Cartilage Tissue Engineering

the effect of different biomaterials, cell types and culture methods

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CHAPTER 1

INTRODUCTION

Cartilage is a tissue with unique properties consisting of a small number of highly specialized cells (chondrocytes) surrounded by a large amount of extracellular matrix.

The inefficient healing response of damaged cartilage was recognized as early as 1743 by Sir William Hunter[1]. Despite extensive experimental and clinical data on the repair of damaged cartilage, none of the current treatment options has led to a repair tissue that histologically, biochemically and functionally closely resembles native cartilage and does not deteriorate over time. Tissue engineering, defined as "techniques that apply the principles of biology and engineering to the development of functional substitutes for damaged tissue"[2], is regarded as a promising treatment option, particularly in the field of (transplantation) surgery. Human skin was one of the first tissues generated and can be used to treat burn victims. Research currently focuses on a variety of tissues, liver, cardiac muscle, bone and cartilage. In theory, cartilage is one of the most promising tissues for clinical application because it consists of only one cell type and lacks vascular and neural tissue.

Based on discrete differences in its composition, cartilage tissue in the human body can be divided in three subtypes. Hyaline cartilage is the predominant type, lines the articulating surfaces in all joints, and is present in large parts of the respiratory tract; elastic cartilage is found in the outer ear and some parts of the respiratory tract; and fibrocartilage is found in the menisci and intervertebral discs.

The work presented in this thesis addresses the problem of damaged cartilage from an orthopaedic point of view and will therefore focus mainly on articular cartilage and articular cartilage defects.

CARTILAGE STRUCTURE AND COMPOSITION

In human adults chondrocytes occupy less than 10% of the tissue volume[3], whereas in the developing embryo cartilage formation starts with a cell density that is many times higher. Chondrocyte precursor cells become closely packed before they start to differentiate into chondrocytes. Thereafter the cells proliferate and by the secretion of extracellular matrix the cells eventually become isolated in individual compartments or lacunae completely surrounded by matrix[4,5]. The chondrocytes are metabolically active and sustain a vast amount of extracellular matrix, which gives cartilage its unique properties.

Hyaline cartilage, found in all diarthrodal joints in the human body, can be divided into four zones with distinct differences in cell shape and volume, collagen fibril diameter and orientation, proteoglycan concentration, and water content.

Closest to the joint cavity lies the superficial zone acting as the gliding surface of the articular cartilage. Collagen fibrils are thin and lie parallel to the surface, thereby enabling low friction

between the gliding surfaces. Chondrocytes of the superficial zone are flat, the proteoglycan content of the extracellular matrix is low, and the water content is high.

Underneath the superficial zone lies the middle or transitional zone. At this level the collagen fibrils are thicker and randomly organized, and the chondrocytes have a more rounded shape.

In the deep zone collagen fibrils are thick and oriented perpendicular to the surface. The chondrocytes in this zone are rounded and lie in vertical columns. The proteoglycan content is high, with a relatively low water content.

The tidemark separates the deep zone from the calcified cartilage zone. The calcified cartilage zone represents the boundary between hyaline cartilage and the subchondral bone. In this particular zone the chondrocytes are small and the extracellular matrix is calcified.

Hyaline cartilage in the nasal septum and respiratory tract shows a more symmetrical structure and is lined by perichondrium on both sides.

Elastic cartilage, found in the ear and respiratory tract, is lined by perichondrium. The extracellular matrix of the tissue contains a network of elastic fibres. In contrast to articular cartilage where the structure changes from top to bottom, the tissue has a more homogenous, symmetrical structure. In this type of cartilage chondrocyte morphology is also different; the cells are larger, often have two nucleoli and contain more lipid vesicles.

Fibrous cartilage is found in the meniscus and intervertebral disc and can be regarded as an intermediate tissue between connective tissue and hyaline cartilage. The tissue has a significant amount of collagen type-I and the chondrocytes more closely resemble fibroblasts. It is the predominant repair tissue found in articular cartilage defects.

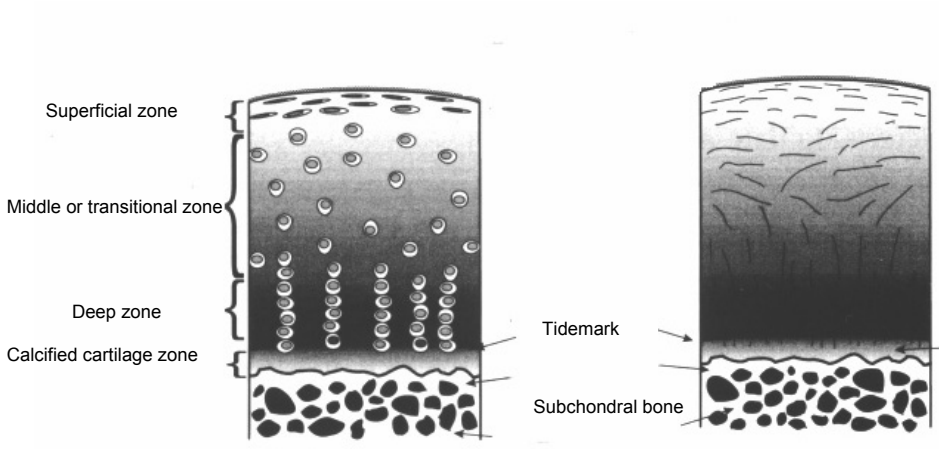

Figure 1. Hyaline cartilage can be divided into four zones with distinct differences in cell shape and volume and collagen fibril orientation.

Extracellular matrix of articular cartilage

Water makes up 65 to 80% of the *wet* weight of articular cartilage.

Collagen is the main structural component of the extracellular matrix, 50% of the tissue's *dry* weight is composed of collagen. Collagen type-II represents 90-95% of the total collagen. Other collagen types present are collagen V, VI, IX, X, and XI, all having a triple-helical structure. The collagen network, stabilized by cross-links gives articular cartilage its tensile and shear properties. Furthermore, the collagen network covalently binds the proteoglycans.

Proteoglycan is the third important constituent of the extracellular matrix. Proteoglycans consist of a protein core with glycosaminoglycan side chains. Three major types of glycosaminoglycans can be distinguished in cartilage: chondroitin sulfate, keratan sulfate and hyaluronic acid. A common feature of all glycosaminoglycans is the repeating carboxyl and sulfate groups; in solution these groups become negatively charged. Water molecules are bound to the negatively charged glycosaminoglycans leading to a Donnan osmotic swelling pressure. Constraining forces of the collagen network counteract this swelling pressure.

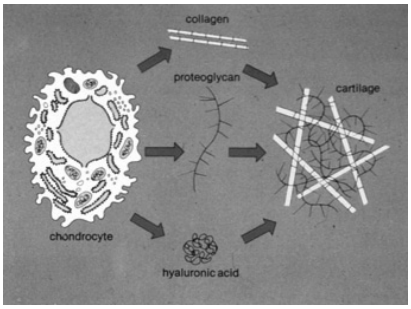

Figure 2. Collagen fibril network with proteoglycans.

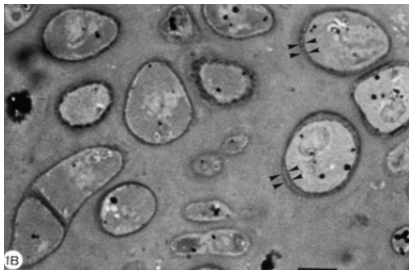

Figure 3. Electronmicroscopy; cell-associated matrix indicated between arrows

About 80 to 90% of the total amount of proteoglycans can be found as large aggregates around a central hyaluronic acid core. These large aggregating proteoglycans are called aggrecan. Other small proteoglycans (e.g. biglycan, decorin, collagen type-IX and fibromodulin) are found in the extracellular matrix as well as non-collagenous proteins, glycoproteins and lipids. However, their functions remain largely unclear and beyond the scope of this thesis.

The extracellular matrix can be divided in two compartments; the cell-associated matrix compartment lies closest to the chondrocyte and consists of the pericellular matrix and the territorial matrix. The second compartment is called the further-removed matrix compartment, also known as the interterritorial matrix.

The cell-associated matrix compartment and the further-removed matrix compartment differ in both their structure and composition. This led researchers to suggest that the two matrix compartments have different functions. The pericellular matrix is a thin layer adjacent to

the cell membrane and completely surrounds the chondrocyte. Proteoglycans are its primary constituent, whereas collagens are virtually absent. The territorial matrix lies between the pericellular and the further-removed matrix. The thin collagen fibrils of the territorial matrix form a network distinct from the surrounding further-removed matrix. The cell-associated matrix is hypothesized to play a regulating role in chondrocyte metabolism[6-8]. The further-removed (interterritorial) matrix is by far the largest compartment and is thought to provide structural support and give cartilage its loadbearing ability[6,9].

Metabolism

Although articular cartilage of human adults has no blood supply, articular chondrocytes show a high level of metabolism. Chondrocytes derive their nutrition mainly from the synovial fluid and to a lesser extent from the underlying bone. They synthesize and assemble extra-cellular matrix components and direct their distribution within the tissue. All this is done in order to maintain the structure and function of the extracellular matrix. The high level of metabolism is mainly due to proteoglycan turnover. Although collagen turnover does take place, its level is much lower.

The extracellular matrix is impermeable to cells and large molecules. Small molecules like growth factors (TGF-ß, bFGF), interleukins, pharmaceutical agents (nonsteroidal anti-inflammatory drugs, ascorbic acid), and (parts of) matrix molecules reach the chondrocytes by diffusion through the extracellular matrix. Physical stimuli like hydrostatic pressure and mechanical loading can also influence the behaviour of the chondrocytes[10-15].

CARTILAGE DEFECTS AND CURRENT SURGICAL TREATMENT OPTIONS

The cartilage lining of the articulations in the human body allows the joints to move freely under almost frictionless conditions. The tissue can withstand numerous repetitive loading cycles, in many individuals for their entire life without failing.

However, the cartilage lining can be disrupted by certain pathologic conditions. Osteochondritis dissecans and traumatic chondral and osteochondral lesions are the main causes of isolated focal articular cartilage lesions and can become invalidating. Disruption of the cartilage lining will alter the delicate equilibrium in the joint and can give rise to pain and loss of function. Repair tissue found in focal cartilage lesions is mechanically and chemically inferior to hyaline cartilage [16-20]. Once established, damage to the articular cartilage tends to accumulate leading to pain, loss of function and secondary degenerative changes in the affected joint and ultimately to arthrosis[16,18,19,21,22].

In an early stage after a focal cartilage lesion, secondary changes to the other structures of the joint have not yet occurred to a gross extent. Therefore efforts to repair articular cartilage

focus on these relatively fresh isolated focal lesions. Knowledge gained from repairing such lesions may lead to repair strategies for larger defects, complete joint 'resurfacing', or surfacing of artificial implants with living articular cartilage.

Cartilage has long been thought to lack progenitor cells. Although the presence of undifferentiated chondrocyte precursor cells in cartilage has been demonstrated [23], articular cartilage has very limited repair capacity. The chondrocytes are virtually locked in the extracellular matrix, leading to a limited capacity to proliferate. The extracellular matrix also limits the ability of the cells to move towards a defect.

The most important factor limiting the response of articular cartilage to injury is, however, the lack of blood supply. When a vascularized tissue is damaged a fibrin clot is formed. Inflammatory cells and undifferentiated stemcells migrate with the blood to the tissue defect. Inflammatory cells remove necrotic tissue, accompanied by proliferation and differentiation of undifferentiated cells leading to the formation of repair tissue[16,24,25].

When studying articular cartilage defects it is therefore essential to discriminate between superficial defects, (limited to the cartilage layer) and osteochondral defects extending into the subchondral bone. In superficial defects no blood clot is formed and as a consequence no repair tissue is formed. Cells surrounding the defect will die in a process called apoptosis[16]. Only when a cartilage injury perforates the subchondral bone can a fibrin clot be formed. Inflammatory and mesenchymal stem cells from the bone marrow can enter the defect and initiate a repair response, influenced by growth factors released from the platelets. But even then the repair is insufficient. The defect is filled with fibrocartilage, the tidemark is not restored and there is no integration with the intact native cartilage. The long-term performance of fibrocartilage is inferior to that of normal hyaline cartilage. It lacks mechanical strength and the collagen fibril network is disorganized[16-22]. Failure of the intrinsic repair capacity has led to several surgical techniques to treat isolated articular cartilage defects.

Intact cartilage from a non-weight bearing area of the joint can be transferred as an **osteochondral plug** to an articular cartilage lesion in techniques like 'OATS' and 'Mosaic plasty'. Although the bone plugs integrate very well with the subchondral bone, there is no integration at the cartilage level[26-28]. Moreover, donor-site morbidity remains a major concern.

Tissue engineering

In 1993 Langer and Vacanti defined tissue engineering as "techniques that apply the principles of biology and engineering to the development of functional substitutes for damaged tissue"[2]. This definition can be interpreted in a broader and a more narrow sense. Adhering to the broadest interpretation of this definition, many techniques available for the repair of damaged articular cartilage can be regarded as tissue engineering techniques.

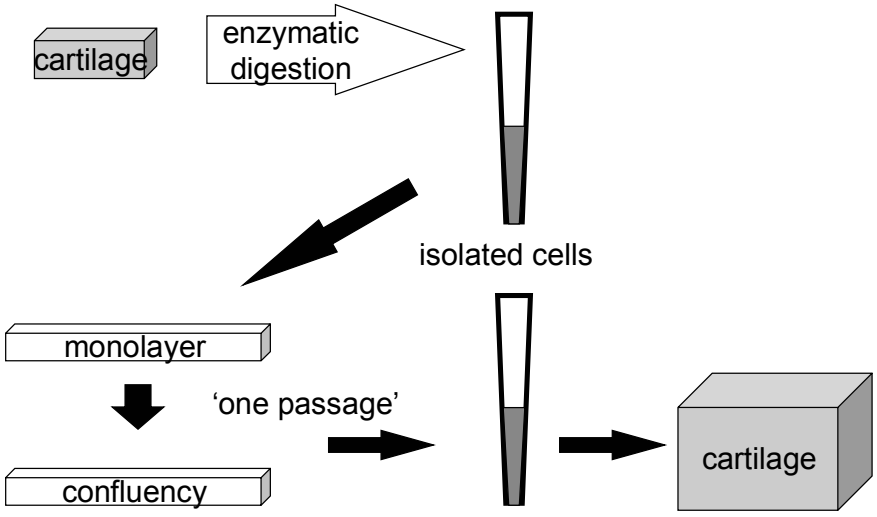

Figure 4. Schematic drawing of tissue engineering process.

Long before Langer and Vacanti's definition Pridie proposed to treat isolated articular cartilage defects by **perforating the subchondral bone**[29,30]. Microfracture is a similar technique using small awls to perforate the subchondral bone[31-34]. Both techniques create access of blood and mesenchymal stemcells from the bone marrow to the defect, leading to formation of fibrocartilage. Although the long-term performance of fibrocartilage is inferior to that of normal hyaline cartilage, clinical results of these bone marrow stimulation techniques are good; up to 80% of patients benefit from the operation[35].

Other early tissue engineering techniques use **perichondrium and periostium**, embryologically closely related tissues that contain stem cells with chondrogenic potential. The chondrogenic potential of perichondrium was recognized by Haebler in 1925[36]. The stem cells originate from the cambium layer i.e., the layer adjacent to the underlying cartilage or bone. After transplantation into a defect, both tissues have the potential to develop into cartilage when the cambium layer faces the joint cavity. However, a well-known drawback of the use of perichondrial grafts is calcification of the repair tissue at long-term follow-up[37-47].

A technique that uses **multiplied chondrocytes suspended at low densities**, was proposed by Grande et al. [48] and introduced to the clinic by Brittberg and co-workers as autologous chondrocyte transplantation[49,50]. Periost is sutured over a cartilage defect and the multiplied cells are injected underneath. Although currently there are no experimental data to support this approach[51], the technique has gained a lot of attention. The authors reported good clinical

results even at long-term follow-up[52,53]. Problems with detachment of the periosteal flaps has led to the "second generation" autologous chondrocyte transplantation [54,55]. Using this technique (which combines multiplied autologous chondrocytes with a biomaterial to repair a cartilage defect) a periosteal flap is no longer needed.

In the context of this thesis we prefer to use a more narrow definition of tissue engineering. When translated literally, tissue engineering can be regarded as the construction of a new tissue, using cells, biomaterials and signaling molecules. To prevent immunological rejection and transmission of infectious diseases, autologous cells are preferred. Undifferentiated stem cells from bone marrow, periost or perichondrium, as well as chondrocytes isolated from a biopsy of healthy cartilage from a non weight-bearing part of the joint, can be used as donor cells. Using isolated cells, the number of cells that can be harvested without donor-site morbidity is usually too small to repair a defect; therefore the cells need to be multiplied. In most cases this is done by monolayer culture. Isolated donor cells suspended in a culture medium are seeded in plastic culture flasks. The cells attach to the bottom and start to multiply until cells cover the entire surface; this sequence is called 'one passage'. The increased number of cells can be detached from the culture flasks by enzymes and used for tissue engineering or repeated passages to obtain the required number of cells. Cell multiplication leads to dedifferentiation, i.e., the cells lose their specific phenotype[56-60]. It is therefore essential to either prevent dedifferentiation, or induce redifferentiation of these cells at some point during the tissue engineering process.

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CHAPTER 2

AIMS OF THE THESIS

The experimental work presented in this thesis is based on the hypothesis that treatment of a cartilage defect with a solid piece of tissue engineered neo-cartilage (with chondrocytes embedded in their extracellular matrix) offers the best chance for success compared with other techniques. Optimal results will be achieved when the neo-cartilage closely resembles native articular cartilage. We investigated the influence of different biomaterials, cell types and culture methods on the tissue engineering process as well as the characteristics of the neo-cartilage.

BIOMATERIALS

Biomaterials used for tissue engineering can be categorized according to their chemical composition into protein-based polymers, carbohydrate-based polymers, artificial materials and combinations of these[1]. (Table 1) In ***Chapters 3 and 4*** different matrices were compared in for their capacity to support neo-cartilage generation. We studied two protein-based matrices: a natural collagen type-I matrix present in its original form (i.e. demineralized bovine bone matrix) and a fleece made of collagen type-I. Collagen type-I is a natural constituent of the body. The degradation products are physiological ones and therefore non-toxic. Good results were obtained with demineralized bovine bone matrix in earlier experiments, however, a collagen type-I fleece is better defined with respect to chemical composition and pore size. A carbohydrate-based polymer widely used for tissue engineering purposes, polylactic acid/ polyglycolic acid fleece (E210) was also evaluated. This latter material is perfectly tailored to accommodate chondrocytes and can be hydrolyzed, however, the degradation products are possibly toxic.

Alginate is a gelatinous carbohydrate which is isolated and purified from brown algae and consists of mannuronic acid and guluronic acid. Gel formation takes place in the presence of calcium ions. Isolated cells suspended in alginate adopt a rounded shape. In vitro culture of dedifferentiated cells in alginate enhanced the expression of a differentiated phenotype. Moreover, alginate was used successfully in vivo, where it is hydrolyzed into mannuronic acid and guluronic acid[2,3]; these two components can then be incorporated in enzymatic pathways for further degradation[4,5].

In this thesis, cell culture in alginate beads is used as the standard culture method for isolated chondrocytes in vitro. In addition we used alginate in vivo to enhance uniform distribution of isolated cells in the biomaterials. The study in ***Chapter 5*** was designed to evaluate the additional effect of alginate in combination with a biodegradable biomaterial.

Table 1. Classification of biomaterials based on chemical composition

1. Protein-based polymers
Fibrin
Collagen Gelatine
2. Carbohydrate-based polymers
Polylactic acid
Polyglycolic acid
Hyaluronan
Agarose
Alginate
3. Artificial polymers
Dacron (polyethylene terephtalates)
Teflon (polytetrafluorethylene)
Carbon fibers
Hydroxyapatite
4. Within/between classes
Crosslinkage
Chemical modifications
Geometrical modifications (to produce fibrillar forms or foams)
Matrix combinations

(adapted from[1])

CELL TYPES AND CULTURE METHODS

Different types of donor cells can be used for tissue engineering. Donor-site morbidity in the joint can be avoided by using progenitor cells from sources other than the joint. In ***Chapter 3*** we used the chondrogenic potential of ear perichondrium to generate neo-cartilage. Progenitor cells from the perichondrium directly colonized different biomaterials in a rabbit model. In this technique (developed by the Department of Otorhinolaryngology of the Erasmus Medical Center) cell multiplication and differentiation takes place in situ[6,7].

Based on the results of ***Chapter 3,*** we hypothesized tissue engineering techniques using isolated cells to be more promising. Therefore, in the subsequent experiments we used isolated chondrocytes to generate neo-cartilage. The in vivo experiments described in C***hapters 4 and 8*** use a nude mouse model to study the redifferentiation capacity of dedifferentiated chondrocytes after sequential passaging in monolayer culture. ***Chapter 4*** also investigates the effect of high cell seeding density (comparable to the situation in the embryonic stage) on redifferentiation capacity. In ***Chapter 5*** the effect of alginate on the tissue engineering process was evaluated. For practical reasons, in our nude mouse model we used differentiated chondrocytes directly after isolation.

Cell multiplication is a crucial step in cartilage tissue engineering. In ***Chapter 6*** chondrocyte multiplication was studied in monolayer culture to establish whether growth factors added to the culture medium during would increase the number of cells generated.

Since cell multiplication in general leads to dedifferentiation, tissue engineering techniques aim at inducing redifferentiation at some point during the process. In ***Chapters 6, 7 and 8*** we evaluated the effect of a defined culture medium on re-expression of the differentiated phenotype in alginate beads. In the tissue engineering process it is essential to be able to assess whether the cells are differentiated or dedifferentiated. The use of histochemical staining, immunostaining and quantitative assays for DNA and glycosaminoglycan production are widely used to assess the differentiation stage and quality of the extracellular matrix. In addition to these techniques in ***chapter 7*** we studied in more detail how differentiated and dedifferentiated cells assemble their extra-cellular matrix. Finally in ***Chapter 8*** we evaluated a monoclonal antibody to characterize cell phenotype.

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CHAPTER 3

THE INFLUENCE OF DIFFERENT BIOMATERIALS ON NEO-CARTILAGE GENERATION BY EAR PERICHONDRIUM AND ABILITY TO RETAIN CARTILAGE-SPECIFIC CHARACTERISTICS AFTER IMPLANTATION IN THE KNEE JOINT

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Submitted

ABSTRACT

Introduction A composite graft of demineralized bone matrix (DBM) enwrapped in ear perichondrium is reported to be an effective method to generate neo-cartilage. However, the wide variation in DBM composition is considered a disadvantage of the technique. Composite grafts have been used in the head and neck region, but have not been studied in articular cartilage defects. This study compared neo-cartilage formation in DBM with two other biomaterials, i.e. polyglycolic/polylactic acid fleece and collagen matrix and evaluated the endurance of this neo-cartilage in an intra-articular environment

Materials and Methods DBM, polylactic/polyglycolic acid fleece and collagen matrix were enwrapped in pedicled ear perichondrium in rabbits and evaluated after 6 weeks. In the second part of the study the endurance of this neo-cartilage was established in an intra-articular environment. After 6 weeks neo-cartilage generated by DBM and ear perichondrium was harvested from the ear and implanted in defects in the medial femoral condyle; defects left untreated served as controls. Femoral condyles were evaluated after 6 or 24 weeks.

Results and Discussion All composite grafts showed similar amounts of neo-cartilage formation. However, in contrast to DBM, polyglycolic/polylactic acid fleece and collagen matrix were not completely resorbed after 6 weeks, leading to an ongoing inflammatory reaction; therefore, they were not used for the intra-articular study. In an intra-articular environment the grafts retained the histological characteristics of hyaline cartilage and developed into mature cartilage. Bone formation was restricted to areas where fibrous tissue had formed during neo-cartilage generation in the ear, but was not found in cartilaginous areas.

INTRODUCTION

The chondrogenic potential of perichondrium was first recognized by Haebler et al[1]. The cambial layer (i.e. the layer facing the cartilage or bone) contains stem cells able to differentiate into chondrocytes[2-6]. Perichondrium and periostium are embryologically closely related tissues, and autologous perichondrial and periosteal flaps have been studied extensively as possible articular cartilage repair strategies [2-4,7-23].

Composite grafts of autologous ear perichondrium and demineralized bovine bone matrix (DBM) have been reported as an effective method to generate hyaline cartilage[24-28]. Composite grafts were reported to be successful in the head and neck region, i.e., defects in nasal septum, ear and cricoid of rabbits[24-27] and children[29]; however, the grafts were never studied in articular cartilage defects.

The use of DBM has certain disadvantages. The material has a wide batch variation in pore size, thickness of the trabeculae, and the presence of osteogenic components. In addition DBM carries a potential risk for pathogen transfer. The first part of the present study was based on the hypothesis that biomaterials with a composition better defined than DBM would yield better neo-cartilage. Three different biomaterials were enveloped in ear perichondrium; collagen matrix and polyglycolic/polylactic acid fleece were compared with DBM, used in the original experiments[25,26].

Using the same rabbit model, perichondrium from the ear was wrapped around a piece of biomaterial and left in the ear for 6 weeks. The newly generated tissue was evaluated histologically for cartilage-specific characteristics, i.e. cell morphology and orientation, glycosaminoglycan and collagen type-II production.

The second part of the study was designed to evaluate whether this neo-cartilage retains cartilage-specific characteristics in an intra-articular environment. Composite grafts were prepared in the ear and after 6 weeks transplanted to a defect in the medial femoral condyle, defects left untreated served as controls. At 6 or 24 weeks after transplantation the femoral condyles were harvested and evaluated macroscopically with focus on possible inflammatory reaction in the joint as well as histologically, with focus on the endurance of specific cartilaginous characteristics, integration with the underlying bone, and the absence of terminal differentiation into bony tissue.

MATERIALS AND METHODS

The experience of our research group with graft preparation in the ear [24-28] and the numerous studies on articular cartilage defects in rabbits[3,7,17,18,30-38] led us to choose a rabbit model for both parts of the present study.

In the first part of the study immature animals were used. However, because in clinical practice patients that may benefit from an articular cartilage transplant (e.g. after a sport injury) will be adolescents or young adults, adolescent rabbits were used for the intra-articular study.

Animal experiments were approved by the local Ethics Committee and carried out as outlined in the "Erasmus Medical Center guidelines for the care and use of laboratory animals", which in general follows the NIH "Guide for the care and use of laboratory animals". Animals were housed at the Erasmus Center for Animal Research. Free cage activity was allowed; food and water were supplied ad libitum. Ultimately, the animals were killed by means of an intravascular injection of 1 ml per kg body weight pentobarbital (Euthesate 200 mg/ml, Apharmo, Arnhem, the Netherlands).

Neo-cartilage generation in the ear using different biomaterials

Nine young female New Zealand White rabbits (body weight: 1200 g) were used. Anesthetics were administered via an intramuscular injection of 10% ketaminehydrochloride (Ketalin, Apharma, Arnhem, the Netherlands) 0.5 ml/kg body weight and 2% xylazinehydrochloride (Rompun, Bayer, Leverkusen, Germany) 0.5 ml/kg body weight. The skin of the ear was elevated and the perichondrium dissected from the underlying cartilage. A pedicled flap was created and wrapped around a piece of biomaterial. The cambium layer, the side of the perichondrium that was dissected from the cartilage, was facing the biomaterial surface. The grafts were left in the ear and harvested after 6 weeks.

Biomaterials were randomly distributed over 18 ears. Six ears with DBM (Osteovit, Braun Gmbh, Melsungen, Germany) (3 mm thick, 25 mm^2), 6 ears with polyglycolic/polylactic acid fleece (E210, Ethicon, Munich, Germany) (3 mm thick, 25 mm^2) and 6 ears with collagen matrix (Datascope, Vaals, the Netherlands) containing 1.8% bovine collagen, pore size 50 - 100 µm (4 mm thick, 25 mm^2).

Endurance of the composite graft in an intra-articular environment

Sixteen adolescent female New Zealand White rabbits (body weight: 2900 g) were used. In the second part of the study DBM was the only biomaterial used because composite grafts with DBM, in contrast to the other two biomaterials, did not show residual inflammatory reaction when retrieved from the rabbit ear.

The cartilage graft was generated as described earlier using a piece of DBM left in situ for 6 weeks. During the first 2 weeks postoperatively, Metacam (meloxicam 1.5 mg/ml, Boehringer Ingelheim, Switzerland) was administered orally at a rate of 8 drops a day as a analgetic and to decrease the risk of calcification[13].

Six weeks after this initial operation, the second operative procedure was performed under general anesthesia. Two ml Depomycine (procainepenicillin-G 200,000 iu/ml with streptomycin 200 mg/ml, Mycopharm, Netherlands) were administered preoperatively. The cartilage grafts were harvested from the ears, the skin of the ears was closed. The medial femoral condyle was

exposed and a defect 3 mm in diameter was created with a manual drill. A circular full-thickness graft with a diameter of 3.2 mm was punched out of the tissue harvested from the ear. The graft thus consisted of neo-cartilage lined by perichondrium on top and bottom sides and was press-fit into the freshly created defect (24 knees). Empty defects (8 knees) served as controls. The tissue not used for implantation, was prepared for histology.

Postoperatively, a single dose of 1 ml Temgesic (buprenorfin 0,3mg/ml, Reckitt and Colman, UK) was administered subcutaneously. Metacam was given during a two-week period orally at a rate of 8 drops a day, again as a analgetic and to minimize calcification.

At both timepoints (i.e. after 6 weeks and 24 weeks) 12 defects with a composite graft and 4 empty defects were evaluated.

Macroscopic characteristics were evaluated, i.e. for intra-articular effusion and adhesions, erosion of surrounding cartilage, osteophyte formation, loose bodies, and appearance of the transplanted cartilage[39].

Histology

Tissue from the ear was fixed in formalin and embedded in paraffin. All knees were fixed in formalin, decalcified in 10% formic acid and subsequently embedded in paraffin.

Histochemical staining

Alcian blue (counterstained with nuclear fast red) and thionin staining was used on 7 μm paraffin sections to evaluate the structural characteristics of the tissue harvested from the ear and the tissue in the articular cartilage defects in the knee.

