Fibrin for Tissue Engineering of Cartilage

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‘If we consult the standard surgical writers from Hippocrates down to present age, we shall find that an ulcerated cartilage is found to be a very troublesome disease [...] and that when destroyed, it is never recovered.’

W. Hunter

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Chapter 1

Goals of the Thesis

Since the beginning of the 1990s a plethora of research approaches towards the engineering of cartilage have been undertaken. However, a general standard method for generation of cartilage tissue equivalent and their clinical application is still lacking. The goal of this thesis is based on the project 'Bavarian Research Cooperation ('Bayerischer Forschungsverbund') for Tissue Engineering and Rapid Prototyping' (ForTEPro), which is a multi-partner network of research groups from university, hospital, and industry supported by a grant from the Bavarian Research Foundation ('Bayerische Forschungsstiftung') in the years 2002 to 2005. The major goal of the overall project was the development of individually customized implants for anatomical cartilage and bone defects of the head and the musculo-skeletal system. A subgroup of the research consortium aimed at the generation of cartilage for facial plastic and reconstructive surgery, especially external ear. In principle, autologous chondrocytes were to be isolated, suspended in a hydrogel, and the gel was subsequently to be injected into a polymeric scaffold that was individually shaped using newly established rapid prototyping technologies.

The overall goal of the thesis was, within the ForTEPro project, the utilization of fibrin for tissue engineering of cartilage. The hydrogel fibrin is a well-investigated medical device and has been used for over 20 years in clinical practice and surgery (**chapter 2**). However, commonly employed commercially available preparation kits often result in gels that are unstable in cell culture, shrink, and dissolve within a few weeks, which makes them unsuitable for many applications in shape-specific tissue engineering [1, 2]. Therefore, as a first major step, fibrin glue parameters were determined influencing appearance and stability of the gel in order to obtain a long-term shape stable scaffold material for culture of cells. Subsequently, these optimized fibrin gels were demonstrated to be suitable for the use in cartilage tissue engineering. Therefore, primary bovine chondrocytes were suspended in the gel system and in detail analyzed regarding cell morphology, cell proliferation as well as extracellular matrix production and distribution to establish a chondrocyte-fibrin culture system. In particular, the influence of fibrinogen concentration, cell density, and different culture conditions on formation of cartilaginous tissue was investigated. With the objective to generate a coherent cartilaginous tissue using primary bovine chondrocytes, the minimum initial cell number required for the formation of an adequate uniform extracellular matrix was determined and the effect of exogenous insulin was examined (**chapter 3 and 4**).

Chondrocytes in monolayer rapidly dedifferentiate from a cartilaginous phenotype towards a more fibroblast-like phenotype accompanied by production of inadequate extracellular matrix molecules. Therefore, as a next step within this thesis, the long-term stable fibrin gels were tested for the use in the proliferation of primary bovine chondrocytes, with specific regard to its potential to retain the ability of the thus expanded chondrocytes to form engineered cartilage (**chapter 5**). Cells that migrated out of fibrin and proliferated on the gel were re-seeded into three-dimensional fibrin gels in order to evaluate differentiation capacity compared to cells proliferated on conventional cell culture surface.

The main goal of **chapter 6** was to transfer the established fibrin culture method from using bovine cells to human cells, with regard to a future clinical application. Human chondrocytes were isolated from small nasal and articular biopsies, and seeded into the established fibrin gels. Based on previous studies in another cartilage engineering culture system [3, 4], a special focus was set on the effect of insulin and insulin-like growth factor-I (IGF-I) to enhance formation of new human cartilaginous tissue.

As an important step within the ForTEPro consortium, the fibrin gels were combined with polymeric scaffolds (**chapter 7**). Hydrogels generally enable a good cell distribution, but often lack adequate mechanical strength [5]. Highly porous solid scaffolds can provide sufficient load-bearing capacity, however, many scaffold systems lack an adequate cell seeding efficiency, cell distribution and a subsequent sufficient extracellular matrix development [6]. In order to overcome the disadvantages associated with either system, bovine chondrocytes were suspended in the optimized fibrin gel and subsequently injected into newly developed polycaprolactone-based scaffolds, based on results from research partners. Additionally, the results were compared with injecting the cell-fibrin suspension into commonly used PGA meshes as well as PLGA scaffolds. With regard to the overall goal of the generation of a prototype of an external ear within ForTEPro, the cell-fibrin suspension was injected into a polycaprolactone-based scaffold in the shape of the cartilage part of an external human ear, and maintenance of ear shape as well as new tissue formation within the scaffold was evaluated.

In a follow-up study, the established composite constructs consisting of bovine chondrocytes suspended in fibrin gel and distributed within a polycaprolactone-based scaffold was further tested in vivo (**chapter 8**). The constructs were implanted into the back of nude mice and examined regarding formation of new cartilaginous tissue after 1, 3, and 6 months. In particular, tissue development was compared to constructs prepared with cells seeded into fibrin gel alone as well as cells seeded directly onto the polymeric

scaffold. Furthermore, a special focus was set on the effect of in vitro pre-cultivation of the constructs prior to implantation on subsequent in vivo development of the cartilaginous tissue.

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Chapter 2

Fibrin in Tissue Engineering

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Introduction

Every year, millions of people suffer loss or failure of tissue or organs due to an accident or clinical condition. A revolutionary strategy to treat these patients is the engineering of the lacking organ or tissue with autologous cells in combination with polymeric matrices [1]. For several years there has been enormous interest in hydrogels as a soft scaffold for tissue engineering [2-4]. In nearly every intact native tissue, cells are held within an extracellular matrix that modulates tissue development, homeostasis and regeneration. Hydrogels are structurally similar to the extracellular matrix of many tissues and are considered to be biocompatible. They have many potential functions in the field of tissue engineering and, thus, must fulfill many different requirements to promote new tissue growth, depending on their application. Hydrogels act as a space filling agent and three-dimensional structure to organize the expanded cells, to maintain a specific shape and structural integrity, and to direct growth and formation for adequate new tissue development. In general, hydrogels can be processed under relatively mild conditions suitable for many cell types. Furthermore, the liquid components of hydrogels may be easily delivered into the patient's defect in a minimally invasive manner.

