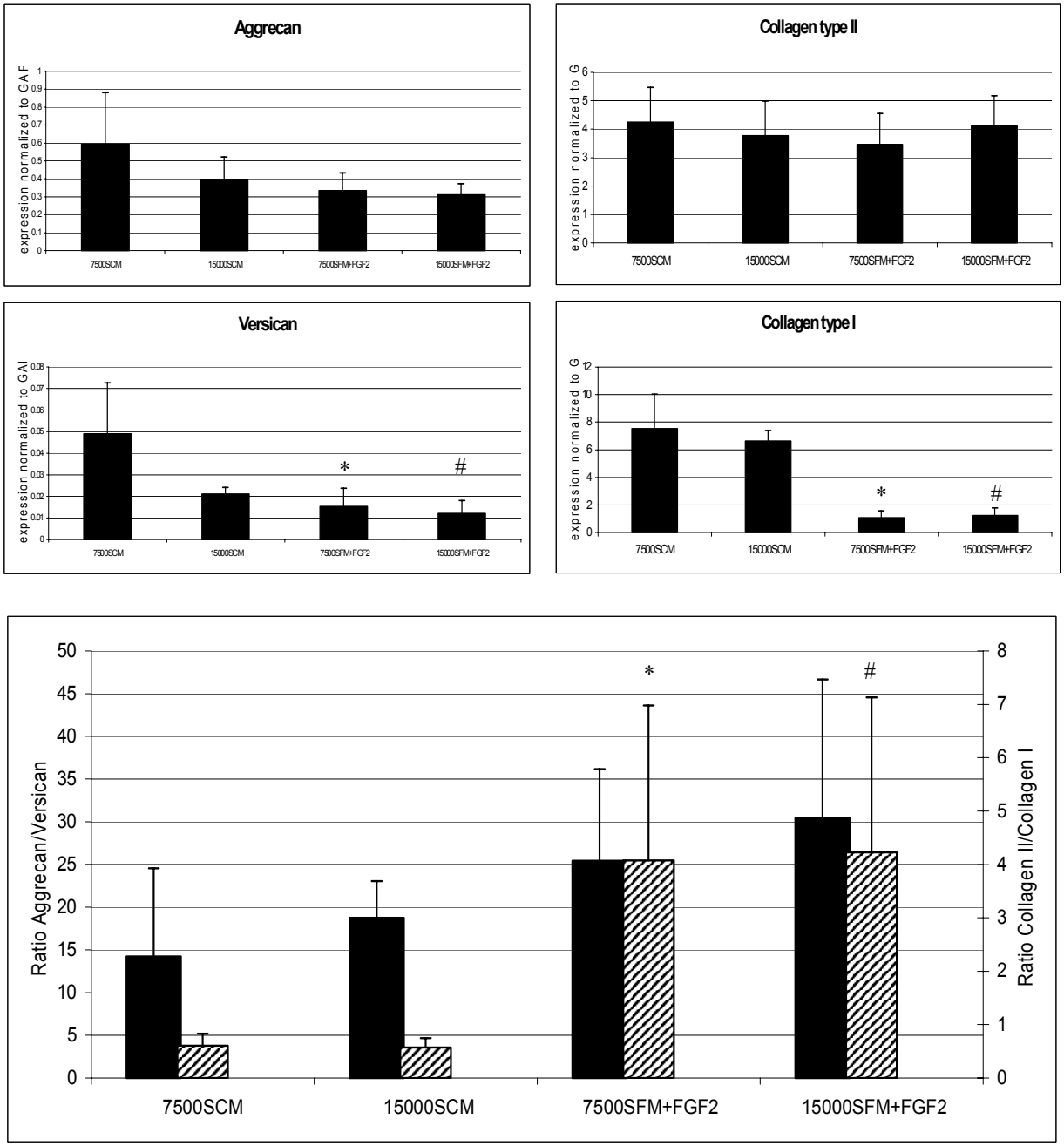


Figure V-3B Gene expression after monolayer (Figure V-3A) and subsequent redifferentiation culture ***in vitro***. In the upper four frames, the expressions of four typical genes (aggrecan, versican, collagen type II and collagen type I) is shown, normalized to *GAPDH*. In the lower frame, the ratios of differentiation, i.e. the expression of the 'differentiated' gene divided by the expression of the 'dedifferentiated' gene are plotted. The left y-axis represent the ratios of differentiation on the proteoglycan level (filled bars), whereas the right y-axis represent the ratios of differentiation on the collagen level (shaded bars). Averages ± SD of 3 donors are presented. For further explanation see preceding Figure V-3A

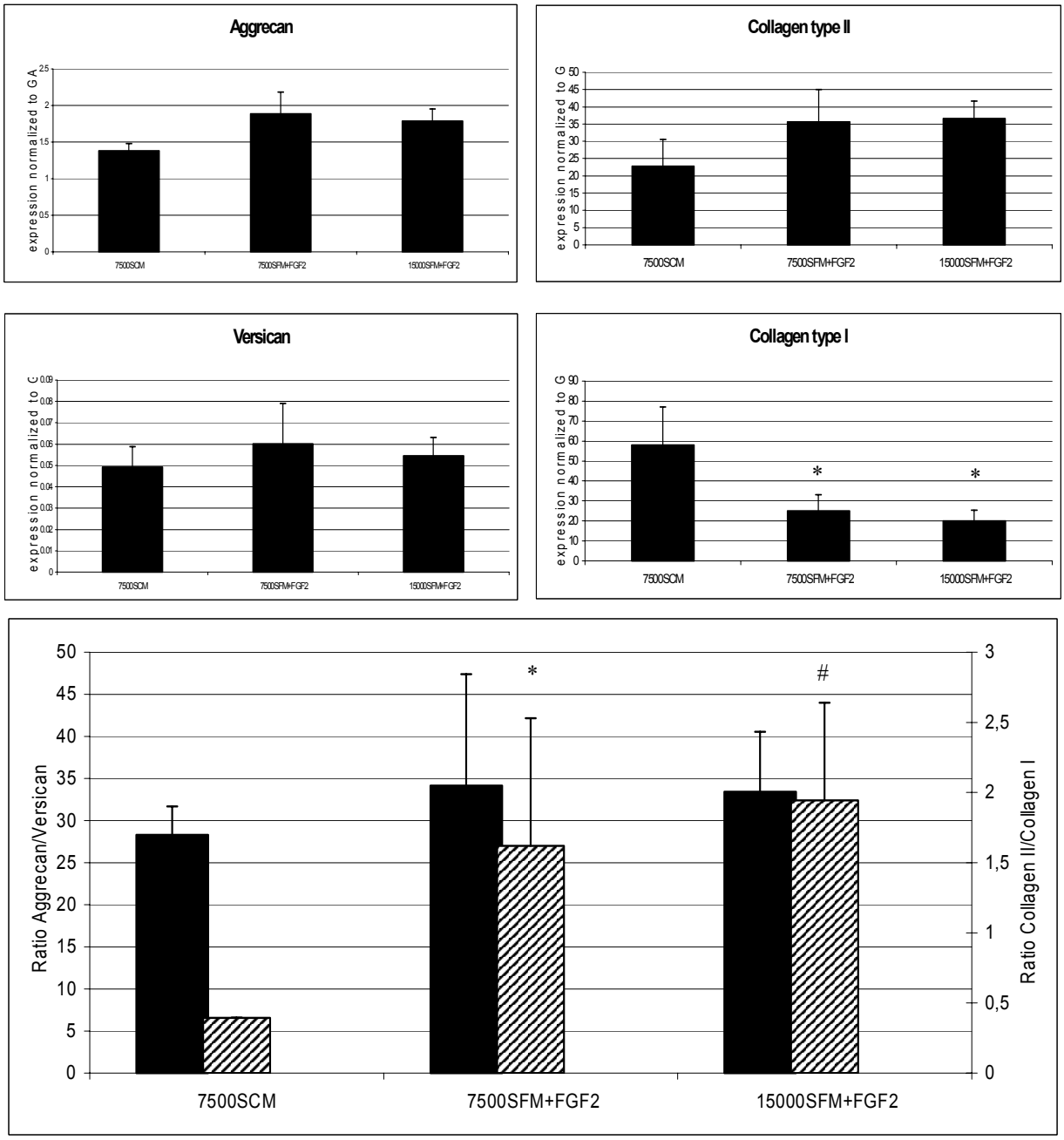
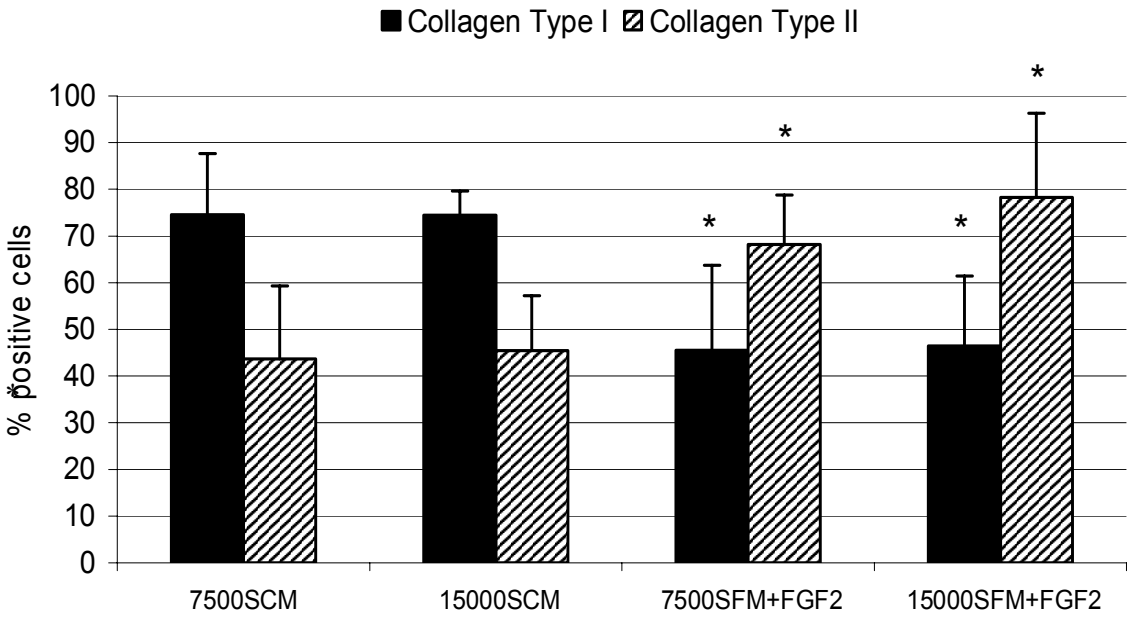
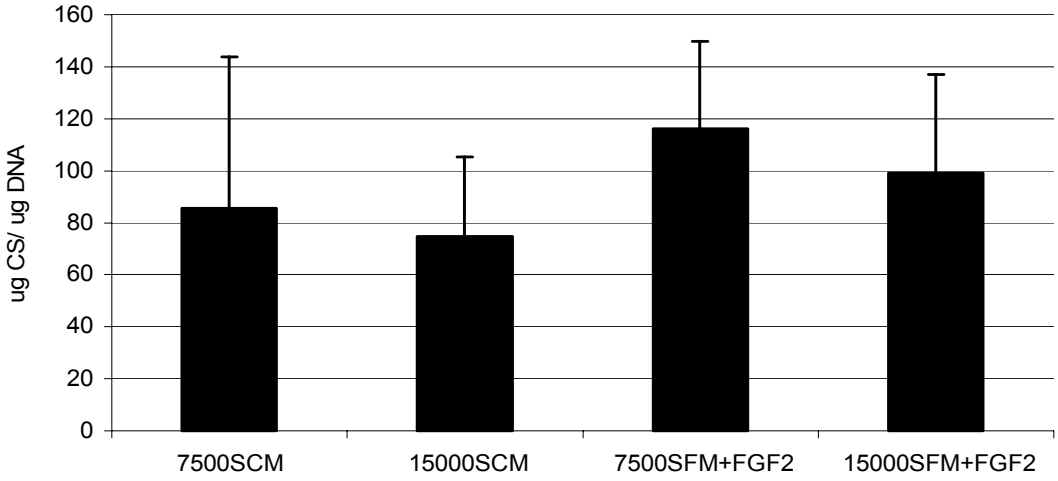


Figure V-3C Gene expression after monolayer and subsequent redifferentiation culture ***in vivo***. In the upper four frames, the expressions of four typical genes (aggrecan, versican, collagen type II and collagen type I) is shown, normalized to *GAPDH*. In the lower frame, the ratios of differentiation, i.e. the expression of the 'differentiated' gene divided by the expression of the 'dedifferentiated' gene are plotted. The left y-axis represent the ratios of differentiation on the proteoglycan level (filled bars), whereas the right y-axis represent the ratios of differentiation on the collagen level (shaded bars). Averages ± SD of 3 donors are presented. For further explanation see Figure V-3A page 58.



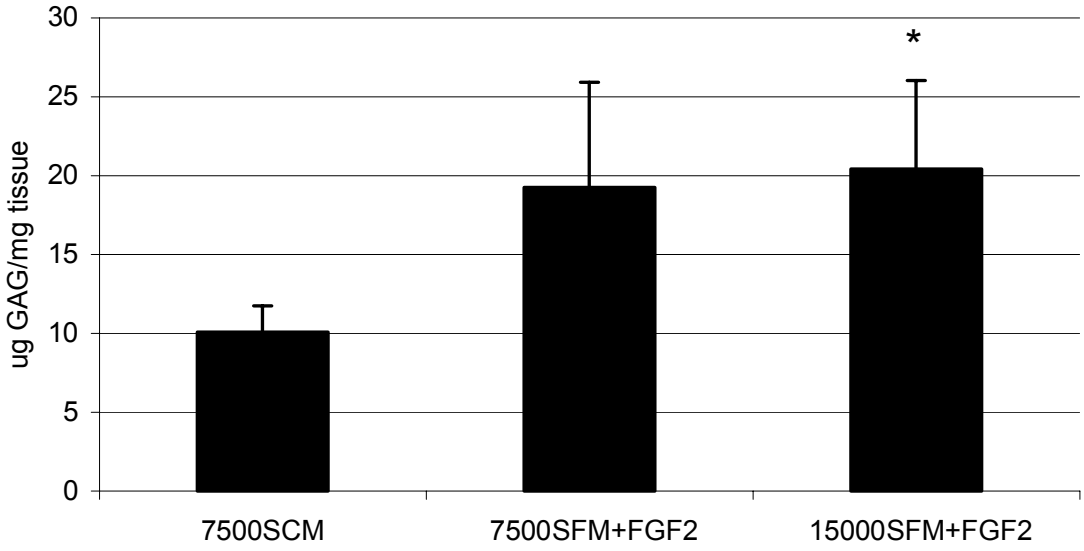
7500SCM = expansion in serum-containing medium at 7500 cells/cm^2.
15000SCM = expansion in serum-containing medium at 15,000 cells/cm^2.
7500SFM+FGF2 = expansion in serum-free medium with FGF2 at 7500 cells/cm^2.
15000SFM+FGF2 = expansion in serum-free medium with FGF2 at 15,000 cells/cm^2.

Figure V-4 The percentage of collagen type I and II positive chondrocytes on cytospins made from the *in vitro* suspension culture. Compared to expansion in serum-containing medium (SCM), the percentage of cells which were positive for collagen type I was significantly lower and for collagen type II significantly higher when chondrocytes had been expanded in a serum-free medium with 100 ng/ml FGF2 (SFM+FGF2). Averages ± sd of three donors are presented.



7500SCM = expansion in serum-containing medium at 7500 cells/cm^2.
15000SCM = expansion in serum-containing medium at 15,000 cells/cm^2.
7500SFM+FGF2 = expansion in serum-free medium with FGF2 at 7500 cells/cm^2.
15000SFM+FGF2 = expansion in serum-free medium with FGF2 at 15,000 cells/cm^2.

Figure V-5 The amount of glycosaminoglycan (GAG) produced during the three weeks *in vitro* alginate culture. Averages ± SD of 3 donors are presented. No significant difference was found between the conditions used during expansion culture.



7500SCM = expansion in serum-containing medium at 7500 cells/cm^2.
15000SCM = expansion in serum-containing medium at 15,000 cells/cm^2.
7500SFM+FGF2 = expansion in serum-free medium with FGF2 at 7500 cells/cm^2.
15000SFM+FGF2 = expansion in serum-free medium with FGF2 at 15,000 cells/cm^2.

Figure V-6 The amount of glycosaminoglycan (GAG) per mg tissue for the alginate/DBM constructs, harvested 8 weeks after subcutaneous implantation in nude mice. Averages ± sd of constructs of 3 donors are presented. The star represents a statistical significant higher GAG/mg tissue in the constructs with chondrocytes expanded with SFM+FGF2 at 15,000 cells/cm^2 compared to constructs with chondrocytes expanded in SCM at 7500 cells/cm^2. Due to an insufficient number of cells the condition '15000SCM' was not included in the *in vivo* redifferentiation experiments.