Immunostaining

Paraffin sections were deparaffinated followed by incubation with 0.1% pronase (Sigma, St. Louis, MO, USA) for 30 minutes and for 30 more minutes with 1% Hyaluronidase (Sigma). Aspecific binding was blocked with 10% normal goat serum. Sections were subsequently incubated with monoclonal antibodies to collagen type-II (II-II 6B3 1:100; DSHB, Iowa City, IA, USA) for 2 hours. This was followed by incubation for 30 minutes with a 1:400 dilution of Goat Fab fragment against mouse conjugated with alkaline phosphatase (GAMAP 1:400; Immunotech, Marseilles, France), which was followed by incubation for 30 minutes with a 1:100 dilution of mouse monoclonal alkaline phosphatase anti alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark). The new fuchsine (Chroma, Kongen, Germany) procedure was used for color development. The sections were counterstained with hematoxylin and mounted in gelatin glycerin. Negative control stainings were done simultaneously by omitting the first antibody.

Evaluation

Representative sections of each specimen were evaluated histologically by two independent observers. A microscopic grid was used to evaluate dimensions of the composite grafts. Neo-cartilage generated in the ear was evaluated at 100 X magnification.

Areas of the graft staining positive with Alcian blue as well as for collagen type-II were regarded to have hyaline cartilage-specific characteristics. These areas were characterized as being young or mature depending on cell morphology. Orientation of the neo-cartilage with respect to the biomaterial or its remnants was evaluated, as well as the presence or absence of inflammatory cells.

Sections of the rabbit knees were evaluated at 50 X magnification. A microscopic grid was used to score the percentage of the tissue in the defect with cartilage-specific characteristics, as well as the percentage of the outer area of the graft that integrated with the surrounding tissue. The transplanted cartilage was characterized on cell morphology as being young or mature. Characteristics unwanted in cartilage tissue engineering were also scored; the percentage of the area with a fibrous appearance, presence of bone, presence of cell clusters, hypertrophic cartilage cells and blood vessels within the grafted tissue.

RESULTS

Neo-cartilage generation in the ear

All composite grafts showed Alcian blue staining regions surrounded by fibrous tissue, although the dimensions and thickness of these regions varied. The histological appearance of the cells in the areas of neo-cartilage showed the morphological properties typical of cartilaginous tissue; rounded cells lying within lacunae surrounded by blue staining matrix. The Alcian blue staining regions were positive for collagen type-II, suggesting a cartilage-specific phenotype for the newly generated tissue. The thickness of the neo-cartilage generated with DBM, collagen matrix and E210 was similar and within the range of normal ear cartilage. No histological signs of bone formation were seen, irrespective of the biomaterial used.

The cartilage generated using collagen matrix and E210 was found on top of the biomaterial (Figure 1 and 2); this neo-cartilage appeared very regular in shape. Neo-cartilage generated with collagen matrix appeared the most immature; the chondrocytes were small with a central nucleus (Figure 3). The neo-cartilage generated with E210 appeared to be the most mature (Figure 4). The chondrocytes lay in lacunae and are larger with vacuoles in the cytoplasm. After 6 weeks, however, in all grafts with E210 and collagen matrix remnants of the biomaterial were still present, evoking an inflammatory response (Figure 1 and 2).

Neo-cartilage generated with DBM showed that perichondrium cells had invaded the pores of the DBM and formed islets of cartilage, mostly fused but sometimes with fibrous tissue in between (Figure 5).

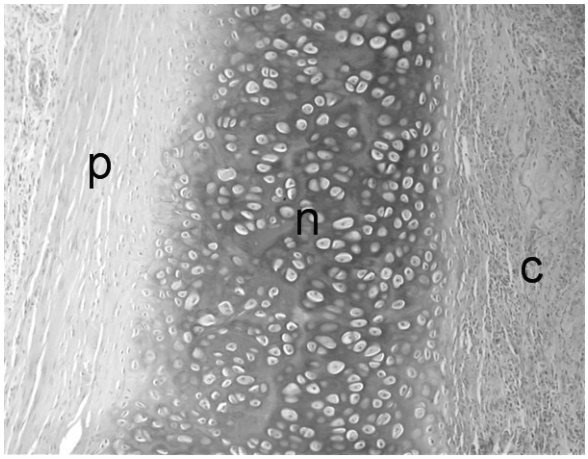

Figure 1.

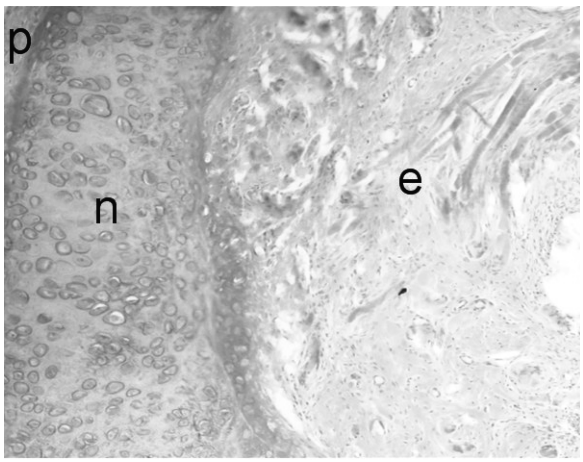

Figure 2.

Figure 1 and 2. Neo-cartilage (n) formed in the ear; evaluation at 6 weeks. Collagen matrix (Figure 1) or E210 (Figure 2) enwrapped in perichondrium (p). Remnants of collagen matrix (c, Figure 1) and E210 (e, Figure 2) are present evoking an inflammatory response. Alcian blue staining, original magnification 100 X.

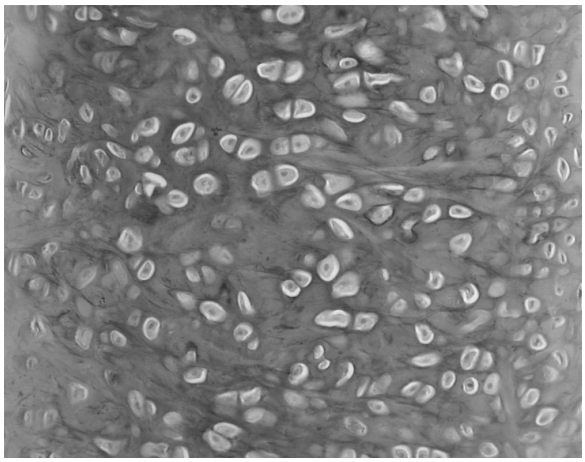

Figure 3. Immature neo-cartilage formed in the ear; evaluation at 6 weeks. Collagen matrix enwrapped in perichondrium. The chondrocytes are small with a central nucleus.

Endurance of the composite graft in an intra-articular environment

There were no differences in the amount of cartilage generated by the three biomaterials. However, because after 6 weeks DBM was the only biomaterial that had degraded completely without ongoing inflammatory reaction we decided to use this biomaterial only in the intra-articular study.

Macroscopic evaluation

The tissue found in the defect was opaque rather than showing the translucent appearance of normal articular cartilage. No effusion, adhesions or erosion of surrounding articular cartilage (indicators of joint inflammation) were found, and no loose bodies or osteophyte formation were observed.

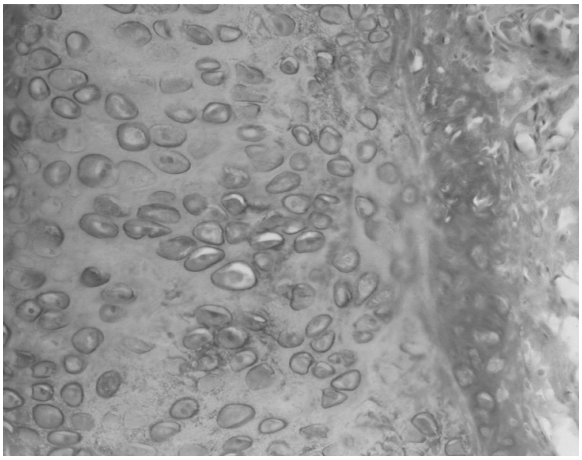

Figure 4. Mature neo-cartilage formed in the ear; evaluation at 6 weeks. E210 enwrapped in perichondrium. The chondrocytes are larger compared to Figure 3, lay in lacunae and show vacuoles in the cytoplasm. Alcian blue staining, original magnification 200 X.

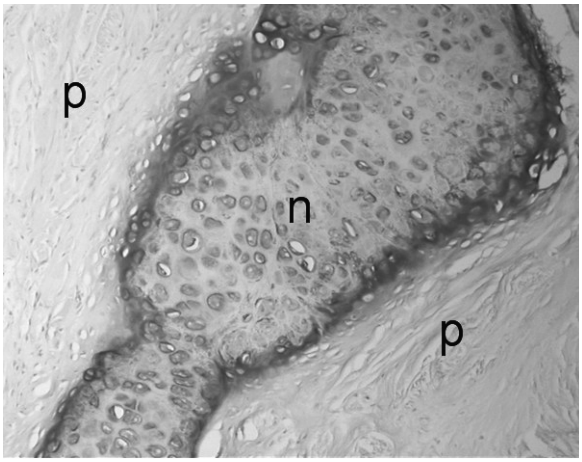

Figure 5. Neo-cartilage (n) formed in the ear, 6 weeks after combining perichondrium (p) with demineralized bovine bone matrix (DBM). Perichondrium cells have invaded the pores of the DBM and formed islets of cartilage, mostly fused but sometimes with fibrous tissue in between. Note that the DBM has disappeared completely, without ongoing inflammatory reaction. Alcian blue staining, original magnification 100 X.

Histology

Six and 24 weeks after implantation of a composite graft, large areas staining positive with Alcian blue, thionin and collagen type-II were visible. The percentage of tissue in the defect with cartilage-specific histological characteristics was similar after 6 and 24 weeks, i.e. 65 ± 28% and 71 ± 20% respectively. The overall aspect was a disorganized tissue that varied from fibrous tissue, to fibrocartilage (Figure 6), to a tissue more closely resembling hyaline cartilage (Figure 7). In the 6-weeks group some cell clusters as well as hypertrophic chondrocytes were seen in the majority of the grafts (Figure 8). In the 24-weeks group, however, cell clusters as well as hypertrophic chondrocytes were observed only occasionally and the cartilage appeared to be more mature and permanent (Figure 9).

The bulk of the graft tissue was implanted well below the original tidemark level, i.e. mainly in a bony environment. Histological signs of bone formation within the neo-cartilage were found occasionally and seemed to be related to the presence of fibrous tissue within the composite

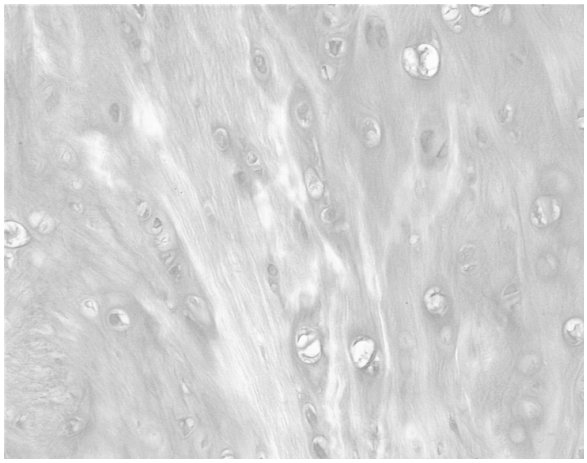

Figure 6.

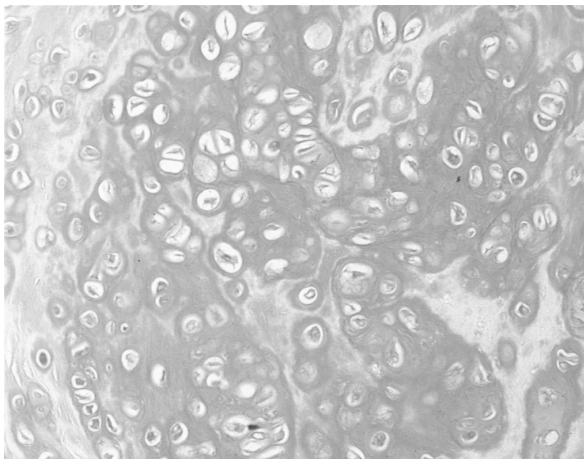

Figure 7.

Figure 6 and 7. Neo-cartilage 6 weeks after implantation in the medial femoral condyle. The overall aspect of the tissue in the defect varies from fibrocartilage (Figure 6), to a tissue more closely resembling hyaline cartilage (Figure 7). Alcian blue staining, original magnification 200 X.

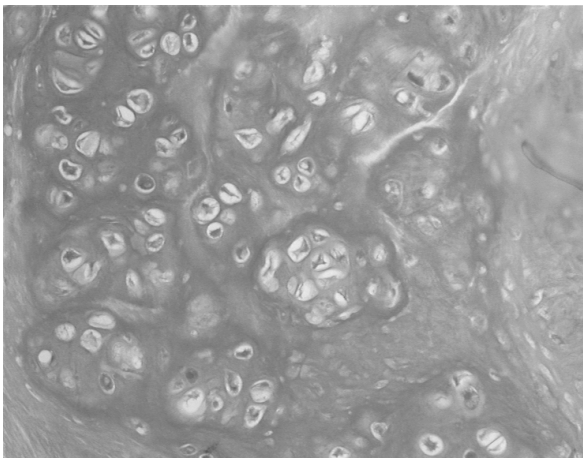

Figure 8.

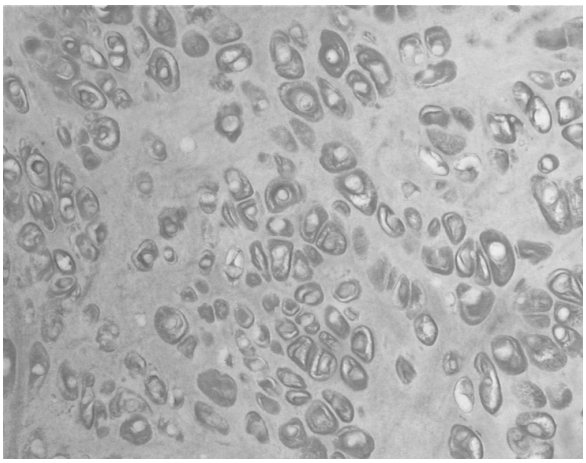

Figure 9.

Figure 8 and 9. Neo-cartilage 6 (Figure 8) and 24 (Figure 9) weeks after implantation in the medial femoral condyle. After 6 weeks cell clusters as well as hypertrophic chondrocytes were seen in the majority of the grafts (Figure 8). After 24 weeks, however, the cartilage appeared to be more mature and permanent, and cell clusters and hypertrophic chondrocytes were seldom observed (Figure 9). Alcian blue staining, original magnification 200 X.

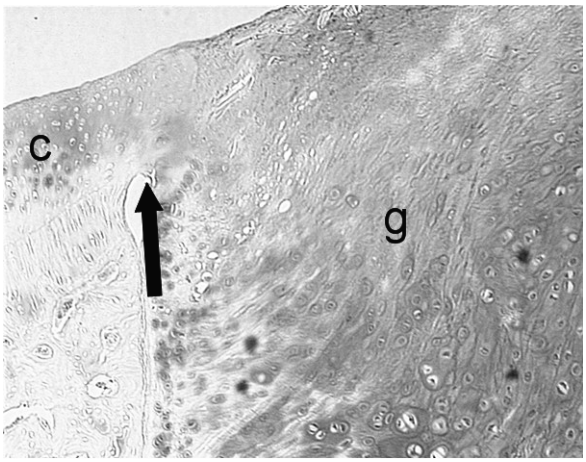

Figure 10. Integration (arrow) between graft tissue (g) and surrounding cartilage (c) observed as an incidental finding. Alcian blue staining, original magnification 100 X.

graft. Blood vessels were present in these fibrous intersections, whereas neither blood vessels nor bone formation were observed in purely cartilaginous areas.

The percentage of the outer area of the graft that integrated with the surrounding tissues was 69 ± 20% after 6 weeks; after 24 weeks this was 93 ±7%. Integration was mainly in the bony area of the defect but, sometimes, integration with the surrounding cartilage could be found as well (Figure 10).

In empty defects a disorganized tissue mainly consisting of trabecular bone, combined with varying amounts of fibrous tissue was found. On top of the tissue, facing the joint cavity a layer of weakly staining fibrocartilage was found. The fibrocartilage lining deteriorated when empty defects after 6 weeks were compared with empty defects after 24 weeks. After 24 weeks Alcian blue staining and collagen type-II immunostaining was further reduced and the fibrocartilage lining became thinner. The tissue found underneath this fibrocartilage matured into trabecular bone.

DISCUSSION

A composite graft of trabecular DBM enwrapped in perichondrium left in situ in the rabbit ear is an effective method to generate solid neo-cartilage, that can be surgically handled[24,27]. In the first part of the present study we evaluated the efficacy of two other biomaterials (E210 and collagen matrix) in this composite graft technique. We hypothesized that the use of better defined biomaterials would improve the characteristics of the neo-cartilage. After 6 weeks in the ear resorption of E210 and the collagen matrix was not complete. Remnants of the biomaterials were still present accompanied by a large number of inflammatory cells. In an intra-articular environment this is a disadvantage because inflammatory cells can cause damage to the joint. In contrast, after 6 weeks in the ear complete resorption of DBM was shown without ongoing inflammatory reaction. This confirmed the results of earlier experiments. Since the thickness of the cartilage formed was similar for the three biomaterials tested, DBM was the material of choice to generate a graft for implantation in the joint in the second part of this study.

In general, grafting of perichondrial and periosteal flaps fails to achieve complete filling of the defect. Another drawback is calcification of the repair tissue at long-term follow-up[13]. The composite graft technique offers certain advantages. Complete filling of the defect is achieved with a solid piece of hyaline cartilage. Press-fit fixation avoids the need for additional gluing or suturing. Good integrative capacity with native cartilage has been reported[40]. The orientation of the different tissue layers in the graft closely fits a logical position described by Hunziker[41]. Perichondrium with the cambium layer down covers the defect, cells from the perichondrium have grown into a porous matrix and perichondrium on the defect floor acts as a structural barrier to impede angiogenic activities[42,43].

The present study shows that even after 24 weeks in a well vascularized bony environment the hyaline cartilage-like regions in the graft retained their cartilage specific characteristics. Bone formation was restricted to the fibrous areas between islets of cartilage where blood vessels were frequently seen. This corresponds with the hypothesis that cartilage contains an anti-angiogenic factor[44]. An effort to generate a homogenous cartilage graft (by reducing the amount of fibrous tissue) would therefore probably contribute to the long-term stability of the neo-cartilage. Previous experiments demonstrated that a homogenous cartilage tissue was formed when the maximum thickness of the DBM was limited to 2 mm; when the thickness of the DBM exceeds 2 mm the graft will have a higher risk to consist of unfused islets of cartilage with fibrous tissue in between (unpublished data).

Composite graft generation has been extensively studied in rabbits [24-27]. In addition, numerous studies on the repair of articular cartilage defects were performed in a rabbit model [3,7,15,18-20,30,32-36,45-49]. We therefore used a rabbit model for the present experiment. A limitation of the rabbit model is that the thin articular cartilage layer prohibits study of the integration between graft and native cartilage. Dynstee et al. showed that dissecting the fibrous perichondrial layer from the cambium layer of native ear cartilage is technically possible, and that removing the fibrous layer enhances cartilage formation at the graft edges[50]. Dissecting the perichondrial layer of the composite graft prior to implantation would bring the composite graft cartilage to the level of the native articular cartilage. We hypothesize that this should enhance the integration of the composite graft with the native cartilage.

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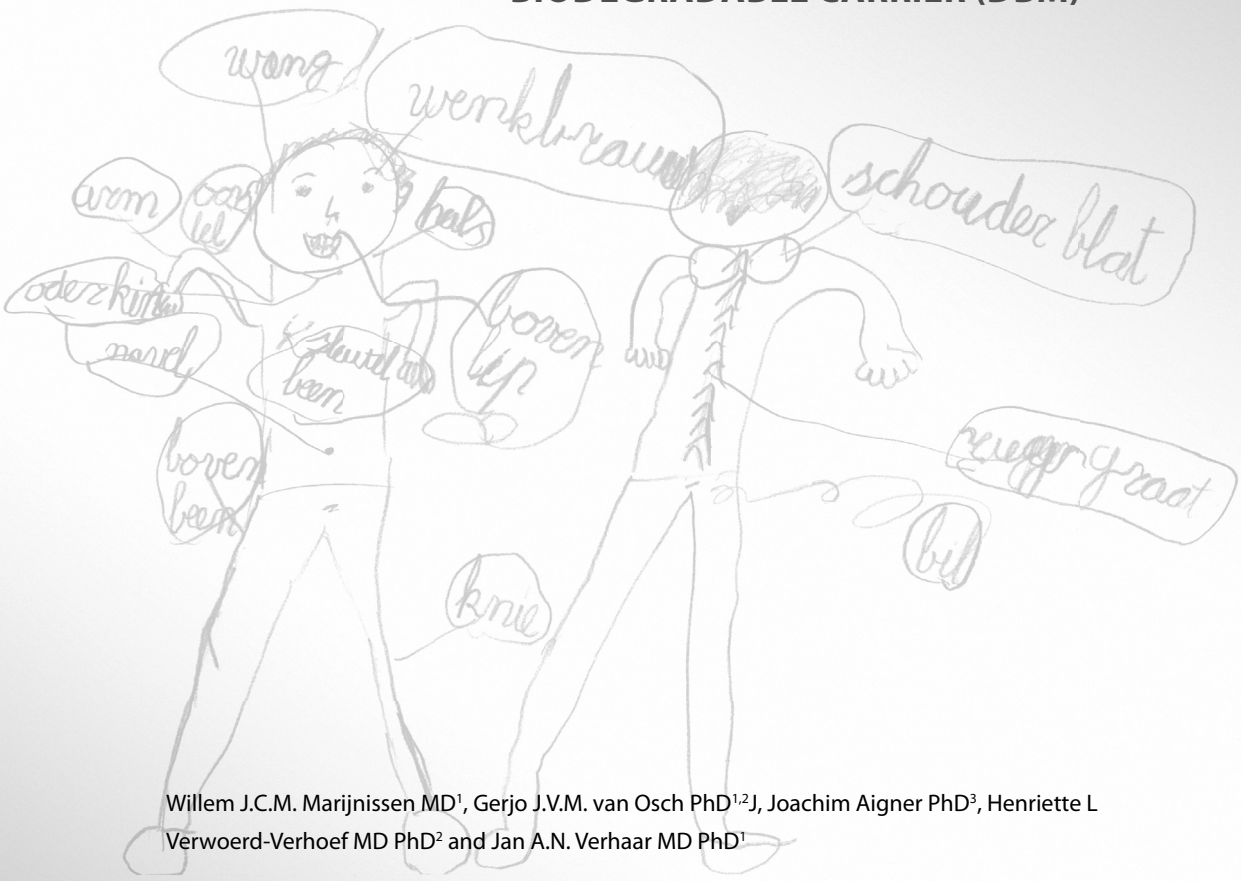
CHAPTER 4

TISSUE-ENGINEERED CARTILAGE USING SERIALLY PASSAGED ARTICULAR CHONDROCYTES. CHONDROCYTES IN ALGINATE, COMBINED IN VIVO WITH A SYNTHETIC (E210) OR BIOLOGIC BIODEGRADABLE CARRIER (DBM)



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ABSTRACT

In vitro multiplication of isolated autologous chondrocytes is required to obtain an adequate number of cells to generate neo-cartilage, but is known to induce cell-dedifferentiation. The aim of this study was to investigate whether multiplied chondrocytes can be used to generate neo-cartilage in vivo. Adult bovine articular chondrocytes, of various differentiation stages, were suspended in alginate at densities of 10 or 50 million/ml, either directly after isolation (P0) or after multiplication in monolayer for one (P1) or three passages (P3). Alginate with cells was seeded in demineralized bovine bone matrix (DBM) or a fleece of polylactic/polyglycolic acid (E210) and implanted in nude mice for 8 weeks. The newly formed tissue was evaluated by Alcian Blue and immunohistochemical staining for collagen type-II and type-I. Structural homogeneity of the tissue, composed of freshly isolated as well as serially passaged cells, was found to be enhanced by high-density seeding (50 million/ml) and the use of E210 as a carrier. The percentage of collagen type-II positive staining P3-cells was generally higher when E210 was used as a carrier. Furthermore, seeding P3-chondrocytes at the highest density (50 million/ml) enhanced collagen type-II expression. This study shows promising possibilities to generate structurally regular neo-cartilage using multiplied chondrocytes in alginate in combination with a fleece of polylactic/polyglycolic acid.

INTRODUCTION

Articular cartilage defects are a major problem in orthopaedic surgery. Cartilage defects do not heal spontaneously with hyaline cartilage. The predominant repair tissue found in such defects is fibrocartilage, which is mechanically and chemically inferior to hyaline cartilage[1-3]. Irregularity of the cartilage lining caused by the defect, may induce pain and mechanical impairment of the injured joint. These joints are predisposed for the development of osteoarthrosis[4,5]. Grafting of tissue engineered cartilage is one of the techniques proposed for early treatment of articular cartilage defects[2,6-10]. Autologous cells are generally preferred to avoid risks of immunological rejection and transmission of infectious diseases. The amount of chondrocytes needed can be generated by in vitro multiplication of isolated chondrocytes. However, multiplication induces cell dedifferentiation[1,11,12]. The cells obtain a more fibroblast-like appearance, produce fewer glycosaminoglycans and switch from production of the hyaline cartilage specific collagen type-II to production of collagen type-I.

Experiments showed that dedifferentiated chondrocytes can re-express the differentiated cartilage phenotype in vitro, when cultured in alginate beads or agarose[13,14]. However after successive passages in monolayer this capability will disappear too[11,13].

Subcutaneous implantation in nude mice of such dedifferentiated chondrocytes can lead to redifferentiation, expressed by formation of cartilage nodules[11]. Although high cell densities are thought to facilitate successful engraftment[15], to date there is no consensus on the optimal cell density to be used. In vivo implantation of cells in alginate can be facilitated by the use of a biodegradable carrier. The carrier provides initial strength to the relatively weak alginate gel and prevents cells from floating out of the graft. An ideal cell carrier can be shaped into almost any desired form. For future clinical application a graft can thus be designed to closely "t into the defect. Many different biodegradable carriers, biological as well as synthetic, are available. In this study we compared demineralized bovine bone matrix (DBM), a collagen type-I matrix, as a biological biodegradable carrier, with a nonwoven fleece of polyglycolic/polylactic acid (E210) as a synthetic biodegradable carrier.

In particular, freshly isolated chondrocytes[6,8,10,16] as well as serially passaged young cells [11] have demonstrated capability to generate neo-cartilage. However, in clinical practice most patients needing a cartilage transplant will be adolescents or adults and, for reasons mentioned earlier, multiplication of autologous chondrocytes is inevitable. To closely mimic the envisioned clinical situation we tested the capability of *articular* chondrocytes from *adult* individuals to generate neo-cartilage after in vitro multiplication. We used a bovine model to study the effect of cell density, cell dedifferentiation and the type of cell carrier used. The study focuses on structural and chemical composition of tissue engineered cartilage after in vivo implantation in nude mice. In vivo redifferentiation capacity was compared with in vitro cultures, using cells in alginate beads.

MATERIALS AND METHODS

Chondrocyte isolation

Full-thickness cartilage slices were harvested, under sterile conditions from the meta-carpophalangeal joints of 12-18 month-old bovine steers, obtained from a local slaughter house, within 8 h after death. Cells were pooled from 4 different steers to exclude interindividual differences. The cartilage was washed with sterile physiological saline and incubated with protease XIV (2 mg/ml; Sigma, St. Louis, MO) for 2 h followed by an overnight incubation with collagenase B (1.5 mg/ml; Boehringer, Mannheim, Germany) in medium (DMEM/Ham's F12; Gibco, Grand Island, NY) with 10% FCS (Bio Whittaker, Verviers, Belgium). Both enzymatic digestions were done at 37^0C. After incubation the undigested cartilage fragments were removed using a 100 µm filter. The isolated chondrocytes were washed with physiological saline and counted using a hemacytometer. Cell viability was tested using the trypan blue exclusion test.

Culture in monolayer

The isolated chondrocytes were seeded in monolayer at a density of 2 x 10^4 cells/cm^2. The culture medium (DMEM/Ham's F12), supplemented with 10% FCS, fungizone (0.5 lg/ml; Gibco) and gentamycin (50 lg/ml; Gibco) was changed twice weekly. When subconfluent, the flasks were trypsinized (Trypsin-EDTA; Gibco). Cells were cultured in monolayer until the first (P1) and third (P3) passage, to obtain chondrocytes in different stages of differentiation.

Preparation of cartilage grafts

To determine the optimal cell density for graft generation freshly isolated or serially passaged cells (P0, P1 and P3) were seeded in alginate at a density of 10 or 50 x 10^6 cells/ml. The preparation of chondrocytes in alginate was done as described by Guo et al.[17], with slight modifications as described by Häuselmann et al.[18]. The cells were suspended in sterile saline containing 1.2% low viscosity alginate gel (Keltone LV, Kelco, Chicago, IL). As a biological biodegradable matrix we used demineralized trabecular bovine bone matrix (Osteovit, Braun-Melsungen GmbH, Melsungen, Germany). As a synthetic biodegradable matrix we used nonwoven polyglactin filaments (a polyglycolic/polylactic-copolymer in a ratio of 90/10 per weight) with a diameter of about 20 µm with punctual polydioxanon adhesions (Ethisorb 210, Ethicon, Norderstedt, Germany). Both matrices were immersed in the alginate-chondrocyte suspension until they were fully saturated. Polymerization of alginate was then achieved by dropping the grafts into a 102 mM CaCl 2 solution. After instantaneous gelation the alginate was allowed to polymerize further for a period of 8-10 min in the $CaCl_2$ solution. Thereafter, all grafts were washed thoroughly with physiological saline.

In vitro cultures

Alginate beads

Alginate beads were cultured to study the in vitro effect of cell density and differentiation stage on collagen type-II production. Preparation of the alginate-chondrocyte suspension was done as described before[17,19] and slowly passed through a 21 Gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution to obtain alginate beads. After instantaneous gelation the beads were allowed to polymerize further for a period of 8-10 min in the $CaCl_2$ solution. They were thereafter washed with physiological saline and finally plated in a 24-well plate. Medium, DMEM/Ham's F12 supplemented with 10%FCS, gentamycin (50 lg/ml), fungizone (0.5 lg/ml) and 25 lg/ml L-ascorbic acid freshly added (Sigma) was changed three times a week. Cultures were done at 37^0 C in a humidified atmosphere of 95% air and 5% CO_2. Beads were harvested after 3 weeks of culture and were dissolved in 55 mM Na-citrate supplemented with 5% FCS. Cells were cyto-centrifuged onto glass slides at 1000 rpm for 7 min. After air drying at room temperature for 1 h the specimens were frozen at -80^0 C until immunostaining took place.