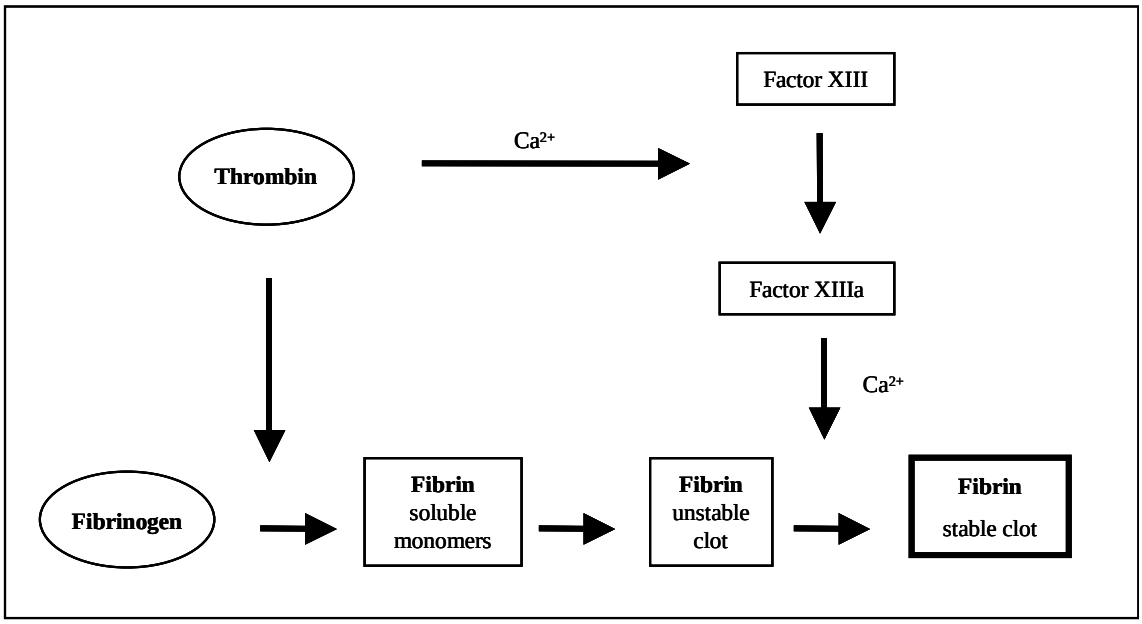

Fig. 1: Scheme of fibrin gel formation.

One of the most widely used hydrogels is fibrin. Fibrin glue is a commonly used surgical haemostatic agent and has been commercially available for over 20 years in surgery and clinical practice. The hydrogel fibrin is a polypeptide consisting of the plasma components fibrinogen and thrombin. Physiologically, fibrin formation occurs as the final step in the natural blood coagulation cascade, producing a clot that assists wound healing. After activation with calcium ions, thrombin cleaves small peptides from the fibrinogen chain to produce soluble fibrin monomers [5]. These monomers are covalently cross-linked through the action of factor XIII to form an insoluble, polymerized fibrin clot [6] (Fig. 1). In recent years, fibrin has been utilized for different applications in the field of tissue engineering with specific physical and biological requirements. The current use of fibrin for each of these categories will be subsequently reviewed.

Fibrin glue in surgery and clinical practice

Several reviews published in recent years have focused on uses of fibrin glue, also referred to as fibrin tissue adhesive or fibrin sealant, in clinical and surgical practice [7-11]. In the literature, fibrin was first mentioned more than 90 years ago. It has been documented that fibrinogen combined with thrombin was used to improve the adhesion of skin grafts of soldiers with burn injuries during the Second World War [7].

A commercial product has been available in Europe and Japan since the 80s, whereas fibrin glue was not FDA approved until 1998 because of possible viral contamination. At the moment, fibrin sealant is considered the most effective physiological tissue adhesive available.

There are a number of commercially available fibrin products with different amounts and origins of the components [7, 9]. The concentration of fibrinogen, varying between 40 and 125 mg/ml, is directly correlated to the tensile strength of the fibrin clot, whereas the concentration of thrombin influences the degree and speed of clotting. The latter proves useful for quick haemostasis to prevent blood loss (e.g., in suturing of vessels) or in surgical procedures involving careful glue adjustment to fit a tissue or organ [9]. Within 3 days of application, a preliminary granulation tissue with a large number of wound healing cells is present and is subsequently replaced with collagen fibers one to two weeks later. During normal wound healing the fibrin glue is absorbed within days to weeks depending on the type of sealant and location of application [12]. The majority of glues contain an anti-fibrinolytic component to reduce the degradation rate. A common agent is the protease

inhibitor aprotinin, which inhibits human trypsin and plasmin by blocking the active sites of the enzymes. Due to the fact that fibrin is a physiological blood component, it is considered to be biocompatible and biodegradable. However, despite a number of rigorous national and international guidelines during manufacturing to assure the high quality of commercial fibrin components, the risk of viral infection or foreign body response, especially due to bovine components, still exists [7]. Additionally, the development of antibodies against coagulation plasma proteins has been documented after application of bovine thrombin resulting in significant anticoagulation [10]. Although most of the commercial products now contain human fibrinogen and thrombin, the majority still contain bovine aprotinin, which was shown to cause hypersensitivity, especially after repeated administration [10]. Autologous preparation methods have been described [13-16] to prevent these foreign body reactions, however, the composition, resulting appearance, and mechanical strength of these gels depend on the patient's physique and constitution. Therefore, there is a great interest in optimizing these methods leading to standardization and validation in clinical practice.

