Cartilage Tissue Engineering

Effects of Interleukin-4, Insulin-like Growth Factor Binding Proteins and Biomaterials

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To my family

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Chapter 1

Introduction
and
Goal of the Thesis

Tissue Engineering

Tissue loss or end-stage organ failure resulting from an injury, disease or due to congenital defects are complex and costly health care problems [1]. The transplantation of tissues or organs is the current treatment which is severely limited by availability of compatible donors, shortage of donor tissue and immune rejection [2;3]. Another problem is that the host may need long-term immunosuppressive medication, with its increased risks of side effects [4]. Additionally, the risk of some viral infections such as AIDS and hepatitis is associated with transplantation of donor organs [5;6]. Also, the currently used alternatives such as artificial prostheses do not repair the tissue or organ function. Additionally, artificial prostheses may be subject to wear upon long-term implantation, and could induce inflammatory response in the host [7;8]. Therefore, tissue engineering is considered effective alternative to overcome the problems and limitations of current therapies, and to develop new substitutes to improve tissue function.

Tissue engineering is an emerging field that aims to regenerate natural tissues and create new tissues using biological cells, biomaterials, biotechnology, and clinical medicine. It is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function [9]. The concept of tissue engineering includes that cells can be isolated from a patient, expanded in cell culture and seeded onto a carrier. The resulting tissue engineering construct is then grafted back into the same patient to function as the introduced replacement tissue. Tissue engineering promises a more advanced approach in which organs or tissues can be repaired, replaced, or regenerated for more targeted solutions. This approach also responds to clinical needs that cannot be met by organ donation alone. In other applications, for example tissue engineering may be used to develop predictive models for toxicity assessment. As research tools, these systems could also be employed as correlates of in vitro and in vivo biological activity.

Since the early 1990s, the tissue engineering field has progressed rapidly and biological substitutes are in development for several tissues in the body. Tissue engineered

products such as bioartificial skin (Apligraf and TranCyte) and cultured autologous chondrocytes (Carticel) have reached the market. Scientists are now engineering cardiovascular tissues such as heart valves, and blood vessels [10-12]. Encapsulated pancreatic islets have been implanted in the patients for the treatment of diabetes [13] and liver assist systems containing encapsulated hepatocytes have been used clinically to provide extracorporeal support to the patients with liver failure [14]. A bioartificial bladder has been developed as a replacement engineered organ [15]. Significant progress has been made in orthopaedic tissue engineering for the repair of bone and cartilage [16-18].

Cartilage

Cartilage and bone are specialized types of connective tissue and are made up of cells, and extracellular matrix (ECM). As connective tissues, they are derived from the mesenchyme of the embryo. Their functions are somewhat similar, but cartilage is more flexible and has less tensile strength than bone. Cartilage is a tissue that supports and protects soft tissues, provides a sliding surface in joints, and functions both in the development of long bones and in the repair of bone breakage. It fulfils its functions without nerves, blood supply, or lymphatic system. Its properties are, in fact, not due to the properties of its cells but of their secretions and of the secondary structuring of water. Cartilage is formed when mesenchymal cells aggregate and secrete intercellular material. There are two types of cartilage growth.

I. Interstitial growth occurs when cartilage cells divide and subsequent chondrification occurs at this site. The trapped cells are termed chondrocytes.

II. Appositional growth occurs when cartilage is added at the periphery of forming cartilage. Peripheral growth is carried out by cells of the perichondrium (a sheath surrounding the cartilage). The inner cells of the perichondrium give rise to new cartilage cells (Fig. 1).

Composition and Anatomy of Cartilage

Cells

Chondroblasts are immature cells (“blasts”) which are actively synthesizing and depositing extracellular matrix materials and fibers but are not yet trapped by this matrix; they

differentiate from mesenchymal cells. Chondrocytes are the mature cells of cartilage and are completely surrounded by cartilage matrix. Hence, they are trapped in small spaces called lacunae (Fig. 1) [19]. In humans, chondrocytes represent only about 1% of the volume of hyaline cartilage but are essential since it is these cells that replace degraded matrix molecules to maintain the correct size and mechanical properties of the tissue. Thus, microscopically, the cells' endoplasmic reticulum and Golgi apparatus are prominent [20].

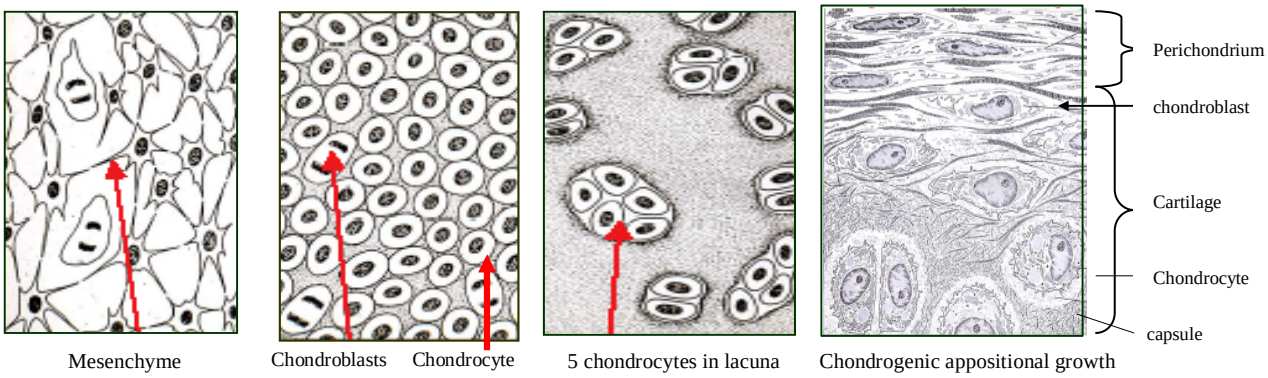

Fig. 1: Chondrogenic interstitial and appositional growth. Cartilage is avascular, gets nutrients by diffusion

Extracellular matrix (ECM)

The ECM is composed mainly of collagen fibers and proteoglycans (Fig. 2). The dominant and typical collagen type of articular cartilage is type II (90-95%) [21;22], in addition there are small portions of types VI, IX, X, and XI. The ECM is mostly free of type I collagen (typical for bone tissue) [23]. Type II collagen content is vital because its concentration is directly related to the tensile strength of the tissue and it is a marker of the hyaline phenotype [24]. Type II collagen has a high amount of bound carbohydrate groups, allowing more interaction with water than some other types. Types IX and XI, along with type II, form fibrils that interweave to form a mesh. This organization provides tensile strength as well as physically entraps other macromolecules. Although the exact function of types IX and XI are unknown, type IX has been observed to bind superficially to the fibers and extending into the inter-fiber space to interact with other type IX molecules, possibly acting to stabilize the mesh structure (Fig. 2). Type X as a marker for hypertrophic chondrocytes is found only near areas of the matrix that are calcified [25;26].

Proteoglycans are composed of about 95% polysaccharide and about 5% protein. The protein core is associated with one or more varieties of glycosaminoglycan (GAG) chains. GAG chains are unbranched polysaccharides made from disaccharides of an amino sugar and another sugar. At least one component of the disaccharide has a negatively charged sulfate or carboxylate group, so the GAGs tend to repel each other and other anions while attracting cations and facilitating interaction with water. Hyaluronic acid, chondroitin sulfate, keratan sulfate, dermatan sulfate and heparan sulfate are some of the GAGs generally found in articular cartilage [20;26;27].

There are both large aggregating monomers and smaller proteoglycans present in articular cartilage. The aggregating proteoglycans, or aggrecans, are composed of monomers with keratan sulfate and chondroitin sulfate attached to the protein core. Aggrecans fill most of the interfibrillar space of the ECM and are thought to be responsible for much of the resilience and stress distribution in articular cartilage through their ability to attract water. There are no chemical bonds between the proteoglycans and collagen fibers; aggregation prevents diffusion of the proteoglycans out of the matrix during joint loading [20;25;26]. The smaller proteoglycans include decorin, biglycan and fibromodulin. They have shorter protein cores and fewer GAG chains than their larger counterparts. Unlike aggrecans, these molecules do not affect physical properties of the tissue, but are thought to play a role in cell function and organization of the collagen matrix [20].

In contrast to proteoglycans, glycoproteins have only a small amount of oligosaccharides associated with the protein core. These polypeptides help to stabilize the ECM matrix and aid in chondrocyte-matrix interactions. Other noncollagenous proteins commonly found in most tissues, such as fibronectin and tenascin, are also observed in articular cartilage and are believed to perform similar functions as the glycoproteins [20].

Tissue fluid is an essential part of hyaline cartilage, comprising up to 80% of the wet weight of the tissue. In addition to water, the fluid contains gases, metabolites and a large amount of cations to balance the negatively charged GAG's in the ECM. It is the exchange of this fluid with the synovial fluid that provides nutrients and oxygen to the avascular cartilage.

In addition, the entrapment of this fluid though interaction with ECM components provides the tissue with its ability to resist compression and return to normal shape after deformation [20;26].

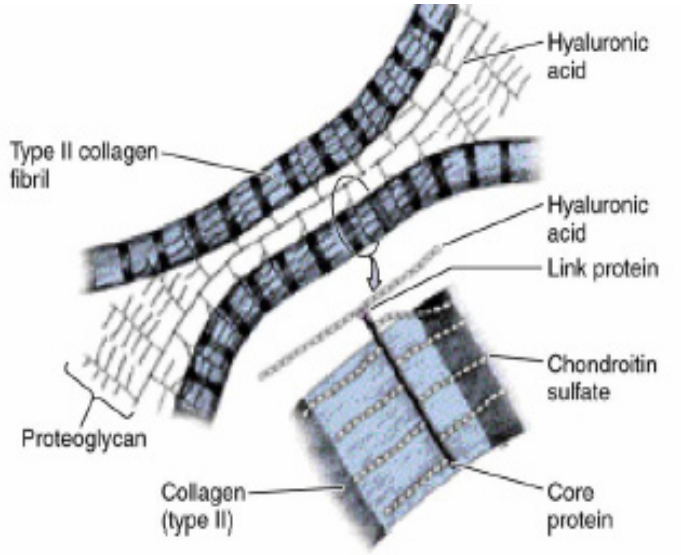

Fig: 2: Illustration of extracellular matrix components of the cartilage

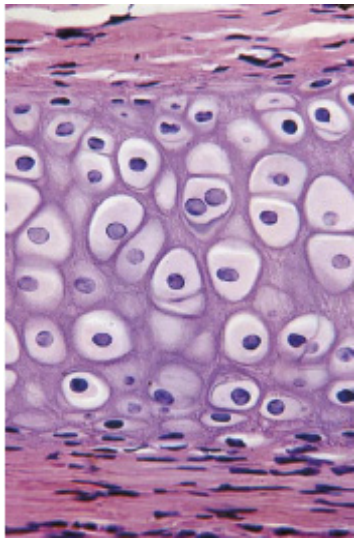

Fig. 3: Hyaline cartilage

Types of Cartilage

- Hyaline cartilage has a white, smooth, glassy appearance, and unlike fibrocartilage, shows no macroscopic evidence of fibers (Fig. 3), this is the most widespread type of cartilage in the body, particularly in the fetus and infant. Most bones are formed by replacing a hyaline cartilage model with bone tissue. In the adult, hyaline cartilage exists in the larynx, trachea, bronchi, ribs, and on the articular surface of bones. A perichondrium is present except on articular surfaces. This cartilage may calcify with age. Hyaline cartilage provides stable movement with less friction than any prosthetic replacement, possesses properties such as resistance to compression and the ability to distribute loads that cannot be fully replaced by any other tissue or device designed to date [20;25].
- Elastic cartilage differs from hyaline cartilage primarily in its high content of elastic fibers which impart resilience. It can be found in the external ear, auditory or eustachian tube, and epiglottis. A perichondrium is present.

- Fibrocartilage differs from hyaline cartilage in possessing prominent bundles of parallel collagen fibers (type I) which give this cartilage high tensile strength. Occasionally described as a “halfway tissue”, between cartilage and connective tissue proper, it arises when connective tissue is subjected to frictional forces. Consequently, it is the only cartilage type that is not surrounded by a perichondrium. It is found in the intervertebral discs and pubic symphysis.

Cartilage tissue engineering

The main functions of articular cartilage, which contains a small number of chondrocytes in an ECM mainly composed of water, collagen type II and proteoglycans are to cover the ends in bone in joints to provide frictionless movement and to distribute loads [28;29]. Articular cartilage damage and regeneration are a major health issue. Only in USA about 21 million suffer from osteoarthritis, degenerative joint disease. Cartilage has very limited capacity for regeneration and self-repair. Its poor ability for self-repair is due to the lack of blood supply and limited number of cells, and mature chondrocytes have a relatively low metabolic activity [20].

Articular cartilage defects are classified as being either partial- or full-thickness. Partial thickness defects demonstrate disruption of the cartilage surface but this does not extend to the subchondral bone. Following the injury, nearby cells begin to proliferate, cellular attempts to fill the defect cease before it is repaired; full thickness defects arise from damage that transverses the entire cartilage thickness and penetrates the subchondral bone. In this type cells from bone marrow migrate to fill the defects [30] leading formation of less stiff and more permeable tissue than native cartilage, which degrade over a period of months [30]; it usually undergoes degeneration within six to twelve months [31]. In the most other tissues, such defects would be rapidly repaired without untoward consequences. However, adult articular cartilage fails to heal and defects will almost certainly enlarge with time [32].

Smaller lesion in cartilage can be attempted to be repaired using a variety of methods. These methods include implanting replacement tissue grafts, or employing techniques that

encourage the native repair process [33]. Autografts are limited by the small amount of cartilage available in the body for transplantation to other sites [30;33]. Allografts induce an immune response once implanted and perichondrial grafts are reported to have about 70% failure at five years [30;33-37]. In cartilage regeneration, many attempts have been made to heal or regenerate existing cartilage, rather than replace it. The proposed techniques have focused on enhancing the intrinsic regenerative properties of the tissue. Unfortunately, neither of these has been completely successful [30;33]. For regeneration enhancement, the most common treatment of cartilage degeneration is to penetrate the subchondral bone, either by abrasion or drilling. This, in essence, creates a full-thickness defect. A clot forms over the bone surface which, can provide a scaffold for migration of MSCs and their eventual differentiation into chondrocyte and osteocytes [31]. Also, this technique and other regeneration enhancement techniques have some limitations [30;33;34]. However, presently these treatments result in limited pain relief and/or restorative tissue function [38-41]. In contrast, tissue engineering has the potential to provide a supply of functional cartilage for the repair and regeneration of compromised native soft tissues [42].

There are two major tissue engineering principles employed for cartilage repair. The first type is the transplantation of chondrocytes to restore lost tissue mass. An optimal approach would be to take a small biopsy of cells, expand the number of cells, and then return them to the defect once the proper mass has been generated. This procedure has been termed autologous chondrocyte transplantation (ACT) and introduced into clinical therapy. However, the success rate has been less than 40%, due to the problem of retaining the cells in the defect for a period of time that would allow them to begin to produce matrix [43;44].

The second tissue engineering principle employs cells in scaffolds. Highly porous scaffolds may be used to maintain differentiated cells in a given area. This design is favorable because it may significantly reduce donor site morbidity and, in addition to simply providing a boundary for retention of cells, the scaffold also acts as a substrate to which the anchorage-dependent chondrocytes can adhere [45]. The tissue engineering strategy applied in this thesis follows this approach, as illustrated in (Fig. 4). We isolated the chondrocytes from bovine knees, and then we dynamically seeded the chondrocytes on polymer scaffolds

using bioreactors. For the cell seeding procedure, a cell suspension was prepared and added to the bioreactors containing the scaffolds, which were fixed on needles that were pinned to the stopper of the spinner flask. Then, the cell suspension was stirred for a period of 48 h to achieve homogeneous cell attachment to the scaffolds. Following cell seeding, the cell-polymer constructs were cultivated in vitro under treatment with different factors in order to engineer cartilage-like tissue. The time point of cultivation was varying from 1 week to 4 weeks.

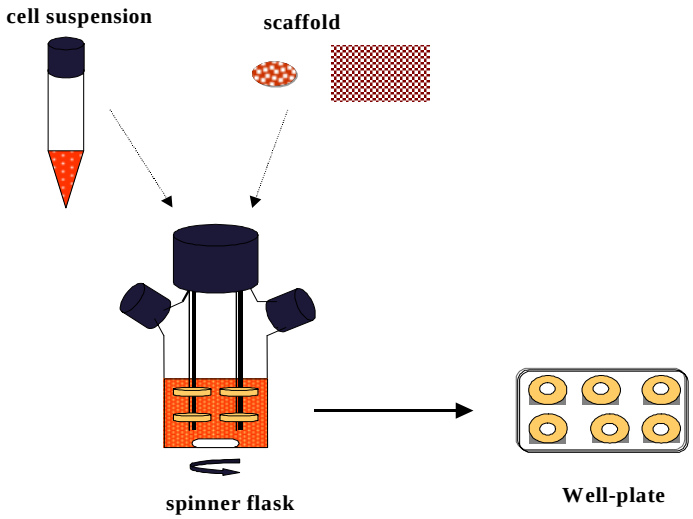

Fig. 4. *Cartilage tissue culture set-up, including dynamic seeding of chondrocytes on scaffolds using spinner flasks and cultivation of cell-polymer constructs in 6-well plates.*

Factors affecting cartilage tissue engineering

Cells

Cells are the most important factor in tissue engineering processes, they are the key to generate and repair the tissue due to their proliferation and differentiation capacities, and formation of ECM. There are several types of cells that can be used in tissue engineering of cartilage. A major sources are primary chondrocytes [46;47] or serially passaged chondrocytes in combination with use of differentiation factors [48;49]. Another promising

possibility is the differentiation of progenitor cells from bone marrow or periosteum called mesenchymal stem cells towards the chondrogenic lineage [50].

Cell carriers (scaffolds)

A porous cell carrier can provide the necessary support for a developing tissue, enabling the 3-D growth. Essential characteristics of scaffolds used for tissue engineering are their chemical, physical and biological properties. Scaffolds designed for this purpose should ideally provide the following characteristics: (a) a 3-D and highly porous structure to support cell attachment, proliferation and ECM production; (b) an interconnected/permeable pore network to promote nutrient and waste exchange; (c) a biocompatible and bioresorbable substrate with controllable degradation rates; (d) a suitable surface chemistry for cell attachment, proliferation and differentiation; (e) mechanical properties to support, or match, those of the tissues at the site of implantation; (f) an architecture which promotes formation of the native anisotropic tissue structure; and (g) a reproducible architecture of clinically relevant size and shape [51-55]. Apart from only being a support for tissue growth, specifically tailored biomaterials can also control the adhesion of cells by integrating extracellular matrix proteins or their synthetic analogous (RGD sequences) on the scaffold's surface [56]. During cultivation and especially after the transplantation of the scaffold in a defect, it can also be used to deliver active growth factors [57].

The most frequently used biodegradable synthetic polymers have been polyglycolic acid (PGA) (Fig. 5A), polylactic acid (PLA), their copolymer of poly ($_{DL}$-lactic-co-glycolic acid) (PLGA), polyvinyl alcohol (PVA) [38;41;58-60] and naturally derived biodegradable polymers are collagen (Fig. 5B) [58;61], alginate [62], hyaluronic acid, fibrin glue and chitosan [55;58;59;61]. Scaffolds are prepared not only with different materials but also with different methods in order to tailor them to a specific applications [51;63-67].

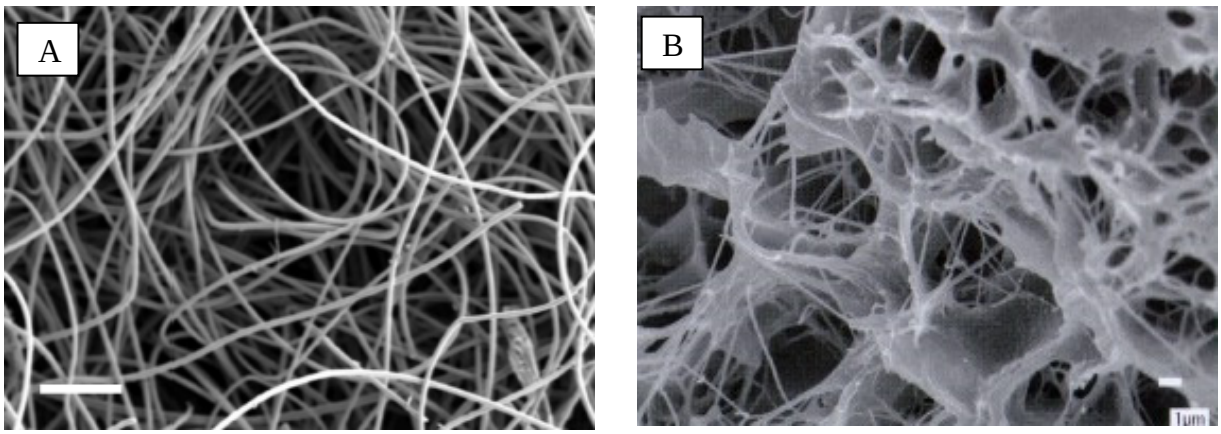

Fig. 5: SEM image: (A) 3-D PGA scaffold mesh (bar indicates 200 µm); (B) 3-D collagen scaffold (bar indicates 1 µm)

Growth factors

The generation of autologous cartilage implants for clinical use would benefit from techniques that (a) decrease the initial number of cells required, thereby minimizing donor site morbidity and (b) increase the rate of tissue growth, thereby shortening the in vitro culture period. The above improvements can be achieved by the judicious application of growth factors, i.e. either, by intermittently supplementing the culture medium with growth factors [68;69], by the use of cells genetically engineered to overexpress growth factors [70], or by polymeric systems that provide controlled release of growth factors [71;72]. Previous studies demonstrated that the exogenous addition of growth factors and some cytokines can be a powerful tool to increase the development and stimulate the formation of ECM of the in vitro grown tissue [68]. Growth factors can stimulate or inhibit cell division, differentiation and migration by binding to their cell specific receptor. They regulate cellular processes such as gene expression, DNA and protein synthesis, and autocrine and paracrine factor release. In natural cartilage tissue, factors such as IGF-I, TGF-ß and PDGF, are known to stimulate chondrocyte proliferation and differentiation and to influence cartilage growth and metabolism [73]. Consequently, some of them have been tested recently in tissue engineering of cartilage [68;74]. However, investigations of the effects of other potentially very potent bioactive protein families or single substances have still to be performed. The increase in growth rates of the engineered tissues due to the use of bioactive substances would be a great benefit especially for clinical applications. There it is very important to generate constructs of

a given size, or alternatively to produce a larger construct from a given number of harvested cells within a given time.

Goals of Thesis

This thesis focused on the evaluation of the effects of bioactive substances on the quality of engineered cartilage in vitro. Also, the potential of novel natural copolymer scaffolds as a 3-D matrix for chondrocytes were investigated. Additionally, the effects of 3-D long-term in vitro culture of chondrocytes were studied.

Bioactive substances

The first aim was to investigate the effects of the cytokine interleukin-4 and insulin-like growth factor binding proteins on the development of tissue engineered cartilage using the dynamic cell seeding system.

Interleukin-4 has been previously reported to have stimulatory effects on the development of cartilage [68;75] inducing cell proliferation and playing a major role in the maintenance of healthy cartilage [75]. Thus it may be a promising candidate for the improvement of engineered cartilage **(Chapter 3)**.

Insulin-like growth factor binding proteins (IGFBPs) have the ability to modulate the actions of the IGFs, either enhancing or inhibiting them, depending largely on their post-translational modifications and tissue localization [76;77]. They have been detected in the culture media of cultured chondrocytes from various species [78;79]. IGFBP-4 has been previously reported to have inhibitory effects on cartilage and bone tissues [80]. The effects of IGFBP-4 on engineered cartilage were studied **(Chapter 4)**. IGFBP-5 is expressed by chondrocytes and is the predominant IGFBP in bone [81;82], where it can either inhibit or increase IGF-I effects [83], in part via an IGF-I-independent mechanism [84]. In the 3-D cartilage engineering culture the hypothesis was evaluated that IGFBP-5 may function as a

growth factor in cartilage, as it has previously been suggested for bone tissue [85] **(Chapter 5)**.

Evaluation of new natural copolymer scaffolds

A copolymer scaffold made from hyaluronic acid and gelatin was previously introduced for cartilage engineering [86], partially resembling ECM components of native cartilage. As these scaffolds initially lacked mechanical stability, they were recently stabilized by chemical crosslinking [87]. These newly generated scaffolds were evaluated with regard to their utility for cartilage engineering **(Chapter 6)**.

Long-term chondrocyte culture

We have been monitoring the growth behaviour of long-term 3-D in vitro chondrocyte culture for 16 months. The focus was on the cartilage markers GAG and collagen type II, in addition the collagen type I and the ossification of the cultivated cartilage was analyzed **(Chapter 7)**.

Chapter 2

Materials and Methods

Materials:

Cells

Knee joints from 2-4 month old bovine calves were obtained from a local slaughter-house within 6-12 hours of slaughter. The fresh articular cartilage was harvested from the surfaces of the femoral patellar groove.

Instruments

Spinner flasks were self-made (250 ml volume, 6 cm bottom diameter, side arms for gas exchange). Silicon stoppers were from Schuber and Weiss (Muenchen, Germany). Silicone tubing was from Cole Palmer (Niles, IL). Needles (4 inch long, 22 gauge) were from Unimed (Lausanne, Switzerland). Magnetic stirrers were from Bellco (Vineland, NJ). 6-well plates were from Corning (Bodenheim, Germany). The Mini orbital shaker SO5 was obtained from Stuart Scientific (Surrey, UK). Mastercycler and centrifuge 5415R were obtained from Eppendorf AG (Hamburg, Germany). The freeze-drier Christ Beta 2-16 was obtained from, Martin Christ Gefriertrocknungsanlagen, GmbH (Osterode am Harz, Germany). The camera DSM 950 was from Zeiss (Oberkochen, Germany). The Kodak EDAS 290 was obtained from Fisher Scientific (Schwerte, Germany). The microtome CM 1900 was obtained from Leica (Nussloch, Germany). The Polaron SC515 was from Fisons surface systems (Grinstead, UK).

Chemicals

Type II collagenase was from Worthington (Freehold, NJ). Papainase was from CellSystem (St. Katharinen, Germany). High glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), non-essential amino acids and calcium- and magnesium-free phosphate buffered saline (PBS) were obtained from Life Technologies GmbH (Karlsruhe, Germany). Ascorbic acid, dimethylmethylene blue, diaminobenzidine (DAB), eosin, glutaraldehyde, hematoxylin, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES buffer), penicillin-streptomycin, proline, glycine, trypan blue, deoxyribonucleic acid and safranin-O were purchased from Sigma-Aldrich (Steinheim, Germany). Formalin 37 %, chloramin-T and p-dimethylaminobenzaldehyde (p-DAB) were purchased from Merck

(Darmstadt, Germany). Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA). L-hydroxyproline was from Fluka (Neu-Ulm, Germany). Chondroitin sulfate was from ICN (Aurora, Ohio, USA). Silver nitrate and OsO_4 were obtained from Carl Roth GmbH & Co. (Karlsruhe, Germany). Trizol reagent was obtained from Invitrogen GmbH (Karlsruhe, Germany). Tissue Tek was obtained from Sakura Finetek (Torrane, CA, USA). Vectastain ABC-kit and diaminobenzidine substrate (DAB) and Normal horse serum were obtained from Vector Laboratories Inc. (Burlingame, CA, USA). Type II collagen monoclonal antibody developed by Rikard Holmdahl/Kristofer Rubin was obtained from the developmental studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, USA) and Type I collagen antibody (monoclonal anti-collagen type I col-1) mouse ascites fluid, was obtained from Sigma-Aldrich (Saint Louis, Missouri 63103, USA).

Investigated proteins

- Interleukin-4 (used in chapter 3) was purchased from R&D Systems (Wiesbaden, Germany)
- Recombinant human IGF-I (used in chapter 3, 4 and 5), recombinant human IGFBP-4 (used in chapter 4) and recombinant human IGFBP-5 (used in chapter 5) were a gift of Roche Pharma Research (Penzberg, Germany)
- Bovine insulin (used in chapter 6 and 7) from bovine pancreas was obtained from Merck (Germany)

Polymer scaffolds

Polyglycolic acid (PGA) meshes

These scaffolds were obtained from Albany Int. Research (Mansfield, MA, USA). They were produced by forming PGA fibers 12-14 µm in diameter into a 96% porous non-woven mesh with a bulk density of 43 mg/cm^3. Scaffolds were prepared by die-punching into discs, which were 5 mm in diameter and 2 mm thick, sterilized 30 minutes with UV light, and pre-wetted with 70% ethanol. After rinsing thoroughly with PBS, the scaffolds were kept in culture medium and used for in vitro cell culture.

Natural copolymer scaffolds

These copolymer scaffolds were prepared in the Department of Interface Chemistry, University of Regensburg, Germany. They were made from three natural biodegradable materials (hyaluronic acid, gelatine and collagen type I) and produced by forming sponges with pore sizes between 350-450 µm. Scaffolds were prepared by die-punching into discs, which were 5 mm in diameter and 2 mm thick, sterilized 30 minutes with UV light, and pre-wetted with 70% ethanol. After rinsing thoroughly with PBS, the scaffolds were kept in culture medium and used for in vitro cell culture. These scaffolds were used only in chapter 6.

Methods

Chondrocyte isolation

Chondrocytes were isolated and cultured using sterile technique as follows [88]. The cartilage was cut into small cubes, put into centrifuge tubes with 10 ml medium per 1 g tissue. The medium contained DMEM with 4.5 g/l glucose, 5% FBS, and 532.5 U/ml of type II collagenase. The tubes were placed horizontally on a mini orbital shaker and kept at 50 rpm for 16 h at 37 °C in the incubator. The digest was repipetted, filtered through a 150 µm filter and centrifuged at 1200 rpm for 5 min. The cell pellet was washed three times with PBS and resuspended in culture medium (DMEM containing 0.4 mM proline, 10 mM HEPES buffer, 0.1 mM non-essential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml ascorbic acid and 10% FBS). The cell number and viability were determined via trypan blue exclusion using a hemocytometer and an inverted phase-contrast light microscope.

The same medium composition used for resuspension of cells was used for cell seeding. The same medium was also used for long-term cultivation of cell polymer constructs with the exception that the concentration of FBS was reduced to 1% (v/v).

In vitro cell culture

Spinner flasks were used for cell seeding [47]. Scaffolds were threaded onto needles (10 cm long, 0.5 mm diameter) and held in place with small segments of silicone tubing (1 mm long). Four needles with two scaffolds apiece were inserted into a silicone stopper, which was in turn placed into the mouth of a spinner flask. The flask was filled with 110 ml culture medium and put on a magnetic stir plate at 80 rpm in a humidified 37 °C / 5 % CO_2 incubator. After 24 h the medium was aspirated and replaced with a cell suspension containing 5 x 10^6 chondrocytes per scaffold in 110 ml culture medium. Stirring at 80 rpm in the incubator for two days allowed cell attachment to the polymer fibers.