Animal experiments

Animal experiments were approved of 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'. Animals were housed, under sterile conditions, at the Center for Animal Research.

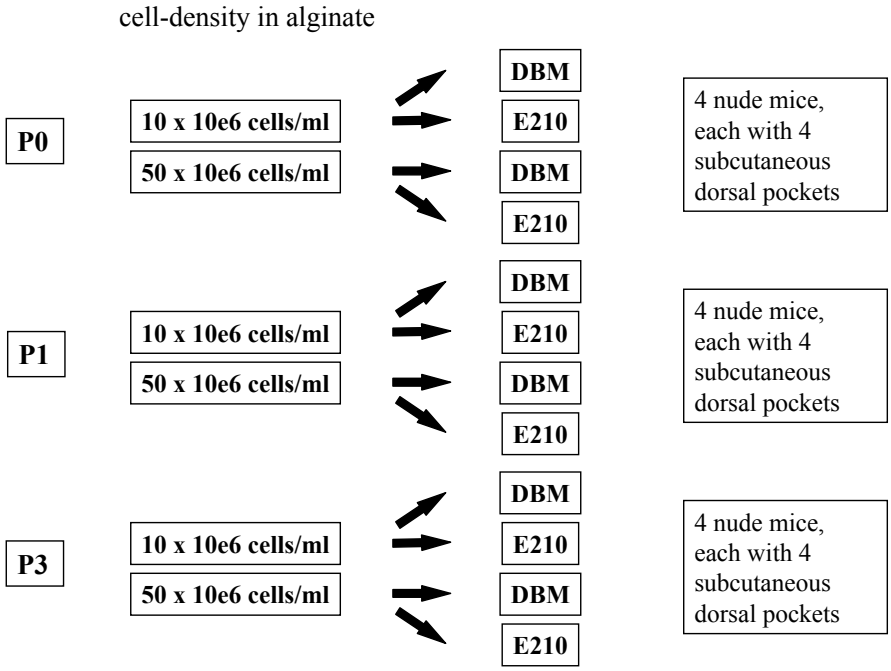
Twelve male athymic mice (NMRI), aged 10-12 weeks, were used for in vivo experiments according to Table 1. To assess the effect of cell characteristics on graft formation, freshly isolated (P0) as well as serially passaged chondrocytes (P1 and P3) were used at densities of 10 and 50 million cells per ml alginate. Two different biodegradable carriers, DBM and E210, were compared with respect to their influence on structural characteristics and matrix components of the neo-cartilage formed. For each passage (P0, P1 and P3) four mice were available. Cells suspended in alginate, at densities of 10 and 50 million/ml were seeded in DBM (8x8x2 mm) or E210 (8x8x2 mm). A sterile surgical procedure was used to implant the grafts in subcutaneous dorsal pockets. Each mouse received one graft of these four experimental conditions. After eight weeks, animals were killed by cervical dislocation. The transplanted tissue was harvested and processed for histology. One-half of each graft was preserved for cryosections, the other half fixed in formalin and embedded in paraffin.

Histology

Modified indirect immunostaining

The method, as described by Hierck et al.[20], was used on 5 µm paraffin sections to detect type-II collagen. The antibody to pro-collagen type-I cannot be used on paraffin sections; therefore

Table 1 . Implantation scheme. For each passage (P0, P1 and P3) 4 mice were used. Cells suspended in alginate, at densities of 10 and 50 million/ml were seeded in DBM (8 x 8 x 2 mm) or E210 (8 x 8 x 2 mm) and implanted in subcutaneous dorsal pockets. Each mouse received one graft of these 4 experimental conditions. After eight weeks the transplanted tissue was harvested and processed for histology.



5 µm cryosections were used to detect type-I pro-collagen. Goat Fab fragment against mouse conjugated with alkaline phosphatase (GAMAP 1:400; Immunotech, Marseilles, France) was allowed to complex overnight with monoclonal antibodies to collagen type-II (II-II 6B3 1:100; DSHB) or to pro-collagen type-I (M38 1:100; DSHB, Iowa City, IA) in PBS at 4^{0}C, in the presence of 1% bovine serum albumin. The next morning 0.1% normal mouse serum was added for 2 h to capture unbound GAMAP. Paraffin sections were deparaffi$nated followed by incubation with 0.1% pronase (Sigma) for 30 min and for 30 more minutes with 1% hyaluronidase (Sigma). Cryosections were initially fixed in acetone for 10 min at room temperature and incubated with 1% hyaluronidase (Sigma) for 30 min at 37^{0}C. Aspecific binding was blocked with 10% normal goat serum. Sections were subsequently incubated with the II-II 6B3/GAMAP or M38/GAMAP complexes for 2 hours, followed by incubation for 30 min with a 1:100 dilution of mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark). The new fuchsin (Chroma, Kongen, Germany) procedure was used for color development. The sections were counterstained with hematoxylin and mounted in gelatin glycerin.

Indirect immunostaining

The protocol, to stain collagen type-II on cytocentrifuged slides of in vitro cultures, differs slightly from the modified immunostaining. Cytocentrifuged slides were initially fixed in acetone for 10 min. Instead of complexing II-II 6B3 and GAMAP prior to incubation, II-II 6B3 and GAMAP were added in separate steps of 2 h and 30 min, respectively. Negative control stainings were done simultaneously by omitting the first antibody.

Histochemical staining

Alcian blue staining was used on 5 lm paraffin sections to evaluate the structural characteristics of the graft. Sections were counterstained with nuclear fast red.

Evaluation

From each specimen 10 histological sections, 50 µm apart, were used for histochemical- and immunostaining. All sections were examined blindly and independently by two observers. Structural characteristics of the median section were scored according to Table 2. To determine the homogeneity of the graft, the number of islets of cartilage-like tissue intersected by remaining strands of biomaterial was counted. Cell clusters were defined as groups of more than five cells. An inflammatory reaction was considered to be present when the tissue was invaded by a monocellular infiltrate. As an indication for cell differentiation, pro-collagen type-I and collagen type-II were scored present or absent in capsule, periphery and center of the graft. The number of collagen type-II producing cells immunostained with II-II 6B3, was counted in representative areas of grafts (Table 3) as well as on cytocentrifuged slides of in vitro cultures.

RESULTS

In vitro data

Each passage in monolayer culture resulted in a 2-3 time increase in cell number. In vitro multiplication led to a rapid decline in positive immunostaining for collagen type-II in alginate beads. When cultured in alginate for 3 weeks immediately after isolation, 50-90% of the cells stained positive for II-II 6B3 (Fig. 1a). When cultured in alginate for 3 weeks after one passage in monolayer 25-50% of cells stained positive, whereas after three passages no collagen type-II positive staining could be detected (Fig 1b). The seeding density in alginate did not influence collagen type-II expression in vitro.

In vivo data

No manifest infections were noted. All grafts were clearly visible underneath the skin and could easily be retrieved. Gross examination of these grafts showed a firm tissue with an opacity resembling normal cartilage,except for grafts based on P3 chondrocytes at a density of 10 x

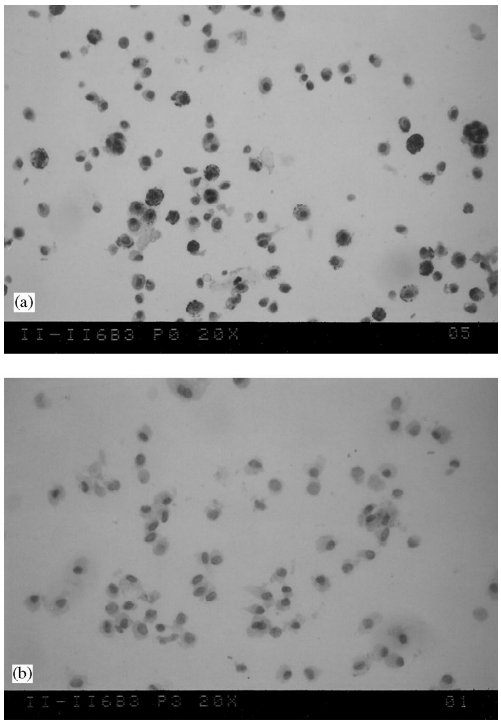


Figure 1. Immunohistochemical staining on cytocentrifuged slides for collagen type-II (II-II6B3), of chondrocytes cultured in alginate. (a) Freshly isolated chondrocytes, cultured in alginate for 3 weeks. 50-90% of cells stain positive. (b) Cells cultured in alginate after multiplication in monolayer until the third passage. Collagen type-II positive staining is absent. Red staining indicates a positive signal. Original magnification x 400.

10^6 cells per ml in combination with DBM. The rectangular shape of the grafts seemed best preserved when E210 was used as a carrier.

Tables 2 and 3 give an overview of histological characteristics. Findings within each group were found to be consistent, with clear differences between different experimental conditions. Grafts based on P0 cells showed 75-90% positive immunostaining for collagen type-II. In contrast to serially passaged chondrocytes cultured in alginate beads in vitro, serially passaged (P3) cells in vivo did produce collagen type-II. The type of cell carrier influenced the percentage of collagen type-II positive staining. Comparing DBM (Fig. 2b) with E210 (Fig. 2a) as a carrier for P3 cells, the percentage of collagen type-II positive staining is generally lower in combination with DBM (Table 3). Cell seeding density was found to influence collagen type-II expression too. When cell seeding density is lowered the percentage of collagen type-II positive staining falls, in combination with DBM even under 25% in all grafts (Fig. 3a and b and Table 3). In grafts based on P3 chondrocytes pro-collagen type-I was found in capsule and periphery if E210 was used as a carrier (Fig. 4a), in combination with DBM(Fig. 4b) the center of the graft was staining positive for pro-collagen type-I too. Intensity of Alcian Blue staining was found to be normal in grafts based on freshly isolated chondrocytes, slightly decreasing with increasing number of passages. No differences in staining intensity were observed between E210 and DBM. All grafts were encapsulated,.most capsules being 5-10 cell layers thick. Vascular ingrowth was found to be absent in all grafts. In four of the grafts a non-specific inflammatory reaction was

Table 2. Number of grafts showing a certain histological structural characteristic. Grafts were composed of chondrocytes directly after isolation (P0), after one (P1), or three (P3) passages in monolayer culture and seeded at a density of 10 x 10^6 cells/ml or 50 x 10^6 cells/ml in DBM or E210.

	P0 DBM 50 x 10^6	DBM 10 x 10^6	E210 50 x 10^6	E210 10 x 10^6	P1 DBM 50 x 10^6	DBM 10 x 10^6	E210 50 x 10^6	E210 10 x 10^6	P3 DBM 50 x 10^6	DBM 10 x 10^6	E210 50 x 10^6	E210 10 x 10^6
Homogeneity of graft												
1-3 islets			4	4		1	4	3	2		4	3
4-10 islets	1	2			2	1		1	2			
>10 islets	3	2			2	2				4		1
Intensity of alcian blue staining												
normal	4		4		1		2				2	
moderate		4		4	3	4	1	3	2	3	2	1
slight							1	1	2	2		3
none												
cluster formation												
none	4		3		3	1	2	2	3	3	3	2
< 25%			1		1	1	1	2	1	1	1	2
25%-75%		2		4		2	1					
> 75%		2										
Degradation of biomaterial												
complete												
moderate	1		4	3			2	1			2	2
slight	3	3		1	2	3	2	3	1	1	2	2
none		1			2	1			3	3		
Vascular ingrowth												
present												
absent	4	4	4	4	4	4	4	4	4	4	4	4
capsule formation												
present	4	4	4	4	4	4	4	4	4	4	4	4
absent												
Inflammatory reaction												
present							1	1	1			1
absent	4	4	4	4	4	4	3	3	3	4	4	3

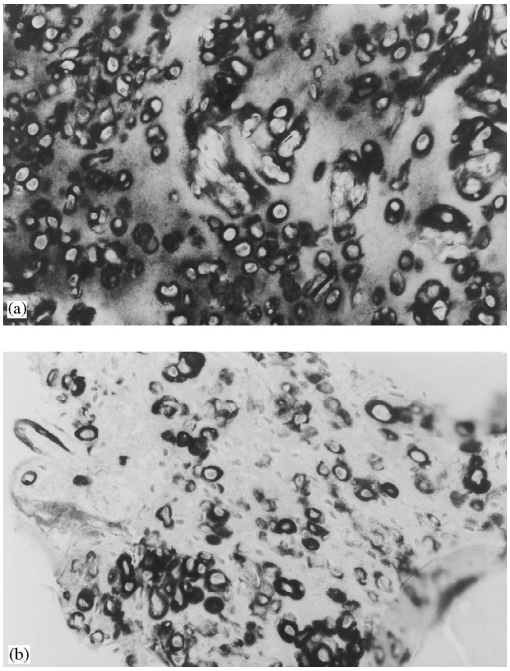


Figure 2. Immunohistochemical staining on paraffin sections for collagen type-II (II-II6B3). Serially passaged chondrocytes (P3) were used at a density of 50 x 106 cells/ml in combination with E210 (a) and DBM (b). 8 weeks after transplantation 90 % of cells is surrounded by an intensely red staining halo, indicating a positive signal. Original magnification x 200.

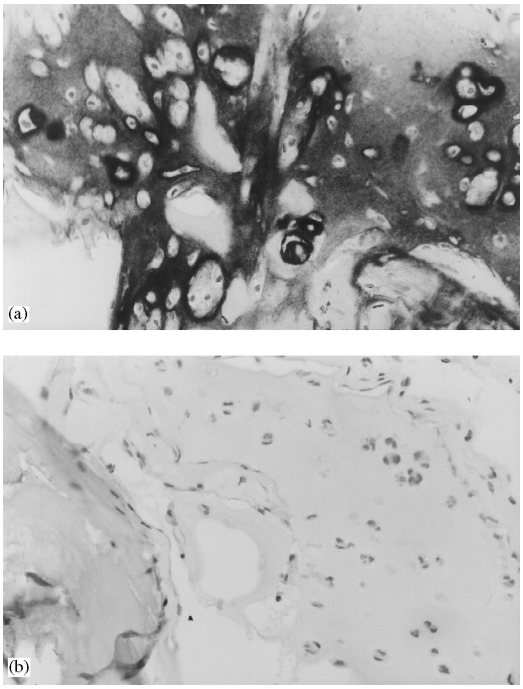


Figure 3. Immunohistochemical staining on paraffin sections for collagen type-II (II-II6B3). Serially passaged chondrocytes (P3) were used at a density of 10 x 106 cells/ml. In combination with E210 (a), 8 weeks after transplantation, approximately 50 % of cells is surrounded by intensely red staining matrix, indicating a positive signal. In combination with DBM (b) the percentage of cells staining positive falls under 25%. Original magnification x 200.

noted (Table 2), giant cells were never seen. Seeded at a density of 50 million/ml, few, small clusters were found in freshly isolated (Fig. 5a) and serially passaged chondrocytes. In contrast, approximately 50% of freshly isolated chondrocytes at a density of 10 million/ml formed clusters (Fig. 5b). With increasing numberof passages cluster formation declined. The type of carrier did not influence cluster formation. Degradation of DBM was observed at the edges of the grafts based on freshly isolated as well as passaged chondrocytes. Strands of elongated cells with stretched nuclei were found at sites where the trabeculae were degraded. In the center of these grafts, remaining parts of DBM, intersected the cartilage-like tissue (Fig. 6). E210 was found to be almost completely degraded after 8 weeks. In contrast to DBM, small remaining parts of the biomaterial did not intersect the cartilaginous tissue but were grouped together (Fig. 7a) and invaded by chondrocytes (Fig. 7b).

Table 3. Number of grafts showing a certain percentage of collagen type-II positive staining. Grafts were composed of chondrocytes after 3 passages in monolayer culture and seeded at a density of 10 x 10^6 cells/ml or 50 x 10^6 cells/ml in DBM or E210.

% type-II positive staining	DBM 50 x 10^6	DBM 10 x 10^6	E210 50 x 10^6	E210 10 x 10^6
>90 %	1		3	
75-90 %			1	
50-75 %				1
25-50 %	2			2
<25 %	1	4		1

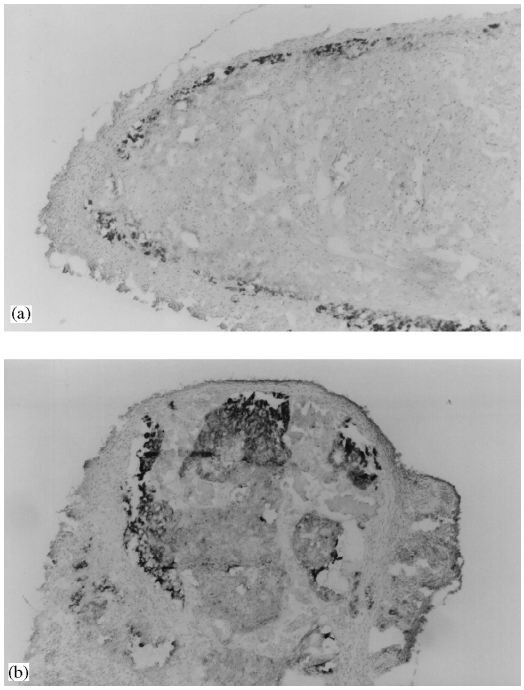


Figure 4. Immunohistochemical staining on cryosections for pro-collagen type-I (M38). Serially passaged chondrocytes (P3) were used at a density of 50 x 106 cells/ml. In combination with E210 (a), 8 weeks after transplantation, pro-collagen type-I positive staining is limited to the capsule and the periphery of the newly generated tissue. In combination with DBM (b) pro-collagen type-I positive staining was found in the capsule, the periphery as well as in the center of the graft. Red staining indicates a positive signal. Original magnification x 50.

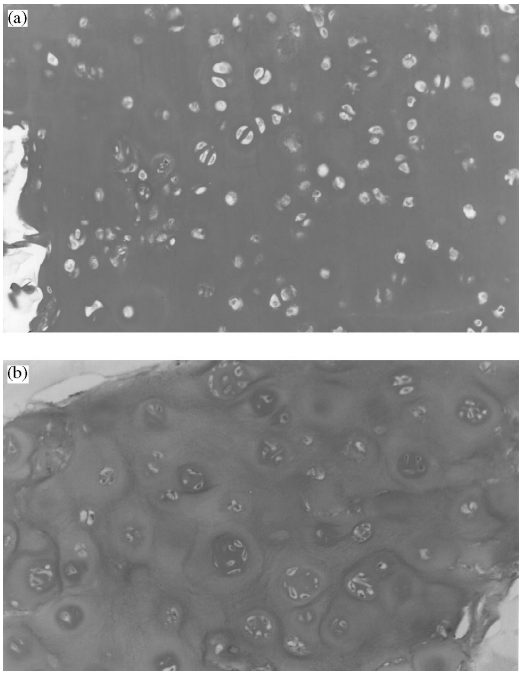


Figure 5. Seeded at the highest density (50 x 106 cells/ml) few and small clusters were found in freshly isolated chondrocytes (a). In contrast at a density of 10 x 106 cells/ml approximately 50% of freshly isolated chondrocytes formed clusters (b). Alcian Blue staining, magnification x 200.

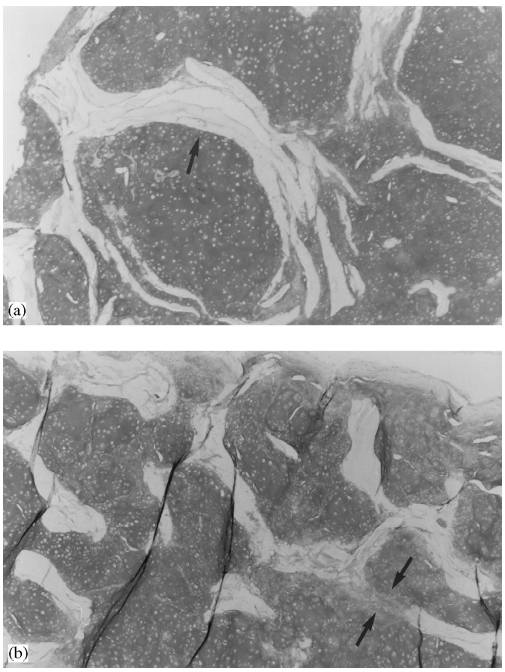


Figure 6. If DBM was used as a carrier, only moderate degradation of the biomaterial was observed in the center of the grafts. These remaining strands of the biomaterial (indicated by arrows, Figure 6a) intersect the cartilage-like tissue causing disintegration of the tissue. Degradation of DBM was observed only at the edges of the grafts. At these sites fibrous strands formed where trabeculae had been located (indicated by arrows, Figure 6b). Alcian Blue staining, magnification x 50.

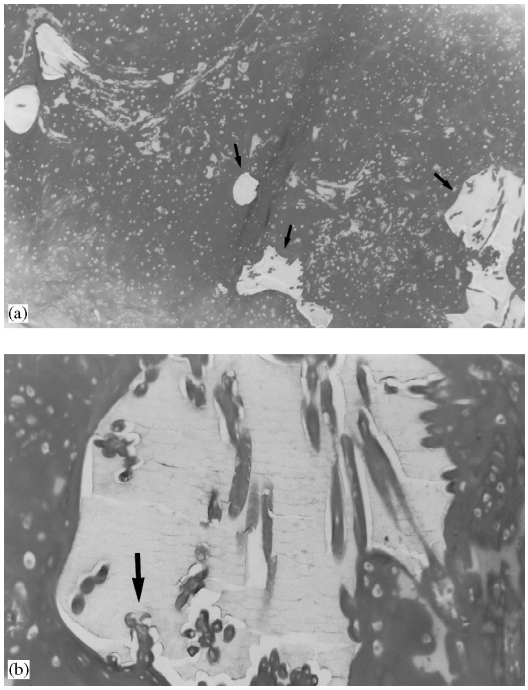


Figure 7. E210 was found to be almost completely degraded after 8 weeks. In contrast to DBM remaining parts of the biomaterial did not intersect the cartilage like tissue, but were grouped together (a) and invaded by chondrocytes (b). Thus forming a more homogenous tissue. Alcian Blue staining., magnification a: x 50 and b: x 200.

DISCUSSION

For clinical applications tissue engineered neo-cartilage should meet certain demands. The mechanical properties of the graft should allow surgical handling and mechanical loading. These mechanical properties will be mainly determined by structural characteristics of the graft. The presence of glycosaminoglycans and collagen type-II is one of the prerequisites for long-term graft survival after intra-articular implantation. This study was designed to investigate whether these demands can be met using freshly isolated, and culture expanded cells and if structural characteristics can be influenced by the type of cell carrier used. Various authors showed that chondrocytes in alginate or agarose synthesize a mechanically functional matrix[21] similar to native articular cartilage[19,22]. We used alginate in this study because, unlike agarose, it gives only minimal inflammatory reaction after in vivo implantation[9,23-25] and in the future might be used for human applications[26].

Since low viscosity alginate gel alone cannot stand mechanical loading nor surgical handling, a biomaterial is required to provide initial strength to the graft. The use of alginate to seed chondrocytes in a biomaterial facilitates a homogenous distribution of identically shaped chondrocytes in the relatively wide pores of the material. If cells are seeded directly into a biomaterial, different cell shapes are seen within the biomaterial. This can lead to different phenotypic expression[16] and negatively influences structural homogeneity. When the alginate

is polymerized the chondrocytes are immobilized within the graft and are prevented from floating out. Absence of vascular ingrowth and lack of, macrophage-mediated, degradation of DBM in the center of the grafts both indicate that alginate prevents cells from entering the graft as well. With time, the transplanted chondrocytes will take over the function of the cell carrier. At this point the alginate and the carrier are no longer needed and can be degraded.

Since all biomaterials are foreign bodies, they carry a permanent risk for infection[27]. They should not stay in place once they have served their purpose. Therefore, we prefer biodegradable to non-biodegradable carriers. A prerequisite for any biodegradable cell carrier is that degradation of the carrier has no adverse effects on cell viability or metabolism[16]. Alginate can be hydrolyzed[24] into mannuronic acid and guluronic acid. These two components can than be incorporated in enzymatic pathways for further degradation[9,25]. No inflammatory reactions against remains of alginate were noted, as has previously been reported by other authors[24,25]. In this study we compared a biological biodegradable carrier and a synthetic biodegradable carrier both with their own advantages and disadvantages. DBM has demonstrated chondrogenic potential[28,29], is composed of collagen type-I and therefore less likely rejected than a synthetic carrier. However, the pore size varies significantly, infectious agents can be transmitted and degradation is macrophage-mediated[30]. In the nude mice model used in this study, no degradation of DBM was noted in the center of the grafts. Apparently macrophages, responsible for degradation, did not reach the center of the transplanted tissue. At the sites where remaining strands of biomaterial intersected the cartilage-like tissue, disintegration of the tissue is obvious and hampers mechanical properties. At the edges of the grafts, where DBM was degraded, fibrous tissue was formed. Clearly, this is an unwanted side effect. The synthetic biodegradable carrier used in this study (E210) is well defined with respect to chemical and physical composition. Whereas biological carriers theoretically could carry infectious agents, like prions, this is not possible with synthetic carriers. Degradation of the biomaterial is by hydrolysis[16,31-33], which is probably the reason why the biomaterial is degraded equally throughout the graft and led to a homogenous tissue. Redifferentiation of multiplied chondrocytes is essential for successful generation of tissue engineered cartilage[34]. In mature cartilage, chondrocytes account for only 2.3 ± 1.2% of the tissue volume[19]. Whereas in the embryo, at sites of cartilage formation, cell density is many times higher[35]. This is the reason why high cell densities are believed to positively influence neo-cartilage generation[36]. However the optimal cell density is as yet unknown. Paige et al. demonstrated gross cartilage formation in samples with an initial cellular density of at least 5 million cells/ml and microscopically in specimens with a cellular density as low as 1 million cells/ml[9]. Puelacher et al., on the other hand, stated that a cell density greater than 20 million cells/ml is required to ensure successful engraftment[15]. Cell shape is believed to determine phenotypic expression of chondrocytes[37]. In a gel like alginate or agarose chondrocytes regain their original rounded shape. This has been reported to induce redifferentiation. In contrast to the results of Bonaventure et al.[14] using fetal human chondrocytes, and Benya and Shaffer[13] using young rabbit chondrocytes, our dedifferentiated chondrocytes did not redifferentiate in vitro, when cultured

in alginate for 3 weeks, irrespective of the cell seeding density that was used. This could indicate that, culture in alginate might not be optimal for redifferentiation of adult articular chondrocytes. Furthermore, Yaeger et al.[38] demonstrated that re-expression of the cartilage phenotype of cells in alginate can vary widely depending on the batch of FCS used. Data from our in vivo experiment indicate that high cell density seeding (50 million cells/ml) and the use of E210 as a carrier enhanced collagen type-II expression of serially passaged chondrocytes. Furthermore, pro-collagen type-I is lacking in the center of these grafts. In conclusion, our data clearly show re-expression of the cartilage phenotype. The present study further demonstrated that, higher cell seeding densities reduced the number of cell clusters formed. Cluster formation, caused by division of individual cells, could possibly indicate an effort of chondrocytes to optimize their cell density. Whereas other authors interpret cluster formation as a positive finding, representing regeneration rather than degeneration[3,39], large cell clusters are no feature of healthy, normal articular cartilage or juvenile repair tissue[40,41] and have to be considered as a negative finding. This study indicates that serially passaged adult articular chondrocytes can be used to generate a cartilage-like tissue that meets some of the demands critical for the envisioned repair of articular cartilage defects. Best histologic results were obtained when serially passaged chondrocytes at 50 million cells per ml alginate were seeded in E210 fleeces, indicating that the type of cell carrier used can influence structural characteristics and phenotypic expression of the newly generated tissue. It has to be kept in mind that the environmental conditions in the joint are distinctly different from a well vascularized bed like a subcutaneous pocket. Therefore, conclusions based on this study possibly can not be extrapolated to application in the joint. However, subcutaneous implantation of the graft prior to intra-articular use, to generate neo-cartilage strong enough to allow surgical handling and mechanical loading, could be a possible clinical application of this technique. Future experiments will investigate the feasibility of this newly formed cartilage for the repair of articular cartilage defects. Experiments will focus on mechanical properties, graft-host interaction and durability of the newly formed cartilage after intra-articular implantation.

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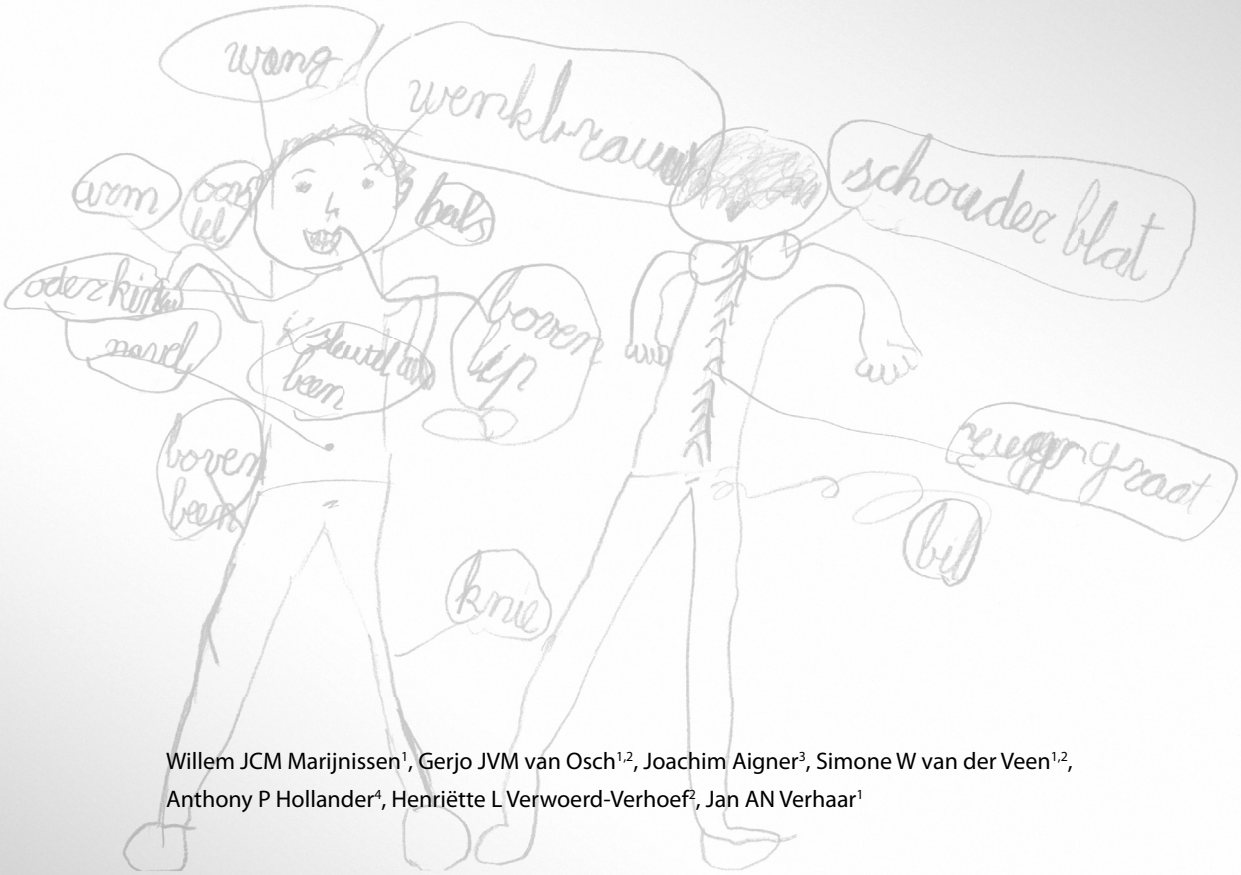
CHAPTER 5

ALGINATE AS A CHONDROCYTE-DELIVERY SUBSTANCE IN COMBINATION WITH A NON-WOVEN SCAFFOLD FOR CARTILAGE TISSUE ENGINEERING



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ABSTRACT

For tissue engineering of cartilage, chondrocytes can be seeded in a scaffold and stimulated to produce a cartilage-like matrix. In the present study, we investigated the effect of alginate as a chondrocyte-delivery substance for the construction of cartilage grafts. E210 (a non-woven fleece of polyglactin) was used as a scaffold.

