Cartilage regeneration using peroral supplementation and artificial scaffolds

PhD Thesis

Mgr. Eva Filová

Charles University in Prague
2nd Faculty of Medicine

Field of study: Medical Biophysics

Supervisor: doc. RNDr. Evžen Amler, CSc.
Supervisor consultant: prof. MUDr. Ctibor Povýšil, DrSc.

Supervising institutes: Institute of Biophysics, 2nd Faculty of Medicine, Charles University, Prague, and Institute of Experimental Medicine of the Academy of Sciences of the Czech Republic, Prague

Prague 2008

Acknowledgements

First of all, I would like to express my gratitude to my supervisors, Doc. RNDr. Evžen Amler, CSc. and Prof. MUDr. Ctibor Povýšil, DrSc. for their many valuable advices and suggestions concerning my experimental work and writing of this manuscript.

My thanks also belong to MUDr. Jaroslav Činátl, CSc. for his advices concerning cell culture. I am also grateful to Mgr. Andriy Lytvynets and MVDr. Dušan Usvald for the performing the surgery of animals. I appreciate the kind help from my colleagues Mgr. Michala Rampichová, Mgr. Lenka Gryčová, RNDr. Ferdinand Varga, and RNDr. Lucie Koláčná, PhD., and from the staff of the lab.

Moreover, I would like to thank all my collaborators from the Institute of Animal Physiology and Genetics, Liběchov, the MR-Unit of the Institute of Clinical and Experimental Medicine in Prague, the Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, Brno, the Orthopedic Clinic, and the Department of Pediatric Hematology and Oncology, University Hospital Motol, Prague, and the Veterinary Histopathological Laboratory, Prague, Czech Republic.

My thanks belong to Prof. RNDr. Arnošt Kotyk, DrSc. for the proofreading of the manuscript. I am grateful to my opponents for revising my thesis and for their comments. My thanks also belong to my family and friends for their help and support.

Content

List of abbreviations

ACI	autologous chondrocyte implantation
ACT	autologous chondrocyte transplantation
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
ECM	extracellular matrix
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAG	glycosaminoglycan
HA	hyaluronic acid
IGF	insulin-like growth factor
IL-1	interleukin-1
IL-8	interleukin-8
ITS	insulin-transferrin-selenium
MACI	matrix-associated autologous chondrocyte implantation
MAP kinase	mitogen-activated protein kinase
MSC	mesenchymal stem cell
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
PG	proteoglycan
PGA	polyglycolic acid
PLGA	polylactide-co-glycolide
PLLA	polylactic acid
RGD	arginine-glycine-aspartic acid
RGDS	arginine-glycine-aspartic acid-serine
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α

1. Introduction

1.1. Cartilage types, composition and function

Cartilage is a specialized tissue characterized by the presence of extracellular matrix enriched with glycosaminoglycans and proteoglycans. These substances are bound to collagen and elastic fibres. The different ratio between the components of extracellular matrix results in different properties of three types of cartilage, i.e. hyaline cartilage, elastic cartilage and fibrocartilage.

Hyaline cartilage is the most common type of cartilage in human body. In a moving joint, an articular cartilage absorbs forces, and enables smooth movement of the joint. Hyaline physeal cartilage is necessary for the growth of bones during the postnatal period (Junqueira *et al.* 1999).

Compared to hyaline cartilage, elastic cartilage contains not only type II collagen fibres but also a dense net of elastic fibres which is responsible for the high elasticity of the elastic cartilage. It is present in auricles, external auditory meatus walls, Eustachian tube, epiglottis, and cuneiform cartilage.

Fibrocartilage forms a transition from fibrous tissue to cartilage. It is characterized by a dense net of type I collagen fibres, which are parallel to applied forces, and a low amount of amorphous ECM. Chondrocytes are usually organized in columns. The fibrocartilage present in intervertebral discs and ligament insertions is able to withstand a high mechanical load.

1.1.1. Articular cartilage

An adult articular cartilage contains only 1 % (V/V) of specialized cells, chondrocytes. Chondrocytes surrounded by pericellular matrix rich in glycosaminoglycans form chondrons, which are surrounded by a basophilic capsule in the middle and deep zone (Lee and Loeser 1998, Ross *et al.* 2006), and create an avascular and alymphatic tissue with no innervation. Chondrocytes from different

zones have different size, shape and metabolic activity but all contain organelles necessary for matrix synthesis, including endoplasmic reticulum, and Golgi membranes (Fig.1). In some chondrocytes, short cilia protrude from the cell to the extracellular matrix. They may play a role in the perception of mechanical changes in the matrix (Buckwalter and Mankin 1998a). Chondrocytes usually do not form cell-to-cell contact as they are surrounded by extracellular matrix. In addition, variabilities of cell number, chondrocyte surface area, and matrix volume per chondrocyte were observed in different locations in the joint. However, a uniform volume per chondron was found (Quinn *et al.* 2005).

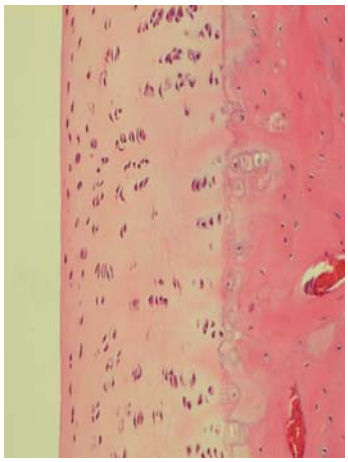

Fig. 1. Histology of native articular cartilage of rabbit. Hematoxilin-eosin staining, magn. × 80.

All components of extracellular matrix are synthesized by chondrocytes. After their synthesis, these components must be organized into a highly ordered framework. Interestingly, chondrocytes are metabolically active but their low density in the cartilage results in a low metabolic activity of the tissue. To maintain the proper structure of cartilage as a response to mechanical loading of the joint, the cartilage requires continuous replacement of degraded matrix components. In skeletally mature cartilage, the volume of tissue does not substantially change but the articular surface can be remodeled (Buckwalter and Mankin 1998a).

In adult cartilage, nutrition of the chondrocytes is provided by a synovial fluid. Diffusion of the synovial fluid through the cartilage is restricted not only by its thickness but also by the charges and specific molecular configuration (Buckwalter and Mankin 1998a).

The major components of the extracellular matrix of hyaline cartilage are type II collagen (≈ 60 % of the dry weight), hyaluronic acid, PGs (≈ 25 %), and other proteins and glycoproteins (≈ 15 %) (Buckwalter and Mankin 1998a). In addition to these components, elastic cartilage contains elastin that allows its excellent elasticity. On the other hand, the dense net of type I collagen permits the fibrocartilage to withstand high mechanical loading in intervertebral discs and ligament insertions (Junqueira *et al.* 1999).

Water contributes to 80 % of the wet weight of articular cartilage. It contains gases, small proteins, metabolites, and high concentrations of cations that balance the negatively charged PGs. The majority of water interacts with structural macromolecules, particularly the large PGs; this helps to maintain water in the matrix.

Articular cartilage is organized in four zones: superficial, transitional, deep, and calcified. The structural macromolecules include collagens, PGs and non-collagenous proteins. The superficial zone contains the highest density of chondrocytes in comparison with deeper zones (Quinn *et al.* 2005). It is rich in collagen; however, in a deep zone, all matrix components are uniformly distributed. Dense matrix of collagen fibrils lies in parallel to the joint surface that gives this zone a greater tensile stiffness and strength, so that they can resist shear stress generated during movement of the joint. The tight mesh of type II, IX and XI collagens throughout the tissue provides the cartilage the tensile stiffness and strength, and contributes to the cohesiveness of the tissue by entrapping the large PGs. The tensile strength depends on intermolecular cross-linking. The role of type IX and XI collagens is probably to form and stabilize the primary collagen fibrils assembled from type II collagen and to connect the meshwork with PGs (Eyre *et al.* 1992, Buckwalter and Mankin 1998a). Type VI collagen is concentrated pericellularly; it is rich in RGD sequences and may help chondrocytes to attach to the matrix. Type X, present only in the calcified zone, probably plays a role in the mineralization of cartilage (Eyre *et al.* 1992, Buckwalter and Mankin 1998a).

PGs consist of a protein core and one or more glycosaminoglycans. Glycosaminoglycans include HA, chondroitin sulfate, keratan sulfate and dermatan sulfate. The concentration of glycosaminoglycans is variable within an articular cartilage and also varies with age, injury or disease, e.g. osteoarthritis (Wang *et al.* 2006).

The highly viscous nature of synovial fluid is important for normal joint fluid. Highly viscous lubricating fluid consists of lubricin and hyaluronic acid (Simon 1999). Hyaluronan is a high-molar-mass linear glycosaminoglycan consisting of a repeating disaccharide unit *N*-acetylglucosamine, and D-glucuronic acid. HA synthesis is linked with the expression of several hormones (e.g. insulin, prostaglandins, interleukin, and somatomedins) and most growth factors (e.g. platelet-derived growth factor, epidermal growth factor, bFGF, and transforming growth factor) as well. Conversely, inhibition of HA in mesenchymal or epithelial cells arrested mitosis *in vitro* even in the presence of growth factors (King *et al.* 1991).

HA plays an important role in many physiological and pathological processes, such as cell proliferation, cell recognition, cell migration, cell differentiation, and inflammation. Most of these responses are mediated through HA-CD 44 interaction (Miyake *et al.* 1990, Laurent and Fraser 1992). Exogenous HA is able to enhance wound healing. The mechanism is still unknown; however, HA is probably a part of feedback that promotes cell proliferation and migration in actively growing tissues. Moreover, the role of HA in both water homeostasis and an increase of tissue hydration can positively influence wound healing (King *et al.* 1991, Maniwa *et al.* 2001). In hamsters, hyaluronic acid, administered topically into the defect, accelerated the wound healing which was connected with a higher microcirculatory perfusion at the site of repair (King *et al.* 1991). In osteoarthritic patients, both the concentration and the molar mass of intraarticular endogenous HA were decreased (Wang *et al.* 2006).

1.1.2. Physeal cartilage

Longitudinal growth of long bones occurs by endochondral ossification. In tubular bones, primary and secondary centers of growth give rise to physes. The primary centers of ossification give rise to proximal and distal metaphyseal physes (Fig.2), (Byers and Brown 2006). The secondary center of ossification expands within epiphysis in all directions; in one direction it comes to underlie the articular cartilage, the articular cartilage physis, in another direction it backs on metaphyseal

physis where it forms a complex with epiphyseal and diaphyseal side. The epiphyseal side ossifies early, while the metaphyseal side remains active till puberty (Byers and Brown 2006, Andrade *et al.* 2007).

The primary and secondary centers develop similarly; chondrocytes are organized in four zones with different proliferation rate, differentiation rate, and cell morphology. The resting zone, adjacent to epiphysis, contains spherical chondrocytes which are distributed single or in pairs, and their proliferation activity is low. They are considered as "stem-like cells" capable of generating new clones of proliferative chondrocytes. In the proliferative zone, chondrocytes are flattened, organized into isogenic rows parallel to the long axis of the bone. The highest proliferation rate is observed in this zone. Columnar arrangement of larger chondrocytes which do not proliferate is typical of the hypertrophic zone. In addition, changes of matrix

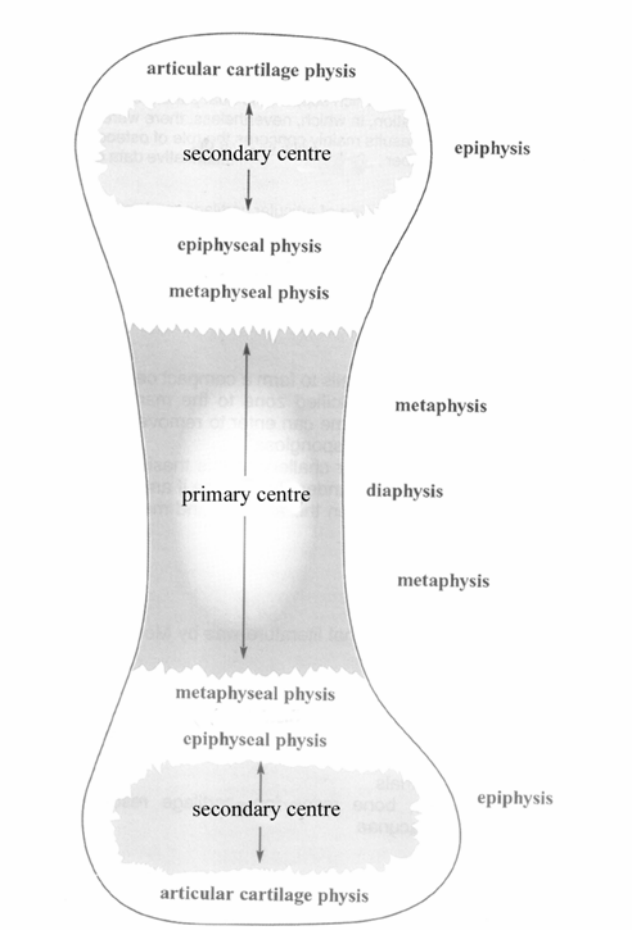

Fig. 2. The nomenclature employed to specify physeal sites.
According to Byers and Brown (2006).

composition including type X collagen synthesis are present. A hypertrophic cartilage in the degenerative zone is invaded by blood vessels and bone cells from adjacent metaphysis, and a newly formed cartilage is remodeled into a bone (Byers and Brown 2006, Andrade *et al.* 2007).

During bone growth, chondrocytes within the growth plate undergo distinct proliferative and differentiation phases. In the proliferative phase, new cells are introduced into the growth plate and matrix synthesis takes place. In the differentiated phase (the hypertrophic phase) the chondrocytes alter the surrounding matrix in anticipation of calcification and vascularization. At the metaphyseal junction, the differentiated chondrocytes undergo apoptosis and osteoblasts infiltrate the matrix scaffold to complete the ossification process (Olney *et al.* 2004).

Endochondral bone formation at the growth plate is regulated by a complex of endocrine network involving growth hormone, IGF-I, thyroid hormone, glucocorticoid, and sex steroids. Moreover, a set of autocrine and paracrine factors that regulate chondrocyte proliferation, differentiation, and ossification, is involved in this process (Andrade *et al.* 2007).

1.1.3. Influence of motion and loading on cartilage

During physiological loading, the cartilage is subjected to hydrostatic pressure in a range from 5 to 15 MPa (Carver and Heath 1999, Darling and Athanasiou 2003). The mechanical stimuli may influence both chemical composition and the structure of the new cartilaginous tissue, and are essential for the nutrition of chondrocytes.

Furukawa *et al.* reported that weight-bearing cartilage of repaired rabbit femoral condyle cartilage exhibits a significantly higher degree of glycosylation of collagens in comparison with non-weight-bearing cartilage (Furukawa *et al.* 1980). Helminen *et al.* reported that immobilization of the knee of young dogs for 11 weeks caused a decrease of the PG content in the cartilage to 38 %, and showed softer cartilage with decreased shear modulus. Partial restoration of the PG content was observed when dogs where subsequently allowed to move freely for 15 weeks. Nonstrenuous running of dogs (4 km/day) showed higher thickness of non-calcified

cartilage and increased both PG synthesis and shear modulus. On the other hand, strenuous running (20 and 40 km/day) decreased the PG content in lateral femoral condyle, the thickness of lateral condyle cartilage, and the shear modulus (Helminen *et al.* 1992).