Each cell-polymer construct was then placed in a well of a 6-well plate in 6 ml culture medium with 1 % FBS, which was placed on an orbital shaker at 50 rpm. After two days the culture medium was changed and from this time point the proteins (IL-4, insulin, IGF-I, IGFBP-4, or IGFBP-5) were added with each medium exchange three times per week for up to four weeks. IL-4 was added at concentrations of 1 and 10 ng/ml, IGF-I at 5 and 50ng/ml, and insulin at 2.5 µg/ml for 4 weeks in chapter 6 and for 16 months in chapter 7. IGFBP-4 was added at concentrations of 15.8, 157.9, 474, 1579 and 15790 ng/ml or in combinations with IGF-I in the following molar ratios 1:0.5, 1:1, 1:3, 1:10 and 1:100 (IGF-I (50 ng/ml):IGFBP-4) in chapter 4. IGFBP-5 was added at concentrations of 9.5, 95, 572 and 1909 ng/ml or in combination with IGF-I in the following ratios 1:0.5, 1:1, 1:3, and 1:10 (IGF-I (50 ng/ml):IGFBP-5) in chapter 5. Three constructs were cultured per group

Analysis of engineered tissues

Biochemical analysis

Cell-polymer constructs were analyzed as previously described [68;89]. The constructs were weighed (= wet weight) and cut in half. One part of the construct was lyophilized, then digested with a papainase solution (3.2 U/ml in buffer) and used for the determination of cell number, collagen content, and glycosaminoglycan content. The number of chondrocytes was determined fluorometrically by measuring the amount of DNA using Hoechst 33258 dye [90].

The total collagen content was determined spectrophotometrically with p-dimethylaminobenzaldehyde and hydroxyproline [91] and calculated using a hydroxyproline to collagen ratio of 1:10 [92]. The amount of sulfated glycosaminoglycans was determined spectrophotometrically at 525 nm as chondroitin sulfate using dimethylmethylene blue [93].

Histology

A part from the cell-polymer construct was prepared as a histological sample by fixing in 2% glutaraldehyde for 30 min and then storing in 10% formalin. After that, the samples were embedded in paraffin and cut into 5 µm sections; deparaffinized sections were stained with safranin-O for the detection of GAG.

Immunohistology

A part from the cell-polymer constructs were prepared for immunohistochemical staining by fixing in methanol-formalin mixture, then successively incubated in different concentrations (5% - 40%) of a sucrose solution followed by Tissue-Tek. Frozen samples were cryosectioned at 10 µm and stained with antibodies for type I collagen (monoclonal anti-collagen type I col-1) mouse ascites fluid, dilution (1: 2000) and type II collagen (DSHB), dilution (1: 6). The samples were dehydrated in PBS containing 0.1% Tween-80, antigen retrieval was performed by pepsin digestion. To prevent non-specific antibody binding, sections were incubated with 5% normal horse serum in PBS. The sections were then incubated overnight at room temperature with the primary antibodies; for control sections, PBS was used instead of the primary antibody. Further incubation with a biotinylated secondary antibody, anti-mouse/rabbit IgG (Vector Laboratories; Inc.; Burlingame, CA, USA), was performed for 30 min at room temperature after washing with PBS. The sections were then stained using a Vectastain ABC-kit and diaminobenzidine substrate (DAB) (Vector Laboratories Inc.; Burlingame, CA, USA) for avidin-biotin-peroxidase complexes formation.

Reverse transcription polymerase chain reaction (RT-PCR):

Total RNA was harvested from the cells (constructs) with the Trizol reagent and isolated according to manufacturer's instructions. First-strand cDNA was synthesized from total RNA by using Oligo $(dT)_{12-18}$ primer (Invitrogen; Karlsruhe, Germany) and Superscript II RNase H- Reverse Transcriptase (Invitrogen GmbH; Karlsruhe, Germany). Samples were incubated at 42°C for 50 min and heated afterwards for 15 min at 70°C to inactivate the enzyme. Subsequently, PCR was performed with Sawady Taq-DNAPolymerase (PeqLab, Erlangen, Germany); initial denaturation occurred at 94°C for 120 sec, final extension at 72°C for 30 sec, and holding at 4°C. The amplification was carried out using specific primers and appropriate conditions for each gene (Tab. 1), primers were purchased from MWG-Biotec AG (Ebersberg, Germany). GAPDH served as control. Reverse transcription and PCR were performed using a Mastercycler.

The PCR products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide. Finally, the gels were subjected to imaging and densitometric scanning of the resulting bands under UV light (λ = 312 nm) using a Kodak EDAS 290.

Table 1: Primer sequences and PCR conditions for the investigated genes

Oligonucleotide sequences of forward (sense) and reverse (antisense) primers. Gene amplification was performed by PCR according to the specified annealing temperatures (AT) and number of cycles for each gene. The reaction conditions of one cycle were as follows: denaturation for 45 sec at 94°C, annealing for 45 sec at the indicated temperatures, and extension for 1 min at 72°C. Table 1: Primer sequences and PCR conditions for the investigated genes.

Gene	Forward and reverse primers of examined genes	AT (°C) / Cycles
Aggrecan	5‘-CAC TGT TAC CGC CAC TTC CC-3‘ (S) 5‘-GAC ATC GTT CCA CTC GCC CT-3‘ (AS)	53/36
Biglycan	5‘-AAC ATG AAC TGC ATT GAG AT-3‘ (S) 5‘-ACT TGG AGT AGC GGA CGA GA-3‘ (AS)	47/36
MMP-1	5’-AGGTTATCCCAAAATGATAG-3’ (S) 5’-TGCAGTTGAACCAGCTATTA-3’ (AS)	47/36
MMP-3	5’-CTTTTGGCGMAAATCYCTCAG-3’ (S) 5’-AAARRAACCCAAATKCTTCAA-3’(AS)	47/36
MMP-13	5’-T(GT)C GGT C(AT)C A (CT)G CTT TTC CTC-3’ (S) 5’-GGT TGG GGT CTT CAT CTC CTG-3’ (AS)	54/36
TIMP-1	5’-CCA CCT TAT ACC AGC GTT AT-3’ (S) 5’- CCT CAC AGC CAA CAG TGT AGG-3’ (AS)	50/36
GAPDH	5‘-TGG TAT CGT GGA AGG ACT CAT-3‘ (S) 5‘-GTG GGT GTC GCT GTT GAA GTC -3‘ (AS)	53/36

Scanning electron microscopy

A part from cell-polymer constructs were rinsed three times with PBS. Afterwards, it was fixed with glutaraldehyde (2.5% in PBS) for 15 min. Cells were further fixed with an aqueous solution of osmium tetroxide (OsO_4) (1%) on ice for 30 min. The excess OsO_4 was removed with several washes with water. Then, the samples were frozen at -80 °C and freeze-dried. For scanning electron microscopy, sample were mounted on aluminium stubs using

conductive carbon tape and coated with gold-palladium (Polaron SC515). Photomicrographs were acquired at 10 kV on a DSM 950.

Von Kossa stain

Matrix mineralization of the cell-polymer constructs of the long term experiment (Chapter 7) was assessed by von Kossa silver nitrate staining. Samples were harvested after 1, 4, 8, 12, and 16 months of dynamic cultivation and fixed and cryosectioned as described in immunohistology. Then the sections were fixed with 10% formalin in PBS for 12 h. They were washed thoroughly with water to remove residuals of PBS, incubated in 5% aqueous silver nitrate solution, and exposed to natural light for 30 min. Then the sections were washed with water to remove excess of von Kossa silver nitrate staining. After that, they were counter-stained with safranin-O.

Statistical analysis

Statistical significance (* = $p<0.05$; ** = $p<0.01$ compared to control groups; + = $p<0.05$; ++ = $p<0.01$ between two groups) was assessed by one-way analysis of variance (ANOVA) in conjunction with Tukey's studentized range test, or a two-tailed unpaired Student's *t* test.

Chapter 3

Effects of Interleukin-4 on Extracellular Matrix Content and Matrix Metalloproteinase Expression in Engineered Cartilage

Introduction

Three-dimensional chondrocyte cultures can be used to investigate the usefulness of growth factors and cytokines for cartilage engineering purposes [68;94;95]. This study was performed in order to evaluate the potential of IL-4 to improve the quality of tissue-engineered cartilage.

IL-4, a 20 kD glycoprotein, is a B cell growth and differentiation factor [96;97] secreted by activated T lymphocytes. The ability of IL-4 to suppress the synthesis of the catabolic factors interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) and the arachidonic acid metabolite prostaglandin E2 in human monocytes has been reported [98]. The effect of IL-4 as an inhibitor for bone resorption has been demonstrated [99]. In human alveolar macrophages, IL-4 suppresses the biosynthesis of both interstitial collagenase and 92 kDa gelatinase [100]. IL-4 can exert its effect through an increase in mRNA levels of the IL-1 type 1 receptor (IL-1RI), a soluble receptor for neutralizing IL-1 activity in polymorphonuclear cells, and by inducing the expression and release of IL-1RII, a decoy target for IL-1 [101;102].

In cartilage explants, IL-4 has been shown to inhibit the degradation of proteoglycans, a major component of cartilage ECM [75], though the mechanisms of action are still controversially discussed [75;103-106]. Yeh et al. [75] found that IL-4 had no effect on TIMP-1 levels in bovine articular cartilage conditioned media by Western blot analysis, while Nemoto et al. [104] found that IL-4 suppresses MMP-3 synthesis in human articular chondrocytes. In contrast, Shingu et al. [103] reported the effects of IL-4 on rheumatoid arthritis chondrocytes in culture and found that IL-4 enhanced TIMP-1 production without affecting MMP-3. Cawston et al. [107] reported that IL-4 increased TIMP-1 secretion and reduced the secretion and activation of MMP-1 by bovine nasal cartilage.

In this study, the effect of IL-4 on tissue engineered cartilage was studied with a focus on the ECM content. A clearly defined 3-D culture system was employed in order to contribute to the elucidation of mechanisms of IL-4 action modulating ECM composition. RT-PCR was

utilized to evaluate the contributions of increased synthesis of various GAG subtypes and inhibition of GAG degradation. The effects of IL-4 were compared to those of IGF-I, which has potent stimulatory effects on chondrocyte proliferation and ECM synthesis and has previously been successfully employed to improve the growth rate of the cells and ECM composition in tissue engineered cartilage [68;94].

Results

Weights of engineered tissues

Wet weights of cell-polymer constructs cultured in medium supplemented with IL-4 (1 and 10 ng/ml) were not affected during the first two weeks of culture, as compared to control constructs. However, IL-4 dose dependently increased the wet weights of the constructs after four weeks of cultivation (1.7-fold increase for 10 ng/ml IL-4, compared to control). The constructs supplemented with IGF-I (50 ng/ml) showed significant increases in the wet weights over the whole course of four weeks culture compared to controls (2.8 -fold increase after 4 weeks of culture, compared to control) (Fig. 6).

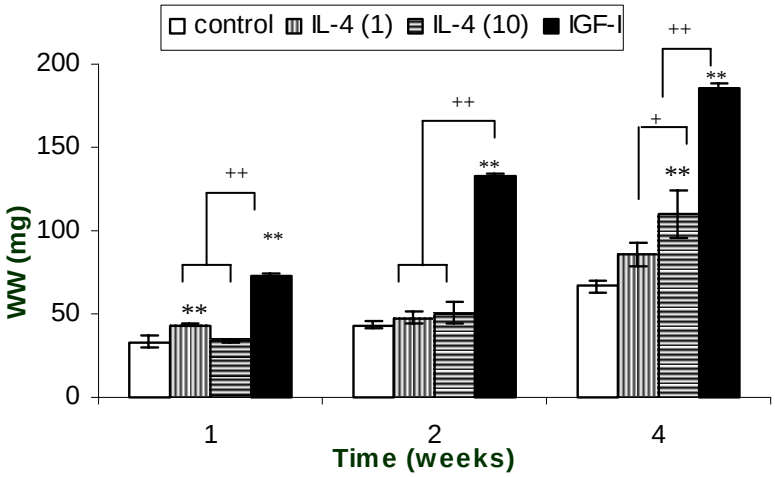

*Fig. 6: Effects of IL-4 (1 and 10 ng/ml) and IGF-I (50 ng/ml) on the wet weight of cell-polymer constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by *, significance between two groups is indicated by +.*

Cell number

The cell number per wet weight of the constructs supplemented with IL-4 (1 and 10 ng/ml) was not affected after one week; however, it was significantly decreased after two and four weeks, as compared to control constructs receiving no additional proteins. The addition of IGF-I (50 ng/ml) significantly decreased the cell number per wet weight of the constructs

throughout the whole culture period, as compared to control constructs (Fig. 7). A lower cell number within the engineered tissues is desirable in order to approach a native-like tissue quality.

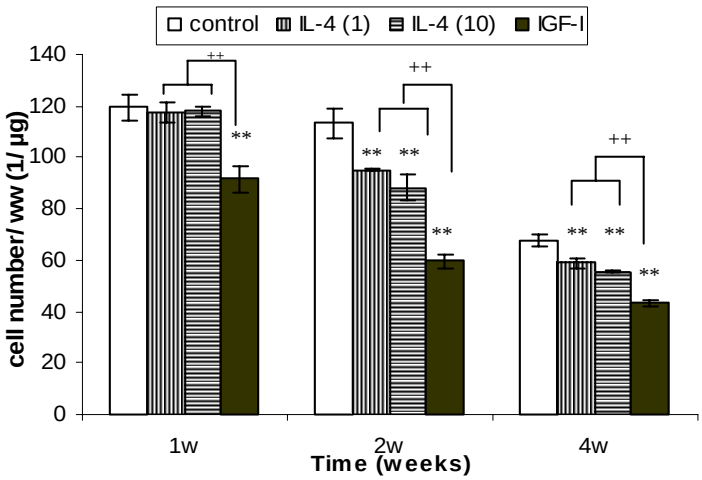

*Fig. 7: Effects of IL-4 (1 and 10 ng/ml) and IGF-I (50 ng/ml) on the cell numbers per wet weight of cell-polymer constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by *, significance between two groups is indicated by +.*

ECM quantification

The amount of GAG per wet weight was significantly increased in the constructs treated either with IL-4 (1 and 10 ng/ml) or IGF-I (50 ng/ml) after one and four weeks (1.4-fold increases after four weeks compared to control). (Fig. 8). The total amount of collagen per wet weight of the constructs was significantly increased with the addition of IL-4 (1 ng/ml) after one and two weeks; the effects were not significant after 4 weeks. In contrast, IL-4 at 10 ng/ml significantly increased the amount of collagen per ww of the constructs after two and four weeks, as compared to control. The addition of IGF-I (50 ng/ml) to the culture medium significantly increased the amount of collagen per wet weight in the constructs after one and two weeks, while after four weeks the effect of IGF-I was not significant, as compared to controls (Fig. 9).

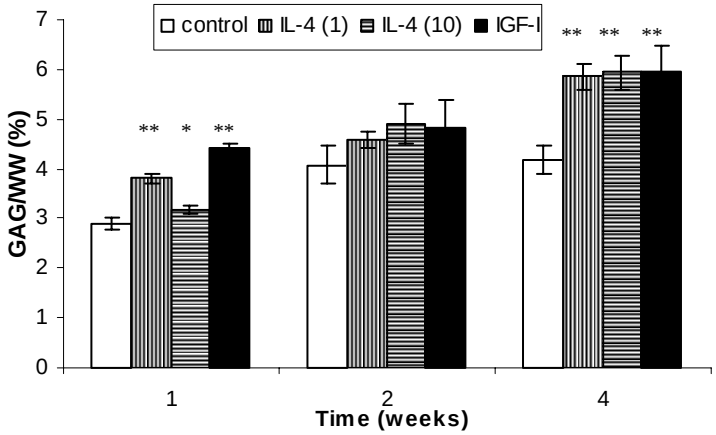

*Fig. 8: Effects of IL-4 (1 and 10 ng/ml) and IGF-I (50 ng/ml) on the GAG per wet weight of cell-polymer constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by *, significance between two groups is indicated by +.*

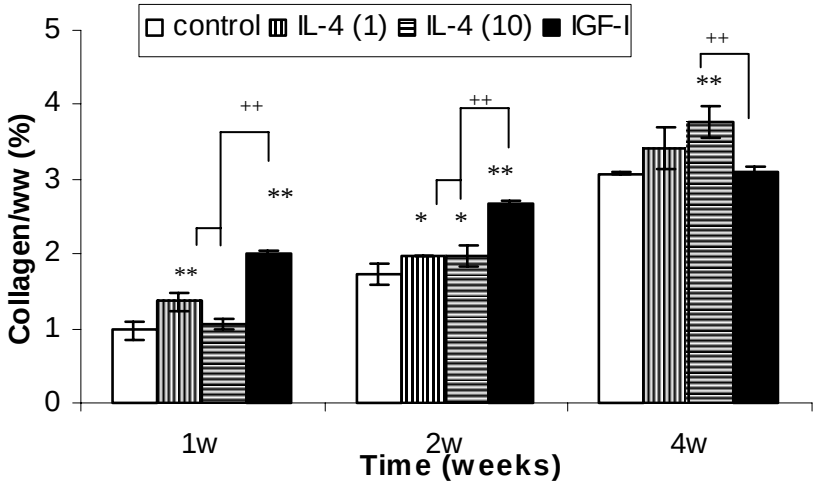

*Fig. 9: Effects IL-4 (1 and 10 ng/ml) and IGF-I (50 ng/ml) on the collagen per wet weight of cell-constructs cultured for four weeks. Three independent long-term cell culture experiments were performed; result from a representative experiment was shown here. Data represent the average ± SD of three. Significance to the control is indicated by *, significance between two groups is indicated by +.*

In another experiment combinations of IL-4 and IGF-I (1:50 and 10:50 ng/ml) were added into the culture medium of the constructs over four weeks of cultivation. The wet weight of the constructs as well as the amounts of GAG and collagen per ww were increased,

as compared to the controls and compared to either IGF-I or IL-4 alone. Also, these combinations decreased the cell number per ww of the constructs (data not shown).

Histology

Safranin-O staining confirmed the results from GAG quantification: Compared to control constructs, the GAG content was considerably higher in constructs supplemented with either IL-4 or IGF-I after 4 weeks. However, only constructs receiving IL-4 exhibited a homogeneous GAG distribution throughout the whole cross-section, i.e., also at the edges of the construct (Fig. 10). In IGF-I supplemented cell-polymer constructs there was a zone at the edges of the sections depleted of GAG, this zone stained green and appeared slightly lights in the black and white print.

Collagen type II, a marker for differentiated chondrocytes was detected immunohistologically at high levels and homogenously distributed in the constructs supplemented with IL-4 (1 and 10 ng/ml) as well as in the constructs supplemented with IGF-I and in control constructs (Fig. 11).

Collagen type I was detected at the surfaces of the control and IGF-I supplemented constructs corresponding to the zones that contained flat, elongated, fibroblast-like cells. This zone almost disappeared in the constructs supplemented with IL-4 (1 ng/ml) and appeared very thin in the constructs supplemented with IL-4 (10 ng/ml) (Fig. 11).

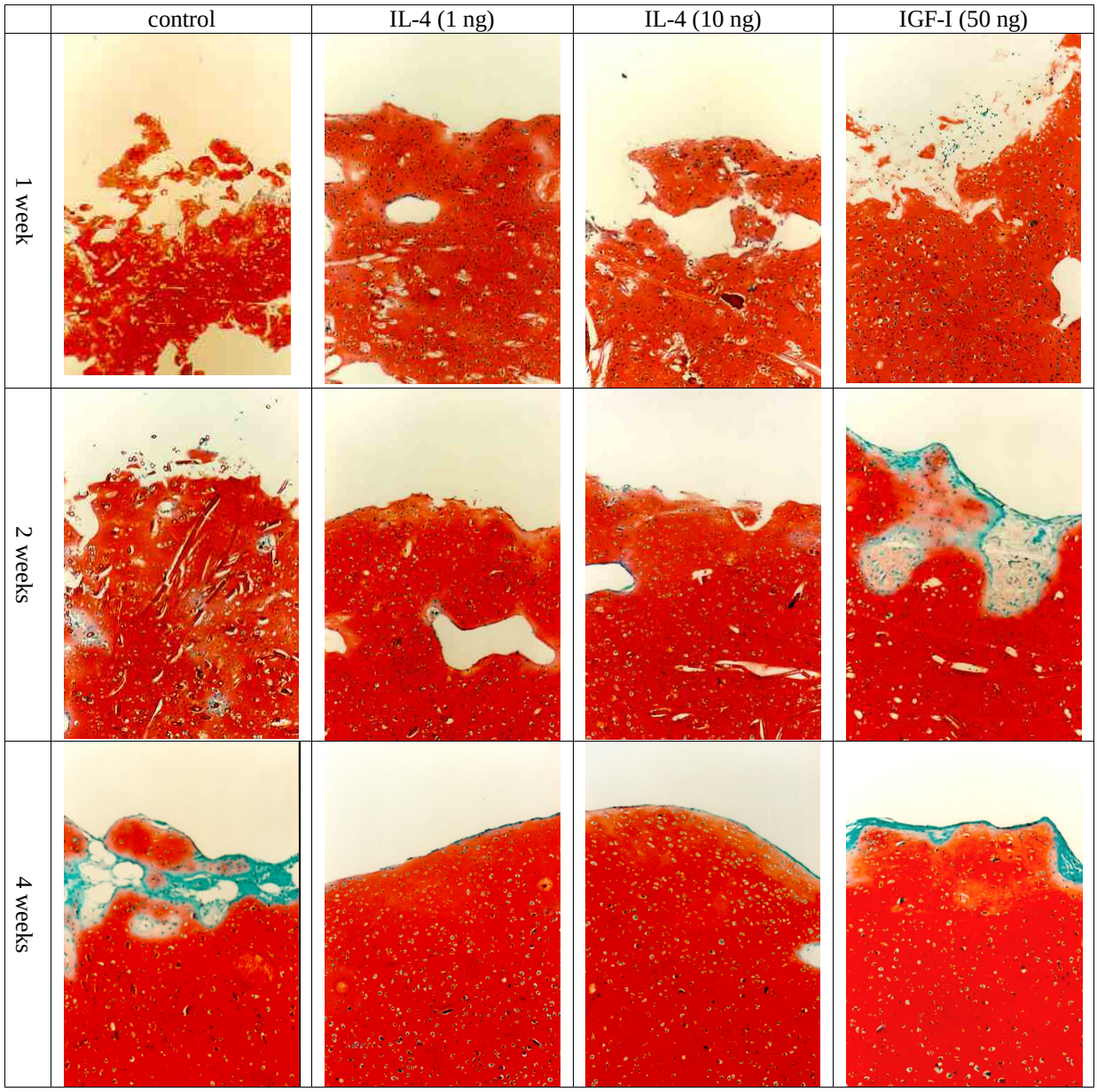

Fig. 10: Histological cross-sections of one, two and four week constructs grown in culture medium with 1% FBS: GAG in ECM was stained red with safranin-O (dark color in the black and white print) (x 100). Three independent long-term cell culture experiments were performed; result from a representative experiment was shown here.

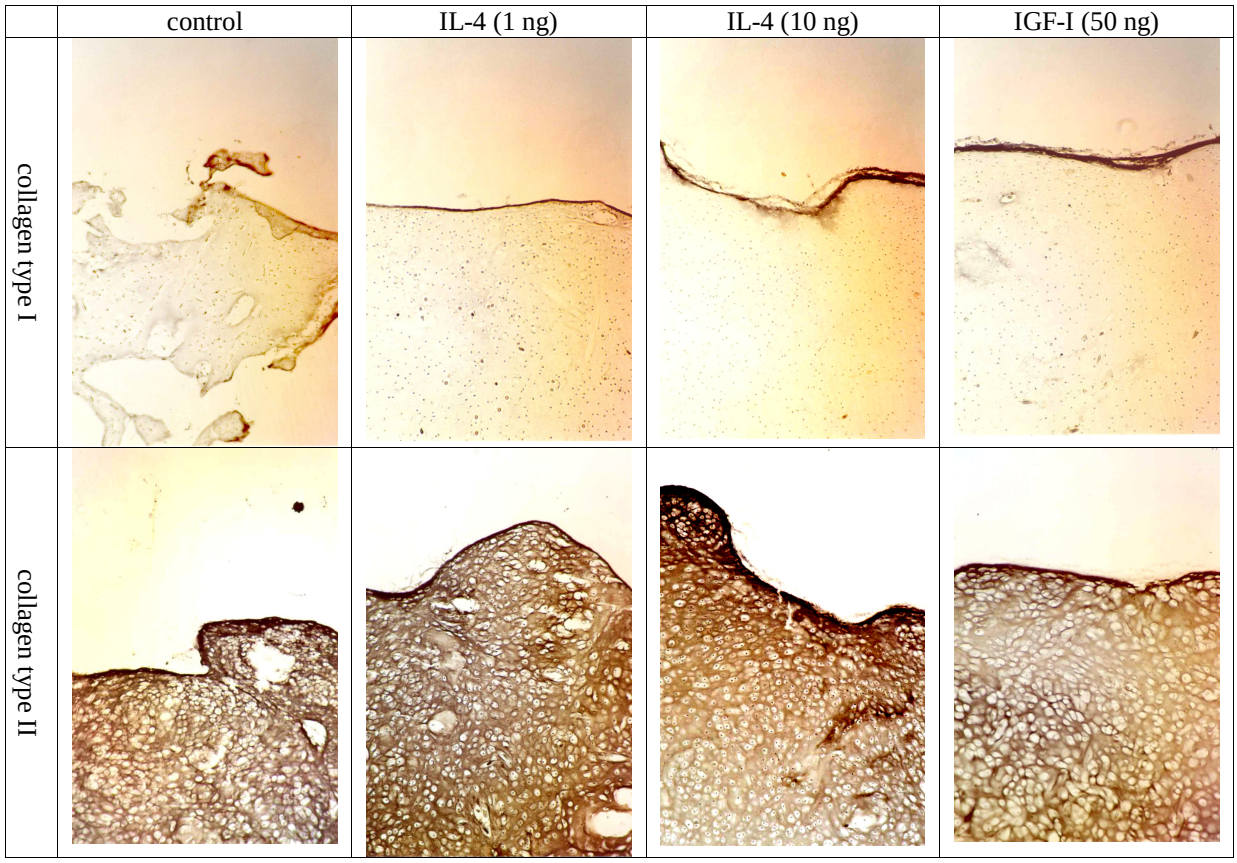

Fig. 11: Immunohistological cross-sections of four week constructs grown in culture medium with 1% FBS: collagen type I and II were stained black with collagen type I and II antibodies (x 100). Immunohistological staining was performed after two independent long-term cell culture experiments; result from a representative experiment was shown here.

Examination of mRNA gene expression

In order to gain further insight into how IL-4 increases the GAG content in the cultivated constructs, investigations into mRNA gene expression were performed by means of RT-PCR at days 0, 7, 14 and 28 of cultivation. We focused on analysis of the GAG subtypes aggrecan and biglycan, the degradatory enzymes matrix metalloproteinases MMP-1, -3 and -13, and the tissue inhibitor of metalloproteinase TIMP-1.

Examination of the expression of the GAG subtypes aggrecan and biglycan revealed that all band intensities were not affected by the addition of IL-4 to the construct cultures during the whole period of cultivation compared to controls. Also, the addition of IL-4 to the construct had no effect on any of the band intensities of TIMP-1. The addition of IL-4 to the culture medium had no effect on the band intensities of MMP-1 and only slightly increased the expression of MMP-3. However, the addition of IL-4 into the culture medium strongly inhibited the expression of MMP-13. The effect of IL-4 on MMP-13 expression was increased with time of exposure to IL-4, i.e., there were moderate effects after one and two weeks, but after four weeks the expression of MMP-13 was strongly inhibited compared to controls and also compared to constructs receiving IGF-I (Fig. 12)

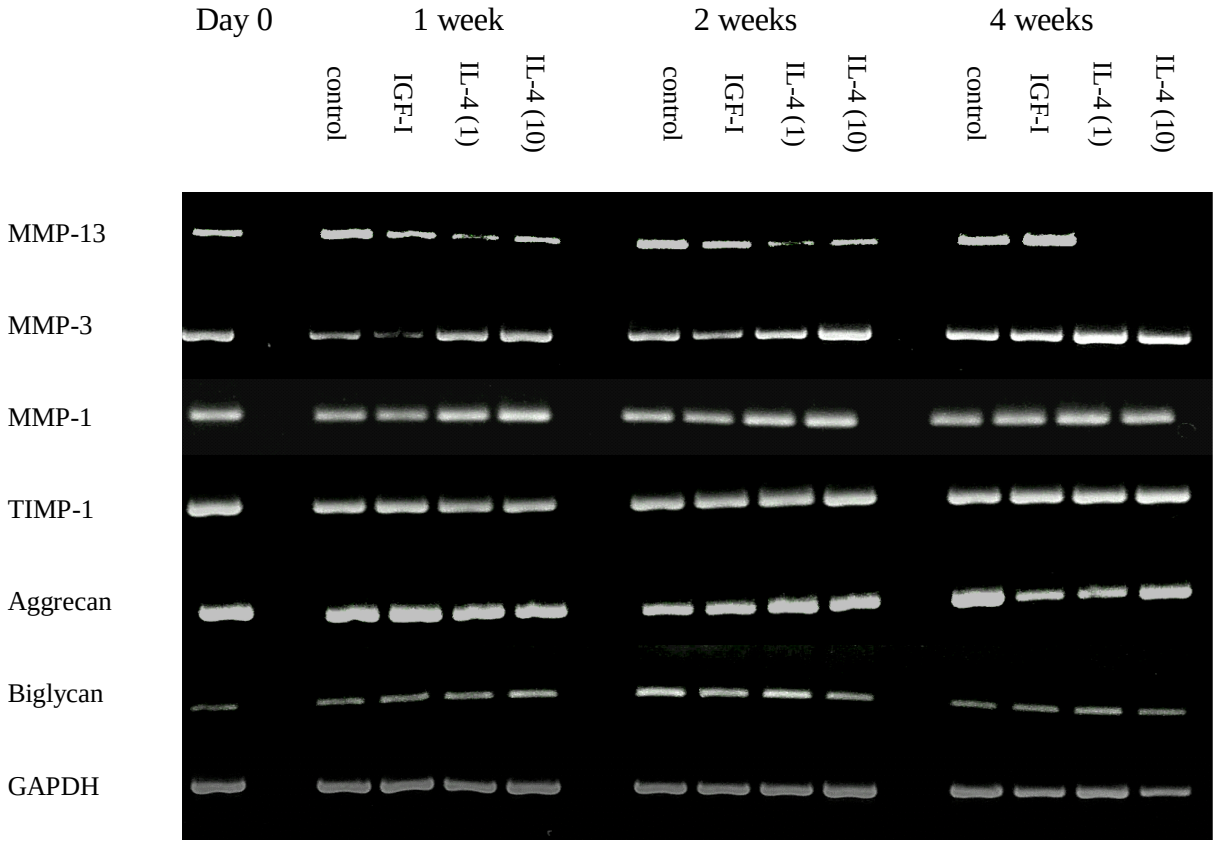

Fig. 12: Evaluation of differential gene expression in engineered cartilage at days 0, 7, 14 and 28 of cultivation in medium containing 1% FBS with and without IGF-I and IL-4 by means of RT-PCR. MMP-1, -3 and -13, TIMP-1, aggrecan, biglycan and GAPDH were investigated. Three independent long-term cell culture experiments were performed; results from a representative experiment were shown here.

Discussion

This study was designed to evaluate the usefulness of IL-4 for tissue-engineered cartilage grown in a well-established in vitro model system, focusing on its ability to increase the amount of ECM, especially the amount of GAG. The effects of IL-4 were compared to those of IGF-I, which has been shown to have potent stimulatory effects on chondrocyte proliferation and ECM synthesis [68;94].