The most prevalent application of fibrin in clinical practice is its use as a haemostatic agent, especially in heparinized or coagulopathic patients, to reduce operative bleeding, e.g., in cardiovascular surgery. The application is most effective when polymerized prior to the onset of bleeding, for example in surgery of a vascular anastomosis [8]. When using fibrin sealants or sprays as adjunct to sutures, a better wound healing and optimal wound integrity results in operative locations where the use of conventional sutures is not feasible or would result in intense bleedings [8, 10].

Fibrin polymers play a key role in tissue and organ sealing, particularly in plastic and reconstructive surgery, including skin grafting [8]. Exact adjustment is possible, bleeding is reduced, fewer sutures are necessary, the length of the operation time is shortened, and fewer post-operative infections occur.

Fibrin glue applications are common in other important fields of clinical practice, including thoracic, orthopedic, neuro-, and oral surgery [7, 8, 12]. Since the recent approval of fibrin glues by the FDA, the number of clinical applications has increased dramatically and companies have started to investigate and improve the use of the sealant in more diverse surgical settings.

Principles and methods of fibrin application in tissue engineering

In order for fibrin gels to be utilized as a tissue scaffold, the material must provide an environment enabling adequate cellular function, e.g., cell migration, proliferation, and differentiation, and must allow for tissue development. For example, it has been shown that chondrocytes in fibrin gel retain their round and vital morphology, do not dedifferentiate, and produce extracellular matrix [17, 18]. The glue components fibrinogen and thrombin are thought to modulate the attachment, migration, and proliferation of different cell types, e.g., chondrocytes, fibroblasts, smooth muscle cells, or keratinocytes [19-23]. Fibrinogen possesses a specific peptide chain, also referred to as a heptide, that contributes to cell attachment and binding primarily of mesenchymal cells, e.g., fibroblasts, endothelial cells, and smooth muscle cells [24]. This data is consistent with a study published by Brown, who investigated the effect of cross-linking various fibrin chains on fibroblast migration [25]. An important factor modulating fibroblast movement into a fibrin gel has been shown to be factor XIII which mediates the cross-linking of the fibrin α-chains [26].

The variation of fibrin parameters can generate gels with different mechanical stiffnesses. These mechanical properties potentially influence the gene expression of different growth factors and cytokines, e.g., of human dermal fibroblasts [27]. Important parameters affecting fibrin characteristics are thrombin, fibrinogen, and calcium concentration, ionic strength, and pH, resulting in either more rigid and stable or more soft and soluble gels [28-30], although the exact contributions of each parameter are still not fully understood. Nevertheless, it may be possible to tailor specific structural fibrin features for specific cell types and for a particular application to modulate individual cell proliferation, migration and differentiation [31].

Another fibrin glue characteristic is an increasing instability and solubility over time, due to fibrinolysis [28]. Commercially available fibrin sealants tend to shrink and disintegrate in vitro and in vivo after a few days and almost completely dissolve within 4 weeks [17, 32-35]. While this could be an advantage in wound sealing or other surgical applications in which dissolution is desired after closure of the defect [7, 9, 12], this can represent a major problem for use as a shape-specific scaffold in tissue engineering. Here, long-term stability is necessary to provide enough time for cell proliferation and matrix production. Therefore, fibrinolysis inhibitors, mostly protease inhibitors, e.g., aprotinin, ε-amino caproic acid or

tranexamic acid, are used within the fibrin gel and/or as a supplement to the cell culture medium; they can help slow down degradation and, thus, stabilize the fibrin gel shape [32, 36, 37]. As a result, degradation of the temporary matrix may be controlled for specific purposes.

The application of fibrin in tissue engineering can be grouped into three main areas: fibrin as cell matrix material alone, fibrin as a cell matrix material combined with a polymeric scaffold, and as delivery system for growth factors or other therapeutic agents.

A simple method for the use of fibrin as a scaffold material involves suspending primary or expanded cells in a liquid component of the fibrin glue with subsequent polymerization in suitable cell culture plates. The resulting three-dimensional construct may be cultivated in vitro to obtain an adequate tissue for re-implantation. In addition to the application as scaffold in vitro, the fibrin system can also be used as cell delivery vehicle in vivo. Cells suspended in fibrin glue can be directly injected into a defect in a minimally invasive procedure with little stress for the patient; the fibrin gel can be polymerized in vivo in the desired three-dimensional shape, at the same time ensuring the retention of the cells at the injection site [22, 36].

An alternative strategy for tissue engineering is the combination of hydrogels with polymeric scaffolds. Highly porous solid scaffolds can provide sufficient load-bearing capacity for the process of implantation and for structural integrity in vivo. However, many scaffold systems lack adequate cell seeding efficiency, sufficient cell distribution and subsequent sufficient extracellular matrix synthesis and deposition. In contrast, fibrin gels generally incorporate all of the applied cells and enable a good cell distribution providing the requirements for a coherent tissue development, but often lack adequate biomechanical strength and volume stability [34, 38-40]. Therefore, the advantages of fibrin glue combined with favorable characteristics of synthetic or naturally derived polymeric scaffolds can be utilized to develop a simple, stable composite for implantation. This way tissue development in the desired three-dimensional shape can be achieved; furthermore the time for tissue development may be reduced as compared to the use of either system alone [41]. This strategy has been successfully applied in tissue engineering of urothelium [42], cartilage [43-45], and cardiovascular engineering [41].