When 'bare' E210 (without alginate and without chondrocytes) was implanted subcutaneously in nude mice for 8 weeks, the explanted tissue consisted of fat and fibrous tissue only. When E210 with alginate but without chondrocytes was implanted in nude mice, small areas of newly formed cartilage were found. Alginate seems to stimulate chondrogenesis of ingrowing cells. When chondrocytes were seeded in E210, large amounts of cartilage were found, independent of the use of alginate. This was expressed by a high concentration of glycosaminoglycans (30 µg/mg wet weight) and the presence of collagen type-II (1.5 µg/mg wet weight). Macroscopically the grafts of E210 without alginate were shrunk and warped, whereas the grafts with alginate had kept their original shape during 8 weeks of implantation. The use of alginate did not lead to inflammatory reactions nor increased capsule formation.

In conclusion, the use of alginate to seed chondrocytes in E210 does not influence the amount of cartilage matrix proteins produced per tissue wet weight. However, it provides retention of the graft shape.

INTRODUCTION

Hyaline cartilage defects still pose a major problem to orthopaedic and facial reconstructive surgeons, since these defects do not heal spontaneously with hyaline cartilage. The use of tissue engineering techniques could be an appropriate way to generate hyaline cartilage grafts for the treatment of cartilage defects in the future. These techniques combine isolated cells with biodegradable scaffolds in order to stimulate production of new cartilage tissue with the typical characteristics of native hyaline cartilage. Autologous cells are generally preferred to avoid risks of immunological rejection and transmission of infectious diseases.

The ideal biodegradable scaffold should provide a preformed three-dimensional shape and initial mechanical strength. In addition it should make uniform cell spreading possible, stimulate the chondrogenic phenotype of the transplanted chondrocytes and prevent cells from floating out of the defect. Combinations of polylactic and polyglycolic acid are frequently used as cell carrier[1-3]. In previous studies, we obtained promising results using Ethisorb 210[4]; a non-woven fleece composed of a polylactic-polyglycolic-acid-copolymer punctually glued with polydioxanon(Ethicon, Norderstedt, Germany)[5,6].

Although isolated chondrocytes can be seeded directly into the scaffold, one could also use a carrier gel for cell-seeding. Nowadays, alginate is frequently applied as cell-carrying gel, both in vitro and in vivo[4,7-12]. The use of alginate may facilitate a uniform distribution of chondrocytes in the relatively wide pores of the scaffold and prevent cells from floating out. Moreover, in vitro experiments showed that alginate can stimulate expression of the chondrogenic phenotype[13]. Whereas alginate has been used in humans for transplantation of pancreatic-islet cells without negative effects[14], alginate or its degradation products could possibly influence chondrocyte matrix neosynthesis.

In this study, we investigated the effect of alginate as a chondrocyte delivery substance for the construction of a cartilage graft. E210 was used as a scaffold and implanted in athymic mice, with and without alginate and with and without differentiated bovine articular chondrocytes.

MATERIALS AND METHODS

Chondrocyte isolation

Full-thickness cartilage slices were harvested under sterile conditions from the meta-carpophalangeal joints of 12-18 months old bovine steers, obtained from a local slaughter house, within eight hours after death. Chondrocytes were pooled from 4 different steers to exclude interindividual differences. The cartilage was washed with sterile physiological saline and incubated with protease XIV (2 mg/ml; Sigma, St. Louis, MO) for 2 hours followed by an overnight incubation with collagenase B (1.5 mg/ml; Boehringer, Mannheim, Germany) in medium (DMEM/Ham's F12; Gibco, Grand Island, NY) with 10% FCS (Bio Whittaker, Verviers,

Belgium). Both enzymatic digestions were done at 37° C. After incubation the undigested cartilage fragments were removed using a 100 μm filter. The isolated chondrocytes were washed with physiological saline and counted using a hemacytometer. Cell viability was tested using the trypan blue exclusion test.

Preparation of implants

For all implants a synthetic biodegradable matrix was used (8x8x2 mm), composed of nonwoven polyglactin filaments (a polylactic-polyglycolic-acid-copolymer in a ratio of 90/10 per weight) with a diameter of about 20 μm with punctual polydioxanon adhesions (Ethisorb 210, Ethicon, Norderstedt, Germany).

Four different conditions were tested, two 'control' conditions:

'bare' E210, without chondrocytes or alginate (F).

E210 saturated with alginate (FA)

And two 'experimental' conditions:

E210 seeded with chondrocytes (FC).

E210 seeded with chondrocytes suspended in alginate (FAC).

For the constructs of the 'experimental' conditions freshly isolated chondrocytes were first suspended at a density of 50 million cells per ml in medium (FC) or in 1.2% alginate (Keltone LV, Kelco, Chicago, IL) (FAC), and then the fleeces were immersed in these suspensions.

For constructs *without alginate*, fleeces of E210 were immersed in medium with (FC) or without (F) chondrocytes. For constructs *with alginate*, the fleeces were immersed in the alginate suspension with (FAC) or without chondrocytes (FA) (1-2 hours with occasionally shaking) until they were fully saturated. Polymerization of alginate was then achieved by dropping the grafts into a 102 mM $CaCl_2$ solution. After instantaneous gelation the alginate was allowed to polymerize further for a period of 8-10 minutes in the $CaCl_2$ solution. Thereafter, grafts were washed thoroughly with physiological saline and stored in medium until implantation.

Animal experiments

Animal experiments were approved of 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". Animals were housed, under sterile conditions, at the Center for Animal Research.

Two experiments were performed. For the first experiment, 3 male athymic mice (NMRI), aged 10-12 weeks, were used. Constructs were implanted in subcutaneous dorsal pockets, each mouse carried all 4 different constructs. Constructs were harvested after 8 weeks and fixed in 4% phosphate-buffered formalin for histological analysis. In the second experiment, 5 male athymic mice (NMRI) aged 10-12 weeks, were used. Constructs of FA, FC and FAC were implanted subcutaneous in dorsal pockets, harvested after 8 weeks and divided into 4 parts. One part was

fixed in 4% phosphate-buffered formalin and embedded in paraffin for histological analysis. One part was used for biochemical analysis of the amount of glycosaminoglycans, one part for quantification of the amount of collagen type-II, and one part was frozen at –80°C to be saved for possible use in the future.

Histology

Paraffin sections (5µm) were stained with Alcian Blue (counterstained with nuclear fast red) to evaluate the structural characteristics of the graft. To evaluate calcification of the graft, Von Kossa staining was performed.

Cryosections were used to detect collagen type-I, and paraffin sections to detect collagen type-II. The method as described by Hierck et al.[15] was used to allow the use of monoclonal antibodies on tissue sections of mouse material. Deparaffinated sections were incubated with 0.1% Pronase (Sigma) for 30 minutes to retain antigenicity. All sections were treated with 1% Hyaluronidase (Sigma) for 30 minutes to unmask the epitopes. Aspecific binding was blocked with 10% normal goat serum. Sections were incubated for 2 hours with monoclonal antibodies to collagen type-II (II-II 6B3 1:100; DSHB) or to procollagen type-I (M38, 1:100; DSHB) that was previously complexed with goat Fab fragment against mouse conjugated with alkaline phosphatase (GAMAP 1:400; Immunotech, Marseilles, France) at 4° C overnight. Unbound GAMAP was captured after incubation of the complexes with 0.1 % normal mouse serum for 2 hours before use. Sections were subsequently incubated for 30 minutes with a 1:100 dilution of mouse monoclonal alkaline phosphatase anti alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark). New fuchsine (Chroma, Kongen, Germany) was used for color development. The sections were counterstained with hematoxylin and mounted in gelatin-glycerin. Control stainings were performed by omitting the primary antibody.

Biochemical analysis

For analysis of the amount of glycosaminoglycans (GAG) whole tissue samples (n=5 for each condition) were digested with 0.5 ml, 0.3mg/ml papain (Sigma, St Louis, MO) overnight at 60°C. Quantification of the amount of glycosaminoglycan was performed using the dimethylmethylene blue assay[16] in microtiter plates. The metachromatic reaction of GAG with dimethylmethylene blue was monitored using a spectrophotometer. The ratio A_{540}/A_{595} was used to calculate the amount of GAG in the samples. Chondroitin sulfate C (isolated from shark; Sigma, St Louis, MO) was used as a standard.

The amount of collagen type-II was quantified using an inhibition ELISA developed by Hollander et al.[17]. In short, the samples were digested overnight in 250µl Proteinase K (1 mg/ml) in 50mM Tris with 1 mM EDTA, 1 mM Iodoacetamide and 10 µg/ml Pepstatin A at 56°C. After inactivation of Proteinase K by boiling for 10-15 minutes the samples were stored at 4°C for at most 7 days, until the ELISA was performed. For the inhibition ELISA a specific mouse monoclonal antibody (Col2-3/4m) was used, which recognizes epitope CB11B in the unwound

α1(II) chain[17]. A goat antibody against mouse conjugated with alkaline phosphatase and paranitrophenolphosphate as substrate were used for quantification with a spectrophotometer at 405 nm. As reference value, heat denaturated bovine collagen type-II (Sigma) was used.

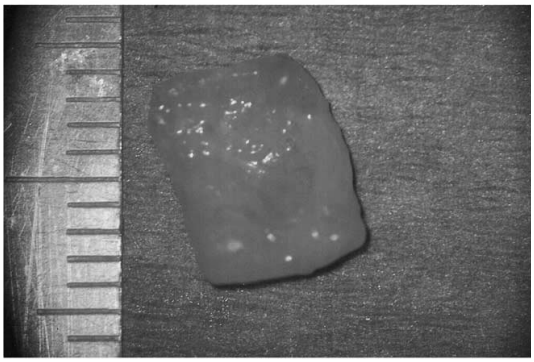
The amount of glycosaminoglycans and the amount of collagen type-II were expressed per tissue wet weight.

RESULTS

Macroscopic analysis

All constructs were retrieved. When handling, the constructs without chondrocytes (construct F&FA) appeared very weak. In particular the 'bare' fleeces (construct F) were too weak to cut in four pieces for separate analysis without destroying the whole construct. The structure of the constructs with chondrocytes without alginate (construct FC) was firm. However, they had shrunk and warped. The constructs with chondrocytes in alginate (construct FAC) had retained their shape, were more elastic than the ones without alginate and had a cartilage-like appearance (Figure 1).

(a)



(b)

Figure 1. Macroscopic picture of constructs of fleece and chondrocytes grown subcutaneously in nude mice for 8 weeks, directly after explantation. (a) construct of E210 with chondrocytes without alginate; (b) construct of E210 with chondrocytes and alginate.

Microscopy

The sections of both experimental conditions grossly appeared similar. After 8 weeks, E210 was largely resorbed in all constructs, only small remnants of the biomaterial remained. All grafts were encapsulated. The capsules were 5-15 cell layers thick and stained postitive for collagen type-I. When 'bare' E210 (without alginate or cells; construct F) was implanted, the explanted tissue consisted of fat and fibrous tissue only. This tissue showed no positive staining with Alcian Blue or for collagen type-II. When alginate was used (construct FA&FAC), no inflammatory reaction nor increased capsule formation was found. Strikingly, when E210 with alginate but without cells (construct FA) was implanted, small areas of Alcian Blue positive tissue were found. These areas stained positive for collagen type-II indicating the presence of hyaline cartilage (Figure 2). No vascular ingrowth was observed.

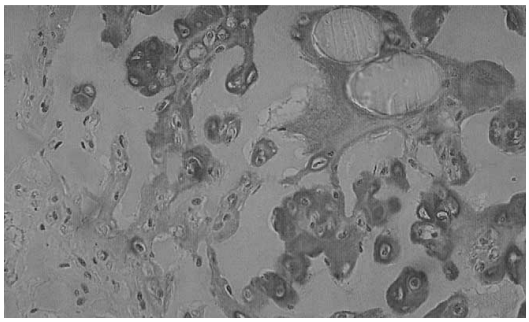


Figure 2. Histological section of construct FA (E210 with alginate without chondrocytes) 8 weeks after implantation. The presence of islets of new cartilage was indicated with immunohistochemistry for collagen type-II. Original magnification 200x.

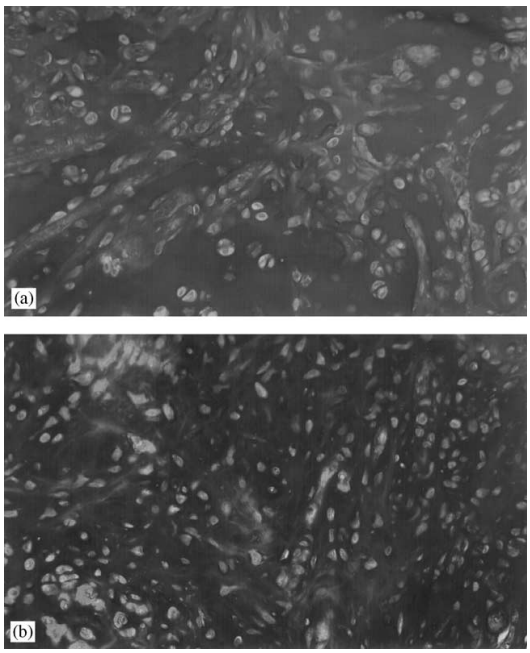


Figure 3. Constructs of E210 with chondrocytes 8 weeks after implantation. (a) construct with alginate (FAC) (b) construct without alginate (FC). Alcian Blue staining, original magnification 200x.

Constructs of E210 seeded with chondrocytes (with or without alginate, constructs FAC&FC respectively) consisted of large amounts of cartilage (Figure 3a and 3b). The matrix stained positive with collagen type-II antibodies. No collagen type-I was detected and again no vascular ingrowth was observed. In constructs without alginate the cell density at the outer borders of the explant was very high. This area stained less intense with Alcian Blue. In constructs with alginate, chondrocytes were more equally dispersed throughout the construct, although cells were lying in small clusters of 2-4 cells. Alginate still seemed present.

Using Von Kossa staining, the extracellular matrix appeared not to be calcified. Although, small calcium deposits were visible at places were matrix was absent and biomaterial had probably been located previously. Such calcium spots were most prominent in constructs with chondrocytes. Two to four spots could be identified per cross-section of approximately 2x2 mm) (Figure 4).

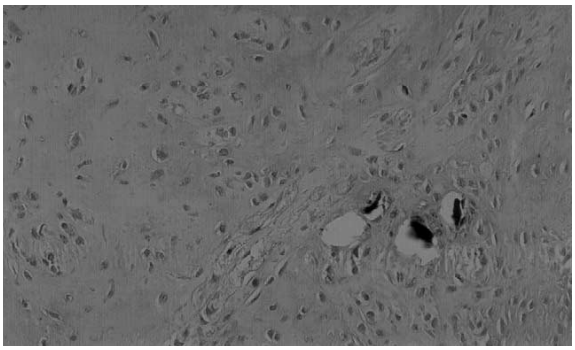


Figure 4. Von Kossa staining of a FC construct 8 weeks after implantation. The extracellular matrix was not stained. Spots of calcium were present at places where biomaterial has been located. Original magnification 200x.

Biochemistry

Biochemical analysis were performed on constructs FA, FC, and FAC. Construct F was too instable to be divided in four parts and was therefore only used for histology. The amount of glycosaminoglycan and collagen type-II is expressed per wet weight of the tissue (Table 1). In E210 with alginate without chondrocytes (construct FA) considerable amounts of GAG and some collagen type-II was found. Although this was surprising, it confirmed the histological results. In the constructs with chondrocytes (FC and FAC) the amount of matrix components was higher. Expressed per wet weight the amount of GAG and collagen II was similar in FC and FAC constructs. However, due to the higher wet weight of the FAC constructs (FAC constructs were twice as heavy as FC and FA constructs), the absolute amount of GAG and collagen II was higher in these constructs.

Table 1. The amount of matrix produced in tissue engineerd constructs after 8 weeks subcutaneous implantation in nude mice. Mean ± SD of 5 mice are presented.

	µg GAG/mg w.w.	µg coll II/mg w.w.
Construct FA E210+alginate	18 ± 0	7 ± 2
Construct FC E210+ cells	30 ± 6	19 ± 3
Construct FAC E210+cells+alginate	31 ± 2	14 ± 4

GAG = glycosaminoglycan, determined by Farndale assay.
Coll II = collagen type-II, determined by ELISA
w.w. = wet weight
Natural calf cartilage contains 70 µg GAG/mg wet weight and 97 µg collagen type-II/mg wet weight[18]

DISCUSSION

For clinical application tissue engineered cartilage should meet certain demands. The mechanical properties of the graft should allow surgical handling and in the long run, the shape of the graft should be retained. In this study, macroscopically the constructs with chondrocytes were all firm. However, the constructs with chondrocytes but without alginate were warped and appeared less elastic than the constructs with alginate. These two types of constructs did not differ in amount of GAG, or collagen per wet weight. Nor were there differences in calcium deposition. The reason for the deforming force of the constructs without alginate might be the zones of high cell density at the margin of the constructs forming a tight layer around the graft. The mechanical properties of the grafts will be determined in part by the main components of cartilage: proteoglycans and collagen. Considering the biochemical analysis, the GAG concentration was close to the value of natural calf cartilage (70 µg/mg wet weight)[18]. The collagen type-II concentration however, was much lower than that of natural calf cartilage (97 µg/mg wet weight)[18]. This can be explained by the slow turnover of collagen (the turnover in human articular cartilage is calculated to be over 100 years[19]) and requires special attention in future studies. Extra stimulation of collagen synthesis, for example by culturing the grafts in a bioreactor[20,21] prior to implantation, might be necessary.

In the present study we used freshly isolated bovine articular chondrocytes directly after harvesting. For clinical purposes, the use of autologous chondrocytes is preferred, because it prevents immunological reactions. However the number of chondrocytes that can be harvested from a patient without donor-site morbidity is limited. Therefore, the cells have to be multiplied in vitro before seeding in a scaffold. Multiplication will inevitably lead to loss of the chondrocyte phenotype, a process called dedifferentiation[22,23]. Redifferentiation will thus be a pre-requisite. In a previous study we demonstrated that E210 in combination with alginate stimulated the redifferentiation of multiplied (dedifferentiated) chondrocytes[4]. Since in that study,

redifferentiation was not found in a demineralized bone matrix in combination with alginate, it was suggested that E210 was more important for stimulation of chondrogenesis than alginate. However, the present study indicates that alginate itself can also stimulate chondrogenesis of cells grown into the grafts of E210 and alginate without multiplied chondrocytes (FA). Cells found in these constructs (after 8 weeks of subcutaneous implantation) probably originate from the subcutaneous connective tissue. Dermal fibroblasts were described to have chondrogenic capacity after arrangement in a three-dimensional culture[24]. It is important to notice that the amount of neo-cartilage formed in this way is highly variable and unpredictable, in contrast to the amount formed with chondrocytes. However this chondro-inductive capacity might be an extra advantage of E210 in combination with alginate.

In this study, only in the control, bare fleeces, blood vessel ingrowth through the capsule that was covering the implant was visible in some areas. We could not demonstrate blood vessels in the newly formed cartilage by differentiated bovine articular chondrocytes, neither in the absence nor in the presence of alginate. In experiments where dedifferentiated human septum chondrocytes or differentiated rabbit auricular chondrocytes were used at a density of 20 million cells/ml in PLA/PGA matrices, blood vessel ingrowth could be demonstrated[6,25]. In similar experiments with dedifferentiated human septal chondrocytes in the presence of alginate, no blood vessels were found (J. Aigner, personal communication, unpublished results). The absence of blood vessels, a condition similar to native cartilage, might be caused by the absence of certain growth factors or the presence of inhibitors of angiogenesis[26,27]. The discrepancy between the present results and other studies might be due to the higher cell densities and the use of alginate, although we can not exclude effects of different species or different cartilage types. Ingrowth of blood vessels in cartilage is undesirable because this is regarded an early sign of mineralization and osteogenesis[28,29].

The use of a gel like alginate to seed chondrocytes in a biomaterial facilitates a homogenous distribution of identically shaped chondrocytes in the relatively wide pores of the material. When the gel is polymerized the chondrocytes are immobilized within the graft and are prevented from floating out. Compared with protein-gels (e.g. fibrin or collagen) less immunoresponse is to be expected against sugar-gels, for instance, alginate or hyaluronic acid. Furthermore, various authors reported that chondrocytes in these gels synthesize a mechanically functional matrix[30] similar to native articular cartilage[7,10]. In the present study alginate was combined with a synthetic biodegradable scaffold. This scaffold is well defined with respect to chemical and physical composition and is clinically used as suture material. Whereas biological scaffolds theoretically could carry infectious agents (e.g. prions) this is not the case with synthetic scaffolds. With time, the transplanted chondrocytes will take over the function of the cell carrier. At this point the alginate and the scaffold are no longer needed and can be degraded. Since all biomaterials are foreign bodies, they carry a permanent risk for infection[31]. They should be degraded once they have served their purpose. A prerequisite for any biodegradable cell carrier is that degradation of the carrier has no adverse effects on cell viability or metabolism[32].

Degradation of E210 is by hydrolysis[5,32,33] (as is true for all scaffolds based on PGA/PLA). This is probably the reason why the biomaterial is degraded equally throughout the graft, leading to a homogenous tissue. Alginate too can be hydrolyzed[34]. The two components (mannuronic acid and guluronic acid) can than be incorporated in enzymatic pathways for further degradation[8,35]. Although the first studies in our laboratory with nude mice and rabbits using alginate as cell-carrier, were very promising, one might question the use of alginate in humans. The degradation pathway of alginate is largely unknown and slow resorption could give rise to foreign body reactions against alginate in the long term. However, no inflammatory reactions against remains of alginate were noted, as has previously been reported by other authors[8,14,34].

The present study demonstrated that the use of alginate in combination with a non-woven scaffold (E210) does not negatively influence the amount of cartilage matrix proteins per tissue wet weight. In fact, it provides retention of the graft shape. Conservation of a preshaped graft is of great importance for clinical use. Future experiments will investigate the feasibility of this newly formed cartilage for the repair of hyaline cartilage defects.

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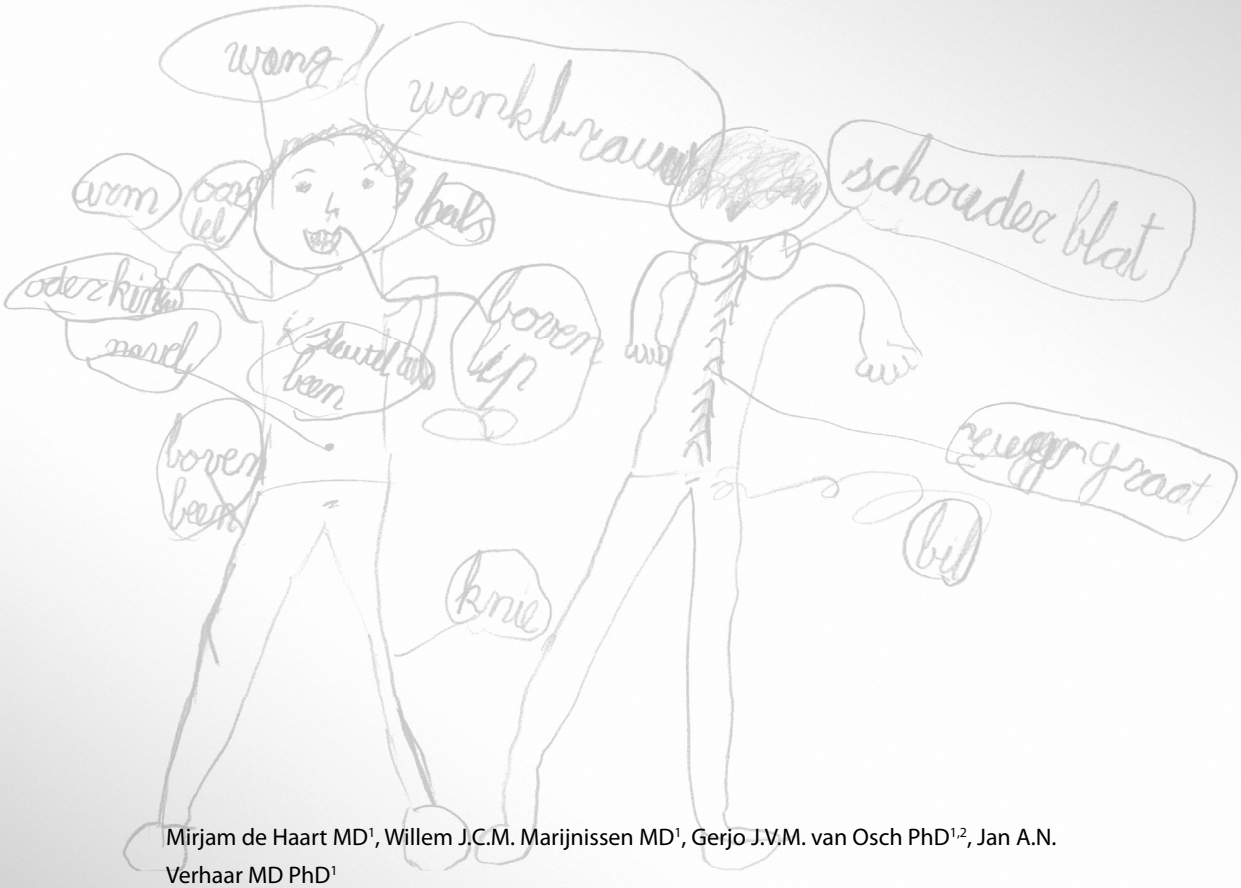
CHAPTER 6

OPTIMIZATION OF CHONDROCYTE EXPANSION IN CULTURE. EFFECT OF TGFSS-2, BFGF AND L-ASCORBIC ACID ON BOVINE ARTICULAR CHONDROCYTES



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ABSTRACT

In vitro multiplication of isolated chondrocytes is needed to repair articular cartilage defects with autologous material. In this study we used monolayer cultures of bovine articular chondrocytes. The effect of transforming growth factor ß-2, basic fibroblast growth factor or L-ascorbic acid on cell-multiplication, in the presence of 10% fetal calf serum, was measured in primary culture, the third and tenth passage. TGFß-2 stimulated the proliferation of chondrocytes in primary culture and L-ascorbic acid stimulated in the third passage. Based on these results we chose an optimal addition scheme in which TGFß-2 was added in primary culture and first passage, followed by addition of L-ascorbic acid in the second and third passage; this resulted in a 7-fold increase in cell number compared to the control group, in about 4 weeks. Our findings stress the importance of adding the right growth factor at the right moment.

Collagen type-II expression was lost after the third passage, in the control as well as in the experimental condition. The ability to produce hyaline cartilage specific matrix components is essential, if multiplied cells are to be used to repair cartilage defects.

INTRODUCTION

Full thickness defects of articular cartilage have a poor capacity for spontaneous repair. The use of cultured autologous cells to repair defects has been considered successful[1-4]. Repair of full-thickness articular cartilage defects has the best chance for success if the defect is isolated, without degenerative changes in the joint. Since defects of this kind are mainly found in young adults, experiments with young adult articular chondrocytes have clinical relevance. Allografts may lead to immunological rejection of the graft and transmission of infectious diseases; this makes autografts preferable. The number of chondrocytes that can be harvested from a patient is limited. Therefore the cells need to be multiplied in vitro. Several research groups have studied the effects of growth factors on this multiplication process. Chondrocytes dedifferentiate during multiplication in monolayer[5]. This can alter the response of chondrocytes to growth factors[6,7].

We used articular chondrocytes from 12-18 month-old bovine steers, which can be considered as young adults. Transforming growth factor ß-2 (TGFß-2), basic fibroblast growth factor (bFGF) and L-ascorbic acid are all able to stimulate chondrocyte proliferation under certain conditions[8-10]. These factors were added to the culture medium in the presence of 10% Fetal Calf Serum (FCS). We studied the effects of the 3 factors on chondrocyte multiplication in primary culture (differentiated chondrocytes), in the third passage (partly dedifferentiated chondrocytes) and in the tenth passage (dedifferentiated chondrocytes). Furthermore a scheme for sequential addition of growth factors was designed to optimize multiplication during four passages.

Dedifferentiated chondrocytes have lost the ability to produce cartilage-specific collagen type-II[5]. Transplantation of in vitro multiplied chondrocytes into an articular cartilage defect can only be successful if the cells can be stimulated to re-express collagen type-II. Culturing in agarose[5,11,12] and in alginate[13,14] has been reported to induce redifferentiation of dedifferentiated chondrocytes. We therefore studied the potential of bovine articular chondrocytes to produce hyaline cartilage matrix after monolayer culture and a subsequent culturing period in alginate.

MATERIALS AND METHODS

Chondrocyte Isolation

Full-thickness cartilage slices were harvested (within eight hours after death under sterile conditions) from the metacarpophalangeal joints of 12-18 months old bovine steers, obtained from a local slaughter house. The cartilage was washed with sterile physiological saline and incubated with protease XIV (2 mg/ml; Sigma, St. Louis, MO) for 2 hours followed by an overnight incubation with collagenase B (1.5 mg/ml; Boehringer, Mannheim, Germany) in medium (DMEM/Ham's F12; Gibco, Grand Island, NY) with 10% Fetal Calf Serum (FCS). Both enzymatic digestions were done at 37° C. After incubation, the undigested cartilage fragments were removed using a 100 μm filter. The isolated chondrocytes were washed with physiological

saline and counted using a hemacytometer. Cell viability was tested with the trypan blue exclusion test. Cells were either seeded in 175 cm^2, 75 cm^2 or 25 cm^2 culture flasks or cultured in alginate.