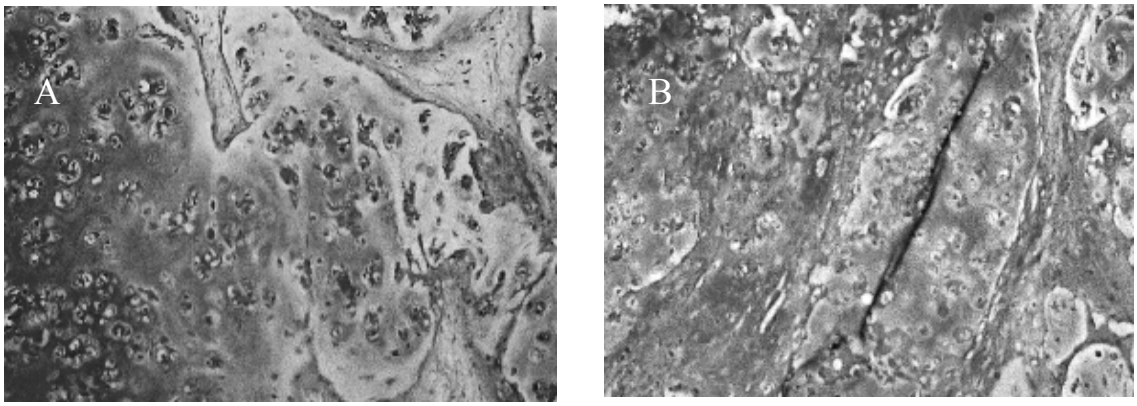


Figure V-7 Collagen type II staining on histological slices from the constructs harvested from the nude mice. A: Slice from a demineralized bone matrix core initially filled with chondrocytes solved in alginate after expansion in serum-containing medium at 7500 cells/cm^2. B: Slice from a demineralized bone matrix core initially filled with chondrocytes solved in alginate after expansion in serum-free medium with FGF2 at 15,000 cells/cm^2.

Discussion

Reducing chondrocyte dedifferentiation during monolayer culture offers a major challenge in cartilage tissue engineering. In the present study we found that expansion of chondrocytes in a defined SFM with a high concentration of FGF2 reduced dedifferentiation and subsequently favored chondrogenic expression during *in vitro* or *in vivo* redifferentiation methods. Alongside, total cell yield after three passages in monolayer was significantly enhanced when SFM+FGF2 had been used instead of SCM. This increase in cell yield could have underestimated the actions of FGF2 on the cellular phenotype, because the process of dedifferentiation during monolayer is enhanced by higher multiplication itself [115, 208, 211]. We have anticipated this, as we expected a difference in cell yield between both media, by using two different seeding densities in monolayer. Total cell yield decreased by doubling the seeding density in the SFM+FGF2 condition, resulting in a non-significant difference in cell yield between expansion in SFM+FGF2 at 15,000 cells/cm^2 and SCM at 7500 cells/cm^2. Comparison of the data of these two conditions is thus less influenced by differences in cell yield (Figure V-3A,B,C pages 58, 59 en 60 and Table V-1 page 56).

Cellular phenotype was assessed by the ratios of mRNA's of COL2/COL1 and AGC/VERS, termed ratios of differentiation. These ratios of differentiation have been demonstrated to decrease with time in monolayer culture [109,110]. Our study shows that the ratios of COL2/COL1 and AGC/VERS are higher after monolayer culture with FGF2 than with 10% FCS. This is mainly due to the lower expression of collagen type I and versican. The possible mechanism for this is assumed as the result of reduction of the dedifferentiation process by FGF2. However, it is possible that it is due to differential effects of FGF2 on proliferation of selective populations in the primary cells, stimulating proliferation of the cells which express collagen type II or suppressing proliferation of the cells, which express collagen type I. Independent of what the mechanism is, we conclude that the cells harvested after expansion in SFM+FGF medium have a less dedifferentiated phenotype. This is in contrast with previous studies by Jakob et al. and Martin et al. [73, 126], in which FGF2 was supplemented to a SCM. These studies showed that FGF2 increased proliferation but decreased gene expression ratios of COL2/COL1 and AGC/VERS during the first two passages in monolayer culture of human articular chondrocytes. Although expression of collagen type I was reduced by FGF2, which is similar to our results, the lower ratio COL2/COL1 must imply that expression of collagen type II was significantly downregulated as compared to SCM alone. It was hypothesized that FGF2 promoted the chondrocytes to dedifferentiate towards a more precursor-like state from which both the ability to multiply and to redifferentiate was enhanced, given the right stimuli. The discrepancy in expression patterns after monolayer culture between our study and the studies by Jakob et al. [73] and Martin et al. [126] may have several reasons. First, these authors

might not have accounted for the higher multiplication rates observed in the condition supplemented with FGF2, which could have increased dedifferentiation during monolayer culture. Furthermore, our experiments were performed with ear chondrocytes, which could respond differently to growth factors than articular chondrocytes. Finally, a serum-supplemented medium with only 5 ng/ml FGF2 was used, whereas our medium contained 20 times more growth factor and no serum.

We conclude that the SFM+FGF2 influenced the chondrocytes in such ways that not only the phenotype at the end of the monolayer culture was improved, but that this effect was sustained during the *in vitro* alginate culture. In contrast, the local conditions in a subcutaneous pocket in a nude mouse and the period of 8 weeks implantation were apparently sufficient to equalize differences in gene expressions we observed directly after the monolayer culture. However, gene expression data were only obtained at the end of monolayer culture and at harvesting of the neo-cartilage after 8 weeks subcutaneous implantation. Whether the differences in gene expression at the end of monolayer culture were equalized instantly after implantation or whether this was a more slowly process is thus not known. Data from the biochemical assays and histology suggest the latter option, as the neo-cartilage produced with SFM+FGF2 expanded chondrocytes contained more GAG and collagen type II.

Next to genes coding for matrix proteins we also determined the expression of *Sox9*, which is a nuclear transcription factor. *Sox9* is an important regulatory factor during the process of differentiation in growth plate chondrocytes, where it is linked to the collagen type II expression [11, 31, 100]. In addition, ectopic *Sox9* has been shown to upregulate collagen type II expression in culture-expanded, and hence dedifferentiated bovine articular chondrocytes [188]. Also, in a recent study by Tew et al. human articular chondrocytes in monolayer culture were transducted with *Sox9* and subsequently (further) passaged [192]. In Sox9-transducted chondrocytes the collagen type II expression was increased compared to controls. In addition, these chondrocytes also showed a sustained higher collagen type II expression during subsequent pellet cultures. Interestingly, Marukami et al. showed that FGF2 stimulated Sox9 expression in murine costal chondrocytes in culture [140]. And ind0eed, our data showed that Sox9 gene expression was higher at the end of monolayer culture when the SFM+FGF2 had been used. However, the higher ratio of differentiation on collagen level we found with SFM+FGF2 was not caused by a (significant) increase in collagen type II expression, but by a significantly lower expression of collagen type I. Therefore, a direct causal correlation between FGF2, Sox9 and collagen type II is not supported by our data. Recently, Schmid et al. also found no correlation between Sox9 and collagen type II expression in normal and osteoarthritic cartilage and suggested that Sox9 is not the key mediator of collagen type II expression [218]. In contrast to collagen type II, our results do suggest a relation between FGF2, Sox9 and aggrecan expression. A relation

between Sox9 and aggrecan expression was previously reported by several other authors [11, 177].

In conclusion, this study provides several important observations. We provide evidence that a defined SFM with FGF2 both increases the cell yield and reduces the dedifferentiation of human ear chondrocytes during monolayer expansion culture compared to the classically SCM. This, together with the possibility to minimize the use of bovine serum during the tissue engineering process by using autologous serum for cell attachment, these positive effects of the SFM with 100 ng/ml FGF2 on ear chondrocytes in monolayer culture is an important step forward in the process of cartilage tissue engineering.

Acknowledgement

The authors would like to thank Dr. G. Nolst-Trenite for providing the ear cartilage. Corrina de Ridder and Cindy Bolder are much appreciated for operating and nursing the nude mice. This work was funded by the Dutch Ministry of Economic Affairs in a grant of Senter (nr. BTS00021). The monoclonal antibodies, II-II6B3 and M38 were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD.

VI

Growth Factors in Cartilage Tissue Engineering

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Biorheology 2002; 39(1-2):215-220

ABSTRACT

Tissue engineering of cartilage consists of two steps. Firstly the cells from a small biopsy of patient's own tissue have to be multiplied. During this multiplication process they lose their cartilage phenotype. In the second step, these cells have to be stimulated to re-express their cartilage phenotype and produce cartilage matrix. Growth factors can be used to improve cell multiplication, redifferentiation and production of matrix. The choice of growth factors should be made for each phase of the Tissue Engineering process separately, taking into account cell phenotype and the presence of extracellular matrix. This paper demonstrates some examples of the use of growth factors to increase the amount, the quality and the assembly of the matrix components produced for cartilage tissue engineering. In addition it shows that the 'culture history' (e.g. addition of growth factors during cell multiplication or preculture period in a 3D environment) of the cells influences the effect of growth factor addition. The data demonstrate the potency as well as the limitations of the use of growth factors in cartilage tissue engineering.

INTRODUCTION

The availability of extra cartilage to reconstruct defects in articular joints or cartilaginous structures in the head and neck area would be of great benefit. Therefore much effort is put into the generation of autologous (patient's own) cartilage using tissue engineering techniques. Generally, tissue engineering involves a two step procedure (Figure VI-1 next page). First cells are isolated from a small biopsy of patient's own cartilage and expanded in-vitro to obtain the cell number required. This process of cell expansion is routinely carried out in monolayer culture. During this culture the cells gradually lose their cartilage phenotype in a process called dedifferentiation [210]. The second step in the tissue engineering process involves seeding of the expanded cells in a 3D carrier and implantation in the defect. An in-vitro culture period can be performed before implantation in-vivo to induce re-expression of the cartilaginous phenotype and to stimulate formation of functional extracellular matrix. The formation of functional extracellular matrix is of utmost importance for the success of the graft. The matrix provides the graft with adequate mechanical properties in order to withstand the forces it is subjected to in-vivo. Formation of functional matrix can be stimulated by the use of growth factors during tissue engineering process.

In-vivo, growth factors are synthesised within a variety of tissues, where they act on their cell of origin (autocrine) or on adjacent cells (paracrine). Certain growth factors are also present in systemic circulation, and the same factor may act both as a systemic and as a local regulator of tissue metabolism. Growth factors are important for the metabolism of chondrocytes. They are present in synovial fluid and reach the chondrocytes by diffusion. Furthermore, growth factors are produced by the chondrocytes themselves. The extracellular matrix of cartilage acts as a store of growth factors that can be released when needed. Various growth factors have been demonstrated to be able to affect chondrocyte multiplication, phenotype and extracellular matrix production and thus can be used during both steps of the tissue engineering process (Figure VI-1 next page). One has to realise however, that growth factors can influence extra-cellular matrix formation and cellular phenotype but that a certain growth factor can have different effects depending on the chondrocyte phenotype and the presence of extracellular matrix. During the cartilage tissue engineering process, both cellular phenotype and the extra-cellular matrix change (Figure VI-2 next page). Thus the choice of growth factors must be made for each stage of the tissue engineering process while taking into account the desired effect as well as the phenotype and presence of extracellular matrix of the cells. We here present a compilation of experiments demonstrating that growth factors can improve the quantity and quality of extracellular matrix components produced in a tissue engineering process. The data show that the effect of a growth factor depends on possible treatments in previous steps of the procedure. The results may help to recognise the potency as well as the limitations of the use of growth factors for cartilage tissue engineering.

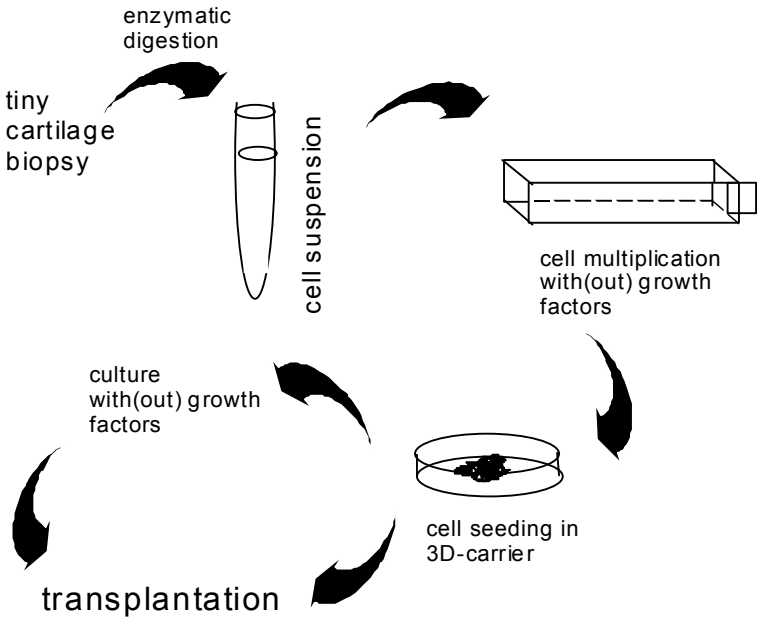

Figure VI-1 Schematic overview of a cartilage tissue engineering process. First cells has to be isolated from a small biopsy of cartilage using enzymatic digestion of the matrix. The cells are expanded in monolayer culture and when sufficient cells are obtained the cells are seeded in a 3 dimensional carrier (biomaterial). The construct can then be transplanted either directly or after in-vitro culture to induce redifferentiation and matrix production.

Small biopsy of cartilage

↓

Cell suspension
Differentiated chondrocytes
No extracellular matrix

↓

Cell Expansion in monolayer
Chondrocytes dedifferentiate = lose their phenotype

↓

3D culture in alginate
Redifferentiation of chondrocytes
Production of extracellular matrix

Figure VI-2 The cellular phenotype and extracellular matrix during different stages of the cartilage tissue engineering process.

MATERIALS AND METHODS

In this paper a compilation of experiments using different types of cartilage is presented. Adult bovine articular cartilage was obtained from metacarpophalangeal joint; Rabbit ear cartilage was prepared from 3 months old New Zealand White rabbits; Adult human articular cartilage was obtained post-mortem from the femoral condyle of road victims without any previous history of joint diseases. Young human ear cartilage was obtained from children undergoing surgery for correction of protruding ears.
In all experiments, cells were isolated from full-thickness cartilage using pronase and collagenase digestion. For multiplication they were cultured in monolayer for 4 passages. To achieve matrix production the chondrocytes were seeded in 1.2% alginate (LV) and cultured for 3 weeks. The amount of GAG (GlycosAminoGlycan) was determined using dimethylmethylene blue (Farndale assay). The amount of DNA was determined using Hoechst 33258 in a fluorimetric assay. Immunohistochemistry was performed on cytospin preparations with antibodies against collagen type II (II-II6B3, Developmental Studies Hybridoma Bank), procollagen type I (m38; Developmental Studies Hybridoma Bank) and a membrane marker for fibroblasts (11-fibrau, Imgen distributed by ITK diagnostics, Uithoorn, the Netherlands).
For some of the monolayer cultures rhFGF2 (Instruchemie, Hilversem, the Netherlands) was used. For cultures in alginate ITS+ (1:100; Becton Dickinson Labware, Bedford, MA), rhIGF1 (Roche Diagnostics, Mannheim, Germany) and rhTGFß-2 (R&D systems, Abingdon, UK) were used.

RESULTS AND DISCUSSION

Matrix production

For production of cartilage matrix a three-dimensional environment is required. Culture in alginate gel is a generally accepted method. To further increase the production of extracellular matrix, growth factors can be added to medium with 10% serum. IGF1 (Insulin-like growth factor 1) is described to be the major anabolic factor for cartilage. TGFß-2 (Transforming Growth Factor ß-2) has been found to be able to have both stimulating and inhibitory effects on cartilage. Addition of IGF1 or TGFß-2 (in a dose of 10 or 25 ng/ml) to freshly isolated, differentiated chondrocytes in alginate increases matrix production with ~50% [197, 198]. However, the data are of limited use for tissue engineering procedures because normally we use multiplied chondrocytes that have lost their phenotype in stead of freshly isolated, differentiated chondrocytes. When added to dedifferentiated chondrocytes, TGFß-2 stimulates GAG production even better than in differentiated cells (2.2x vs 1.6x;) [198]. However, it does not stimulate re-expression of cartilage specific collagen type II.

Serum-free culture

In the serum used for previous studies, many growth factors are present in variable and unknown concentrations. In addition, many inhibitors of growth factors are also present. It has been demonstrated that the presence of serum can influence the effect of growth factors [207]. Therefore, the use of serum in experiments with growth factors creates a very badly defined system with results that are difficult to predict and reproduce. This is why we prefer a serum-free culture system for tissue engineering procedures, especially when growth factors are supplemented. When serum in our alginate culture experiments is replaced by 10 ng/ml IGF1 and 10 ng/ml TGFß-2, production of collagen type II in dedifferentiated cells can be stimulated. This stimulation of redifferentiation in SFM (Serum-Free Medium) with IGF1 and TGFß-2 is shown for various types of chondrocytes (references for rabbit ear [201]; rabbit nasal septum [196]; human ear [201]; human nasal septum [196]; human articular [215]), although the amount of stimulation was variable.