In this study IL-4 (1 and 10 ng/ml) increased the weight of constructs and increased the formation of extracellular matrix, particularly GAG content after long-term treatment (4 weeks). IL-4 also affected the GAG distribution in the constructs: In IL-4 supplemented constructs GAG was distributed throughout the whole cross-section of the construct, whereas both control and IGF-I supplemented groups lacked GAG at the edge of the constructs.

IL-4 has been reported to affect GAG content in cartilage in previous studies, however, as of yet the precise mechanism of action of IL-4 on GAG is not fully understood. IL-4 has been shown to have the ability to inhibit the proteoglycan degradation in cartilage [75]. IL-4 appeared to either suppress MMP-3 synthesis [104] or downregulate other cartilage degradatory systems, but without affecting TIMP-1 levels [75]. In contrast, Shingu et al. [103] investigated the effects of IL-4 using rheumatoid arthritis (RA) chondrocytes in culture and found that IL-4 enhanced TIMP-1 production without affecting MMP-3. The contrasting reports may be explained on the basis of differential species or disease responsiveness. As a further potential mechanism it has been reported that IL-4 interferes with IL-1 or TNF-alpha, inhibitors of proteoglycan synthesis [108]. IL-4 can potentially shift the balance between GAG synthesis and degradation. In many cell types IL-4 was shown to diminish IL-1 gene expression and/or to promote the expression of the IL-1 receptor antagonist protein that blocks the action of IL-1 [105;106;109]. Suppression of IL-1 synthesis may lead in turn to blocking of the IL-1 stimulated MMP-3 production. Furthermore, the potent anti-inflammatory properties of IL-4 in various cell types indicate that IL-4 has a protective role in the catabolic pathway of osteoarthritis (OA) [110;111].

RT-PCR was used to contribute to the elucidation of the mechanisms by which IL-4 increased the GAG content in the cultivated cell-polymer constructs. The mechanisms of enhancing effects of IL-4 on the ECM formation might include one of the following: 1) inhibiting the release of the matrix metalloproteinases (MMP-1, MMP-3 and MMP-13); 2) increasing the release of the tissue inhibitor matrix metalloproteinase (TIMP-1); and 3) increasing GAG subtype synthesis (aggrecan and biglycan). Whereas effects of IL-4 on MMP-1, MMP-3, and TIMP-1 expression have been suggested previously, this study was the first to investigate the effects of IL-4 on MMP-13 expression. The results of this study indicated that IL-4 had distinct inhibitory effects on MMP-13 gene expression without significantly affecting the other factors. These inhibitory effects on MMP-13 expression increased with time of IL-4 treatment; the most dramatic effects were observed at late stages of the culture, corresponding well with the late increase of GAG content provoked by IL-4.

MMP-13 has been shown to degrade collagen type I, II and III, preferably type II, which is the primary collagen found in articular cartilage [112]. The primary role of MMPs , particularly MMP-1 and MMP-13, in the specific cleavage of the triple helix of type II collagen in arthritis is well-established [113;114]. MMP-13 also degrades the cartilage proteoglycan aggrecan. It cleaves aggrecan at three sites, that is, in the interglobular domain (IGD) at the same site identified for other members of the MMP family, and also at another site not observed for other MMPs [115]. Little et al. [116] reported that MMPs were responsible for C-terminal catabolism of aggrecan and the generation of chondroitin sulfate (CS). Recombinant MMP-1, -3 and –13 were all capable of C-terminally truncating aggrecan with at least two cleavage sites on the N-terminal side of the CS attachment domains of aggrecan. All three MMPs resulted in a very similar pattern of metabolites, suggesting that they cleave similar sites within the aggrecan C-terminus, although MMP-1 appeared to be the most efficient at generating the 75 kDa metabolite. Digestion with MMP-13 for 16 h resulted in an intermediate band of approximately 100 kDa, suggestive of an additional C-terminal cleavage site by this enzyme when compared with the two other MMPs tested. It has been reported that MMPs also have cleavage sites within the CS attachment regions of aggrecan as well as the IGD [117;118].

Summary

The study was designed to evaluate the effects of interleukin-4 (IL-4) on tissue engineered cartilage focusing on its ability to modulate the extracellular matrix (ECM) content. Bovine articular chondrocytes were seeded on polyglycolic acid scaffolds in spinner flask for two days, after which cell-polymer constructs were transferred into 6-well plates. Constructs were cultured for up to four weeks in medium containing 1% FBS either with or without supplemented proteins.

IL-4 exhibited profound stimulatory effects on engineered cartilage. It increased the wet weight of the cell-polymer constructs and decreased the cell number per wet weight. As detected by biochemical analysis and histological staining, IL-4 significantly increased the fractions of glycosaminoglycans (GAG) per wet weight and improved the GAG distribution minimizing GAG depleted areas at the construct edges which were observed in control constructs.

The mechanism by which IL-4 increases the GAG content might be either inhibition of GAG degradation or increased GAG subtype synthesis. In order to elucidate the respective contributions, RT-PCR was employed to investigate the expression of (1) matrix metalloproteinases MMP-1, MMP-3, MMP-13; (2) tissue inhibitor of metalloproteinases-1 (TIMP-1); and (3) GAG subtypes aggrecan and biglycan. IL-4 was demonstrated to have distinct inhibitory effects on MMP-13 expression without significantly affecting any of the other factors investigated. Our results suggest that IL-4 increases the GAG fractions in engineered cartilage at least in part by decreasing the MMP-13 expression and thus inhibiting GAG degradation.

Chapter 4

Effects of Insulin-like Growth Factor Binding Protein-4 on Engineered Cartilage

Introduction

IGFBPs are a family of six or more related proteins with a high affinity for IGF-I [119-121]. This group of proteins have the ability to modulate the actions of the IGFs, either enhancing or inhibiting them, depending largely on their post-translational modifications and tissue localization [76;77]. IGFBPs-1 to -6 have been isolated and their cDNAs have been cloned [122]. They have been detected in culture media of cultured articular chondrocytes [78;79;81;123] and in intact bovine cartilage [124]. The predominant IGFBPs produced by rat articular chondrocytes were IGFBP-3, -4 and -5 [125]. An increase in IGFBP mRNAs in osteoarthritic chondrocytes and in the culture medium of osteoarthritic cartilage slices and in monolayer culture has been observed [126-128]. In osteoarthritic cartilage, the normal anabolic function of IGF-I may be disrupted. Chondrocytes from animals with experimental arthritis and from patients with osteoarthritis are non-responsive to IGF-I, although they express increased levels of IGF-I and the IGF-I receptor [128;129]. These findings may be explained by the hypothesis that the IGFBPs play an inhibitory role during the disease, blocking the actions of IGF-I [126-128].

IGFBP-4 has been proposed to play an important role in the regulation of cartilage and bone formations. It has been shown to inhibit the growth of embryonic chick pelvic cartilage in vitro [80]. It is a potent inhibitor of IGF-induced cell proliferation in a number of cell types in vitro [130]. In vitro studies on the mechanism by which IGFBP-4 inhibits osteoblast cell proliferation show that IGFBP-4 may inhibit IGF actions in osteoblasts by preventing the binding of IGF ligand to its membrane receptors [130;131].

In this study, the hypothesis that IGFBP-4 inhibits the growth of cartilage in absence of IGF-I was evaluated in a well-established 3-D culture system. In addition, the inhibitory effects of IGFBP-4 on the actions of IGF-I, which has potent stimulatory effects on chondrocyte proliferation and extracellular matrix synthesis [68], were investigated. Specifically, we investigated if exogenous IGFBP-4 was able to inhibit the growth rate and compromise the concentrations of ECM components of cell-polymer constructs. Also, the effects of IGFBP-4 in combination with IGF-I on in vitro engineered cartilage were studied in the same model.

Results

Wet weight

The addition of IGF-I (5 and 50 ng/ml) to the culture medium increased the construct wet weights by up to 1.8-fold for 50 ng/ml, compared to control values, over 4 weeks of cultivation (Fig. 13A and Table 2). The addition of IGFBP-4 (15.8–15790 ng/ml) to the culture medium significantly decreased construct wet weights in a dose-independent manner to as low as 15 % of control values over 4 weeks of cultivation. In detail, IGFBP-4 at 15.8 ng/ml did not change the construct wet weight significantly, while at higher concentrations of 158, 474, 1579, and 15790 ng/ml, it significantly decreased the construct wet weights to 78, 56, 29 and 15% of control values, respectively (Fig. 13A and Table 2).

The combination of IGF-I (50 ng/ml) and IGFBP-4 in different molar ratios (MRs) decreased construct wet weights over 4 weeks of cultivation, as compared to IGF-I alone. In detail, 1:0.5, 1:1 and 1:3 MRs decreased the weights to 54-62%, whereas at 1:10 and 1:100 the weights were decreased to 42% and 12% of IGF-I values (Fig. 13B).

(Please note: The molecular weight of IGFBP-4 is 24 kDa, that of IGF-I is 7.6 kDa. Therefore, in order to employ molar ratios of 1:0.5, 1:1, 1:3, 1:10 and 1:100, IGFBP-4 concentrations of 78.9, 157.9, 474, 1579 and 15790 ng/ml had to be used in combination with 50 ng/ml of IGF-I (Fig. 13B). Based on these concentrations, the concentrations for the experiments employing IGFBP-4 alone were chosen (Fig. 13A)).

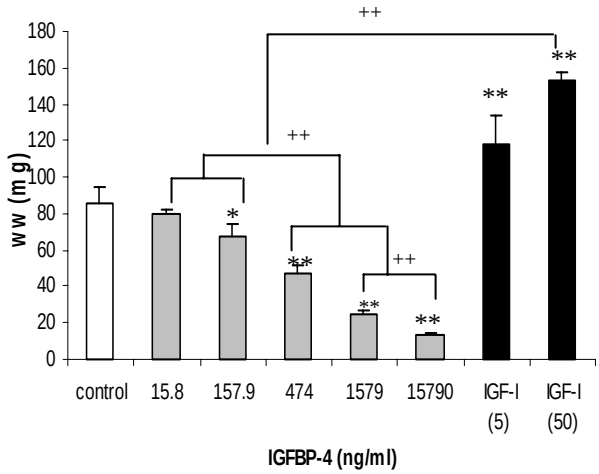

*Fig. 13A: Effects of IGFBP-4 (15.8-15790 ng/ml) and IGF-I on the wet weight of constructs cultured for four weeks. Data represent the average ± SD of three independent measurements. Significance to the control is indicated by *, Significance between two groups is indicated by +.*

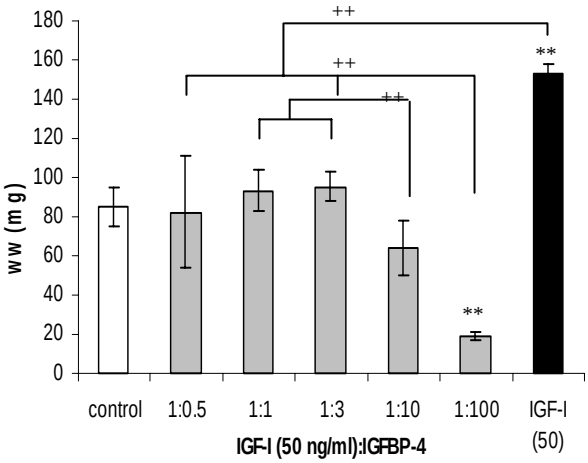

*Fig. 13B: Effects of IGF-I (50 ng/ml) and IGF-I: IGFBP-4 in different molar ratios on the wet weight of constructs cultured for four weeks. Data represent the average ± SD of three independent measurements. Significance to the control is indicated by *, significance between two groups is indicated by +.*

Cell number

The addition of IGF-I at 5 and 50 ng/ml to the culture medium of the cell-polymer constructs decreased the cell number per wet weight of the constructs (to 72% for 50 ng/ml of control values) (Fig. 14A and Table 2). Addition of IGFBP-4 (15.8–15790 ng/ml) to the culture medium of the cell-polymer constructs increased the cell number per wet weight in a dose-independent manner (up to 3.9-fold increase for 15790 ng/ml compared to control values) (Fig. 14A and Table 2).

The combination of IGF-I (50 ng/ml) and IGFBP-4 in different MR significantly increased the cell number per construct wet weight as compared to IGF-I alone, that is, MR of 1:0.5 to 1:10 led to 1.5-fold to 2.1-fold increases, whereas a MR of 1:100 increased the cell number per weight 4.5-fold, as compared to IGF-I (Fig. 14B).

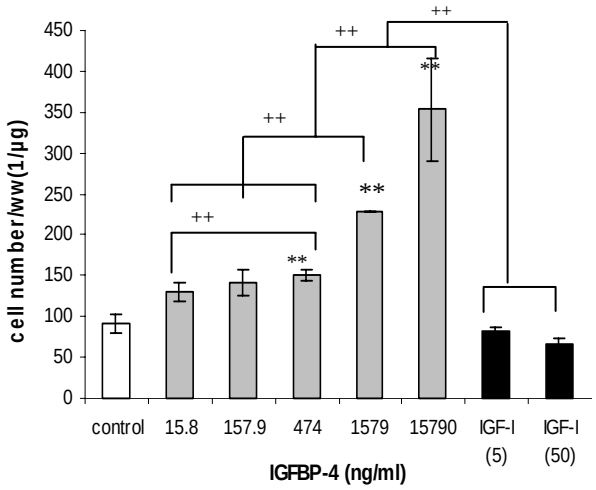

*Fig. 14A: Effects of IGFBP-4 (15.8-15790 ng/ml) and IGF-I on the cell number per wet weight of constructs cultured for four weeks. Data represent the average ± SD of three independent measurements. Significance to the control is indicated by *, significance between two groups is indicated by +.*

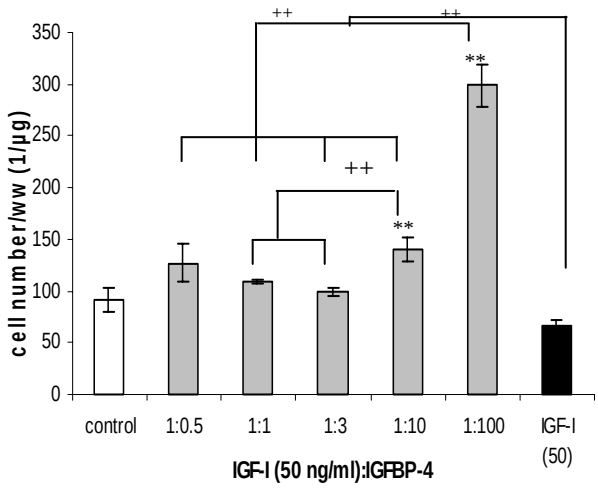

*Fig. 14B: Effects of IGF-I (50 ng/ml) and IGF-I: IGFBP-4 in different molar ratios on the cell number per wet weight of constructs cultured for four weeks. Data represent the average ± SD of three independent measurements. Significance to the control is indicated by *, significance between two groups is indicated by +.*

Major ECM components

Collagen fractions

IGF-I (50 ng/ml) increased the amount of total collagen in the cell-polymer constructs 3.5-fold, as compared to controls; the fraction of collagen per wet weight was increased 1.9-fold. The addition of IGFBP-4 (15.8–15790 ng/ml) to the culture medium of the cell-polymer constructs decreased the amount of collagen in a dose-dependent manner. In detail, IGFPB-4 at 15.8 ng/ml did not affect the amount of collagen per construct, and at higher concentrations of 158, 474, 1579, and 15790 ng/ml, IGFBP-4 decreased the amount of collagen per construct to 84, 57, 28 and 11% of the control values, respectively. The fraction of collagen per wet weight was only significantly changed at 15790 ng/ml (decrease to 73%) (Fig. 15A and Table 2).

The combination of IGF-I (50 ng/ml) and IGFBP-4 drastically decreased collagen amounts per construct. In detail, at 1:0.5, 1:1 and 1:3 molar ratios, collagen amounts where

reduced to 38-39%, whereas at 1:10 and 1:100, collagen amounts were reduced to 23% and 5.2%, respectively, as compared to IGF-I alone. The collagen fraction per wet weight was also reduced at all molar ratios, as compared to IGF-I alone (44% at 1:100) (Fig. 15B and Table 3).

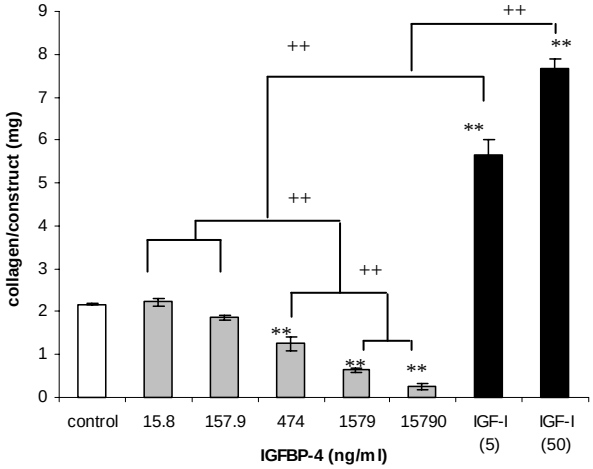

*Fig. 15A: Effects of IGFBP-4 (15.8-15790 ng/ml) and IGF-I on the amount of total collagen per construct cultured for four weeks. Data represent the average ± SD of three independent measurements. Significance to the control is indicated by *, significance between two groups is indicated by +.*

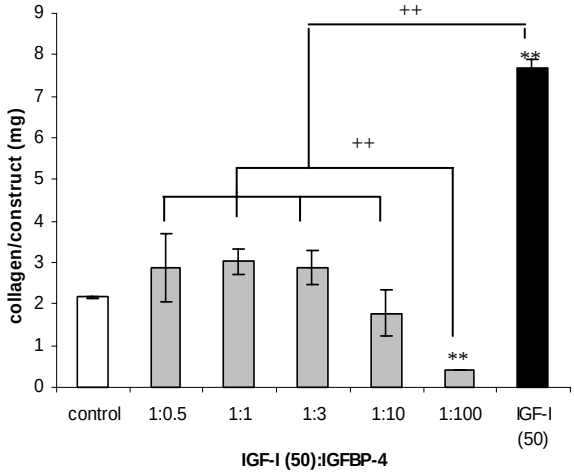

*Fig. 15B: Effects of IGF-I (50 ng/ml) and IGF-I: IGFBP-4 in different molar ratios on the amount of total collagen per construct cultured for four weeks. Data represent the average ± SD of three independent measurements. Significance to the control is indicated by *, significance between two groups is indicated by +.*

GAG fractions

IGF-I at 50 ng/ml increased the amount of GAG per cell-polymer construct 3-fold and the fraction of GAG per wet weight 1.6-fold, as compared to control constructs. The addition of IGFBP-4 (15.8–15790 ng/ml) to the culture medium dose-dependently decreased the amounts of GAG per construct. In detail, IGFBP-4 at 15.8 and 158 ng/ml did not significantly affect the amount of GAG, while at higher concentrations (474, 1579, and 15790 ng/ml) it significantly decreased the amount of GAG per construct to 72, 38, and 13% of control values, respectively. The GAG fraction per wet weight was significantly decreased (to 72%) only at a concentration of 15790 ng/ml (Fig. 16A and Table 2).

The combination of IGF-I (50 ng/ml) and IGFBP-4 drastically decreased the amounts of GAG per construct, as compared to IGF-I alone. Whereas molar ratios of 1:0.5 to 1:3

resulted in decreases to 43-46%, at molar ratios of 1:10 and 1:100, GAG per construct was reduced to 24% and 6.1%, respectively, of IGF-I values. The GAG fraction per wet weight was also reduced at all molar ratios, as compared to IGF-I alone (47% at 1:100) (Fig. 16B and Table 3).

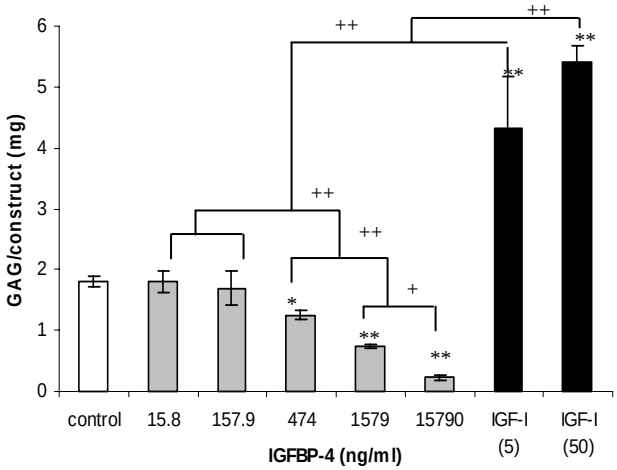

*Fig. 16A: Effects of IGFBP-4 (15.8-15790 ng/ml) and IGF-I on the amount of glycosaminoglycan per construct cultured for four weeks. Data represent the average ± SD of three independent measurements. Significance to the control is indicated by *, significance between two groups is indicated by +.*

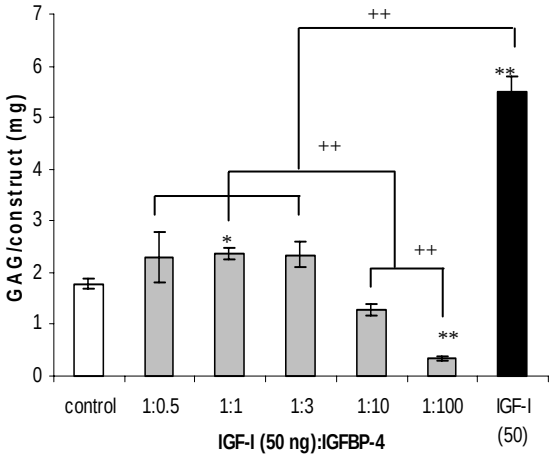

*Fig. 16B: Effects of IGF-I (50 ng/ml) and IGF-I: IGFBP-4 in different molar ratios on the amount of glycosaminoglycan per construct cultured for four weeks. Data represent the average ± SD of three independent measurements. Significance to the control is indicated by *, significance between two groups is indicated by +.*

Table 2: Comparison of four week cell-polymer constructs cultured without or with supplemental IGF-I (5 and 50 ng/ml) or supplemental IGFBP-4 (15.8-15790 ng/ml).

	conc.	ww	cell number per ww	collagen/ww	collagen/ construct	GAG/ww	GAG/ construct
	ng/ml	mg	1/µg	%	mg	%	mg
control	**0**	85 ± 9.8	91 ± 12	2.6 ± 0.06	2.2 ± 0.02	2.2 ± 0.23	1.8 ± 0.09
IGF-I	**5**	117± 16	82 ± 4	4.7± 0.37	5.6 ± 0.35	3.7 ± 0.48	4.3 ± 0.86
	50	153 ± 5	66 ± 6.8	5.0 ± 0.08	7.66 ± 0.24	3.6 ± 0.05	5.4 ± 0.27
IGFBP-4	**15.8**	80 ± 2	130 ± 11	2.8 ± .04	2.22 ± 0.09	2.3 ± 0.29	1.8 ± 0.19
	158	67 ± 7	142 ± 16	2.8 ± 0.22	1.86 ± 0.04	2.6 ± 0.15	1.7 ± 0.28
	474	47 ± 4.4	150 ± 6	2.7 ± 0.38	1.26 ± 0.16	2.7 ± 0.13	1.3 ± 0.08
	1579	25 ± 2	230 ± 0.2	2.5 ± 0.28	0.63 ± 0.04	2.8 ± 0.21	0.7 ± 0.04
	15790	13 ± 2	353 ± 61	1.9 ± 0.21	0.25 ± 0.06	1.7 ± 0.34	0.23 ± 0.04

Table 3: Comparison of four week cell-polymer constructs cultured without or with supplemental IGF-I (50 ng/ml) or supplemental IGF-I (50 ng/ml): IGFBP-4 (1:0.5, 1:1, 1:3, 1:10 and 1:100 molar ratio).

	conc.	ww	cell number per ww	collagen/ww	collagen/ construct	GAG/ww	GAG/ construct
	ng/ml	mg	1/µg	%	mg	%	mg
control	**0**	85 ± 9.8	91 ± 12	2.6 ± 0.06	2.2 ± 0.02	2.2 ± 0.23	1.8 ± 0.09
IGF-I	**50**	153 ± 5	66 ± 6.8	5.0 ± 0.08	7.7 ± 0.24	3.6 ± 0.05	5.4 ± 0.27
IGF-I: IGFBP-4	**1:0.5**	82 ± 28	126 ± 18	3.53 ± 0.16	2.9 ± 0.82	2.8 ± 0.7	2.3 ± 0.48
	1:1	93 ± 10	108 ± 2.1	3.3 ± 0.06	3.0 ± 0.3	2.6 ± 0.18	2.5 ± 0.11
	1:3	95 ± 7	99 ± 3.2	3.1 ± 0.27	2.9 ± 0.4	2.5 ± 0.17	2.4 ± 0.12
	1:10	64 ± 14	140 ± 12.3	2.8 ± 0.35	1.8 ± 0.5	2.0 ± 0.2	1.3 ± 0.12
	1:100	19 ± 1.7	299 ± 20	2.2 ± 0.2	0.4 ± 0.01	1.7 ± 0.09	0.33 ± 0.04

Histology

Constructs grown under control conditions and in IGF-I–supplemented medium appeared histologically cartilaginous with round chondrocytes in lacunae and an ECM that stained positively with safranin-O (Fig. 17A and B). The distribution of GAG was spatially uniform throughout the constructs of the IGF-I (50ng/ml) group. Constructs grown in the presence of IGFBP-4 appeared histologically partially un-cartilaginous especially at higher concentrations (1579 and 15790 ng/mL) with an elevated number of small un-differentiated cells (Fig. 17 D and E) and only small amounts of GAG in the core of the construct (Fig. 17 E) Also, constructs grown in the presence of combinations of IGF-I (50 ng/mL) and IGFBP-4, at higher MRs (1:100) appeared histologically partially un-cartilaginous with small undifferentiated cells (Fig. 17, G and H) and only a small amount of GAG appeared in the core of the construct (Fig. 17 H)

We can summarize that the addition of IGF-I and IGFBP-4 combinations to the culture medium at 1:0.5, 1:1 and 1:3 MR slightly increased construct wet weights and ECM components, while at 1:10 and especially at 1:100 MR, the construct wet weights and/or ECM components were decreased in comparison to the control group of constructs. When we compared these results with the constructs supplemented only with IGF-I, we found that the combinations have significant inhibitory effects on the construct wet weights and ECM components at all molar ratios.

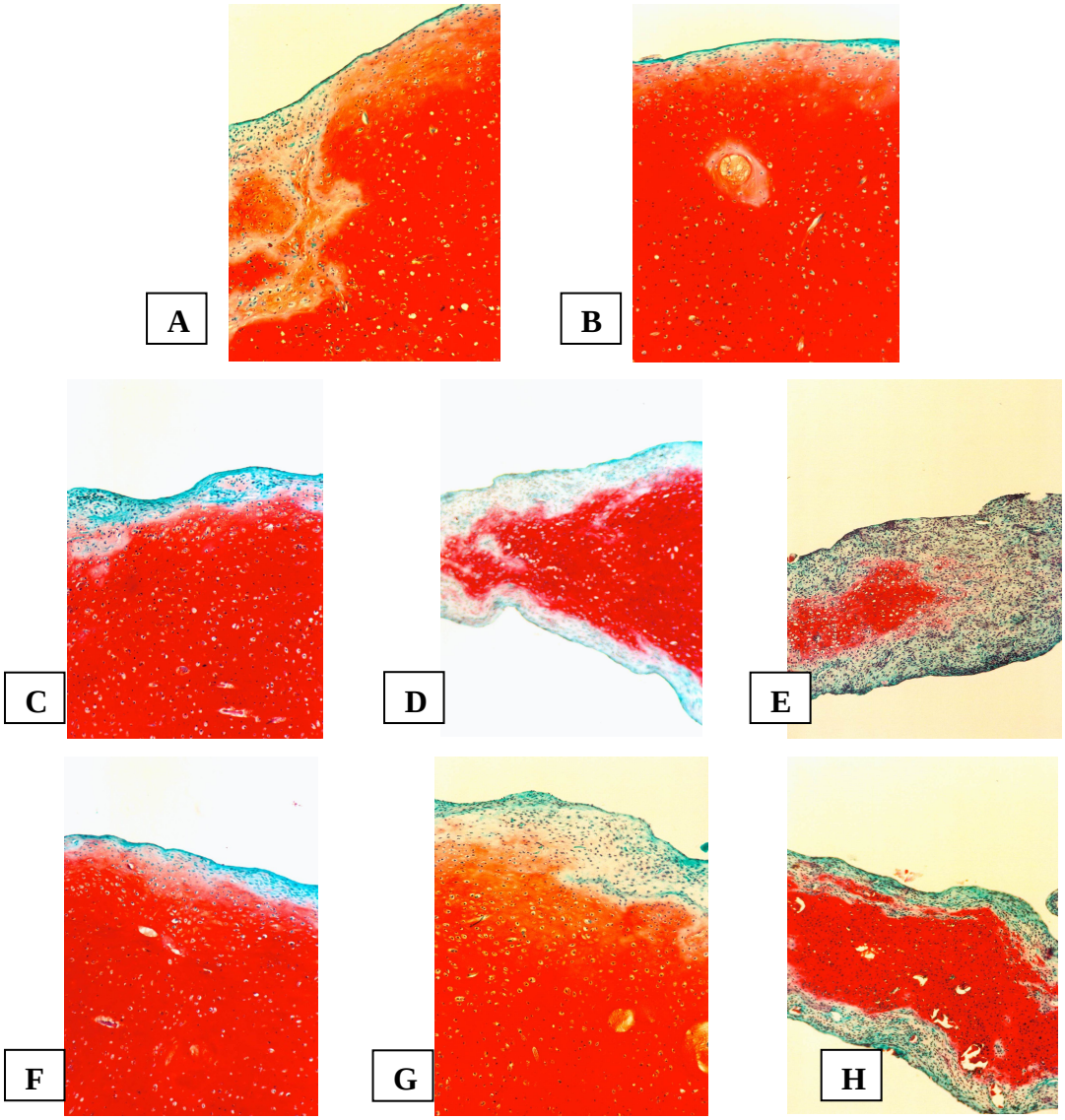

Fig. 17: Glycosaminoglycan (GAG) distribution in histological cross-sections of four week constructs grown in culture medium with (A) no supplements, (B) IGF-I 50ng/ml, (C) , IGFBP-4 (15.8 ng/ml) (D) IGFBP-4 (1579 ng/ml), (E) IGFBP-4 (15790 ng/ml),(F) 1:1 (IGF-I:IGFBP-4), (G) 1:10 (IGF-I:IGFBP-4) and (H)1:100 (IGF-I:IGFBP-4). GAG was stained red with safranin-O (dark color in the black and white print). (x 100).

Discussion

The present study was designed to investigate the effects of IGFBP-4 either in the absence or presence of IGF-I on tissue-engineered cartilage grown in a well-established in vitro model system (bovine articular chondrocytes dynamically seeded onto biodegradable polymer mesh and cultured in medium containing 1% serum) [46;132] with a specific focus on the growth rate and ECM components.

IGFBP-4 is the smallest of the six IGFBPs (233 amino acids in rats, 237 amino acids in humans). It contains one N-linked glycosylation site and commonly exists in biological fluids as a doublet: a 24-kDa non-glycosylated form and a larger 28-kDa glycosylated form [133;134]. While IGFBP-4 appears to interact specifically with connective tissues in vivo [135], there is no evidence for membrane association of IGFBP-4 in cellular systems, suggesting that IGFBP-4 exists primarily as a soluble, extracellular IGFBP.