Culture in Monolayer

The isolated chondrocytes were seeded in monolayer at a density of 2 x 10^4 cells/cm^2. The culture medium (DMEM/Ham's F12), supplemented with 10% FCS, Fungizone (0.5 μg/ml) and Gentamycin (50 μg/ml) was changed twice weekly.

In an initial experiment the multiplication of bovine chondrocytes in response to 1.0 ng/ml TGFß-2 (Sandoz, Basel, Switzerland), 1.0 ng/ml or 10 ng/ml bFGF (Serva, Heidelberg, Germany) and 25 μg/ml L-Ascorbic Acid (Sigma) was tested in primary culture (P0), in the third passage (P3) and in the tenth passage (P10). Three culture flasks of 25 cm^2 were used to test each growth factor and 3 flasks were used as controls. When one of the monolayer cultures reached the subconfluent phase, the flasks of all experimental groups were trypsinized (Trypsin-EDTA; Gibco). After washing, the cells were resuspended and two samples of each flask were counted twice using a hemacytometer. Since the interest of this study was cell proliferation over a prolonged time period, i.e. one passage, incorporation of radiolabeled thymidine was not used. The cells were reseeded in monolayer or suspended in alginate (1.2%; Keltone LV, Kelco, Chicago, IL). Because cell multiplication was stimulated by TGFß-2 in primary culture and by L-ascorbic acid in the third passage a dose response experiment was performed. The culture medium was supplemented with 0.1 ng/ml, 1.0 ng/ml and 10 ng/ml TGFß-2 in primary culture and supplemented with 2.5 μg/ml, 25 μg/ml and 100 μg/ml L-ascorbic acid in the third passage.

Using the results of the above described experiments, an optimal scheme to administer growth stimulating factors to chondrocyte monolayer cultures was designed. We investigated the response of chondrocytes to 1.0 ng/ml TGFß-2 in primary culture and the first passage and 25 μg/ml L-ascorbic acid in the second and third passage. Chondrocytes not treated with growth factors in former passages (native chondrocytes) served as controls (Figure 1).

Culture in Alginate Beads

Cells were encapsulated in alginate beads at a density of 2 - 4 x 10^6 cells/ml. The preparation of chondrocytes in alginate beads was done as described by Guo et al[13], with slight modifications as described by Häuselmann et al[15]. The cells were suspended in sterile saline containing 1.2% low viscosity alginate gel, then slowly passed through a 21 Gauge needle in a dropwise fashion into a 102 m*M* $CaCl_2$ solution. After instantaneous gelation the beads were allowed to polymerize further for a period of ten minutes in the $CaCl_2$ solution. They were thereafter washed with physiological saline and finally plated in a 24-well plate with 7-10 beads/well in 1 ml DMEM/Ham's F12 medium supplemented with 10% FCS, Gentamycin (50 μg/ml), Fungizone (0.5 μg/ml) and 25 μg/ml L-ascorbic acid freshly added and cultured at 37° C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed 3 times a week. After 7, 14, 21

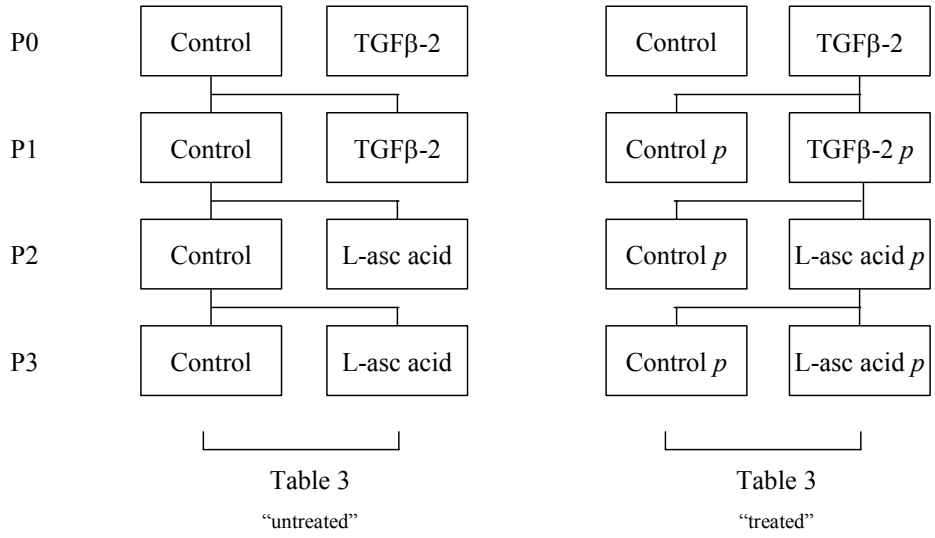


Figure 1. Experimental design of in vitro multiplication of chondrocytes, first treated with TGFß-2, followed by L-ascorbic acid. P0; primary culture, P1; first passage, P2; second passage, P3; third passage. TGFß-2 1.0 ng/ml, L-ascorbic acid 25 µg/ml. p = previously incubated.

and 28 days alginate beads were removed from culture and dissolved in 55 m*M* Na-Citrate, supplemented with 5% FCS. After 10 minutes the cells were cyto-centrifuged onto glass slides at 1000 rpm during 7 minutes. After air drying at room temperature for 1 hour the specimens were frozen at -80° C until immunohistochemical staining took place.

Immunohistochemical Staining

For detection of type I and type II collagen all slides were initially fixed in acetone for 10 minutes at room temperature and incubated with 1% hyaluronidase (Sigma) for 30 minutes at 37° C. Aspecific binding was blocked with 10% normal goat serum. Subsequently the cells were incubated for two hours with monoclonal antibodies to pro-collagen type-I (M38 1:100; DSHB, Iowa City, IA) or collagen type-II (II-II6B3 1:100; DSHB) at room temperature. The slides were incubated for 30 minutes with a 1:100 dilution of goat Fab-fragment against mouse conjugated with alkaline phosphatase (GAMAP; Immunotech, Marseille, France) in PBS-BSA (1%) supplemented with 10% FCS, followed by incubation for 30 minutes with a 1:100 dilution of mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark). The new fuchsine (Chroma, Kongen, Germany) procedure was used for color development. The slides were counterstained with hematoxylin and mounted in gelatin-glycerin. Negative control stainings were done simultaneously by omitting the first antibody. The slides were examined blindly and independently by two observers. The staining for both collagen type-I and collagen type-II was classified as: (1) less than 10% of the total number of cells staining

positive, (2) 10-40% of the total number of cells staining positive, (3) 40-60% positive staining, (4) 60-90% positive staining and (5) more than 90% positive staining.

Data analysis

The experiment to test the response of TGFß-2, L-ascorbic acid and bFGF was performed 3 times. Each experiment consisted of 3 culture flasks per group. The mean and standard deviation (SD) of the experiments were calculated. The dose response experiment was performed once with 3 flasks per group, to check literature data. The experiment in which addition of TGFß-2 in the first 2 passages was followed by L-ascorbic acid in the next passages was performed 3 times, with 3 flasks per group. Differences between individual samples were analyzed. One way ANOVA was used to compare multiple groups, a p-value <0.001 was considered significant. If statistical significant differences were found, the rank sum test was used to compare two experimental conditions, a p-value < 0.05 was considered significant.

RESULTS

After isolation, more than 95% of the cells were viable. In the initial experiment, the time for cells to reach the subconfluent phase was 9 ± 4 days for the primary culture, 5 ± 1 days for the third passage and 7 ± 0 days for the tenth passage. Each passage corresponded to a cell multiplication of 2.4 ± 1.0 times. One experiment was kept in culture until the twenty-second passage. Those cells kept on multiplying with an approximate subconfluent time of one week. TGFß-2 (1.0 ng/ml) significantly stimulated the proliferation of primary bovine chondrocytes (p=0.008). This stimulation decreased in the third passage (p=0.04) and had disappeared by the tenth passage (Table 1). bFGF (1.0 ng/ml and 10 ng/ml) had no significant effect on the proliferation rate of chondrocytes in any of these passages (Table 1). L-ascorbic acid (25 μg/ml) had an inhibitory effect (p=0.01) on the proliferation of primary chondrocytes, but became a stimulator in the third passage (p=0.0004) (Table 1).

Table 1. Effect of TGFß-2, bFGF and L-ascorbic acid on cell multiplication in monolayer (% of control

Passage	TGFß-2 1.0 ng/ml	bFGF 1.0 ng/ml	L-ascorbic acid 25 µg/ml
Primary (P0)	139 (20) P=0.008	95 (16)	85 (4) P=0.01
Third (P3)	110 (7) P=0.04	107 (14)	147 (9) P=0.0004
Tenth (P10)	91 (17)	89 (9)	124 (20)

TGFß-2 (1.0 ng/ml), bFGF (1.0 ng/ml) or L-ascorbic acid (25 µg/ml) was added in primary culture (P0), in the third passage (P3) or in the tenth passage (P10) until subconfluency was reached. Data represent mean (SEM) of 3 experiments.

The dose-response experiment (Table 2) revealed stimulation of multiplication in monolayer using 0.1 and 1.0 ng/ml TGFß-2 and no effect if 10 ng/ml TGFß-2 was used. Addition of 1.0 ng/ml TGFß-2 was chosen because it is widely used in literature. This experiment also confirmed that the optimal dose of L-ascorbic acid is 25 μg/ml in the third passage.

Table 2. Dose response of TGFß-2 on cell multiplication in primary culture and L-ascorbic acid in the third passage (% of control)

	Control	TGFß-2 0.1 ng/ml	TGFß-2 1.0 ng/ml	TGFß-2 10 ng/ml
P0	100 (11)	139 (7)	134 (27)	77 (36)
	Control	L-asc acid 2.5 μg/ml	L-asc acid 25 μg/ml	L-asc acid 100 μg/ml
P3	100 (5)	114 (44)	143 (20)	105 (18)

P0; primary culture. P3; third passage. Cell number of control flasks is set at 100%. Data represent mean (SD) of three culture flasks. v

These results led to the design of an optimal scheme for the sequential addition of multiplication stimulating factors, which was tested in the following experiments. As in the previous experiments TGFß-2 stimulated cell-proliferation in primary culture (p=0.01), and L-ascorbic acid stimulated proliferation in the third passage of native (not previously incubated) cells (p=0.001) (Table 3; 'untreated'). In the scheme of sequential addition (Table 3; 'treated'), TGFß-2 stimulated in primary culture (p=0.01). In the first passage, two experiments showed stimulation whereas one did not (p=0.2). A significant stimulating effect of L-ascorbic acid was seen in the second passage (p=0.0007) and in the third passage (p=0.009) when the chondrocytes had previously been incubated with TGFß-2.

Table 3. Effect (on cell multiplication in monolayer culture) of the addition of TGFß-2 and L-ascorbic acid on native cells (untreated) or in a sequential addition scheme (treated)

Passage	Untreated	Treated
Primary (P0)	130 (9) P=0.01	130 (9) P=0.01
First (P1)	98 (15)	131 (25)
Second (P2)	112 (9)	161 (12) P=0.0007
Third (P3)	132 (9) P=0.001	128 (13) P=0.009

For experimental design see Figure 1. Untreated: Native cells (not previously incubated) were cultured in medium with TGFß-2 (1.0 ng/ml) in primary culture, or in the first passage. In the second or third passage L-ascorbic acid (25 μg/ml) was added to the culture medium. Treated: TGFß-2 (1.0 ng/ml) was added in primary culture, continued in the first passage and followed by the addition of L-ascorbic acid (25 μg/ml) in the second and third passage. Controls were set at 100 %. Data represent mean (SEM) of 3 experiments.

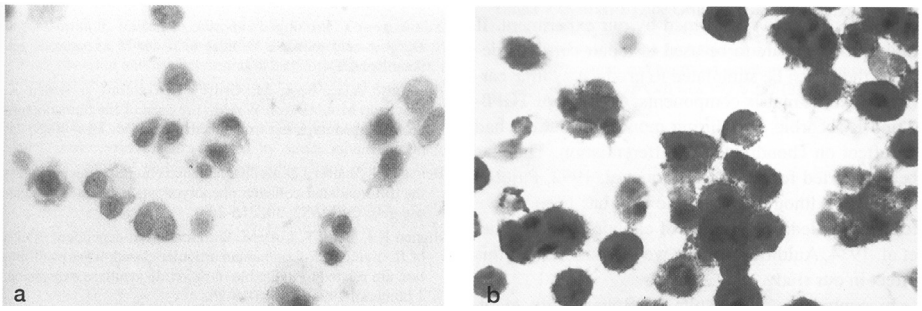
The total number of cells in the native, control group was 15.5 times as many as the number of cells to begin with. In the group treated with the sequential addition scheme, the cell number was 106 times higher than the original number of cells (Table 4). This means that 7 times more cells were acquired after treatment with TGFß-2 followed by L-ascorbic acid.

Table 4. Cumulative multiplication factors of chondrocytes after treatment with TGFß-2 and L-ascorbic acid, compared to native (not previously incubated) chondrocytes

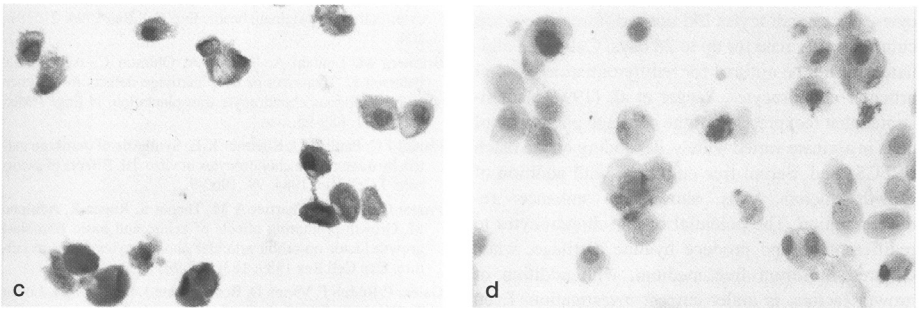
Passage	Native	treated
Onset	1.0	1.0
Primary	1.9 (0)	2.5 (0.2)
First	3.1 (0.4)	5.8 (0.8)
Second	8.5 (1.3)	29.5 (3.2)
Third	16 (2.3)	106 (20)

Chondrocytes were treated with TGFß-2 (1.0 ng/ml) primary culture and in the first passage, followed by the addition of L-ascorbic acid (25 µg/ml) in the second and third passage. Data represent mean (SEM) of 3 experiments.

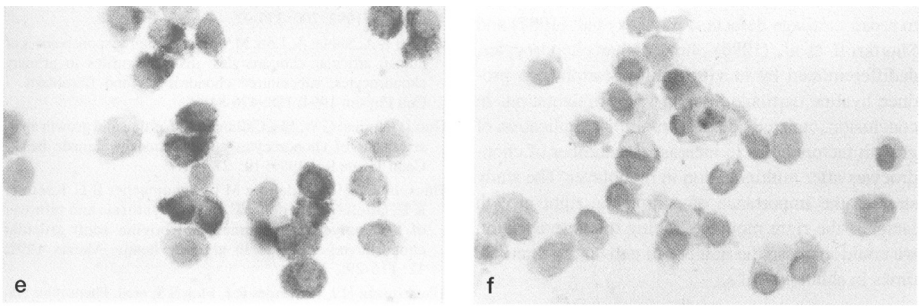
Immunohistochemistry showed that freshly isolated (P0) bovine chondrocytes cultured in alginate, nearly all stained positive for the antibody to collagen type-II (II-II6B3) (Figure 2b). None of the cells stained positive for the antibody to procollagen type-I (M38) (Figure 2a), except for cells in the cell-clusters that formed occasionally. Chondrocytes suspended in alginate after one passage in monolayer (P1) showed collagen type-I to be as frequent as collagen type-II; 50% of the cells stained positive for M38 (Figure 2c) and just under 50% of the cells stained positive for II-II6B3 (Figure 2d). After 3 passages more than 50% of the cells stained positive for M38 (Figure 2e) and none of the cells stained positive for II-II6B3 (Figure 2f). Chondrocytes suspended in alginate after ten or twenty-two passages in monolayer showed only collagen type-I staining. The PBS control sections were all negative. No difference was found between 7, 14, 21 and 28 days of culture in alginate. Addition of growth factors during monolayer did not influence collagen expression in alginate cultures.



Freshly isolated chondrocytes, cultured in alginate, stained with M38 (a) and with II-II 6B3 (b)



Cells cultured in alginate, after multiplication in primary monolayer, stained with M38 (c) and with II-II6B3 (d)



Cells cultured in alginate after multiplication in monolayer until the third passage, stained with M38 (e) and with II-II6B3 (f). Original magnification of all slides 200 X. Red staining indicates a positive signal

Figure 2. Immunohistochemical staining for pro-collagen type-I (M38) and collagen type-II (II-II6B3), on chondrocytes cultured in alginate. Illustrating the gradual shift from collagen type-II to type-I, during the process of cell multiplication.

DISCUSSION

Repair of cartilage defects with autologous chondrocytes requires multiplication of chondrocytes. In this in vitro study TGFß-2, bFGF and L-ascorbic acid were tested for their ability to stimulate chondrocyte multiplication in monolayer culture. One of the findings of the present study is that the response of chondrocytes to TGFß-2 and L-ascorbic changes during cell multiplication. TGFß-2 stimulated the growth of freshly isolated chondrocytes whereas L-ascorbic acid stimulated chondrocytes in the third passage and inhibited freshly isolated ones. Although we used bovine chondrocytes, we think that these results have important implications for the choice of growth factors to stimulate proliferation of human chondrocytes.

In agreement with our results, the stimulation of chondrocyte proliferation by TGFß has been reported to decrease with the number of in vitro subcultures[7] and may eventually become inhibitory[9]. In contrast with our results, bFGF has been reported to stimulate the proliferation of chondrocytes. This conflict may be caused by the different origins of chondrocytes and different culture circumstances[8,16]. Our study showed an inhibition of the proliferation of primary chondrocytes by L-ascorbic acid and a stimulation in the third passage which have not been described before.

To obtain as many cells as possible in 4 passages, we designed an optimal sequential addition scheme for growth factors. TGFß-2 was added to the culture medium in the first 2 passages and L-ascorbic acid was added in the subsequent 2 passages. This led to a more than 100-fold increase in the number of cells after 4 passages over 4 weeks. This is 7 times as many cells as obtained in the native, control group. TGFß-2 indeed stimulated proliferation in the first two passages. The cells which were treated with TGFß-2 in primary culture and in the first passage, were stimulated by L-ascorbic acid in the second passage and in the third passage.

During monolayer culture chondrocytes dedifferentiate and the number of collagen type-II producing cells decreases[7,17]. This was confirmed by our experiment. If multiplied chondrocytes are to be used to repair cartilage defects, they must be stimulated to produce hyaline cartilage specific matrix components. Addition of TGFß-2 and L-ascorbic acid during monolayer culture had no effect on chondrocyte dedifferentiation. This has been reported for TGFß[6,18]. Although L-ascorbic acid has been shown to increase dedifferentiation of chondrocytes[19,20], we did not observe this in our study.

In contrast to the results of Bonaventure et al. using fetal human chondrocytes[14], and Benya and Shaffer using immature rabbit chondrocytes[5], our chondrocytes did not redifferentiate when cultured in alginate for up to 28 days. Culture in alginate may not be optimal for redifferentiation of adult articular chondrocytes. Yaeger et al. demonstrated that reexpression of the cartilage specific phenotype of cells in alginate varied widely, depending on the batch of FCS used. Serum free culturing, with addition of growth factors, was shown to enhance redifferentiation[21]. The potential of our chondrocytes to redifferentiate and produce hyaline cartilage is under current investigation. Even if multiplied chondrocytes can not be stimulated

to redifferentiate in vitro, the cells may still be used to repair cartilage defects. Takigawa et al.[22] and Shortkroff et al.[12] showed that chondrocytes, dedifferentiated by in vitro multiplication, can produce hyaline cartilage after in vivo implantation.

In conclusion, our study demonstrates that application of growth factors helps to increase the number of chondrocytes after multiplication in monolayer. The study stresses the importance of adding the right growth factor at the right moment. Taking this into account, we were able to increase the amount of cells more than 100 times in about 4 weeks.

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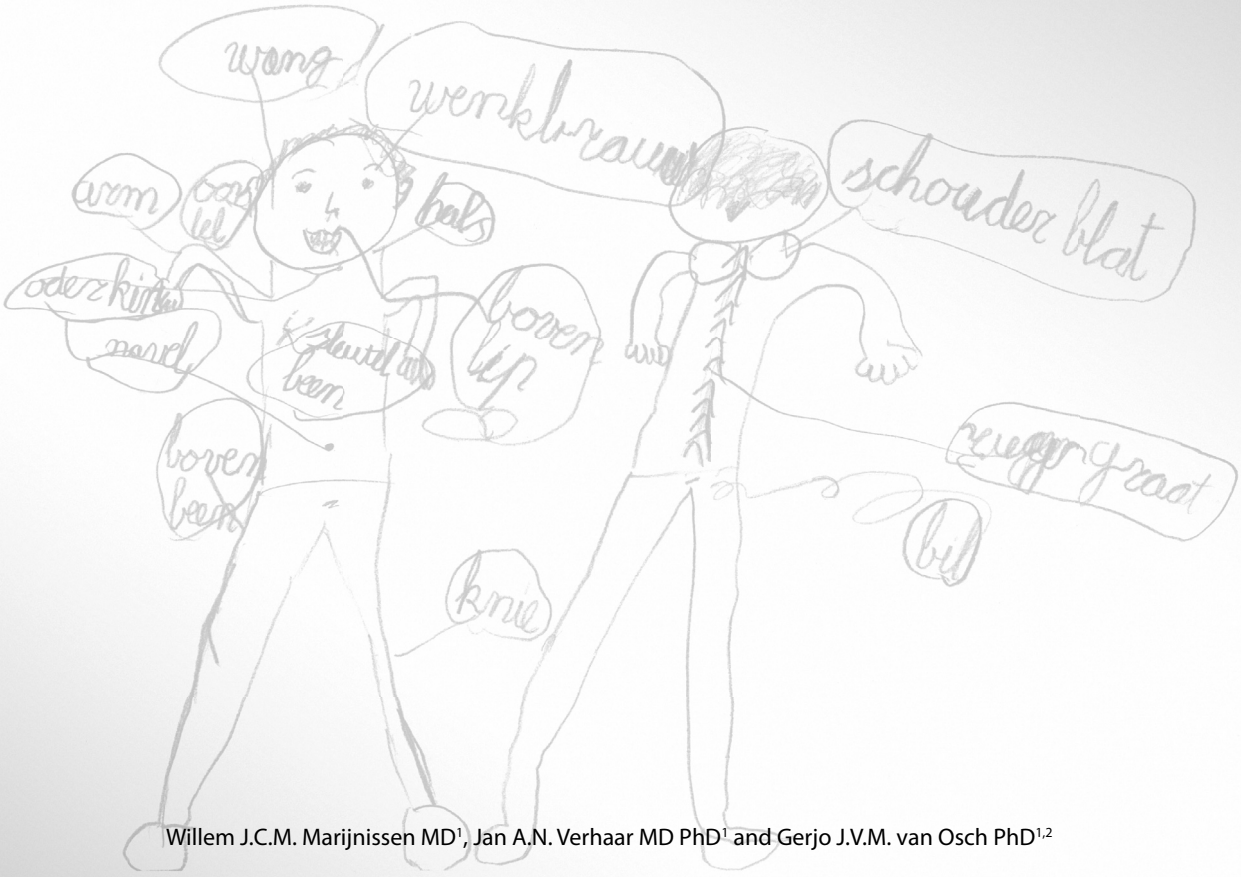
CHAPTER 7

CHONDROCYTE DIFFERENTIATION STAGE IN RELATION TO GLYCOSAMINOGLYCAN ASSEMBLY IN CELL-ASSOCIATED AND FURTHER-REMOVED MATRIX COMPARTMENT IN ALGINATE CULTURES



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ABSTRACT

Chondrocyte multiplication is needed to generate the number of cells required for cartilage tissue engineering. The multiplication process leads to chondrocyte dedifferentiation, clearly demonstrated when multiplied chondrocytes are cultured in alginate; collagen type-II expression is lost and the cells produce reduced amounts of glycosaminoglycan. In the present study we demonstrated that the amount of glycosaminoglycan deposited in the cell-associated matrix compartment is similar for differentiated and dedifferentiated chondrocytes. Thus, the decrease in the absolute amount of glycosaminoglycan synthesized by dedifferentiated cells can be attributed to a decrease in the amount deposited in the further-removed (interterritorial) matrix compartment.

We cultured multiplied chondrocytes in alginate in a defined culture medium to induce redifferentiation and evaluated whether redifferentiation was accompanied by normalized matrix assembly. The results demonstrated that the increased amount of glycosaminoglycans synthesized by chondrocytes in a defined medium was found exclusively in the further-removed matrix compartment. This led to a ratio of glycosaminoglycans in the cell-associated matrix compartment versus further-removed matrix compartment that resembles the value of differentiated cells. Based on these findings, we believe that in addition to quantitative analysis of the synthesis of specific matrix components, the organisation of extracellular matrix may provide useful information on chondrocyte phenotype and should also be taken into consideration.

INTRODUCTION

Tissue engineered cartilage has been proposed as a possible treatment for isolated articular cartilage defects[1-3]. Immunological rejection and transmission of infectious diseases can be avoided by the use of autologous cells. Tissue engineering of hyaline-like neo-cartilage requires a certain number of chondrocytes. The number required is substantially larger than the amount of chondrocytes that can be isolated from an articular cartilage biopsy. To generate the amount of chondrocytes needed, isolated chondrocytes can be multiplied in-vitro. However, multiplication induces cell dedifferentiation[4-6]. The cells obtain a more fibroblast-like appearance, produce fewer glycosaminoglycans and switch from production of the cartilage specific collagen type-II to production of collagen type-I.

In alginate gel, cells regain their original rounded shape. This is hypothesized to be prerequisite for (re)expression of the cartilage phenotype[7,8]. However, redifferentiation capacity of adult articular chondrocytes in alginate can vary widely, depending on the specific batch of fetal calf serum added to the culture medium. Yaeger et al. showed the potential of a defined serum-free medium, with addition of IGF-1 and TGF-β, to stimulate collagen type-II expression and aggrecan synthesis of dedifferentiated chondrocytes[9].

The type of collagen and the amount of glycocaminoglycans (GAG) synthesized are generally considered to be key indicators of chondrocyte phenotype. However, the organization of these components in the extracellular matrix might be equally important, because this organisation is likely to determine cell function and mechanical properties of the matrix. The extracellular matrix in articular cartilage and the extracellular matrix of chondrocytes cultured in alginate beads can be divided into two distinct compartments[10-13]. The cell-associated matrix compartment (CM) lies closest to the chondrocyte and consists of the pericellular matrix and the territorial matrix. The second compartment is called the further-removed matrix compartment (also known as the interterritorial matrix). The cell-associated matrix compartment and the further-removed matrix compartment differ in both their structure and composition. Noonan et al. demonstrated that growth plate chondrocytes from the upper proliferative zone differ from lower hypertrophic zone chondrocytes with respect to matrix assembly in cell-associated and further-removed matrix[14]. His findings indicate that changes in the distribution of matrix components between the two matrix components may reflect differences in phenotypical expression of the chondrocytes.

In the present study we studied the effect of chondrocyte dedifferentiation on matrix assembly. Moreover, we investigated whether increased glycosaminoglycan production and re-expression of collagen type-II of chondrocytes in alginate in a defined medium (as described by Yaeger et al.[9]) was accompanied by normalized matrix assembly.

MATERIALS AND METHODS

Chondrocyte isolation

Full-thickness cartilage slices were harvested, under sterile conditions from the metacarpophalangeal joints of 12-18 months old bovine steers, obtained from a local slaughter house, within eight hours after death. Cells were pooled from 4 different steers to exclude interindividual differences. The cartilage was washed with sterile physiological saline and incubated with protease XIV (2 mg/ml; Sigma, St. Louis, MO) for 2 hours, followed by an overnight incubation with collagenase B (1.5 mg/ml; Boehringer, Mannheim, Germany) in medium (DMEM/Ham's F12; Gibco, Grand Island, NY) with 10% FCS (Bio Whittaker, Verviers, Belgium). Both enzymatic digestions were done at 37° C. After incubation, the undigested cartilage fragments were removed using a 100 μm filter. The isolated chondrocytes were washed with physiological saline and counted using a hemacytometer. Cell viability was tested using the trypan blue exclusion test. These chondrocytes are referred to as P0 cells.

Culture in monolayer

Isolated chondrocytes were seeded in monolayer at a density of 2 x 10^4 cells/cm^2. The culture medium (DMEM/Ham's F12), supplemented with 10% FCS, Fungizone (0.5 μg/ml; Gibco) and Gentamycin (50 μg/ml; Gibco) was changed twice a week. When subconfluent, the flasks were trypsinized (Trypsin-EDTA; Gibco). Cells were cultured in monolayer until the third passage (P3), to induce dedifferentiation.

Culture in Alginate Beads

Freshly isolated (P0) or serially passaged cells (P3) were encapsulated in alginate beads at a density of 10 x 10^6 cells/ml as described by Guo et al.[15] with slight modifications as described by Häuselmann et al.[16]. The cells were suspended in sterile physiological saline containing 1.2% low viscosity alginate gel, then slowly passed through a 21 Gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution. After instantaneous gelation the beads were allowed to polymerize further for a period of 10 minutes in the $CaCl_2$ solution. They were thereafter washed with physiological saline and finally plated in a 24-well plate with 8 beads/well in 1 ml DMEM/Ham's F12 medium, with Fungizone (0.5 μg/ml) and Gentamycin (50 μg/ml). L-ascorbic acid (25 μg/ml) was added freshly. In the experimental, serum free (SF), condition this culture medium was supplemented with 0.1% bovine serum albumin (BSA), 10 ng/ml Insulin-like Growth Factor-1 (Boehringer Mannheim Biochemica) and 10 ng/ml Transforming Growth Factor β2 (R&D Systems Europe Ltd) as described by Yaeger et al.[9]. Control cultures were done in DMEM/Ham's F12, with 25 μg/ml L-ascorbic acid supplemented with 10% FCS. Cultures were performed at 37° C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed three times a week. After 3 weeks alginate beads were removed from culture.