1.2. Osteoarthritis

Osteoarthritis (OA), which is also described as osteoarthrosis, degenerative joint disease, degenerative OA or hypertrophic OA, is characterized by retrogressive changes in both cells and matrix that result in the loss of both structure and function of the articular cartilage. According to the extent of degenerative changes, three stages of OA have been described.

The first stage of OA includes a disruption of the macromolecular framework of the matrix, a decreased aggregation of PGs, a lower concentration of aggrecan, and a lower length of glycosaminoglycan chains. At the same time, increased water content, altered collagenous organization, and induced collagen degradation lead into swelling of aggrecan molecules, increase of permeability of the tissue, and reduced cartilage stiffness. PGs of the osteoarthritic joint contained different chondroitin sulfate chains (Caterson *et al.* 1992, Bank *et al.* 2000). In another study on osteoarthritic articular cartilage, the authors observerd decreased aggrecan expression (Eid *et al.* 2006).

During the second stage of OA, the following changes stimulate a cellular response: tissue damage, changes in osmolarity, density, strain, and release of mediators. The cellular response includes both synthesis and degradation of the matrix, and induced chondrocyte proliferation. This in turn can lead to restoration of the tissue, maintaining the tissue in an altered state, or increase the volume of cartilage; these changes can persist for years.

Failure to stabilize or restore the tissue leads to the third stage of OA which includes a progressive loss of cartilage as well as a decline in the chondrogenic anabolic and proliferative response. It can result from both a mechanical damage of chondrocytes and a down-regulation of chondrocytic response to anabolic cytokines. The loss of cartilage is accompanied by both remodeling and sclerosis of the

subchondral bone, and the formation of both bone cysts and marginal osteophytes. Bone remodeling that is combined with the loss of cartilage changes the shape of the joint, and may lead to shortening of the involved limb deformity and instability (Buckwalter and Mankin 1998b). Clinical symptoms include pain, a restriction of motion, crepitus with motion, joint effusion, and deformity of the joint. OA occurs most frequently in foot, knee, hip, spine, and hand joints.

OA most frequently develops in the absence of a known cause (primary OA), or as a result of joint injury, infection, or hereditary, developmental, metabolic or neurological disorders (secondary OA). The prevalence of primary OA increases from 5 % of people younger than twenty-five years to 80 % of people more than seventy-five years old (Buckwalter and Mankin 1998b). This is going to be an economical problem.

1.3. Therapy of osteoarthritis

1.3.1. Non-invasive cartilage regeneration and hyaluronan supplementation

Cartilage lesions are often treated in a conservative manner (Towheed 2002). NSAIDs are often used in the therapy of OA but they do not affect the cause of osteoarthritis. Moreover, people who might have a significant improvement from the anti-inflammatory and analgesic effects of these drugs are also those who are at the greatest risk of developing potentially serious NSAID-induced gastrointestinal side effects. The new generation of cyclooxygenase-selective inhibitors includes rofecoxib, and valdecoxib, which effectively relieve pain in OA, but are known for potentially severe cutaneous, and cardiovascular adverse reactions, respectively (Moskowitz *et al.* 2006). More recently, various substances have proved their chondroprotective effect in the treatment of OA. They include chondroitin sulfate, D-glucosamine sulfate, and hyaluronic acid. After a long-term supplementation of glucosamine sulfate, a significant reduction of symptoms as well as positive radiological changes were observed in patients with OA (Reginster *et al.* 2001, Pavelka *et al.* 2002).

Intraarticular supplementation of HA with molar-mass average from 1×10^6 to 7×10^7 Da is often used in the therapy of symptomatic OA of patients who do not respond to nonpharmacological treatment and analgesic drugs (Adam and Ghosh 2001). Viscosupplementation with high-molar-mass HA used in the therapy of patients with OA of both the knee (Wobig *et al.* 1999) and the ankle (Sun *et al.* 2006) significantly relieved the patient`s pain. This effect increased with higher molar-mass hyaluronan viscosity (Wobig *et al.* 1999). Recently, the down-regulation of OA-associated enzyme aggrecanase-2, OA-associated genes TNF-α, IL-8, and inducible nitric oxid synthase were observed in synoviocytes by the high molar-mass HA (Wang *et al.* 2006).

1.3.2. Standard surgical therapy of osteoarthritis

Isolated chondral or osteochondral defects may result from traumas that leave most of the articular surface intact. They mostly occur in young patients and can cause joint pain, effusion, and mechanical dysfunction. When they are left untreated, they fail to heal. Moreover, large defects can progress to a symptomatic degeneration of the joint. Therefore, treatment of selected isolated defects can prevent or delay the development of OA.

Standard surgical techniques, such as debridement, penetration of subchondral bone, osteotomy, joint distraction, transplantation of autographs from non-weight-bearing zone into a defect, and soft tissue grafts, such as perichondrial, periosteal flap, joint capsule, or fascia have the potential to stimulate the formation of new articular surface, and may decrease symptoms and improve joint function (Niedermann *et al.* 1985, Hoikka *et al.* 1990, Buckwalter and Lohmander 1994, Outerbridge *et al.* 1995, Buckwalter and Mankin 1998b). However, they are not able to restore the articular cartilage (Buckwalter and Lohmander 1994, Levy *et al.* 1996, Buckwalter and Mankin 1998b).

1.3.2.1. Penetration of subchondral bone

The penetration of subchondral bone is a method developed to stimulate the formation of a new articular surface. In regions with a full-thickness loss or an advanced degeneration of articular cartilage, penetration of the exposed subchondral bone disrupts subchondral blood vessels and leads to the formation of fibrin clot over the bone surface. If the surface is protected from excessive loading, undifferentiated mesenchymal cells migrate into the clot, proliferate, and differentiate into cells with morphological features of chondrocytes. In some instances they form fibrocartilaginous tissue, but in others no repair was observed (Shapiro *et al.* 1993, Buckwalter and Lohmander 1994).

Surgeons developed various methods for the penetration of subchondral bone, including a debridement of degenerated articular cartilage, drilling into subchondral bone through arthrotomy, arthroscopic abrasion of articular surface, and creation of multiple small-diameter defects or fractures, referred to as a microfracture. Unfortunately, the bone marrow stimulation techniques are less successful in patients older than 40 years (Buckwalter and Lohmander 1994, Levy *et al.* 1996, Steadman *et al.* 1997, Minas 2001, Dorotka *et al.* 2005a).

Compared to drilling, microfracture is reported to produce less heat and thus, less necrosis, and to maintain the integrity of the subchondral bone. Failure of the microfracture treatment was observed in patients with chronic lesions, advancing age, preoperative space narrowing, isolated chondral defects, or no continuous passive motion after surgery. Rehabilitation after surgery is considered as an important factor of cartilage regeneration (Steadman *et al.* 1997).

1.3.2.2. Soft-tissue grafts

The treatment of osteoarthritic joints with soft-tissue grafts most often involves debridement of the joint and interposition of soft-tissue grafts consisting of fascia, joint capsule, muscle, tendon, periosteum, or perichondrium between the debrided or resected articular surface (Niedermann *et al.* 1985, O'Driscoll *et al.* 1988,

Hoikka *et al.* 1990). Periosteal graft implantation was able to regenerate hyaline-like tissue in the osteochondral defects in rabbits in a long-term experiment (Rubak 1982). In another experiment Rubak *et al*.(1982) showed that cartilage tissue in the defect originated from the periosteal graft and not from the subchondral bone. Periosteal graft implantation was successful in patients younger than 35 years (Niedermann *et al.* 1985), or 50 years (Hoikka *et al.* 1990), but failed in the treatment of a 55-year-old man with a severe patellar arthrosis (Hoikka *et al.* 1990).

1.3.3. Autologous chondrocyte transplantation

One of the modern techniques involves autologous chondrocytes injected into the defect that is covered by periosteal flap sealed with a fibrin glue (Brittberg *et al.* 1994, Breinan *et al.* 1997, 2001). This teqnique is based on a combination of two chondrogenic factors: cambium cells of the periosteum and suspension of chondrocytes. However, in animal models, the positive effect of ACI was observed only three months after the implantation (Breinan *et al.* 2001), but no effect was observed after 12 and 18 months (Breinan *et al.* 1997). That may be ascribed to the lack of retention of the cells in the defect due to a possible displacement of the periosteal flap, uneven distribution of cells, dedifferentiation of chondrocytes, and the location of the defect. Erggelet *et al.* (2003) decribed the arthroscopic ACI technique for a treatment of osteochondral defects. In clinical practice, the ACI technique has been successfully performed on patients aged 14 to 58 years since 1987 (Brittberg *et al.* 1994, Minas 2001, Marlovits *et al.* 2006).

Although ACI demonstrated an improved function and a diminished pain, several complications, such as periosteal hyperthrophy, delamination of the transplant, arthrofibrosis and transplant failure have also occurred (Minas 2001, Marlovits *et al.* 2006). Higher improvement was observed in patients with lower severity of the defects (Minas 2001).

The second generation of ACI technique (or ACT) involves the injection of autologous chondrocytes under a three-dimensional biodegradable scaffold, such as a collagen bilayer membrane (Chondro-Gide^{TM} membrane, Geistlich Biomaterials)

(Marlovits *et al.* 2006). The advantage of this technique in comparison with ACT is no hypertrophy.

1.3.4. Matrix-associated autologous chondrocyte implantation

The third generation of ACT is based on autologous chondrocyte-seeded biomaterials, such as cell carriers (matrix-associated autologous chondrocyte transplantation). The biomaterials are used as three-dimensional constructs; they secure cells in the defect area, and enhance their proliferation and differentiation. After debridement, the biomaterials seeded with cells are trimmed to match the size of the defect and implanted into the defect without the use of the periosteal flap. Fibrin is often used to fix the scaffold (Trattnig *et al.* 2005).

The fleece of hyaluronan derivative (Hyalograft C) (Pavesio *et al.* 2003, Trattnig *et al.* 2006), type I/III collagen membrane (Verigen, Genzyme; Igor, André), bilayer membrane consisting of type I/III and II collagen (Dorotka *et al.* 2005b), and polylactin and polyglactin (BioTissue, Germany) were intensively studied in clinical (Cherubino *et al.* 2003, Pavesio *et al.* 2003, Marlovits *et al.* 2005, Trattnig *et al.* 2005, Marlovits *et al.* 2006), and animal studies (Willers *et al.* 2005, Dorotka *et al.* 2005a, Dorotka *et al.* 2005b). Dorotka reported improved a healing of chondral defects in sheep and a formation of hyaline cartilage compared to microfracture treatment or microfracture with unseeded collagen matrix implantation.

In comparison with the second generation of ACT, this technique allows chondrocytes to redifferentiate and to produce their own extracellular matrix. Subjective and objective improvement has been observed in 76 % to 100 % of patients. These results suggest that MACI is the appopriate technique for treatment of chondral/osteochondral defects, and can be used in combination with other techniques, such as microfracture. MACI can be used for the therapy of larger defects with satisfactory results.

1.4. Physeal fractures and their therapy

In children, physeal fractures belong to serious bone injuries. Mann and Rajmaira reported that 30 % of 2,650 long-bone fractures in children involved a physeal growth plate (Mann and Rajmaira 1990). Traumas of proximal growth plate of humerus, distal physes of tibia, fibula, humerus and femur represent 2−10 % of all bone injuries (Ashcraft *et al.* 2005). Limb long-bones injuries, except for humerus, are often accompanied by a damage of physis, which leads to a bony bridge formation at the site of physeal injury. According to the extent and localization of the physeal defect, the affected limb may become shorter, may display angular deformity, or both. Although 25−35 % of the physeal fractures in humans lead to some shortening or an angular deformity, only about 10 % of the fractures resulted in functional problems (Mizuta *et al.* 1987, Mann and Rajmaira 1990).

Many experimental and clinical attempts for surgical correction of bone deformities have been described, only varying degrees of success have been achieved. They include stapling, epiphyseodesis, osteotomy, excision of bony bridge and insertion of interpositioning materials such as bone cement, fat, or polymeric silicone (Lee *et al.* 1993). Chondrocytes, perichondrial cells, or MSCs used either alone or embedded in a three-dimensional scaffold have shown the potential to repair physeal defects in animal models, and they represent a promising approach to the treatment of physeal defects. The aim of a novel therapeutical approach is to prevent a bony bridge formation and thus to avoid the further problems with both a shortening and a deformation of the limb (Park *et al.* 1994, Lee *et al.* 1998, Ahn *et al.* 2004, Yoo, W. J. *et al.* 2005).

1.5. Cell culture

Autologous chondrocytes are widely used in clinical practice. They are well characterized and their proliferation and differentiation is known to be tailored by modifying both the culture medium composition and the cultivation systems, such as two- or three-dimensional sytems, or dynamic cultivation. However, some problems

concerning harvesting of sufficient amount of healthy cartilage led to using mesenchymal stem cells in tissue engineering of cartilage.

1.5.1. Chondrocyte culture and differentiation

Cartilage contains only 1 % (V/V) of chondrocytes. To obtain an adequate number of chondrocytes for subsequent therapy of osteochondral or chondral defects, they have to be enzymically digested from the piece of non-weight-bearing cartilage and then multiplied *in vitro* into a few millions in a few weeks (Lee and Loeser 1998).

The two-dimensional culture of chondrocytes allows their multiplication, but causes chondrocyte dedifferentiation into fibroblast-like cells after a few passages as well (Fig.3). The dedifferentiation of chondrocytes is gradual and is accompanied by the decrease of type II collagen synthesis and increase of type I collagen synthesis. Moreover, distinct differences were found in chondrocytes obtained from the superficial zone and the growth zone; the latter exhibited 20-fold higher type II collagen expression than the superficial layer (Darling and Athanasiou 2005). However, this dedifferentiation process can be reduced using culture media containing growth factors and a low concentration of fetal bovine serum, or serum free media (Mandl *et al.* 2002).

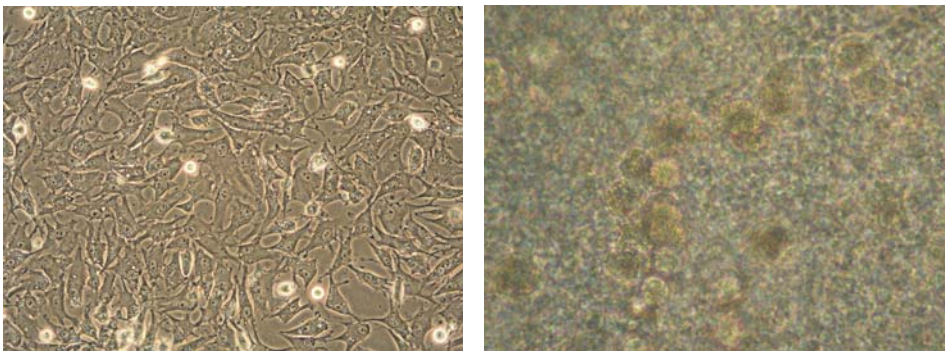

Fig. 3. Dedifferentiated fibroblast-like chondrocytes cultured on the tissue culture polystyrene for 7 days (left) compared with round redifferentiated chondrocytes embedded in fibrin scaffold (right).