We studied the effects of IGFBP-4 in the presence and absence of IGF-I on in vitro engineered cartilage for the following reasons: Firstly, it has been previously reported that IGFBPs inhibit the proliferation and growth rate of many cell types, in vivo and in vitro, through the modulation of IGF-I actions [130] and through IGF independent action [136]. Secondly, reports indicate that both IGF-I and IGFBPs levels are elevated in the rheumatic diseases [137]. Therefore, we investigated the effects of IGFBP-4 both in the presence and absence of external IGF-I.

This is the first time that the in vitro effect of IGFBP-4 on tissue-engineered cartilage has been studied. Exogenous IGFBP-4 (15.8-15790 ng/ml) in the absence of external IGF-I dose-dependently reduced the tissue growth rate and the ECM components of the grown constructs. Whereas low concentrations (15.8, 158 ng/ml) did not significantly affect the grown tissues, high concentrations (1579, 15790 ng/ml) strongly decreased wet weights and amounts of GAG and collagen in the constructs. However, fractions of the ECM components per weight were only reduced at the highest concentration (15790 ng/ml). Histological cross-

sections also revealed strongly decreased GAG amounts at the highest concentration, corresponding well with the biochemical analysis.

In contrast to IGFBP-4, the addition of IGF-I 50 ng/ml into the culture medium of engineered cartilage increased the growth rate and ECM deposition, consistent with previous results [68;138;139]. IGF-I is one of the most important growth-stimulating factors for cartilage, stimulating proliferation and ECM biosynthesis of chondrocytes in vivo and cultured chondrocytes and cartilage explants from various species in vitro [68;138;139]. The addition of a combination of IGF-I (50 ng/ml) and IGFBP-4 in different MRs into the culture medium, however, demonstrated that even at a 1:3 MR, IGF-I can overcome the inhibitory effects of IGFBP-4. At the same time, IGFBP-4 can also diminish the stimulatory effects of IGF-I. At all molar ratios investigated, the wet weight and the fractions per weight of GAG and collagen were reduced when combinations of IGF-I and IGFBP-4 were used, as compared to IGF-I alone.

These results support the concept that IGFBPs may play an important role in the regulation of cartilage growth. The logical mechanisms for these effects could be, as previously reported, that IGFBPs elicit their effects by their ability to modulate IGF actions and by IGF independent action. The findings that IGFBP-4 reduces growth rate and ECM content of engineered cartilage may be explained by IGF independent action. Alternatively, IGFBP-4 may form complexes with endogenous IGF-I leading either to an inability of IGF-I to bind to the type I IGF receptor and/or to the blocking of the receptor by the complex [133;134].

The results that combinations of IGFBP-4 and IGF-I reduce growth rate and ECM content, as compared to IGF-I alone, can be easily explained by reduced concentrations of free IGF-I in the medium, as it is sequestrated by complex formation with IGFBP-4. Additionally, the formed complex may have blocked the type I IGF receptor rendering it inaccessible for exogenous as well as endogenous IGF-I. At high concentrations of IGFBP-4 (MR 1:10 and 1:100), IGF-I-independent actions of excess IGFBP-4 likely contributed to the results. In agreement with our results, to date there have been no reports that IGFBP-4 enhances IGF-I effects at the cellular level. IGFBP-4 was initially isolated on the basis of its inhibition of IGF-stimulated cell proliferation [140]. Furthermore, the ability of IGFBP-4 to

inhibit both basal and IGF-mediated chick pelvic cartilage growth in vitro has been reported [80].

In summary, we have demonstrated that IGFBP-4 had significant inhibitory effects on the growth rate and ECM components of in vitro engineered cartilage in the absence of IGF-I. When a combination of IGF-I (50 ng/ml) and IGFBP-4 in different molar ratios was applied, the inhibitory effects of IGFBP-4 could be overcome by IGF-I even at a 1:3 MR; at the same time IGFBP-4 diminished the stimulatory effects of IGF-I.

Summary

The effects of insulin-like growth factor binding protein-4 (IGFBP-4) in the presence and absence of external IGF-I on in vitro engineered cartilage were investigated. The specific focus was on the ability of IGFBP-4 to influence the growth rate of the constructs and concentrations of major extracellular matrix components, i.e., collagen and glycosaminoglycans (GAG), and on the effects on the morphological appearance of the tissue. Bovine articular chondrocytes were cultured on biodegradable polyglycolic acid (PGA) scaffolds in medium with 1% fetal bovine serum (FBS) with and without the addition of IGFBP-4 and IGF-I as well as combinations of IGFBP-4 and IGF-I. IGF-I (50 ng/m)l had significant stimulatory effects on the engineered cartilage, as compared to control constructs. IGFBP-4, in the absence of external IGF-I, had significant inhibitory effects on the engineered cartilage compared to control constructs in a dose-dependent manner, as demonstrated by an inhibited growth rate, a reduction in the of GAG and collagen production and an increased cell number per wet weight. The addition of IGFBP-4 to the engineered constructs in the presence of external IGF-I (50 ng/ml) in different molar ratios had significant inhibitory effects at all the molar ratios (1:0.5 - 1:100), as compared to constructs treated only with IGF-I (50 ng/ml). In contrast, compared to the control group receiving no supplemental protein, the combinations showed no inhibitory effects on the growth rate and ECM components of the engineered constructs at low IGFBP-4 concentrations (IGF-I: IGFBP-4 molar ratio of 1:3), whereas at high concentrations (especially 1:100 molar ratio) these parameters were significantly reduced.

Chapter 5

Effects of Insulin-like Growth Factor Binding Protein-5 on Engineered Cartilage

Introduction

In cartilage, IGF-I has been identified as one of the major stimulatory factor of proteoglycan synthesis [141;142]. Also, it has been reported that IGFs have important role in skeletal growth [143] and in the maintenance of mature articular cartilage matrix metabolism [144;145]. In recent years, a family of six or more related proteins termed insulin-like growth factor binding proteins (IGFBPs) has been discovered that have a high affinity for IGF-I [119-121]. Their ability to modulate the distribution, function and activities of IGFs in various cell tissues and body fluids (either antagonize or augment IGF action) [146;147] points to the necessity for understanding this regulatory axis in cartilage.

It has been reported that IGFBP-5 is one of the three predominant IGFBPs (IGFBP-3, -4 and -5) produced by articular chondrocytes [125]. However, most of the studies reporting on IGFBP-5 have been conducted in bone tissue, where it is acknowledged as the predominant IGFBP [148;149]. IGFBP-5 has been suggested to act like a growth factor as it stimulates bone formation, at least in part via IGF-independent mechanisms [85;150]. It can either inhibit or increase IGF-I effects [83], again in part via an IGF-I-independent mechanism [84]. It has been reported that IGFBP-5 is secreted by osteoblast-like cells [151]. It is produced as a 252-amino acid protein of about 29 kD [83] and it has IGF-independent stimulatory effects in mouse and human osteoblast-like cells [130;152]. Subsequent studies have shown that recombinant human IGFBP-5 stimulated DNA synthesis in osteoblasts [152] It has been demonstrated that the presence of IGFBP-5 in cell culture medium potentiated the growth stimulatory effects of IGF-I on fibroblasts [148]. So far, only a few studies exist that have examined the role of IGFBP-5 in cartilage. Increased levels of IGFBPs 2, 3, and 5 were detected in the culture media of osteoarthritic compared to normal cartilage; in addition, mRNA levels of IGFBPs 3 and 5 were increased in freshly excised diseased cells [126]. The concentration of IGFBP-5 in medium conditioned by articular chondrocytes was increased by IGF-I in a dose-dependent manner by a transcriptional mechanism via the type 1 IGF receptor [153]. Furthermore, it has been reported that the exposure of growth plate chondrocytes to IGFBP-5 increased cell proliferation, in the absence or presence of exogenous IGF-I [154].

However, the hypothesis that IGFBP-5 may function as a growth factor in cartilage, as shown in bone tissue, has not been evaluated. Therefore, in this study, the effects of IGFBP-5 were investigated in an established three-dimensional cell culture for the in vitro engineering of cartilage. Additionally, the effects of IGFBP-5 in combination with IGF-I were studied. The specific focus was on the IGFBP-5 effects on growth and ECM composition of the chondrocyte-polymer constructs.

Results

Wet weights

The wet weights of the cell-polymer constructs after 4 weeks of culture in medium containing 1% FBS were significantly increased (1.6, 1.8, and 1.7-fold compared to control) with exogenous addition of IGFBP-5 (at 9.5, 95 and 572 ng/ml, respectively). IGFBP-5 at a very high concentration (1909 ng/ml) did not affect the wet weight of the cell-polymer construct. In the same experiment, the addition of IGF-I at 5 and 50 ng/ml significantly increased the wet weights of the construct after 4 weeks of culture to an even higher extent (2.6- and 4.8-fold compared to control, respectively) (Fig. 18A). At the same time, the addition of a combination of IGF-I (50 ng/ml) and IGFBP-5 at different molar ratios (1:0.5, 1:1, 1:3 and 1:10) resulted in wet weights of the cell-polymer constructs that were in between those of the control and the IGF-I group, i.e., these combinations decreased the wet weights of the cell-polymer constructs, as compared to IGF-I (Fig. 18B). (The molecular weight of IGFBP-5 is 29 kDa that of IGF-I is 7.6 kDa. Therefore, in order to employ molar ratios of 1:0.5, 1:1, 1:3, and 1:10, IGFBP-5 concentrations of 95, 190, 572 and 1909 ng/ml had to be used in combination with 50 ng/ml of IGF-I (Fig. 18B). Based on these concentrations, the concentrations for the experiments employing IGFBP-5 alone were chosen (Fig. 18A)).

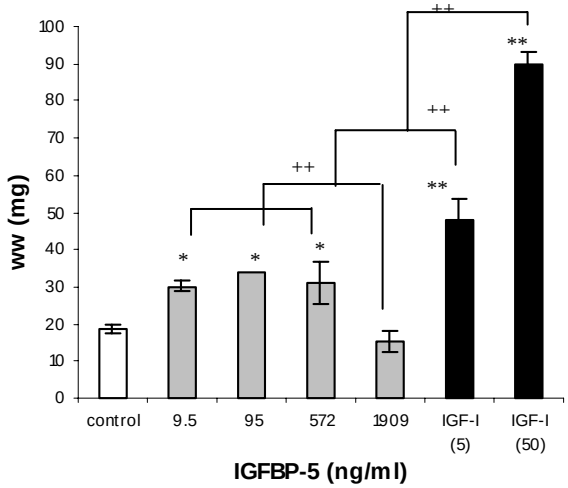

Fig. 18A: Effects of IGFBP-5 (9.5-1909ng/ml) on the wet weight of constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by, significance between two groups is indicated by +.*

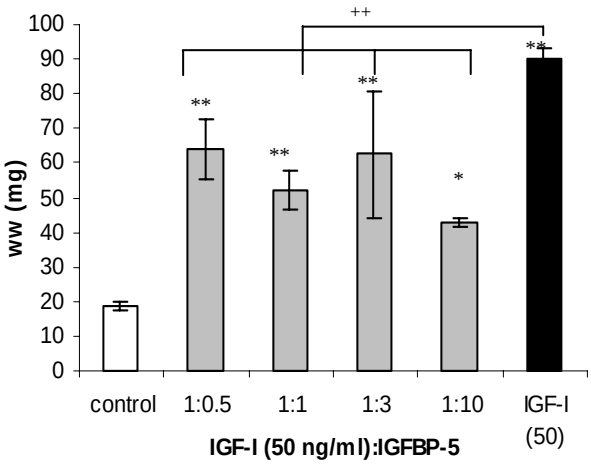

Fig. 18B: Effects of IGF-I (50 ng/ml) and IGF-I: IGFBP-5 in different molar ratios on the wet weight of constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by significance between two groups is indicated by +.*

GAG

The amount of GAG per ww of cell-polymer constructs after 4 weeks culture were either slightly decreased (9.5, 1909 ng/ml) or not affected (95, 572 ng/ml) with the exogenous addition of IGFBP-5. The addition of IGF-I at 5 ng/ml did also not affect GAG per ww, in contrast, 50 ng/ml IGF-I significantly increased the amount of GAG per ww, as compared to controls (Fig. 19A). At the same time, the addition of the combination of IGF-I (50 ng/ml) and IGFBP-5 at different molar ratios (1:0.5, 1:1, 1:3, and 1:10) significantly decreased the amount of GAG per ww of the cell-polymer construct, as compared to IGF-I (Fig. 19B).

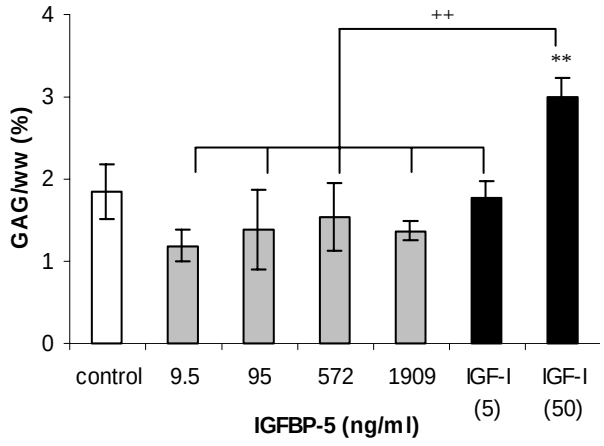

*Fig. 19A: Effects of IGFBP-5 (9.5-1909 ng/ml) on the fraction of GAG per ww in constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by *, significance between two groups is indicated by +.*

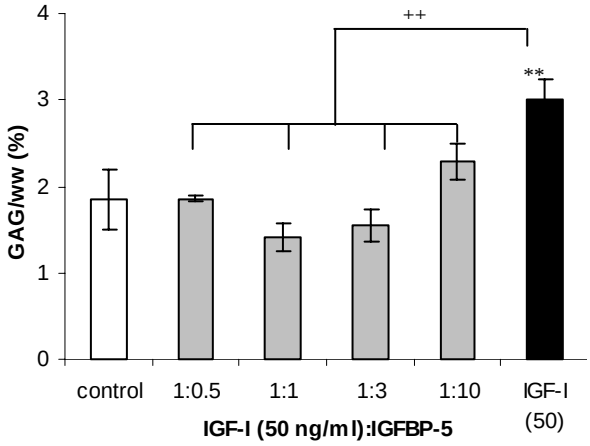

*Fig. 19B: Effects of IGF-I (50 ng/ml) and IGF-I:IGFBP-5 in different molar ratios on the fraction of GAG per ww in constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by *, significance between two groups is indicated by +.*

Collagen

Exogenous addition of IGFBP-5 (9.5, 95, 572 and 1909 ng/ml) into the culture medium increased the amount of collagen per ww of the cell-polymer constructs slightly (about 1.3-fold for all concentrations, compared to control), but not significantly at all concentrations. The addition of IGF-I at 5 and 50 ng/ml significantly increased the amount of collagen per ww (1.5- and 1.9-fold compared to controls, respectively) (Fig. 20A). At the same time, the addition of the combinations of IGF-I (50 ng/ml) and IGFBP-5 decreased the collagen content per ww of the cell-polymer constructs compared to IGF-I (Fig. 20B); at molar ratios of (1:0.5, 1:1 and, 1:3) the amounts of collagen per ww were in between those of the control and the IGF-I group, whereas at a molar ratio of 1:10 the values were reduced to control levels.

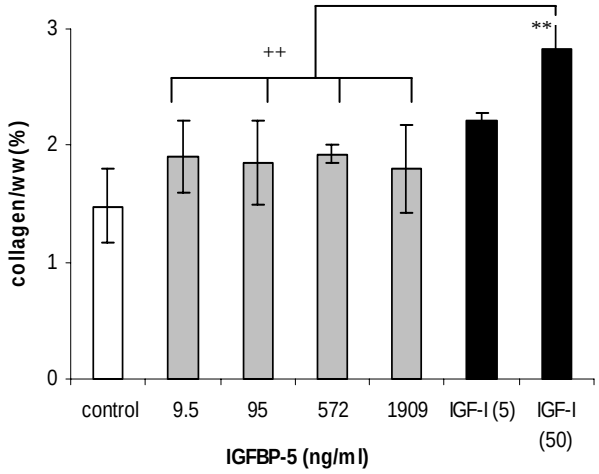

*Fig. 20A: Effects of IGFBP-5(9.5-1909 ng/ml) on the fraction of collagen per ww in constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by *, significance between two groups is indicated by +.*

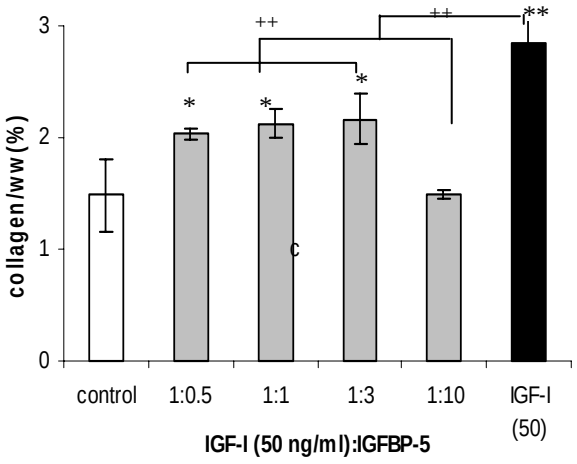

Fig. 20B: Effects of IGF-I (50 ng/ml) and IGF-I: IGFBP-5 in different molar ratios on the fraction of collagen per ww in constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by, significance between two groups is indicated by +.*

Cell number

The cell number per ww of cell-polymer constructs after 4 weeks culture was not significantly affected with the exogenous addition of IGFBP-5 at all concentrations tested. The addition of IGF-I significantly decreased the cell number per ww of the construct (56% and 38% of control values) (Fig. 21A). At the same time, the addition of the combination of IGF-I (50 ng/ml) and IGFBP-5 at different molar ratios (1:0.5, 1:1, 1:3 and 1:10) yielded cell numbers similar to that of IGF-I alone and significantly lower as control values (47%, 46%, 50% and 50% of control values) (Fig. 21B).

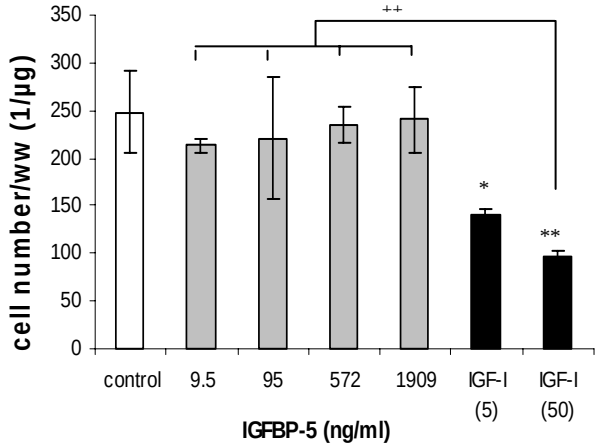

*Fig. 21A: Effects of IGFBP-5 (9.5-1909 ng/ml) on the cell number per ww of constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by *, significance between two groups is indicated by +.*

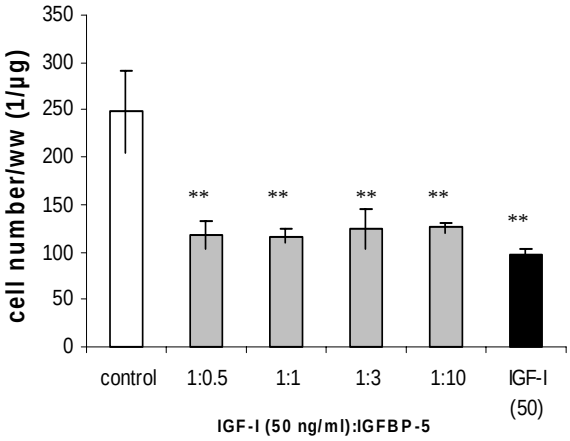

*Fig. 21B: Effects of IGF-I (50 ng/ml) and IGF-I: IGFBP-5 in different molar ratios on the cell number per ww of constructs cultured for four weeks. Data represent the average ± SD of three constructs. Significance to the control is indicated by *, significance between two groups is indicated by +.*

Histology

Safranin-O staining for the detection of the GAG distribution in the constructs after 4 weeks of culture indicated the following results: control constructs (no protein supplemented) stained weakly for GAG; scattered areas stained red for GAG alternated with GAG-depleted areas indicated by the green colour which was especially pronounced at the edge of the construct (Fig. 22). Compared to controls, cell-polymer constructs supplemented with IGFBP-

5 exhibited a more coherent tissue formation and relatively high amounts of GAG (Fig. 22.) The constructs supplemented with IGF-I showed the strongest staining for GAG. The constructs supplemented with combinations of IGF-I and IGFBP-5 exhibited higher amounts of GAG than the control, but, at the same time, in these constructs the Safranin-O staining appeared to be dose-dependently decreased, as compared to IGF-I constructs (Fig. 22)

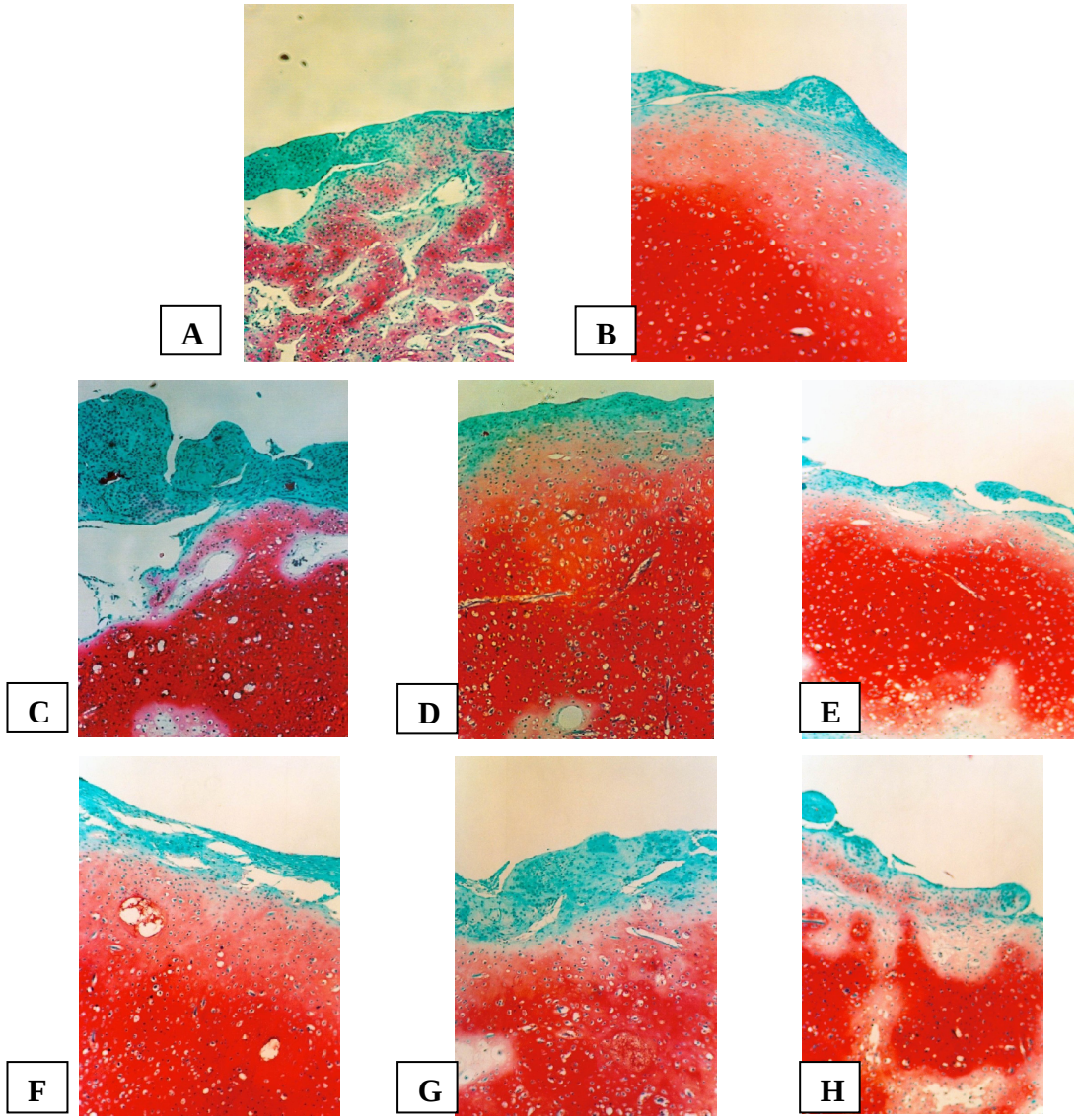

Fig. 22: Glycosaminoglycan (GAG) distribution in histological cross-sections of four week constructs grown in culture medium with (A) no supplements (control), (B) IGF-I 50ng/ml, (C) , IGFBP-5 (9.5 ng/ml) (D) IGFBP-5 (95 ng/ml), (E) IGFBP-5 (1909 ng/ml),(F) 1:1 (IGF-I:IGFBP-5), (G) 1:3 (IGF-I:IGFBP-5) and (H)1:10 (IGF-I:IGFBP-5). GAG was stained red with safranin-O (dark color in the black and white print). (x 100).

Discussion

This study was conducted to evaluate the hypothesis that IGFBP-5 may function as a growth factor in cartilage, as it has previously been suggested for bone tissue [85;150]. The experiments were designed to investigate the effects of IGFBP-5 on tissue engineered cartilage grown in a well-established in vitro model system. The effects of IGFBP-5 alone or in combination with IGF-I on engineered cartilage were studied, focusing on its ability to increase tissue growth and the amount of ECM.

Exogenous IGFBP-5 alone clearly stimulated the growth of cartilaginous tissue constructs. The amounts of the ECM components GAG and collagen per construct were also increased; for collagen even the fraction per wet weight of the constructs was increased. Histological cross-sections revealed a more coherent tissue formation after application of IGFBP-5, as compared to control constructs; glycosaminoglycans were more evenly distributed throughout the sections in IGFBP-5 constructs. Although these significant effects of IGFBP-5 were clearly observable, their extent did not equal the results yielded by IGF-I, which is known as one of the major growth factors in cartilage and which exerted stronger effects in all parameters investigated. In contrast, IGFBP-4 exerted rather detrimental effects on tissue-engineered cartilage (**see chapter 4**) demonstrating within the 3-D culture system that IGF binding proteins differentially act on anabolic processes of cartilage.

To date, only few studies exist on direct effects of IGFBP-5 in cartilage tissue ([126;153;154]**,** see introduction). More data is available on bone tissue, in which IGFBP-5 has been described to function as a growth factor that stimulates bone formation, at least in part via IGF-independent mechanisms [85]. The mechanism by which IGFBP-5 was suggested to exert its growth factor effects on osteoblasts involves binding to a specific site on osteoblast cell surfaces, i.e., a putative receptor for IGFBP-5 [130;150]. This putative receptor is said to be stimulated by IGFBP-5, but not by IGF-I or TGF-ß, thus suggesting that the IGFBP-5 putative receptor is functionally different from the IGF-I receptor [155]. Another potential mechanism by which IGFBP-5 can exert IGF-independent effects is based on

transcriptional activation of genes by IGFBP-5 transported into the nucleus via its nuclear localization signal [156].

IGFBP-5, as the other members of the IGFBP family, has been shown to associate with IGF-I under physiological conditions [157]. Consequently, besides its direct effects IGFBP-5 may modulate the effects of IGF-I when added into the culture medium in combination. An increase of the IGF-I activity by IGFBP-5 may be caused either by targeting IGF-I to the receptor-containing cell surface, by an IGF-independent effect of the binding protein [158].

In this study, IGFBP-5 was mixed with IGF-I before the addition into the culture medium because the high affinity of IGFBP-5 to ECM structures has been reported to lead to an 8-17-fold decrease of the IGFBP-5 affinity for IGF-I [148]. In general, the addition of combinations of IGF-I and IGFBP-5 in different molar ratios, into the culture medium of the engineered cell-polymer constructs stimulated the growth rate and the formation of ECM compared to control; at the same time, these combinations significantly reduced the stimulatory effects of IGF-I, i.e., the stimulatory effects on the engineered cartilage resulting from this combination were smaller than the stimulatory effects resulting from the addition of IGF-I alone. In previous studies, similar results, i.e., that IGFBP-5 can inhibit the stimulatory effects of IGF-I have been reported. The addition of a molar excess of IGFBP-5 to cell lines that do not secrete IGFBP-5 protease resulted in an inhibition of IGF-I actions [146]. Keifer et al showed that IGFBP-5 can inhibit IGF-I stimulation of DNA and glycogen synthesis in human osteosarcoma cells [134]. Also, an inhibition of IGF-I-induced steroidogenesis by IGFBP-5 in granulosa cells was reported [159]. The mechanism by which IGFBP-5 may reduce IGF-I effects was investigated using radiolabelled IGFs in ligand binding assays. It was demonstrated that IGFBP-5 is able to inhibit the binding of IGFs to the IGF-I receptor, resulting in reduction of receptor stimulation and autophosphorylation [160]. IGFBP-5 inhibits IGF-stimulated autophosphorylation of the receptor very efficiently and complete inhibition was reported to be achieved when IGFBP-5 is present in excess of IGFs [160].

Summary

This study was conducted to evaluate the hypothesis that insulin-like growth factor binding protein-5 may function as a growth factor in cartilage, as it has previously been suggested for bone tissue. The effects of IGFBP-5 in the presence and absence of exogenous IGF-I on in vitro engineered cartilage were investigated, with a specific focus on the ability of IGFBP-5 to influence the growth rate and the composition of the extracellular matrix of the cell-polymer constructs. Bovine articular chondrocytes were cultured on biodegradable polyglycolic acid (PGA) scaffolds without and with the addition of IGFBP-5 (9.5 -1909 ng/ml) as well as in combination with IGF-I, in medium with 1% fetal bovine serum (FBS).

Exogenous IGFBP-5 alone clearly stimulated the growth of cartilaginous tissue constructs. The fraction of collagen per wet weight of the constructs was slightly increased. Histological cross-sections revealed a more coherent tissue formation after application of IGFBP-5, as compared to control constructs; glycosaminoglycans were more evenly distributed throughout the sections in IGFBP-5 constructs. Though the impact of IGFBP-5 on the cartilaginous tissues was obvious, in comparison, IGF-I alone (50 ng/ml) exerted stronger effects in all parameters investigated. In combinations with IGF-I, IGFBP-5 generally significantly reduced the effects of IGF-I at all molar ratios investigated (1:0.5 – 1:10).

Chapter 6

New Natural Biodegradable Copolymer Scaffolds for Cartilage Tissue Engineering

Introduction

A major factor for successful cartilage engineering is the choice of a suitable 3-D matrix that can provide the initial structural support and retain cells in the defective area and is then degraded when the cells secrete their own matrix. The desirable characteristics of the 3-D matrix material are biocompatibility, biodegradability, appropriate surface chemistry to promote cell attachment, proliferation, and tissue development, and adequate mechanical properties to maintain the structure and function after implantation and during the remodelling of the implants [53;161;162]. Potential materials with these characteristics include natural and synthetic polymers. Although used in many tissue engineering approaches, synthetic polymers have the inherent disadvantage that they do not possess a surface which is familiar to the cells to support their growth and secretion of extracellular matrix (ECM) [163]. Furthermore, the degradation of synthetic polymers often releases toxic [164] or acidic products, such as lactic acid from PLLA [164;165], into surrounding tissues, reducing the pH, which may affect cellular function [166] and accelerate the degradation rate due to autocatalysis [167]. In contrast, among natural polymers are promising candidates to overcome these limitations [50;168;169]. For cartilage engineering, collagen, gelatin (denatured collagen), and hyaluronic acid appear to be a logical choice, as they are major components of the ECM of cartilage. However, scaffolds made from these polymers often lack mechanical stability, the scaffold contracts in the culture medium [170;171] leading to reduction in the pore size that could restrict cell proliferation and tissue development. Furthermore, in vivo deformation of the matrix could result in a loss of contact between the implanted device and the host tissue.