Matrix assembly

Besides the amount of matrix produced, the assembly of matrix components is also important for the functionality of the matrix. The extra-cellular matrix of cartilage is structured in a pericellular compartment (~1-2 µm surrounding the cell), a territorial matrix (~5 µm) and an interterritorial matrix. The alginate culture system offers the unique possibility to quantify the accumulation of matrix components in cell-associated compartment (which is believed to correspond to the pericellular and the territorial matrix) and further-removed compartment (believed to correspond to the interterritorial matrix) [53]. The cell-associated matrix is in close contact with the cell and therefore largely influences cell behaviour. The interterritorial matrix is supposed to be most important for the gross mechanical properties of the cartilage. Addition of growth factors to medium with serum has been demonstrated to change the assembly of GAG. After addition of IGF1 to differentiated bovine articular chondrocytes in alginate relatively more of the produced GAG is laid down in the cell-associated matrix than in the further-removed matrix compartment. On the contrary, addition of TGFß-2 shifts the deposition of GAG from the cell-associated matrix more towards the further-removed matrix compartment [197]. Furthermore, dedifferentiated chondrocytes cultured in alginate in medium with 10% serum divide the newly synthesised GAG differently over the two compartments than differentiated chondrocytes do; relatively more GAG is laid down in the cell-associated matrix of dedifferentiated cells [119]. This might be a way for the cell to keep its direct environment as optimal as possible. When serum is replaced by IGF1 and TGFß-2, the division of GAG over the two compartments by dedifferentiated cells is comparable with differentiated cells [119]. This demonstrates that growth factors not only affect the production of matrix components, but also the assembly of these components in the matrix.

'Culture history'

The effect of a growth factor not only depends on cellular phenotype but also in a more general way the 'culture history' of the cells before addition of a growth factor is important for the effect of this growth factor. This not only involves the difference between freshly isolated, differentiated cells and culture expanded, dedifferentiated cells, but also more subtle differences. For example, if cells are surrounded by extracellular matrix, the response of the cell to growth factor addition can be altered. This might be explained, for example, by an interaction between integrins and growth factor receptors [56, 179]. In the experiments described above, growth factors were added starting from the moment the cells were cultured in alginate. Before the cells were seeded into alginate they were either freshly isolated from the cartilage tissue or multiplied in monolayer culture in medium with 10% serum. In another set of experiments we demonstrated that TGFß-2 stimulates matrix production when supplemented to alginate cultures immediately, but has no effect on matrix production when addition starts off for the first time after 3 weeks culture in alginate [198]. This appears to be due to the presence of extracellular matrix surrounding the cell, produced during the 3 weeks pre-culture in alginate. Removing this previously synthesised matrix, makes the cells again respond to TGFß-2 by increased matrix production.

Besides the 3D culture history, the history in 2D monolayer can also influence the response to growth factors during alginate culture. Addition of FGF2 (Fibroblast Growth Factor 2) in monolayer has been demonstrated to affect the capacity of the cells to re-express their phenotype [73, 127]. We added 5 ng/ml FGF2 to SCM (Serum-Containing Medium) for expansion of human articular chondrocytes. In the subsequent alginate culture, these cells have increased production of GAG and collagen II upon stimulation with IGF1 and TGFß-2, compared to cells expanded in medium with serum alone. Finally, the effect of IGF1 and TGFß-2 on GAG and collagen II production in alginate culture is identical for cells expanded in medium in which serum had been replaced by ITS+ and 100 ng/ml FGF2. This shows that ITS+ and FGF2 can imitate the effect of serum. However, next to evaluating chondrocyte phenotype using the most common determinants (GAG and collagen II) we have previously demonstrated the usefulness of additional markers, i.e. the anti-fibroblast marker 11-fibrau, that can be thought of as a dedifferentiation marker [200]. The 11-fibrau antibody does not bind to differentiated chondrocytes and binding is increased with time in monolayer culture when cells dedifferentiate. 11-fibrau still binds to chondrocytes that have been expanded in medium with serum and subsequently 'redifferentiated' in alginate in SFM with IGF1 and TGFß-2. This indicates that these cells, although expressing collagen type II, are not (yet) fully redifferentiated. However, when using a SFM with ITS+ and 100 ng/ml FGF2 to expand the chondrocytes, expression of fibroblast marker 11-fibrau is absent after alginate culture. This implicates that addition of FGF2 during expansion culture influences the response of cells to IGF1/TGFß-2 during alginate culture.

A relation between the group of FGF2, Sox9 and chondrocyte matrix production is suggested [31]. Sox9 is a transcription factor, which is responsible for the differentiation of mesenchymal stem cells to chondrocytes. Also, Sox9 promotes upregulation of several chondrocyte-specific marker genes including the genes for collagen type II and aggrecan. FGF2, including FGF2, can enhance the expression of Sox9 and can alter the response of cells to other growth factors.

Conclusion and future perspectives

In summary, growth factors can be used in different phases of the tissue engineering process to stimulate the formation of a functional tissue to obtain better results for cartilage grafting procedures. The aim is to improve mechanical properties of the graft and of the interface between graft and adjacent cartilage. This is achieved by stimulating both the formation and proper assembly of matrix components. The optimal choice of growth factors will vary with time during the tissue engineering process and depends on the changing phenotype and presence of extracellular matrix components. This is a highly complex process.

Until now all in-vitro experiments to evaluate the effect of growth factors are performed in free-floating (unloaded) cultures. This provides valuable information for the use of growth factors during such an in-vitro procedure. However, this does not necessarily mean that these growth factors will be valuable for in-vivo use in the joint, for example coupled on a biomaterial or in a gene delivery system. In the joint, the construct will be mechanically loaded. Although nothing is known about the effects of growth factors during simultaneous loading it is very likely that there will be some effects. This will be an important topic of future studies in our laboratory.

Acknowledgements

This work was partly funded by the Dutch Technology Foundation (STW), applied science division of NWO and the technology programme of the Ministry of Economic Affairs and partly by the Dutch Ministry of Economic Affairs in a grant of Senter (BTS 00021).

VII

Considerations on the use of ear chondrocytes as donor chondrocytes for cartilage tissue engineering

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Biorheology 2004; 41(3-4): 411-21

Abstract

Articular cartilage is often used for research on cartilage tissue engineering. However, ear cartilage is easier to harvest, with less donor-site morbidity. The aim of this study was to evaluate whether adult human ear chondrocytes were capable of producing cartilage after expansion in monolayer culture.

Cell yield per gram ear cartilage was twice as high as for articular cartilage. Moreover, ear chondrocytes proliferated faster. Cell proliferation could be further stimulated by the use of SFM (Serum-Free Medium) with FGF2 instead of medium with 10% serum. To evaluate the chondrogenic capacity, the multiplied chondrocytes were suspended in alginate and implanted subcutaneously in athymic mice. After 8 weeks the constructs demonstrated a proteoglycan-rich matrix that contained collagen type II. Constructs of ear chondrocytes showed a faint staining for elastin. Quantitative RT-PCR revealed that expression of collagen type II was 2-fold upregulated whereas expression of collagen type I was 2-fold down regulated in ear chondrocytes expanded in SFM with FGF2 compared to SCM (Serum-Containing Medium). Expression of alkaline phosphatase and collagen type X were low indicating the absence of terminal differentiation.

We conclude that ear chondrocytes can be used as donor chondrocytes for cartilage tissue engineering. Furthermore, it may proof to be a promising alternative cell source to engineer cartilage for articular repair.

Introduction

Tissue engineering techniques might offer possibilities of restoring missing or damaged cartilage structures. Although significant progression has been made during the last decade, some major problems still have to be addressed before widespread clinical use can be expected. The chondrocytes, being the key factor of tissue engineering, will largely determine the quality of the engineered tissue. Thus the choice of donor cell source is of utmost importance. For clinical tissue engineering, an autologous cell source is a prerequisite, which will limit the risk of infection, disease transmission and potential immune rejection. Consequently, treatment of cartilage defects with tissue engineering techniques will most often be performed with donor tissue of mature origin, as cartilage defects are most commonly found in adult patients [17]. However, compared to immature tissues, mature tissues are often characterised by lower cell yield and lower metabolism, which could have disadvantageous effects on the quality of the engineered repair tissue [3, 80, 134, 196].

Cartilage itself represents the most obvious choice as autologous cell source. From the various cartilage subtypes existing in the body, articular cartilage is most frequently used for research on cartilage tissue engineering, although some experiments have been published on the use of nasal septum cartilage and ear cartilage [19, 20, 93, 201]. However, articular cartilage as a donor cell source has some obvious disadvantages: it is frequently harvested from a diseased joint and harvesting itself may induce further degenerative changes in the joint. In this respect, ear cartilage has the advantages that it is healthy, easy to harvest and has little donor-site morbidity [50, 133, 144]. However, ear cartilage is elastic cartilage and amongst other things differs from hyaline cartilage by the presence of elastin. It is our goal to investigate the possibility of using ear chondrocytes as donor source for reconstruction of articular cartilage. The aim of this study was to evaluate whether adult human ear chondrocytes were capable of producing cartilage after expansion in monolayer culture. The performance of human ear chondrocytes was compared to human articular chondrocytes using data of various experiments previously performed in our laboratory. Also, porcine chondrocytes were used to compare ear and articular chondrocytes from the same individual cultured at similar conditions. Furthermore, addition of FGF2 (Fibroblast Growth Factor 2) during expansion culture has been demonstrated to support chondrogenic capacity of bone marrow stromal chondrocytes [128]. Therefore, in this study the effect of addition of FGF2 during expansion on chondrogenic capacity of expanded ear chondrocytes after subcutaneous implantation in mice, is evaluated.

Materials & Methods

Cell sources

Adult human cartilage from the ear (three donors) obtained as leftover material from patients undergoing reconstructive surgery of the nose using ear cartilage, was used.

For comparative studies on ear and articular cartilage, adult human cartilage from healthy knees was obtained from road victims (aged 19-40 years) and osteoarthritic knee cartilage was obtained from donors (aged 35-70 years) undergoing surgery for total knee replacement. For additional data on adult human cartilage from the ear, material was used that was obtained as left-over material from donors (aged 26-52 years) undergoing meatoplasty or reconstructive surgery on the nasal septum using ear cartilage. In addition we have used cartilage from healthy knees and ears of porcine origin (approxemately. 4 months old).

Cell isolation

Slices of cartilage were washed with saline and subsequently incubated in Pronase E (Sigma, St Louis, MO, 2 mg/ml) for 2 hours at 37 °C and in Collagenase B (Boehringer Mannheim, Germany;1.5 mg/ml DMEM and 10% fetal calf serum) overnight. The next day cell suspensions were filtered through a 100 μm filter, centrifuged and washed with saline

Monolayer culture

Adult human ear chondrocytes were seeded at 7500 chondrocytes/cm^2 or 15000 chondrocytes/cm^2 and cultured in DMEM (Life Technologies, Breda, the Netherlands) with 50 μg/ml gentamycin and 0.5 μg/ml fungizone with either 10% FCS (Fetal Calf Serum) or in a SFM (Serum-Free Medium) containing ITS+ (1:100, Becton Dickinson, Bedford, MA) and 100 ng/ml FGF2 (Recombinant Human, Instruchemie, Hilversum, the Netherlands) (SFM+FGF2). Porcine chondrocytes were seeded at 7500 chondrocytes/cm^2 and cultured in medium with 10% FCS. Human chondrocytes used for the additional data were seeded at a density of 40,000 chondrocytes/cm^2 and cultured in medium with 10% FCS. When chondrocytes reached confluency they were trypsinized with trypsin-EDTA (Life Technologies).

Subcutaneous implantation in vivo

The expanded chondrocytes were suspended in 1.8% alginate (LV, Keltone) at a concentration of $20 \cdot 10^6$ chondrocytes/ml [149]. Constructs of approximately 3 mm height and 8 mm diameter were prepared by polymerisation of alginate in 102 mM $CaCl_2$ and implanted subcutaneously in athymic mice. This experiment was performed with cartilage of two healthy adult human knee donors, three adult human ear donors, one immature human ear donor and both ear and knee cartilage of one pig.

Animals were housed under sterile conditions at the Center for Animal Research. After eight weeks the animals were killed by cervical dislocation and the constructs were harvested. Animal experiments were approved by the University Ethics Committee and carried out as outlined in the University guidelines for the care and use of laboratory animals, which in general follows the NIH 'Guide for the care and use of laboratory animals'.

Analyses

Constructs after in-vivo implantation were digested in proteinase K solution (1 mg/ml Proteinase K; Boehringer Mannheim, Germany) overnight at 56 °C. The amount of DNA in the beads was measured fluorometrically using Hoechst 33258 dye, using calf thymus DNA (Sigma, St Louis, MO) as standard.

The amount of GAG (GlycosAminoGlycan) was quantified spectrophotometrically by the absorption ratio A_{540}/A_{595} using a metachromatic reaction of GAG with dimethylmethylene blue, with chondroitin sulfate C (Shark; Sigma, St Louis, MO) as a standard.

Histology

Evaluation of tissue engineered constructs after subcutaneous implantation in athymic mice was performed using paraffin sections. Sections were stained with thionin and immunohistochemically stained for collagen II (using mab II-II6B3, Developmental Studies Hybridoma Bank, University of Iowa, under contract N01-HD-6-2915) and elastin (using mab BA4, Sigma, St Louis MO). For procollagen type I (using mab M38, DSHB) and a fibroblast membrane marker (11-Fibrau, Imgen, distributed by ITK diagnostics, Uithoorn, the Netherlands) cryosections were prepared. For all immunohistochemical stainings the primary antibodies were coupled overnight to a secondary antibody directed against mouse, before incubation on the sections.

Gene expression of expanded adult human ear chondrocytes after subcutaneous implantation

Constructs were snap frozen in LiN2 and disrupted in a Microdismembrator-S (B. Braun Biotech International GmbH, Melsungen, Germany). Chondrocytes were then homogenized in RNABee, the RNA was isopropanol precipitated and purified using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany).

Quantities and purity of extracted total RNA were determined spectrophotometrically at 260/280 nm. Ratios between 1.7 and 2.0 indicated reasonable quality. 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAid$^{TM.}$ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany).

Primer and probes were designed using PrimerExpress1.5 software (Applied Biosystems, Foster City, CA, USA) Primer sets were first tested in reverse transcription PCR (RT-PCR). Real-time quantitative PCR analysis was

performed using the ABI 7700 Sequence Detection System (Applied Biosystems) with the following probes:

Aggrecan: 5'-GGCTACCCCGACCCCTC-3' (hAGC1_F),
5'-TCCACAAAGTCTTCACCTCTGTGT-3' (hAGC1_R),
5'-TCCCGCTACGACGCCATCTGC-3' (hAGC1_FAM);

alkaline phosphatase: 5'-GACCCTTGACCCCCACAAT-3' (hALPL_F),
5'-GCTCGTACTGCATGTCCCCT-3' (hALPL_R),
5'-TGGACTACCTATTGGGTCTCTTCGAGCCA-3' (hALPL_FAM);

elastin: 5'-AGCCGCTAAGGCAGCCA-3' (hELN_F),
5'-ACCAACTACTCCCGGGCC-3' (hELN_R),
5'-TATGGAGCTGCTGCAGGCTTAGTGCC-3' (hELN_FAM);).

The primer and probe nucleotide sequences, as well as assay concentrations, for collagen type I, collagen type II, collagen type X, versican, and GAPDH were adopted from [124] All Taqman assays were performed using qPCR™ Core Kit (Eurogentec Nederland B.V., Maastricht, Nederland B.V.) according to user's manual. Per reaction, 300 pg of gene-specific cDNA (assuming 3% mRNA) was amplified in 40 cycles (15 sec at 95 °C, 1 min at 60 °C) after initially activating *Taq* DNA polymerase 10 min at 95 °C.
The Ct value for each sample was defined as the cycle number in PCR at which the fluorescence intensity reached a value significantly above the background. The Ct value of the genes of interest was subtracted from the Ct value of GAPDH. The level of expression for each gene was then calculated as $2^{Ct'gapdh'-Ct'gene\ of\ interest'}$. The efficiency of amplification of each set of primers and probes was always higher than 90%.

Results

Cell expansion in monolayer
Adult human ear chondrocytes (four donors) cultured for 3-4 passages at relatively high seeding densities (40,000 chondrocytes/cm^2) in medium with 10% FCS, showed 3.2 ± 1.6 times multiplication in 32 ± 10 days. Chondrocytes of healthy knees (three donors), expanded 1.3 ± 0.4 times in 42 ± 2 days, whereas chondrocytes of osteoarthritic knees (5 donors) expanded 5.1 ± 4.8 times in 52 ± 13 days. In general, articular chondrocytes more often showed problems with cell attachment and sometimes suddenly ceased to grow at various time points in the monolayer culture.

Using animal experiments we can compare the capacities of ear and knee chondrocytes of the same animal to proliferate under exactly similar conditions. Seeded at 7500 chondrocytes/cm^2 and cultured for 28 days in medium with 10% FCS, porcine ear chondrocytes were passaged three times and yielded 47 times multiplication, whereas knee chondrocytes were passaged only two times in 28 days and cell number increased only 13 times.
Human ear chondrocyte seeded at high densities (40,000 chondrocytes/cm^2) and cultured in SFM with FGF2 (SFM+FGF2), expanded similar to serum containing conditions [150]. When lower seeding densities were used (either 7500 chondrocytes/cm^2 or 15,000 chondrocytes/cm^2), expansion of human ear chondrocytes during four weeks was significantly increased. Furthermore, with lower seeding densities, the use of SFM+FGF2 further increased cell expansion on average four times compared to SCM (Serum-Containing Medium). When cultured in SFM+FGF2, initial cell number was expanded 150 times when chondrocytes seeded at a density of 7500 chondrocytes/cm^2 whereas seeded at a density of 15,000 chondrocytes/cm^2, cell number expanded 60 times.