Addition of TGF-β1 or TGF-β2 into the serum-free medium supplemented with insulin-containing supplement ITS+ or IGF-I induce a dose-dependent redifferentiation of chondrogenic markers. However, neither insulin nor TGF-β alone can induce chondrocyte redifferentiation (Yaeger *et al.* 1997).

FBS is often used in cell culture for a stimulation of cell adhesion and proliferation but its variable composition causes variability of results. The inhibitory or activating effect of FBS on cell proliferation depends on both its amount in a culture medium and the time (Vivien *et al.* 1990, Olney *et al.* 2004). Human articular chondrocytes suspended in alginate gel were able to re-express their chondrogenic phenotype if they were cultured in a medium supplemented with specific lots of FBS. Other lots of FBS were not efficient, but if they were used in the presence of IGF-I and ascorbate, increased mRNA synthesis of both aggrecan and type II collagen was observed (Yaeger *et al.* 1997).

Various schemes optimizing chondrocyte proliferation and redifferentiation have been published that used different combinations of growth factors and other additives, and their time-dependent supplementation. In addition, a combination of monolayer culture followed by three-dimensional culture was more efficient than the two-dimensional system alone (de Haart *et al.* 1999, Kuriwaka *et al.* 2003, Miot *et al.* 2006).

The use of growth factors in the cartilage defect repair needs proper understanding of the role of various growth factors in cartilage metabolism and knowledge of their potential side effects on cells and tissues in a joint. Moreover, it is reported that the effects of growth factors on chondrocytes from osteoarthritic or inflamed cartilage are different; for example, osteoarthritic chondrocytes do not respond to treatment by IGF-I, which is a homeostatic factor regulating matrix synthesis and degradation. Enhanced proteoglycan synthesis in osteoarthritic cartilage is caused by the higher sensitivity of the tissue to TGF-β (van den Berg *et al.* 2001).

Chondrocyte culture in three-dimensional scaffolds

Benya and Shaffer (1982) were the first to describe the ability of chondrocytes cultivated in a monolayer to re-express their differentiated phenotype in agarose gel. Chondrocytes, cultivated in a monolayer from the third to the sixth passages, were embedded in an agarose gel. Consequently, the protein and collagen

synthesis by chondrocytes increased to levels higher than those in a monolayer. The chondrocytes in the third passage rapidly increased the synthesis of both collagen and proteoglycan but chondrocytes from higher passages showed a transient decrease before the protein and collagen synthesis increased. Re-expression of proteoglycan synthesis was less sensitive to the passage number. The third passage monolayer culture of chondrocytes expressed dedifferentiated phenotype for a shorter time, and this was probably essential for the redifferentation process.

The encapsulation of chondrocytes into alginate beads increased type II collagen expression but full redifferentiation was not achieved even after two weeks (Darling and Athanasiou 2005). The chondroitin sulfate synthesis by freshly isolated chondrocytes embedded in Atellocollagen gel was higher than that in a monolayer culture. Ultrastructural evaluation revealed than collagen network was organized even after one week in a three-dimensional Atelocollagen gel. After three weeks of cultivation, the collagen network was well organized with interconnecting collagen fibers and more resembled normal cartilage structure (Kuriwaka *et al.* 2003).

Similar reddiferentiation of dedifferentiated chondrocytes was observed in many types of three-dimensional scaffolds including hydrogels, porous scaffolds, and textile scaffolds (Fig.3). Chondrocyte differentiation and cartilaginous matrix formation in non-woven PGA mesh was influenced by a sequential supplementation of growth factors in the presence of 10 % FBS. A ten-day supplementation with both TGF-β1 and FGF-2 followed by an eighteen-day supplementation with IGF-I yielded the highest and the best differentiated construct (Pei *et al.* 2002).

Chondrocyte growth plate culture

The growth hormone is considered to play a key role in the regulation of longitudinal bone growth The stimulatory effect of the growth hormone on cell proliferation of chondrocytes and other cells is known. It is probably mediated by circulating levels of IGF-I or by stimulation of local synthesis and secretion of IGF-I, which subsequently induces chondrocyte proliferation through a paracrine or autocrine mechanism (Rosselot *et al.* 1994). The other growth factors, including TGF-β, IGF-I and FGFs, may also be involved in autocrine or paracrine control of cartilage growth and differentiation. This theory can be supported by the fact that they are produced by chondrocytes and are present in growth plate tissue, and they

are able to induce chondrocyte proliferation and differentiation (Rosselot *et al.* 1994).

In contrast with mammalian chondrocytes, the proliferation of avian growth plate chondrocytes under serum-free conditions seems not to be affected by the growth hormone but bFGF displays mitogenic activity that can be enhanced by TGF-β1 or TGF-β1 + IGF-I. IGF-I was found to be a key factor for matrix synthesis which can be enhanced by adding TGF-β1, TGF-β1 + bFGF, or serum (Rosselot *et al.* 1994).

1.5.2. Mesenchymal stem cells

1.5.2.1. Mesenchymal stem cell culture

The characteristics of stem cells are their self-renewal and their ability to differentiate into one or more specialized cell types. Traditionally, stem cells can be divided into two major groups: embryonic stem cells and tissue-specific stem cells. Embryonic stem cells can differentiate into all cell types and can be maintained indefinitely *in vitro* without loss of their differentiation potential. However, they are still used only in experimental studies because of ethical and legal problems concerning the use of human embryos.

Many adult tissues contain stem cell populations that have a capacity for tissue renewal after trauma, disease or aging. Multipotential MSCs are precursor cells localized in the stromal compartment of bone marrow (Pittenger *et al.* 1999). They are of interest for tissue engineering because of their easy multiplication and multilineage potential (Huang *et al.* 2005). They are able to differentiate into tissues of mesenchymal lineages including cartilage, bone, fat, tendon, muscle, neuronal tissue and stroma; they can also differentiate into cardiomyocytes and hepatocytes. Their number inversely correlates with age and depends on the site of extraction and systemic disease state. Besides bone marrow, MSCs are present in blood, synovial membrane, periosteum, adipose tissue, trabecular bone, dermis, cartilage, and

muscle. MSCs from these sources have a more limited differentiation potential (Tuan *et al.* 2003, Alsalameh *et al.* 2004).

MSCs from bone marrow are isolated using Percoll gradient centrifugation, and adherent cells are multiplied *in vitro*. When cultured long-term *in vitro*, they remain in a stable undifferentiated state. They are characterized by a set of markers on their surface including SH2, SH3, CD29 (integrin β1), CD44, CD90, CD106 (VCAM-1), CD120a, CD124, etc. In contrast, MSCs are negative for other markers of hematopoietic lineage such as lipopolysaccharide receptor CD14, CD34, and the leukocyte common antigen CD45. After long-term cultivation, MSCs exhibit finite life spans (Pittenger *et al.* 1999) A fraction of isolated human stromal MSCs, characterized by negative CD34, CD117 (c-kit), and CD140α markers, exhibited a chondrogenic potential (Imabayashi *et al.* 2003). In the same study, CD29, CD44, CD90, CD105, and CD106 were detected in both dedifferentiated chondrocytes and stromal MSCs.

1.5.2.2. Mesenchymal stem cell differentiation

MSC differentiation *in vitro* is strongly influenced by culture conditions including cell density, cell adhesion and growth factors (Tuan *et al.* 2003). Cartilage differentiation is induced by TGF-β superfamily of proteins, such as TGF-β, BMPs, other growth factors including bFGF, IGF-1, and other substances, such as dexamethasone, ascorbic acid, and hyaluronic acid, but without the presence of fetal bovine serum. TGF-β1 seems to play the pivotal role in chondrogenic differentiation of MSC cultures from various species. The potential effect of TGF- β is mediated by MAP kinases through the cell-cell adhesion molecule N-cadherin, a requisite cell-cell interaction in developmental chondrogenesis, which was previously shown to mediate embryonic mesenchymal condensation (Johnstone *et al.* 1998, Ringe *et al.* 2002a, Imabayashi *et al.* 2003, Tuan *et al.* 2003, Indrawattana *et al.* 2004). However, TGF-β1 alone was not able to induce complete chondrogenic differentiation in human MSC but a combination of TGF-β1 either with BMP-6 or IGF-1 enhanced their chondrogenic potential accompanied by increased synthesis of aggrecan and type II collagen (Indrawattana *et al.* 2004).

In addition, Wnt and Wnt-related family of signaling proteins are involved in adult cartilage homeostasis. Wnt-1-inducible signaling pathway protein 3 (WISP-3) is expressed in adult human synoviocytes and articular cartilage, and other Wnts, such as Wnt-11, are expressed in developing cartilage and are upregulated during MSC chondrogenesis. This suggests the involvement of Wnt signalization in chondrogenic differentiation. Interestringly, a higher expression of Wnt-5 by synoviocytes in rheumatoid arthritis was observed. Both TGF-β and Wnt signaling are probably mediated via MAP kinases (Tuan *et al.* 2003, Hegewald *et al.* 2004).

In the tissue, the differentiation of MSC is influenced by environmental factors, such as oxygen tension. For example, high oxygen tension supports osteogenesis, whereas in areas with low oxygen tension chondrogenesis is favored (Boyan *et al.* 1996).

Moreover, a high-density culture of MSCs, such as in pellets or chondrospheres, strongly promotes cartilaginous tissue formation accompanied with glycosaminoglycans and type II collagen synthesis even after 10 days; this synthesis persisted for 3 months (Pittenger *et al.* 1999). This system allows cell-cell interactions analogous to those that occur in precartilage condensation during embryonic development. In comparison to pellet culture, chondrosphere culture did not require a scaffold for chondrogenesis, and is sufficient for chondrogenesis of dedifferentiated chondrocytes and stromal MSCs (Imabayashi *et al.* 2003). The best chondrogenic differentiation was obtained in the pellet culture with TGF-β and dexamethasone (Johnstone *et al.* 1998). Ringe *et al.* (2002a) reported type II synthesis after 20 days of culture. However, in MSC culture from rabbit postnatal bone marrow, chondrogenic differentiation was accompanied by alkaline phosphatase activity and type X collagen expression; they indicate that cells differentiated into their terminal phenotype, the hypertrophic chondrocytes (Johnstone *et al.* 1998). The more recent study introduced new approaches to MSC culture including cycling induction of chondrogenesis by growth factors (Indrawattana *et al.* 2004).

Osteoarthritic cartilage contains an increased number of CD105+/CD166+ positive MSCs, which may result from proliferation of resident progenitor cells or from recruitment from other sites; they may be involved in the pathogenesis of arthrosis (Alsalameh *et al.* 2004). In arthritic joints, the inflammatory mediators,

such as IL-1 or tumor necrosis factor, may lead to abnormal differentiation of the progenitor cells and subsequently to fibrocartilage formation.

The treatment of large defects in osteoatrhritic patients may be negatively influenced by the decreased ability of MSCs to undergo chondrogenic differentiation (Murphy *et al.* 2002). However, this problem has been solved and chondrogenic differentiation in pellets was increased, when MSCs were cultured in the presence of bFGF in a monolayer culture (Akaogi *et al.* 2004).

Mesenchymal stem cell culture in three-dimensional scaffolds

Two major strategies that use MSCs in tissue engineering are appplied: (1) using MSCs for protein delivery by inserting genes coding for a secreted protein and implanting the cells into the defect (Lee *et al.* 2001), or (2) using MSCs as multipotential cells (Ringe *et al.* 2002a, 2002b) In this case the differentiation is controlled by a specific differentiation medium and the scaffold environment; e.g. hydroxyapatite ceramics which enhances osteogenic differentiation of MSCs compared to tissue-culture polystyrene (Kotobuki *et al.* 2005). The expression of cartilage-specific genes for aggrecan, type II collagen, and Sox-9 mRNA is enhanced when they are embedded in PLA-alginate (Caterson *et al.* 2001) or type II collagen scaffold, and reduced when they are embedded in type I collagen (Chen *et al.* 2005). MSCs isolated from bone marrow and periosteum were embedded in a type I collagen gel and implanted into the osteochondral defect. After 24 weeks, both cell-seeded scaffolds were able to repair osteochondral bone and cartilage, but with low mechanical properties of the articular cartilage (Wakitani *et al.* 1994). Similarly, in a clinical study, MSC-seeded type I collagen showed only partially repaired articular cartilage (Wakitani *et al.* 2002). Ahn *et al*. (2004) reported that MSC-seeded onto gelatin scaffolds and implanted into physeal defects prevented bony bridge formation with varus deformity, the effect was higher in the presence of the TGF-β.

1.5.3. Dynamic cultivation of cells

Cyclic mechanical loading is important for chondrocyte function in native cartilage. Recently, dynamic cell cultivation has been developed to improve cell culture and production of ECM matrix by cells in three-dimensional scaffolds. Bioreactor utilizing a dynamic laminar flow (Vunjak-Novakovic *et al.* 1999), intermittent pressure and perfusion significantly stimulated both the collagen and GAG synthesis by chondrocytes (Carver and Heath 1999), or GAG synthesis alone (Lu *et al.* 2005). Consequently, the scaffolds showed better mechanical properties. In contrast, in a wavy-walled bioreactor or spinner flask, the shear stress applied to chondrocytes caused increased synthesis of type I collagen, and the fibroblast-like morphology of chondrocytes at the scaffold perifery (Vunjak-Novakovic *et al.* 1999, Bueno *et al.* 2005).

1.6. Scaffold formulation

The scaffolds used in tissue engineering must be biocompatible and nonimmunogenic. Bioresorbable materials allow controlled replacement of the material by regenerated tissue. The chemical composition, structure and surface properties, as well as the biomechanical properties of the scaffold are crucial for its interaction with cells and tissues and subsequently, for tissue regeneration.

1.6.1. Biocompatibility

Synthetic polymers implanted in the body may be recognized as foreign by the immune system of the host. In the first step, this process includes the adsorption of bioactive proteins, such as complement, fibronectin, fibrinogen, or plasma proteins which then act as a matrix for the interaction with the cells of the immune system. The adsorption starts immediately after an implantation and after a few hours

the material is coated with a chaotic layer of denatured or partially denatured proteins. The immobilization is influenced by the chemical compostion of the material, the presentation, quantity and conformation of funtional groups. An acute inflammatory reaction is reflected by fagocytic cell accumulation. The macrophages that adhere on the surface of the implant are derived mostly from circulating monocytes. Monocyte activation is mediated by several factors including adhesive contacts, and leads to macrophage formation, and activation of immediate-early response genes, such as genes for IL-1 and IL-8, tumor necrosis factor, and transcription-associated proteins, such as c-fos or c-jun (Kao *et al.* 1995, Tang and Eaton 1995, Smetana *et al.* 1997, Hu *et al.* 2001).