Immunohistochemical Staining

Cells from an additional sample of 3-5 beads were cyto-centrifuged onto glass slides at 1000 rpm during 7 minutes. After drying at room temperature for 1 hour the specimens were frozen and stored at -80° C, until immunostaining took place. For detection of type-I and type-II collagen all slides were initially fixed in acetone for 10 minutes at room temperature and incubated with 1% hyaluronidase (Sigma) for 30 minutes at 37° C. Non-specific binding was blocked with 10% normal goat serum. Subsequently the cells were incubated for two hours with monoclonal antibodies to pro-collagen type-I (M38 1:100; DSHB, Iowa City, IA) or collagen type-II (II-II6B3 1:100; DSHB) at room temperature. The slides were incubated for 30 minutes with a 1:100 dilution of goat Fab-fragment against mouse conjugated with alkaline phosphatase (GAMAP; Immunotech, Marseille, France) in PBS-BSA (1%) supplemented with 10% FCS, followed by incubation for 30 minutes with a 1:100 dilution of mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark). The new fuchsine (Chroma, Kongen, Germany) procedure was used for color development. The slides were counterstained with hematoxylin and mounted in gelatin-glycerin. Negative control stainings were done simultaneously by omitting the first antibody. The percentage of chondrocytes staining positive for collagen type-II and pro-collagen type-I was measured using a microscopic grid. These percentages were used to calculate the ratio of collagen type-II/pro-collagen type-I producing cells for each condition, thereby assessing the chondrocyte differentiation stage.

Biochemical analysis

Beads were dissolved by adding 150 μl 55mM sodium citrate in 150 mM NaCl per bead for 10 minutes at room temperature.

Determination of the amount of DNA

Papain (Sigma, St Louis, MO) was added to the sodium-citrate/EDTA dissolved beads to a final concentration of 125 μg/ml and incubated overnight at 60°C. The amount of DNA in the beads was measured using Hoechst 33258 dye[17] and compared with calf thymus DNA (Sigma, St Louis, MO). Extinction (365nm) and emission (440nm) were measured with a fluorometer (Perkin-Elmer LS-2B).

Quantification of the amount of glycosaminoglycan

Centrifugation (10 minutes; 1000 rpm) of dissolved beads was used to separate cells and cell-associated matrix (CM) in the pellet, from the further-removed matrix (FRM) in the supernatant (as described by Häuselmann et al.[16]). Glycosaminoglycan (GAG) was extracted dissociatively using 4M GuHCl for 48 hours at 4°C. The amount of GAG was determined in CM and FRM separately using the dimethylmethylene blue assay[18] in microtiter plates. The metachromatic reaction of GAG with dimethylmethylene blue was monitored using a spectrophotometer. The

ratio A_{540}/A_{595} was used to calculate the amount of GAG in the samples. Chondroitin sulfate C (Shark; Sigma, St Louis, MO) was used as a standard. For each condition, the amount of GAG was corrected for the amount of DNA.

Data analysis

Experiments were performed 4 times. Three samples of 2 beads were used for the DNA assay and the dimethylmethylene blue assay. Statistical analysis was done using SPSS. One way ANOVA was used to test statistical differences between groups. Wilcoxon Signed Rank test was used to test the change in amount of DNA over time. Mann-Whitney U test was used to compare individual groups. $P<0.05$ was considered significant.

RESULTS

After isolation more than 95% of chondrocytes were viable. During monolayer culture chondrocytes (initially all with a rounded shape) gradually changed their form to a more fibroblastlike, spindle-shaped appearance. Monolayer cultures reached subconfluency after an average of 7 days, this time being approximately 2 days longer in the first passage. Each passage in monolayer culture resulted in a 2-3 times increase in cell number.

Immunohistochemical staining

In vitro multiplication in monolayer led to a rapid decline in the percentage of cells staining positive for collagen type-II in alginate beads. When differentiated cells (P0) were cultured in alginate in the presence of FCS for 3 weeks, 42 ± 29% (range 21-85) of the cells stained positive for collagen type-II and 17 ± 8% (range 11-26) stained positive for collagen type-I. When cultured in defined medium 74 ± 13% (range 61-92) of the cells were positive for collagen type-II and 9 ±7 % (range 1-15) for collagen type-I.

When dedifferentiated cells (P3) were cultured in alginate in the presence of 10% FCS for 3 weeks, hardly any of the cells stained positive for collagen type-II (1 ± 1%, range 0-1) and 18 ± 8% (range 8-27) for collagen type-I. If alginate cultures of dedifferentiated chondrocytes were performed in a defined medium, the percentage of cells staining positive for collagen type-II showed an increase to 40 ± 31% (range 16-84). Next to this, the number of cells staining positive for collagen type-I was noted to increase to 56 ± 2% (range 54-57). However, the ratio of collagen type-II over collagen type-I producing cells was 14 times higher when dedifferentiated chondrocytes were cultured in a defined medium compared to medium with 10% fetal calf serum.

Biochemical analysis

Amount of DNA

In the presence of FCS, freshly isolated chondrocytes showed an increase in the amount of DNA per bead during a three-week culture period in alginate (p=0.018). Dedifferentiated chondrocytes in the presence of FCS however, showed a decrease in the amount of DNA per bead (p=0.004).

In a defined culture medium the amount of DNA did not significantly change over time, neither for freshly isolated, nor for dedifferentiated chondrocytes (p=0.398 and p=0.233 respectively).

Amount of GAG

In the presence of 10% FCS dedifferentiated chondrocytes in alginate beads synthesized significantly less GAG compared to differentiated chondrocytes (p=0.008) (Figure 1). The absolute amount of GAG deposited in the CM of dedifferentiated cells did not differ from the absolute amount of GAG deposited in the CM of differentiated cells. Thus, the decrease in the absolute amount of GAG synthesized by dedifferentiated cells can be attributed to a decrease in the amount of GAG deposited in the FRM (p=0.001). This leads to a relative increase in the percentage of GAG deposited in the CM of the dedifferentiated cell (P=0.001) (Figure 2).

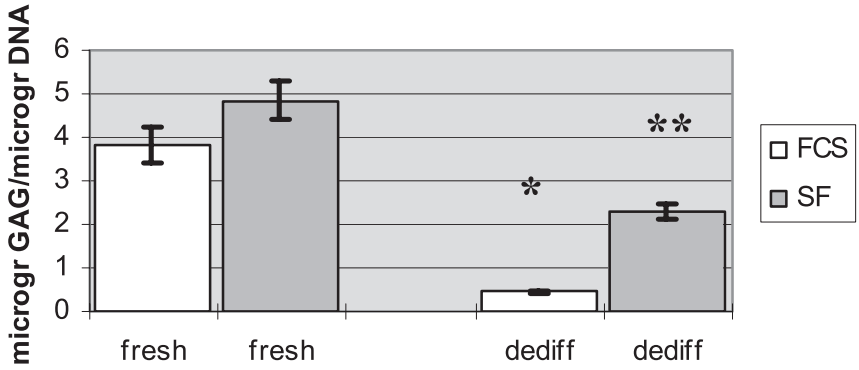

Figure 1. GAG production of freshly isolated (fresh) and serially passaged (dediff) chondrocytes was calculated by correcting for the amount of DNA. Data represent mean and SEM of 4 experiments. *=sign different from 'fresh' FCS (p=0.008), **=sign different from 'dediff' FCS (p=0.009). FCS= cultured in standard medium with 10% FCS, SF= cultured in serum free medium.

Differentiated cells showed that the total amount of GAG synthesized as well as the distribution over CM and FRM was similar in 10% FCS and SF (Figure 2).

Dedifferentiated cells cultured in defined medium synthesized an increased amount of GAG compared to the standard culture method with FCS (p=0.009) (Figure 1). This increased amount

of GAG was unequally distributed over CM and FRM. The increase was found exclusively in the FRM (p=0.006), while the amount of GAG deposited in the CM remained unchanged (p=0.14). This lead to a reduced percentage of total GAG deposited in the cell-associated matrix compartment. A ratio that resembles the value of differentiated cells (Figure 2).

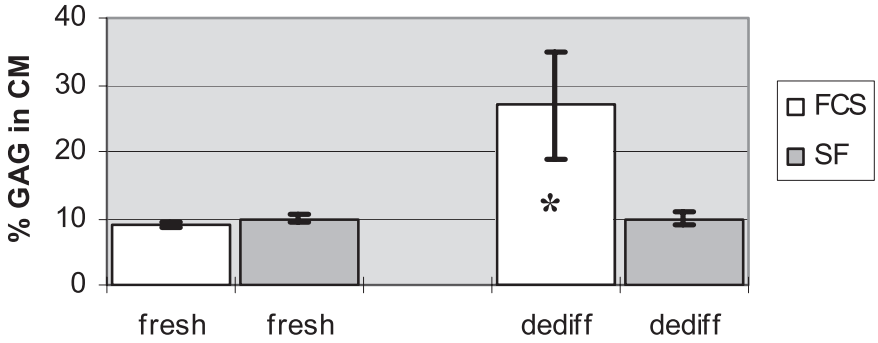

Figure 2. The percentage of GAG deposited in the CM of freshly isolated (fresh) and serially passaged (dediff) chondrocytes is calculated by division of the amount of GAG in the CM, by the total amount GAG per bead. Data represent mean and SEM of 4 experiments. *=sign different from 'fresh' FCS (p=0.001). FCS= cultured in standard medium with 10% FCS, SF= cultured in serum free medium.

DISCUSSION

Chondrocyte multiplication is needed to generate the number of cells required for tissue engineering purposes. Dedifferentiation can not be avoided during the multiplication process, therefore redifferentiation of multiplied chondrocytes is essential for successful neo-cartilage generation[19].

Yaeger et al.[9] demonstrated re-expression of the cartilage phenotype of multiplied chondrocytes in alginate cultured in a defined medium, with addition of growth factors. After 4 weeks of culture in such a defined medium, 40% of the cells produced collagen type-II.

In the present study we assessed the differentiation stage immunohistochemically by the amount of cells synthesizing collagen type-II. We demonstrated earlier that culturing multiplied bovine articular chondrocytes in aginate beads in medium with FCS was not sufficient to induce redifferentiation[20]. In the present study, using a defined medium, the percentage of cells synthesizing collagen type-II lies within the range described by Yaeger et al. for human articular chondrocytes[9]. The increased ratio of collagen type-II/pro-collagen type-I synthesizing cells indicates that our chondrocytes did redifferentiate.

Lohmander proposed that the cartilage matrix in vivo is composed of two compartments of glycosaminoglycans, turning over at different rates[10]. Mok et al. and Häuselmann et al. showed that chondrocytes cultured in alginate beads form an extracellular matrix, composed of two distinct matrix pools similar to the in vivo situation[11,12]. Different functional aspects have been suggested for the two matrix compartments. The further-removed matrix is thought to provide structural support and give cartilage its loadbearing ability[14,21]. The cell-associated matrix is hypothesized to play a regulating role in chondrocyte metabolism[21,22]. The present study shows that chondrocyte dedifferentiation in the presence of FCS leads not only to a decrease in total amount of GAG synthesized by the cell, but to a change in distribution of GAG over the different matrix components as well. The decrease in GAG-synthesis per cell is found mainly in the FRM, whereas the CM remains less affected. We hypothesize that the chondrocyte preferably rebuilds the cell-associated matrix, initially paying less attention to the further-removed matrix. All this in an effort to optimize cell function, being, at least in part, determined by the cell-associated matrix[22].

Compared to the 'standard' culture condition (with 10% FCS) the defined culture medium only had a minor effect on differentiated chondrocytes. However it did have large effects on dedifferentiated chondrocytes. It enhanced the total amount of GAG synthesized, and restored the assembly of glycosaminoglycans in the cell-associated and further-removed matrix to a value that resembles the values measured in cultures of differentiated chondrocytes.

Our findings indicate that next to quantitative analysis of synthesis of specific matrix components, the organization of glycosaminoglycans in the extracellular matrix can provide additional information on chondrocyte phenotype. Proper assembly of matrix components is important in tissue engineering of cartilage, for it determines cartilage function and its response to mechanical and chemical stimuli.

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CHAPTER 8

MONOCLONAL ANTIBODY 11-FIBRAU: A USEFUL MARKER TO CHARACTERIZE CHONDROCYTE DIFFERENTIATION STAGE

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ABSTRACT

The aim of this study was to determine the feasibility of discriminating between differentiated and dedifferentiated chondrocytes by using the Mab 11-fibrau. Mab 11-fibrau did not bind to differentiated chondrocytes in cartilage of human knee joint, auricle, or nasal septum. During monolayer culture, when cells dedifferentiate, the number of 11-fibrau positive cells gradually increased and reached up to 100% after 4 passages. When differentiated chondrocytes were cultured in alginate, most (90–95%) of the cells remained 11-fibrau negative, in accordance with previous studies demonstrating that differentiated chondrocytes cultured in alginate keep their phenotype. Dedifferentiated (11-fibrau positive) cells were subjected to different redifferentiation regimes. As a well-known fact, cultures in alginate in medium where FCS was replaced by IGF1 and TGFß2 results in increased collagen type-II formation, indicative for redifferentiation. However, these cells remained 11-fibrau positive, suggesting they are not (yet) fully redifferentiated. On the other hand, when dedifferentiated cells (after 4 passages in monolayer culture) were seeded in a biomaterial and implanted subcutaneously in a nude mouse, the newly formed cartilage matrix contained collagen type-II and the 11-fibrau staining on the cells had disappeared. Our results indicate that 11-fibrau may be a reliable and sensitive marker of chondrocyte phenotype.

INTRODUCTION

A functionally well described cell membrane marker for chondrocyte differentiation does not exist. This is in contrast to cells of the immune system, where CD-markers (Cluster of Differentiation antigens) are of common use. Determination of chondrocyte phenotype is important in cartilage tissue engineering where the goal is to produce cartilage matrix of proper quality using isolated cells. Usually the isolated cells are multiplied in vitro to obtain the high number of cells required. This multiplication inevitably leads to loss of phenotype of the cells, in a process called dedifferentiation. Much research nowadays aims at redifferentiation of these multiplied cells[1-4], because only cells with differentiated phenotype can be expected to produce cartilage matrix of proper quality. Therefore, fast and sensitive characterization of cellular phenotype is required.

The phenotype of chondrogenic cells is usually demonstrated by the type of matrix components that the cells produce, either at protein or at mRNA level. Expression of aggrecan and collagen type-II, typical for differentiated chondrocytes, reduces during the process of dedifferentiation, and cells start to express collagen type-I and III[5-7]. An important disadvantage of analyses at the mRNA level is that expression of RNA does not prove that the functional protein is synthesized. Moreover, although collagen type-I is not synthesized by differentiated chondrocytes, they appear to express mRNA for collagen type-I[8]. Measurements at the protein level using biochemistry or immunohistochemistry have the disadvantage that it provides an overall result of synthesis and breakdown of matrix components from the past. An antibody that recognizes a cell surface marker would enable us to directly assess cellular phenotype. The possibility to discriminate between differentiated and dedifferentiated cells would be of great help finding the optimal growth conditions for tissue engineering. Furthermore, isolation of the cells with the right phenotype before implantation could be established with such cell marker if we apply this antibody to the cell sorter (FACS) or bind it to magnetic beads.

In this study we choose the monoclonal antibody 11-fibrau (clone D7-fib) as possible negative marker for chondrocyte differentiation. The antibody is described to recognize a 112-kD molecule expressed on the cell surface of human fibroblasts. The antigen was not expressed by peripheral blood cells and only by a minority of melanoma cell lines (datasheet Imgen). Other cell types were not tested, as far as we know. The aim of this study was to determine the value of 11-fibrau to discriminate between differentiated and dedifferentiated chondrocytes under different experimental conditions.

MATERIALS AND METHODS

Description of Mab 11-fibrau

The monoclonal antibody 11-fibrau was produced by Imgen (clone number D7-fib, distributed by ITK Diagnostics BV, The Netherlands). It recognizes a 112-kD molecule expressed on the cell surface of human fibroblasts (the immunogen was human foreskin). The antigen is not expressed by peripheral blood cells and is found at low levels on a minority of melanoma cell lines. Studies up to now have shown that the antibody is sensitive to SDS but not to trypsin, tunicamycin and collagenase. The epitope is resistant to fixation with 1% buffered formaldehyde (datasheet Imgen).

Tissue Sections and Cytospin Preparations

A biopsy of normal human skin tissue was obtained from the arm of a healthy volunteer. Human cartilage was obtained from the operation theatre of the departments of Otorhinolaryngology, Plastic and Reconstructive surgery and Orthopaedic surgery. In addition, normal healthy articular cartilage from the Dutch Bone Bank, from road-accident victims, was used. Rabbit ear cartilage was obtained from New Zealand White rabbits (age 6–12 weeks) used for other experiments. Bovine articular joint cartilage was obtained from MCP joints from a local slaughterhouse.

Cryosections (5 µm) were made from this material and stored at -80°C until further use. For Cytospin preparations alginate beads, dissolved in sodium-citrate buffer, and cells after trypsin treatment of the monolayer were spinned down on slides, coated with poly-llysine. Each spot contained about 50,000 cells. Slides were stored at -80°C until further use.

Chondrocyte Cultures

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

Monolayer culture

Cells were seeded at an initial density of 4 x 10^4 cells/cm^2 in DMEM (Life Technologies) with the addition of 10% FCS, 50 µg/ml gentamycin and 0.5 µg/ml fungizone. When cells reached confluency they were trypsinized with trypsin–EDTA (Life Technologies). Monolayer cultures were performed 7 times with ear chondrocytes and 6 times with articular chondrocytes.

After four passages in monolayer, the cells were seeded in alginate and used for the redifferentiation experiments, in vivo or in vitro.

Alginate cultures

Cells were solved in 1.2% alginate (LV, Keltone) at a concentration of 4 x 10^6 cells/ml and slowly passed through a 23 Gauge needle in a drop wise fashion into a 102 mM $CaCl_2$ solution. After instantaneous gelation the beads were allowed to polymerize further for a period of 10 min. They were thereafter washed with saline and plated in a 24-well plate and cultured in DMEM/Ham's F12 medium with 10% FCS and 25 µl L-ascorbic acid. The medium was changed three times a week. Alginate cultures with freshly isolated differentiated chondrocytes were performed 2 times with ear chondrocytes and 2 times with articular joint chondrocytes.

Redifferentiation Experiments

After multiplication in monolayer culture, cells have lost their cartilage phenotype and are called "dedifferentiated." In vitro and in vivo experiments were done in order to "redifferentiate" the cells, meaning the cells regain their cartilage phenotype and become "differentiated" chondrocytes again.

In vitro.

Redifferentiation was stimulated by culturing the cells in alginate beads. Beads were cultured in a 24-well plate, 5–10 beads/well, in DMEM/Ham's F12 with or without 10% FCS and refreshed three times a week. Medium without FCS was supplemented with 10 ng/ml rhTGFβ2 (R&D systems, Oxon UK), 10 ng/ml rhIGF1 (Boehringer Mannheim, Germany) ITS^+ premix and 0.1 µg/ml Hydrocortisone (Becton Dickinson, Bedford, MA), as described previously[1,3,4]. In both conditions L-ascorbic acid (25 µg/ml) was added.

This experiment was performed 6 times with ear chondrocytes and 5 times with articular joint chondrocytes.

In vivo.

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." Animals were housed under sterile conditions at the Center for Animal Research. One male athymic mouse (NMRI), aged 12 weeks, was used for the in vivo experiment.

After 4 passages in monolayer, the cells were suspended in 1.2% alginate, at a concentration of 50 x 10^6 cells/ml, and seeded in the biodegradable carrier E210 (Ethicon, Norderstedt, Germany; 5x5x2 mm), as described previously[9]. Two grafts were placed in subcutaneous dorsal pockets. This experiment was performed with ear cartilage of one human donor (9 years old). These cells were simultaneously cultured in vitro in alginate for 3 weeks, according to our standard procedures (described previously). After eight weeks the animal was killed by cervical dislocation and the transplants were harvested. Each graft was divided in two pieces, one-half was preserved for cryosection, and one-half was processed for histology in paraffin.

Immunohistochemistry

Cryosections and cytospins were fixed in acetone for 10 min. After incubation with the blocking buffer (Boehringer, with 10% normal goat serum), for 30 min the specific antibody 11-fibrau (Imgen; 21 µg/ml PBS with 2% normal goat serum) for 2 h (RT) was added. Fab-fragments against mouse conjugated with alkaline phosphatase (Immunotech, Marseille, France; 1:100) were added and amplified with mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP: Dakopatts, Copenhagen, Denmark; 1:100). Visualization was performed by incubation with New Fuchsin substrate (Chroma, Kongen, Germany), resulting in a red signal. Sections were counterstained with Gill's haematoxylin and embedded in gelatine-glycerine. Negative controls were performed by omitting the primary antibody.

For collagen type-I and type-II staining the same procedure was followed but cells were treated with 1% hyaluronidase (Sigma, St. Louis, MO) before the blocking agent was added. The primary antibody for collagen type-II; II-II6B3 (DSHB) or for procollagen type-I; M38 (DSHB) was used in a concentration between 0.5–1.0 µg/ml.

For collagen type-II staining of the nude mouse experiments, paraffin-embedded sections were used. These were first deparaffinated followed by treatment with pronase (1%) and hyaluronidase. Further staining was performed first coupling primary and secondary antibodies as described previously[9].

Results were counted as the percentage of cells staining positive. Three times hundred cells were counted at a magnification of 200x.

RESULTS

Tissue Sections

In cryosections of the skin, the fibroblasts of the dermis stained 11-fibrau positive, the epidermis negative. Cryosections of cartilage of the external ear, the nasal septum and the knee joint always appeared 11-fibrau negative. Perichondrium present in sections of the ear and nasal septum contained 11-fibrau positive cells (Figure 1).

Isolated Cells in Culture

Isolated chondrocytes were cultured in monolayer culture for expansion. The number of 11-fibrau positive cells increased with time in monolayer culture. Nearly all cells (of all the different cartilage types tested) stained positive for 11-fibrau after 4 passages (Figure 2). The gradual increase in 11-fibrau positive cells after each passage in monolayer culture is shown for a typical sample of chondrocytes from the external ear in Figure 3. When these chondrocytes were cultured in alginate, immediately after isolation from the cartilage, after 1 week 5–10% of the cells were 11-fibrau positive. This percentage did not change over the total culture period of 3 weeks (Figure 3). The same was found with chondrocytes of the human knee joint (data not shown).

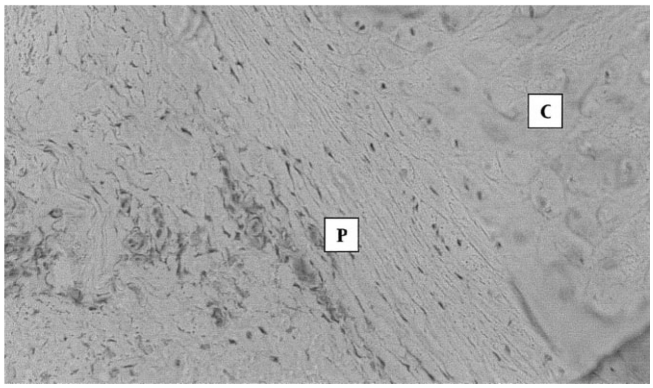
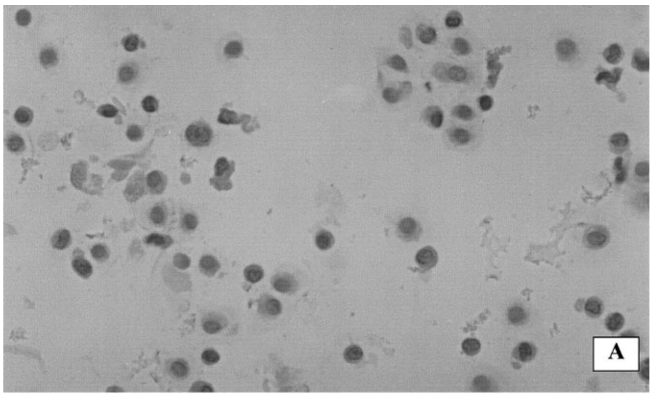


Figure 1. Cryosection of human ear cartilage with surrounding perichondrium stained with Mab 11-fibrau. The chondrocytes stain negative. Positive staining appeared in the fibrous perichondrium cells.



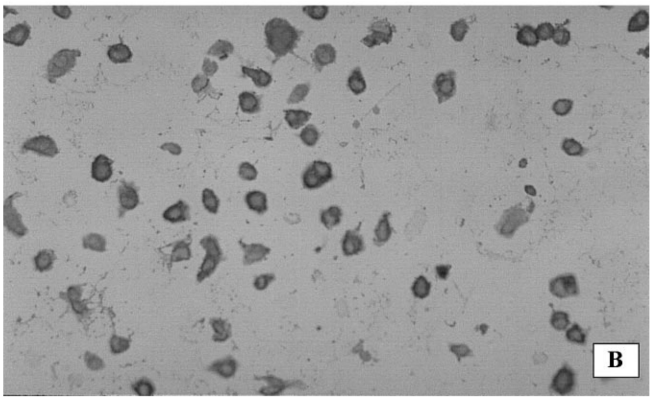


Figure 2. Cytospin preparations of isolated ear chondrocytes stained with Mab 11-fibrau directly after isolation from the cartilage (A) and after 4 passages in monolayer culture (B).

When cells after 1 passage in monolayer culture (about 30% of the cells were 11-fibrau positive) were cultured in alginate for 3 weeks, the amount of 11fibrau positive cells was unaltered during culture.

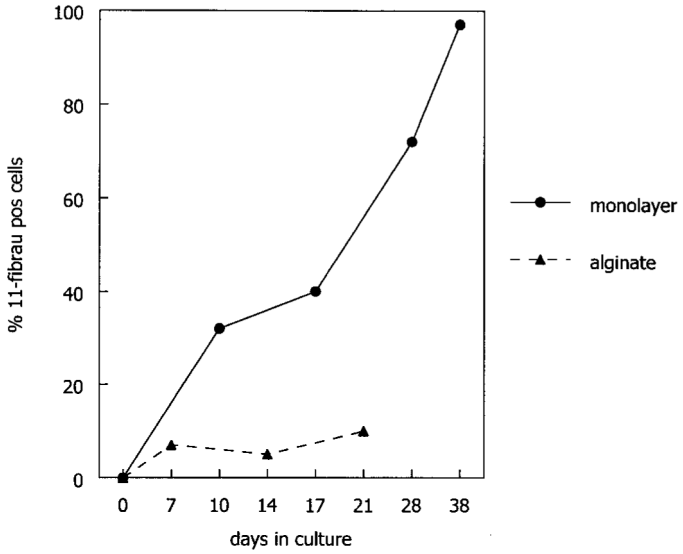


Figure 3. The amount of 11-fibrau positive cells in time in a typical example of ear chondrocytes cultured in monolayer up to 4 passages (11-fibrau is tested after each passage) and differentiated chondrocytes in alginate cultured in alginate for 3 weeks (11-fibrau is tested each week).

Redifferentiation Studies

After multiplication in monolayer culture for 4 passages, the cells were completely dedifferentiated and close to 100% stained 11-fibrau positive. Subsequently they were cultured in alginate in medium with 10% serum and in medium where serum was replaced by IGF1 and TGFβ. The replacement of serum by IGF1 and TGFβ increased the number of collagen type-II positive cells. However, it did not have a significant effect on the number of 11-fibrau positive cells (Table 1).

Dedifferentiated ear chondrocytes from one of the donors were, next to in vitro culture in alginate, seeded in E210 and implanted subcutaneously in nude mice. After 8 weeks cartilage tissue had been formed in the graft. The graft stained positive for collagen type-II and the expression of 11-fibrau had disappeared (Figure 4).

Table 1. Percentage of ear chondrocytes (mean ± SD; typical sample of the cells also used for the in vivo experiment) staining positive for 11-fibrau, collagen type-II, and collagen type-I when cultured in alginate for 3 weeks after being multiplied for 4 passages in monolayer

Culture condition	11-Fibrau	collagen type-II	collagen type-I
10% FCS	91 ± 1	0 ± 0	10 ± 3
'Serum free'	88 ± 2	70 ± 6	23 ± 4

Note. During alginate culture two different types of medium were used: medium with 10% FCS and 'serum free' where FCS was replaced by ITS+, cortisol, 10 ng/ml IGF1 and 10 ng/ml TGFβ2.

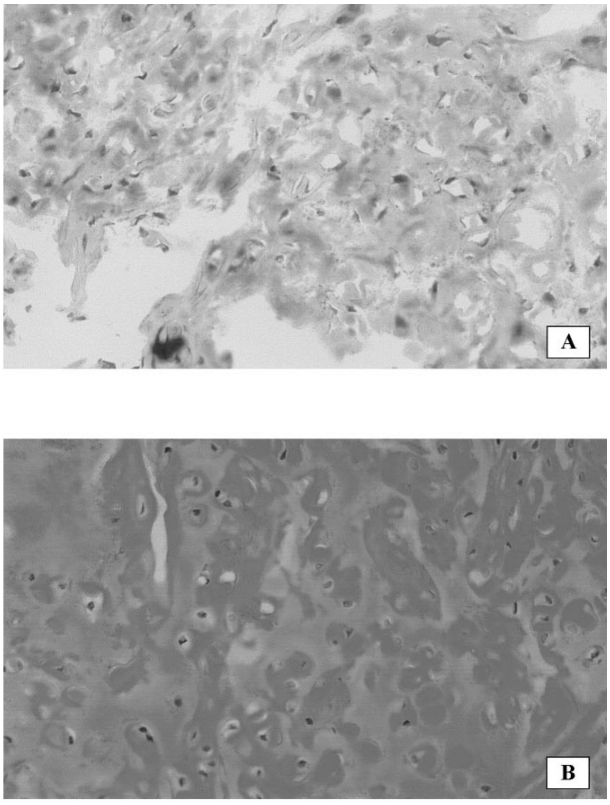


Figure 4. Cartilage graft of multiplied ear chondrocytes solved in alginate and seeded in E210 8 weeks after subcutaneous implantation in nude mouse stained with Mab against collagen type-II (A) and Mab 11-fibrau (B).

Species Cross-Reactivity

Dedifferentiated chondrocytes of rabbit ear and bovine articular joint stained negative for 11-fibrau, suggesting no cross-reactivity with rabbit and bovine.

DISCUSSION

Our results demonstrate the possibility to discriminate between differentiated and dedifferentiated chondrocytes using the monoclonal antibody 11-fibrau.