Comparison of cell yields from donor tissue
Besides a better capacity to proliferate, the number of vital chondrocytes isolated per gram of healthy mature ear cartilage was higher than per gram of healthy mature articular cartilage. Average cell yield of human adult healthy ear cartilage (three donors) was $9.7 \pm 0.8 \cdot 10^6$ chondrocytes/gram of tissue, but from human adult healthy knee cartilage (four donors) only $4.9 \pm 1.4 \cdot 10^6$ chondrocytes/gram of tissue were obtained. We propose that 0.5 gram of ear cartilage can be easily harvested from a human ear with minimal cosmetic problems, from which $5 \cdot 10^6$ donor chondrocytes can be routinely obtained for tissue engineering purposes. Previously we have calculated that a minimum of $50 \cdot 10^6$ chondrocytes ($20 \cdot 10^6$ chondrocytes/cm^3 is considered to be a minimum) is needed to repair an average defect of 5 cm^2 and thickness of 5 mm. This implies that the amount of donor chondrocytes has to be expanded approximately 10 times. Expansion from a seeding density of 7500 chondrocytes/cm^2 in medium with 10% FCS would take approximately 3 weeks to reach 10 times multiplication, whereas it would take approximately 2 weeks using SFM+FGF2, considering cell expansion rates we found in this study.

Cartilage matrix production in constructs
At harvesting after eight weeks subcutaneous implantation in athymic mice the constructs with human ear chondrocytes had a cartilage-like macroscopic appearance. On average, the constructs contained 20 µg GAG/mg wet weight, being half the amount present in native articular or nasal septum cartilage [168]. To get a fair comparison, we compared chondrocytes that had been through a similar expansion. Chondrocytes expanded in monolayer at a seeding density of 7500 chondrocytes/cm^2 in SCM had a similar multiplication as chondrocytes

expanded from 15,000 chondrocytes/cm^2 in SFM+FGF2. Chondrocytes that had been expanded in SFM+FGF2 produced 1.5 times more GAG per milligram tissue than constructs with chondrocytes expanded in SCM. On histology, abundant GAG was visible by thionin staining. Collagen type II was expressed in all graft. In constructs of chondrocytes expanded in SCM the expression of collagen type II was lower and appeared to be mainly associated with chondrocytes embedded in alginate (Figure VII-1A below). In the constructs of chondrocytes expanded in SFM+FGF2, expression occurred though out the construct (Figure VII-1B below) with exception of the capsule surrounding the construct.

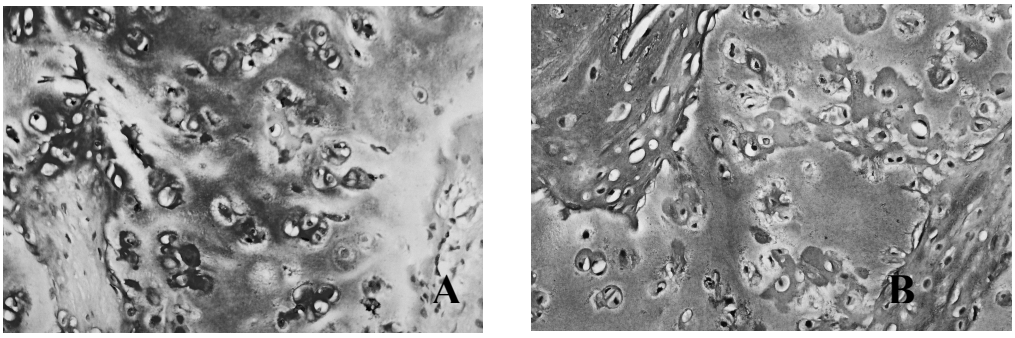

Figure VII-1 Immunohistochemical staining for collagen type II of constructs generated by human adult ear chondrocytes expanded in monolayer from seeding density of 7500 chondrocytes/cm^2 in medium with 10% FCS (A) and from seeding density of 15000 chondrocytes/cm^2 in serum-free medium with 100 ng/ml FGF2 (B). (Magnification 200x)

Procollagen type I staining with antibody M38 was found in the capsule and in the dense, more fibrous-appearing tissue in the construct, but was absent in chondrocytes associated with alginate. Immunohistochemical staining for elastin was only faintly positive. However, differences could be found within and between the constructs. In general, dense more fibrous-appearing tissue between alginate remnants showed the most intense staining for elastin (Figure VII-2A next page). On locations were alginate was still present (showing by amorphous substance between the chondrocytes), hardly any staining was found when chondrocytes were expanded with SFM+FGF2 (Figure VII-2B next page), whereas chondrocytes expanded in SCM stained positive (Figure VII-2C next page). In contrast, construct of human articular chondrocytes were negative for elastin (Figure VII-2D next page).

Gene expression was determined to evaluate cellular phenotype of the neo-cartilage harvested from the athymic mice in more detail. In human ear chondrocytes expanded in SCM, expression of collagen type I was always higher than expression of collagen type II, resulting in an expression ratio of

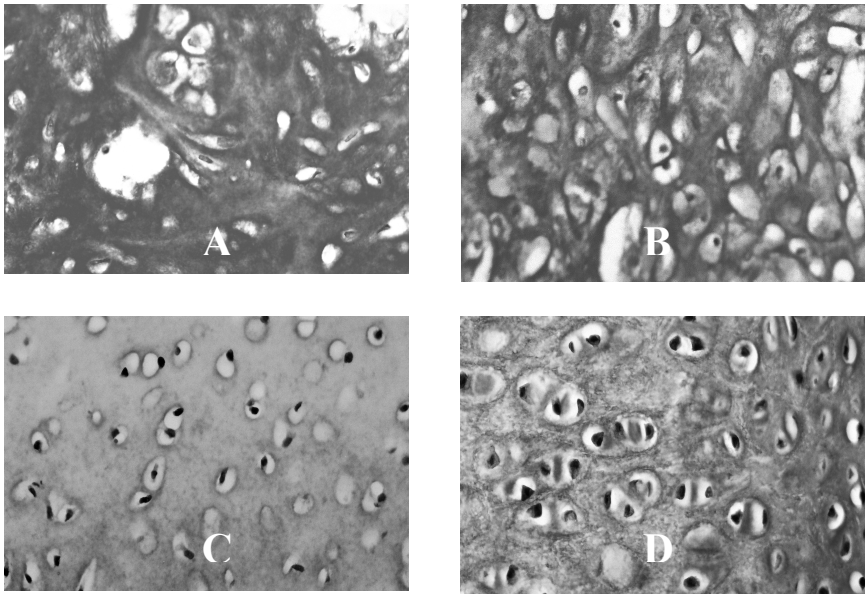

Figure VII-2 **(A):** Immunohistochemical staining for elastin, **(B):** Example of dense fibrous tissue from a construct generated by ear chondrocytes; **(C):** Ear chondrocytes still surrounded by alginate, that has been expanded in monolayer from seeding density of 7500 chondrocytes/cm^2 in medium with 10% FCS, **(D):** Ear chondrocytes still surrounded by alginate, that have been expanded in monolayer from seeding density of 15000 chondrocytes/cm^2 in serum-free medium with 100 ng/ml FGF2. (Magnification 400X)

collagen type II to collagen type I of 0.39. Expression ratio of collagen type II to collagen type I was 1.9 for chondrocytes expanded in SFM+FGF2. Chondrocytes expanded in SFM+FGF2 actually expressed two times more collagen type II and two times less collagen type I than chondrocytes expanded in SCM. Expressions of collagen were 20-40 times as high as that of GAPDH. Elastin expression of chondrocytes expanded in SFM+FGF2 and SCM were similar. Expression of elastin was 5-10 times as high as that of GAPDH. Aggrecan and versican were not differently expressed in the different expansion media. Expression of chondrocyte hypertrophy markers, such as alkaline phosphatase and collagen type X, were low (Table VII-1 next page).

With the porcine cartilage it was possible to harvest enough chondrocytes to study the behaviour of differentiated ear and knee chondrocytes in constructs.

This enabled us to compare the performance of differentiated chondrocytes with that of chondrocytes, which were culture-expanded in monolayer before seeding in a construct. It appeared that differentiated (seeded immediately after isolation) ear chondrocytes produced more GAG than differentiated articular chondrocytes. However, in similar experiments with ear and articular chondrocytes that had been expanded in monolayer culture for 28 days, this difference was absent. It should be noted that the ear chondrocytes were expanded more (47 times) than the knee chondrocytes (13 times) and were therefore probably more dedifferentiated.

	AGC	versican	Col 1	Col 2	Eln
7500SCM	1.38±0.10	0.05±0.01	57.99±19.12	22.83±7.68	10.48±4.94
7500SFM+FGF2	1.89±0.30	0.06±0.02	25.05±8.13	35.63±9.44	6.25±3.37
15000SFM+FGF2	1.79±0.16	0.05±0.01	20.06±5.33	36.58±5.12	8.36±2.33

AGC= aggrecan; Col 1=collagen type I
Col 2= collagen type II; Eln=elastin.
Collagen type X and alkaline phosphatase were not demonstrated because gene expression was at highest 0.001.

Table VII-1 Expression of genes normalized for GAPDH (mean ± sd of chondrocytes from three donors) in constructs of human ear chondrocytes expanded in monolayer from seeding density of 7500 chondrocytes/cm^2 in medium with 10% FCS and from seeding density of 7500 chondrocytes/cm^2 and 15,000 chondrocytes/cm^2 in serum-free medium with 100 ng/ml FGF2. Data on chondrocytes expanded from15,000 chondrocytes/cm^2 in SCM are missing because not enough chondrocytes were obtained to generate a construct.

On histology, constructs generated from ear chondrocytes and constructs generated from articular chondrocytes were both positive for collagen type II (Figure VII-3 below).

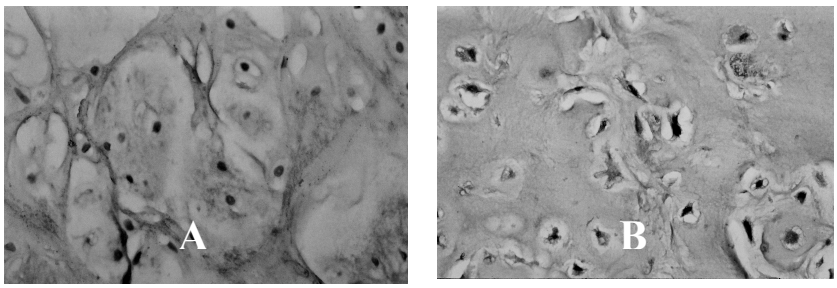

Figure VII-3 **(A):** Immunohistochemical staining for elastin in constructs generated from differentiated porcine ear chondrocytes, **(B):** and monolayer expanded porcine ear chondrocytes. (Magnification 400x)

Constructs made from previously expanded chondrocytes appeared less positive than constructs from differentiated chondrocytes. Constructs of ear chondrocytes demonstrated elastin, especially in the more fibrous-like areas, whereas these areas did not stain for elastin in constructs of articular chondrocytes. Constructs with expanded ear chondrocytes showed less elastin than those initially seeded with differentiated ear chondrocytes. Also these constructs with differentiated ear chondrocytes more often contained the typical 'big' chondrocytes which are characteristic for the central part of ear cartilage.

Discussion

It has been demonstrated that ear chondrocytes can be used to generate cartilage. However, these studies used immature cartilage, mostly from porcine origin and only rarely from human tissues. Furthermore, these studies were usually performed with primary, differentiated chondrocytes. However, in a clinical setting, the donor tissue most often used will be mature and, due to the limited amount of donor chondrocytes, the chondrocytes have to be culture-expanded. Our study showed that ear chondrocytes of adult human origin were able to multiply in monolayer culture. Even more, the cell yields after cultures were always higher compared to experiments with articular chondrocytes. A serious side effect of monolayer expansion culture is chondrocyte dedifferentiation. During expansion, chondrocytes change the expression pattern from the cartilage specific collagen type II and aggrecan towards less functional molecules like collagen type I and versican. The ability to re-express the differentiated phenotype is, besides the ability to multiply *in vitro*, a crucial prerequisite to be a suitable donor cell source for cartilage tissue engineering. To assess whether ear chondrocytes possessed this ability, we subcutaneously implanted culture-expanded human ear chondrocytes in nude mice. Subsequent histology revealed cartilage–like tissue that was highly positive for collagen type II and GAG.
Recently, Kafienah et al. [79] compared the capacity of chondrocytes from articular cartilage and nasal septum cartilage and concluded that human adult nasal chondrocytes proliferate approximately four times faster than human articular chondrocytes in monolayer culture. Evenmore, they possessed a markedly higher chondrogenic capacity, as assessed by the mRNA and protein analysis of *in vitro*-engineered constructs. Our study, together with the study by Kafienah et al.[79], clearly show a growing interest, as well as the possibilities, for the use of alternative cartilage types as donor tissue for articular cartilage engineering. We hypothesise that chondrocytes exhibit certain plasticity in the expression of their phenotype, which means that ear chondrocytes could be persuaded to express a more hyaline-like phenotype. Local environmental factors within a joint might provide the necessary stimuli to accomplish this. Whether ear chondrocytes can respond to these stimuli is not known. Furthermore, as mechanical loading is not physiological for ear chondrocytes it remains questionable whether these chondrocytes and its produced extracellular matrix can prevail itself within a joint. In pilot experiments we found that immature bovine ear chondrocytes seeded in agarose discs remained vital after four consecutive days of 5-10% cyclic compression. In future experiments we will investigate whether ear chondrocytes subjected to a mechanical stimulus will produce a more hyaline-like matrix. So far, no data on mechanical loading of ear cartilage or ear chondrocytes seeded in a biomaterial are present in literature.
Obviously, the ultimate test to assess the feasibility of using ear chondrocytes for articular cartilage repair would be the implantation of dedifferentiated ear chondrocytes in an articular cartilage defect. Again, no data are found in

literature. However, in reconstructive surgery of the nose, ear cartilage transplants have been used with success for many years to repair nasal septum defects, which is in fact hyaline cartilage. In a study on the fate of the implanted ear cartilage within the nasal septum, Min et al. [217] showed that hyaline-like neo-cartilage was formed after 4 weeks from within the transplanted ear cartilage. In our opinion, this study might represent an example on how local environmental factors can alter cellular phenotype.

A repair tissue that exactly resembles the native tissue might not be obligatory for a functional cartilage repair. In other words, cartilage repair is not similar to cartilage regeneration. Therefore, a sustained expression of elastin might be of less importance. Mechanical characteristics similar to the native tissue and good integrative capacities could be more important. Data on the mechanical characteristics of cartilage generated by ear chondrocytes are lacking and even data on mechanical characteristics of native ear cartilage are scarce. In experiments comparing characteristics of the different cartilage subtypes in rabbits, Naumann et al. [142] found that the aggregate modulus of ear cartilage was higher and the permeability was lower than that of articular cartilage. In our experiments we did not assess the mechanical characteristics of the neo-cartilage engineered with adult human ear chondrocytes.

Summarising, in this paper we combined data from previous experiments in our laboratory with articular and ear chondrocytes as donor chondrocytes for cartilage tissue engineering. We found that both the cell yield of the donor tissue and monolayer expansion culture favoured the use of ear chondrocytes. Cell yield and performance could further be increased by the use of SFM with 100 ng/ml FGF2 for the expansion culture in monolayer. Furthermore, we speculated on the use of ear chondrocytes to engineer repair tissue for articular cartilage defects. To make more solid statements on this issue, the mechanical properties of engineered cartilage with ear chondrocytes should be assessed in future. Furthermore, experiments in which culture expanded ear chondrocytes, seeded onto a scaffold, are implanted in articular cartilage defects will be performed.

Acknowledgements

The authors would like to thank Corrina de Ridder and Cindy Bolder for technical assistance with the animal experiments. This work was funded by the Dutch Ministry of Economic Affairs in a grant of Senter (nr. BTS00021).

VIII

The pericellular matrix reduces the effects of cyclic mechanical loading on proteoglycan synthesis in chondrocyte-seeded agarose gels

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Submitted

Abstract

Mechanical loading of cell-seeded scaffolds could prove to be an important factor in cartilage tissue engineering. However, for this purpose, long-term loading experiments on chondrocytes are required. In this study we investigated the effects of preculture and subsequent pericellular matrix formation on the synthesis of proteoglycans by chondrocytes and the chondrocyte deformation as a response to mechanical loading. Agarose discs seeded with bovine chondrocytes were dynamically loaded in an unconfined set-up at 0.5 Hz with 10% peak strain for 90 minutes twice a day, for a period of four days. When no preculture was performed, the dynamic compression significantly increased GAG content by 34% compared to control. However, starting the loading regime after four days of preculture did not result in an increase in GAG content. Using confocal laser-scanning microscopy, chondrocyte deformation during an applied static 10% mechanical gel-deformation was measured in the agarose discs cultures with and without preculture. The precultured chondrocytes showed significantly reduced cell-deformation. We conjecture that this reduction in cell deformation is due to pericellular matrix formation in the precultured conditions, which was demonstrated by histology. The produced pericellular matrix stiffens the cell-matrix complex and consequently protects the cell from deformation. By extrapolating the results from the confocal microscopy experiments to the dynamic loading experiments, we hypothesise that the reduction in cell-deformation in the precultured agarose discs largely explains the lack of increase in proteoglycan production during the dynamic loading regime.

Introduction

Mechanical loading is a key regulatory factor in cartilage homeostasis and may influence the development of a functional tissue during tissue engineered repair [22, 85, 169]. However, tissue engineering of cartilage is almost always performed *in vitro*, without physiological mechanical stresses and loads. Accordingly, the introduction of mechanical conditioning into the tissue engineering process could enhance the ultimate quality of the repair tissue.