Fibrin can also be applied as delivery system for the release of growth factors, cytokines or other bioactive molecules to control cell adhesion, proliferation, migration, differentiation, and matrix production. Many growth factors bind to fibrin, bFGF and VEGF are even supposed to bind specifically. Alternatively, such proteins can be incorporated into the gel

during polymerization [46-48]. Fibrin is known to protect growth factors against denaturation and proteolysis in vitro and in vivo [49]. Furthermore, when applied in vivo the presence of the growth factor in the defect is maintained over a long time. Additionally, the kinetics of growth factor release can be controlled by varying fibrinogen and thrombin concentration as well as by the addition of degradation inhibitors. Factors released from fibrin gels used for tissue regeneration include bFGF, VEGF, NTF, ECGF, GDNF and NGF [48, 50-62]. Hubbell et al. developed an innovative technology for growth factor delivery on the basis of a combination of fibrin and heparin, utilizing the ability of heparin to stabilize the bioactivity of growth factors and control their release [49]. A fibrin gel was modified by covalently binding exogenous bi-domain peptides with a heparin-binding domain using the transglutaminase activity of factor XIIIa during coagulation. These peptides can bind heparin and subsequently heparin-binding growth factors (Fig. 2). This approach was successfully applied in the controlled release of different growth factors [53, 54, 62-67] either by passive release or facilitated by enzymatic factors secreted by migrating cells.

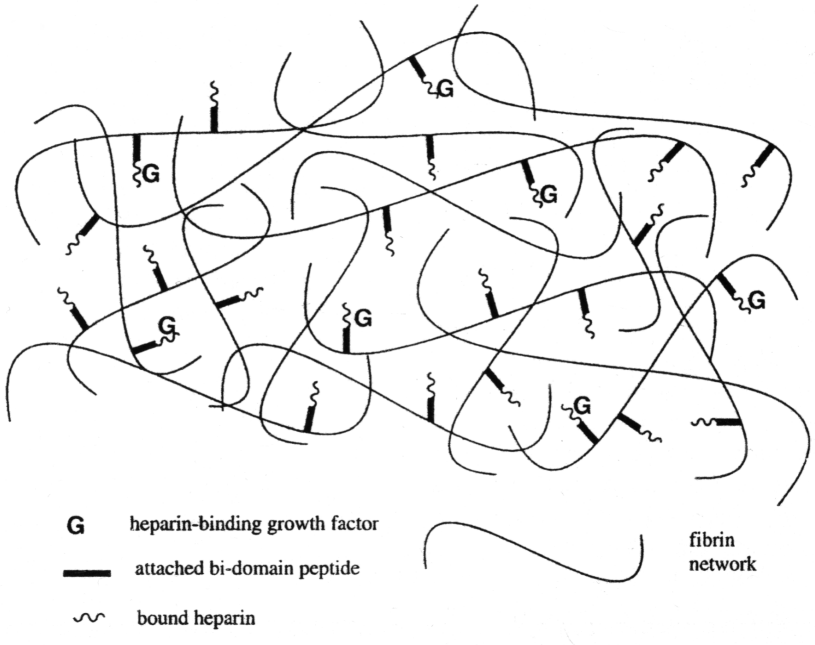

Fig. 2: Fibrin matrix as gel for delivery system utilizing the growth factor binding properties of heparin. A bi-domain peptide, containing a factor XIIIa substrate and a heparin-binding domain, is covalently cross-linked to the fibrin matrix during coagulation. Heparin is immobilized to the heparin-binding domain of the peptide by electrostatic interactions. Heparin-binding growth factors are immobilized by binding to the immobilized heparin within the fibrin matrix (with permission of Elsevier; Ref: 62).

Andree et al. established a method to deliver EGF expression plasmids from a fibrin matrix to a human keratinocyte culture system. These plasmids can enhance keratinocyte proliferation during expansion in vitro as well as directly after transplantation of the cells in combination with a fibrin matrix into a skin defect in vivo [68].

Besides release of growth factors, the local delivery of antibiotics and chemotherapeutic agents from a fibrin gel could be beneficial in clinical applications [69, 70]. Skin replacement therapies, a common application in tissue engineering, carries a high risk of infection during the implantation procedure that could be moderated by the release of antibiotics from the tissue replacement itself. Unfortunately, the time span of release from fibrin hydrogels is rather short due to the fast diffusion of the small molecules. Release may be prolonged, however, by varying drug concentration or by use of fibrin insoluble antibiotics [71]; these drugs dissolve slowly inside the defect due to low hydrophilicity.

Fibrin in the engineering of specific tissues

Skin tissue

Numerous authors have investigated fibrin glue for skin tissue engineering during the last years. Not only can fibrin be used as a graft sealant as well as a haemostatic and antibacterial agent [72], fibrin is a functional scaffold material for engineering of skin tissue. It has been shown that fibrinogen stimulates the migration of epidermal cells and keratinocytes [73-75]. Fibronectin, a specific glycoprotein in fibrin glue, enhances cellular migration during wound healing [76, 77]. A common clinical application consists of a single cell suspension of in vitro expanded autologous human keratinocytes in fibrin sealant and delivery directly into the skin defect. The cell-glue system adheres and spreads over the defect resulting in re-epithelialization within a few weeks [76, 78, 79]. Additionally, cultivation of keratinocytes onto a fibrin layer in vitro maintains the status of differentiation [80].

An alternative therapy for chronic wounds and severe burns includes isolation of a small biopsy of the patient’s skin, expansion of the resultant single keratinocytes in vitro onto a supportive 3T3 feeder layer and transfer of the developed epidermal sheet directly to the wound. However, this is an expensive and time-consuming process and careful enzymatic detachment and handling of the cell sheet is critical. The application of fibrin as culture bed during expansion and transfer of the cell-fibrin construct facilitates and accelerates the

operative procedure [81]. Meana et al. investigated a fibrin gel either with or without human fibroblasts as a base for a dermal equivalent. Only in the group with fibroblasts, cultivation of even of low cell numbers of primary keratinocytes on this gel system resulted in a newly developed epithelium within 10-14 days (Fig. 3). The cell layer was anually detached from the culture flask without enzymatic treatment and could be easily transplanted into the skin defect [82]. Gorodetsky et al. developed an innovative technology to deliver cells from fibrin-derived microbeads instead of conventional fibrin gel systems [83]. These biodegradable microbeads, 50-200µm in diameter, represent a simple provisional matrix and cell carrier with good attachment properties for different cell types. Fibroblasts seeded at low density can proliferate in vitro on the fibrin particles and may be easily transferred from the culture plate into specific defects for wound healing. The cell-seeded microbeads showed improved formation of granulation tissue in pig wound healing as compared to fibrin-derived microbeads alone. This strategy may be transferable to the engineering of other tissues as well.