Monocyte adhesion and spreading depend on the chemical composition of the scaffold. Smetana *et al.* (1993, 1997) demonstrated that dimethylamino groups in dimethylaminoethyl metacrylate copolymer with poly(2-hydroxyethyl methacrylate) lead to increased macrophage spraeding and subsequent macrophage fusion into foreign-body giant multinucleate cells. In contrast, the expression of carbohydrate binding sites for galectins, *β*-*N*-acetylgalactosamine, *α*-mannoside, specific lectin for heparin, and lymphokine-macrophage migration inhibitory factor was not dependent on the chemical structure of the polymers (Smetana *et al.* 1997).

Chronic inflammatory reactions are accompanied by the accumulation of macrophages and/or foreign-body giant cells. The biomaterial-associated phagocytes are responsible for biomaterial-mediated adverse responses, including inflammation surrounding many types of implants, implant degradation and stress-cracking, tissue fibrosis surrounding mammary prosthesis, joint and other types of implants, and device-centered infection (Hu *et al.* 2001).

Both collagen and HA are the major components of ECM and tissues and they should not be immunogenic, provided they have not been contaminated during processing and there are no cross-species issues. Similarly, natural derived chitosan has been shown to be nonimmunogenic if it is purified, although it has a chemotactic effect on neutrophils (Drury and Mooney 2003).

1.6.2. Porosity

Porosity is defined as the percentage of void space in a solid, and it is a morphological property independent of material (Karageorgiou and Kaplan 2005). In bone formation, pores are necessary for migration and proliferation of osteoblasts, mesenchymal stem cells, and vascularization, while large pores improve bone ingrowth. Both chondrocyte migration and proliferation are increased in scaffolds with a high porosity. Interconnected pores provide cells with nutrition, growth factors and cytokine supplementation. In addition, porous surface (higher roughness) allows mechanical interlocking between the implant and the surrounding bone tissue which provides better mechanical stability of the implant and leads to better osseointegration (D'Lima *et al.* 1998, Karageorgiou and Kaplan 2005).

The specific surface area, which is the surface area per unit volume, was found to correlate linearly with cell atachment and viability. On the other hand, avarage pore size above 100 μm caused decreased attachment of mouse clonal osteogenic cells (O'Brien *et al.* 2005).

The pore scaffolds can be prepared by various techniques, including salt leaching, molding, porogen melting, melt blowing, sintering, gas foaming, porogen dissolving, emulsion polymerization, and electrospinning (Fig.4). The pore size in scaffolds varies from 2 to 800 μm, and can be tailored for different applications (Karageorgiou and Kaplan 2005).

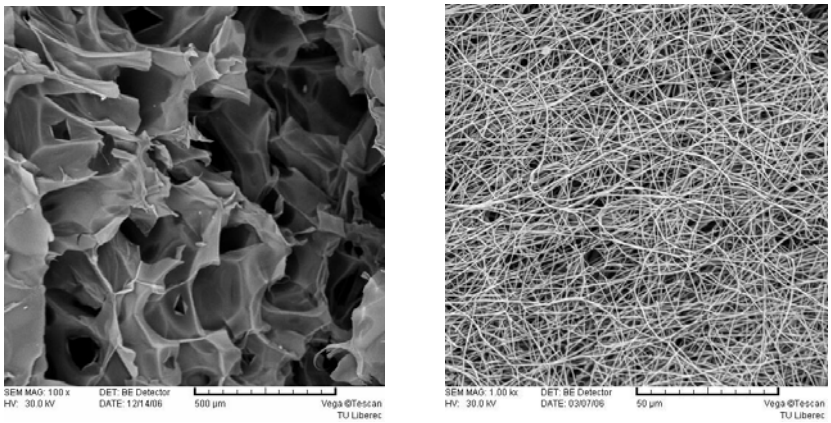

Fig. 4. Different structure and porosity of gelatine foam (left) and nanofibre PLGA scaffold (right).

1.6.3. Surface properties

The surface characteristics determine the adsorption of biological materials. The nature of conditioning materials as well as their orientation on the surface directly affect cell recruitment, attachment, spreading, proliferation, and differentiation (Fig.5). Thus, chemotactic factors are necessary for cell recruitment, specific binding proteins on the surface allow cell attachment, and growth factors and cytokines are essential for cell proliferation and differentiation (Boyan *et al.* 1996).

Cells interact with the surface material through integrin receptors that assemble into focal adhesion plaques. The type and geometry of focal adhesions determine the cell shape and changes in the cell shape alter the transduction of extracellular signals to the cell via integrin receptors and thus influence their phenotypic expression (Enomoto *et al.* 1993, Aplin *et al.* 1998). Chondrocytes cultured in a monolayer become flattened, with the morphology of fibroblast-like cells, and dedifferentiate. However, when they are embedded in a three-dimensional scaffold, they become round and redifferentiation occurs (Benya and Shaffer 1982).

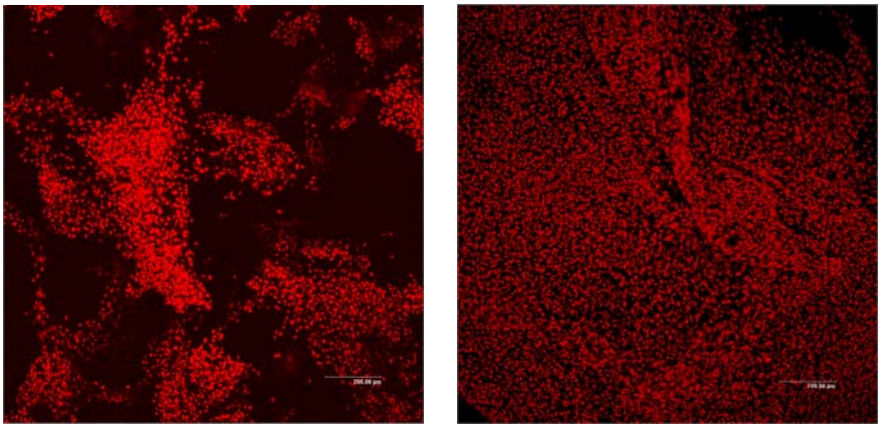

Fig. 5. Chondrocyte growth on microstructured surface of gelatin foam (left) and nanostructured surface of PLGA nanofibre scaffold (right) 14 days after seeding (confocal microscope, propidium iodide staining, obj. × 10).

Bone cells, epithelial cells, and fibroblasts are sensitive to the gross morphology of the material. The optimized pore diameter in the range of 200-400 μm is known to enhance osteoblast attachment, proliferation and migration. This curvature may provide optimum compression and tension on cell´s mechanoreceptors.

Chondrocyte and osteoblast attachment, proliferation and extracellular matrix synthesis is influenced by surface roughness. Immature osteoblast-like MG63 cells showed decreased cell number, [^{3}H]-thymidine incorporation and alkaline specific activity, when grown on rough cpTi surfaces. Resting chondrocytes showed the same results as MG63 cells, while growth zone chondrocytes, which are more mature than resting chondrocytes, displayed different results. This indicates that the cell response to the surface roughness is influenced by the maturation state of the cells. In another experiment, different spreading of MSCs on smooth and rough surfaces was observed (Boyan *et al.* 1996). Topography of the substratum influences the cell shape and movement. The cell alignment on multiple grooved substrate increased with the groove depth (Clark *et al.* 1990).

Nanostructured and microstructured surfaces of PLGA/titanium and PLGA improved chondrocyte and osteoblast adhesion in comparison with conventional polymer films but the nanostructure of the surface was more effective (Kay *et al.* 2002).

The surface energy is defined by its general charge density and the net polarity of the charge. Thus, a surface with a net positive or negative charge may be hydrophilic in character, and a surface with neutral charge may be more hydrophobic. The net effect of the surface charge creates a local environment with a specific surface tension, surface free energy, and energy of adhesion. The biological response is determined by the relative strength of the serum–surface interaction. This interaction can be defined as interfacial free energy that is the most important for the process of cell adhesion (Boyan *et al.* 1996).

1.6.4. Biomechanical properties of scaffolds

After the implantation of the scaffold into the weight-bearing part of the knee joint, the scaffold is subjected to a cyclic loading. Chondrocytes embedded in the scaffold are able to withstand such high forces only if the scaffold possesses adequate biomechanical properties, and if it is able to transmit the loading in an appropriate manner to the cells and tissues. Therefore, for optimum scaffold characterization, it is necessary to cover the whole extent of physiologically possible strains, and to characterize the basic material parameters, such as dynamic stress-strain relationship, Young's modulus strain dependence, and energy losses.

From the mechanical point of view the cartilage can be regarded as a so-called poroelastic material, hence a biphasic model has been developed to describe its basic mechanical properties. The mechanical parameters, such as stiffness or Young's modulus and viscoelastic characteristics, are highly strain-rate-dependent. In order to better understand the real dynamic behavior of the material, several attempts have been made both to measure and to simulate the impact loading stress state (Ateshian *et al.* 1997, Mow and Guo 2002).

Physical properties of the scaffolds are influenced by a gel formation mechanism and dynamics, mechanical characteristics and degradation behavior. The mechanical properties of hydrogels are affected by polymer concentration and characteristics, by cross-linking agent and its characteristics (e.g.concentration, type, size of molecules), gelling conditions, such as temperature and pH, and by swelling and degradation (Drury and Mooney 2003). Higher polymer concentration increases the compression modulus and the equilibrium shear modulus (LeRoux *et al.* 1999). The gelling conditions must be in the range of physiological conditions so that the cells survive.

Hydrogel swelling and degradation lead to weakening of the gel. The weakening can be reduced if cells produce extracellular matrix or if the surrounding tissue grows into the scaffold. The desired degradation kinetics may vary due to specific scaffold application. The optimum scaffold degradation copies the rate of the new tissue formation. On the other hand, the controlled release of bioactive substances needs controlled degradation rate. The hydrogels degrade by hydrolysis, enzymic cleavage, and dissolution. The natural derived polymers, such as chitosan, collagen and hyaluronic acid, are degraded enzymically, and the degradation rate depends on the number of cleavage sites in the polymer and enzyme concentration in the scaffold environment. On the other hand, synthetic polymers are usually degraded by hydrolysis of ester linkages; the hydrolysis is constant *in vitro* and *in vivo* (Drury and Mooney 2003).

1.6.5. Natural and synthetic hydrogel scaffolds used in cartilage regeneration

Natural derived scaffolds

Hydrogels from biopolymers permit three-dimensional immobilization of the cells and provide the proper environment that supports chondrocyte and MSC differentiation (Benya and Shaffer 1982, Brittberg *et al.* 1997, Radice *et al.* 2000, Drury and Mooney 2003, Ng *et al.* 2005). Due to their biodegradability and biocompatibility they are widely used as tissue substitutes in many applications, such as cartilage, vascular, skin, liver, intestine, and urological tissue repair (Vacanti *et al.* 1998). However, their limitations include batch-to-batch variation upon isolation from biological tissues as well as restricted possibility to design scaffolds with specific biomechanical properties.

Hydrogels mimic the natural highly hydrated cartilage that contains above 90 % of water (Buckwalter and Mankin 1998a). The high water content facilitates transport of nutrients. They are prepared from naturally derived biopolymers, such as fibrin, collagen, chitosan, agarose, alginate, and hyaluronic acid derivatives.

Fibrinogen and fibrin play an important role in blood clotting, fibrinolysis, cellular and matrix interactions, inflammation, wound healing, and neoplasia. The fibrin network is formed from fibrils that undergo further lateral associations and form branches by two types of branching. The tetramolecular or bilateral branch consisting of large fiber bundles and more condensed bilateral brand structure confers strength and rigidity to the network fibers. Trimolecular or equilateral branch is formed by coalescence of three fibrin molecules that connect three double-stranded fibrils of equal width. The architecture is influenced by various factors, such as thrombin concentration, and the availibality of intermediate fibrin units (Mosesson *et al.* 2001) .

At the site of injury, the fibrin clot serves as a provisional scaffold for the recruitment of fibroblasts, endothelial cells and monocytes. To recognize the fibrin clot, the migrating cells use integrin receptors for fibrin, fibronectin and vitronectin (Clark 2001). The activated macrophages play an important role in fibrin degradation by production of proteolytic enzymes, such as plasmin, plasminogen activators, collagenase and elastase. Moreover, the degradation is influenced by the presence of protease inhibitors, and fibrin fragments. Fibrin is gradually replaced by collagen and

other ECM components produced by cells (Bense and Woodhouse 1999, Clark 2001).

Synthetic scaffold

In situ gel-forming hydrogels, such as modified polyethylene oxide (PEO), have been succesfully used in chondrocyte encapsulating. Some limitations, however, exist with respect to controlling the mechanics and uniformity of the gel. Synthetic polymers, e.g. polyvinyl alcohol and polyethylene glycol have been studied (Bryant and Anseth 2001, Stammen *et al.* 2001, Bryant and Anseth 2002, Kobayashi *et al.* 2003). Cross-linked hydrogels (Bryant and Anseth 2002), or porous scaffolds prepared from hydrogels by cross-linking and/or by freeze-drying exhibit increased stiffness and reduced degradation velocity (Chang *et al.* 2003). The chondrocyte viability and extracellular matrix synthesis in the photocross-linked PEO scaffolds was not affected by the high stiffness of the scaffold (Bryant and Anseth 2001). They allow preparation of scaffolds of variable composition, biological, and biomechanical properties, especially when they are combined with other hydrogels, woven or non-woven scaffolds, and other polymer scaffolds (Minoura *et al.* 1998, Caterson *et al.* 2001, Park *et al.* 2004).

Synthetic polyesters, such as PLLA, PGA and copolymer PLGA, have been intensively studied; they have been already used in clinical practice as sutures and medical devices. Porous synthetic scaffolds demonstrated a higher stiffness compared with hydrogels but also an increased fragility and low elasticity. Therefore, they are not suitable for cartilage regeneration. Conversely, the textile woven scaffolds with improved elastic properties can be utilized in tissue engineering.

1.6.6. Hydrogel applications

In tissue engineering, hydrogels are applied as space-filling agents which are used in many applications including bulking, adhesion prevention, or bioadhesives, and biological glue for soft tissues. The scaffolds used as space-filling agents should maintain the desired volume and structural integrity for the desired time. Many scaffolds including alginate, chitosan, and collagens have the potential for use as a

bulking agent. They have been used in the treatment of urinary incontinence, vesicoureteral reflux, and for both plastic and reconstructive surgery. The antiadhesive polymer PEG is used as a prevention of post-operative adhesion. Conversely, adhesive polymers, such as fibrin and chitosan, can seal soft tissues and allow air and body fluid leakage from the wound, which improves wound dressing (Wang *et al.* 1995). Hydrogels can serve as delivery vehicles for bioactive substances, such as TGF-β, vascular endothelial growth factor and bFGF, which promote cell differentiation or angiogenesis (Park, H. *et al.* 2005). Moreover, hydrogels supply the wound with antibiotics (Park *et al.* 2004). Controlled drug delivery is in demand in many applications, including wound healing and tissue regeneration. The incorporated bioactive molecules are released by diffusion, mechanical stimulation, and degradation (Ruel-Gariepy *et al.* 2000, Saito *et al.* 2001). Finally, hydrogel scaffolds seeded with transplanted cells are able to regenerate the majority of tissues including skin, cartilage, bone, muscle, fat, liver, and neurons (Drury and Mooney 2003). To improve cell adhesion, cell non-adhesive synthetic hydrogel scaffolds can be modified with cell adhesion peptides such as RGDS. In addition, the incorporation of degradation sites increased the proliferation of vascular smooth muscle cells and their penetration into the scaffold (Schmedlen *et al.* 2002).