The most commonly used marker for chondrocyte dedifferentiation is collagen type-I expression. However, this has several drawbacks. Firstly, collagen type-I mRNA expression is not very specific, because differentiated cells are also described to express collagen type-I mRNA. Secondly, protein detection is not a good indication of the differentiation stage of the cells at that very moment, but rather shows what was produced in the past. Finally, using isolated cells for example for cytospins or FACS analysis, the problem using collagen type-I as a marker is that it can be produced but not necessarily stays attached to the cell. If secreted in the medium, the cell can not be detected anymore.

The present study demonstrates that dedifferentiated chondrocytes express a 112-kD protein on their surface, like fibroblasts do. The function of this protein is currently not known. The protein appears when chondrocytes are cultured in monolayer. The number of cells positive for 11-fibrau increases with time in monolayer culture and after 4 passages in culture almost all cells were positive. The antibody 11-fibrau bound to dedifferentiated human chondrocytes of all types of cartilage (ear, nose and joint) investigated.

Assessment of chondrocyte phenotype is mainly performed by analysis of the matrix components produced. However, unique (unidentified) differentiation antigens have been demonstrated on the surface of human chondrocytes, which appear to be able to induce immunologic responses in rheumatologic diseases[10]. Specific monoclonal antibodies against human chondrocytes have been described for immunohistological analysis of different cartilage tissues and cultured chondrocytes[11]. These antibodies showed a positive reaction in the cytoplasm. The advantage of 11-fibrau is that it recognizes an epitope at the cell membrane. This makes 11-fibrau suitable to use as cell sorter. To select cells to be used for tissue engineering applications a negative marker for chondrocyte phenotype (e.g. 11-fibrau) as a tool to discard the nonchondrocytic cells is preferred. Moreover, 11-fibrau is commercially available.

Culture of chondrocytes in alginate has been reported to preserve cellular phenotype. This was assessed by protein analysis for collagen types and aggrecan[12]. The present study demonstrates that the majority of the chondrocytes cultured in alginate stay negative for 11-fibrau. In agreement with previous studies, a small percentage of the cells cultured in alginate become fibroblast like[12]. These cells have a spindle-like appearance and produce collagen type-I. This strongly suggests that the cells with fibroblast morphology expressing collagen type-I at the margins of the alginate beads are the cells that become 11-fibrau positive.

To our surprise multiplied cells that re-express collagen type-II after culturing in defined medium stayed 11-fibrau positive. This indicates that the cells have not fully redifferentiated; they may produce collagen type-II but at the same time still express the fibroblast-like protein on their cell membrane. This is confirmed by the observation that after subcutaneous

implantation of the cells in nude mice the expression of 11-fibrau was absent, indicating the cells did only fully redifferentiate after in vivo implantation. This study indicates that 11-fibrau is a very useful marker of cellular differentiation.

Apart from the importance of cellular phenotype for tissue engineering, cellular phenotype also has implications for the response of cells to external stimuli like growth factors[13-15] and mechanical loading[16,17]. Furthermore, evidence exists for alterations on chondrocyte phenotype in cartilage pathology[7,18]. Taken together we state that it is very important to have a sensitive and reliable tool to determine chondrocyte phenotype. The present study shows that 11-fibrau can be used as cell membrane marker to characterize chondrocyte differentiation stage.

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CHAPTER 9

SUMMARY

Chapter 1 outlines the normal structure and composition of articular cartilage and the inefficient spontaneous healing response after focal damage. Current surgical treatment options are briefly discussed and tissue engineering techniques for the repair of articular cartilage defects are addressed in more detail. The aims of the work presented in this thesis are outlined in ***Chapter 2***: namely to investigate the influence of different biomaterials, cell types and culture methods on the tissue engineering process, as well as characteristics of the tissue engineered cartilage.

In ***Chapter 3*** we evaluated a composite graft technique in which a biomaterial was colonized directly by chondrocyte precursor cells to generate tissue engineered cartilage.

The first part of ***Chapter 3*** is based on the hypothesis that biomaterials with a composition that was better defined than demineralized bovine bone matrix (DBM) would yield neo-cartilage with improved histological and structural characteristics. We compared the biological biomaterial used in the original experiments, i.e. DBM, with two other biomaterials well defined with respect to pore size and chemical composition. Like DBM, one of these biomaterials was a protein-based material, collagen type-I fleece, whereas the other was a carbohydrate-based polymer, polylactic/polyglycolic acid fleece (E210).

The amount of neo-cartilage generated was similar for all three biomaterials. DBM was completely resorbed after 6 weeks in the ear, whereas resorption of neither collagen fleece nor E210 was completed after 6 weeks. Remnants of the material accompanied by many inflammatory cells were still present in the graft. This high level of inflammation was assumed to be a disadvantage in an intra-articular environment, because inflammatory cells can cause damage to the joint. For this reason in our view DBM was the material of choice to generate a graft for implantation in the joint.

The second part of the composite graft study was designed to evaluate whether neo-cartilage generated by the composite graft technique retained its cartilage-specific characteristics after transplantation into an articular cartilage defect. The grafts demonstrated good integration with the bony part of the defects and histologically retained their cartilaginous characteristics.

In ***Chapter 4*** isolated chondrocytes suspended in alginate were seeded in DBM or E210 and implanted in subcutaneous pockets in a-thymic mice. The effect on the expression of cartilage-specific characteristics was evaluated. After multiplication in monolayer culture, adult articular chondrocytes were used in two different cell seeding densities.

The data indicated that seeding cells at high density (50 million cells/ml) leads to large amounts of cartilage in both biomaterials. The use of E210 enhanced collagen type-II expression of serially passaged chondrocytes. Furthermore pro-collagen type-I was lacking in the centre of these grafts. The data clearly show re-expression of the cartilage phenotype. In contrast to the study in ***Chapter 3*** in which biomaterials were colonized directly by cells from

the perichondrium, in ***Chapter 4*** large areas of remnants of DBM were present, whereas E210 had been resorbed almost completely, leading to a more homogenous neo-cartilage.

In the former experiment E210 seeded with cultured chondrocytes in high density in alginate appeared to be the optimal condition. However, because this combination of fleece and alginate had not been described previously, the study in ***Chapter 5*** evaluated the influence of alginate on the tissue engineering process was evaluated. For practical reasons freshly isolated adult articular chondrocytes were used. The study demonstrated that the use of alginate in combination with E210 did not negatively influence the amount of cartilage matrix proteins per tissue wet weight. In fact, it provided retention of the graft shape. Conservation of a pre-shaped form is of great importance for clinical use.

Thus far, seeding of isolated chondrocytes in alginate appeared to be a good method for cartilage tissue engineering. It was also demonstrated that for successful tissue engineering a high number of cells is needed. Therefore, in ***Chapter 6*** we investigated whether the number of cells obtained after monolayer culture (our standard method for chondrocyte multiplication) could be optimized by adding growth-factors to the culture medium. TGFß-2 was added in the first two passages and L-ascorbic acid was added in the subsequent two passages. After four passages this led to a significant increase in the number of generated cells compared to the control group. However, when these multiplied chondrocytes were cultured in alginate beads in standard culture medium (with fetal calf serum) they did not redifferentiate in-vitro.

It was earlier demonstrated that re-expression of the cartilage phenotype occurs when chondrocytes (dedifferentiated after multiplication in monolayer) were cultured in a defined medium, with addition of growth factors. After 4 weeks of culture in such a defined medium, 40% of the cells produced collagen type-II. In ***Chapter 7*** we investigated whether that method could be reproduced in our laboratory and in what way defined medium affected the assembly of extracellular matrix components. We demonstrated an increased ratio of collagen type-II/ pro-collagen type-I synthesizing multiplied chondrocytes in defined medium, indicating that these cells did redifferentiate. The increase in the amount of glycosaminoglycan produced by redifferentiated cells was unequally distributed over the cell-associated and the further-removed matrix compartment. The decrease in the absolute amount of glycosaminoglycan synthesized by dedifferentiated chondrocytes was found to be due to a decrease in the amount deposited in the further-removed (interterritorial) matrix compartment. This means that redifferentiation led to recovery of the normal, differentiated ratio of glycosaminoglycan in the cell-associated matrix compartment versus the further-removed matrix compartment.

In ***Chapter 8*** we studied a new marker for chondrocyte dedifferentiation. In vitro redifferentiation using serum-free culturing was compared with in vivo redifferentiation in nude mice. It was

demonstrated that dedifferentiated chondrocytes express the protein 11-fibrau on their surface, which can also be found on fibroblasts. We found that multiplied cells that re-express collagen type-II after culturing in defined medium stayed 11-fibrau positive. This indicates that the cells did not completely redifferentiate. After subcutaneous implantation of the cells in nude mice the expression of 11-fibrau was absent, indicating that the cells only fully redifferentiated after in-vivo implantation.

CHAPTER 10

DISCUSSION

The natural course of articular cartilage lesions is known to be troublesome[1]. In general articular cartilage lesions do not heal, or heal only partially under certain biological conditions. Articular cartilage lesions commonly affect active adolescents and adults and are frequently associated with disability and invalidating symptoms such as joint pain, locking phenomena and reduced or disturbed function. Moreover, such lesions may progress to arthrosis[2-4]. In recent decades many attempts, both experimental and clinical, have been made to induce the healing of articular cartilage lesions. These repair strategies have mainly aimed at generating a durable repair tissue that structurally and functionally closely resembles native cartilage.

The transfer of osteochondral plugs from a non weightbearing area of the joint to an articular cartilage defect was put forward by Bobic[5,6] and Hangody[7,8]. Using this technique donor-site morbidity and secondary degenerative changes remain an important matter of concern. Restoration of the exact surface level is generally extremely difficult and although bony integration is achieved, no integration is found at the cartilage level. Surprisingly, however, good to excellent clinical results are reported in a substantial number of patients[9].

Tissue engineering[10], using cells, biomaterials and signalling substances to generate cartilage for the repair of cartilage defects, seems to be a promising method to address some of the shortcomings of the techniques mentioned above[11,12].

One of the earliest strategies techniques was that of Pridie, who proposed drilling of the subchondral plate as a treatment for focal articular cartilage lesions[13,14]. Both Pridie drilling and microfracturing[15,16], aim to stimulate a spontaneous repair reaction. These interventions are relatively cheap, can be performed by arthroscopy and can easily be repeated. Good results, improved joint function and relief from pain are reported in over 75% of cases. However, since the defects are resurfaced with fibrocartilaginous tissue, deteriorating long-term effects can be expected.

Other authors, in a number of experimental and clinical studies, advocated the transplantation of perichondrial flaps[17-21] or periosteal flaps[22-25] to the floor of an isolated cartilage defect. Although incomplete filling of the defect and calcification of the grafts in the long-term are regarded to be important drawbacks of these techniques[18], the clinical outcome is reported to be good[18,26,27].

Autologous chondrocyte transplantation was proposed by Grande et al.[28] and introduced to the clinic by Brittberg et al.[29]. The technique has quickly gained wide acceptance and good to excellent clinical results are reported even at 9-10 years follow-up[30,31]. However several questions remain unanswered. Do the transplanted cells redifferentiate? Is the number of cells large enough? Can detachment of the periosteal flap be prevented? Moreover, the method failed to yield better results in a randomized trial[32]. In addition, the very high costs compared to drilling or microfracturing may not be justified.

The work presented in this thesis focuses on improving the experimental aspects of tissue engineering for the repair of articular cartilage defects. We hypothesize that treatment of a

cartilage defect with a solid piece of tissue engineered neo-cartilage (with chondrocytes embedded in their extracellular matrix) offers the best chance for success. Optimal results are probably achieved when the neo-cartilage closely resembles native articular cartilage. We investigated the influence of different biomaterials, cell types and culture methods on the tissue engineering process, as well as the characteristics of the neo-cartilage.

Chapter 3 of this thesis elaborated on a method for cartilage tissue engineering that was used in earlier studies by our research group. In this technique demineralized bovine bone matrix (DBM) is enveloped in perichondrium and left in situ in the ear. Chondrocyte precursor cells from the cambium layer colonize the biomaterial and generate a solid piece of young developing cartilage. In earlier experiments young cartilage showed a good capacity to integrate with the native cartilage surrounding a defect[33]. This is an advantage of the current technique since insufficient integration is considered one of the most important reasons for failure of cartilage transplantation[34]. Moreover, young cartilage can mature 'on site' and adapt to local conditions. The technique proved to be capable to repair defects in the nasal septum, ear and cricoid of rabbits[35-38] and children[39], but had never been tested in an intra-articular environment.

Although DBM demonstrated chondrogenic potential[40,41] it probably acts merely as a spacer[42]. The drawback of DBM is the significant variation in pore size and chemical composition, and the possible risk for transmission of infectious agents. The study in ***Chapter 3*** was designed to optimize the 'composite graft' technique and to study the young developing cartilage thus generated in an intra-articular environment.

The first part of ***Chapter 3*** was based on the hypothesis that biomaterials with a composition better defined than DBM would yield neo-cartilage with improved histological and structural characteristics. However, we could not prove that any one of the biomaterials was superior the other. The high level of inflammation in the two better defined biomaterials led us to revert to DBM as the material of choice to generate a graft for implantation in the joint. Using DBM the grafts demonstrated good integration with the bony part of the defects while also retaining their cartilaginous characteristics. Although the composite graft technique proved to be a reliable method to generate neo-cartilage, in its current form the technique can not be used to repair articular cartilage defects. The orientation of the different tissue layers in the graft closely fits a logical position described by Hunziker[34]. Perichondrium with the cambium layer down covers the defect, cells from the perichondrium have grown into a porous matrix, and perichondrium on the defect floor acts as a structural barrier to impede angiogenic activities[43,44]. Nevertheless the composite graft technique offered certain advantages and we envisioned that changes could be made to the original technique to make it applicable to larger animals, possibly even to humans. For example, removal of the upper perichondrium layer prior to implantation[45] would bring the neo-cartilage to the level of the native articular cartilage. Despite these theoretical advantages, it seemed that some important problems (e.g., donor-site morbidity and maximum thickness of the biomaterial to obtain a homogenous cartilage graft) could probably not be

addressed. Therefore we decided to focus on isolated chondrocytes seeded in biomaterials to generate neo-cartilage.

The use of isolated cells involves different biomaterial requirements. The material is not colonized by cells growing in and therefore should allow a homogenous distribution of the isolated cells. We hypothesized that the use of alginate to seed chondrocytes would facilitate a homogenous distribution of identically-shaped chondrocytes in the relatively wide pores of the DBM. In addition, we assumed that polymerisation of the alginate would prevent the chondrocytes from floating out of the graft.

In ***Chapter 4*** we compared a natural protein-based matrix (DBM) with a carbohydrate-based carrier (E210). In contrast to the findings of ***Chapter 3*** where cells colonize the biomaterial from the outside, ***Chapter 4*** showed that when isolated chondrocytes are used, E210 performs better than DBM. Large areas with remnants of DBM were present in the centre of the grafts. Macrophages, responsible for degradation of the DBM[38], were apparently unable to reach the centre of the grafts. Because degradation of E210 is by hydrolysis[46-49], the biomaterial had to great extent been resorbed leading to a homogenous tissue. Alginate used in combination with DBM and E210 can be hydrolyzed into mannuronic acid and guluronic acid[50]. These two components can then be incorporated in enzymatic pathways for further degradation[51,52]. We did not observe any inflammatory reactions against alginate, thus confirming the data from previous studies[50,51].

Chapter 4 demonstrated that serially passaged adult articular chondrocytes can be used to generate neo-cartilage. Seeding multiplied cells at high density (50 million cells/ml), mimicking the situation in the developing embryo, and the use of E210 as a carrier enhanced the expression of collagen type-II. Furthermore pro-collagen type-I was absent in the centre of grafts composed of multiplied chondrocytes. This indicates that the type of cell carrier used can influence structural characteristics and phenotypic expression of the newly generated tissue. It has to be noted, however, that because the environmental conditions in the joint are very different from a subcutaneous pocket, conclusions based on this study may not be directly extrapolated to application in the joint. On the other hand, subcutaneous implantation of the graft prior to intra-articular use could prove to be a clinical application of this technique.

Carbohydrate-based matrices (combinations of polylactic and polyglycolic acid) are frequently used as cell carrier[53-56], but in the latter experiments isolated chondrocytes were seeded directly into the biomaterial. In ***Chapter 4***, we obtained promising results using E210 combined with serially passaged chondrocytes in alginate gel[57]. Although alginate is frequently applied as a cell-carrying gel, both in vitro and in vivo[33,51,57-61], the combination of fleece and alginate has never been described before. Therefore in ***Chapter 5*** we investigated the effect of alginate as a chondrocyte-delivery substance on macroscopic, histological and chemical characteristics of tissue engineered cartilage[62]. E210 was used as a scaffold and implanted in athymic mice,

with and without alginate, and with and without differentiated bovine articular chondrocytes. In this study, macroscopically the constructs with chondrocytes were all firm. However, the constructs with chondrocytes but without alginate were warped and appeared less elastic than the constructs with alginate. Both types of constructs with chondrocytes showed no difference in the amount of glycosaminoglycans, or collagen per wet weight, and no differences were found in calcium deposition. The reason for the deforming force of the constructs without alginate might be the zones of high cell density at the margin of the constructs, forming a tight layer around the graft. Considering the biochemical analysis, the glycosaminoglycan concentration was close to the value of natural calf cartilage (70 g/mg w.w.)[63]. The collagen type-II concentration, however, was much lower than that of natural calf cartilage (97 g/mg w.w.)[63]. This can be explained by the slow turnover of collagen (the turnover in human articular cartilage is calculated to be over 100 years[64]) and requires more attention in future studies. Extra stimulation of collagen synthesis, for example by culturing the grafts in a bioreactor[65,66] prior to implantation using growth factors and/or mechanical stimulation, might be necessary. In ***Chapter 4*** the redifferentiation capacity of dedifferentiated chondrocytes in alginate was enhanced by E210 compared to DBM. This led to the hypothesis that E210 was more important in inducing redifferentiation than alginate. However, the study in ***Chapter 5*** showed cells with chondrogenic characteristics in constructs of E210 and alginate without seeded chondrocytes; these cells probably originated from the subcutaneous connective tissue. After arrangement in a three-dimensional culture, dermal fibroblasts were described to have chondrogenic capacity [67]. It is important to note that the amount of neo-cartilage thus formed is small, highly variable and unpredictable, in contrast to the amount formed in constructs with E210, alginate and chondrocytes. This chondro-inductive capacity might be an extra advantage of E210 in combination with alginate. In this study, only in the control bare fleeces was blood vessel ingrowth through the capsule covering the implant visible in some areas. No blood vessels were found in the newly formed cartilage by differentiated bovine articular chondrocytes seeded at a density of 50 million cells/ml, neither in the absence nor in the presence of alginate. In experiments where dedifferentiated human septum chondrocytes or differentiated rabbit auricular chondrocytes were used at a density of 20 million cells/ml in PLA/PGA matrices blood vessel ingrowth could be demonstrated[48,68]. In comparable experiments with dedifferentiated human septal chondrocytes in the presence of alginate, no blood vessels were found (J. Aigner, personal communication, unpublished results). Ingrowth of blood vessels in cartilage is undesirable because this is regarded as an early sign of mineralisation and osteogenesis[69,70]. At present we can only speculate that the absence of blood vessels (a condition similar to native cartilage) is caused by the absence of certain growth factors or the presence of inhibitors of angiogenesis[71,72]. The discrepancy between the present results and other studies might be due to our use of higher cell densities and the use of alginate, although we cannot exclude effects of different species or different cartilage types.

Although the first studies in our laboratory with nude mice using alginate in combination with a carbohydrate-based matrix were very promising, one might question the use of alginate in humans. The degradation pathway of alginate is largely unknown and slow resorption could give rise to foreign body reactions against alginate in the long term. However, no inflammatory reactions against remains of alginate were noted, as also reported by others[50,51,73]. ***Chapter 5*** demonstrated that the use of alginate in combination with a non-woven scaffold (E210) does not negatively influence the amount of cartilage matrix proteins per tissue wet weight. In fact, the combination provides retention of the graft shape, which is regarded to be of great importance for clinical use.

Chapters 4 and 5 revealed that high cell seeding density enhances the characteristics of neo-cartilage formed by isolated cell-based tissue engineering techniques. These high cell numbers can only be achieved by multiplication in monolayer of isolated chondrocytes. In ***Chapter 6*** TGFß-2, bFGF and L-ascorbic acid were tested for their ability to stimulate chondrocyte multiplication in monolayer culture. We showed that the use of an optimal addition scheme of growth factors led, after four passages, to 100 times the amount of cells originally started with; this is 7 times as many cells as obtained in the native, control group. Growth-factors proved to be potent simulators of chondrocyte multiplication in monolayer culture. The effect of a certain growth-factor depends on the differentiation stage of the chondrocytes. The results of this study stress the need to add the right growth-factor at the right moment. Using medium with fetal calf serum, the addition of TGFß-2, bFGF and L-ascorbic acid during monolayer culture could not prevent chondrocyte dedifferentiation. In contrast to the results of Bonaventure et al.[74] using fetal human chondrocytes, and Benya and Shaffer[75] using immature rabbit chondrocytes, our chondrocytes did not redifferentiate when cultured in alginate for a period up to 28 days. Studies by our group demonstrated beneficial effects of low initial seeding densities and the use of a defined medium, without fetal calf serum with bFGF, for monolayer culture. Both the number of cells yielded as well as the redifferentiation capacity was found to improve[76-78]. In the future we hope to further optimize multiplication and redifferentiation capacity by combining these protocols.

It is well established that cell multiplication leads to dedifferentiation i.e., the cells lose their specific phenotype. Dedifferentiated chondrocytes fail to express collagen type-II and produce reduced amounts of glycosaminoglycan. Yaeger et al. [79]demonstrated re-expression of the cartilage phenotype when chondrocytes (dedifferentiated by multiplication in monolayer) were cultured in a defined medium, with addition of the growth factors IGF1 and TGF-β. After 4 weeks of culture in such a defined medium, 40% of the cells produced collagen type-II[79]. In ***Chapter 7*** we used this medium and assessed the differentiation stage immunohistochemically by the amount of cells synthesizing collagen type-II. Culturing multiplied bovine articular chondrocytes in alginate beads in the presence of fetal calf serum was not sufficient to induce

redifferentiation, as shown in ***Chapter 6***[80]. In the defined medium in the present study the percentage of cells synthesizing collagen type-II lies within the range described by Yaeger et al. for human articular chondrocytes[79]. The increased ratio of collagen type-II/pro-collagen type-I synthesizing cells indicates that these cells did redifferentiate.

Lohmander[81] proposed that the cartilage matrix in-vivo is composed of two compartments of glycosaminoglycans, turning over at different rates[81]. Mok et al.[82] and Häuselmann et al.[60] showed that chondrocytes cultured in alginate beads form an extracellular matrix, composed of two distinct matrix pools similar to the in-vivo situation. Different functional aspects have been suggested for the two matrix compartments. The further-removed matrix is thought to provide structural support and give cartilage its loadbearing ability[83,84]. The cell-associated matrix is hypothesized to play a regulating role in chondrocyte metabolism[84,85]. The data from ***Chapter 7*** showed that dedifferentiated chondrocytes cultured in alginate in the presence of fetal calf serum synthesized less glycosaminoglycans and in addition changed the distribution of glycosaminoglycans over the different matrix compartments. This decrease in glycosaminoglycan synthesis per cell is found mainly in the further-removed matrix, whereas the cell-associated matrix remains less affected. We hypothesized that the chondrocyte preferably rebuilds the cell-associated matrix, initially paying less attention to the further-removed matrix; all this in an effort to optimize cell function being (at least in part) determined by the cell-associated matrix[86].

Compared to the 'standard' culture condition, in the presence of 10% fetal calf serum, the defined culture medium only had a minor effect on differentiated chondrocytes; however, it did have a large effect on dedifferentiated chondrocytes. It not only enhanced the total amount of glycosaminoglycans synthesized, but also restored the assembly of glycosaminoglycans in the cell-associated and further-removed matrix to a value that resembled those measured in cultures of the differentiated chondrocytes. Based on our findings, we believe that in addition to quantitative analysis of the synthesis of specific matrix components, the organisation of glycosaminoglycans in the extracellular matrix may provide useful information on chondrocyte phenotype and should also be taken into consideration. Proper assembly of matrix components is important in tissue engineering of cartilage because it determines cartilage function and its response to mechanical and chemical stimuli.

Chapter 8 demonstrated the possibility to discriminate between differentiated and dedifferentiated chondrocytes using the monoclonal antibody 11-fibrau[87]. Collagen type-I expression is a common marker for chondrocyte dedifferentiation; however, this marker has several drawbacks. In the first place, collagen type-I mRNA expression is not very specific because differentiated cells are also described to express collagen type-I mRNA. Secondly, protein detection does not represent the status of the cells at that very moment, but rather shows what is produced in the past. Finally, collagen type-I can detach from the cell membrane after it is produced. In that case cytospins and FACS analysis fail to identify dedifferentiated

chondrocytes. The present study demonstrated that dedifferentiated chondrocytes express a 112 kD protein on their surface, as do fibroblasts. The protein appears when chondrocytes are cultured in monolayer. After four passages in culture almost all cells were positive. The cellular phenotype in general and chondrocyte phenotype in particular, is usually assessed by indirect measurements, i.e. the type (sometimes combined with the quantity) of extracellular matrix components produced by the cells. 11-fibrau recognises an epitope at the cell membrane. Results from ***Chapter 8*** demonstrated that the monoclonal antibody 11-fibrau allows to discriminate between differentiated and dedifferentiated chondrocytes. This implies that this monoclonal antibody can be used to select cells that can be used for tissue engineering purposes, or rather to discard dedifferentiated, fibroblast-like cells. To our surprise multiplied cells that re-express collagen type-II after culturing in defined medium stayed 11-fibrau positive; this indicates that the chondrocytes did not fully redifferentiate. Only after subcutaneous implantation of the cells in nude mice was the expression of 11-fibrau absent, indicating full redifferentiation.

FUTURE RECOMMENDATIONS

In conclusion, tissue engineering techniques have the potential to generate a solution to the well-established problem of articular cartilage defects. However, at present no data are available to support any treatments other than bone marrow stimulation techniques. Autologous chondrocyte transplantation has gained wide acceptance based on very few experimental data. The same applies to the newer autologous chondrocyte transplantation technique (MACI). One should bear in mind a statement by Hunziker that; "*good to excellent results (improved joint functionality and relief from pain in 70-80% of patients) are invariably yielded when a single group of surgeons favours a particular procedure.*" [34] This implies the need for controlled randomized clinical trials.

New experimental data will probably influence future articular cartilage repair strategies. In our laboratory research focuses on the improvement of cell culture methods to generate higher cell numbers accompanied by less dedifferentiation. In our experiments (***chapter 4***) high cell seeding densities proved to enhance the characteristics of tissue engineered cartilage. Cyclic mechanical loading is currently being investigated as a means of improving the mechanical properties of tissue engineered cartilage grafts prior to transplantation. In theory, better mechanical properties will enhance long-term performance of the transplanted tissue. Another factor under investigation that may enhance long-term survival of transplanted cartilage is the enzymatic treatment of cartilage aiming to achieve integration of native cartilage and tissue engineered cartilage[88,89].

A drawback of all techniques using isolated multiplied chondrocytes is donor-site morbidity. Alternative chondrocyte sources have the same problem, but perhaps to a lesser extent. Mesenchymal stem cells isolated from a bone-marrow aspirate might offer a solution.

Alternative techniques emerging from a completely different point of view (e.g. prevention and early diagnosis protocols, cartilage protective medication, and gene-therapy) might contribute to solving a problem that, although some authors claim otherwise, still remains largely unsolved.

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CHAPTER 11

SUMMARY AND DISCUSSION IN DUTCH

SAMENVATTING EN DISCUSSIE

Het natuurlijke beloop van beschadigd gewrichtskraakbeen is in het algemeen ongunstig. In 1743 werd dit al beschreven door Sir William Hunter[1]. Afhankelijk van het type defect herstellen kraakbeen-defecten in het geheel niet of slechts gedeeltelijk. Vooral bij adolescenten en jong volwassenen leiden kraakbeen-defecten regelmatig tot invaliditeit. Pijn, slotverschijnselen en bewegingsbeperking van het aangedane gewricht komen vaak voor. Tevens is er een verhoogde kans op het ontwikkelen van arthrose van het aangedane gewricht[2-4]. De laatste decennia is er veel, experimenteel en klinisch, onderzoek gedaan naar dit type letsels. Deze onderzoeken richten zich op herstel van het defect met een duurzaam weefsel dat in zoveel mogelijk opzichten lijkt op het oorspronkelijke gewrichtskraakbeen.

Het als één geheel transplanteren van kraakbeen met het onderliggend bot van een niet belast deel van het gewricht naar een kraakbeen-defect (zoals beschreven door Bobic[5,6] en Hangody[7,8]) lijkt aan de zojuist genoemde voorwaarde te voldoen. Echter, schade die optreedt op de plaats waar de 'osteochondrale pluggen' worden geoogst en secundaire degeneratieve veranderingen in het gewricht worden gezien als belangrijke nadelen van deze techniek. Daarnaast is het technisch zeer moeilijk het niveau van het gewrichtsvlak exact te herstellen en hoewel het benige deel van de plug goed integreert met subchondrale bot, treedt op kraakbeen niveau geen integratie op. Desondanks worden goede klinische resultaten gerapporteerd met deze technieken[9].

Tissue engineering[10] (letterlijk te vertalen als 'cel- en weefseltechnologie') is een nieuwe veelbelovende techniek, waarbij gebruik wordt gemaakt van cellen, biomaterialen en 'signaal stoffen' (zoals groeifactoren) om kraakbeenweefsel te genereren. Wanneer dit weefsel kan worden gebruikt om een defect in gewrichtskraakbeen te herstellen kan een aantal nadelen van de hierboven beschreven techniek worden vermeden[11,12].

Eén van de oudste technieken die voldoet aan de definitie van tissue engineering is het perforeren van de subchondrale plaat zoals door Pridie werd geïntroduceerd[13,14]. Microfracturering volgens Steadman is een techniek die hier sterk op lijkt[15,16]. Wanneer de subchondrale plaat wordt geopend krijgen stamcellen uit het beenmerg toegang tot het defect. De cascade die hierop volgt leidt tot herstel van het defect met fibreus kraakbeen. Goede klinische korte termijn resultaten worden beschreven in 75% van de behandelde patiënten[16]. Echter aangezien de eigenschappen van fibreus kraakbeen in vergelijking met het oorspronkelijke gewrichtskraakbeen inferieur zijn, verslechteren de resultaten op lange termijn.