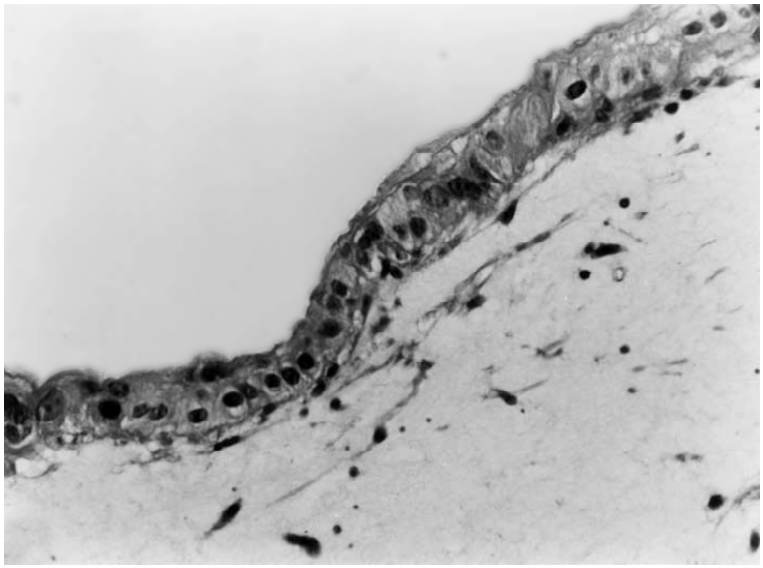

Fig. 3: Fibrin as an approach to generate a dermal equivalent: keratinocytes were cultured on a fibroblast-containing fibrin gel. Histological appearance after 15 days of culture (H&E staining, x 250) (with permission of Elsevier; Ref: 82).

Vascular tissue

There is a tremendous demand for blood vessel repair, especially in cardiovascular surgery due to the high number of patients with arteriosclerosis. The investigation of fibrin gel parameters and the effects of exogenous factors added to fibrin scaffolds on inducing cells

to form vascular tissue within these gels are important first steps towards a clinical solution [84, 85]. Koolwijk et al. used in vitro experiments to analyze the effect of TNFα, bFGF and VEGF on human microvascular endothelial cells seeded on fibrin gels to form capillary-like tubular structures for angiogenesis [84]. The data showed that the inflammatory mediator TNFα is necessary in addition to bFGF and/or VEGF for development of capillary-like structures by endothelial cells.

Moreover, fibrin gels can serve as a three-dimensional matrix molded to the exact structure of vessels. Jockenhoevel et al. developed a method towards the engineering of valve conduits [86]. Fibroblasts were suspended in a fibrin gel that was polymerized in silicone-coated aluminum molds. 2 mm thick constructs were cultivated in vitro and cells subsequently produced collagen bundles, an element of valve conduits. In another study, the same group investigated methods to prevent the shrinkage of fibrin gels so they could be used in cardiovascular engineering. In addition to supplementing protease inhibitors to the culture medium, the mechanical and chemical fixation of gels onto culture plates was tested [40]. Matrix analysis and histology showed the best collagen synthesis and tissue development using a chemical border fixation onto culture plates. In contrast, Mol et al. discussed the possible advantage of shrinking fibrin gels for vascular engineering leading to higher mechanical forces inside the construct and thereby potentially enhancing collagen production [41].

Furthermore, fibrin gels were tested as a scaffold for cardiovascular tissue engineering using myofibroblasts. Ye et al. suspended human myofibroblasts in a fibrin matrix [39]. Microscopy showed homogenous cell growth and collagen synthesis. Additionally, a higher concentration of aprotinin supplemented to the medium resulted in improved gel stability and enhanced tissue development. Cummings et al. investigated the morphological and mechanical properties of fibrin gel, collagen type I gel, and a combination of both for vascular engineering using rat aortic smooth muscle cells. A combination of rigid, less elastic collagen with a weaker, more instable fibrin gel resulted in higher ultimate tensile stress, increased toughness, and increased gel compaction compared to each system alone [87]. As an alternative, the variation in fibrin parameters resulting in more stable and rigid gels may be a means to avoid the more complex combinations with another gel system for this kind of application.

Bone tissue

Another potential field of application of fibrin is the healing of critical size defects of bone, although there are only a few papers published on the topic so far. One strategy, again, is the delivery of cells suspended in a fibrin gel directly into the bone defect. Isogai et al. injected a periosteal cell-fibrin mixture into the dorsum of athymic mice. Histology showed new bone development and western blot assay demonstrated production of osteopontin, a specific protein in bone tissue [88]. Ng et al. investigated the potential of suspending cells derived from four different sites of the body in fibrin glue for three-dimensional bone constructs in vivo [89]. Osteoprogenitor cells isolated from periosteum showed best results, whereas using cells derived from cancellous and cortical bone as well as bone marrow resulted in less bone-forming activity.

Bensaid et al. tested the potential of fibrin as a scaffolding structure for mesenchmal stem cells in bone tissue engineering in vitro [22]. They varied fibrinogen concentration and thrombin activity and analysed the effect on cell spreading and proliferation in vitro. Perka et al. compared the cultivation of periosteal cells on PLGA polymer fleece and on fibrin beads. Both groups were cultivated in vitro for 14 days and subsequently implanted into metadiaphyseal ulna defects of white rabbits [90]. Histological and radiological analysis showed intense bone formation in both groups.