1.6.7. Future trends in cartilage tissue engineering

The first generation of cartilage regeneration used an autologous chondrocyte implantation with a periosteal flap. The second one was based on the implantation of three-dimensional scaffolds or osteochondral transplants, and the delivery of bioactive substances.

The future development of the third generation of scaffolds involves the cellular and molecular basis of tissue regeneration. The gene-activating biomaterials should be tailored for specific patients and disease states. The construct seeded with the patient`s own cells should provide the optimum treatment. Another approach includes gene-activating biomaterials that prevent further denegenerative processes in the tissue, or even improve a tissue function (Hench and Polak 2002).

1.7. Objectives of the study

My experiments were focused on cartilage defect regeneration. They included the following stages:

1. Improvement of healing of osteochondral defects using oral supplementation of GAGs and antioxidants.
2. Development of three-dimensional biocompatible and biodegradable artificial scaffold for articular cartilage regeneration, which possesses appropriate biomechanical properties.
3. Evaluation of osteochondral defect regeneration using a composite scaffold seeded with autologous chondrocytes.
4. Evaluation of physeal defect regeneration using a composite scaffold seeded with autologous mesenchymal stem cells.

This PhD Thesis is composed of four attached publications where applied methods and experimental details can be found.

2. Results

2.1. Peroral supplementation of GAGs and antioxidants

Histological analysis showed a higher organization level of the cartilage-like repair tissue in the healed osteochondral defect in the GAG group in comparison with the placebo group. The amount of the sulfated GAG in the osteochondral defects of the GAG group was significantly higher ($p < 0.03$) than in the control group (Table 1). In both groups the total GAG content in the cartilage of the operated knee joint was also significantly higher ($p < 0.05$) than that in the non-operated knee joint.

Histological analysis showed a higher organization level of the cartilage-like repair tissue in the healed osteochondral defect in the GAG group in comparison with the placebo group.

The amount of the sulfated GAG in the osteochondral defects of the GAG group was significantly higher than in the control group (Table 1). In both groups the total GAG content in the cartilage of the operated knee joint was also significantly higher ($p < 0.05$) than that in the non-operated knee joint.

	Defect [%]	**Cartilage - operated knee [%]**	**Cartilage - non-operated knee [%]**
GAG group	8.8 ± 1.1 *	9.6 ± 1.7 +	5.9 ± 1.7
Control	6.0 ± 1.6	6.8 ± 1.5 +	3.6 ± 1.6

*Table 1. The amount of sulfated GAG as share of the total weight of the regenerated tissue in the osteochondral lesions (mean ± SEM, * p< 0,001, +p < 0,05)*

The amount of synovial fluid was increased in the placebo group while the viscosity of the synovial fluid was significantly enhanced in the GAG group (90.0 ± 2.9 vs. 69.4 ± 4.9 poise, $p < 0.001$). (Fig. 1)

2.2. Development of composite scaffold for cartilage regeneration

We developed the shock dynamic method for biomechanical characterization of cartilage. With regard to the distinct poroelastic and viscoelastic material properties of cartilages, blunt impact response evaluation seems to be the most promising method to obtain comprehensive mechanical data. Simple laboratory

realization, repeatability and sufficient accuracy are the main features of this approach.

The reaction of poroelastic material to static, dynamic and impact loading differs substantially. An example of cartilage biomechanical characteristic comparison obtained by both the proposed impact dynamic method and static loading test using strain rates of 5 mm min–1 and 10 mm min–1 is shown in Fig. 1.

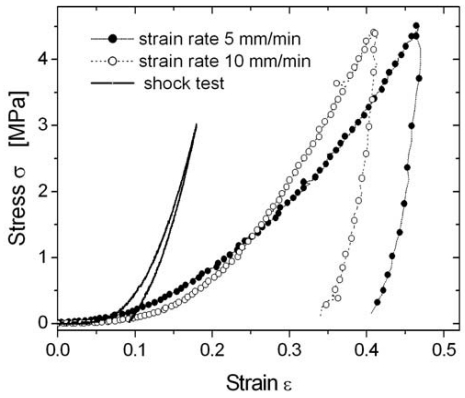

Fig. 1. *Comparison of single pig joint cartilage sample stress-strain curves for static (strain rate 5 mm/min and 10 mm/min) and impact (shock test) loading.*

The nonlinear shape of each loading curve results predominantly from large strains of unconfined compression, as the interstitial fluid flow within the tissue is sufficient for its escape from the material structure at low loading rate.

Healthy joint cartilage response consists of a nearly linear part characterizing elastic matrix, followed by steep nonlinear part caused mainly by viscous fluid. In damaged cartilage, the diagram changes usually occur in favor of linearity due to damaged porous structure.

Four different chondrocyte-seeded hyaluronan-type I collagen-fibrin composite scaffolds displayed excellent viability of chondrocytes (84 % to 97 %) after a 7- and 14-day cultivation. The stiffness of the tested composite matrix (scaffold 1) was significantly higher than that of the fibrin gel (scaffold 4) but also better than that of scaffold 2, or 3 (Fig. 2).

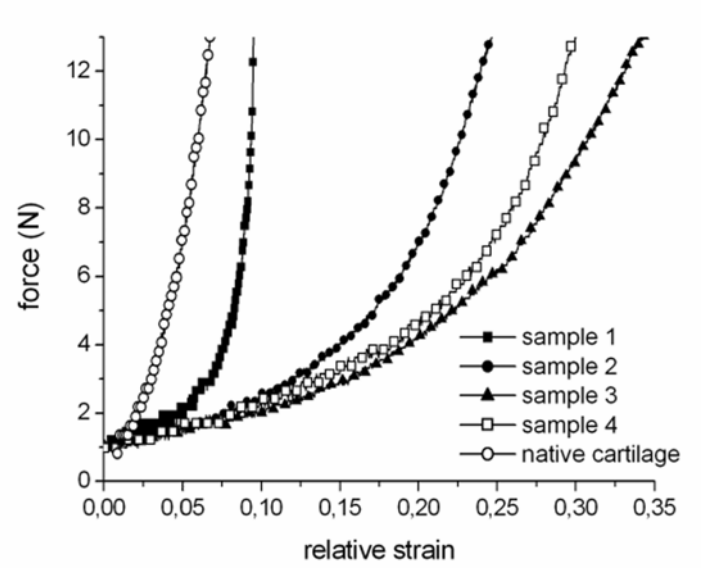

Fig. 2. *Loading diagrams of different composite scaffolds in comparison with native rabbit cartilage. Four different composite scaffolds (six pieces of each) were prepared, using 3 × 10^4 cells per scaffold. Hyaluronan/collagen volume ratio was 12.5/18.8 (sample 1), 16.3/13.6 (sample 2), 8.8/24.2 (sample 3). Sample 4 was prepared from Tissucol and Thrombin solutions without hyaluronan and collagen. Fibrin scaffold was recently used in clinical application (Handl et al. 2006).*

2.3. Regeneration of osteochondral defects in miniature pigs

The composite scaffold 1 with the best biomechanical properties was subsequently implanted in osteochondral defects in miniature pigs.

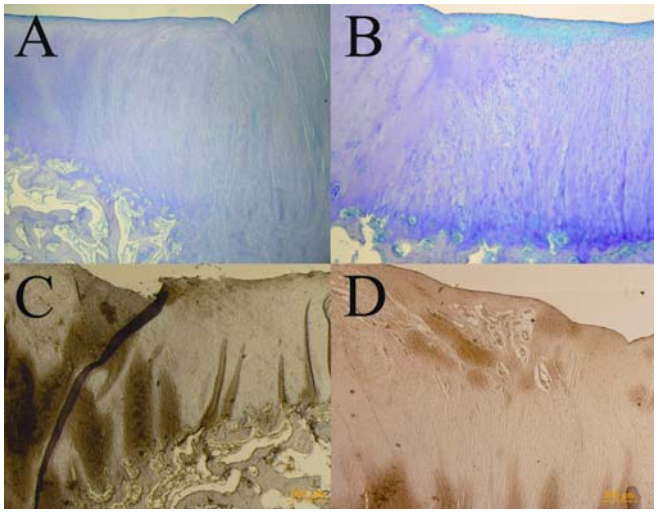

Fig. 3. *Histology of osteochondral defects A of the chondrocyte-seeded scaffold group (A, C) and non-seeded group (B, D) after 24 weeks using Alcian blue staining and PAS reaction at pH 2.5 (A, B), and immunohistochemical staining using monoclonal antibody against type II collagen (C, D). Magnification × 100 (B), × 40 (A, C, D).*

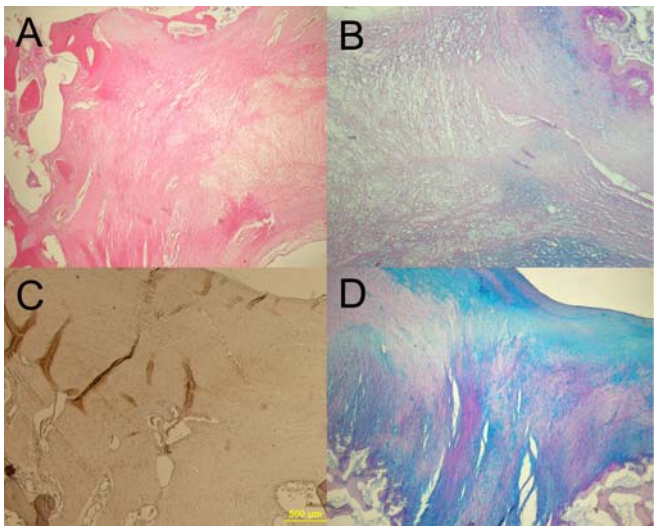

Fig. 4. *Histology of osteochondral defects of the control group after 24 weeks using HE staining (A), Alcian blue staining and PAS reaction at pH 2.5 (B, D), and immunohistochemical staining using monoclonal antibody against type II collagen (C) which showed chaotic sparse fibrous tissue with negative both GAG and type II collagen staining (A,B,C). Magnification × 40 (A, C, D), × 100 (B).*

In the A defects of chondrocyte-seeded scaffold group, hyaline cartilage and fibrocartilage was formed, containing type II collagen, acidic and neutral glycosaminoglycans while the non-seeded scaffold group was predominantly filled with fibrocartilage (Fig. 3). Defects in the control group were predominantly filled with fibrous tissue (Fig. 4).

Histomorphometric analysis of photomicrographs revealed a significantly higher amount of hyaline cartilage in the cell-seeded scaffold group in A defects than in other groups. Both scaffold groups in A defects showed significantly less fibrous tissue than cell-seeded defects B and the control group.

2.4. Regeneration of physeal defects in rabbits

Prophylactic transplantation of autogenous mesenchymal stem cells to iatrogenically damaged distal growth plate of rabbit femur prevented bone bridge formation and resulted in healing of the physeal defect with hyaline cartilage (Fig. 5). Immunofluorescence examination showed that the chondrocytes newly formed in the growth zone are the result of implanted MSCs differentiation.

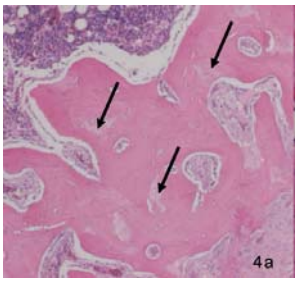

Fig.5. *Histological examination of the distal femoral physis in rabbits after autogenous MSCs transplantation (HE stain, magnification × 40), arrows point to isolated cartilage islets in the defect of growth cartilage.*

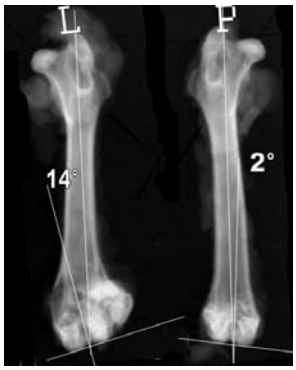

Fig. 6 *Valgus deformity of the distal section of the left (L) femur (without transplantation of MSCs into the growth zone defect) and the right (P) femur in rabbit. Measured from X-ray photographs of femurs in CC projection after euthanasia (4 months after transplantation of MSCs).*

Femur growth in traumatized physis was maintained even after transplantation of autogenous MSCs (Fig. 6). As compared with the opposite femur (with physeal defect but without transplanted MSCs), the bone showed no significant shortening or valgus deformity ($p = 0.018$).

3. Discussion

3.1. Regeneration of cartilage using non-invasive methods

Proteoglycans form a viscoelastic layer which lubricates and protects the cartilage surface (Buckwalter and Mankin 1998a). Changes of both structure and chemical composition of glycosaminoglycans observed in OA result in reduced biomechanical properties of the cartilage (O'Driscoll 1998). Proteoglycans, as well as other substances, have exhibited a potential chondroprotective effect in the conservative treatment of OA (McAlindon *et al.* 2000, Reginster *et al.* 2001, Raynauld *et al.* 2002, Towheed 2002). In clinical studies, the peroral supplementation of GAG could improve clinical symptoms and radiological parameters in patients with cartilage damage or OA (Reginster *et al.* 2001, Pavelka *et al.* 2002). However, no link between peroral supplementation of these drugs and structural changes in cartilage was demonstrated.

Peroral supplementation of a mixture of GAGs, enzymically extracted from fish cartilage to a size between 1 500 and 2 500 Da, and antioxidants (vitamin E and selenium) used in our study improved healing of osteochondral defects in many respects. Regenerated fibrocartilage displayed a significantly higher amount of sulfated GAGs. However, the surrounding tissue of both tested and control groups contained a significantly higher amount of total GAGs, which was probably caused by activation of chondrocytes by cytokines released as a consequence of the operation. In addition, the viscosity of the synovial fluid was increased in the operated joint. In accordance with a previous observation, peroral supplementation of GAGs reduced the effusion in the GAG-treated joints. This may be explained by the antiinflammatory potential of GAGs (McAlindon *et al.* 2000).

3.2. Articular cartilage regeneration using autologous chondrocytes

The operative treatment of cartilage lesions and OA depends on the degree of cartilage damage, and the age of the patient. Although the old patient can profit from joint replacement, the limited life of the prosthesis does not allow to use this technique in younger patients (Sharkey *et al.* 2002). Standard surgical techniques, such as debridement, soft-tissue grafts or penetration of the subchondral bone can improve joint function but they are not able to restore normal articular cartilage (Buckwalter and Lohmander 1994, Levy *et al.* 1996, Buckwalter and Mankin 1998b). Therefore, the cell-based therapy of chondral and osteochondral lesions was developed.

In our study on miniature pigs we used autologous chondrocytes from non-weight-bearing cartilage. Autologous chondrocytes have been used in ACI and MACI techniques with good results (Brittberg *et al.* 1994, Minas 2001, Marlovits *et al.* 2006). However, multiplication of chondrocytes in older donors or patients with OA may be difficult because their chondrocytes were reported to have a low proliferative capacity and exhibited the signs of degeneration.