Angele et al. previously introduced a copolymer scaffold made from hyaluronic acid and gelatin; the scaffolds allowed for cell proliferation and ECM synthesis indicating their potential for cartilage engineering [86]. In order to increase their mechanical stability, recently Mueller et al. modified similar scaffolds made from hyaluronic acid, gelatine, and collagen type I [87] through the use of selected chemical crosslinking using glutaraldehyde (GTA) [172-174], carbodiimides (EDC) [174-176], and genipin [177], respectively. However, different crosslinking protocols are reported to possibly exert strong negative effects on degradation rate [174;178], cell proliferation [174;176], and biosynthesis [179].

Therefore, the objective of this study was to evaluate the effects of the selected crosslinking treatment on the quality of in vitro engineered cartilage. Specifically, we seeded primary bovine chondrocytes on different derivatives of these copolymer scaffolds that is either non-crosslinked or crosslinked using GTA, EDC, or genipin, respectively. Cell-polymer constructs were cultivated over 4 weeks in medium containing 1% FBS, either without or with insulin, which has been previously demonstrated to improve engineered cartilage [89]. The quality of the engineered cartilaginous tissue was assessed on the basis of construct weight, cell number, quantitative content of the major ECM components collagen and glycosaminoglycans (GAG), and histological and imunohistological staining for ECM components.

Results

Cell adhesion to the scaffolds after seeding

Determination of the cell number per construct performed after 48 h of seeding, i.e., before cultivation, indicated that the chondrocytes adhered to all copolymer scaffolds to a similar extent **(**Fig. 23). Virtually all of the chondrocytes applied in the spinner flask were detected on the scaffolds (5 mio cells). The SEM images confirmed that the chondrocytes were attached to the surface of the scaffolds as well as showed that in all scaffolds chondrocytes appear in the interior of the copolymer scaffold. The latter was especially pronounced in EDC crosslinked polymers **(**Fig. 24**)**.

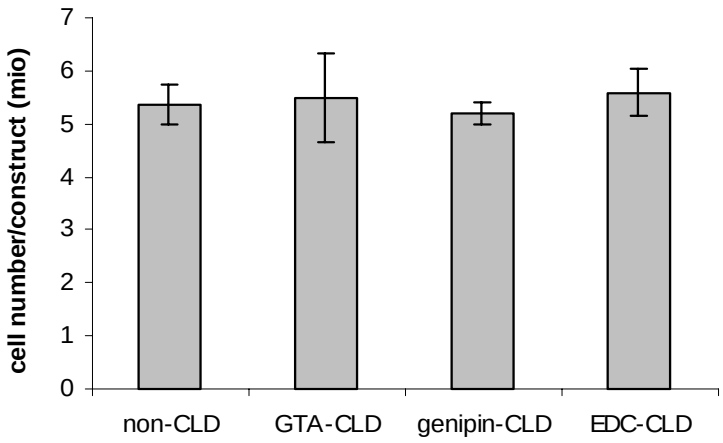

Fig. 23: Effects of crosslinking on the cells which attached to the construct after two days seeding in spinner flask with 5 million cells per scaffold in medium with 10% FBS. Data represent the average ± SD of 4 independent measurements. Significance between two groups is indicated by +.

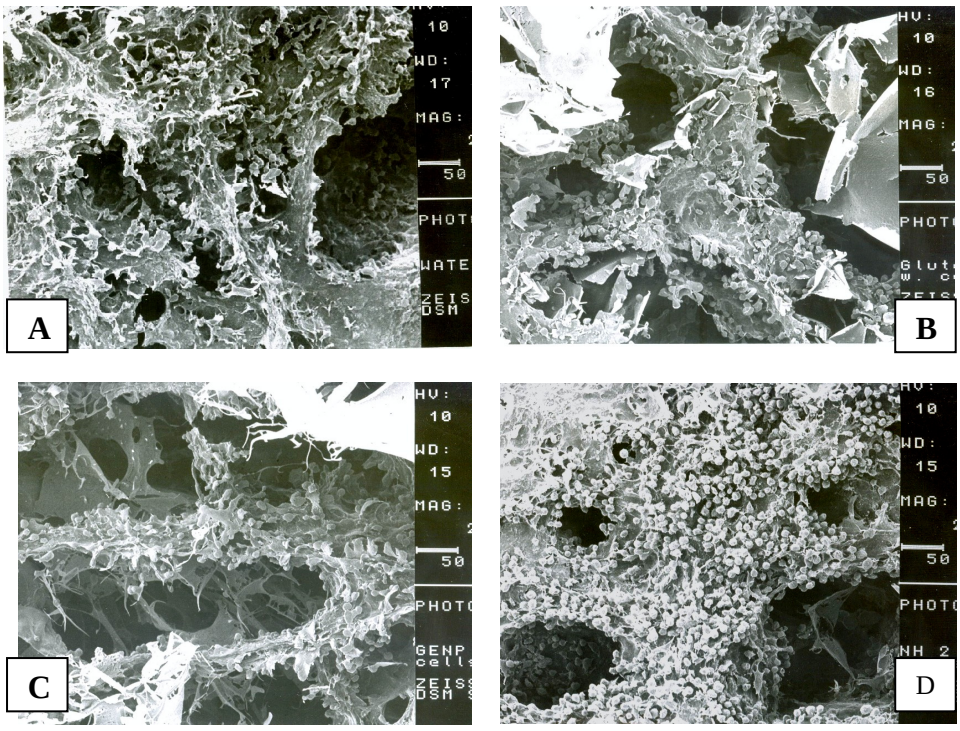

Fig. 24: SEM pictures of cell-polymer constructs after 2 days of seeding in spinner flask in cell suspension of 5 million cells per scaffold in medium with 10 % FBS. (a) non-crosslinked (b) glutaraldehyde crosslinked (c) genipin crosslinked (d) EDC crosslinked.

Wet weight

After 4 weeks of cultivation in basal medium containing no insulin (control groups), the wet weights (ww) in all groups were comparatively small; among the controls the genipin and EDC crosslinked scaffolds yielded the largest weights, i.e., 70 and 57 mg, respectively (Fig. 25). The ww of the constructs supplemented with insulin (2.5 µg/ml) were strongly increased for all four different scaffolds, especially for the EDC crosslinked group (212 ± 22 mg) (Fig. 25). Additionally, the macroscopical appearance demonstrating the size of the constructs corresponded well with the obtained weights (Fig. 26).

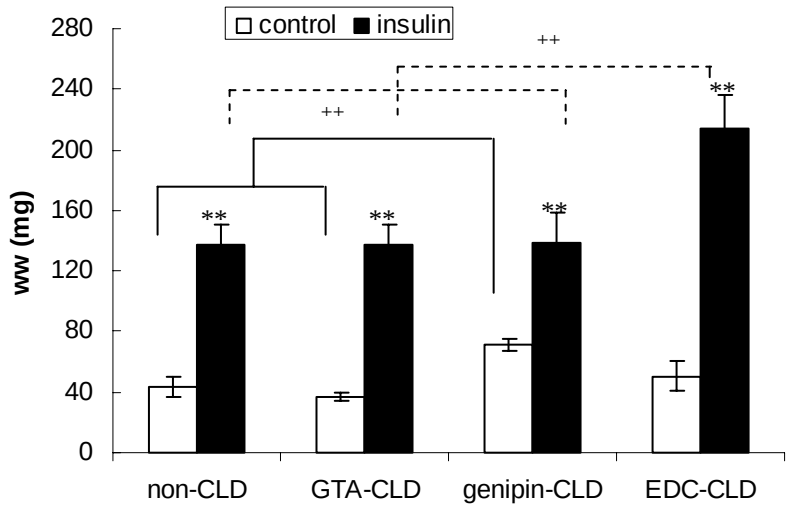

*Fig. 25: Effects of crosslinking and insulin on the wet weights of cell-polymer constructs cultured for four weeks in medium with 1% FBS. Data represent the average ± SD of three constructs. Significance to the controls is indicated by *, significance between two groups is indicated by +.*

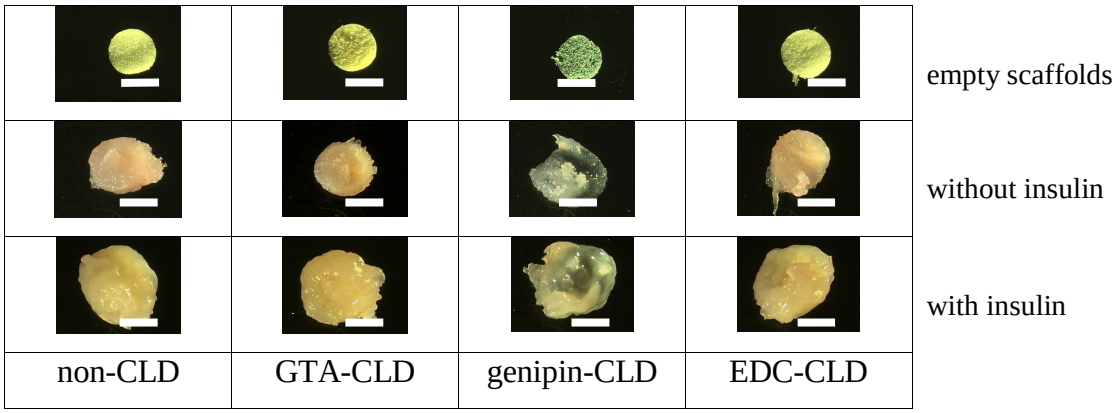

Fig. 26: Cell-polymer constructs cultured with or without supplemented insulin (2,5 µg/ml) after 4weeks of cultivation in medium with 1%FBS (bars indicates 5 mm)

Cell number

The cell numbers per ww of the constructs in the control groups (no insulin) for the non-crosslinked and glutaraldehyde crosslinked scaffolds were 60 and 65 cells/µg, respectively. In contrast, distinctly lower cell numbers per construct were determined in the control constructs of the genipin and EDC crosslinked groups (35 and 47 cells/µg). The numbers of cells per ww found in the insulin-supplemented constructs were clearly decreased

(20, 19, 21 and 14 cell/µg), as compared to their controls. (As cell numbers are commonly rather high in engineered cartilage, as compared to native tissue, generally smaller cell numbers per wet weight are desired in newly developed tissue) (Fig. 27A).

With regard to total cell number per construct, there were no significant differences between all control groups; also between the insulin-supplemented groups, no differences were detected. Values for the insulin groups were slightly higher than for controls, the cell numbers for all groups were between 2.4 and 3 million cells per construct (Fig. 27B).

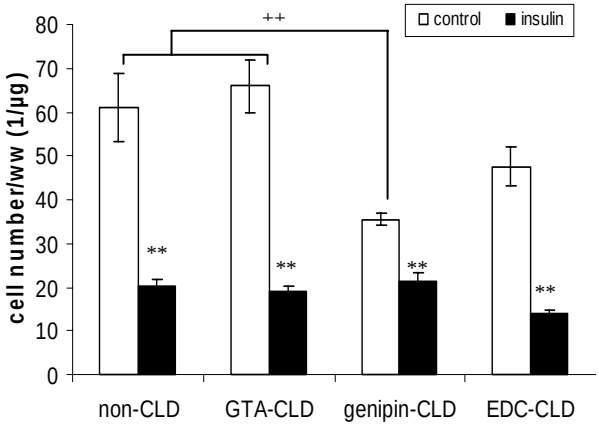

*Fig. 27A: Effects of crosslinking and insulin on the cell number per ww of constructs cultured for four weeks in medium with 1% FBS. Data represent the average ± SD of three constructs. Significance to the controls is indicated by *, significance between two groups is indicated by +.*

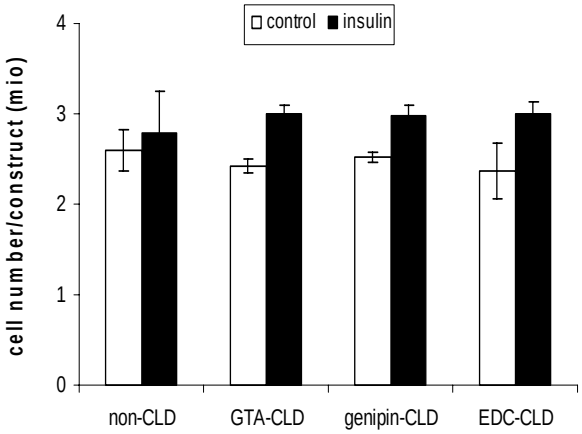

*Fig. 27B: Effects of crosslinking and insulin on the cell number per constructs cultured for four weeks in medium with 1% FBS. Data represent the average ± SD of three constructs. Significance to the controls is indicated by *, significance between two groups is indicated by +.*

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Collagen

In order to assess a possible contribution of the scaffold compounds gelatin and collagen type I to the results of the quantitative assay for total collagen, blank scaffolds were assayed that had been either not incubated at all or incubated in culture medium for four weeks. Whereas unincubated scaffolds resulted in very low absorbance in the UV spectrophotometric assay, incubated scaffolds did not result in any reading at all (Fig. 28A).
After 4 weeks of cultivation, only for the control groups (no insulin) with non-crosslinked scaffolds and with glutaraldehyde crosslinked scaffolds a low absorbance was observed (0.51

and 0.38 mg collagen per construct) which was in the same order of magnitude as for the blank scaffolds, the values of all other groups were distinctly higher (Fig. 28A). The control groups of the genipin and EDC crosslinked copolymer scaffolds showed collagen amounts of 0.91 and 0.71 mg, respectively (Fig. 28A). The amount of collagen per construct in the insulin-supplemented groups was strongly increased in all groups to 3.4, 3.6, 3.6, and 5.2 mg per construct for non-crosslinked, glutaraldehyde, genipin and EDC crosslinked derivatives, respectively (Fig. 28A).

With regard to the collagen content, i.e., the percentage of collagen per ww, among the control groups, the scaffolds crosslinked with genipin and EDC yielded the highest values (1.3 and 1.4 %, respectively (Fig. 28B)). For insulin-receiving groups, the percentages of collagen per ww were greatly increased (2.4, 2.3, 2.68, and 2.5 mg), as compared to their controls.

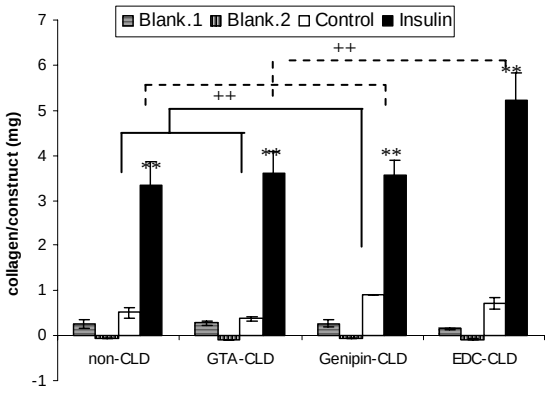

*Fig. 28A: Effects of crosslinking and insulin on the collagen fractions per constructs cultured for four weeks in medium with 1% FBS. Data represent the average ± SD of three constructs. Significance to the controls is indicated by *, significance between two groups is indicated by +.*

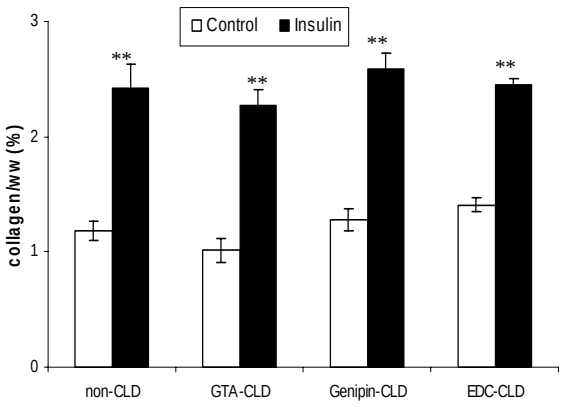

*Fig. 28B: Effects of crosslinking and insulin on the collagen fractions per ww of the constructs cultured for four weeks in medium with 1% FBS. Data represent the average ± SD of three constructs. Significance to the controls is indicated by *, significance between two groups is indicated by +.*

GAG

Theoretically, hyaluronic acid, a major component of the scaffolds, is not detected by the quantitative assay for glycosaminoglycans [93]. This was confirmed by assaying scaffolds which were not incubated as well as scaffolds which were incubated for four weeks in culture medium: No absorbance in the UV spectrophotometric assay was observed in either case.
In the cultured cell-polymer constructs, after 4 weeks of cultivation in medium containing no insulin, the amount of GAG per construct was very low (0.07 and 0.08 mg) for the non-crosslinked and glutaraldehyde crosslinked copolymer scaffolds and increased for genipin and EDC crosslinked copolymer scaffolds (0.6 and 0.2 mg) (Fig. 29A)**.** Also, the percentages of GAG per ww were lowest for the non-crosslinked and glutaraldehyde crosslinked copolymer scaffolds (0.36 and 0.44 %) and distinctly increased in genipin and EDC crosslinked copolymer scaffolds (1.3 and 0.8 %) (Fig. 29B).

For the insulin-supplemented constructs, the amount of GAG per construct was distinctly increased in all groups with the EDC crosslinked group exhibiting the highest value (3.86 mg) (Fig. 29A). The percentage of GAG per ww for the insulin-receiving constructs was strongly increased, as compared to their controls groups, with genipin and EDC crosslinked scaffolds showing the highest content (3.4 and 3.7%) (Fig. 29B).

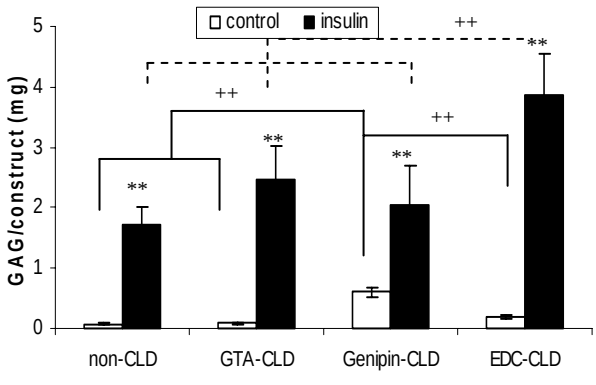

*Fig. 29A: Effects of crosslinking and insulin on the GAG fractions of constructs cultured for four weeks in medium with 1% FBS. Data represent the average ± SD of three constructs. Significance to the controls is indicated by *, significance between two groups is indicated by +.*

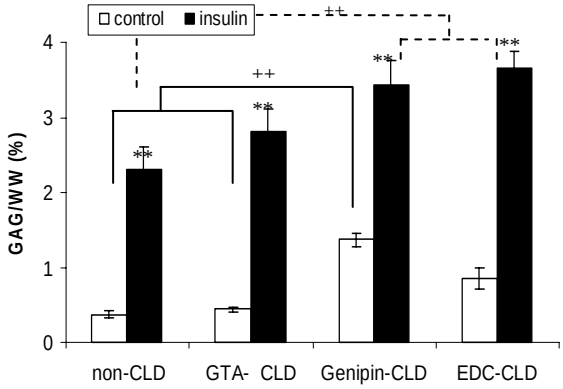

*Fig. 29B: Effects of crosslinking and insulin on the GAG per ww of constructs cultured for four weeks in medium with 1% FBS. Data represent the average ± SD of three constructs. Significance to the controls is indicated by *, significance between two groups is indicated by +.*

Histology

Safranin-O staining also supported the results obtained from the quantitative assay, generally indicating the presence of small amounts of GAG in the control constructs of the four copolymer scaffolds (Fig. 30). In contrast, the constructs supplemented with insulin possessed high amounts of GAG indicated by a strong staining, especially within the EDC cross-linked group large coherent areas were detected (Fig. 30). In addition, within the cross-sections the safranin-O staining indicated for all four control groups only scattered areas of coherent tissue formation (Fig. 30). In the insulin-receiving groups, large areas of coherent tissue were obtained, especially in the genipin and EDC crosslinked scaffolds. Only in the centre of the constructs areas containing only few cells and hardly any ECM were detected, especially in the non-crosslinked and glutaraldehyde crosslinked scaffolds (Fig. 30).

Immunohistochemistry with type I and type II collagen antibodies indicated that collagen type I was recognized in very small amounts, especially at the edges of the constructs, in all scaffold types, regardless of insulin supplementation. Collagen type II was detected in all control constructs in all areas where tissue development was observed; however, considerable holes within the cross-sections were also detected. In contrast, collagen type II was detected in larger and more coherent areas in the insulin-supplemented groups, especially in the genipin and EDC crosslinked scaffolds (Fig. 30).

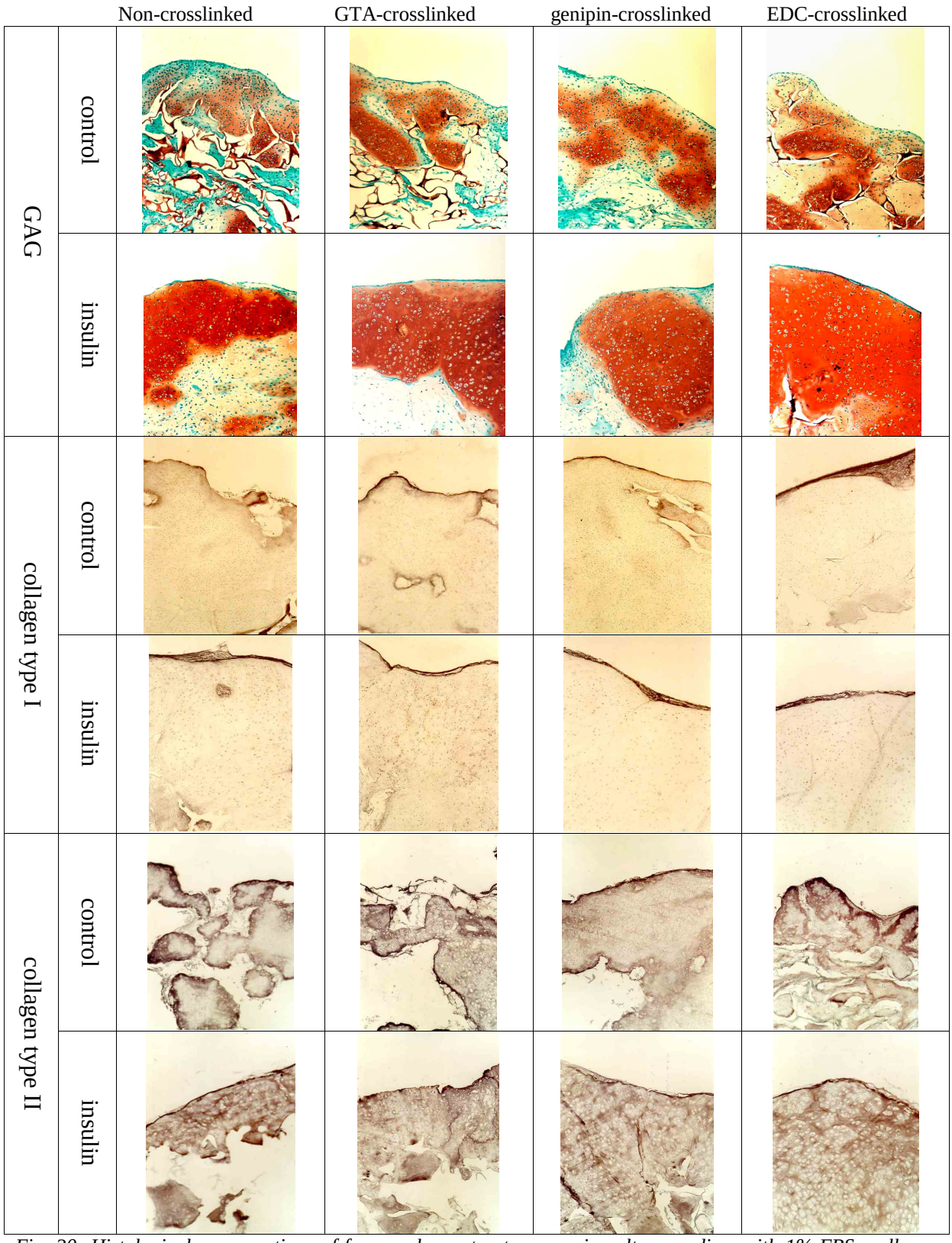

Fig. 30: Histological cross-sections of four week constructs grown in culture medium with 1% FBS: collagen type II was stained black with collagen type II antibody, collagen type I was stained black with collagen type I antibody, and GAG in ECM was stained red with safranin-O (dark color in the black white and print) (x 100).

Discussion

Scaffolds made from ECM-derived polymers have been repeatedly suggested to serve as suitable cell carriers for cartilage engineering [4-6]. The commonly observed disadvantage of a low mechanical stability can be overcome by polymer crosslinking. However, serious drawbacks, that are impaired cell function and tissue development, have been encountered after chemical crosslinking. Therefore, thorough investigations into possible effects of such procedures on the quality of engineered cartilage are indispensable.

In this study, we demonstrate that crosslinking of recently developed scaffolds made from hyaluronic acid, gelatine, and collagen type I [87] did not impair the development of cartilaginous tissues in vitro. Whereas crosslinking using glutaraldehyde resulted in cell-polymer constructs which were very similar to constructs grown on non-crosslinked scaffolds, crosslinking by EDC as well as genipin even resulted in partial improvement of the grown constructs.

We seeded bovine chondrocytes on scaffolds made from either non-crosslinked or crosslinked copolymers and cultivated them over four weeks in medium containing 1% FBS with or without insulin. Cell seeding resulted in no significant differences in cell number between the different scaffolds.

After four weeks of cultivation, among the cell-polymer constructs cultured only in basal medium (no insulin supplementation), the constructs of the genipin group had the largest wet weight and a distinctly higher GAG per weight content than all other groups, the EDC group being second. The collagen content of these two groups were only slightly higher than that of the non-crosslinked group. Histological cross-sections showed no significant differences between all groups receiving basal medium; all constructs exhibited considerable amounts of type II collagen and only little amounts of type I collagen, however, coherent tissue formation was only detected in scattered areas.

In general, all cell-polymer constructs receiving insulin showed a clearly improved development of cartilaginous tissue, as compared to the respective groups receiving no insulin. The constructs of the insulin-receiving EDC group were considerably larger than those of all other groups. The GAG per weight contents were considerably larger in the EDC and genipin group than in the group of the non-crosslinked scaffolds, the collagen per weight content showed no meaningful differences between all groups. Histological cross-sections confirmed the larger ECM content in all insulin-receiving groups. Large areas of coherent tissue formation were detected, which was the most pronounced in the EDC and genipin group.

The differences between non-crosslinked and crosslinked groups, and the favourable effects of crosslinking on the growth rate and on the formation of ECM may, at least in part, be explained with previously reported results [180]: Articular chondrocytes produce α-smooth muscle actin, enabling them to contract in culture, which was reported to reduce the pore size of scaffolds made of collagen and glycosaminoglycans, restricting cell proliferation and preventing synthesis of additional type II collagen [29]. Crosslinking was considered to be an effective method to minimize the contraction [29]. In addition, the crosslinking of collagen with EDC and N-hydroxysuccinimide was shown to be the superior method of crosslinking when compared to glutaraldehyde and UV radiation [180]. These findings are well in agreement with our study, in which EDC cross-linking yielded advanced tissue development as compared to non-cross-linked and glutaraldehyde crosslinked scaffolds. In a study employing acellular biological tissue made from bovine pericardia, genipin was used as a crosslinker [181] Tissue ingrowth after in vivo implantation was observed demonstrating the general usefulness of genipin in tissue engineering applications, again in agreement with the results obtained with cartilage in the present study.

In summary, the hyaluronic acid-gelatine-collagen copolymer scaffolds, especially when crosslinked using EDC, appear to be suitable candidates for cartilage engineering. Under favourable conditions employing insulin as potent effector molecule almost the complete cross-section exhibited well-developed cartilaginous tissue, demonstrating that the process of crosslinking can be advantageous for cartilage engineering. Future studies should

be undertaken to further improve tissue development also in the very centre of the constructs, possibly by altering scaffold pore size and interconnectivity.

Summary

The scaffold used in the process of cartilage engineering is considered as one of the most important factors affecting the quality of the engineered tissue. Scaffolds made from natural polymers appear to be useful candidates as they can provide a similar matrix as present in native tissue. As scaffolds made from natural polymers often lack mechanical stability, chemical crosslinking can be employed in order to improve the mechanical performance. However, crosslinking has been reported to have serious disadvantages with regard to cellular function and tissue development. Therefore, it is indispensable to investigate into the effects on tissue quality exerted by the employed crosslinking procedures.

Recently, a new natural biodegradable copolymer scaffold has been developed consisting of hyaluronic acid, gelatin, and collagen [87]. Within this study, scaffolds made from different derivatives of this copolymer, that is non-crosslinked, genipin crosslinked, EDC crosslinked and glutaraldehyde crosslinked, were investigated with regard to their suitability for in vitro cartilage engineering. Scaffolds were seeded with bovine chondrocytes; the seeding efficiency was found to be equal for all scaffolds. Cell-polymer constructs were cultivated in medium containing 1% fetal bovine serum (FBS) over four weeks without and with insulin supplementation. Thereafter, the weight, size and shape of each construct were evaluated and the amount of extracellular matrix (ECM) and the cell number were estimated. In addition, GAG and collagen types I and II were detected histologically and immunohistologically.

It was demonstrated that neither of the crosslinking procedures impaired the development of cartilaginous tissues In general, whereas crosslinking using glutaraldehyde resulted in cell-polymer constructs which were very similar to constructs grown on non-crosslinked scaffolds, crosslinking by EDC as well as genipin even resulted in partial improvement of the grown constructs. The most advanced cell-polymer constructs were those grown on EDC crosslinked scaffolds in the presence of insulin. They exhibited a distinctly

larger wet weight and larger glycosaminoglycan content per weight, and larger areas of coherent ECM and tissue formation, as compared to the non-crosslinked scaffolds.

The study suggests the scaffolds made from hyaluronic acid, gelatine, and collagen, and crosslinked with EDC as suitable candidate for cartilage engineering.

Chapter 7

Effects of Long-term in vitro Culture on Tissue Engineered Cartilage

Introduction

Most of the research on the generation of engineered cartilage has focused on (a) the cell carrier, i.e., the nature, the quality and the method of preparations of scaffolds [39;40;55;56;62;182;183], (b) the effects of exogenous growth factors on chondrocyte proliferation and ECM synthesis [68;73;89;95;184-190], (c) the effects of physical stimuli on extracellular matrix synthesis [94;191-193], and (d) the cell source (mainly chondrocytes or mesenchymal stem cells) and the possibility of expanding the cells in order to obtain a sufficient number of cells to graft or to seed in a scaffolds [49;194-196]. However, most of the studies have investigated these parameters in a short term culture (two - six weeks) and up to now only very few studies were conducted to study the effects of long-term culture on engineered cartilage.