Een andere benadering voor het herstel van defecten in gewrichtskraakbeen is het gebruik van vrije transplantaten van perichondrium[17-21] en periost[22-25]. Er is een ruime hoeveelheid experimentele en klinische data beschikbaar met betrekking tot deze technieken. In het algemeen worden de defecten niet volledig opgevuld met herstelweefsel. Daarnaast worden

verkalkingen in het herstelweefsel[21] beschouwd als een nadeel van deze techniek. Toch zijn ook hier de klinische resultaten redelijk goed te noemen[21,26,27].

Transplantatie van autologe chondrocyten (ACT) werd voor het eerst in een experimentele studie beschreven door Grande et al.[28] en later in de kliniek geïntroduceerd door Brittberg et al.[29]. De techniek heeft veel in de belangstelling gestaan en wordt al op relatief grote schaal toegepast. Dit lijkt te worden gesteund door de goede klinische resultaten die door de auteurs worden beschreven, zelfs na een follow-up van negen tot tien jaar[30,31]. Toch kunnen bij deze techniek nog altijd grote vraagtekens worden geplaatst. Is de hoeveelheid getransplanteerde chondrocyten groot genoeg en zijn ze in staat om kraakbeen te vormen? Kan loslating van de periostflap, waaronder de cellen worden geïnjecteerd, worden voorkómen? Bovendien waren de resultaten van deze techniek in een gerandomiseerde studie niet beter dan die van microfracturering[32]. Het lijkt dan ook onwaarschijnlijk dat de hoge kosten die autologe chondrocyten transplantatie met zich meebrengt op dit moment te rechtvaardigen zijn.

Dit proefschrift bestudeert de experimentele aspecten van kraakbeen tissue engineering en op welke wijze deze kunnen worden verbeterd. Hierbij is uitgegaan van de hypothese dat voor het herstel van een articulair kraakbeen-defect het gebruik van een homogeen kraakbeen transplantaat de beste kans op succes biedt. Voor een optimaal resultaat dient het getransplanteerde kraakbeen in zoveel mogelijk aspecten te lijken op het oorspronkelijke gewrichtskraakbeen. Het effect van verschillende biomaterialen, cel types en kweekmethoden op het tissue engineering proces en op de karakteristieken van het nieuw gevormde kraakbeen wordt bestudeerd.

In ***hoofdstuk 3*** wordt een techniek gebruikt die werd ontwikkeld door de afdeling KNO van het Erasmus MC. Deze techniek maakt gebruik van gedemineraliseerd bot dat wordt gewikkeld in een gesteelde perichondrium-flap en wordt achtergelaten onder de huid van een konijnenoor. Vanuit het perichondrium koloniseren immature kraakbeencellen het gedemineraliseerde bot, zodat een jong, zich nog ontwikkelend, kraakbeen transplantaat ontstaat. Dit jonge kraakbeen heeft in eerdere studies bewezen goed te integreren met het oorspronkelijke kraakbeen[33]. Het is juist het gebrek aan integratie dat wordt beschouwd als één van de belangrijkste oorzaken voor het falen van kraakbeentransplantatie[34]. Bovendien kan het jonge kraakbeen in het defect verder uitrijpen en zich aanpassen aan de locale situatie. Met deze techniek werden in kraakbeen-defecten in het neustussenschot, het cricoid en het oor bij konijnen goede resultaten behaald[35-38]. De techniek werd zelfs met succes klinisch toegepast in jonge kinderen[39]. Ondanks deze bemoedigende resultaten werden nooit defecten in gewrichtskraakbeen met deze techniek behandeld.

Van gedemineraliseerd bot is bekend dat het chondrogene factoren bevat[40,41]. In de genoemde techniek creëert het gedemineraliseerde bot ruimte tussen perichondrium-flappen en geeft steun aan het bloedstolsel[42]. De bijdrage van de chondrogene factoren is waarschijnlijk

zeer gering. Nadelen van gedemineraliseerd bot zijn de grote variatie in zowel de chemische samenstelling als de afmetingen van de poriën. Tevens zouden ziektekiemen kunnen worden overgebracht, wat door de BSE crisis weer zeer actueel is geworden. In ***hoofdstuk 3*** is getracht de techniek, zoals die werd ontwikkeld door de afdeling KNO, te optimaliseren en geschikt te maken voor toepassing in het gewricht.

Het eerste deel van ***hoofdstuk 3*** gaat uit van de hypothese dat een biomateriaal waarvan de samenstelling beter gecontroleerd kan worden beter reproduceerbaar kraakbeen vormt. Ofwel, dat in combinatie met perichondrium nieuw kraakbeen wordt gevormd met betere histologische en structurele eigenschappen. E210 (een biomateriaal gebaseerd op koolhydraten) en een collageen matrix werden vergeleken met het materiaal uit de oorspronkelijke studies; gedemineraliseerd bot. De hypothese werd echter door de experimentele resultaten niet gesteund.

In de transplantaten met de beter gedefinieerde matrices werden veel meer ontstekingscellen gezien. Voor implantatie in het gewricht werd daarom gekozen voor gedemineraliseerd bot. Met gedemineraliseerd bot werd een transplantaat verkregen dat goede integratie liet zien met het subchondrale bot. Het nieuw gegenereerde kraakbeen behield bovendien zijn kraakbenige eigenschappen in het gewricht.

Hoewel de techniek waarbij gebruik wordt gemaakt van een perichondrium-flap een betrouwbare manier is gebleken om nieuw kraakbeen te genereren, kan deze nog niet toegepast worden in het gewricht. De perichondrium-flap (met de cambiumlaag naar het defect gericht) dekt het defect af, stamcellen zijn vanuit het perichondrium in een poreuze matrix gegroeid en de bodem van het defect wordt bedekt door perichondrium, dat vaat-ingroei vanuit het subchondrale bot voorkomt[43,44]. Deze oriëntatie van de verschillende weefsellagen wordt door Hunziker beschreven als optimaal[34]. De techniek heeft dus een aantal voordelen en zou beter geschikt kunnen worden gemaakt voor toepassing in grotere proefdieren en mogelijk ook mensen. Bijvoorbeeld, wanneer de bovenste perichondrium laag wordt verwijderd voordat het kraakbeen wordt geïmplanteerd, komt het nieuw gevormde kraakbeen op dezelfde hoogte als het oorspronkelijke kraakbeen[45]. Dit zou de integratie tussen transplantaat en oorspronkelijke kraakbeen gunstig kunnen beïnvloeden. Toch kunnen belangrijke problemen, zoals schade op de plaats waar het donor weefsel wordt geoogst en de beperkte dikte van het biomateriaal die nog tot een homogeen transplantaat leidt waarschijnlijk niet worden opgelost. Deze overwegingen hebben ertoe bijgedragen dat in de overige experimenten in dit proefschrift de nadruk meer is komen te liggen op geïsoleerde cellen in combinatie met een biomateriaal voor het genereren van nieuw kraakbeen.

Wanneer gebruik wordt gemaakt van geïsoleerde cellen worden er andere eisen gesteld aan de gebruikte biomaterialen. Er vindt geen langzame ingroei van cellen plaats en dus moet het biomateriaal geschikt zijn voor een homogene verdeling van de geïsoleerde cellen. In dit proefschrift is ervan uitgegaan dat het gebruik van alginaat gel de verdeling van chondrocyten van gelijke vorm en grootte binnen gedemineraliseerd bot vereenvoudigt. Daarbij is tevens

aangenomen dat polymerisatie van de gel voorkomt dat getransplanteerde cellen het biomateriaal verlaten.

In ***hoofdstuk 4*** van dit proefschrift werd een natuurlijke eiwit matrix (gedemineraliseerd bot) vergeleken met een op koolhydraten gebaseerde matrix (E210). In tegenstelling tot de resultaten in ***hoofdstuk 3*** (waar cellen van buiten af het biomateriaal koloniseren) werd in ***hoofdstuk 4*** aangetoond dat wanneer geïsoleerde gekweekte chondrocyten worden gebruikt E210 tot betere resultaten leidt. Bij gebruik van gedemineraliseerd bot werden in de kern van het nieuw gegenereerde kraakbeen resten van het biomateriaal gevonden. Blijkbaar kunnen macrofagen, die verantwoordelijk zijn voor de afbraak van gedemineraliseerd bot[38], niet doordringen tot in de kern van het biomateriaal. Afbraak van E210 vindt plaats door hydrolyse[46-49]. Dit biomateriaal bleek bij evaluatie grotendeels geresorbeerd, waardoor een homogeen weefsel verkregen werd. De alginaat gel, gebruikt in combinatie met beide biomaterialen, wordt eveneens afgebroken middels hydrolyse[50]. De bestanddelen; mannuronzuur en guluronzuur die op deze wijze ontstaan, kunnen vervolgens worden op genomen in enzymatische cycli en verder worden afgebroken[51,52]. Er werd geen onstekings-reaktie tegen alginaat gel waargenomen, hetgeen overeenstemt met gegevens uit de literatuur[50,51].

In ***hoofdstuk 4*** werd aangetoond dat volwassen chondrocyten na vermenigvuldiging in monolayer kweek gebruikt kunnen worden voor het genereren van nieuw kraakbeen. Hoge celdichtheden (50 miljoen cellen per ml), zoals die ook voorkomen in het ontwikkelende embryo, en het gebruik van het biomateriaal E210 leidden tot een verhoogde expressie van collageen type-II. Bovendien werd in de kern van het nieuw gegenereerde kraakbeen geen pro-collageen type-I aangetoond. De resultaten suggereren dat het type biomateriaal dat wordt gebruikt de structurele eigenschappen van het nieuwe weefsel en de expressie van het cel-fenotype kan beïnvloeden. Vanzelfsprekend moet hierbij in aanmerking worden genomen dat de condities in een gewricht duidelijk verschillen van de condities in het subcutane weefsel. De conclusies van deze studie kunnen dan ook niet rechtstreeks worden vertaald naar gebruik van dergelijke technieken in het gewricht. Toch zou onderhuidse implantatie voorafgaand aan transplantatie naar een articulair kraakbeen-defect een klinische toepassing kunnen zijn.

Op koolhydraten gebaseerde biomaterialen worden vaak toegepast in tissue engineering technieken, een veel voorkomende combinatie is; poly-melkzuur/poly-glycolzuur(PLA/PGA)[53-56]. In ***hoofdstuk 4*** leek de combinatie van E210 (eveneens een combinatie van PLA en PGA) met vermenigvuldigde chondrocyten in alginaat gel veelbelovend[57]. Alginaat gel wordt vaak gebruikt als drager voor geïsoleerde cellen zowel in-vitro als in-vivo, echter de combinatie van E210 en alginaat gel werd nooit eerder beschreven[33,51,57-61]. De studie in ***hoofdstuk 5*** bestudeert daarom het effect van alginaat als cel-drager op macroscopische, histologische en chemische eigenschappen van nieuw gegenereerd kraakbeen[62]. E210 werd gebruikt in vier condities (met en zonder alginaat gel en met en zonder gedifferentieerde chondrocyten) en geïmplanteerd in naakte muizen. Alle transplantaten die chondrocyten bevatten bleken stevig te zijn, echter het nieuw gegenereerde kraakbeen zonder alginaat had een meer geschrompeld aspect. Het

gebruik van alginaat leidde tot een veel gladder transplantaat dat ook elastischer aanvoelde. In beide condities (chondrocyten met én zonder alginaat) werd kwantitatief een vergelijkbare hoeveelheid glycosaminoglycanen en collageen type-II per nat gewicht gevonden. Ook de hoeveelheid calcium neerslag verschilde niet in beide condities. De vervorming in de conditie zonder alginaat moet waarschijnlijk worden toegeschreven aan de dichte laag van cellen die een strak kapsel vormen rond het transplantaat.

De hoeveelheid glycosaminoglycanen in het nieuw gegenereerde kraakbeen (met én zonder alginaat) benadert de hoeveelheid glycosaminoglycanen die wordt gevonden in kalfs-kraakbeen[63]. De concentratie collageen type-II was veel lager dan die in kalfs-kraakbeen[63]. Een mogelijke verklaring hiervoor is de lange halfwaarde tijd van collageen in levende wezens[64]. In de toekomst zal hier nadere aandacht aan worden besteed. Collageen synthese zou kunnen worden gestimuleerd door kweken van de transplantaten in een bioreactor voordat tot implantatie in een gewricht wordt overgegaan[65,66]. Het kweken in een bioreactor zou nog verder kunnen worden verbeterd door het toepassen van mechanische belasting en groeifactoren.

In ***hoofdstuk 4*** werd aangetoond dat het redifferentiatie-vermogen van gekweekte chondrocyten in alginaat versterkt werd door E210 in vergelijking met gedemineraliseerd bot. Deze bevinding leidde tot de hypothese dat E210 een belangrijker factor was voor redifferentiatie dan alginaat. Echter, in ***hoofdstuk 5*** werden chondrogene cellen aangetroffen in transplantaten die bestonden uit E210 en alginaat zonder toegevoegde chondrocyten. Deze cellen zijn waarschijnlijk ingegroeid vanuit het onderhuidse bindweefsel. Van huid-fibroblasten is bekend dat zij kraakbenige eigenschappen kunnen ontwikkelen wanneer zij worden gekweekt in een driedimensionaal kweek-systeem[67]. Een belangrijke kanttekening is dat de hoeveelheid kraakbeen die hierbij ontstaat zeer variabel en onvoorspelbaar is. Dit in tegenstelling tot de conditie waarbij gekweekte chondrocyten worden gebruikt in combinatie met E210 en alginaat. Dit chondro-inductieve vermogen zou een extra voordeel van de combinatie van E210 en alginaat kunnen blijken.

In het huidige experiment werd vaatingroei slechts gezien in de controle conditie ('leeg' E210). Wanneer chondrocyten werden toegevoegd (50 miljoen cellen per ml)werden in het nieuw gegenereerde kraakbeen geen bloedvaten aangetoond, ongeacht of er wel of geen alginaat werd gebruikt. In experimenten waarbij gekweekte menselijke chondrocyten uit het neustussenschot werden gebruikt (20 miljoen cellen per ml) werden daarentegen wél bloedvaten in het transplantaat aangetoond[48,68]. Wanneer menselijke cellen werden gebruikt in condities vergelijkbaar met de condities in ***hoofdstuk 5*** bleef vaatingroei achterwege (J. Aigner ongepubliceerde data). Vaatingroei in kraakbeen is ongewenst, aangezien het wordt beschouwd als een vroeg teken van mineralisatie en botvorming[69,70]. Mogelijk berust het achterwege blijven van vaatingroei in de experimenten van ***hoofdstuk 4 en 5*** op de afwezigheid van bepaalde groeifactoren, danwel op de aanwezigheid van remmers van bloedvat-vorming in de transplantaten[71,72]. Bewijzen hiervoor ontbreken echter vooralsnog. De verschillen met de resultaten van andere auteurs zijn mogelijk te verklaren door de hogere celdichtheden en het

gebruik van alginaat in ***hoofdstuk 4 en 5***, hoewel ook soort specifieke en kraakbeen specifieke verschillen niet geheel kunnen worden uitgesloten.

Hoewel het gebruik van alginaat gel gecombineerd met een biomateriaal op koolhydraatbasis in naakte muizen in ***hoofdstuk 4 en 5*** veel belovend lijkt, kunnen bij het gebruik van alginaat gel in mensen vraagtekens worden geplaatst. Het afbraakproces van alginaat is nog niet geheel opgehelderd en vertraagde afbraak van alginaat zou kunnen leiden tot vreemdlichaam reakties op de lange termijn. Ontstekingsreakties tegen alginaat werden echter niet gevonden, hetgeen in overeenstemming is met data van andere auteurs[50,51,73]. In ***hoofdstuk 5*** werd aangetoond dat alginaat gel gecombineerd met een biomateriaal op koolhydraatbasis (E210) geen negatieve invloed heeft op de hoeveelheid kraakbeen-matrix-eiwitten per nat gewicht van het weefsel. Het behoud van de vorm van het transplantaat wordt gezien als een belangrijk voordeel van de combinatie; E210 met alginaat, zeker ook met het oog op eventuele klinische toepassing van de techniek.

De ***hoofdstukken 4 en 5*** tonen aan dat wanneer voor tissue engineering hoge celdichtheden worden gebruikt, dit de eigenschappen van het nieuw gegenereerde kraakbeen gunstig beïnvloedt. Deze hoge celdichtheden kunnen slechts worden bereikt wanneer geïsoleerde chondrocyten in celkweek vermenigvuldigd worden. Het effect van TGFß-2, bFGF en vitamine-C op celvermenigvuldiging in kweek werd getest in ***hoofdstuk 6***. Het initiële aantal chondrocyten was na 4 weken verhonderdvoudigd wanneer deze groeifactoren in een optimaal schema aan de monolayer kweek werden toegevoegd. Dit was 6.4 maal zoveel dan wanneer geen groeifactoren werden toegevoegd (controle kweek). Het gebruik van groeifactoren blijkt de opbrengst van chondrocyten in monolayer kweek te kunnen verhogen. Het effect van een bepaalde groeifactor is afhankelijk van het differentiatie stadium van de doel-cellen, in dit geval de chondrocyten. De huidige studie benadrukt dan ook het toevoegen van de juiste groeifactor op het juiste moment. Wanneer kweekmedium wordt gebruikt waar foetaal kalfs-serum aan is toegevoegd kunnen TGFß-2, bFGF en vitamine-C de dedifferentiatie van de chondrocyten niet voorkómen; er werd geen redifferentiatie gezien wanneer de vermenigvuldigde chondrocyten gedurende 28 dagen in alginaat gel werden gekweekt. Bonaventure et al.[74] en Benya en Schaffer[75] beschreven daarentegen wél redifferentiatie in alginaat gel. Zij maakten echter gebruik van foetale menselijke chondrocyten en immature konijnen chondrocyten. Recente experimenten van onze groep hebben aangetoond dat een hogere celopbrengst ook kan worden bereikt door een lagere initiële celdichtheid in monolayer kweek toe te passen en kweekmedium zonder foetaal kalfsserum te gebruiken. Ook het vermogen van de cellen om te redifferentiëren neemt onder deze condities toe[76-78]. Het combineren van deze kweekprotocollen zal in de toekomst mogelijk leiden tot verdere optimalisatie van het kweken van chondrocyten in monolayer.

Dedifferentiatie is een algemeen bekend nadelig effect van het vermenigvuldigen van chondrocyten in celkweek, de cellen verliezen hun specifieke kraakbenige eigenschappen.

Gedediffertieerde chondrocyten brengen geen collageen type-II meer tot expressie en produceren aanzienlijk minder glycosaminoglycanen. Yaeger et al.[79] hebben aangetoond dat vermenigvuldigde (gededdifferentieerde) chondrocyten opnieuw kraakbeen specifieke eigenschappen tot expressie brengen, wanneer zij worden gekweekt in een gedefinieerd medium zonder foetaal kalfsserum, waaraan de groeifactoren IGF1 en TGFß zijn toegevoegd. Na 4 weken in dit gedefinieerde medium produceerde 40% van de cellen collageen type-II[79]. In ***hoofdstuk 7*** van dit proefschrift werd ditzelfde gedefinieerde medium gebruikt. Het differentiatie stadium van de cellen werd vastgesteld door het aantal collageen type-II producerende cellen te bepalen met behulp van immuunhistochemie. Het kweken van gededdifferentieerde runder gewrichts-chondrocyten in alginate in medium waaraan foetaal kalfsserum werd toegevoegd leidde niet tot redifferentiatie, zoals in ***hoofdstuk 6*** werd aangetoond[80]. Gebruikmakend van het gedefinieerde medium in ***hoofdstuk 7*** was het aantal collageen type-II producerende cellen vergelijkbaar met de studie die door Yaeger et al. werd verricht met menselijke gewrichts-chondrocyten[79]. De toegenomen verhouding van collageen type-II ten opzichte van pro-collageen type-I producerende cellen duidt erop dat redifferentiatie is opgetreden.

De kraakbeen matrix kan worden verdeeld in twee compartimenten; het 'cel-geassocieerde' matrix-compartiment en het 'verder-verwijderde' matrix-compartiment, ieder met een verschillende halfwaarde-tijd van de glycosaminoglycanen[81]. Mok et al.[82] en Häuselmann et al.[60] toonden aan dat ook chondrocyten in alginaat gel deze twee verschillende matrix-compartimenten vormen. Waarschijnlijk verschillen de 'cel-geassocieerde' matrix en de 'verder-verwijderde' matrix ook functioneel van elkaar. De 'verder-verwijderde' matrix draagt vooral bij aan de stevigheid en belastbaarheid van de kraakbeen matrix, terwijl de 'cel-geassocieerde' matrix een belangrijke rol speelt in het reguleren van het kraakbeen-metabolisme[83-85]. In ***hoofdstuk 7*** werd aangetoond dat gededdifferentieerde chondrocyten gekweekt in alginaat met toevoeging van foetaal kalfs-serum minder glycosaminoglycanen produceerden. Daarnaast veranderde ook de verdeling van de glycosaminoglycanen over de matrix-compartimenten. De verminderde productie van gyclosaminoglycanen is nagenoeg geheel toe te schrijven aan een afname in de 'verder-verwijderde' matrix. De afname in de 'cel-geassocieerde' matrix daarentegen was veel geringer. Gebaseerd op deze resultaten luidt de hypothese; dat de chondrocyten bij voorkeur hun 'cel-geassocieerde' matrix op peil houden en in eerste instantie minder aandacht besteden aan de 'verder-verwijderde' matrix. Dit alles in een poging om de cel functie, die in ieder geval deels bepaald wordt door de 'cel-geassocieerde' matrix[86], te optimaliseren.

Het gedefinieerde kweekmedium had, in vergelijking met de standaard kweekmethode (met 10% foetaal kalfs-serum), slechts een gering effect op gedifferentieerde chondrocyten. Op gededdifferentieerde chondrocyten was het effect veel duidelijker. Niet alleen nam de totale hoeveelheid geproduceerde glycosaminoglycanen toe, ook de verdeling van de glycosaminoglycanen over de matrix-compartimenten herstelde zich tot een verdeling die ook bij gedifferentieerde cellen werd gevonden. Naast de kwantitatieve analyse van de

verschillende matrix-componenten, verdient ook de verdeling van deze componenten over de 'cel-geassocieerde' en de 'verder-verwijderde' matrix de aandacht, aangezien deze bruikbare informatie kan verschaffen over het fenotype van de gekweekte chondrocyten. De juiste opbouw van de matrix is belangrijk voor het nieuw gegenereerde kraakbeen; het is bepalend voor de functie en de reaktie op fysische en chemische stimuli.

In ***hoofdstuk 8*** werd aangetoond dat met een nieuw monoclonaal antilichaam (11-fibrau) onderscheid kan worden gemaakt tussen gedifferentieerde en gededifferentieerde chondrocyten[87]. Een veel gebruikte indicator voor gededifferentieerde chondrocyten is expressie van collageen type-I, echter hieraan kleven een aantal nadelen. In de eerste plaats is aantonen van mRNA voor collageen type-I niet specifiek, omdat ook door gedifferentieerde cellen dit mRNA tot expressie wordt gebracht. In de tweede plaats is het aantonen van een eiwit niet altijd representatief voor de status van een cel op dat bepaalde moment, maar toont het eerder aan wat tot dan toe door de cel is geproduceerd. Tenslotte kan collageen type-I loslaten van de celmembraan nadat het werd geproduceerd. Cytospins en FACS analyse kunnen in dat geval de gededifferentieerde chondrocyten niet identificeren. De huidige studie laat zien dat gededifferentieerde chondrocyten en fibroblasten een 112 kD eiwit tot expressie brengen aan het cel-oppervlak. Het eiwit verschijnt wanneer de cellen worden gekweekt in monolayer; na 4 passages zijn nagenoeg alle cellen positief voor dit eiwit. Het fenotype van cellen in het algemeen en van chondrocyten in het bijzonder wordt doorgaans vastgesteld door indirecte meetmethoden, dat wil zeggen; het type (al dan niet in combinatie met de hoeveelheid) componenten van de extra-cellulaire matrix die door de cel is geproduceerd. 11-fibrau is in staat een eiwit op de celmembraan te herkennen en maakt het mogelijk onderscheid te maken tussen gedifferentieerde en gededifferentieerde chondrocyten. Dit houdt in dat met dit monoclonale antilichaam cellen die geschikt zijn voor tissue engineering toepassingen kunnen worden geselecteerd en gededifferentieerde (fibroblast-achtige) cellen kunnen worden verwijderd. Een opvallende bevinding in ***hoofdstuk 8*** was dat gekweekte chondrocyten die in gedefinieerd medium opnieuw collageen type-II tot expressie brachten toch 11-fibrau positief bleken, er trad dus geen volledige redifferentiatie op. Na subcutane implantatie bij naakte muizen werd geen 11-fibrau meer aangetoond, duidend op volledige redifferentiatie.

TOEKOMSTPERSPECTIEVEN

Tissue engineering zou een oplossing kunnen vormen voor het lang bestaande probleem van defecten in gewrichtskraakbeen. Op dit moment echter kan op basis van literatuur gegevens voor geen enkele techniek, anders dan de beenmergstimulatie technieken, voldoende steun worden gevonden voor klinische toepassing. Desondanks, is het transplanteren van gekweekte autologe chondrocyten (ACT) inmiddels op basis van beperkte experimentele gegevens doorgedrongen tot de klinische praktijk. Hetzelfde geldt voor de hierop door-ontwikkelde MACI

techniek (waarbij gekweekte chondrocyten in een biomateriaal worden getransplanteerd). De lange termijn resultaten van ACT zijn beschreven in een goed gedocumenteerde, maar niet gerandomiseerde en niet gecontroleerde studie. Hunziker merkte over dergelijke studies op dat; "wanneer één bepaalde groep chirurgen een bepaalde techniek uitvoert, dit altijd tot goede (verbeterde bewegingsmogelijkheid en vermindering van pijnklachten in 70-80% van de gevallen) resultaten leidt[34]. Dit geeft nog eens duidelijk de behoefte aan gerandomiseerde, gecontroleerde klinische studies aan.

Nieuwe experimentele data zullen waarschijnlijk hun invloed hebben op toekomstige behandel strategieën. Het kraakbeen onderzoek van de afdelingen Orthopaedie en KNO van het Erasmus MC richt zich op dit moment onder andere op celkweek methoden waarmee een grotere hoeveelheid cellen kan worden verkregen. Tevens wordt getracht het optreden van dedifferentiatie zoveel mogelijk te reduceren en redifferentiatie te verbeteren. Hoge celdichtheden hebben in ***hoofdstuk 4*** bewezen de eigenschappen van kraakbeen, verkregen door tissue engineering, gunstig te beïnvloeden. Cyclische mechanische belasting wordt bestudeerd als additionele conditie om de mechanische eigenschappen van het nieuw gegenereerde kraakbeen te verbeteren voorafgaand aan implantatie in het gewricht. In theorie wordt het lange termijn functioneren van getransplanteerd kraakbeen verbeterd door optimale mechanische eigenschappen.

Een ander probleem dat op dit moment wordt bestudeerd is de slechte integratie van getransplanteerd kraakbeen met omliggende gewrichtskraakbeen. Enzymatische voorbehandeling van het kraakbeen zou een betere integratie kunnen bewerkstelligen en de lange termijn overleving van het transplantaat bevorderen[88,89].

Een belangrijk nadeel van alle technieken die gebruikmaken van gekweekte chondrocyten is de schade die wordt aangericht op de donorplaats, waar de cellen worden geoogst. Alternatieve bronnen voor het kraakbeen zijn geopperd, maar leveren vergelijkbare problemen op, hoewel misschien op een kleinere schaal. Stamcellen uit het beenmerg brengen mogelijk de oplossing.

Ook volledig andere benaderingen van het probleem, uitgaande van preventie en vroege onderkenning, kraakbeen beschermende medicatie en gen-therapie zouden oplossingen kunnen bieden voor een probleem dat nog altijd grotendeels onopgelost blijft.

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DANKWOORD


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DANKWOORD

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CURRICULUM VITAE

CURRICULUM VITAE

Willem Jan Marijnissen is geboren op 7 september 1966 in 'de parel van het zuiden' Breda. De lagere school werd doorlopen aan de "Hertoginne van Brabant" en de middelbare school aan het "Onze Lieve Vrouwe Lyceum" beiden te Breda. In de 'grote stad' Rotterdam volgde de auteur de medische opleiding aan de Erasmus Universiteit; van 1984 tot het diploma basisarts in april 1992. De keuze was toen al, onder andere door het 'Pandak onderzoek' o.l.v. Prof. Dr. B. van Linge, op de orthopaedie gevallen. Toch startte de medische carrière aan de poorten van het Schieland Ziekenhuis. Na twee jaar werd de stap naar de orthopaedie gemaakt in het Reinier de Graaf Ziekenhuis te Delft, onder de bezielende leiding van Dr. B.R.H. Jansen. Weer twee jaar later werd de basis voor het onderzoek, dat leidde tot dit proefschrift, gelegd op het kraakbeenlab o.l.v. dr. Gerjo van Osch. Het kraakbeenlab bestond destijds, wat de orthopaedie betreft, uit één bureau met computer. Voor het praktische deel werd uitgeweken naar het kraakbeenlab van de afdeling KNO, o.l.v. Dr. H.L. Verwoerd-Verhoef.

In januari 1999 startte de auteur met de chirurgische vooropleiding in het Merwede ziekenhuis (inmiddels Albert Schweitzer Ziekenhuis) te Dordrecht, afdelingshoofd Dr. K.G. Tan. Aansluitend werd de opleiding tot orthopaedisch chirurg vervolgd gedurende drie jaar in het Dijkzigt ziekenhuis (inmiddels Erasmus Medisch Centrum) te Rotterdam, afdelingshoofd Prof. Dr. J.A.N. Verhaar en één jaar in het Leyenburg Ziekenhuis (inmiddels Haga Ziekenhuis) te den Haag, afdelingshoofd Dr. L.N.J.E.M. Coene.

Daarna werkte de auteur 4 maanden als chef de clinique in het Erasmus Medisch Centrum gevolgd door 6 maanden als chef de clinique (terug waar het ooit begon) in het Reinier de Graaf, afdelingshoofd Dr. R.M. Bloem.

Sinds 1 januari werkt de auteur als orthopaedisch chirurg in het Albert Schweitzer Ziekenhuis te Dordrecht in een maatschap met C.J. Tseng, W.R.J. Pepels, B. Hylkema, P. de Bruin en sinds kort ook dr. A.J. vanKoeveringe.

Willem Jan Marijnissen woont samen met Linda van den Bent, samen hebben zij drie kinderen; Wander, Julie en Femke.

Het monster was nu heel beroemd, maar de volgende dag ging hij weer gewoon aan het werk.

Uit: Het goedige monster en de rovers. Max Velthuijs, 1996