The formulation of the ideal scaffold should respect chemical, biological and biomechanical properties of the cartilage. This difficult task has not been accomplished yet. There may be many causes for this: cell proliferation is limited by low diffusion of the nutrients through the scaffold; the nutrition depends on the scaffold structure, thickness, porosity, pore size and interconnectivity, and the conditions of cultivation.

Fibrin clot plays an important role in wound healing. It serves as a provisional matrix for migration of many cell types, such as monocytes, fibroblasts and endothelial cells into the matrix. Fibrin clot serves as a reservoir of growth factors that regulate cell functions (Clark 2001). *In vitro*, the advantage of using fibrin and other hydrogels is the homogeneous cell seeding during scaffold preparation. The fibrin gels produced from patients promoted ECM matrix synthesis as a consequence of releasing TGF-β and PDGF; the newly synthesized matrix is accumulated in the original matrix (Jockenhoevel *et al.* 2001). Similarly, the homologous fibrin gel prepared from rabbit and human blood supported cell ingrowth but, when fibrin gel from commercially available Tisseel® was used, no cell

ingrowth was observed (Brittberg *et al.* 1997). Another advantage is that the fibrinogen/thrombin concentration was able to control gel rigidity that was critical for the viability of human MSCs (Bensaid *et al.* 2003). Moreover, fibrin gel can be prepared *in situ* in a desired shape, and it serves as glue which does not require further suturing.

The disadvantages of fibrin include its inapropriate mechanical properties, such as low viscoelasticity and stiffness, rather quick shrinkage and degradation of the gel (Jockenhoevel *et al.* 2001, Bensaid *et al.* 2003). However, the shrinkage can be reduced by chemical or mechanical fixation. The mechanical fixation of the gel using poly-L-lysine induced collagen synthesis and improved the mechanical properties of the fibrin scaffold (Jockenhoevel *et al.* 2001). The other means of improving the mechanical properties include preparation of the composite scaffold. For example, the viscoelastic behavior of our composite fibrin scaffold was positively influenced by high-molar-mass hyaluronic acid, and the stiffness was increased by the content of collagen.

The desired scaffold should possess mechanical properties similar to native cartilage. Small diameter and thickness, low material stiffness as well as highly nonlinear and strain-rate-dependent properties of the scaffolds tested should be taken into account for using the appropriate testing method. Cartilage mechanical properties have already been evaluated by both static and dynamic tests (Repo and Finlay 1977, Kerin *et al.* 1998, LeRoux *et al.* 1999, Duda *et al.* 2000). In a static test, the sample is continuously being adapted to applied stress, while in the dynamic test, the strain rate is too fast, and adaptation to applied stress is not possible. Under physiological conditions, an impact loading is applied on the joint. Therefore, we developed a novel shock dynamic method.

The poroelasticity and viscoelasticity are complex nonlinear properties that cannot be described by a simple characteristic value, e.g. Young's modulus and Poisson's ratio. However, loading diagrams, such as stress-strain or force-strain diagrams appear to be suitable for characterization of poro- and visco-elastic properites of the cartilage or our scaffolds. The nearly linear part of the stress-strain diagram corresponds to the elastic properties of the matrix, and the following nonlinear part corresponds to viscous fluid (Mow and Guo 2002). However, the nonlinear shape of the dynamic loading curve results predominantly from large strains of unconfined compression because the interstitial fluid within the tissue is

not able to escape at these high loading rates. The loading diagram of pure fibrin glue exhibited a more linear curve compared with both pig cartilage and woven PGA scaffold due to the different structure of fibrin and the lack of viscous fluid inside the fibrin gel. The loading diagrams of both native pig cartilage and the composite scaffold had a similar nonlinear course that indicated outstanding viscoelastic properties of the tested materials as well as dynamic response resembling the properties of native cartilage.

It has been reported that pure fibrin sealant Tisseel® implanted into osteochondral defects in rabbits did not allow any cell migration into the gel after eight days. After four months, only incomplete irregular fibrous or fibrocartilaginous tissue formation were observed. This finding is in agreement with *in vitro* tests (Brittberg *et al.* 1997). The implanted fibrin sealant probably created a strong hemostatic barrier that prevented cell migration from the endogenous fibrin clot formed in the base of the osteochondral defect. The commercially available products Tisseel® and Tissucol® contain a twenty-fold higher concentration of fibrinogen and a fifty-fold higher concentration of factor XIII, a fibrin cross-linking factor, compared to plasma. This probably led to the formation of fibrin mesh of a structure differing from normal fibrin clot (Brittberg *et al.* 1997).

Heterologous chondrocyte-seeded fibrin implanted into the ostechochondral defect in the weight-bearing zone was reported to repair the defect to a limited extent. After 13, 26, and 52 weeks post implantation, the fibrocartilage was formed in both chondrocyte-seeeded and non-treated control groups. Moreover, in the chondrocyte-seeded group, many dead cells were observed in the central part of the defects. This was accompanied by the migration of leukocyte-like inflammatory cells into the progressively degraded fibrin (van Susante *et al.* 1999).

Composite scaffolds prepared from two or more biopolymers are supposed to utilize the benefits of both components. Biocompatible, biodegradable polymers, such as collagen, alginate, fibrin or hyaluronic acid, support cell attachment, proliferation or control cell differentiation (Chang *et al.* 2003, Park *et al.* 2004). Hyaluronic acid as a natural component of the cartilage plays the most important role in chondrocyte differentiation (Park, S. H. *et al.* 2005, Yoo, H. S. *et al.* 2005).

Our composite scaffold, composed of hyaluronan, type I collagen and fibrin, led to hyaline and fibrocartilaginous cartilage formation in the autologous chondrocyte-seeded group. We suppose that the superior biomechanical properties of

our scaffold were essential to resist the dynamic loading during movement. This prevented the damage of the cells inside the scaffold, supported their survival, proliferation and cartilage formation, and improved healing compared to pure fibrin scaffolds used by the authors mentioned above (Brittberg *et al.* 1997, van Susante *et al.* 1999). The biomechanical properties of the artificial matrices can be improved by synthetic components, e.g. PLGA copolymer with well defined structure, physical and chemical properties (Chen *et al.* 2003).

3.3. Physeal cartilage regeneration using autologous mesenchymal stem cells

The injuries of long bones are very often associated with damage of physis, such as distal physis of ulna or distal physis of femur. Physes do not heal spontaneously and may result in the formation of a bony bridge at the site of injury. If the bony bridge takes 7 to 9 % of the physis area, a shortening and/or deformity of the bone is observed (Gál *et al.* 2002). Clinical therapy of physeal injuries has been targeted as therapy of bone shortnening and deformity (Lee *et al.* 1993). In our study we used a distal femoral physis defect as a model, because the distal femoral physis is critical for longitudinal growth of the femur. The second reason was that the injuries of the distal femur were more frequent than in the proximal femur (Maretta and Schrader 1983, Mann and Rajmaira 1990).

Recently, a new approach to the prevention of bony bridge formation has been found. It includes an implantation of autologous chondrocytes, MSCs or perichondrial cells either in suspension or in a three-dimensional scaffold. Suspension of chondrocytes injected into a partial physeal defect of proximal epiphyseal plate of tibia (50 % of physis) leaked out from the defect and growth arrest was observed. On the other hand, chondrocytes embedded in agarose gel prevented the bony bridge formation and growth arrest. Moreover, they were able to regenerate physeal cartilage even if the bony bridge had been formed before transplantation (Lee *et al.* 1998). Gál *et al.* (2002) reported that articular chondrocyte-seeded fibrin graft prevented the formation of bony bridge. In addition, perichondrium-derived chondrocytes embedded in alginate-fibrin beads were able to partially restore the growth plate (Yoo, W. J. *et al.* 2005).

In our study MSCs were used for the repair of physeal cartilage. Adult MSCs can differentiate into the chondrocytes and thus have a therapeutical potential for the cartilage and growth plate repair. They can be obtained from bone marrow without destroying the healthy cartilage. In addition, the ethical, moral and legal issues associated with the use of embryonic stem cells are avoided. The chondrogenic stimulation of the MSCs and their seeding on the appropriate scaffold are the key factors in cartilage regeneration. Ahn *et al.* (2004) reported that after the embedding MSCs in different gelatin scaffolds, significant physis repair was observed only in the group of Gelfoam scaffold, probably due to the leakage of the cells from the scaffolds made from 5 and 10 % gelatin.

In our study on rabbits, MSCs were embedded into composite hyaluronan-type I collagen-fibrin scaffold and implanted into the distal growth plate of the femur by arthroscopic instruments without destroying the scaffold. After four months, the structure and morphology of regenerated physis resembled both hyaline cartilage and fibrocartilage. Morevoer, the regenerated physeal cartilage prevented the bony bridge formation and valgus deformation of the femur. Before the implantation, vimentin in MSCs was stained with a lipophilic fluorescent dye. The staining was present in physeal chondrocyte-like cells even 4 months after the implantation. The use of MSCs in the therapy of physis trauma is promising for young patients, because it prevents the closure of long bone growth plates causing both bone shorterning and deformities.

4. Conclusions

Peroral supplementation of a mixture of both GAGs and radical scavengers (vitamin E and selenium) resulted in higher amount of sulfated GAGs in the repaired tissue of the osteochondral defect of rabbit compared to the contralateral joint. This was accompanied by the increased viscosity of the synovial fluid in the treated joint. The combination of GAGs and radical scavengers can be used as supporting therapy in the treatment of osteochondral defects.

The viscoelastic properties of the cartilage cannot be characterized by a simple value of Young´s modulus or Poisson´s ratio. We introduced the novel dynamic shock method to characterize highly nonlinear and strain-rate-dependent properties of cartilage. The advantages of the method include measurement of samples of small size and thickness as well as fast and reproducible measurement of complex nonlinear characteristics of the sample during a simple measurement. This method was used to characterize newly developed scaffolds. The biomechanical properties of fibrin gel were far from the native cartilage. We have developed composite type I collagen-hyaluronan-fibrin scaffold which showed a similar course of loading diagram as native cartilage. This indicates viscoelastic properties similar to those of native cartilage. This scaffold has been subsequently used in tests *in vivo.*

Chondrocytes embedded in a three-dimensional hydrogel scaffold maintain their differentiated phenotype or even re-differentiate. Autologous chondrocyte-seeded fibrin scaffold implanted into osteochondral defects of goat resulted in incomplete regeneration of the defect. The autologous chondrocyte-seeded type I collagen-hyaluronan-fibrin scaffold, characterized by improved biomechanical properties, implanted into the osteochondral defects in miniature pigs, resulted in improved healing of the defects and hyaline cartilage or fibrocartilage formation while the non-seeded composite scaffold led predominantly to fibrocartilage formation. Moreover, no shrinkage was observed probably due to better viscous properties of the sample.

MSCs isolated from bone marrow have shown the potential to differentiate into many cell types including cartilage and bone under specific culture conditions. In our study, autologous MSC-seeded composite type I collagen-hyaluronan-fibrin

scaffold implanted into iatrogenic physeal defect in lateral portion of distal growth plate prevented bone bridge formation and resulted in healing of physeal defect with both hyaline cartilage and fibrocartilage. This was not accompanied by any significant valgus deformity and shortening of the bone. The therapy of physeal defects using MSC-seeded composite scaffold can avoid surgical intervention in the treatment of consequences of physeal closure, such as bone deformities and shortening of bone.

5. Reprints of the papers published by the author

M. Handl, E. Amler, K. Bräun, J. Holzheu, T. Trč, A. B. Imhoff, A. Lytvynets, E. Filová, H. Kolářová, A. Kotyk, V. Martínek: Positive Effect of Oral Supplementation with Glycosaminoglycans and Antioxidants on the Regeneration of Osteochondral Defects in the Knee Joint. Physiol. Res. 56: 243-249, 2007, IF = 2.093(06)

E. Filová, M. Rampichová, M. Handl, A. Lytvynets, R. Halouzka, D. Usvald, J. Hlucilová, R. Procházka, M. Dezortová, E. Rolencová, E. Košťáková, T. Trč, E. Šťastný, L. Koláčná, M. Hájek, J. Motlík, E. Amler: Composite Hyaluronate-type I Collagen-Fibrin Scaffold in the Therapy of Osteochondral Defects in Miniature Pigs. Physiol. Res. 2007; 56 (Suppl. 1): S5-S16, IF = 2.093(06)

F. Varga, M. Držík, M. Handl, J. Chlpík, P. Kos, E. Filová, M. Rampichová, T. Trč, E. Amler: Biomechanical Characterization of Cartilages by a Novel Approach of Blunt Impact Testing. Physiol. Res. 2007; 56 (Suppl. 1): S61-S68, IF = 2.093(06)

Plánka L., A. Nečas, P. Gál, H. Kecová, E. Filová, L. Křen, P. Krupa: Prevention of Bone Bridge Formation using Transplantation of the Autogenous Mesenchymal Stem Cells to Physeal Defects: An Experimental Study in Rabbits. Acta Vet Brno 2007, 76: 253-63, IF = 0.491(06)

Article not related to the topic

M. Handl, E. Filová, M. Kubala, Z. Lánský, L. Koláčná, J. Vorlíček, T. Trč, M. Pach, E. Amler: Fluorescent Advanced Glycation End Products in the Detection of Factual Stages of Cartilage Degeneration. Physiol. Res. 2007; 56: 235-242, IF = 2.093(06)

6. References

ADAM N, GHOSH P: Hyaluronan molecular weight and polydispersity in some commercial intra-articular injectable preparations and in synovial fluid. *Inflamm Res* **50**: 294-299, 2001.

AHN JI, TERRY CANALE S, BUTLER SD, HASTY KA: Stem cell repair of physeal cartilage. *J Orthop Res* **22**: 1215-1221, 2004.

AKAOGI H, AKIMOTO T, MIYAKI S, USHIDA T, OCHIAI N, TATEISHI T, TANAKA J: Basic fibroblast growth factor supports in vitro chondrogenesis of bone marrow-derived mesenchymal stem cells from patients with osteoarthritis. *Material science and engineering C* **24**: 403-406, 2004.

ALSALAMEH S, AMIN R, GEMBA T, LOTZ M: Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. *Arthritis Rheum* **50**: 1522-1532, 2004.

ANDRADE AC, NILSSON O, BARNES KM, BARON J: Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation. *Bone* **40**: 1361-1369, 2007.

APLIN AE, HOWE A, ALAHARI SK, JULIANO RL: Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev* **50**: 197-263, 1998.

ASHCRAFT KW, HOLCOMB GW, MURPHY JP: *Pediatric surgery*.Saunders, W.B., Philadelphia, 2005, 229-231.

ATESHIAN GA, WARDEN WH, KIM JJ, GRELSAMER RP, MOW VC: Finite deformation biphasic material properties of bovine articular cartilage from confined compression experiments. *J Biomech* **30**: 1157-1164, 1997.

BANK RA, SOUDRY M, MAROUDAS A, MIZRAHI J, TEKOPPELE JM: The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. *Arthritis Rheum* **43**: 2202-2210, 2000.