It has been reported that some of the most age-related changes in articular cartilage in adult humans and animals are an increase in the degradation of matrix components [197]. and a decrease in the number of chondrocytes [198] Calcification occurs in the ECM of the hypertrophic zone of the growth plate, the ECM volume is reduced to a minimum and alkaline phosphatase content is maximal [199]. Total collagen initially increases through the proliferating and maturing zones but then rapidly decreases in the hypertrophic zone [199]. Chondrocyte play a signalling role in the determination of bone growth through their function in the endochondral ossification process [200]. In this process the avascular cartilage template is replaced with a highly vascularized, mineralized tissue. In addition, hypertrophic chondrocytes secrete matrix vesicles containing enzymes that actively degrade and mineralize their surrounding matrix [201]; these cells undergo apoptosis as this area is invaded by blood vessels, osteogenic cells and mesenchymal precursors. The residual calcified cartilage matrix act as scaffolds for the deposition of mineralized bone matrix, resulting in the production of new trabecular bone [202;203].

The purpose of this experiment was the monitoring of the effects of in vitro ageing on engineered cartilage. Bovine chondrocytes were seeded on PGA scaffolds and the cell-polymer constructs were cultivated in medium containing 1% FBS for up to 16 months with

and without insulin. We focused on the extracellular matrix content and the calcification of the engineered cartilage after long-tem in vitro culture.

Results

Time course of tissue development

In this study conducted large PGA meshes (6 x 8 mm, 2 mm thick) were seeded with chondrocytes. Two separate studies were performed; in the first one, large scaffolds were cultivated and harvested at four different time points, that is, after 1, 4 , 8, and 12 months in order to evaluate the time course of tissue development. In the second experiment, scaffolds were harvested only after 16 months, mainly to investigate calcification of the tissue.
This study was planned as a preliminary investigation.Therefore, only one large construct was used per group. Parts of the construct were used for histology, other parts were used for GAG, collagen, and cell number assays. Standard deviations in the GAG, collagen, and cell number assays result from the investigation of two different parts of the construct.

First, the growth rate of cell-polymer constructs cultured in medium containing 1% FBS either supplemented with insulin (2.5 µg/ml) or without insulin was monitored. The cell-polymer constructs strongly and continuously increased in size and weight over the course of 12 months of in vitro culture. After one month the wet weights were 232 mg (without insulin) and 508 mg (with insulin), after 4 months the cell-polymer constructs had grown to 474 mg and 1877 mg, after eight months the weights were 1090 mg and 3500 mg (see also Fig. 31) and after twelve months the weights were (1667 mg and 4660 mg) (Fig. 32).

In the second study, cell-polymer constructs harvested after 16 months were smaller and the weights of the constructs were 940 mg and 2600 mg without and with insulin supplementation, respectively (Fig. 32).

Macroscopically, at four months and thereafter the appearance of the cell-polymer constructs highly resembled that of native cartilage, i.e., glossy and white (Fig. 31A). However, also at four months, there was a zone recognizable in the middle of the construct cross-sections that appeared slightly darker and, upon gross examination using forceps and scalpel, mechanically weaker (Fig 31B).

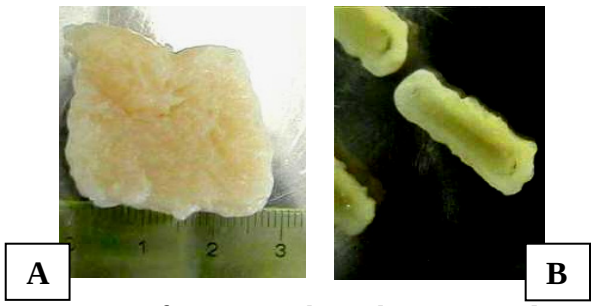

Fig. 31: Cell-polymer construct after 8 months culture in medium containing 1% FBS and supplemented with insulin (2.5 µg/ml). A: Whole construct. B: Cross-section.

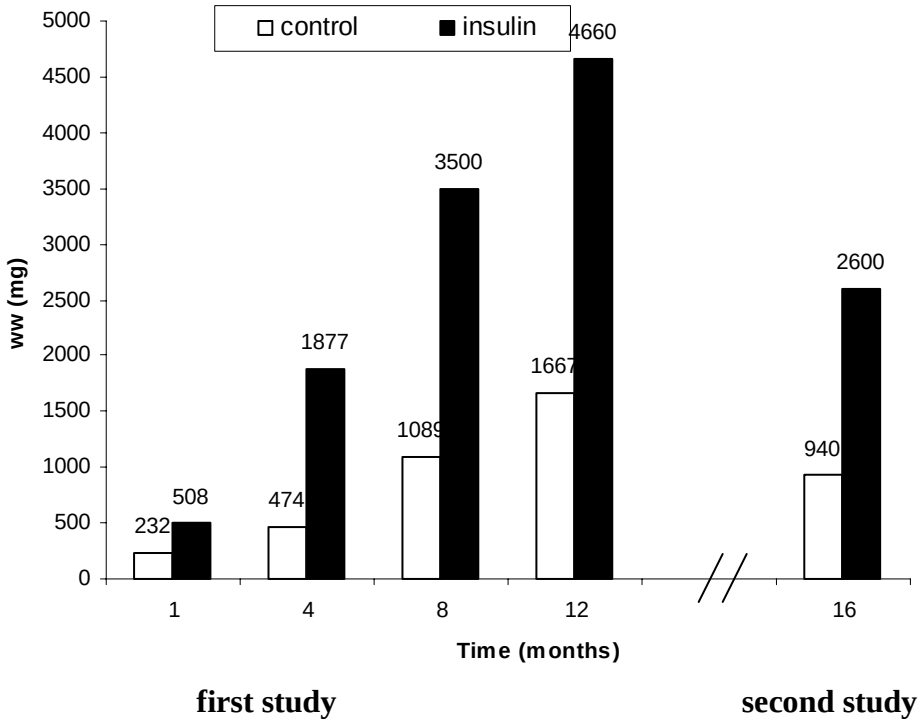

Fig. 32: Wet weights of the constructs at different points of time of in vitro culture. Medium contained 1% FBS with and without insulin.

Cell number

The cell number per wet weight of the constructs cultured for four months with or without insulin was highly decreased, as compared to after one month (67 and 36 per µg

without insulin, 34 and 23 per µg with insulin). After that the cell numbers per weight did not change significantly (Fig. 33), it was always lower in insulin constructs, as compared to the constructs receiving no insulin.

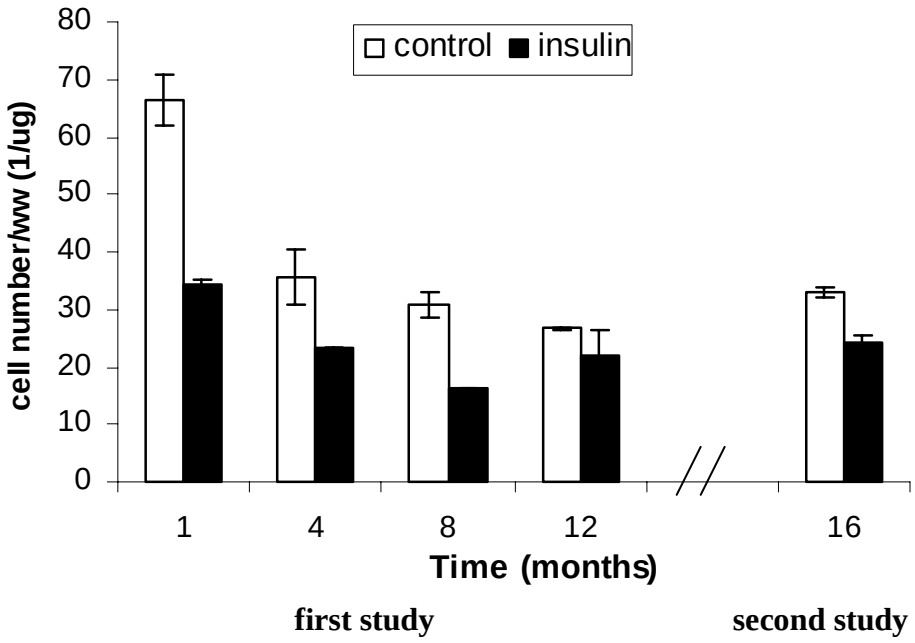

Fig. 33: Cell number per wet weight of the constructs at different points of time of in vitro culture. Medium contained 1% FBS with and without insulin

ECM quantification

GAG

The amount of GAG per wet weight increased in constructs supplemented either with or without insulin up to eight months. After one month the amount of GAG per wet weight in both groups (with and without insulin) was about 3.8% and after four months about 6%. However, after eight months GAG per weight was 7.6% for the construct without insulin and 10% for the construct with insulin. After one year in culture the amount of GAG per wet weight was hardly changed in the group without insulin (6.8%) and decreased in the group supplemented with insulin (to 7%) (Fig. 34).

In the second study, after sixteen months the amount of GAG per wet weight was very low in the construct without insulin (3%), the insulin-supplemented construct had about 6% (Fig. 34).

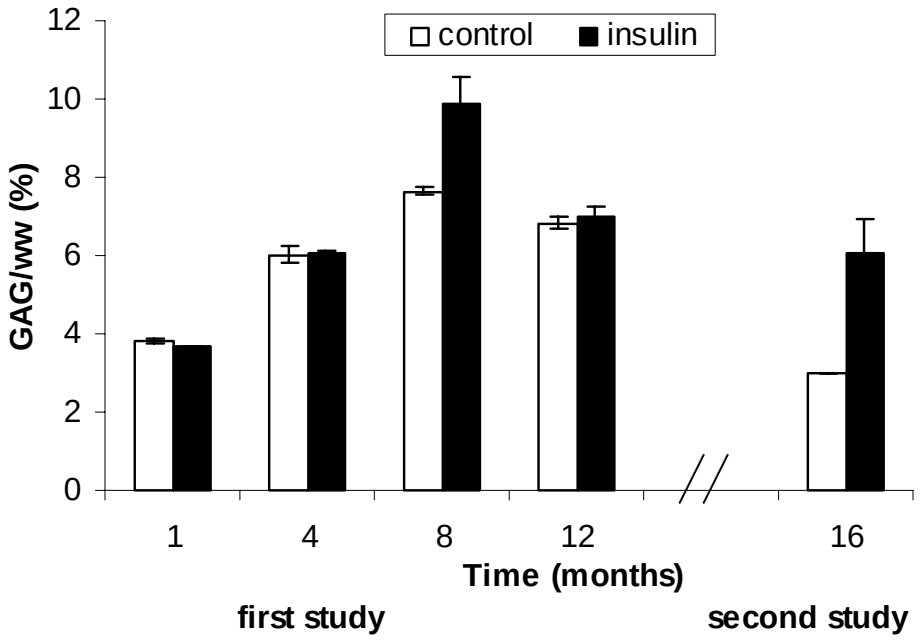

Fig. 34: GAG per wet weight of the constructs at different points of time of in vitro culture. Medium contained 1% FBS with and without insulin.

Total collagen

The amount of collagen per wet weight of the constructs increased in both the insulin-receiving group and in the group without insulin over the whole course of the experiment. After 1 month the collagen content was about 2% in both groups and after 12 months it was 6.1% in the non-insulin group and 8% in the insulin-receiving group. (Fig. 35).

In the second study, after 16 month also high values were reached, i.e., 7.4% in the non-insulin group and 8.1% in the insulin-receiving group (Fig. 35).

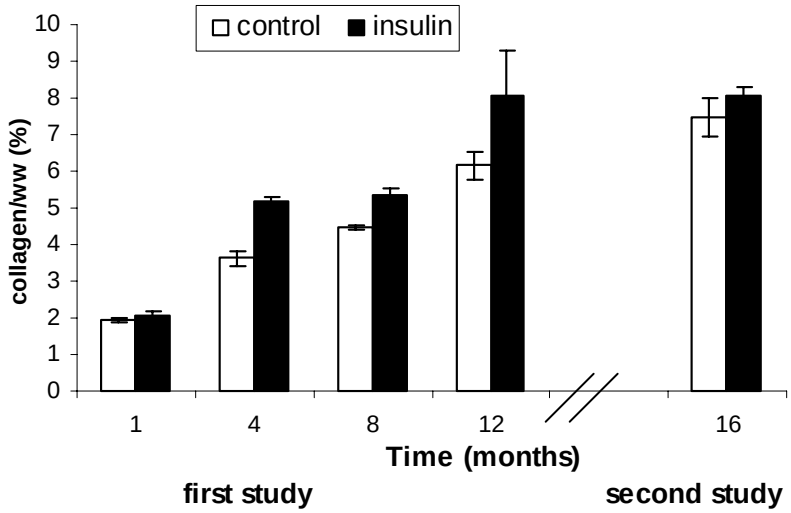

Fig. 35: Collagen per wet weight of the constructs at different points of time of in vitro culture. Medium contained 1% FBS with and without insulin.

Histology

GAG

The histological staining with safranin-O for the detection of GAG distribution throughout the constructs confirmed the results obtained in the biochemical assay. After one month, the construct without insulin exhibited a considerable amount of GAG but with large holes in the cross-sections (Fig. 36). After four months, cross-sections of these constructs also contained large areas with GAG, but also GAG-depleted regions preferentially at the edges; in addition, the chondrocytes in the GAG-containing areas appeared more rounded than after one month (Fig. 36). After eight and twelve months the staining for GAG appeared even more intensive and continuously distributed throughout the whole constructs (Fig. 36).

The insulin-supplemented group, after one month, contained a considerable amount of GAG. The amount of GAG was increased after four and eight months, when an intensive red staining was observed and chondrocytes appeared large and rounded and lay in lacunae. At one year, whereas the inner area of the cross-section appeared similar to the constructs at earlier time points, there was a large outer area in the cross-section which contained little GAG (pink color) and hardly any cells in lacunae (Fig. 36).

In the separate study after sixteen months, the amount of GAG in the non-insulin group appeared very small and poorly distributed throughout the construct. There were different zones observable within the cross-section: The first zone at the constructs edge appeared completely depleted of GAG and contained a very small number of cells, the second zone contained cells surrounded by matrix stained with green or pink colour, and the third zone contained large, rounded chondrocytes embedded in little GAG, indicated by the light red staining (Fig. 36). In the insulin-receiving group, after sixteen months, the cross-sections were similar as at twelve months in the first study, only the area at the very edge of the construct, which was depleted of GAG and was also observed at all time points before, was even thicker and more obvious, which was similar to the non-insulin group (Fig. 36).

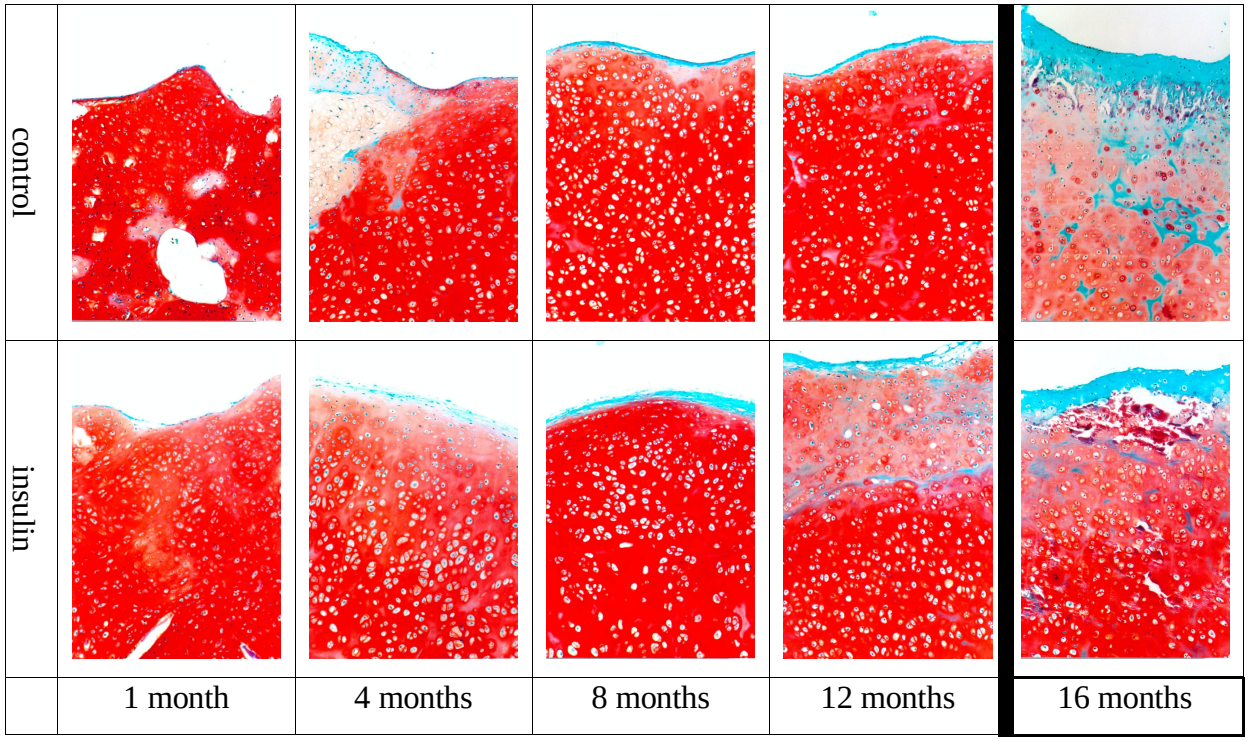

Fig. 36: Histological cross-sections of constructs grown in culture medium with 1% FBS. GAG in extracellular matrix was stained red with safranin-O (dark color in the black and white print) (x 100).

Collagen type II and type I

Collagen type II, a marker for differentiated chondrocytes, was detected at high levels in all constructs either supplemented with insulin or without insulin. The chondrocytes appeared large, i.e. probably hypertrophic, especially after 4, 8, and 12 months (Fig. 37). Collagen type I was clearly detected, at all time points, at the surface of the constructs supplemented either with or without insulin, corresponding to the flat, elongated fibroblast-like cells (Fig. 37).

In the second study, after sixteen months of in vitro culture, the zone at the edge of the cross-section that stained dark with collagen type I antibody was broader corresponding to the broad GAG-depleted areas observed in the Safranin-O stain (Fig. 37).

Calcification

Von Kossa staining for the detection of calcium phosphate as black spots in the construct cross-sections revealed that there was no calcium phosphate in both groups of constructs, either with or without insulin, at 1, 4, 8, and 12 months of cultivation.

In contrast, in the second study after sixteen months, in the cross-sections of constructs supplemented with insulin there were small spots stained with black colour beneath the superficial zone. In the non-insulin group, the black spots appeared more intensive and were more widely spread into the constructs (Fig. 38).

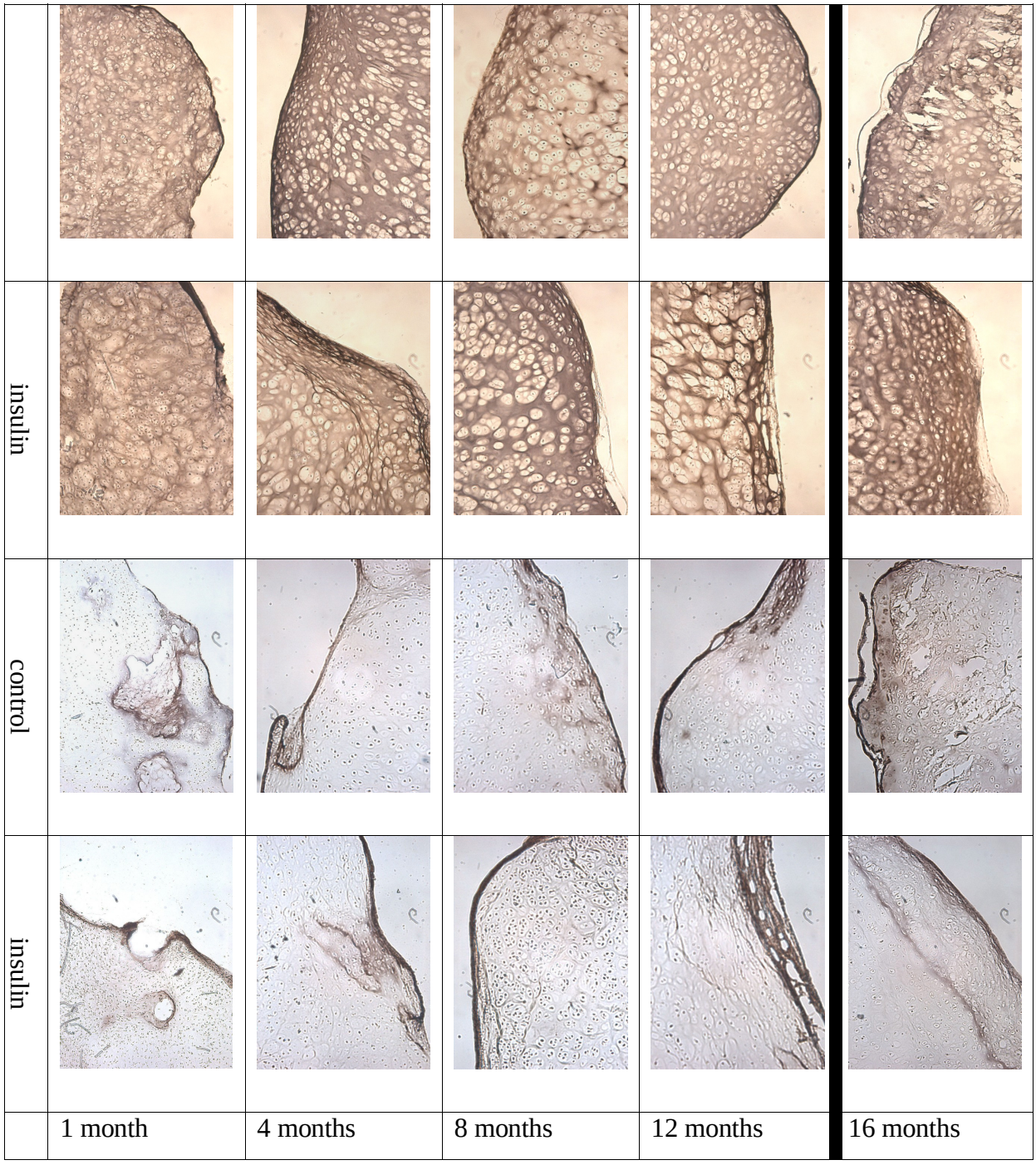

Fig. 37: Immunohistological staining of cross-sections of constructs grown in culture medium with 1% FBS. Collagen type II (first and second row) and type I (third and fourth row) was stained dark brown with collagen type II and type I antibodies (dark color in the black and white print) (x 100).

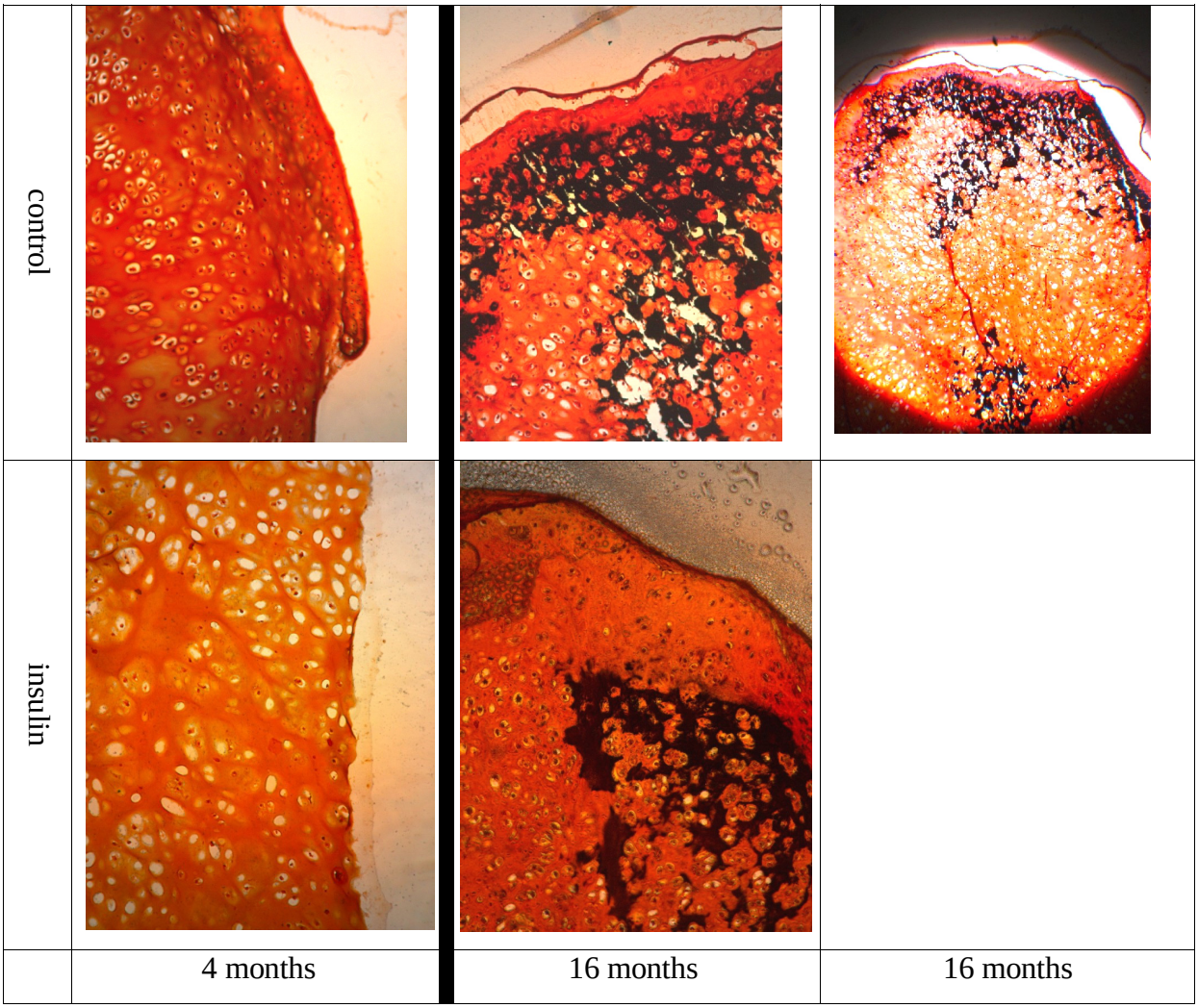

Fig. 38: Von Kossa staining of cross-sections of constructs grown in culture medium with 1% FBS at two time points. Calcified zones stained black (x 100 or x 40)

Discussion

This study was performed to evaluate the effects long-term in vitro culture on tissue-engineered cartilage grown in a well-established in vitro model system with and without insulin. It focused on the growth rate, the amount of ECM, cell number and the calcification of the engineered cartilage. So far, many studies have been undertaken investigating parameters affecting engineered cartilage in a short term culture (mainly two to six weeks), however, few data exist on long-term engineering culture. Here, two separate studies were performed; in the first one, tissue constructs were harvested after 1, 4, 8, and 12 months, and in the second study the constructs were harvested after 16 months.

The first study illustrated that it is possible to culture tissue-engineered cartilage over one year. The results obtained after 1, 4, 8, and 12 months showed that the cartilage grown without insulin (control) continuously increased throughout this period of in vitro culture in weight and size; also high amounts of the ECM components GAG and collagen were accumulated. At the same time, exogenous addition of insulin dramatically increased construct weight and size, as compared to the control constructs, and even increased fractions per weight of GAG and collagen (10% GAG and 8% collagen, compared to 8% GAG and 6% collagen of control constructs, after 8 and 12 months, respectively). All constructs contained type II collagen in abundance, as determined by immunohistochemistry; type I collagen was mainly detected in thin areas at the edges of the construct cross-sections. Especially after 4, 8, and 12 months enlarged cells appeared which had likely undergone hypertrophy. Previous studies have shown that chondrocytes undergo hypertrophy in vitro and express a number of bone factors that contribute to bone formation [204]. In constructs of both groups, after four month, a zone was recognized at the construct core that appeared mechanically weaker upon gross examination. Due to the large construct size, a lack of nutrient diffusion and/or oxygen supply possibly contributed to this phenomenon.

The second study used cells from a different cell isolation and results were only obtained after sixteen months. The constructs were smaller than the ones of the first study after twelve month. Insulin-receiving constructs contained similar fractions of GAG and

collagen than constructs of the first study, whereas control constructs contained smaller GAG fractions and larger collagen fractions. Differences between the studies can likely be attributed to different primary cell material. The most important results in this study is the development of scattered zones of calcification in the constructs. Von Kossa staining demonstrated that these zones were rich in calcium; at the same time very small amounts of GAG and collagen type II were detected with safranin-O stain and collagen type II antibody, respectively. The calcified zones appeared more intensive and more widely spread into the construct receiving no insulin, as compared to the insulin-receiving construct. This finding may be explained by insulin keeping the chondrocytes in the differentiated state, i.e., synthesizing cartilage-specific ECM, GAG and collagen. Thus, it may have minimized the calcification of the engineered cartilage in this experiment. Additionally, insulin may have been suppressing the release of proteases such as MMPs which can play a major role in the ossification process.

It has been reported that in vivo after terminal differentiation of chondrocytes into the hypertrophic state, these chondrocytes secrete large amounts of a specialized matrix rich in collagen type X, as well as some proteases such as matrix metalloproteinase (MMPs) [205;206]. MMPs can cleave ECM components and may facilitate mineralization; the absence of these proteases resulted in an enlargement of the hypertrophic zone and subsequent delay of the ossification process [202].In the future, further studies would be valuable that investigate the levels of proteases such as MMPs in engineered cartilage in order to decide if the calcification of the engineered cartilage after long-term in vitro culture resulted from the elevation of these enzymes or not. Also, further studies are needed to detect the distribution of collagen type X as a marker for the hypertrophic zone.

The results of this study raise questions, if the development of the calcified zones in the engineered cartilage after long-term culture is to be regarded as a positive or negative effect and also if similar effects will appear in vivo after implantation of such constructs. The latter may lead to serious problems in zones where pure cartilaginous tissue is required. Therefore, further research has to be conducted into possibilities to avoid undesired calcification of engineered cartilage. For other applications, these properties of the constructs

may be turned into an advantage. Case et al (2003) studied the ability of tissue engineered cartilage constructs to support bone formation. They seeded articular chondrocytes onto PGA disks, cultured for 4 weeks in vitro, and then transferred them to empty bone chambers previously implanted into rabbit femoral metaphyses. After 4 weeks in vivo, the result demonstrated that tissue-engineered cartilage constructs implanted into a well-vascularized bone defect will support direct appositional bone formation [207]. One can consider the results of our experiment as positive if it is hypothesized that after implantation of such a construct into a cartilage defect at the junction between the engineered cartilage and the host bone tissue it will perform the same physiological functions (endochondral ossification) of native cartilage, especially in children, to promote normal bone development and maintain longitudinal bone growth. However, further studies are needed to evaluate this hypothesis.

Summary

The objective of this study was to evaluate the effects of long-term in vitro culture on tissue-engineered cartilage with and without insulin focusing on the amount of ECM and the calcification of the engineered cartilage. In two separate studies bovine articular chondrocytes were seeded on polyglycolic acid scaffolds in spinner flask for two days, after which cell-polymer constructs were transferred into 6-well plates. In the first study, constructs were cultured for up to 12 months in medium containing 1% FBS either with or without exogenous insulin; the cell-polymer construct were harvested after 1, 4, 8, and 12 months. In the second study, the cell-polymer constructs were cultured at the same condition and harvested after 16 months.