Many studies have shown effects of *in vitro* loading on cartilage explants. In general, static mechanical loading resulted in a decrease of chondrocyte metabolism [22-24, 30, 48, 66, 92, 97, 169], whereas dynamic mechanical loading with certain loading regimes enhanced chondrocyte metabolism or extracellular matrix production [23, 25, 92, 97, 130, 150, 185-187]. The majority of these experiments were short-term, using either gene expression levels or incorporation of radioactive labels into the matrix as an outcome measure of the effects of dynamic compression. To date, few studies have focussed on long-term loading experiments and on its effects on extracellular matrix content and mechanical characteristics of the cell-seeded scaffolds [130, 131]. During long-term loading experiments several factors may become significant which are of limited consequence during short-term loading experiments, complicating such experiments. Firstly, introducing cell-seeded scaffolds into a loading device involving fluid-impermeable indentors results in a reduction in area available for medium diffusion, by means of placing indentors on top of the constructs. The subsequent retardation of nutrient/metabolite transport could be inhibitory for appropriate extracellular matrix production. Secondly, in long-term loading experiments the production of extracellular matrix may influence the metabolic response to loading, as it has been shown that the elaborated matrix can influence the cell-deformation [49, 87].

To explore the effects of dynamic compression in cartilage tissue engineering, the authors have utilised a custom-designed loading device in which cell-seeded constructs can be placed under separate indentors. In the present study we report on the effects of dynamic compression applied for four consecutive days to bovine chondrocyte-seeded agarose gels, starting at time 0 or after 4 days of free-swelling culture.

Materials and Methods

Chondrocyte Isolation

Articular chondrocytes were isolated from the metacarpalphalangeal joints of 6-7 months old calves. Within 4 hours after slaughter slices of cartilage were harvested and washed three times in physiological saline. The cartilage was digested by incubation in 2 mg/ml pronase (Sigma, St Louis, Mo) in physiological saline for 1.5 hours, followed by overnight incubation in 2 mg/ml collagenase B (Boehringer Mannheim, Germany) in Dulbecco's modified eagle

medium (Life Technologies, Breda, the Netherlands; Glutamax-I) with 10% FCS (Fetal Calf Serum). The released chondrocytes were subsequently washed and cell viability was determined using trypan blue staining. More than 95% of the chondrocytes were viable. For each experiment isolated chondrocytes from three different joints were pooled.

Agarose suspension

Agarose (type VII, low melting point, Sigma) was added to PBS at a concentration of 6% (w/v) and sterilised using heating to 120 ^{o}C. The sterile agarose gel was cooled to 37 ^{o}C and a suspension of chondrocytes in DMEM + 10% FCS was added, to yield a final concentration of 3% agarose with $4 \cdot 10^{6}$ cells/ml. The agarose/chondrocyte suspension was transferred into a petridish and was incubated for 15 minutes at 4 ^{o}C. Cores were punched out of the gel using a biopsy punch, thus creating cell-seeded agarose discs of 8 mm in diameter and 3.0-3.4 mm in height.

The loading device

A loading device was designed and custom-built. Briefly, below a central air cylinder four (edge-welded) bellows (Hositrad Holland bv, Hoevelaken, The Netherlands) were mounted, permitting four cell-seeded agarose discs to be simultaneously subjected to dynamic loading during an experiment. A linear voltage displacement transducer (LVDT) was placed within each of the bellows. Changing the air pressure within the cylinder resulted in a displacement of the bellows, which was measured by the LVDT's. A computer-controlled air valve regulated the air pressure within the cylinder, which was measured by a pressure transducer (AE Sensors, Dordrecht, The Netherlands). Labview software was used to control the air valve and to acquire the output of the four LVDT's and the pressure transducer to a prescribed frequency and amplitude.

To transduce the displacement from the bellows onto the agarose discs indentors were used, which were placed on top of the cell-seeded agarose gels and were guided by a custom-made cover-plate that fitted a 24-wells plate (Figure VIII-1 next page). Accordingly, no lift-off was possible between the indentor and the agarose gel. During dynamic compression, the lift-off occurred between the bellow and the indentor (Figure VIII-1 next page). The indentors were manufactured from unfilled PEEK (PolyEtherEtherKetone) and had a weight of less than 1 gram per indentor. Negative effects of the PEEK on cell viability and GAG (GlycosAminoGlycan) production were ruled out in pilot experiments testing the feasibility to use this material in direct vicinity of cell-seeded agarose discs (data not shown). The indentors were 1 mm less in diameter than the wells, resulting in an unconfined set-up (Figure VIII-1 next page).

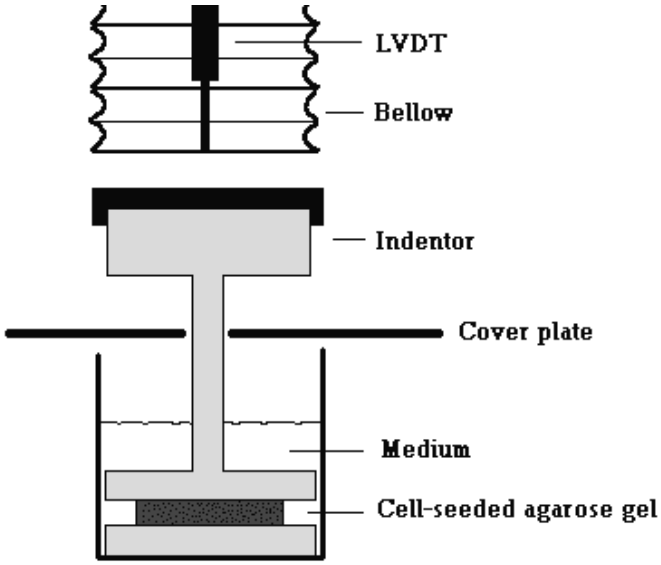


Figure VIII-1 A schematic overview of the set-up below the loading device. On top of the cell-seeded agarose disc a PEEK indentor was placed, which was guided by a macrolon cover plate that fitted a 24-wells plate. The indentor was displaced by the bellow of the loading device.

Loading protocols

A methodology involving two experimental groups was adopted, as outlined in Figure VIII-2 next page. In group I the loading regime was initiated two hours after the cell-seeded agarose discs were produced. The loading regime consisted of 90 minutes dynamic compression twice a day, with an one-hour break in between, for four consecutive days. The dynamic compression was applied with a sinusoidal waveform at frequency of 0.5 Hz and with an initial peak strain and dynamic strain of 10%. However, the actual displacement of the agarose discs resembled a mixture of a sinusoidal and a block-wave (Figure VIII-3 page 93). And due to incomplete gel-height recovery during dynamic compression, associated with the viscoelastic properties of the agarose, the actual dynamic strain on the cell-seeded agarose gels was between 5-10%, whereas the peak strain (measured from the initial gel-height) was not allowed to be higher than 10% (Figure VIII-3 page 93). After four days the medium was changed (1 ml per disc) and the cell seeded-agarose gels were cultured for an additional three days in a free swelling state before harvesting. In group II, the cell-seeded agarose discs were cultured in a free swelling state for four days prior to initiation of the same loading regime as in group I. Therefore, total time in culture for cell-seeded agarose discs in group II was 11 days. The medium used during the experiments consisted of DMEM with 10% FCS and 25 µg/ml L-ascorbic acid. Two control conditions were employed (figure 2); one group of discs were maintained with the indentor on top within the loading device, but without superimposing dynamic compression, and

a second control group of cell-seeded agarose discs were cultured throughout in a free swelling state. Experiment I was performed with a total of 12 constructs per condition, whereas experiment II was performed with 8 constructs per condition.

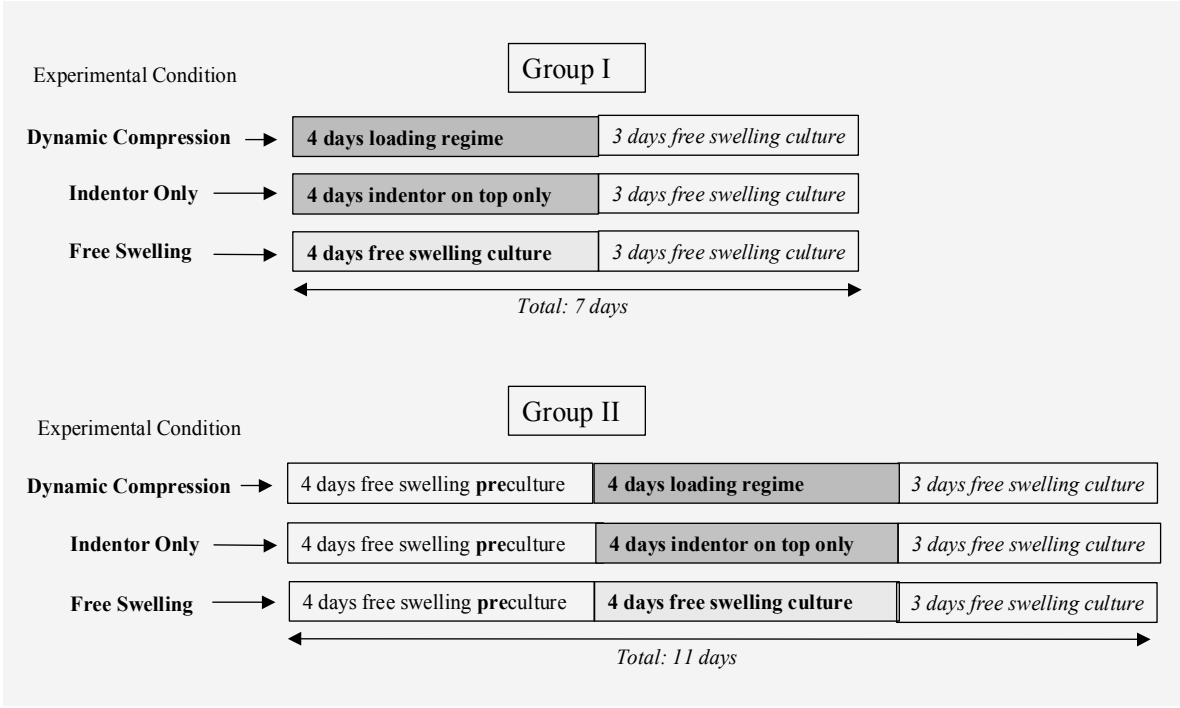


Figure VIII-2 A schematic overview of the experiments.
Group I: Dynamic compression, in which cell-seeded agarose gels were dynamically compressed at 0.5 Hz, with 5-10% peak strain, for two times 90 minutes per day. The loading regime was started two hours after the cell-seeded agarose discs were made and lasted four days. After four days the agarose gels were cultured 'free swelling' for an additional three days before harvesting. Two control conditions were made: **(1)** Indentor only, in which cell-seeded agarose discs were cultured for 4 days inside the compression device and with an indentor on top, but without superimposing dynamic compression. After four days the agarose gels were cultured 'free swelling' for an additional three days before harvesting. **(2)** Free Swelling control, in which cell-seeded agarose discs were cultured in a 'free swelling' state during the 7 days the experiments lasted.
Group II: In these experiments the cell-seeded agarose discs were cultured in a free swelling state for four days before similar experiments as in group I were performed. As a result, the total duration of the experiments in group II lasted 11 days.during the experiments consisted of DMEM with 10% FCS and 25 µg/ml L-ascorbic acid.
Two control conditions were employed; one group of discs were maintained with the indentor on top within the loading device, but without superimposing dynamic compression, and a second control group of cell-seeded agarose discs were cultured throughout in a free swelling state. Experiment I was performed with a total of 12 constructs per condition, whereas experiment II was performed with 8 constructs per condition.

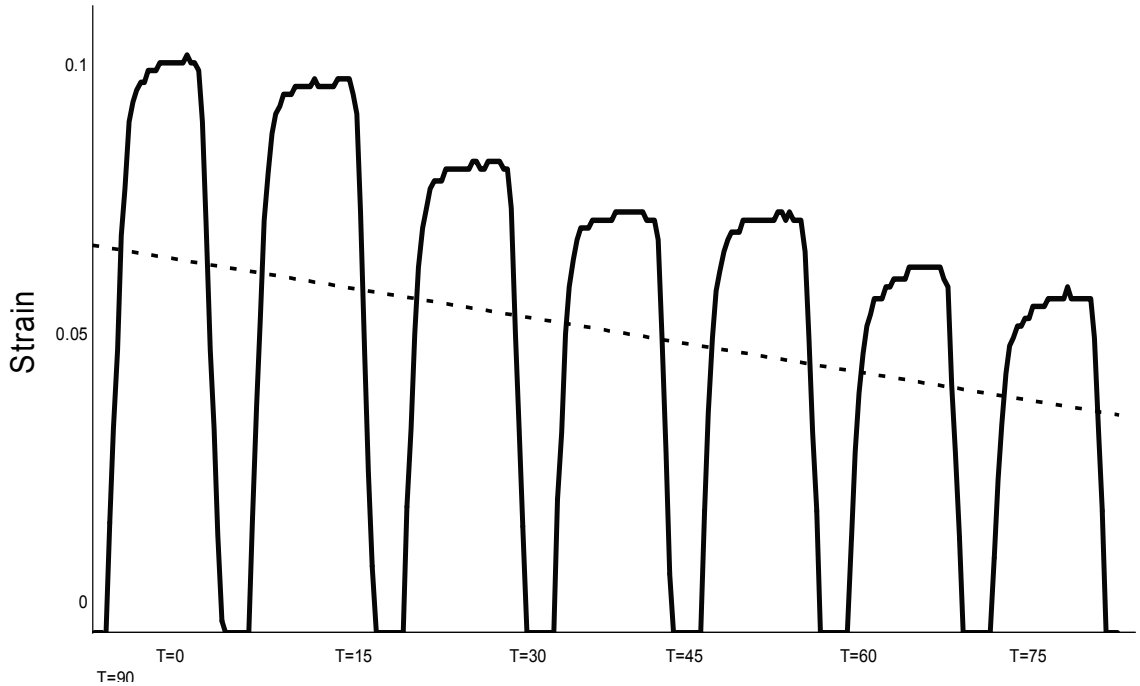


Figure VIII-3 The dynamic deformation of an agarose gel of 3 mm height in time is shown for an initial peak strain 0.1 (=10%). During dynamic compression for 90 minutes at 0.5 Hz typical curves of gel deformation with an interval of 15 minutes are plotted (the actual interval of unloading during each cycle is not plotted). Note that from t = 0 to t = 90 the initial gel height and the dynamic amplitude decreases. A trend-line is plotted to underscore the decrease in dynamic amplitude.

Live/dead staining

Calcein AM and ethidium homodimer-1 (live/dead viability/cytotoxicity kit (l-3224), Molecular Probes, Leiden, the Netherlands) were used to assess cell viability 24 hours after casting of the gel and at harvesting of the cell-seeded agarose discs. A cylindrical specimen, 2 mm in diameter, was cored out of the middle of each disc and washed in physiological saline before incubation in DMEM supplemented with 5 μM each of Calcein AM and ethidium homodimer-1 for 45 minutes at 37 ^{o}C. The percentage of dead cells was assessed from a random selection of 300 cells from three separate specimens.

Biochemical assays

The samples were digested using the protocol previously described by Knight et al. [86]. Briefly, the samples were incubated at 70 ^{o}C with L-cysteine hydrochloride and EDTA until molten. The samples were cooled to 40 ^{o}C before agarase (10 IE/ml, Sigma) and papain (2.8 IE/ml, Sigma) were added for incubation at 40 ^{o}C overnight. The total content of DNA per sample was assessed using a fluorimetric assay based on the Hoechst 33258 dye as previously described (Chapter III, materials and methods). GAG content was measured using the dimethylmethylene blue assay [40]. Using a microplate reader the ratio of

absorption at 540 nm and absorption at 595 nm was measured. The amount of GAG was calculated using a standard of chondroitin sulfate C (shark; Sigma).

Histology

Cell-seeded agarose discs were removed from culture at time 0 and after 4 or 8 days of free swelling culture and fixed in formalin and embedded in paraffin. Sections, 5 μm in thickness, were prepared and stained with thionin according to Bulstra et al. [21]. For collagen type II staining, the sections were incubated with 1 mg/ml pronase and subsequently with 1% hyaluronidase. Next, normal goat serum was added followed by incubation with monoclonal antibodies against collagen type II (1:100 II-II6B3; Developmental Studies Hybridoma Bank) for two hours. Anti-mouse F_{ab}-fragments conjugated with alkaline phosphatase (GAMAP; Immunotech, Marseille, France, 1:100) were added and detected with mouse monoclonal alkaline phosphate anti-alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark; 1:100). Freshly prepared neo-fuchsin substrate was used to achieve a positive staining for collagen type II.

Confocal Microscopy

Cell deformation at different time-points was measured using confocal microscopy. At time points 0, 4 and 8 days cell-seeded agarose gels, cultured in free-swelling conditions were cut longitudinally into half-cores and incubated with 10 μM Calcein-AM in DMEM+10% FCS for 45 minutes at 37 ^{0}C. Constructs were then mounted in a specially designed loading rig, based on that described by Knight et al. [86]. The rig was placed on the stage of an inverted microscope, which was coupled to a confocal laser-scanning microscope (LSM 410, Zeiss, Germany). Viable, calcein labelled cells were visualised using laser excitation at a wavelength of 488 nm and fluorescence detection at wavelengths of 500 nm and higher. Confocal images of individual cells were taken in the central region of the construct, using a 40x/0.95 objective, 8x confocal zoom and a confocal slit of 20 microns, resulting in images with a pixel-size of 0.078 microns.