Karp et al. combined fibrin glues containing different thrombin concentrations with interconnecting, macroporous PLGA scaffolds for use in bone engineering [91]. However, no difference was seen between the control group (only PLGA scaffold, no fibrin) and the group with low thrombin concentration, whereas scaffolds filled with fibrin gel containing high thrombin concentrations showed less bone formation. Fibrin parameters may be optimized for this kind of application to obtain improved cell migration and matrix production.

Finally, Haisch et al. investigated a method to induce transdifferentiation of articular chondrocytes into bone-forming cells with addition of corticosteroids [92]. Auricular rabbit chondrocytes were suspended in fibrin or agarose and the mixture was injected into polymer fleeces. Constructs were subsequently implanted subcutaneously into the ridge of New Zealand rabbits with and without methylprednisolone treatment. Histology showed that the simple injection of corticosteroids prevented fibrous tissue formation and enhanced bone development.

Cartilage tissue

Fibrin is widely used in approaches to tissue engineering of cartilage. Fortier et al. tested fibrin as a matrix for engineering articular cartilage in vitro using equine chondrocytes. Positive effects of exogenously applied IGF-I and TGF-β on chondrocyte matrix synthesis was shown [93, 94]. In another study, autologous fibrinogen was demonstrated to better maintain differentiation of chondrocytes compared to commercially available fibrinogen [18]. To further enhance cartilage development, Hunter et al. tested the influence of oscillatory compression on development of chondrocyte-fibrin constructs [95]. Though dynamic compression has frequently been shown to stimulate matrix production and gene expression in tissue, mechanical stimulus of the fibrin constructs resulted in the inhibition of cartilage matrix production. The effect of mechanical stimulation may depend on the structure and mechanical properties of the fibrin gel itself, which in turn depends on the concentration of the individual components and has to be tested for specific applications.

Several studies were published showing tissue engineering of cartilage after subcutaneous implantation in mice. Silverman et al. suspended swine chondrocytes in fibrin gel, injected the cell-fibrin mixture directly into the subcutaneous back of nude mice and determined the optimal fibrinogen and cell concentration required for adequate tissue development [38]. Sims et al. were the first who reported about the successful tissue engineering of cartilage in the back of nude mice after construct preparation in vitro [96]. Bovine chondrocytes were isolated and suspended in fibrinogen; after polymerization with bovine thrombin, the constructs were implanted subcutaneously into mice. Quantitative analysis and histology demonstrated that differentiated cells were producing cartilaginous extracellular matrix after 6 and 12 weeks. Using the same method, the growth and development of swine chondrocytes from different sites of the body as well as the volume stability of the constructs were explored [97]. Cultivation of articular chondrocytes resulted in decreased construct volume, whereas auricular chondrocytes produced high amounts of extracellular matrix resulting in construct overgrowth. These results indicate the importance of cell source.

Furthermore, the cultivation of chondrocytes in fibrin gels with expanded highly porous polytetrafluoroethylene as a stabilizing pseudoperichondrial layer was suggested as an intelligent functional composite for repair of craniofacial defects by Xu et al. The pseudoperichondrium was either placed in the center of the construct with the cell-fibrin mixture on both sides or on both surfaces with the cell-fibrin mixture in the middle.

Implantation of these constructs without pre-cultivation in vitro into the dorsal subcutaneous pocket of nude mice for 8 months resulted in good infiltration of the transplanted chondrocytes into the microporous structure of the polymer, the creation of a stable connection to the pseudoperichondrium and the development of an elastic construct for cartilage engineering [98, 99].

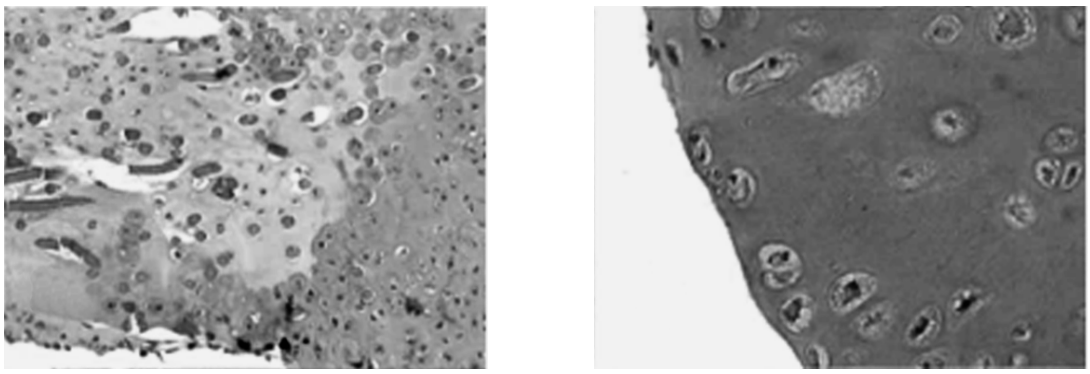

Fig. 4: Fibrin combined with a PGA non-woven mesh for cartilage engineering employing swine chondrocytes. Distributions of sulphated GAG, assessed with safranin-O staining within a composite cell construct after 7 days (left) and after 28 days (right) of in vitro culture. Dark segmented lines (left) are polymer fibers (with permission of Elsevier; Ref: 43).

Cartilage is still the most important application of fibrin in tissue engineering. However, several publications complain about fast fibrin shrinkage and disintegration during chondrocyte cultivation. To overcome these problems, protease inhibitors or higher fibrinogen concentrations were employed as mentioned before [32, 36]. Other strategies include combinations with other hydrogels and polymeric scaffolds, respectively. Perka et al. mixed fibrin with stabilizing alginate, which can be easily removed prior to implantation [100]. Histology of human chondrocytes cultivated in this mixture for 30 days in vitro showed differentiated cells and formation of cartilaginous matrix. In an approach using a combination of fibrin and a polymeric scaffold, a high number of swine chondrocytes were suspended in fibrin gel and added to a PGA mesh [43]. It was reported that this combination already resulted in more mechanically stable constructs directly after cell seeding, i.e., the gel injected into the scaffold was more stable than the scaffold alone. After 4 weeks of cultivation in vitro, the combination of fibrin and polymeric scaffold resulted in a higher amount of glycosaminoglycans, an effect that was partially attributed to increased matrix retention in the fibrin gel, and advanced mechanical stability of constructs for implantation compared to polymeric scaffold alone (Fig. 4).