The monitoring of in vitro engineered cartilage for 12 months in the first study revealed that the engineered tissues grew continuously and contained high amounts of ECM. Insulin-receiving constructs were dramatically larger in size and contained larger fractions of GAG and collagen (up to 10% and 8%, respectively). Type II collagen was detected in abundance in all constructs. In the second study, after sixteen months of in vitro culture, scattered zones of calcification developed in the engineered cartilage, especially in constructs cultivated without insulin. Future studies may investigate the events associated with

chondrocytes within the constructs undergoing hypertrophy and calcification of the constructs. Furthermore it appears important to evaluate the long-term performance of tissue engineered cartilage in vivo in dependence of the implantation site.

Chapter 8

Summary and Conclusions

Summary and Conclusions

Tissue engineering of cartilage holds the promise to improve treatment strategies to overcome the problems arising from the loss or failure of tissue occurring through diseases or accidents. Engineered cartilage is considered to be one of the first engineered tissues that will reach a broad market. The optimization of the in vitro culture conditions to get an ideal tissue, highly resembling native tissue, is a very important step in the process of tissue engineering. The goal of this thesis was to study different factors affecting the development of in vitro engineered cartilage.

Chondrocytes were seeded on synthetic or natural biodegradable scaffolds. Then the cell-polymer constructs were cultivated over different periods of time to develop a cartilage-like tissue. The cells proliferated and produced ECM molecules, at the same time the polymer scaffolds degraded. After the in vitro culture period, the cell-polymer construct was biochemically, histologically and immunohistochemically investigated. In the period of in vitro culture the effects of exogenously applied cytokines and growth factor binding proteins (IGFBPs) on the growth rate and content of ECM components (glycosaminoglycans (GAG) and collagen) of the engineered cartilage were analyzed **(chapter 3, 4 and 5);** investigations into the mechanism of action of interleukin-4 (IL-4) was conducted (**chapter 3)**. Furthermore, the potential of new natural biodegradable copolymer scaffolds to generate cartilage-like tissue was evaluated (**chapter 6)**. An additional experiment was designed to monitor the development of in vitro engineered cartilage for 16 months with a special focus on the ECM components and a possible calcification of the engineered cartilage **(chapter 7).**

Interleukin-4 was investigated in order to evaluate its potential to improve the quality of engineered cartilage **(chapter 3)**. IL-4 was previously shown to inhibit degradation of proteoglycans, a major component of cartilage ECM [75], through mechanisms of action are still controversially discussed [75;103-106]. In the present study, IL-4 had positive effects on the growth rate, ECM composition, and GAG distribution of the engineered cartilage. Thus, IL-4 appeared as promising candidates to improve the development and composition of engineered cartilage. The mechanism by which IL-4 increases the GAG content may be either inhibition of GAG degradation or increased GAG subtype synthesis. In order to elucidate the

respective contributions, RT-PCR was employed to investigate the expression of (1) matrix metalloproteinases MMP-1, MMP-3, MMP-13; (2) tissue inhibitor of metalloproteinases-1 (TIMP-1); and (3) GAG subtypes aggrecan and biglycan. Whereas effects of IL-4 on MMP-1, MMP-3 and TIMP-1 expression have been suggested previously, this study was the first to evaluate the effects of IL-4 on MMP-13 expression. IL-4 was demonstrated to have distinct inhibitory effects on MMP-13 expression without significantly affecting any of the other factors investigated (slight increase of MMP-3 and decrease of aggrecan). Our results suggest that IL-4 increases GAG fractions in engineered cartilage at least in part by decreasing MMP-13 expression and thus inhibiting GAG degradation.

Insulin-like growth factors binding proteins (IGFBPs) are a family of six or more related proteins that affect the growth rate of cartilage, have a high affinity for IGF-I [119-121] and modulate its actions [76;77]. They have been detected in the culture media of cultured chondrocytes from various species [78;79] and in intact bovine cartilage [124]**.** In **chapter 4,** the hypothesis that IGFBP-4 inhibits the growth of cartilage in absence of IGF-I was evaluated in a well-established 3-D culture system. In addition, the inhibitory effects of IGFBP-4 on the actions of IGF-I, which has potent stimulatory effects on chondrocyte proliferation and extracellular matrix synthesis [68], were investigated. The results demonstrated that exogenous IGFBP-4, in the absence of exogenous IGF-I, had significant inhibitory effects on the engineered cartilage compared to control constructs in a dose-dependent manner, as demonstrated by an inhibited growth rate, a reduction in the GAG and collagen production and an increased cell number per wet weight. The addition of IGFBP-4 to the engineered constructs in the presence of exogenous IGF-I (50 ng/ml) in different molar ratios had significant inhibitory effects at all molar ratios (1:0.5 - 1:100), as compared to constructs treated only with IGF-I (50 ng/ml). In contrast, compared to the control group receiving no supplemental protein, the combinations showed no inhibitory effects on the growth rate and ECM components of the engineered constructs at low IGFBP-4 concentrations (IGF-I: IGFBP-4 molar ratio of 1:3), demonstrating that IGF-I can overcome the inhibitory effects of IGFBP-4.

IGFBP-5 is expressed by chondrocytes [81;82] and is the predominant IGFBP in bone [148] where it can either inhibit or increase IGF-I effects [83], in part via an IGF-I-

independent mechanism [84]. The hypothesis that insulin-like growth factor binding protein-5 may function as a growth factor in cartilage, as it has previously been suggested for bone tissue [85], was evaluated in **chapter 5.** The effects of IGFBP-5 on in vitro engineered cartilage were investigated in the presence and absence of exogenous IGF-I. The results demonstrated that exogenous IGFBP-5 in absence of IGF-I clearly stimulated the growth of cartilaginous tissue constructs. The fraction of collagen per wet weight of the constructs was slightly increased. Histological cross-sections revealed a more coherent tissue formation after application of IGFBP-5, as compared to control constructs; glycosaminoglycans were more evenly distributed throughout the sections in IGFBP-5 constructs. Though the impact of IGFBP-5 on the cartilaginous tissues was obvious, in comparison, IGF-I alone (50 ng/ml) exerted stronger effects in all parameters investigated. In combinations with IGF-I, IGFBP-5 generally significantly reduced the effects of IGF-I at all molar ratios investigated.

Scaffolds made from natural polymers appear to be useful candidates for tissue engineering purposes as they can provide a similar matrix as present in native tissue. Recently, a new natural biodegradable copolymer scaffold has been developed consisting of hyaluronic acid, gelatin, and collagen [87]. In order to improve mechanical stability of scaffolds made from natural polymers, chemical crosslinking can be employed [175-177]. However, as chemical crosslinking processes may lead to cell toxic effects or impairment of tissue development [174;175], such scaffolds have to be carefully investigated in cell culture. In **chapter 6**, scaffolds made from different derivatives of this copolymer, that is non-crosslinked, genipin-crosslinked, EDC-crosslinked and glutaraldehyde-crosslinked, were investigated with regard to their suitability for in vitro cartilage engineering. The results revealed that the seeding efficiency was found to be equal for all scaffolds after seeding with chondrocytes for two days. After 4 weeks of cultivation without and with insulin supplementation the results demonstrated that neither of the crosslinking procedures impaired the development of cartilaginous tissues. In general, whereas crosslinking using glutaraldehyde resulted in cell-polymer constructs which were very similar to constructs grown on non-crosslinked scaffolds, crosslinking by EDC as well as genipin even resulted in partial improvement of the grown constructs. The most advanced cell-polymer constructs were those grown on EDC crosslinked scaffolds in the presence of insulin. They exhibited a

distinctly larger wet weight and larger glycosaminoglycan content per weight, and larger areas of coherent ECM and tissue formation, as compared to the non-crosslinked scaffolds.

Many studies on in vitro engineering of cartilage have been conducted; however, most of those studies have been conducted in short-term cultures, that is, over two to six weeks. Hardly any data exist on long-term in vitro culture so far. Therefore, in **chapter 7** the development of engineered cartilage was monitored in two separate studies over 12 and 16 months, respectively. In the first study, the engineered tissues grew continuously over one year and contained high amounts of ECM. Insulin-receiving constructs were dramatically larger in size and even contained larger fractions of GAG and collagen (up to 10% GAG and 8% collagen). Type II collagen was detected in abundance in all constructs. In the second study, after sixteen months of in vitro culture, scattered zones of calcification developed in the engineered cartilage, especially in constructs cultivated without insulin. Future studies may investigate the events associated with the chondrocytes undergoing hypertrophy and the calcification of the constructs. Furthermore it appears important to evaluate the long-term performance of tissue engineered cartilage in vivo in dependence of the implantation site.

To conclude, this thesis contributed to the development of strategies for engineering cartilage tissues. Specific bioactive molecules (IL-4, IGFBP-5) were demonstrated to enhance the quality of engineered cartilage; another protein (IGFBP-4) was shown to reduce the growth rate and compromise the quality of engineered cartilage. Additionally, a novel mechanism of action of IL-4 in cartilage was suggested. All of these studies also demonstrated the employed 3-D engineering culture as a useful model to investigate the effects and/or mechanisms of specific molecules in developing cartilage. The potential of novel scaffolds made from natural crosslinked copolymers to generate new cartilage was illustrated. Furthermore, the effects of long-term in vitro culture up to 16 months on engineered cartilage were monitored, proving, on the one hand, the feasibility of generating tissue constructs with high ECM content, but, on the other hand, showing calcification of the constructs and contributing to the discussion about the potential applications of such tissues in vivo.

Chapter 9

References

[1] Lalan, S., Pomerantseva, I., Vacanti, J.P.(**2001**) "Tissue engineering and its potential impact on surgery" in *World Journal of Surgery* 25(11) p. 1458-1466

[2] Marler, J.J., Upton, J., Langer, R., Vacanti, J.P.(**1998**) "Transplantation of cells in matrices for tissue regeneration" in *Advanced Drug Delivery Reviews* 33(1-2) p. 165-182

[3] Freed, L.E. and Vunjak-Novakovic, G. (**1995**) "Tissue Engineering of Cartilage" in *The Biomedical Engineering Handbook* (Ed.: Bronzino,J.D.; CRC Press; Boca Raton, Florida)

[4] Fuchs, J.R., Nasseri, B.A., Vacanti, J.P.(**2001**) "Tissue engineering: a 21st century solution to surgical reconstruction" in *The Annals of Thoracic Surgery* 72(2) p. 577-591

[5] Patrick, C.W., Mikos, A.G., McIntire, L.V. (**1998**) "Prospectus of Tissue Engineering" in *Frontiers in Tissue Engineering* (Ed.: Patrick,C.W., Mikos, A. G., and McIntire, L. V.; Pergamon; Oxford)

[6] Laurencin, C.T., Ambrosio, A.M.A., Borden, M.D., Cooper, J.A., Jr.(**1999**) "Tissue engineering: orthopedic applications" in *Annual Review of Biomedical Engineering* 1 p. 19-46

[7] Benjamin, J.B., Bosco, I.I.I., Wallace, D.(**1994**) "Morphologic and qualitative analysis of polyethylene wear debris in synovial fluid of patients with total knee arthroplasty" in *The Journal of Arthroplasty* 9(1) p. 111-112

[8] Wright, T.M. and Rimnac, C.M. (**1991**) "Ultra-high-molecular-weight polyethylene" in *Joint Replacement Arthroplasty* (Ed.: Morrey,B.F.; Churchill Livingstone; New York)

[9] Langer, R. and Vacanti, J.P.(**1993**) "Tissue engineering" in *Science* 260(5110) p. 920-926

[10] Fabiani, J.N., Dreyfus, G.D., Marchand, M., Jourdan, J., Aupard, M., Latremouille, C., Chardigny, C., Carpentier, A.F.(**1995**) "The autologous tissue cardiac valve: a new paradigm for heart valve replacement" in *Annal of Thoracic Surgery* 60(2 Suppl) p. S189-S194

[11] Niklason, L.E., Gao, J., Abbott, W.M., Hirschi, K.K., Houser, S., Marini, R., Langer, R.(**1999**) "Functional arteries grown in vitro" in *Science* 284(5413) p. 489-493

[12] Huynh, T., Abraham, G., Murray, J., Brockbank, K., Hagen, P.O., Sullivan, S.(**1999**) "Remodeling of an acellular collagen graft into a physiologically responsive neovessel" in *Nature Biotechnology* 17(11) p. 1083-1086

[13] Lanza, R. and Chick, W.L.(**1997**) "Bioartificial organs: Science, medicine, and technology" in *Annals of the New York Academy of Sciences* 831 p. 323-331

[14] Chen, S.C., Mullon, C., Kahaku, E., Watanabe, F., Hewitt, W., Eguchi, S., Middleton, Y., Arkadopoulos, N., Rozga, J., Solomon, B., Demetriou, A.A.(**1997**) "Treatment of severe liver failure with a bioartificial liver" in *Annals of the New York Academy of Sciences* 831 p. 350-360

[15] Oberpenning, F., Meng, J., Yoo, J.J., Atala, A.(**1999**) "De novo reconstitution of a functional mammalian urinary bladder by tissue engineering" in *Nature Biotechnology* 17(2) p. 149-155

[16] Stading, M. and Langer, R.(**1999**) "Mechanical shear properties of cell-polymer cartilage constructs" in *Tissue Engineering* 5(3) p. 241-250

[17] Ashiku, S.K., Randolph, M.A., Vacanti, C.A.(**1997**) "Tissue engineered cartilage" in *Materials Science Forum* 250 p. 129-150

[18] Einhorn, T.A. (**1999**) "Clinically applied models of bone regeneration in tissue engineering research" in *Clinical Orthopaedics and Related Research*(367 Suppl) p. 59-67

[19] Ham, A.W. (**1974**) "Dense Ordinary Connective Tissue and Cartilage" in *Histology* (Ed.: Ham,A.W.; Lippincott; Philadelphia)

[20] Buckwalter, J.A. and Mankin, H.J. (**1998**) "Articular cartilage: tissue design and chondrocyte-matrix interactions" in *Instructional Course Lectures* 47 p. 477-486

[21] Freed, L.E., Hollander, A.P., Martin, I., Barry, J.R., Langer, R., Vunjak-Novakovic, G.(**1998**) "Chondrogenesis in a Cell-Polymer-Bioreactor System*1" in *Experimental Cell Research* 240(1) p. 58-65

[22] Hollander, A.P., Heathfield, T.F., Webber, C., Iwata, Y., Bourne, R., Rorabeck, C., and Poole, A.R.(**1993**) "Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay" in *J.Clin.Invest.* 93 p. 1722-1732

[23] Fawcett, D.W. (**1994**) "Cartilage" in *A textbook of Histology* (Ed.: Fawcett,D.W.; Chapman; Hall; New York)

[24] Kempson, G. (**1980**) "The mechanical properties of articular cartilage" in *The Joints and Synovial Fluid* (Ed.: Sokoloff,L.; Academic Press; New York)

[25] Cohen, N.P., Foster, R.J., Mow, V.C. (**1998**) "Composition and dynamics of articular cartilage: structure, function, and maintaining healthy state" in *The Journal of Orthopaedic and Sports Physical Therapy* 28(4) p. 203-215

[26] Buckwalter, J.A.(**1983**) "Articular cartilage" in *Instructional Course Lectures* 32 p. 349-370

[27] Wirth, C.J. and Rudert, M.(**1996**) "Techniques of cartilage growth enhancement: a review of the literature" in *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 12(3) p. 300-308

[28] Huber, M., Trattnig, S., Lintner, F.(**2000**) "Anatomy, biochemistry, and physiology of articular cartilage" in *Investigative Radiology* 35(10) p. 573-580

[29] Wilkins, R.J., Browning, J.A., Ellory, J.C.(**2000**) "Surviving in a matrix: membrane transport in articular chondrocytes" in *The Journal of Membrane Biology* 177(2) p. 95-108

[30] Buckwalter, J.A. and Mankin, H.J.(**1998**) "Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation" in *Instructional Course Lectures* 47 p. 487-504

[31] Shapiro, F., Koide, S., Glimcher, M.J.(**1993**) "Cell origin and differentiation in the repair of full-thickness defects of articular cartilage" in *The Journal of Bone and Joint Surgery. American Volume* 75(4) p. 532-553

[32] Hunziker, E.B.(**2002**) "Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects" in *Osteoarthritis and Cartilage* 10(6) p. 432-463

[33] O'Driscoll, S.W.(**1998**) "The healing and regeneration of articular cartilage" in *The Journal of Bone and Joint Surgery .American Volume* 80(12) p. 1795-1812

[34] Minas, T.(**1999**) "The role of cartilage repair techniques, including chondrocyte transplantation, in focal chondral knee damage" in *Instructional Course Lectures* 48 p. 629-643

[35] Hunziker, E.B.(**1999**) "Articular cartilage repair: are the intrinsic biological constraints undermining this process insuperable?" in *Osteoarthritis and Cartilage* 7(1) p. 15-28

[36] Goldberg, V.M. and Caplan, A.I.(**1999**) "Biologic restoration of articular surfaces" in *Instructional Course Lectures* 48 p. 623-627

[37] Gillogly, S.D., Voight, M., Blackburn, T.(**1998**) "Treatment of articular cartilage defects of the knee with autologous chondrocyte implantation" in *The Journal of Orthopaedic and Sports Physical Therapy* 28(4) p. 241-251

[38] Ma, P.X. and Langer, R.(**1999**) "Morphology and mechanical function of long-term in vitro engineered cartilage" in *Journal of Biomedical Materials Research* 44(2) p. 217-221

[39] Suh, J.K. and Matthew, H.W.(**2000**) "Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review" in *Biomaterials* 21(24) p. 2589-2598

[40] Sherwood, J.K., Riley, S.L., Palazzolo, R., Brown, S.C., Monkhouse, D.C., Coates, M., Griffith, L.G., Landeen, L.K., Ratcliffe, A.(**2002**) "A three-dimensional osteochondral composite scaffold for articular cartilage repair" in *Biomaterials* 23(24) p. 4739-4751

[41] Gugala, Z. and Gogolewski, S.(**2000**) "In vitro growth and activity of primary chondrocytes on a resorbable polylactide three-dimensional scaffold" in *Journal of Biomedical Materials Research* 49(2) p. 183-191

[42] Elisseeff, J.H., Lee, A., Kleinman, H.K., Yamada, Y.(**2002**) "Biological response of chondrocytes to hydrogels" in *Annals of the New York Academy of Sciences* 961 p. 118-122

[43] Wakitani, S., Goto, T., Young, R.G., Mansour, J.M., Goldberg, V.M., Caplan, A.I.(**1998**) "Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collagen gel" in *Tissue Engineering* 4(4) p. 429-444

[44] Aston, J.E. and Bentley, G.(**1986**) "Repair of articular surfaces by allografts of articular and growth-plate cartilage" in *The Journal of Bone and Joint Surgery.British Volume* 68(1) p. 29-35

[45] Thomson, R.C., Wake, M.C., Yaszemski, M.J., Mikos, A.G.(**1995**) "Biodegradable polymer scaffolds to regenerate organs" in *Advanced Polymer Science* 122 p. 245-274

[46] Freed, L.E., Grande, D.A., Lingbin, Z., Emmanual, J., Marquis, J.C., Langer, R.(**1994**) "Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds" in *Journal of Biomedical Materials Research* 28(8) p. 891-899

[47] Vunjak-Novakovic, G., Obradovic, B., Martin, I., Bursac, P.M., Langer, R., Freed, L.E.(**1998**) "Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering" in *Biotechnology Progress* 14(2) p. 193-202

[48] Marijnissen, W.J.C.M., van Osch, G.J.V.M., Aigner, J., Verwoerd-Verhoef, H.L., Verhaar, J.A.N.(**2000**) "Tissue-engineered cartilage using serially passaged articular chondrocytes. Chondrocytes in alginate, combined in vivo with a synthetic (E210) or biologic biodegradable carrier (DBM)*1" in *Biomaterials* 21(6) p. 571-580

[49] Martin, I., Vunjak-Novakovic, G., Yang, J., Langer, R., Freed, L.E.(**1999**) "Mammalian Chondrocytes Expanded in the Presence of Fibroblast Growth Factor 2 Maintain the Ability to Differentiate and Regenerate Three-Dimensional Cartilaginous Tissue" in *Experimental Cell Research* 253(2) p. 681-688

[50] Caplan, A.I., Elyaderani, M., Mochizuki, Y., Wakitani, S., Goldberg, V.M.(**1997**) "Principles of cartilage repair and regeneration" in *Clin.Orthop.* p. 254-269

[51] Woodfield, T.B.F., Malda, J., de Wijn, J., Peters, F., Riesle, J., van Blitterswijk, C.A.(**2004**) "Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique" in *Biomaterials* 25(18) p. 4149-4161

[52] Woodfield, T.B.F., Bezemer, J.M., Pieper, J.S., van Blitterswijk, C.A., Riesle, J.(**2002**) "Scaffolds for tissue engineering of cartilage" in *Critical Reviews in Eukaryotic Gene Expression* 12(3) p. 209-236

[53] Yang, S., Leong, K.F., Du, Z., Chua, C.K.(**2001**) "The design of scaffolds for use in tissue engineering. Part I. Traditional factors" in *Tissue Engineering* 7(6) p. 679-689

[54] Yang, S., Leong, K.F., Du, Z., Chua, C.K.(**2002**) "The design of scaffolds for use in tissue engineering. Part II. Rapid prototyping techniques" in *Tissue Engineering* 8(1) p. 1-11

[55] Hutmacher, D.W.(**2000**) "Scaffolds in tissue engineering bone and cartilage" in *Biomaterials* 21(24) p. 2529-2543

[56] Quirk, R.A., Chan, W.C., Davies, M.C., Tendler, S.J.B., Shakesheff, K.M.(**2001**) "Poly(-lysine)-GRGDS as a biomimetic surface modifier for poly(lactic acid)" in *Biomaterials* 22(8) p. 865-872

[57] Jansen, J.A., Vehof, J.W.M., Ruhe, P.Q., Kroeze-Deutman, H., Kuboki, Y., Takita, H., Hedberg, E.L., Mikos, A.G. "Growth factor-loaded scaffolds for bone engineering" in *Journal of Controlled Release* In Press, Corrected Proof

[58] Lee, K.Y. and Mooney, D.J.(**2001**) "Hydrogels for tissue engineering" in *Chemical Reviews* 101(7) p. 1869-1879

[59] Temenoff, J.S. and Mikos, A.G.(**2000**) "Review: tissue engineering for regeneration of articular cartilage" in *Biomaterials* 21(5) p. 431-440

[60] Stammen, J.A., Williams, S., Ku, D.N., Guldberg, R.E.(**2001**) "Mechanical properties of a novel PVA hydrogel in shear and unconfined compression" in *Biomaterials* 22(8) p. 799-806

[61] Seal, B.L., Otero, T.C., Panitch, A.(**2001**) "Polymeric biomaterials for tissue and organ regeneration" in *Materials Science and Engineering: R: Reports* 34(4-5) p. 147-230

[62] Caterson, E.J., Li, W.J., Nesti, L.J., Albert, T., Danielson, K., Tuan, R.S.(**2002**) "Polymer/alginate amalgam for cartilage-tissue engineering" in *Annals of the New York Academy of Sciences* 961 p. 134-138

[63] Kawanishi, M., Ushida, T., Kaneko, T., Niwa, H., Fukubayashi, T., Nakamura, K., Oda, H., Tanaka, S., Tateishi, T.(**2004**) "New type of biodegradable porous scaffolds for tissue-engineered articular cartilage" in *Materials Science and Engineering: C* 24(3) p. 431-435

[64] Smith, L.A. and Ma, P.X. "Nano-fibrous scaffolds for tissue engineering" in *Colloids and Surfaces B: Biointerfaces* In Press, Corrected Proof

[65] Grad, S., Kupcsik, L., Gorna, K., Gogolewski, S., Alini, M.(**2003**) "The use of biodegradable polyurethane scaffolds for cartilage tissue engineering: potential and limitations" in *Biomaterials* 24(28) p. 5163-5171

[66] Stendahl, J.C., Li, L., Claussen, R.C., Stupp, S.I.(**2004**) "Modification of fibrous poly(-lactic acid) scaffolds with self-assembling triblock molecules" in *Biomaterials* 25 p. 5847-5856

[67] Svensson, A., Nicklasson, E., Harrah, T., Panilaitis, B., Kaplan, D.L., Brittberg, M., Gatenholm, P.(**2005**) "Bacterial cellulose as a potential scaffold for tissue engineering of cartilage" in *Biomaterials* 26 p. 419-431

[68] Blunk, T., Sieminski, A.L., Gooch, K.J., Courter, D.L., Hollander, A.P., Nahir, A.M., Langer, R., Vunjak-Novakovic, G., Freed, L.E.(**2002**) "Differential effects of growth factors on tissue-engineered cartilage" in *Tissue Engineering* 8(1) p. 73-84

[69] Fortier, L.A., Mohammed, H.O., Lust, G., Nixon, A.J.(**2002**) "Insulin-like growth factor-I enhances cell-based repair of articular cartilage" in *The Journal of Bone and Joint Surgery. British Volume* 84(2) p. 276-288

[70] Evans, C.H. and Robbins, P.D.(**1999**) "Genetically augmented tissue engineering of the musculoskeletal system" in *Clinical Orthopaedics and Related Research*(367) p. S410-S418

[71] Mahoney, M.J. and Saltzman, W.M.(**2001**) "Transplantation of brain cells assembled around a programmable synthetic microenvironment" in *Nature Biotechnology* 19(10) p. 934-939

[72] Shea, L.D., Smiley, E., Bonadio, J., Mooney, D.J.(**1999**) "DNA delivery from polymer matrices for tissue engineering" in *Nature Biotechnology* 17(6) p. 551-554

[73] van-Susante, J.L., Buma, P., van-Beuningen, H.M., van-den-Berg, W.B., Veth, R.P.(**2000**) "Responsiveness of bovine chondrocytes to growth factors in medium with different serum concentrations" in *J.Orthop.Res* 18 p. 68-77

[74] Weiser, L., Bhargava, M., Attia, E., Torzilli, P.A.(**1999**) "Effect of serum and platelet-derived growth factor on chondrocytes grown in collagen gels" in *Tissue Engineering* 5(6) p. 533-544

[75] Yeh, L.A., Augustine, A.J., Lee, P., Riviere, L.R., Sheldon, A.(**1995**) "Interleukin-4, an inhibitor of cartilage breakdown in bovine articular cartilage explants" in *Journal of Rheumatology* 22(9) p. 1740-1746

[76] Jones, J.I. and Clemmons, D.R.(**1995**) "Insulin-like growth factors and their binding proteins: biological actions" in *Endocrine Reviews* 16(1) p. 3-34

[77] Collett-Solberg, P.F. and Cohen, P.(**1996**) "The role of the insulin-like growth factor binding proteins and the IGFBP proteases in modulating IGF action" in *Endocrinology and Metabolism Clinics of North America* 25(3) p. 591-614

[78] Froger-Gaillard, B., Hossenlopp, P., Adolphe, M., Binoux, M.(**1989**) "Production of insulin-like growth factors and their binding proteins by rabbit articular chondrocytes: relationships with cell multiplication" in *Endocrinology* 124(5) p. 2365-2372

[79] Sunic, D., Belford, D.A., McNeil, J.D., Wiebkin, O.W.(**1995**) "Insulin-like growth factor binding proteins (IGF-BPs) in bovine articular and ovine growth-plate chondrocyte cultures: their regulation by IGFs and modulation of proteoglycan synthesis" in *Biochimica et Biophysica Acta* 1245(1) p. 43-48

[80] Schiltz, P.M., Mohan, S., Baylink, D.J.(**1993**) "Insulin-like growth factor binding protein-4 inhibits both basal and IGF-mediated chick pelvic cartilage growth in vitro" in *Journal of Bone and Mineral Research* 8(4) p. 391-396

[81] Sunic, D., McNeil, J.D., Rayner, T.E., Andress, D.L., Belford, D.A.(**1998**) "Regulation of insulin-like growth factor-binding protein-5 by insulin-like growth factor I and interleukin-1alpha in ovine articular chondrocytes" in *Endocrinology* 139(5) p. 2356-2362

[82] Matsumoto, T., Tsurumoto, T., Goldring, M.B., Shindo, H.(**2000**) "Differential effects of IGF-binding proteins, IGFBP-3 and IGFBP-5, on IGF-I action and binding to cell membranes of immortalized human chondrocytes" in *The Journal of Endocrinology* 166(1) p. 29-37

[83] Conover, C.A. and Kiefer, M.C.(**1993**) "Regulation and biological effect of endogenous insulin-like growth factor binding protein-5 in human osteoblastic cells" in *Journal of Clinical Endocrinology and Metabolism* 76(5) p. 1153-1159

[84] Richman, C., Baylink, D.J., Lang, K., Dony, C., Mohan, S.(**1999**) "Recombinant human insulin-like growth factor-binding protein-5 stimulates bone formation parameters in vitro and in vivo" in *Endocrinology* 140(10) p. 4699-4705

[85] Miyakoshi, N., Richman, C., Kasukawa, Y., Linkhart, T.A., Baylink, D.J., Mohan, S.(**2001**) "Evidence that IGF-binding protein-5 functions as a growth factor" in *Journal of Clinical Investigation* 107(1) p. 73-81

[86] Angele, P., Kujat, R., Nerlich, M., Yoo, J., Goldberg, V., Johnstone, B.(**1999**) "Engineering of osteochondral tissue with bone marrow mesenchymal progenitor cells in a derivatized hyaluronan-gelatin composite sponge" in *Tissue Engineering* 5(6) p. 545-554

[87] Müller, R., Sarhan, H., Blunk, T., Kujat, R., Angele, P.(**2004**) "Chemische Stabilisierung einer dreidimensionalen Hyaluronsäure-Gelatine-Kompositmatrix und ihre Auswirkung auf die in vitro-Kultivierung von bovinen Knorpelzellen" in *DGBM Jahrestagung 2004*

[88] Freed, L.E., Marquis, J.C., Nohria, A., Emmanual, J., Mikos, A.G., Langer, R.(**1993**) "Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers" in *Journal of Biomedical Materials Research* 27(1) p. 11-23

[89] Kellner, K., Schulz, M.B., Gopferich, A., Blunk, T.(**2001**) "Insulin in tissue engineering of cartilage: a potential model system for growth factor application" in *Journal of Drug Targeting* 9(6) p. 439-448

[90] Kim, Y.J., Sah, R.L., Doong, J.Y.a.G.A.J.(**1988**) "Fluorometric assay of DNA in cartilage explants using Hoechst 33258" in *Anal.Biochem.* 174(168) p. 176

[91] Woessner, J.F.(**1961**) "The Determination of Hydroxyproline in Tissue and Protein Samples containing Small Proportions of this Imino Acid" in *Arch.Biochem.Biophys.* 93 p. 440-447

[92] Hollander, A.P., Heathfield, T.F., Webber, C., Iwata, Y., Bourne, R., Rorabeck, C., Poole, R.(**1994**) "Increased Damage to Type II Collagen in Osteoarthritic Articular Cartilage Detected by a New Immunoassay" in *Journal of Clinical Investigation* 93(4) p. 1722-1732

[93] Farndale, R.W., Buttle, D.J., Barrett, A.J.(**1986**) "Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue" in *Biochimica et Biophysica Acta (BBA) - General Subjects* 883(2) p. 173-177

[94] Gooch, K.J., Blunk, T., Courter, D.L., Sieminski, A.L., Bursac, P.M., Vunjak-Novakovic, G., Freed, L.E.(**2001**) "IGF-I and Mechanical Environment Interact to Modulate Engineered Cartilage Development" in *Biochemical and Biophysical Research Communications* 286(5) p. 909-915