For each construct approx. 6 individual cells were imaged in the unstrained state. The position of the centre of the cell was selected by adjusting the position of the focal plane during real time confocal imaging. A 10% uniaxial compressive strain was then applied to the construct at a strain rate of 10 %.s^{-1}, by means of a plunger, which was driven by a computer controlled stepping linear actuator with a step size of 2.5 microns. For each sample 12 cells were imaged 8-10 minutes following loading. At each time-point this procedure was repeated for 3 samples.

Ellipsoid morphology of the chondrocytes was analysed from the confocal images measuring the X/Y-diameter ratio of a best fitting ellipsoid (Matlab, The Mathworks Inc., USA). From the resulting diameter ratio's the X-strain was calculated using the formula previously described [88], which is based on the

assumption that no change in cell-volume occurs, as has been demonstrated previously for isolated gels in agarose [98].

Assessment of molecular diffusion

Cell-free agarose discs were prepared using the methodology described above. The discs were incubated for 15 minutes in a solution containing trypan blue in free-swelling conditions or within the loading device, with or without the application of dynamic compression at 0.5 Hz. At the end of the incubation period the discs were removed and molecular diffusion was assessed qualitatively by observation of the extent of staining across the disc.

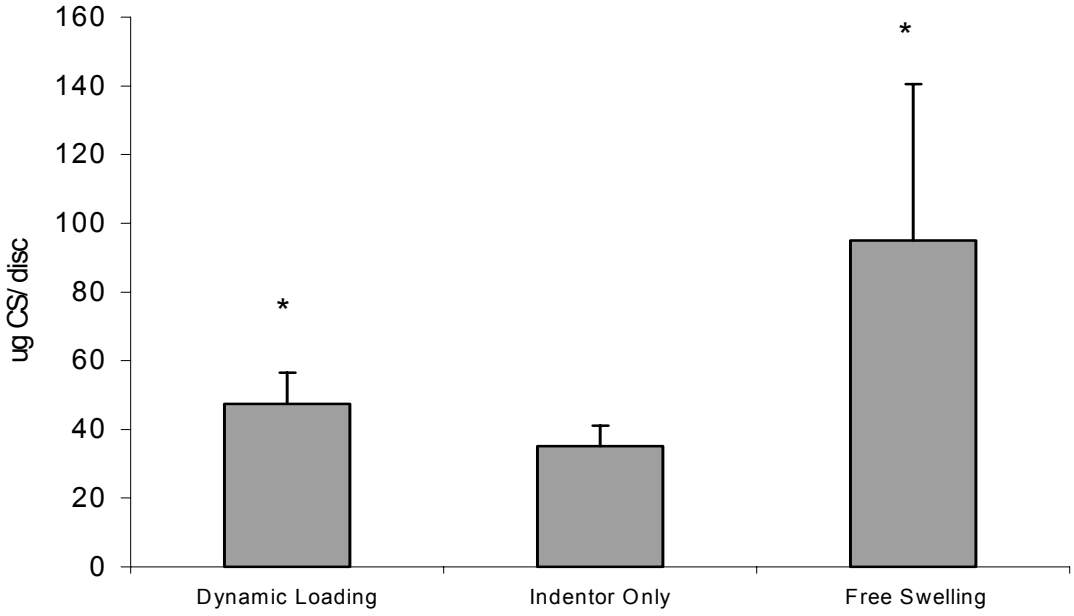
Data Analysis

Results from the biochemical assays on GAG, DNA and the live/dead staining were analysed using the Mann-Whitney U test. For the results from the confocal laser the student's T test was used. A p-value less than 0.05 was considered to show a significant difference.

Results

The application of dynamic compression at 0.5 Hz to cell-seeded agarose discs without preculture (group I) induced a significant increase in GAG content of 34% compared to the 'indentor-only' control group ($p = 0.015$, Figure VIII-4 next page).

However, when a 4 days preculture was employed prior to application of dynamic compression (group II), dynamic compression did not alter GAG content compared to the 'indentor-only' control ($p = 0.6$, Figure VIII-5 next page. In all cases GAG content was highest in the free swelling group compared to the other experimental conditions ($p < 0.01$). In group I, the live/dead staining demonstrated that the lowest percentage of non-viable cells were present in the free swelling conditions (average = 5%, SD = 0.5%) . The percentage of non-viable cells was similar in both the dynamic compression condition and the 'indentor-only' control (average = ± SD respectively 10% ± 0.5% and 9% ± 1%). Although the live/dead staining in group II showed a similar pattern to group I, the percentage of non-viable cells was higher in both the dynamic compression condition and the 'indentor-only' control compared to group I (average ± SD respectively 26% ± 14% and 16% ± 6%). Interestingly, DNA content was not significantly different between experimental conditions in both group I and II (data not shown). Representative micrographs, prepared from histological sections of cell-seeded agarose discs fixed at t=0 and after 4 or 8 days of free-swelling culture are presented in Figure VIII-6 page 97. The data demonstrate that at t=0 no extracellular matrix was present. After four and eight days of free swelling culture an increasing amount of extracellular matrix, which stained for



. * = sign for a statistical significant difference to the 'indentor-only' control.

Figure VIII-4 The average amount of GAG per disc is shown for the experiments in group I. Dynamic compression significantly increased GAG production in the dynamically loaded agarose discs compared to the 'indentor-only' control. Free swelling cultures contained most GAG.

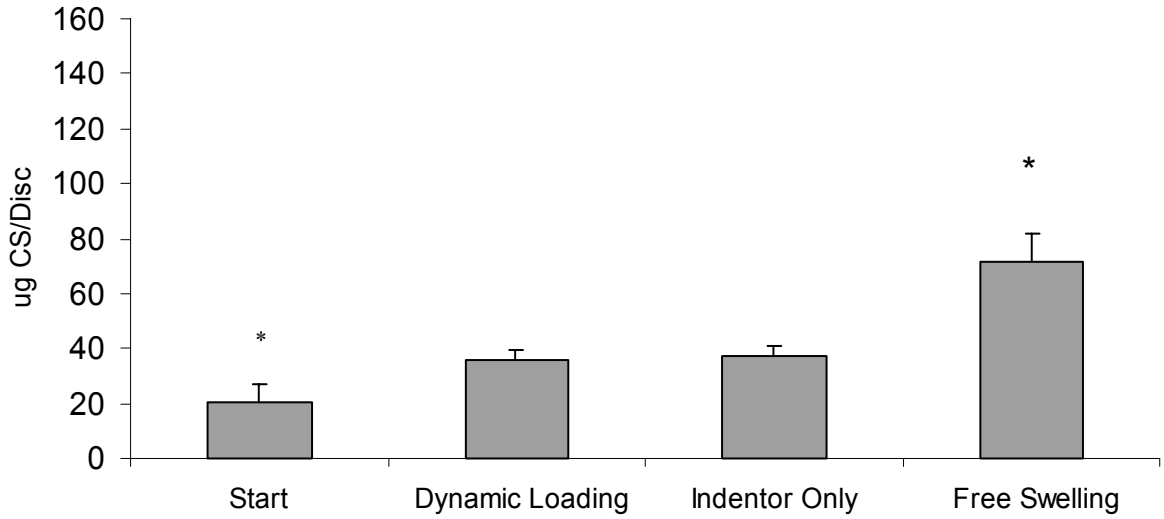


Figure VIII-5 The average amount of GAG per disc is shown for the experiments in group II. No difference in GAG production was observed between the dynamically loaded agarose discs and discs from the 'indentor-only' control. The most left bar represents the GAG content of the agarose discs at the start of the loading regime, which was after four days of free swelling culture. * = sign for a statistical significant difference to the 'indentor-only' control.

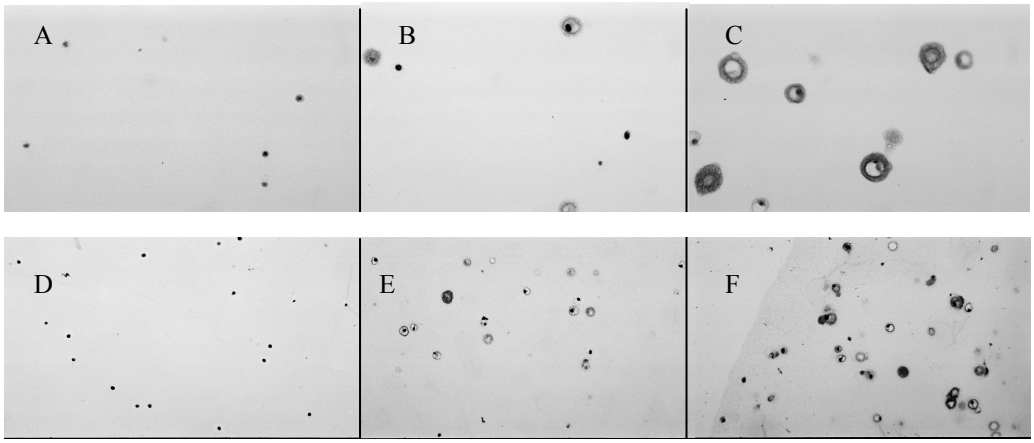


Figure VIII-6 Stainings on free swelling cultured cell-seeded agarose discs. The top three photos **(A,B,C)** represent the thionin staining on glycosaminoglycan at day 0, day 4 and day 8 respectively. The bottom three photos **(D,E,F)** represent the staining for collagen type II at the same time points and maginifaction.

both proteoglycans and collagen type II, was evident, primarily concentrated in the pericellular region (Figure VIII-6 above). Data from experiments to determine cell deformation in discs at t=0 and after 4 or 8 days of free-swelling culture are presented in Table VIII-1 below. The application of 10% strain to cell-seeded agarose discs at t=0 resulted in an mean cell deformation index (X-axis/Y-axis) of 0.85. Following free-swelling culture for 4 or 8 days mean values for cell deformation index has increased to 0.92 and 0.96 respectively. These cell deformation indices corresponded to a cell-strain of 10.6% at t = 0 days, 5.4% at t = 4 days and 2.9% at t = 8 days, based an assumption of maintenance of cell volume during the application of strain.

	Unstrained	T=0 strained	T=4 strained	T=8 strained
Cell Deformation index	1.01±0.07	0.85±0.05 *	0.92±0.04 *, #	0.96±0.04 *, #
Cell Strain in %	0.77±4.7	10.6±3.7 *	5.4±3.6 *, #	2.9±3.0 *, #

* = sign for a statistical significant difference compared to unstrained agarose.
# = sign for a statistical significant difference compared to the previous time point in strained agarose.

TableVIII-1 The results of confocal microscopy experiments to assess the cell deformation in 10% strained cell-seeded agarose discs at time points representing the dynamical loading experiments. The average value ± SD are given. The agarose discs had been cultured in a free swelling condition

Representative images of cell free discs, incubated for 15 minutes in a solution containing trypan blue under free-swelling conditions or within the loading device, with or without the application of dynamic compression at 0.5 Hz are presented in Figure VIII-7 below. The trypan blue, a molecule of approximately 1 kDa was observed to diffuse throughout the agarose disc in the free swelling condition after 15 minutes. However, for both the dynamically compressed disc and the disc from the 'indentor-only' control the only available area for diffusion was via the outer ring of the gel. This was clearly shown by the fact that the dye only stained a 2 mm thick ring at the periphery of the gel. No enhanced dye distribution towards the centre of the gel was observed in the dynamically compressed (for 15 minutes) discs.

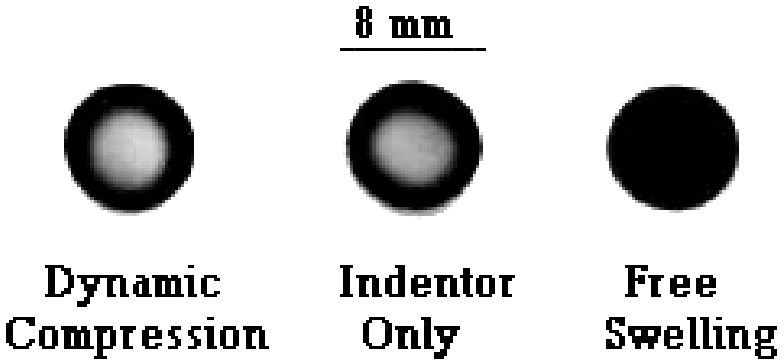


Figure VIII-7 Agarose discs were cultured for 15 minutes in trypan blue to show the effects of introducing cell-seeded agarose discs into a loading device on the area available for medium diffusion. The disc in the free swelling culture was thoroughly stained within 15 minutes, whereas the discs in the loading device showed staining only in the peripheral ring. Superimposing dynamic compression did not result in better staining (comparing the 'indentor-only' control with the dynamic compression).

Discussion

The relative absence of studies describing the effect of long-term loading on chondrocyte-seeded constructs compared to the enormous pool of published data on short-term loading effects reflects, in our opinion, the complexity of long term *in vitro* loading of cell-seeded scaffolds. Dynamic compression of cartilage, cell-seeded scaffolds and cell-seeded hydrogels typically results in cell deformation and alterations in fluid pressure, fluid flows and electrochemical flows [47, 139]. These effects, individually or in combination may mediate alterations in metabolic cell response. For example, it has been suggested that for dynamic compression at higher frequencies than 0.1 Hz and strains up to 10% the increase in matrix production is related to changes in cell shape and/or fluid flows [84].

However, introducing cell-seeded constructs into a loading device may impose constraints on the construct that could influence cell activity in the absence of loading. For example, the configurations used in the majority of loading systems reduce the area available for medium diffusion, due to the presence of a fluid-impermeable indentor, which is placed on top of the constructs. It may be estimated that approximately 70% of the total surface are of the discs, representing the upper surface and peripheral surface, is available for free diffusion in the free swelling condition. By contrast less than 45% of the total surface area is available for free diffusion when the discs are mounted into the loading system. Indeed, qualitative analysis of molecular diffusion confirmed that the diffusion was impaired in the 'indentor-only' and 'dynamic loading' conditions, as compared to the free swelling cultures (Figure VIII-7 previous page). This major reduction in area available for medium diffusion is likely to be responsible for the significantly lower GAG content in the 'indentor-only' and 'dynamic loading' conditions, as compared to the free swelling cultures, measured after 7 and 11 day protocols associated with groups I and II respectively.

The loading systems used in a number of previous studies may induce alterations in the surface area available for diffusion, due to lift-off of the indentor from the construct during a proportion of the loading cycle. This phenomenon is related to the viscoelastic nature of hydrogels such as agarose. The system used in the current study overcomes this problem by ensuring that the indentor remains in contact with the agarose disc throughout the loading cycle. Lift-off occurs between the indentor and the bellows. Accordingly, alterations in metabolic response between 'dynamic loading' and 'indentor-only' conditions may not be attributed to alterations in basic diffusion profiles. It should be acknowledged, however, that the dynamic loading may alter the uptake of biomolecules through loading-induced fluid flow. No alteration in the pattern of uptake of trypan blue was observed, qualitatively, in this study on application of dynamic compression, although previous studies have reported that the uptake of radiolabelled thymidine and proline was enhanced by dynamic compression [97].

In this study, an increase in GAG content was observed in response to dynamic compression when no preculture was performed prior to initiation of the loading regime. When the agarose discs were cultured for four days before the loading experiments started, no effect of dynamic compression on GAG production was observed. Biochemical analysis indicated a significant increase of GAG content with time in culture. Therefore, it is likely that the presence or absence of extracellular matrix played a crucial role in mediating mechanotransduction processes. To examine this effect further histological sections were prepared from the cell-seeded agarose discs at different time points and demonstrated formation of extracellular matrix, which was mainly concentrated in the pericellular region. In previous experiments by Knight et al. [87], the elaborate matrix was shown to decrease the cell deformation from day 1 to day 6 at 20%

gel compression. However, in the current study a maximum peak strain of 10% was used. Accordingly, cell deformation experiments were performed with parameters that replicated the loading regime adopted, to allow correlation between the results of the confocal experiments and dynamic compression experiments. A significant decrease in cell deformation was observed from t = 0 days to t = 4 days and a further decrease was apparent between days 4 and 8. These data suggest that a reduction in dynamic cell deformation would be expected during the dynamic loading of group II discs compared to group I discs, even though the dynamic strain regimes are identical.
It should be appreciated, however, that the cell deformation experiments were performed using static strain. Knight et al. [86] performed similar confocal experiments in association with dynamic gel-compression and determined that the resulting cell-deformation significantly decreased during the first 20 minutes of dynamic strain, while cell-deformation within statically compression gels remained constant. Knight et al. attributed this effect to permanent deformation of the gel constructs, which occurs following dynamic compression of viscoelastic materials at high strains and dynamic frequencies. A decrease in gel height was also observed during the dynamic compression experiments in the current study, which consequently resulted in a decrease dynamic gel strain, as the peak strain was not allowed to overshoot 10% (Figure VIII-5 page 96).
Taken together, the lower cell deformation after 4 days of preculture, the decreasing cell deformation during dynamic gel compression observed by Knight *et al.* and the decrease in gel height during dynamic compression may result in insufficient cell deformation to elicit an increase in GAG production in group II. This might suggest that a higher peak strain should be used during later time periods in culture. For instance, D'Lima et al. showed an increase in GAG content in cell-seeded agarose discs after 7 days of preculture with a different loading regime [35]. Although the peak strain was similar, frequency and the duration of the dynamic compression were 1 Hz and 8 hours respectively. The above might indicate that a loading regime needs constant adjustments as time progresses and conditions change, in order to obtain a sustained positive effect of dynamic compression on cell-seeded scaffolds. However, as regimes of virtually an endless combination of frequency, strain and duration of the loading can be thought off, finding the optimal regime for a given time point in culture is a difficult task, if not impossible. This underscores the need for a more scientifically based approach towards loading regimes and unravelling the mechanotransduction pathways in chondrocytes, which remain largely unknown yet.