Conclusion

We have summarized a wide range of fibrin applications in tissue engineering approaches to date. Fibrin glue can serve as a scaffold material alone, in combination with other hydrogels or porous polymeric scaffolds, and as delivery system for growth factors. However, fibrin gels have a complex composition. Their appearance and mechanical strength varies enormously due to different components and concentrations. Therefore, it is extremely important to determine the exact matrix parameters necessary for a specific application. A soft gel is necessary for easy migration of cells inside the scaffold. The fast degradation of fibrin may be useful in sealants or for cell or growth factor delivery. In contrast, a strong and durable gel is essential for tissue engineering in vitro, where cells need enough time and sufficient mechanical integrity to produce their tissue-specific matrix. Also for an earlier implantation after cell-fibrin construct preparation, i.e. to shorten the in vitro culture period, mechanically stronger gels appear desirable. By varying the fibrin composition, cell number, and cultivation time, fibrin gels with different properties can be developed that are suitable for many different applications in clinical practice and the engineering of tissues. Such manipulations of fibrin properties may even eliminate the need for the more complex combinations with polymeric scaffolds or other hydrogels and supplements like protease inhibitors, in turn minimizing the risk of undesired side effects.

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Chapter 3

Factors Influencing Chondrocyte Behavior and Development of Cartilaginous Tissue in Three-Dimensional Fibrin Gel

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Abstract

Despite several disadvantages, fibrin glue is a common material for three-dimensional cultivation of chondrocytes in the field of tissue engineering. Within this study, a modified long-term stable fibrin gel was tested for its general potential as scaffold in cartilage engineering using primary bovine chondrocytes in vitro. Cells suspended in 500 µl fibrin gels and cultured for 5 weeks in medium containing 5 % or 10 % FBS maintained their round and vital appearance and produced extracellular matrix containing GAG and collagen, though primarily concentrated in a small area around cells. In order to generate a uniform and coherent cartilaginous tissue, a lower fibrinogen concentration was found to improve homogenous distribution of extracellular matrix, however, at the same time reducing gel stability, compared to fibrin gels prepared with higher concentrations of fibrinogen. Furthermore, increasing cell density resulted in an increasingly coherent and homogenous extracellular matrix. However, when using a cell number of $40*10^6$ or more per construct, matrix development decreased in the center of the gel, compared to the periphery of the constructs and to seeding a lower cell number. This effect was attributed to the large construct size resulting in insufficient diffusion and/or increased consumption of oxygen or nutrients. Moreover, a dynamic cultivation on an orbital shaker had enhancing effect neither on production of extracellular matrix components GAG and collagen nor on distribution of matrix, compared to statical cultivation. In contrast, addition of bioactive insulin to the culture medium containing 5 % FBS resulted in increased growth rate and development of extracellular matrix, even higher compared to cultivation with 10 % FBS in medium. However, this effect was still insufficient for the formation of a uniform and coherent cartilaginous tissue when employing 500 µl gels and a relatively low cell density. On the other hand, the chondrocyte culture system can be used as a test system for delivery of growth factors or other bioactive molecules from controlled release devices. As an example, the model protein insulin slowly released from lipid microparticles enhanced production of cartilaginous extracellular matrix of cells suspended in fibrin gel and cultured in medium containing 5 % FBS. Taken together, these investigations clearly confirm the suitability of long-term stable fibrin gels for the use in cartilage tissue engineering, however, further investigations have to be conducted with regard to the generation of an adequate homogenous cartilaginous tissue.

Introduction

The aim of tissue engineering is to generate a new functional tissue by controlling the growth, differentiation and behavior of cell [1]. Besides the use of polymeric scaffolds, there has been enormous interest in using hydrogels as scaffold system [2-4]. Many hydrogels are similar to the extracellular matrix of various intact tissues, therefore, they are considered to be biocompatible and biodegradable. Hydrogels act as space filling agent and three-dimensional structure to organize the expanded cells, to maintain a specific shape and structural integrity, and to direct growth and formation for adequate new tissue development. They are suitable for many different cell types, as they are processed under relatively mild conditions, and may be easily delivered into the patient's defect in a minimally invasive manner.

A common hydrogel in tissue engineering is fibrin [5]. Fibrin glue has been commonly used as a sealant and an adhesive in surgery, and has been commercially available for over 20 years in surgery and clinical practice [6-9]. In order for fibrin gels to be utilized as a tissue scaffold, the material must provide an environment enabling adequate cellular function, e.g. cell migration, proliferation, and differentiation, and must allow for tissue development. It has been shown that chondrocytes in fibrin gel retain their round and vital morphology, do not dedifferentiate, and produce extracellular matrix [10, 11]. The glue components fibrinogen and thrombin are thought to modulate the attachment, migration, and proliferation of different cell types, e.g. chondrocytes [12-16]. However, a fibrin characteristic is an increasing instability and solubility over time [17]. Commercial fibrin glues tend to shrink and disintegrate in vitro and in vivo after a few days and almost completely dissolve within 4 weeks, therefore they cannot be used in many applications in tissue engineering, where a shape-specific scaffold is preferred [10, 18-20]. Therefore, in modifying various fibrin parameters, our group has recently developed a transparent gel, that is stable in vitro for at least one year. Fibrin optimization as well as mechanical and rheological properties of the new gel will be discussed in detail in chapter 4.