BENSAID W, TRIFFITT JT, BLANCHAT C, OUDINA K, SEDEL L, PETITE H: A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. *Biomaterials* **24**: 2497-2502, 2003.

BENSE CA, WOODHOUSE KA: Plasmin degradation of fibrin coatings on synthetic polymer substrates. *J Biomed Mater Res* **46**: 305-314, 1999.

BENYA PD, SHAFFER JD: Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**: 215-224, 1982.

BOYAN BD, HUMMERT TW, DEAN DD, SCHWARTZ Z: Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials* **17**: 137-146, 1996.

BREINAN HA, MINAS T, HSU HP, NEHRER S, SHORTKROFF S, SPECTOR M: Autologous chondrocyte implantation in a canine model: change in composition of reparative tissue with time. *J Orthop Res* **19**: 482-492, 2001.

BREINAN HA, MINAS T, HSU HP, NEHRER S, SLEDGE CB, SPECTOR M: Effect of cultured autologous chondrocytes on repair of chondral defects in a canine model. *J Bone Joint Surg Am* **79**: 1439-1451, 1997.

BRITTBERG M, LINDAHL A, NILSSON A, OHLSSON C, ISAKSSON O, PETERSON L: Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* **331**: 889-895, 1994.

BRITTBERG M, SJOGREN-JANSSON E, LINDAHL A, PETERSON L: Influence of fibrin sealant (Tisseel) on osteochondral defect repair in the rabbit knee. *Biomaterials* **18**: 235-242, 1997.

BRYANT SJ, ANSETH KS: The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels. *Biomaterials* **22**: 619-626, 2001.

BRYANT SJ, ANSETH KS: Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels. *J Biomed Mater Res* **59**: 63-72, 2002.

BUCKWALTER JA, LOHMANDER S: Operative treatment of osteoarthrosis. Current practice and future development. *J Bone Joint Surg Am* **76**: 1405-1418, 1994.

BUCKWALTER JA, MANKIN HJ: Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr Course Lect* **47**: 477-486, 1998a.

BUCKWALTER JA, MANKIN HJ: Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. *Instr Course Lect* **47**: 487-504, 1998b.

BUENO EM, BILGEN B, BARABINO GA: Wavy-walled bioreactor supports increased cell proliferation and matrix deposition in engineered cartilage constructs. *Tissue Eng* **11**: 1699-1709, 2005.

BYERS PD, BROWN RA: Cell columns in articular cartilage physes questioned: a review. *Osteoarthritis Cartilage* **14**: 3-12, 2006.

CARVER SE, HEATH CA: Influence of intermittent pressure, fluid flow, and mixing on the regenerative properties of articular chondrocytes. *Biotechnol Bioeng* **65**: 274-281, 1999.

CATERSON B, HUGHES CE, JOHNSTONE B, MORT JS (1992). Immunological markers of cartilage proteoglycan metabolism in animal and human osteoarthritis. Articular cartilage and osteoarthritis. K. Kuettner. New York, Raven Press, Ltd.**:** 415-428.

CATERSON EJ, NESTI LJ, LI WJ, DANIELSON KG, ALBERT TJ, VACCARO AR, TUAN RS: Three-dimensional cartilage formation by bone marrow-derived cells seeded in polylactide/alginate amalgam. *J Biomed Mater Res* **57**: 394-403, 2001.

CHANG CH, LIU HC, LIN CC, CHOU CH, LIN FH: Gelatin-chondroitin-hyaluronan tri-copolymer scaffold for cartilage tissue engineering. *Biomaterials* **24**: 4853-4858, 2003.

CHEN CW, TSAI YH, DENG WP, SHIH SN, FANG CL, BURCH JG, CHEN WH, LAI WF: Type I and type II collagen regulation of chondrogenic differentiation by mesenchymal progenitor cells. *J Orthop Res* **23**: 446-453, 2005.

CHEN G, SATO T, USHIDA T, HIROCHIKA R, SHIRASAKI Y, OCHIAI N, TATEISHI T: The use of a novel PLGA fiber/collagen composite web as a scaffold for engineering of articular cartilage tissue with adjustable thickness. *J Biomed Mater Res A* **67**: 1170-1180, 2003.

CHERUBINO P, GRASSI FA, BULGHERONI P, RONGA M: Autologous chondrocyte implantation using a bilayer collagen membrane: a preliminary report. *J Orthop Surg (Hong Kong)* **11**: 10-15, 2003.CLARK P, CONNOLLY P, CURTIS AS, DOW JA, WILKINSON CD: Topographical control of cell behaviour: II. Multiple grooved substrata. *Development* **108**: 635-644, 1990.

CLARK RA: Fibrin and wound healing. *Ann N Y Acad Sci* **936**: 355-367, 2001.

D'LIMA DD, LEMPERLE SM, CHEN PC, HOLMES RE, COLWELL CW, JR.: Bone response to implant surface morphology. *J Arthroplasty* **13**: 928-934, 1998.

DARLING EM, ATHANASIOU KA: Articular cartilage bioreactors and bioprocesses. *Tissue Eng* **9**: 9-26, 2003.

DARLING EM, ATHANASIOU KA: Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J Orthop Res* **23**: 425-432, 2005.

DE HAART M, MARIJNISSEN WJ, VAN OSCH GJ, VERHAAR JA: Optimization of chondrocyte expansion in culture. Effect of TGF beta-2, bFGF and L-ascorbic acid on bovine articular chondrocytes. *Acta Orthop Scand* **70**: 55-61, 1999.

DOROTKA R, BINDREITER U, MACFELDA K, WINDBERGER U, NEHRER S: Marrow stimulation and chondrocyte transplantation using a collagen matrix for cartilage repair. *Osteoarthritis Cartilage* **13**: 655-664, 2005a.

DOROTKA R, WINDBERGER U, MACFELDA K, BINDREITER U, TOMA C, NEHRER S: Repair of articular cartilage defects treated by microfracture and a three-dimensional collagen matrix. *Biomaterials* **26**: 3617-3629, 2005b.

DRURY JL, MOONEY DJ: Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* **24**: 4337-4351, 2003.

DUDA GN, HAISCH A, ENDRES M, GEBERT C, SCHROEDER D, HOFFMANN JE, SITTINGER M: Mechanical quality of tissue engineered cartilage: results after 6 and 12 weeks in vivo. *J Biomed Mater Res* **53**: 673-677, 2000.

EID K, THORNHILL TS, GLOWACKI J: Chondrocyte gene expression in osteoarthritis: Correlation with disease severity. *J Orthop Res* **24**: 1062-1068, 2006.

ENOMOTO M, LEBOY PS, MENKO AS, BOETTIGER D: Beta 1 integrins mediate chondrocyte interaction with type I collagen, type II collagen, and fibronectin. *Exp Cell Res* **205**: 276-285, 1993.

ERGGELET C, SITTINGER M, LAHM A: The arthroscopic implantation of autologous chondrocytes for the treatment of full-thickness cartilage defects of the knee joint. *Arthroscopy* **19**: 108-110, 2003.

EYRE DR, WU J-J, WOODS P (1992). Cartilage-Specific collagens. Structural studies. Articular Cartilage and Osteoarthritis. K. Kuettner. New York, Raven Press, Ltd**:** 119-131

FURUKAWA T, EYRE DR, KOIDE S, GLIMCHER MJ: Biochemical studies on repair cartilage resurfacing experimental defects in the rabbit knee. *J Bone Joint Surg Am* **62**: 79-89, 1980.

GÁL P, NEČAS A, ADLER J, TEYSCHL O, FABIÁN P, BIBROVÁ Š: Transplantation of the autogenous chondrocyte graft to physeal defects: an experimental study in pigs *Acta Vet Brno* **71**: 327-332, 2002.

HEGEWALD AA, RINGE J, BARTEL J, KRUGER I, NOTTER M, BARNEWITZ D, KAPS C, SITTINGER M: Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study. *Tissue Cell* **36**: 431-438, 2004.

HELMINEN HJ, KIVIRANTA I, SÄÄMÄNEN A-M, JURVELIN JS, AROKOSKI J, OETTMEIER R, ABENDROTH K, ROTH AJ, TAMMI IM (1992). Effect of Motion and Load on Articular Cartilage in Animal Models. Articular cartilage and osteoarthritis. K. Kuettner. New York, Raven Press, Ltd**:** 501-510.

HENCH LL, POLAK JM: Third-generation biomedical materials. *Science* **295**: 1014-1017, 2002.

HOIKKA VE, JAROMA HJ, RITSILA VA: Reconstruction of the patellar articulation with periosteal grafts. 4-year follow-up of 13 cases. *Acta Orthop Scand* **61**: 36-39, 1990.

HU W-J, EATON JW, TANG L: Molecular basis of biomaterial-mediated foreign body reactions. *Blood* **98**: 1231-1238, 2001.

HUANG JI, KAZMI N, DURBHAKULA MM, HERING TM, YOO JU, JOHNSTONE B: Chondrogenic potential of progenitor cells derived from human bone marrow and adipose tissue: a patient-matched comparison. *J Orthop Res* **23**: 1383-1389, 2005.

IMABAYASHI H, MORI T, GOJO S, KIYONO T, SUGIYAMA T, IRIE R, ISOGAI T, HATA J, TOYAMA Y, UMEZAWA A: Redifferentiation of dedifferentiated chondrocytes and chondrogenesis of human bone marrow stromal cells via chondrosphere formation with expression profiling by large-scale cDNA analysis. *Exp Cell Res* **288**: 35-50, 2003.

INDRAWATTANA N, CHEN G, TADOKORO M, SHANN LH, OHGUSHI H, TATEISHI T, TANAKA J, BUNYARATVEJ A: Growth factor combination

for chondrogenic induction from human mesenchymal stem cell. *Biochem Biophys Res Commun* **320**: 914-919, 2004.

JOCKENHOEVEL S, ZUND G, HOERSTRUP SP, CHALABI K, SACHWEH JS, DEMIRCAN L, MESSMER BJ, TURINA M: Fibrin gel -- advantages of a new scaffold in cardiovascular tissue engineering. *Eur J Cardiothorac Surg* **19**: 424-430, 2001.

JOHNSTONE B, HERING TM, CAPLAN AI, GOLDBERG VM, YOO JU: In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* **238**: 265-272, 1998.

JUNQUEIRA LC, CARNEIRO J, O. KELLEY R (1999). Chrupavka. Základy histologie, Nakladatelství a vydavatelství H&H: 124-132

KAO WJ, MCNALLY AK, HILTNER A, ANDERSON JM: Role for interleukin-4 in foreign-body giant cell formation on a poly(etherurethane urea) in vivo. *J Biomed Mater Res* **29**: 1267-1275, 1995.

KARAGEORGIOU V, KAPLAN D: Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials* **26**: 5474-5491, 2005.

KAY S, THAPA A, HABERSTROH KM, WEBSTER TJ: Nanostructured polymer/nanophase ceramic composites enhance osteoblast and chondrocyte adhesion. *Tissue Eng* **8**: 753-761, 2002.

KERIN AJ, WISNOM MR, ADAMS MA: The compressive strength of articular cartilage. *Proc Inst Mech Eng [H]* **212**: 273-280, 1998.

KING SR, HICKERSON WL, PROCTOR KG: Beneficial actions of exogenous hyaluronic acid on wound healing. *Surgery* **109**: 76-84, 1991.

KOBAYASHI M, TOGUCHIDA J, OKA M: Preliminary study of polyvinyl alcohol-hydrogel (PVA-H) artificial meniscus. *Biomaterials* **24**: 639-647, 2003.

KOTOBUKI N, IOKU K, KAWAGOE D, FUJIMORI H, GOTO S, OHGUSHI H: Observation of osteogenic differentiation cascade of living mesenchymal stem cells on transparent hydroxyapatite ceramics. *Biomaterials* **26**: 779-785, 2005.

KURIWAKA M, OCHI M, UCHIO Y, MANIWA S, ADACHI N, MORI R, KAWASAKI K, KATAOKA H: Optimum combination of monolayer and

three-dimensional cultures for cartilage-like tissue engineering. *Tissue Eng* **9**: 41-49, 2003.

LAURENT TC, FRASER JR: Hyaluronan. *Faseb J* **6**: 2397-2404, 1992.

LEE EH, GAO GX, BOSE K: Management of partial growth arrest: physis, fat, or silastic? *J Pediatr Orthop* **13**: 368-372, 1993.

LEE EH, CHEN F, CHAN J, BOSE K: Treatment of growth arrest by transfer of cultured chondrocytes into physeal defects. *J Pediatr Orthop* **18**: 155-160, 1998.

LEE GM, LOESER RF: Interactions of the chondrocyte within its pericellular matrix. *Cells and Materials* **8**: 135-149, 1998.

LEE K, MAJUMDAR MK, BUYANER D, HENDRICKS JK, PITTENGER MF, MOSCA JD: Human mesenchymal stem cells maintain transgene expression during expansion and differentiation. *Mol Ther* **3**: 857-866, 2001.

LEROUX MA, GUILAK F, SETTON LA: Compressive and shear properties of alginate gel: effects of sodium ions and alginate concentration. *J Biomed Mater Res* **47**: 46-53, 1999.

LEVY AS, LOHNES J, SCULLEY S, LECROY M, GARRETT W: Chondral delamination of the knee in soccer players. *Am J Sports Med* **24**: 634-639, 1996.

LU L, ZHU X, PEDERSON LG, JABBARI E, CURRIER B, O'DRISCOLL S, YASZEMSKI M: Effects of dynamic fluid pressure on chondrocytes cultured in biodegradable poly(glycolic acid) fibrous scaffolds. *Tissue Eng* **11**: 1852-1859, 2005.

MANDL EW, VAN DER VEEN SW, VERHAAR JA, VAN OSCH GJ: Serum-free medium supplemented with high-concentration FGF2 for cell expansion culture of human ear chondrocytes promotes redifferentiation capacity. *Tissue Eng* **8**: 573-580, 2002.

MANIWA S, OCHI M, MOTOMURA T, NISHIKORI T, CHEN J, NAORA H: Effects of hyaluronic acid and basic fibroblast growth factor on motility of chondrocytes and synovial cells in culture. *Acta Orthop Scand* **72**: 299-303, 2001.

MANN DC, RAJMAIRA S: Distribution of physeal and nonphyseal fractures in 2,650 long-bone fractures in children aged 0-16 years. *J Pediatr Orthop* **10**: 713-716, 1990.

MARETTA SM, SCHRADER SC: Physeal injuries in the dog: A review of 135 cases. *J Am Vet Med Assoc* **182**: 708-710, 1983.

MARLOVITS S, STRIESSNIG G, KUTSCHA-LISSBERG F, RESINGER C, ALDRIAN SM, VECSEI V, TRATTNIG S: Early postoperative adherence of matrix-induced autologous chondrocyte implantation for the treatment of full-thickness cartilage defects of the femoral condyle. *Knee Surg Sports Traumatol Arthrosc* **13**: 451-457, 2005.

MARLOVITS S, ZELLER P, SINGER P, RESINGER C, VECSEI V: Cartilage repair: generations of autologous chondrocyte transplantation. *Eur J Radiol* **57**: 24-31, 2006.