Acknowledgements

The authors would like to thank T. Boer & Zn in Nieuwerkerk a/d IJssel for providing the bovine legs. Ruud Niesing, Cor Goedegebure and Wim Boender

are appreciated for their help in designing and building the loading device. We would also like to thank Andre Nederlof and Judd Day for their help with the confocal microscopy set-up. Finally, we would like to thank Wendy Koevoet and Nicole Kops for their support with the various assays. This work was funded by the Dutch Ministry of Economic Affairs in a grant of Senter (nr. BTS00021). The monoclonal antibody, II-II6B3, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD.

IX

General Discussion

To date, surgical treatments of cartilage defects often result in fibrocartilage and fail to produce long-term functional cartilage repair tissue [62, 68]. With tissue engineering techniques, opportunities have arisen to provide new treatment methods for cartilage repair. First generation cartilage tissue engineering techniques, the ACT (Autologous Chondrcyte Transplantation), have shown promising clinical results [15, 156, 157]. However, as yet, there have been no reports that ACT is superior to other surgical techniques for articular cartilage repair, like subchondral plate drilling or mosaic plasty [62,89]. For cartilage tissue engineering to become a superior technique for cartilage repair many problems have to be addressed. In this thesis we focussed on two issues concerning cartilage tissue engineering. Firstly, we conducted several experiments to enhance the chondrocyte phenotype and extracellular matrix production during the tissue engineering process. Secondly, we tested the feasibility of using ear chondrocytes as donor cells for cartilage.

Chondrocyte dedifferentiation during monolayer expansion culture, searching ways to prevent this and develop methods to regain the differentiated phenotype concern a substantial part of published studies in the field of cartilage tissue engineering [8, 32, 36, 37, 60, 61, 73, 126, 127, 174, 206]. These studies are performed under the assumption that less dedifferentiated, or better differentiated, chondrocytes will produce a better quality repair tissue. Surprisingly, only one study can be found in literature in which cartilage repair with differentiated chondrocytes is compared to cartilage repair with culture-expanded, and hence dedifferentiated, chondrocytes [81]. This study supports the above mentioned assumptions. In fact, we also found that the ability to generate cartilage in a nude mouse model was better for both porcine knee and ear chondrocytes when primary cells were implanted, as opposed to culture-expanded cells (Chapter VII). Thus, extrapolated to cartilage tissue engineering, optimizing the monolayer expansion culture in such ways that chondrocyte dedifferentiation is reduced seems justified. To achieve this, we focussed on two important factors in monolayer

expansion culture: the culture medium and seeding density of cells onto the culture flask. Regarding the first, we used a SFM (Serum-Free Medium) with a serum-substitute (ITS+) and FGF2 (Fibroblast Growth Factor 2). Throughout the various experiments the SFM with FGF2 proved to enhance total cell yield significantly and to improve the cellular phenotype during monolayer expansion culture compared to the commonly used SCM (Serum-Containing Medium), Chapter III and V. Furthermore, we showed that the beneficial effects of the SFM with FGF2 were sustained during suspension cultures *in vitro* and in a nude mouse model (Chapter V). We can therefore conclude that the SFM with FGF2 greatly improved our monolayer expansion culture, compared to SCM. Naturally, the ultimate goal will be implantation in an actual cartilage defect. How chondrocytes that have been expanded in the SFM with FGF2 will behave in such an environment, *in situ*, we do not know as yet. Also, we supplemented our SFM with only one growth factor: FGF2. It is, however, not unlikely that for optimal performance other growth factors should be added. Concurrently, several research groups are developing new expansion media. Besides FGF2, growth factors like TGFß-2 (Transforming Growth Factor β-2), EGF (Epithelial Growth Factor) and IGF1 (Insulin-like Growth Factor 1) are under investigation [20, 37, 73, 162]. Unfortunately, insufficient data have yet been published to compare the different expansion media. Another complicating factor is that the different research groups use different cell sources, i.e. nasal chondrocytes, articular chondrocytes, ear chondrocytes and bone marrow stromal cells, to develop and test their expansion media [20, 37, 73, 162].
Next to the higher cell yield and positive actions on the chondrocyte phenotype, using the SFM during monolayer culture largely abolished the use of serum in our culture system. However, bovine serum was needed for 24 hours to promote cell-attachment onto the culture flask after each passage. Autologous serum or special pre-coated culture flasks could be an alternative for bovine serum as a promoter for cell-attachment and will have to be introduced in future cultures.

Besides optimizing the monolayer expansion culture, the quality of the engineered cartilage can also be enhanced by using bioactives during subsequent cultures *in vitro*, *in vivo* or *in situ*. Previously, using the combination of IGF1 and TGFß-2 during *in vitro* suspension cultures enhanced both the re-expression of collagen type II and proteoglycans [199]. Experiments in this thesis underscored these findings (Chapter III, IV, V and VII). Compared to SCM without additives a SFM with IGF1 and TGFß-2 increased redifferentiation of dedifferentiated chondrocytes.

An environment with mechanical loads and stresses is physiological for articular cartilage *in situ* [171, 204, 219]. Therefore, introducing controlled mechanical loading during the *in vitro* cultures for cartilage tissue engineering purposes might function as a promoter of redifferentiation and extracellular matrix formation.

Evenmore, we hypothesized that mechanical loading could push the phenotype of dedifferentiated ear chondrocytes towards a more articular-like phenotype and extracellular matrix production, so-called 'transphenotyping'.
Since the beginning of cartilage tissue engineering much research has been performed on mechanical loading on cartilage and chondrocytes *in vitro*. However, during this period of about 15 years, little significant progress has been made and the introduction of controlled mechanical loading as a useful tool in cartilage tissue engineering is far from real. To investigate controlled mechanical stimuli as a bioactive in the tissue engineering process, we designed and built a loading device that could dynamically compress cartilage constructs or cell-seeded biomaterials. In experiments with primary (differentiated) bovine articular chondrocytes in agarose gels we were able to enhance the proteoglycan production with controlled mechanical stimuli compared to control in experiments lasting several days (Chapter VII). Being able to perform longer-term loading experiments was an important criterion, as controlled mechanical stimuli should very likely be applied over a longer period in order to be an effective bioactive. However, we also showed that during longer-term loading experiments several factors become increasingly important. The elaborate matrix, for instance, will influence the reaction on the proteoglycan production of the chondrocyte to mechanical deformation. And to have a sustained positive effect of mechanical loading, the loading regime might need constant adjustments to changing conditions. This might well explain why so little progress has been made in the last 15 years to introduce mechanical loading as a bioactive in cartilage tissue engineering. Most loading regimes reported in literature have been formed by 'trial and error' or by modification of a previous published and successful loading regime, which is exactly what we used for our experiments. We propose the introduction of subtle and soundly based loading regimes. Future mechanical loading experiments for cartilage tissue engineering purposes should be performed in close harmony with research on chondrocyte biomechanics and mechanotransduction pathways. Only then significant progress can be made and controlled mechanical loading might be introduced as a useful tool in cartilage tissue engineering.

Another specific aim of this thesis was to test the feasibility of using ear chondrocytes for articular repair. Therefore, the majority of the experiments in this thesis were performed with ear chondrocytes. Pursuing alternative donor tissues for articular repair has several generally accepted (theoretical) grounds. Firstly, filling one articular cartilage defect by creating another articular cartilage defect does not seem logical. In fact, harvesting donor cartilage from the already diseased joint is likely to inflict additional morbidity. Surprisingly, published data concerning for instance the ACT procedure and mosaic plasty fail to recognize and discuss the issue of donor-site morbidity. However, a recent publication by van Susante et al. showed that osteochondral defects, which are

made in mosaicplasty for harvesting purposes, did not heal and that the surrounding cartilage and bone deteriorated [203]. A second reason to pursue alternative cell-sources for articular repair, which is in line with the previous mentioned, is the limited amount of articular cartilage that can be used for transplantation or donor purposes.
Our data showed that ear cartilage yielded a higher cell amount per mg tissue compared to articular cartilage. Furthermore, during expansion culture a higher cell yield could be obtained with ear chondrocytes of both human and porcine origin. This applied for both our experimental SFM with FGF2 as with the conventional SCM (Serum-Containing Medium). Also, these culture-expanded ear chondrocytes proved to retain the capacity to redifferentiate during *in vitro* and *in vivo* suspension cultures. Thus, we can state that in our experiments ear chondrocytes performed at least as well as articular chondrocytes and on several aspects were superior to articular chondrocytes. However, with our present knowledge we can only speculate whether ear chondrocytes are suitable for articular repair. To obtain successful and long-term articular cartilage repair the engineered tissue might not have to resemble the native tissue. Small differences in matrix content, i.e. the presence of elastin, could well be of less importance. Probably more important is whether the mechanical properties are consistent with the native tissue. Because ear cartilage has only a supportive function, no systematic research has been performed on its mechanical properties. In fact, a shortage in our experiments is that we did not assess the mechanical properties of our engineered cartilage. Future studies in our laboratory will therefore comprise research on mechanical properties of the tissue-engineered neo-cartilage from nude mice studies. Furthermore, the mechanical properties of native articular cartilage and ear cartilage will have to be assessed in order to relate the properties of the different tissues.

At present, growth factors like FGF2 have only limited clinical use. FGF2 has been used as a topical agent in chronic venous ulcera, with varying results [152, 163]. Also, FGF2 is under current investigation for treatment of acute and chronic lesions in the tympanic membrane [109]. Until now, no adverse effects have been reported with respect to these clinical applications of FGF2. However, several considerations regarding tissue engineering and the use of growth factors should be made. With tissue engineering techniques cells are extracted from their natural environment, enzymatically isolated and subsequently artificially modified *in vitro* using culture media, scaffold matrices and growth factors. Although no reports of cell aberrations or metaplasia have been reported in literature regarding cartilage tissue engineering, we should always bare in mind that the whole process could have negative effects on the chondrocyte. For instance, studies on cell senescence showed that *in vitro* expansion culture of chondrocytes resulted in telomere shortening similar to 30 years of aging [151]. Furthermore, as FGF2 significantly increased cell expansion in our studies, it is likely to further increase

telomere shortening. In fact, this has also been suggested for skin fibroblasts, which were cultured in the presence of FGF2 [176]. Thus, accelerated cell senescence could have implications on the long-term survival of the engineered repair tissue. Systemic side effects of growth factors is another issue regarding tissue engineering. At present, growth factors in cartilage tissue engineering are mostly used in an *in vitro* setting, making systemic effects at the time of implantation *in situ* very unlikely. However, with the development of so-called smart scaffolds [142] (scaffold matrices pre-loaded with growth factors) systemic side effects of the growth factors in the scaffold-cell construct could become relevant.

Concluding, the main aims of this thesis, improving the chondrocytes phenotype and extracellular matrix production during the tissue engineering process together with the feasibility of using ear chondrocytes as donor cells have been met, although some in a preliminary state. However, with our present results and new knowledge we can initiate new experiments to fulfill the stated aims in a more advanced level. Ultimately, this should lead to a durable repair of cartilage defects using tissue engineering techniques.

X

Summary

Articular cartilage defects pose a difficult problem in orthopaedic surgery. Unlike vascularized tissues such as skin and bone, cartilage has limited intrinsic repair capacity. Current surgical options to treat articular cartilage defects are limited and have a sub optimal long-term outcome. However, since 15 years new and promising techniques, named *Tissue Engineering*, have been developed to overcome the general shortage in donor material and organs. For orthopaedic surgery tissue engineering could provide a serious alternative to treat articular cartilage defects. First generation cartilage tissue engineering techniques, based upon the technique of ACT (Autologous Chondrocyte Transplantation), have been clinically employed in Sweden and the US for the last 13 years. Although the first long-term results (2-9 years) are promising, ACT has not yet been proven to be superior to subchondral plate perforation and osteochondral plug transplantation (and mosaic plasty). The resulting repair tissue resembles fibrocartilage at best and frequently deteriorates within time. This thesis mainly discusses several interventions into the cartilage tissue engineering process that are aimed at the improvement of the chondrocyte phenotype and extracellular matrix production during and after the *in vitro* process. Also, several experiments were performed with ear chondrocytes as a possible alternative cell source for articular cartilage tissue engineering.

Chapter I is an overview of the structure and function of articular cartilage and ear cartilage. Furthermore, the process of cartilage tissue engineering is introduced and discussed.
Chapter II deals with the specific aims of this thesis. The first aim was to improve the chondrocyte phenotype and extracellular matrix production during the tissue engineering process. The second aim was to test the feasibility of ear cartilage as donor tissue for cartilage tissue engineering.
In **Chapter III** immature human ear chondrocytes were culture expanded using a defined SFM+FGF2 (Serum-Free Medium + Fibroblast Growth Factor 2). As a control the classically used SCM (Serum-Containing Medium, fetal calf serum) was used. Although total multiplication was comparable, the ability to regain the differentiated phenotype and express functional extracellular matrix molecules

during subsequent suspension culture in alginate was significantly enhanced when the SFM with FGF2 had been used during expansion culture.

Chapter IV describes the effect of lowering the seeding densities during monolayer culture and its effect on total multiplication and the redifferentiation capacity of immature human ear chondrocytes. A relatively low seeding density (7500 cells/cm^2) requires less time and fewer passages to reach a certain cell yield during monolayer culture compared to high seeding densities (30,000 cells/cm^2). Surprisingly, the subsequent redifferentiation capacity is at least comparable and probably better when lower seeding densities were used. This latter observation can probably be attributed to less time in expansion culture and fewer passages needed.

In **Chapter V** adult human ear chondrocytes are culture expanded in the SFM with FGF2 at lower seeding densities (7500 cells/cm^2 or 15,000 cells/cm^2) and compared to expansion in SCM (10% FCS (Fetal Calf Serum)). Unlike the results of the study discussed in Chapter III, total cell yield was significantly higher when SFM+FGF2 had been used. With real-time PCR techniques it was shown that the chondrocyte dedifferentiation was less with SFM+FGF2 as compared to expansion in SCM, despite the higher multiplication. Next, the culture-expanded chondrocytes were encapsulated in alginate and cultured *in vitro* or subcutaneously implanted in nude mice. After either culture had been stopped the gene expressions and biochemical assays on (GlycosAminoGlycan) and DNA, next to immunohistochemical stainings, still favored chondrocytes that had been expanded with SFM+FGF2.

In **Chapter VI** several considerations on the use of ear chondrocytes as donor cells for cartilage tissue engineering are discussed. Experiments with articular chondrocytes and ear chondrocytes are compared. Per mg wet tissue more cells could be isolated from ear cartilage than from articular cartilage. Furthermore, monolayer expansion culture yielded more cells in the same amount of time when ear chondrocytes were used. During subsequent culture in alginate beads both cell types re-expressed collagen II and GAG (GlycosAminoGlycan). We conclude that ear chondrocytes are suitable for cartilage tissue engineering in general and have several advantages over articular chondrocytes. However, whether ear chondrocytes are suitable for articular repair has to be assessed in future research.

Chapter VII discusses the role of several growth factors in cartilage tissue engineering.

A compilation of experiments are presented which show that the effects of several typical growth factors in cartilage tissue engineering FGF2, TGFβ-2 and IGF1 (respectively Fibroblast Growth Factor 2, Transforming Growth Factor β-2 and Insulin-like Growth Factor 1) are dependant upon the expressed phenotype and culture history of the chondrocytes.

In **Chapter VIII** the effects of *in vitro* mechanical loading on the expressed extracellular matrix were investigated. Isolated bovine articular chondrocytes

were encapsulated in an agarose gel and subjected to dynamic mechanical loading via a custom-made loading device. Compared to control the dynamically loaded cell-seeded agarose gels produced more GAG when the loading regime (two times 90 minutes loading per day for four days with a frequency of 0.5 Hz and a peak strain of 10%) was started 2 hours after the gels had been made. When a preculture of 4 days preceded the loading regime no additional effect of the dynamic loading on the GAG production was observed compared to the static control. After 4 days of preculture a pericellular matrix was formed and the cell-strain decreased. We hypothesized that the decreased cell-strain after 4 days of preculture was responsible for the absent effect of dynamic loading on the GAG production. Therefore, we propose that loading regimes should be adjusted to the changing conditions (i.e. extracellular matrix deposition) during mechanical loading experiments.

Chapter IX discusses the overall finding of this thesis. We conclude that the aims of this thesis have been met, although in a preliminary state. By using a defined SFM with FGF2 in combination with low seeding densities during monolayer expansion culture we have been able to enhance our cell yield and at the same time improve the chondrocyte phenotype. Furthermore, we were able to stimulate extracellular matrix production with *in vitro* mechanical loading. Taken together, for cartilage tissue engineering purposes this could imply a better quality repair tissue. Alongside, we found that ear chondrocytes are a suitable cell source for cartilage tissue engineering. Whether ear chondrocytes are suitable for articular repair will have to be assessed in future experiments.