[95] Blunk, T., Sieminski, A.L., Appel, B., Croft, C., Courter, D.L., Chieh, J.J., Goepferich, A., Khurana, J.S., Gooch, K.J.(**2003**) "Bone morphogenetic protein 9: A potent modulator of cartilage development in vitro" in *Growth Factors* 21(2) p. 71-77

[96] Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hamaoka, T., Paul, W.E.(**1982**) "Identification of a T cell-derived b cell growth factor distinct from interleukin 2" in *Journal of Experimental Medicine* 155(3) p. 914-923

[97] Carding, S.R. and Bottomly, K.(**1988**) "IL-4 (B cell stimulatory factor 1) exhibits thymocyte growth factor activity in the presence of IL-2" in *Journal of Immunology* 140(5) p. 1519-1526

[98] Te Velde, A.A., Huijbens, R.J.F., Heije, K., De Vries, J.E., Figdor, C.G.(**1990**) "Interleukin-4 (IL-4) inhibits secretion of IL-1.beta., tumor necrosis factor a, and IL-6 by human monocytes" in *Blood* 76(7) p. 1392-1397

[99] Watanabe, K., Tanaka, Y., Morimoto, I., Yahata, K., Zeki, K., Fujihira, T., Yamashita, U., Eto, S.(**1990**) "Interleukin-4 as a potent inhibitor of bone resorption" in *Biochemical and Biophysical Research Communications* 172(3) p. 1035-1041

[100] Lacraz, S., Nicod, L., Galve-de Rochemonteix, B., Baumberger, C., Dayer, J.M., Welgus, H.G.(**1992**) "Suppression of metalloproteinase biosynthesis in human alveolar macrophages by interleukin-4" in *Journal of Clinical Investigation* 90(2) p. 382-388

[101] Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J.G., Dower, S.K., Sims, J.E., Mantovani, A.(**1993**) "Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4" in *Science (Washington, DC, United States)* 261(5120) p. 472-475

[102] Re, F., Muzio, M., De Rossi, M., Polentarutti, N., Giri, J.G., Mantovani, A., Colotta, F.(**1994**) "The type II "receptor" as a decoy target for interleukin 1 in polymorphonuclear leukocytes: characterization of induction by dexamethasone and ligand binding properties of the released decoy receptor" in *Journal of Experimental Medicine* 179(2) p. 739-743

[103] Shingu, M., Miyauchi, S., Nagai, Y., Yasutake, C., Horie, K.(**1995**) "The role of IL-4 and IL-6 in IL-1-dependent cartilage matrix degradation" in *British Journal of Rheumatology* 34(2) p. 101-106

[104] Nemoto, O., Yamada, H., Kikuchi, T., Shinmei, M., Obata, K., Sato, H., Seiki, M., Shimmei, M.(**1997**) "Suppression of matrix metalloproteinase-3 synthesis by

interleukin-4 in human articular chondrocytes" in *Journal of Rheumatology* 24(9) p. 1774-1779

[105] Sone, S., Orino, E., Mizuno, K., Yano, S., Nishioka, Y., Haku, T., Nii, A., Ogura, T.(**1994**) "Production of IL-1 and its receptor antagonist is regulated differently by IFN-gamma and IL-4 in human monocytes and alveolar macrophages" in *European Respiratory Journal* 7(4) p. 657-663

[106] Orino, E., Sone, S., Nii, A., Ogura, T.(**1992**) "IL-4 up-regulates IL-1 receptor antagonist gene expression and its production in human blood monocytes" in *Journal of Immunology* 149(3) p. 925-931

[107] Cawston, T.E., Ellis, A.J., Bigg, H., Curry, V., Lean, E., Ward, D.(**1996**) "Interleukin-4 blocks the release of collagen fragments from bovine nasal cartilage treated with cytokines" in *Biochimica et Biophysica Acta* 1314(3) p. 226-232

[108] Verschure, P.J., Van Noorden, C.J.F., Van Marle, J., Van Den Berg, W.B.(**1996**) "Articular cartilage destruction in experimental inflammatory arthritis: insulin-like growth factor-1 regulation of proteoglycan metabolism in chondrocytes" in *Histochemical Journal* 28(12) p. 835-857

[109] Wong, H.L., Costa, G.L., Lotze, M.T., Wahl, S.M.(**1993**) "Interleukin (IL) 4 differentially regulates monocyte IL-1 family gene expression and synthesis in vitro and in vivo" in *Journal of Experimental Medicine* 177(3) p. 775-781

[110] Suzuki, H., Sugiyama, E., Tunru, I.S., Yamashita, N., Matsuno, H., Hamazaki, T., Kobayashi, M.(**1993**) "Suppressive effect of interleukin-4 (IL-4) on IL-6 production by adherent rheumatoid synovial cells" in *Clinical Immunology and Immunopathology* 66(1) p. 67-72

[111] Miossec, P., Briolay, J., Dechanet, J., Wijdenes, J., Martinez-Valdez, H., Banchereau, J.(**1992**) "Inhibition of the production of proinflammatory cytokines and immunoglobulins by interleukin-4 in an ex vivo model of rheumatoid synovitis" in *Arthritis & Rheumatism* 35(8) p. 874-883

[112] Knauper, V., Lopez-Otin, C., Smith, B., Knight, G., Murphy, G.(**1996**) "Biochemical characterization of human collagenase-3" in *The Journal of Biological Chemistry* 271(3) p. 1544-1550

[113] Vankemmelbeke, M., Dekeyser, P.M., Hollander, A.P., Buttle, D.J., Demeester, J.(**1998**) "Characterization of helical cleavages in type II collagen generated by matrixins" in *Biochemical Journal* 330(2) p. 633-640

[114] Billinghurst, R.C., Wu, W., Ionescu, M., Reiner, A., Dahlberg, L., Chen, J., Van Wart, H., Poole, A.R.(**2000**) "Comparison of the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1 and the selective inhibition of type II collagen cleavage by collagenase" in *Arthritis & Rheumatism* 43(3) p. 664-672

[115] Fosang, A.J., Last, K., Knauper, V., Murphy, G., Neame, P.J.(**1996**) "Degradation of cartilage aggrecan by collagenase-3 (MMP-13)" in *FEBS Letters* 380(1-2) p. 17-20

[116] Little, C.B., Hughes, C.E., Curtis, C.L., Janusz, M.J., Bohne, R., Wang-Weigand, S., Taiwo, Y.O., Mitchell, P.G., Otterness, I.G., Flannery, C.R., Caterson, B.(**2002**) "Matrix metalloproteinases are involved in C-terminal and interglobular domain processing of cartilage aggrecan in late stage cartilage degradation" in *Matrix Biology* 21(3) p. 271-288

[117] Hughes, C., Murphy, G., Hardingham, T.E.(**1991**) "Metalloproteinase digestion of cartilage proteoglycan. Pattern of cleavage by stromelysin and susceptibility to collagenase" in *Biochemical Journal* 279(3) p. 733-739

[118] Tortorella, M.D., Pratta, M., Liu, R.Q., Austin, J., Ross, O.H., Abbaszade, I., Burn, T., Arner, E.(**2000**) "Sites of aggrecan cleavage by recombinant human aggrecanase-1 (ADAMTS-4)" in *Journal of Biological Chemistry* 275(24) p. 18566-18573

[119] Ferry, R.J., Jr., Katz, L.E.L., Grimberg, A., Cohen, P., Weinzimer, S.A.(**1999**) "Cellular actions of insulin-like growth factor binding proteins" in *Hormone and Metabolic Research* 31(2/3) p. 192-202

[120] Baxter, R.C.(**2000**) "Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities" in *American Journal of Physiology. Endocrinology and Metabolism* 278(6) p. E967-E976

[121] Collett-Solberg, P.F. and Cohen, P.(**2000**) "Genetics, chemistry, and function of the IGF/IGFBP system" in *Endocrine* 12(2) p. 121-136

[122] Shimasaki, S. and Ling, N.(**1991**) "Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and -6)" in *Progress in Growth Factor Research* 3(4) p. 243-266

[123] Olney, R.C., Smith, R.L., Kee, Y., Wilson, D.M.(**1993**) "Production and hormonal regulation of insulin-like growth factor binding proteins in bovine chondrocytes" in *Endocrinology* 133(2) p. 563-570

[124] Morales, T.I.(**1997**) "The Role and Content of Endogenous Insulin-Like Growth Factor-Binding Proteins in Bovine Articular Cartilage*1" in *Archives of Biochemistry and Biophysics* 343(2) p. 164-172

[125] Martin, J.A., Miller, B.A., Scherb, M.B., Lembke, L.A., Buckwalter, J.A.(**2002**) "Co-localization of insulin-like growth factor binding protein 3 and fibronectin in human articular cartilage" in *Osteoarthritis and Cartilage* 10(7) p. 556-563

[126] Olney, R.C., Tsuchiya, K., Wilson, D.M., Mohtai, M., Maloney, W.J., Schurman, D.J., Smith, R.L.(**1996**) "Chondrocytes from osteoarthritic cartilage have increased expression of insulin-like growth factor I (IGF-I) and IGF-binding protein-3 (IGFBP-3) and -5, but not IGF-II or IGFBP-4" in *The Journal of Clinical Endocrinology and Metabolism* 81(3) p. 1096-1103

[127] Tardif, G., Reboul, P., Pelletier, J.P., Geng, C., Cloutier, J.M., Martel-Pelletier, J.(**1996**) "Normal expression of type 1 insulin-like growth factor receptor by human osteoarthritic chondrocytes with increased expression and synthesis of insulin-like growth factor binding proteins" in *Arthritis & Rheumatism* 39(6) p. 968-978

[128] Dore, S., Pelletier, J.P., DiBattista, J.A., Tardif, G., Brazeau, P., Martel-Pelletier, J.(**1994**) "Human osteoarthritic chondrocytes possess an increased number of insulin-like growth factor 1 binding sites but are unresponsive to its stimulation. Possible role of IGF-1-binding proteins" in *Arthritis & Rheumatism* 37(2) p. 253-263

[129] Middleton, J.F. and Tyler, J.A.(**1992**) "Upregulation of insulin-like growth factor I gene expression in the lesions of osteoarthritic human articular cartilage" in *Annals of the Rheumatic Diseases* 51(4) p. 440-447

[130] Mohan, S., Nakao, Y., Honda, Y., Landale, E., Leser, U., Dony, C., Lang, K., Baylink, D.J.(**1995**) "Studies on the mechanisms by which insulin-like growth factor (IGF)

binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells" in *Journal of Biological Chemistry* 270(35) p. 20424-20431

[131] Qin, X., Strong, D.D., Baylink, D.J., Mohan, S.(**1998**) "Structure-function analysis of the human insulin-like growth factor binding protein-4" in *Journal of Biological Chemistry* 273(36) p. 23509-23516

[132] Freed, L.E. and Vunjak-Novakovic, G. (**2000**) "Tissue culture bioreactors" in *Principles of Tissue Engineering* (Ed.: Lanza,R., Langer, R., and Vacant, J. P.; Academic Press; San Diego)

[133] Cheung, P.T., Smith, E.P., Shimasaki, S., Ling, N., Chernausek, S.D.(**1991**) "Characterization of an insulin-like growth factor binding protein (IGFBP-4) produced by the B104 rat neuronal cell line: chemical and biological properties and differential synthesis by sublines" in *Endocrinology* 129(2) p. 1006-1015

[134] Kiefer, M.C., Schmid, C., Waldvogel, M., Schlapfer, I., Futo, E., Masiarz, F.R., Green, K., Barr, P.J., Zapf, J.(**1992**) "Characterization of recombinant human insulin-like growth factor binding proteins 4, 5, and 6 produced in yeast" in *Journal of Biological Chemistry* 267(18) p. 12692-12699

[135] Boes, M., Booth, B.A., Sandra, A., Dake, B.L., Bergold, A., Bar, R.S.(**1992**) "Insulin-like growth factor binding protein (IGFBP)4 accounts for the connective tissue distribution of endothelial cell IGFBPs perfused through the isolated heart" in *Endocrinology* 131(1) p. 327-330

[136] Yamanaka, Y., Wilson, E.M., Rosenfeld, R.G., Oh, Y.(**1997**) "Inhibition of insulin receptor activation by insulin-like growth factor binding proteins" in *Journal of Biological Chemistry* 272(49) p. 30729-30734

[137] Matsumoto, T., Gargosky, S.E., Iwasaki, K., Rosenfeld, R.G.(**1996**) "Identification and characterization of insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), and IGFBP proteases in human synovial fluid" in *The Journal of Clinical Endocrinology and Metabolism* 81(1) p. 150-155

[138] Fortier, L.A., Lust, G., Mohammed, H.O., Nixon, A.J.(**1999**) "Coordinate upregulation of cartilage matrix synthesis in fibrin cultures supplemented with exogenous insulin-like growth factor-I" in *Journal of Orthopaedic Research* 17(4) p. 467-474

[139] Sah, R.L., Chen, A.C., Grodzinsky, A.J., Trippel, S.B.(**1994**) "Differential Effects of bFGF and IGF-I on Matrix Metabolism in Calf and Adult Bovine Cartilage Explants" in *Archives of Biochemistry and Biophysics* 308(1) p. 137-147

[140] Mohan, S., Strong, D.D., Linkhart, T.A., Baylink, D.J. (**1994**) "Regulation and the actions of insulin-like growth factor-binding protein (IGFBP)-4 and IGFBP-5 in bone, physiological and clinical implications" in *The Insulin-like Growth Factors and Their Regulatory Proteins* (Ed.: Baxter,R.C., Gluckman, P. D., and Rosenfeld, R. G.; Elsevier; Amesterdam)

[141] McQuillan, D.J., Handley, C.J., Campbell, M.A., Bolis, S., Milway, V.E., Herington, A.C.(**1986**) "Stimulation of proteoglycan biosynthesis by serum and insulin-like growth factor-I in cultured bovine articular cartilage" in *The Biochemical Journal* 240(2) p. 423-430

[142] Schalkwijk, J., Joosten, L.A., van den Berg, W.B., van Wyk, J.J., van de Putte, L.B.(**1989**) "Insulin-like growth factor stimulation of chondrocyte proteoglycan synthesis by human synovial fluid" in *Arthritis & Rheumatism* 32(1) p. 66-71

[143] Wood, T.L.(**1995**) "Gene-targeting and transgenic approaches to IGF and IGF binding protein function" in *The American Journal of Physiology* 269(4, Part 1) p. E613-E622

[144] Tyler, J.A.(**1989**) "Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines" in *The Biochemical Journal* 260(2) p. 543-548

[145] Trippel, S.B.(**1995**) "Growth factor actions on articular cartilage" in *The Journal of Rheumatology. Supplement* 43 p. 129-132

[146] Clemmons, D.R.(**1997**) "Insulin-like growth factor binding proteins and their role in controlling IGF actions" in *Cytokine & Growth Factor Reviews* 8(1) p. 45-62

[147] Rechler, M.M.(**1993**) "Insulin-like growth factor binding proteins" in *Vitamins and Hormones* 47 p. 1-114

[148] Jones, J.I., Gockerman, A., Busby, W.H., Jr., Camacho-Hubner, C., Clemmons, D.R.(**1993**) "Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I" in *The Journal of Cell Biology* 121(3) p. 679-687

[149] Bautista, C.M., Baylink, D.J., Mohan, S.(**1991**) "Isolation of a novel insulin-like growth factor (IGF) binding protein from human bone: a potential candidate for fixing

IGF-II in human bone" in *Biochemical and Biophysical Research Communications* 176(2) p. 756-763

[150] Andress, D.L.(**1998**) "Insulin-like growth factor-binding protein-5 (IGFBP-5) stimulates phosphorylation of the IGFBP-5 receptor" in *American Journal of Physiology* 274(4 Pt 1) p. E744-E750

[151] Andress, D.L. and Birnbaum, R.S.(**1991**) "A novel human insulin-like growth factor binding protein secreted by osteoblast-like cells" in *Biochemical and Biophysical Research Communications* 176(1) p. 213-218

[152] Andress, D.L., Loop, S.M., Zapf, J., Kiefer, M.C.(**1993**) "Carboxy-truncated insulin-like growth factor binding protein-5 stimulates mitogenesis in osteoblast-like cells" in *Biochemical and Biophysical Research Communications* 195(1) p. 25-30

[153] Matsumoto, T., Gargosky, S.E., Oh, Y., Rosenfeld, R.G.(**1996**) "Transcriptional and post-translational regulation of insulin-like growth factor-binding protein-5 in rat articular chondrocytes" in *Journal of Endocrinology* 148(2) p. 355-369

[154] Kiepe, D., Andress, D.L., Mohan, S., Standker, L., Ulinski, T., Himmele, R., Mehls, O., Tonshoff, B.(**2001**) "Intact IGF-binding protein-4 and -5 and their respective fragments isolated from chronic renal failure serum differentially modulate IGF-I actions in cultured growth plate chondrocytes" in *Journal of the American Society of Nephrology* 12(11) p. 2400-2410

[155] Andress, D.L.(**1999**) "Insulin-like growth factor binding protein-5 (IGFBP-5)-receptor phosphorylation is specific for IGFBP-5 and IGFBP-5 related peptides" in *Growth Hormone & IGF Research* 9 p. 319

[156] Schedlich, L.J., Young, T.F., Firth, S.M., Baxter, R.C.(**1998**) "Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells" in *Journal of Biological Chemistry* 273(29) p. 18347-18352

[157] Zapf, J.(**1995**) "Physiological role of the insulin-like growth factor binding proteins" in *European Journal of Endocrinology / European Federation of Endocrine Societies* 132(6) p. 645-654

[158] Gill, Z.P., Perks, C.M., Newcomb, P.V., Holly, J.M.P.(**1997**) "Insulin-like growth factor-binding protein (IGFBP-3) predisposes breast cancer cells to programmed cell

death in a non-IGF-dependent manner" in *Journal of Biological Chemistry* 272(41) p. 25602-25607

[159] Ling, N.C., Liu, X.J., Malkowski, M., Guo, Y.L., Erickson, G.F., Shimasaki, S.(**1993**) "Structural and functional studies of insulin-like growth factor binding proteins in the ovary" in *Growth Regulation* 3(1) p. 70-74

[160] Kalus, W., Zweckstetter, M., Renner, C., Sanchez, Y., Georgescu, J., Grol, M., Demuth, D., Schumacher, R., Dony, C., Lang, K., Holak, T.A.(**1998**) "Structure of the IGF-binding domain of the insulin-like growth factor-binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions" in *EMBO Journal* 17(22) p. 6558-6572

[161] Cohen, S., Bano, M.C., Cima, L.G., Allcock, H.R., Vacanti, J.P., Vacanti, C.A., Langer, R.(**1993**) "Design of synthetic polymeric structures for cell transplantation and tissue engineering" in *Clinical Materials* 13(1-4) p. 3-10

[162] Langer, R.(**1997**) "Tissue engineering: a new field and its challenges" in *Pharmaceutical Research* 14(7) p. 840-841

[163] Alberts, B., Bary, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D.(**1994**) "Molecular Biology of the Cell" in 3rd p. 971-1000

[164] Sittinger, M., Reitzel, D., Dauner, M., Hierlemann, H., Hammer, C., Kastenbauer, E., Planck, H., Burmester, G.R., Bujia, J.(**1996**) "Resorbable polyesters in cartilage engineering: affinity and biocompatibility of polymer fiber structures to chondrocytes" in *Journal of Biomedical Materials Research* 33(2) p. 57-63

[165] Reed, A.M. and Gilding, D.K.(**1981**) "Biodegradable polymers for use in surgery -- poly(glycolic)/poly(Iactic acid) homo and copolymers: 2. In vitro degradation" in *Polymer* 22(4) p. 494-498

[166] Kohn, D.H., Sarmadi, M., Helman, J.I., Krebsbach, P.H.(**2002**) "Effects of pH on human bone marrow stromal cells in vitro: implications for tissue engineering of bone" in *Journal of Biomedical Materials Research* 60(2) p. 292-299

[167] Vert, M., Mauduit, J., Li, S.(**1994**) "Biodegradation of PLA/GA polymers: increasing complexity" in *Biomaterials* 15(15) p. 1209-1213

[168] Solchaga, L.A., Dennis, J.E., Goldberg, V.M., Caplan, A.I.(**1999**) "Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage" in *Journal of Orthopaedic Research* 17(2) p. 205-213

[169] Grande, D.A., Halberstadt, C., Naughton, G., Schwartz, R., Manji, R.(**1997**) "Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts" in *Journal of Biomedical Materials Research* 34(2) p. 211-220

[170] Lee, C.R., Breinan, H.A., Nehrer, S., Spector, M.(**2000**) "Articular cartilage chondrocytes in type I and type II collagen-GAG matrices exhibit contractile behavior in vitro" in *Tissue Engineering* 6(5) p. 555-565

[171] Nehrer, S., Breinan, H.A., Ramappa, A., Shortkroff, S., Young, G., Minas, T., Sledge, C.B., Yannas, I.V., Spector, M.(**1997**) "Canine chondrocytes seeded in type I and type II collagen implants investigated in vitro" in *Journal of Biomedical Materials Research* 38(2) p. 95-104

[172] Petite, H., Frei, V., Huc, A., Herbage, D.(**1994**) "Use of diphenylphosphorylazide for cross-linking collagen-based biomaterials" in *Journal of Biomedical Materials Research* 28(2) p. 159-165

[173] Yannas, I.V., Lee, E., Orgill, D.P., Skrabut, E.M., Murphy, G.F.(**1989**) "Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin" in *Proceedings of the National Academy of Sciences of the United States of America* 86(3) p. 933-937

[174] Weadock, K., Olson, R.M., Silver, F.H.(**1983**) "Evaluation of collagen crosslinking techniques" in *Biomaterials, Medical Devices, and Artificial Organs* 11(4) p. 293-318

[175] Osborne, C.S., Reid, W.H., Grant, M.H.(**1999**) "Investigation into the biological stability of collagen/chondroitin-6-sulphate gels and their contraction by fibroblasts and keratinocytes: the effect of crosslinking agents and diamines" in *Biomaterials* 20(3) p. 283-290

[176] Osborne, C.S., Barbenel, J.C., Smith, D., Savakis, M., Grant, M.H.(**1998**) "Investigation into the tensile properties of collagen/chondroitin-6-sulphate gels: the effect of crosslinking agents and diamines" in *Medical & Biological Engineering & Computing* 36(1) p. 129-134

[177] Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N., Rubini, K.(**2002**) "Stabilization of gelatin films by crosslinking with genipin" in *Biomaterials* 23(24) p. 4827-4832

[178] Weadock, K.S., Miller, E.J., Keuffel, E.L., Dunn, M.G.(**1996**) "Effect of physical crosslinking methods on collagen-fiber durability in proteolytic solutions" in *Journal of Biomedical Materials Research* 32(2) p. 221-226

[179] Chevallay, B., Abdul-Malak, N., Herbage, D.(**2000**) "Mouse fibroblasts in long-term culture within collagen three-dimensional scaffolds: influence of crosslinking with diphenylphosphorylazide on matrix reorganization, growth, and biosynthetic and proteolytic activities" in *Journal of Biomedical Materials Research* 49(4) p. 448-459

[180] Lee, C.R., Grodzinsky, A.J., Spector, M.(**2001**) "The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis" in *Biomaterials* 22(23) p. 3145-3154

[181] Liang, H.C., Chang, Y., Hsu, C.K., Lee, M.H., Sung, H.W.(**2004**) "Effects of crosslinking degree of an acellular biological tissue on its tissue regeneration pattern" in *Biomaterials* 25(17) p. 3541-3552

[182] Chen, V.J. and Ma, P.X.(**2004**) "Nano-fibrous poly(-lactic acid) scaffolds with interconnected spherical macropores" in *Biomaterials* 25(11) p. 2065-2073

[183] Hacker, M., Tessmar, J., Neubauer, M., Blaimer, A., Blunk, T., Gopferich, A., Schulz, M.B.(**2003**) "Towards biomimetic scaffolds: Anhydrous scaffold fabrication from biodegradable amine-reactive diblock copolymers" in *Biomaterials* 24(24) p. 4459-4473

[184] Kellner, K., Lang, K., Papadimitriou, A., Leser, U., Milz, S., Schulz, M.B., Blunk, T., Goepferich, A.(**2002**) "Effects of hedgehog proteins on tissue engineering of cartilage in vitro" in *Tissue Engineering* 8(4) p. 561-572

[185] Toolan, B.C., Frenkel, S.R., Pachence, J.M., Yalowitz, L., Alexander, H.(**1996**) "Effects of growth-factor-enhanced culture on a chondrocyte-collagen implant for cartilage repair" in *Journal of Biomedical Materials Research* 31(2) p. 273-280

[186] Weisser, J., Rahfoth, B., Timmermann, A., Aigner, T., Brauer, R., der Mark, K.(**2001**) "Role of growth factors in rabbit articular cartilage repair by chondrocytes in agarose" in *Osteoarthritis and Cartilage* 9(Supplement 1) p. S48-S54

[187] Loeser, R.F. and Shanker, G.(**2000**) "Autocrine stimulation by insulin-like growth factor 1 and insulin-like growth factor 2 mediates chondrocyte survival in vitro" in *Arthritis & Rheumatism* 43(7) p. 1552-1559

[188] Stewart, M.C., Saunders, K.M., Burton-Wurster, N., Macleod, J.N.(**2000**) "Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation" in *Journal of Bone and Mineral Research* 15(1) p. 166-174

[189] Yaeger, P.C., Masi, T.L., de Ortiz, J.L.B., Binette, F., Tubo, R., McPherson, J.M.(**1997**) "Synergistic Action of Transforming Growth Factor-[beta] and Insulin-like Growth Factor-I Induces Expression of Type II Collagen and Aggrecan Genes in Adult Human Articular Chondrocytes*1" in *Experimental Cell Research* 237(2) p. 318-325

[190] Quarto, R., Campanile, G., Cancedda, R., Dozin, B.(**1997**) "Modulation of commitment, proliferation, and differentiation of chondrogenic cells in defined culture medium" in *Endocrinology* 138(11) p. 4966-4976

[191] Lee, R.B., Wilkins, R.J., Razaq, S., Urban, J.P.G.(**2002**) "The effect of mechanical stress on cartilage energy metabolism" in *Biorheology* 39(1-2) p. 133-143

[192] Wang, C.C.B., Guo, X.E., Sun, D., Mow, V.C., Ateshian, G.A., Hung, C.T.(**2002**) "The functional environment of chondrocytes within cartilage subjected to compressive loading: a theoretical and experimental approach" in *Biorheology* 39(1-2) p. 11-25

[193] Kim, Y.J., Sah, R.L.Y., Grodzinsky, A.J., Plaas, A.H.K., Sandy, J.D.(**1994**) "Mechanical Regulation of Cartilage Biosynthetic Behavior: Physical Stimuli" in *Archives of Biochemistry and Biophysics* 311(1) p. 1-12

[194] Domm, C., Schunke, M., Christesen, K., Kurz, B.(**2002**) "Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension" in *Osteoarthritis and Cartilage* 10(1) p. 13-22

[195] Bonaventure, J., Kadhom, N., Cohen-Solal, L., Ng, K.H., Bourguignon, J., Lasselin, C., Freisinger, P.(**1994**) "Reexpression of Cartilage-Specific Genes by Dedifferentiated Human Articular Chondrocytes Cultured in Alginate Beads" in *Experimental Cell Research* 212(1) p. 97-104

[196] Martin, I., Suetterlin, R., Baschong, W., Heberer, M., Vunjak-Novakovic, G., Freed, L.E.(**2001**) "Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation" in *Journal of Cellular Biochemistry* 83(1) p. 121-128

[197] Ribault, D., Riera, H., Mitrovic, D.R.(**1992**) "Characterization of proteoglycans extracted from articular cartilage with smooth and deeply-fissured surface from a single human patello-femoral joint" in *European Journal of Experimental Musculoskeletal Research* 1(3-4) p. 135-143

[198] Mitrovic, D., Quintero, M., Stankovic, A., Ryckewaert, A.(**1983**) "Cell density of adult human femoral condylar articular cartilage. Joints with normal and fibrillated surfaces" in *Laboratory Investigation* 49(3) p. 309-316

[199] Alini, M., Matsui, Y., Dodge, G.R., Poole, A.R.(**1992**) "The extracellular matrix of cartilage in the growth plate before and during calcification: changes in composition and degradation of type II collagen" in *Calcified Tissue International* 50(4) p. 327-335

[200] Hunziker, E.B.(**1994**) "Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes" in *Microscopy Research And Technique* 28(6) p. 505-519

[201] Kirsch, T., Harrison, G., Worch, K.P., Golub, E.E.(**2000**) "Regulatory roles of zinc in matrix vesicle-mediated mineralization of growth plate cartilage" in *Journal of Bone and Mineral Research* 15(2) p. 261-270

[202] Ortega, N., Behonick, D., Stickens, D., Werb, Z.(**2003**) "How proteases regulate bone morphogenesis" in *Annals of the New York Academy of Sciences* 995 p. 109-116

[203] Wagner, E.F. and Karsenty, G.(**2001**) "Genetic control of skeletal development" in *Current Opinion in Genetics & Development* 11(5) p. 527-532

[204] McFarland, C.D., Brown, R.A., McLaughlin, B., Ali, S.Y., Weiss, J.B.(**1990**) "Production of endothelial cell stimulating angiogenesis factor (ESAF) by chondrocytes during in vitro cartilage calcification" in *Bone and Mineral* 11(3) p. 319-333

[205] Enomoto, H., Enomoto-Iwamoto, M., Iwamoto, M., Nomura, S., Himeno, M., Kitamura, Y., Kishimoto, T., Komori, T.(**2000**) "Cbfa1 is a positive regulatory factor in chondrocyte maturation" in *The Journal of Biological Chemistry* 275(12) p. 8695-8702

[206] Sternlicht, M.D. and Werb, Z.(**2001**) "How matrix metalloproteinases regulate cell behavior" in *Annual Review of Cell and Developmental Biology* 17 p. 463-516

[207] Case, N.D., Duty, A.O., Ratcliffe, A., Muller, R., Guldberg, R.E.(**2003**) "Bone formation on tissue-engineered cartilage constructs in vivo: effects of chondrocyte viability and mechanical loading" in *Tissue Engineering* 9(4) p. 587-596

Appendices

Abbreviations

3-D	three-dimensional
ANOVA	analysis of variance
AT	annealing temperature
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CLD	crosslinked
CS	chondroitin sulfate
DAB	diaminobenzidine
DMEM	Dulbecco´s Modified Eagle´s Medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDC	carbodiimides
exog.	exogenous
FBS	fetal bovine serum
FCS	fetal calf serum
GAG	glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GTA	glutaraldehyde
H&E	hematoxylin and eosin
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
IGD	interglobular domain
IGFBP-4	insulin-like growth factor binding protein-4
IGFBP-5	insulin-like growth factor binding protein-5
IGF-I	insulin-like growth factor-I
IL-1	Interleukin-1
IL-1RI	IL-1 type 1 receptor
IL-4	interleukin-4
kDa	kilodalton

MMP	matrix metalloproteinase
MMP-1	matrix metalloproteinase-1
MMP-13	matrix metalloproteinase-13
MMP-3	matrix metalloproteinase-3
MW	molecular weight
NMR	nuclear magnetic resonance
OsO_4	osmium tetroxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
p-DAB	p-dimethylaminobenzaldehyde
PGA	polyglycolic acid
PLA	polylactic acid
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SEM	scanning electron microscopy
TE	tissue engineering
TIMP-1	tissue inhibitor of metalloproteinase-1
TNFα	tumor necrosis factor α
UV	ultraviolet light
ww	wet weight

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