MCALINDON TE, LAVALLEY MP, GULIN JP, FELSON DT: Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. *Jama* **283**: 1469-1475, 2000.

MINAS T: Autologous chondrocyte implantation for focal chondral defects of the knee. *Clin Orthop Relat Res*: S349-361, 2001.

MINOURA N, KOYANO T, KOSHIZAKI N, UMEHARA H, NAGURA M, KOBAYASHI K: Preparation, properties, and cell attachment/growth behavior of PVA/chitosan-blended hydrogels *Mater Sci Eng C* **6**: 275-280 1998.

MIOT S, SCANDIUCCI DE FREITAS P, WIRZ D, DANIELS AU, SIMS TJ, HOLLANDER AP, MAINIL-VARLET P, HEBERER M, MARTIN I: Cartilage tissue engineering by expanded goat articular chondrocytes. *J Orthop Res* **24**: 1078-1085, 2006.

MIYAKE K, UNDERHILL CB, LESLEY J, KINCADE PW: Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J Exp Med* **172**: 69-75, 1990.

MIZUTA T, BENSON WM, FOSTER BK, PATERSON DC, MORRIS LL: Statistical analysis of the incidence of physeal injuries. *J Pediatr Orthop* **7**: 518-523, 1987.

MOSESSON MW, SIEBENLIST KR, MEH DA: The structure and biological features of fibrinogen and fibrin. *Ann N Y Acad Sci* **936**: 11-30, 2001.

MOSKOWITZ RW, SUNSHINE A, HOOPER M, OLSON NZ, CAWKWELL GD: An analgesic model for assessment of acute pain response in osteoarthritis of the knee. *Osteoarthritis Cartilage* **14**: 1111-1118, 2006.

MOW VC, GUO XE: Mechano-electrochemical properties of articular cartilage: their inhomogeneities and anisotropies. *Annu Rev Biomed Eng* **4**: 175-209, 2002.

MURPHY JM, DIXON K, BECK S, FABIAN D, FELDMAN A, BARRY F: Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. *Arthritis Rheum* **46**: 704-713, 2002.

NG KW, WANG CC, MAUCK RL, KELLY TA, CHAHINE NO, COSTA KD, ATESHIAN GA, HUNG CT: A layered agarose approach to fabricate depth-dependent inhomogeneity in chondrocyte-seeded constructs. *J Orthop Res* **23**: 134-141, 2005.

NIEDERMANN B, BOE S, LAURITZEN J, RUBAK JM: Glued periosteal grafts in the knee. *Acta Orthop Scand* **56**: 457-460, 1985.

O'BRIEN FJ, HARLEY BA, YANNAS IV, GIBSON LJ: The effect of pore size on cell adhesion in collagen-GAG scaffolds. *Biomaterials* **26**: 433-441, 2005.

O'DRISCOLL SW: The healing and regeneration of articular cartilage. *J Bone Joint Surg Am* **80**: 1795-1812, 1998.

O'DRISCOLL SW, KEELEY FW, SALTER RB: Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion. A follow-up report at one year. *J Bone Joint Surg Am* **70**: 595-606, 1988.

OLNEY RC, WANG J, SYLVESTER JE, MOUGEY EB: Growth factor regulation of human growth plate chondrocyte proliferation in vitro. *Biochem Biophys Res Commun* **317**: 1171-1182, 2004.

OUTERBRIDGE HK, OUTERBRIDGE AR, OUTERBRIDGE RE: The use of a lateral patellar autologous graft for the repair of a large osteochondral defect in the knee. *J Bone Joint Surg Am* **77**: 65-72, 1995.

PARK H, TEMENOFF JS, HOLLAND TA, TABATA Y, MIKOS AG: Delivery of TGF-beta1 and chondrocytes via injectable, biodegradable hydrogels for cartilage tissue engineering applications. *Biomaterials* **26**: 7095-7103, 2005.

PARK JS, AHN JI, OH DI: Chondrocyte allograft transplantation for damaged growth plate reconstruction. *Yonsei Med J* **35**: 378-387, 1994.

PARK SH, PARK SR, CHUNG SI, PAI KS, MIN BH: Tissue-engineered cartilage using fibrin/hyaluronan composite gel and its in vivo implantation. *Artif Organs* **29**: 838-845, 2005.

PARK SN, KIM JK, SUH H: Evaluation of antibiotic-loaded collagen-hyaluronic acid matrix as a skin substitute. *Biomaterials* **25**: 3689-3698, 2004.

PAVELKA K, GATTEROVA J, OLEJAROVA M, MACHACEK S, GIACOVELLI G, ROVATI LC: Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. *Arch Intern Med* **162**: 2113-2123, 2002.

PAVESIO A, ABATANGELO G, BORRIONE A, BROCCHETTA D, HOLLANDER AP, KON E, TORASSO F, ZANASI S, MARCACCI M: Hyaluronan-based scaffolds (Hyalograft C) in the treatment of knee cartilage defects: preliminary clinical findings. *Novartis Found Symp* **249**: 203-217; discussion 229-233, 234-208, 239-241, 2003.

PEI M, SEIDEL J, VUNJAK-NOVAKOVIC G, FREED LE: Growth factors for sequential cellular de- and re-differentiation in tissue engineering. *Biochem Biophys Res Commun* **294**: 149-154, 2002.

PITTENGER MF, MACKAY AM, BECK SC, JAISWAL RK, DOUGLAS R, MOSCA JD, MOORMAN MA, SIMONETTI DW, CRAIG S, MARSHAK DR: Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143-147, 1999.

QUINN TM, HUNZIKER EB, HAUSELMANN HJ: Variation of cell and matrix morphologies in articular cartilage among locations in the adult human knee. *Osteoarthritis Cartilage* **13**: 672-678, 2005.

RADICE M, BRUN P, CORTIVO R, SCAPINELLI R, BATTALIARD C, ABATANGELO G: Hyaluronan-based biopolymers as delivery vehicles for bone-marrow-derived mesenchymal progenitors. *J Biomed Mater Res* **50**: 101-109, 2000.

RAYNAULD JP, TORRANCE GW, BAND PA, GOLDSMITH CH, TUGWELL P, WALKER V, SCHULTZ M, BELLAMY N: A prospective, randomized, pragmatic, health outcomes trial evaluating the incorporation of hylan G-F 20 into the treatment paradigm for patients with knee osteoarthritis (Part 1 of 2): clinical results. *Osteoarthritis Cartilage* **10**: 506-517, 2002.

REGINSTER JY, DEROISY R, ROVATI LC, LEE RL, LEJEUNE E, BRUYERE O, GIACOVELLI G, HENROTIN Y, DACRE JE, GOSSETT C: Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. *Lancet* **357**: 251-256, 2001.

REPO RU, FINLAY JB: Survival of articular cartilage after controlled impact. *J Bone Joint Surg Am* **59**: 1068-1076, 1977.

RINGE J, KAPS C, BURMESTER GR, SITTINGER M: Stem cells for regenerative medicine: advances in the engineering of tissues and organs. *Naturwissenschaften* **89**: 338-351, 2002b.

RINGE J, KAPS C, SCHMITT B, BUSCHER K, BARTEL J, SMOLIAN H, SCHULTZ O, BURMESTER GR, HAUPL T, SITTINGER M: Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. *Cell Tissue Res* **307**: 321-327, 2002a.

ROSS JM, SHERWIN AF, POOLE CA: In vitro culture of enzymatically isolated chondrons: a possible model for the initiation of osteoarthritis. *J Anat* **209**: 793-806, 2006.

ROSSELOT G, VASILATOS-YOUNKEN R, LEACH RM: Effect of growth hormone, insulin-like growth factor I, basic fibroblast growth factor, and transforming growth factor beta on cell proliferation and proteoglycan synthesis by avian postembryonic growth plate chondrocytes. *J Bone Miner Res* **9**: 431-439, 1994.

RUBAK JM: Reconstruction of articular cartilage defects with free periosteal grafts. An experimental study. *Acta Orthop Scand* **53**: 175-180, 1982.

RUBAK JM, POUSSA M, RITSILA V: Chondrogenesis in repair of articular cartilage defects by free periosteal grafts in rabbits. *Acta Orthop Scand* **53**: 181-186, 1982.

RUEL-GARIEPY E, CHENITE A, CHAPUT C, GUIRGUIS S, LEROUX J: Characterization of thermosensitive chitosan gels for the sustained delivery of drugs. *Int J Pharm* **203**: 89-98, 2000.

SAITO N, OKADA T, HORIUCHI H, MURAKAMI N, TAKAHASHI J, NAWATA M, OTA H, NOZAKI K, TAKAOKA K: A biodegradable polymer as a cytokine delivery system for inducing bone formation. *Nat Biotechnol* **19**: 332-335, 2001.

SHAPIRO F, KOIDE S, GLIMCHER MJ: Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. *J Bone Joint Surg Am* **75**: 532-553, 1993.

SHARKEY PF, HOZACK WJ, ROTHMAN RH, SHASTRI S, JACOBY SM: Why are total knee arthroplasties failing today? *Clin Orthop Relat Res* **404**: 7-13, 2002.

SCHMEDLEN RH, MASTERS KS, WEST JL: Photocrosslinkable polyvinyl alcohol hydrogels that can be modified with cell adhesion peptides for use in tissue engineering. *Biomaterials* **23**: 4325-4332, 2002.

SIMON LS: Viscosupplementation therapy with intra-articular hyaluronic acid. Fact or fantasy? *Rheum Dis Clin North Am* **25**: 345-357, 1999.

SMETANA K, JR., VACÍK J, SOUČKOVÁ D, PITROVÁ Š: The influence of chemical functional groups on implant biocompatibility. *Clinical Materials* **13**: 47-49, 1993.

SMETANA K, JR., LUKAS J, PALECKOVA V, BARTUNKOVA J, LIU FT, VACIK J, GABIUS HJ: Effect of chemical structure of hydrogels on the adhesion and phenotypic characteristics of human monocytes such as expression of galectins and other carbohydrate-binding sites. *Biomaterials* **18**: 1009-1014, 1997.

STAMMEN JA, WILLIAMS S, KU DN, GULDBERG RE: Mechanical properties of a novel PVA hydrogel in shear and unconfined compression. *Biomaterials* **22**: 799-806, 2001.

STEADMAN JR, RODKEY WG, SINGLETON SB, BRIGGS KK: Microfracture techniques for full-thickness chondral defects: technique and clinical results *Operative Techniques in Orthopaedics* **7**: 300-304, 1997.

SUN SF, CHOU YJ, HSU CW, HWANG CW, HSU PT, WANG JL, HSU YW, CHOU MC: Efficacy of intra-articular hyaluronic acid in patients with osteoarthritis of the ankle: a prospective study. *Osteoarthritis Cartilage* **14**: 867-874, 2006.

TANG L, EATON JW: Inflammatory responses to biomaterials. *Am J Clin Pathol* **103**: 466-471, 1995.

TOWHEED TE: Published meta-analyses of pharmacological therapies for osteoarthritis. *Osteoarthritis Cartilage* **10**: 836-837, 2002.

TRATTNIG S, BA-SSALAMAH A, PINKER K, PLANK C, VECSEI V, MARLOVITS S: Matrix-based autologous chondrocyte implantation for cartilage repair: noninvasive monitoring by high-resolution magnetic resonance imaging. *Magn Reson Imaging* **23**: 779-787, 2005.

TRATTNIG S, PINKER K, KRESTAN C, PLANK C, MILLINGTON S, MARLOVITS S: Matrix-based autologous chondrocyte implantation for cartilage repair with HyalograftC: two-year follow-up by magnetic resonance imaging. *Eur J Radiol* **57**: 9-15, 2006.

TUAN RS, BOLAND G, TULI R: Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther* **5**: 32-45, 2003.

VACANTI JP, LANGER R, UPTON J, MARLER JJ: Transplantation of cells in matrices for tissue regeneration. *Adv Drug Deliv Rev* **33**: 165-182, 1998.

VAN DEN BERG WB, VAN DER KRAAN PM, SCHARSTUHL A, VAN BEUNINGEN HM: Tissue engineering, cells scaffolds, and growth factors. *Clinical orthopaedics and related research* **392S**: S244-250, 2001.

VAN SUSANTE JL, BUMA P, SCHUMAN L, HOMMINGA GN, VAN DEN BERG WB, VETH RP: Resurfacing potential of heterologous chondrocytes suspended in fibrin glue in large full-thickness defects of femoral articular cartilage: an experimental study in the goat. *Biomaterials* **20**: 1167-1175, 1999.

VIVIEN D, GALÉRA P, LEBRUN E, LOYAU G, PUJOL JP: Differential effects of transforming growth factor-beta and epidermal growth factor on the cell cycle of cultured rabbit articular chondrocytes. *J Cell Physiol* **143**: 534-545, 1990.

VUNJAK-NOVAKOVIC G, MARTIN I, OBRADOVIC B, TREPPO S, GRODZINSKY AJ, LANGER R, FREED LE: Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. *J Orthop Res* **17**: 130-138, 1999.

VUNJAK-NOVAKOVIC G, MARTIN I, OBRADOVIC B, TREPPO S, GRODZINSKY AJ, LANGER R, FREED LE: Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. *J Orthop Res* **17**: 130-138, 1999.

WAKITANI S, GOTO T, PINEDA SJ, YOUNG RG, MANSOUR JM, CAPLAN AI, GOLDBERG VM: Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg Am* **76**: 579-592, 1994.

WAKITANI S, IMOTO K, YAMAMOTO T, SAITO M, MURATA N, YONEDA M: Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. *Osteoarthritis Cartilage* **10**: 199-206, 2002.

WANG CT, LIN YT, CHIANG BL, LIN YH, HOU SM: High molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis. *Osteoarthritis Cartilage* **14**: 1237-1247, 2006.

WANG MC, PINS GD, SILVER FH: Preparation of fibrin glue: the effects of calcium chloride and sodium chloride. *Mater Sci Eng C* **3**: 131-135 1995.

WILLERS C, CHEN J, WOOD D, XU J, ZHENG MH: Autologous chondrocyte implantation with collagen bioscaffold for the treatment of osteochondral defects in rabbits. *Tissue Eng* **11**: 1065-1076, 2005.

WOBIG M, BACH G, BEKS P, DICKHUT A, RUNZHEIMER J, SCHWIEGER G, VETTER G, BALAZS E: The role of elastoviscosity in the efficacy of viscosupplementation for osteoarthritis of the knee: a comparison of hylan G-F 20 and a lower-molecular-weight hyaluronan. *Clin Ther* **21**: 1549-1562, 1999.

YAEGER PC, MASI TL, DE ORTIZ JL, BINETTE F, TUBO R, MCPHERSON JM: 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. *Exp Cell Res* **237**: 318-325, 1997.

YOO HS, LEE EA, YOON JJ, PARK TG: Hyaluronic acid modified biodegradable scaffolds for cartilage tissue engineering. *Biomaterials* **26**: 1925-1933, 2005.

YOO WJ, CHOI IH, CHUNG CY, CHO TJ, KIM IO, KIM CJ: Implantation of perichondrium-derived chondrocytes in physeal defects of rabbit tibiae. *Acta Orthop* **76**: 628-636, 2005.