XI

Samenvatting

Kraakbeendefecten in gewrichten herstellen blijft een uitdaging in de orthopaedische chirurgie. In tegenstelling tot bijvoorbeeld huid en bot heeft kraakbeen geen bloedvoorziening en daardoor beperkte intrinsieke capaciteit tot genezing. Naast pijn en functiebeperking kunnen kraakbeendefecten aanleiding geven tot gegeneraliseerde artrose binnen het gewricht. Huidige chirurgische behandeltechnieken bestaan uit het opboren van de subchondrale plaat en het transplanteren van osteochondrale pluggen (zogenaamde mozaïekplastiek).
Echter, sinds 15 jaar worden nieuwe technieken vanuit de technische en medische wetenschap gebundeld om nieuwe weefsels te genereren in het laboratorium. Deze technieken worden samengevat onder de naam 'tissue engineering'. Sinds een kleine tien jaar worden tissue-engineeringproducten toegepast om kraakbeen defecten te behandelen (voornamelijk in Zweden en de Verenigde Staten). Deze techniek, de autologe chondrocytentransplantatie ACT (Autologous Chondrcyte Transplantation), wordt beschouwd als de eerste generatie tissue engineering van kraakbeen. Bij deze behandelmethode worden autologe gewrichtschondrocyten in het laboratorium vermenigvuldigd en in het kraakbeendefect achter een periostlapje teruggeplaatst. Hoewel de eerste langetermijnresultaten bemoedigend zijn, zijn er nog geen bewijzen dat ACT superieur is aan chirurgische behandeltechnieken voor kraakbeendefecten zoals het opboren van de subchondrale plaat en de mozaïekplastiek met osteochondrale pluggen. Meestal bestaat het weefsel uit fibreus kraakbeen, met inferieure kwaliteiten ten opzichte van hyalien kraakbeen. Een belangrijk aspect van zowel dierproeven als hernieuwde kijkoperaties na de ACT-procedure is dat er een relatie bestaat tussen de kwaliteit van het 'littekenweefsel' en de functionele en histologische resultaten op langere termijn. Het optimaliseren van de tissue-engineeringtechnieken om de kwaliteit van het te genereren herstelweefsel te verhogen lijkt dus zinvol; dit is het hoofddoel van dit proefschrift. Het tweede doel van dit proefschrift is de haalbaarheid van een alternatieve bron van donorcellen voor het repareren van gewrichtskraakbeen: oorchondrocyten.

Hoofdstuk I geeft een algemene inleiding tot de structuur en samenstelling van kraakbeen. Daarnaast worden huidige chirurgische technieken voor het herstel van gewrichtskraakbeen besproken. Als laatste wordt het proces 'tissue engineering van kraakbeen' geïntroduceerd en nader belicht.
In **Hoofdstuk II** worden de specifieke doelstellingen van dit proefschrift besproken. Ten eerste het verbeteren van het fenotype en de daarmee samenhangende extracellulaire matrixproductie van de chondrocyten gedurende het proces van tissue engineering. En als tweede het gebruik van oorchondrocyten voor tissue engineering van kraakbeen in het algemeen en gewrichtskraakbeen in het bijzonder.
In **Hoofdstuk III** worden experimenten besproken waarin onvolwassen oorchondrocyten werden vermenigvuldigd in een serumvrij kweekmedium met FGF2 (Fibroblast Growth Factor 2). Dit werd vergeleken met vermenigvuldiging in het standaard serumhoudende kweekmedium. Hoewel de totale celopbrengst vergelijkbaar was, was tijdens additionele kweken de capaciteit tot re-expressie van kraakbeen-specifieke moleculen hoger wanneer het serumvrije medium met FGF2 was gebruikt tijdens de expansiekweek.
In **Hoofdstuk IV** komen expansiekweken aan de orde, waarbij het aantal cellen per oppervlakte-eenheid bij het begin van de kweek, kortweg de inzaaidichtheid genoemd, verschillend is. Het gaat daarbij om kweken met onvolwassen, menselijke oorchondrocyten. Het doel van deze experimenten was het verkrijgen van voldoende cellen gedurende de expansiekweek om kraakbeen te genereren, uitgaande van een bepaalde hoeveelheid donorcellen. Om een gemiddeld kraakbeendefect te vullen zal ongeveer een 20-voudige vermenigvuldiging nodig zijn. Sinds lange tijd is bekend dat bij lagere inzaaidichtheden de cellen zich vaker vermenigvuldigen voordat ze in de kweek tegen elkaar aan groeien. Echter, vaker vermenigvuldigen geeft ook meer dedifferentiatie. Uit de experimenten beschreven in Hoofdstuk IV blijkt dat met een lage inzaaidichtheid (3500 en 7500 cellen/cm^2) de vereiste 20-voudige vermenigvuldiging in kortere tijd wordt bereikt dan met een hogere inzaaidichtheid (15.000 en 30.000 cellen/cm^2). Daarnaast blijkt dat de capaciteit tot re-expressie van het gedifferentieerde fenotype (dat zijn de cellen als product van hun erfelijke aanleg en de door hen ondergane invloeden uit hun leefomgeving) groter is voor de cellen die zijn vermenigvuldigd met een lagere inzaaidichtheid. Voor tissue engineering van kraakbeen met chondrocyten lijkt het gebruik van korte expansiekweken met een lagere inzaaidichtheid en minder passages gunstig voor het behoud van fenotype.
In **Hoofdstuk V** worden volwassen humane oorchondrocyten vermenigvuldigd met lage inzaaidichtheden in serum-vrij kweekmedium met FGF2 of in serumhoudend kweekmedium. In tegenstelling tot de experimenten beschreven in Hoofdstuk III wordt nu wel een significante hogere celopbrengst (met dezelfde inzaaidichtheid) bereikt met het serum-vrije medium met FGF2. Met behulp van real-time PCR technieken blijken de cellen die met behulp van het serum-vrije medium met FGF2 zijn vermenigvuldigd minder te zijn gededifferentieerd aan

het eind van de expansiekweek, ondanks de significante hogere vermenigvuldiging. Na de expansiekweek worden de chondrocyten uit beide groepen (serumvrij met FGF2 of serumhoudend) gesuspendeerd in alginaat en verder *in vitro* gekweekt onder gelijke condities en ook subcutaan geimplanteerd in naakte muizen. Het blijkt dat de cellen die zijn vermenigvuldigd in het serum-vrije medium met FGF2 beter in staat zijn om een kraakbeen-achtige matrix te produceren dan cellen die zijn vermenigvuldigd in serumhoudend kweekmedium. Ergo, het gunstiger (minder gededifferentieerde) fenotype aan het eind van de expansiekweek wordt tot expressie gebracht dan wel vastgehouden gedurende verdere kweken *in vitro* en tevens na implantatie *in vivo*.

Hoofdstuk VI bepreekt overwegingen omtrent het gebruik van oorchondrocyten als donorcellen voor het generen van gewrichtskraakbeen. Experimenten met beide typen kraakbeencellen worden vergeleken. Per mg weefsel levert oorkraakbeen meer donor cellen op dan gewrichtskraakbeen. Daarnaast blijkt dat expansiekweken met oorchondrocyten een grotere celopbrenst hebben. Beide celtypen vertonen een vergelijkbare capaciteit tot re-expressie van de juiste extracellulaire matrix componenten (bijvoorbeeld collageen type II). Wij concluderen dat oorchondrocyten geschikt zijn voor tissue engineering van kraakbeen. Of oorchondrocyten geschikt zijn voor het herstel van gewrichtskraakbeen zal moeten blijken uit toekomstige experimenten.

Het gebruik van groeifactoren, zoals onder andere FGF2, IGF1 (Insulin-like Growth Factor 1) en TGFβ-2 (Transforming Growth Factor β-2), staat centraal in **Hoofdstuk VII**. Verschillende experimenten met deze groeifactoren worden besproken. Groeifactoren kunnen zeer waardevol zijn voor tissue engineering van kraakbeen. Belangrijk is dat het effect van de diverse groeifactoren afhankelijk kan zijn van factoren zoals bijvoorbeeld eerdere kweken of aanwezigheid van extracellulaire matrixcomponenten.

In **Hoofdstuk VIII** wordt een zelf ontworpen en vervaardigd belastingapparaat geïntroduceerd waarmee kraakbeen of chondrocyten in een biomateriaal mechanisch kunnen worden belast door middel van compressie. De achterliggende gedachte is dat mechanische prikkels belangrijk zijn voor de homeostase van kraakbeen en chondrocyten. Mechanische prikkels gedurende het proces van tissue engineering zouden de (gededifferentieerde) chondrocyt kunnen verleiden tot de expressie van de juiste (redifferentiatie) en meer extracellulaire matrix componenten. In dit kader werd begonnen met experimenten waarbij gewrichtschondrocyten van kalveren in een schijfje agarosegel werden ingebracht en vervolgens cyclisch werden gecomprimeerd (tot 90% van de oorspronkelijke hoogte) gedurende vier dagen. De hoeveelheid aanwezige GAG (GlycosAminoGlycan) aan het eind van de experimenten was hoger dan in de controlegroep. Dit werd overigens alleen waargenomen als aan het begin van de experimenten nog geen extracellulaire matrix aanwezig was in de agarose. Na een voorkweek van vier dagen, die resulteerde in een pericellulaire matrix productie, was de resulterende celcompressie als gevolg van

de 10% compressie van het agaroseschijfje significant lager (ongeveer 4%) en werd geen effect meer gezien van cyclische compressie. Uit deze experimenten blijkt dat de aanwezige extracellulaire matrix en de productie hiervan een grote invloed kan hebben op mechanische belasting experimenten met kraakbeen of chondrocyten. Toekomstige experimenten zullen moeten uitwijzen of mechanische prikkels gedurende het proces van tissue engineering een waardevolle aanvulling kunnen zijn als modulator van het fenotype en de daarmee samenhangende extracellulaire matrixproductie.

Hoofdstuk IX is de discussie. De gestelde doelen van dit proefschrift zijn bereikt, waarbij sommige voornamelijk in een beginfase. Door het gebruik van lage inzaaidichtheden in combinatie met het serum-vrije kweekmedium met FGF2 gedurende de expansiekweek zijn we in staat gebleken om zowel de celopbrengst te verhogen als de mate van dedifferentiatie te verkleinen. Met behulp van het zelf ontworpen en vervaardigde belastingapparaat en met de resultaten van de eerste reeks experimenten hebben wij een aanzet gegeven tot een nieuwe reeks experimenten in ons laboratorium waaruit moet blijken of mechanische compressie de kwaliteit van het gegenereerde kraakbeen kan verbeteren.

Een substantieel deel van de experimenten in dit proefschrift zijn uitgevoerd met oorchondrocyten. Gedurende deze experimenten is gebleken dat oorchondrocyten geschikt zijn voor tissue engineering van kraakbeen. Of met behulp van oorchondrocyten een succesvol herstel van gewrichtskraakbeen kan worden bewerkstelligd zal nog moeten blijken.

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Dankwoord

‘De laatste loodjes wegen het zwaarst’. Tot voor een aantal maanden heb ik mij eigenlijk nooit gerealiseerd hoeveel werk en inspanning het vervaardigen van een proefschrift en alle randfenomenen met zich meebrengt. Zeker naast een full-time baan en een gezin met twee jonge kinderen. Daarom wil ik in de eerste en voornaamste plaats jou, lieve Shilo, bedanken voor jouw steun zodat ik alle tijd had voor het afronden van deze beproeving. Daarnaast wil ik mijn ouders bedanken voor de kansen die door hen gecreëerd zijn om in feite alles te doen wat ik wilde. In het bijzonder wil ik mijn vader bedanken voor het verzorgen van de layout, een noodzakelijk kwaad.
Mijn schoonouders; Henk en Miep, veel dank zijn wij jullie verschuldigd voor het feit dat Henk nu al 3 jaar het kinderdagverblijf ‘buiten de deur houdt’ en ons ontlast van veel taken.

Mijn promotor, Prof. dr. J.A.N. Verhaar. Via Den Haag ben ik uiteindelijk toch in Rotterdam beland. Dank u voor de geboden kans tot het verrichten van dit promotieonderzoek. Het was altijd verbazingwekkend hoe snel (meestal na 1 dag) de gecorrigeerde manuscripten klaarlagen. Met het laboratorium heeft u voor Nederland een unieke mogelijkheid tot fundamenteel orthopaedisch onderzoek in handen. Tijdens mijn vervolgopleiding in het Erasmus Medisch Centrum hoop ik het onderzoek weer voort te zetten.

Harrie Weinans. Beste Harrie, jouw enthousiasme en harde werken heeft ervoor gezorgd dat het orthopaedisch lab snel groter is geworden en internationaal op de kaart staat. Bedankt voor het altijd logische en heldere commentaar op het onderzoek.

Mijn co-promotor, Gerjo van Osch. Beste Gerjo, binnen het lab ben jij zonder meer de belangrijkste factor in het totstandkomen van dit proefschrift. Veel dank ben ik jou verschuldigd voor de directe begeleiding in mijn onderzoek. Zorg ervoor dat de kraakbeenlijn binnen het lab in leven blijft.

Mijn lotgenoten op het laboratorium, Jarno, Elian en Justus. Ook jullie zijn alweer bijna klaar op het lab. Bedankt voor de gezellige tijd in de nadagen van mijn verblijf op het laboratorium. Lang leve BMW (not).
Koen en Willem-Jan, veel succes met het afronden van jullie promotieonderzoeken.

Simone, Nicole en in het bijzonder Wendy, veel dank voor jullie hulp met het kweken en uitvoeren van vaak saaie bepalingen en kleuringen.

Holger(meister), jouw ‘Gründlichkeit’ is een voorbeeld voor alle arts-onderzoekers in het laboratorium. Veel dank voor je hulp en nooit afwezige enthousiasme.
Jacqueline, bedankt voor het medeontwikkelen van het belastingsapparaatje en de altijd ongezouten kritieken. Erwin en Judd, succes met het afronden van jullie promotieonderzoek.
Mijn kamergenotes, eerst Franci en later Irene, bedankt voor jullie geduld. Het zal af en toe niet meegevallen zijn om mijn kamergenoot te zijn...
Alex Kleinjan, eerst bij de KNO en later bij Longziekten, dank voor jouw hulp en opvoeding ‘hoe te werken (en te gedragen) in een lab’. Esther, Inesz en Barbera, ook jullie hebben daar zeker in bijgedragen.
De afdeling Urologie, en vooral Corrina de Ridder en Cindy Bolder, bedankt voor jullie hulp bij het opereren en verzorgen van de muizen.
Daarnaast wil ik Ruud Niesing en Cor Goedegebuure bedanken voor hun hulp bij het vervaardigen van het belastingsapparaatje.

De partners van het project ‘Finding novell cell sources for cartilage tissue engineering’: de afdeling Orthopaedie van het Academisch Ziekenhuis Maastricht en Isotis. Roel, Edwin (schlappenbar), Pieter, Jens, Tim en Jos, bedankt voor de wetenschappelijke input, noodzakelijke meetings en gezellige diners.

Mijn paranimfen, Anne van Lievenoogen en Jarno van de Breevaart Bravenboer. Anne wij kennen elkaar nu alweer ongeveer 16 jaar, met ups en downs. Ik ben blij dat jij naast mij staat tijdens de verdediging en hoop nog lang vrienden te blijven. Jarno, dat ook jij naast mij staat tijdens de verdediging lijkt mij niet meer dan logisch. Als mijn opvolger heb jij veel bijgedragen aan dit proefschrift en ken jij als geen ander de tegenslagen van wetenschappelijk onderzoek (lees: pneumatisch aangedreven apparaten). Ik zal de unieke tijd in San Francisco en Sausalito nooit vergeten.

Lieve Lucas en Jasmijn, jullie liefde en vrolijkheid zijn het alle belangrijkste wat bestaat. Jasmijn, je zit nu naast mij terwijl ik dit schrijf, je bent bijna alweer 1 jaar. Wat worden jullie toch snel groot. Soms gaat het te snel, zeker de laatste maanden.
Tot slot, nogmaals, lieve Shilo, ik had dit zeker niet zonder jou kunnen doen.

Curriculum Vitae

Erik William Mandl werd op 22 oktober 1973 geboren te Groningen. In 1992 behaalde hij het diploma Atheneum B aan het Drachtster Lyceum in Drachten. Na aanvankelijk te zijn uitgeloot voor de studie Geneeskunde werd hij alsnog nageplaatst in Rotterdam, alwaar het artsexamen in september 1998 werd afgelegd. Van september 1998 tot januari 2000 was hij werkzaam als AGNIO Heelkunde/Orthopaedie in het Ruwaard van Putten ziekenhuis te Spijkenisse. Hierna was hij AGNIO orthopaedie in het Leyenburg ziekenhuis te Den Haag tot oktober 2000. Vervolgens startte hij met promotieonderzoek in het Orthopaedic Research Laboratory aan de Erasmus Universiteit Rotterdam onder leiding van prof. dr. J.A.N Verhaar, dr. H. Weinans en dr. G.J.V.M van Osch. In juli 2003 startte hij met de vooropleiding Heelkunde in het Medisch Centrum Rijnmond Zuid (MCRZ, opleider dr. J.F. Lange). De specialisatie tot orthopaedisch chirurg zal worden afgerond in het Erasmus Medisch Centrum te Rotterdam (opleider prof. dr. J.A.N. Verhaar).