Within this study, the newly developed fibrin gel was tested for its general potential for the use in cartilage tissue engineering. Therefore, primary bovine chondrocytes were suspended in fibrin, and cell behavior, cell morphology, as well as development of cartilaginous extracellular matrix were evaluated. With the objective to optimize chondrocyte culture, the effect of dynamical cultivation as well as of exogenous insulin on cartilaginous tissue development was investigated. Furthermore, in order to obtain a

uniform and coherent new tissue, influence of fibrinogen concentration as well as initially seeded cell number on cell behavior as well as matrix development and distribution were analyzed. Finally, the established chondrocyte culture system was tested for the use in evaluating controlled release of bioactive molecules from microparticles, using insulin as a potent cartilage-effective model drug. We tested the effect of insulin slowly released from incorporated lipid microparticles on chondrocytes grown within the long-term stable fibrin gels.

Materials and Methods

Materials

Aprotinin solution (Trasylol®) was bought from Bayer (Leverkusen, Germany). Thrombin (as a part of Tissucol®), thrombin dilution buffer and the commercially available fibrin glue kit Tissucol® was kindly provided by Baxter (Unterschleißheim, Germany). Bovine fibrinogen was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Knee joints from three-months-old bovine calves were obtained from a local abattoir within 12-18 hours of slaughter. Type II collagenase and papainase were purchased from Worthington (CellSystem, St. Katharinen, Germany). Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, fetal bovine serum (FBS), MEM non-essential amino acid solution, penicillin, streptomycin, N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES buffer), phosphate buffer solution (PBS buffer) and trypsin EDTA were obtained from Gibco (Karlsruhe, Germany). 149 µm pore size polypropylene filters were purchased from Spectrum (Rancho Dominguez, CA, USA).

Ascorbic acid, deoxyribonucleic acid, diaminobenzidine, dimethylmethylene blue, glutaraldehyde, glycine, hematoxylin, proline and safranin-O were purchased from Sigma-Aldrich (Taufkirchen, Germany). Bovine insulin from bovine pancreas, chloramin-T, formalin 37%, and p-dimethylaminobenzaldehyde (p-DAB) were from Merck (Darmstadt, Germany). Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA) and L-hydroxyproline from Fluka (Neu-Ulm, Germany). Chondroitin sulfate was from ICN (Aurora, Ohio, USA) and glycerol tripalmitate (Dynasan116®) was provided by Sasol AG (Witten, Germany).

Cell culture plastics were purchased from Corning Costar (Bodenheim, Germany).

Cell isolation

Primary chondrocytes were isolated from the surface of the femoral patellar groove using sterile technique. The cartilage was cut into small pieces and enzymatically digested over night in complete chondrocyte medium, DMEM containing 4.5 g/l glucose, 584 mg/l glutamine, 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.4 mM proline, 0.1 mM MEM non-essential amino acids and 50 µg/ml ascorbic acid, with addition of 470 U/ml of type II collagenase. The digest was re-pipetted, filtered through a 149 µm filter and washed three times with PBS. The cell number was determined by cell counting using a hemocytometer and an inverted phase-contrast microscope.

Cell seeding and culture

Fibrin gels were prepared with 100 mg/ml fibrinogen in 10,000 KIE/ml aprotinin solution and 500 U/ml thrombin in 40 mM $CaCl_2$, diluted to 5 U/ml with dilution buffer. For investigation of the effect of fibrinogen concentration, a fibrinogen solution containing 150 mg/ml was diluted with aprotinin solution to obtain 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml fibrinogen.

For standard culture, $1.5*10^6$ freshly isolated bovine chondrocytes were suspended in 250 µl fibrinogen solution; subsequently, gels were prepared adding the same volume of 5 U/ml thrombin in a stabilizing silanized glass ring to obtain a fibrin disc of 10 mm diameter and 6.4 mm thickness (500 µl gel). The fibrin gels were allowed to gel for 45 min at 37 °C before removing the glass ring and covering the cell-fibrin constructs with 4 ml complete chondrocyte culture medium. Medium contained either 10 % FBS or 5 % FBS, and was replaced three times per week. Constructs were statically cultured in 6-well-plates in an incubator at 37 °C, 5 % CO^2 and 95 % humidity, and harvested after 2 or 5 weeks.

For dynamical cultivation, constructs in 6-well-plates were placed on an orbital shaker at 50 rpm (Dunn Labortechnik GmbH, Ansbach, Germany). For constructs containing various initial cell densities, cell numbers in the range from $5*10^6$ to $60*10^6$ were suspended in 250 µl fibrinogen solution (100 mg/ml fibrinogen). The same volume of 5 U/ml thrombin was added in a silanized glass ring to obtain 500 µl fibrin discs. For investigation of the effect of insulin in medium, 2.5 µg/ml bovine insulin was added to the culture medium containing 5 % FBS, and medium was replaced three times per week ($1.5*10^6$ cells per 500 µl fibrin gel).

For constructs containing lipid microparticles loaded with insulin for controlled release, 0.375 mg particles were introduced into the gel together with the cell suspension before gelling. Lipid microparticles loaded with 2 % insulin and median particle size of 250 µm were prepared in our group by spray congealing method [21]. Briefly, the molten lipid (Dynasan®) including insulin was sprayed with a temperature-controlled single substance nozzle into a customized spray congealing apparatus, resulting in complete encapsulation of insulin into particles. An in vitro study demonstrated a release of about 20 % of the encapsulated insulin into release buffer within 28 days [21].

Histological and biochemical analysis

Constructs were analyzed as previously described [22, 23]. Briefly, the constructs were weighed (= wet weight) and cut in half. One part of the construct was lyophilized and digested with 1 ml of a papainase solution (3.2 U/ml in buffer) for 18 h at 60 °C for determination of cell number and content of glycosaminoglycans and total collagen.

The number of cells per cell-fibrin construct was assessed from the DNA content using Hoechst 33258 dye and a conversion factor of 7.7 pg DNA per chondrocyte [24]. The amount of sulfated glycosaminoglycans was determined spectrophotometrically at 525 nm as chondroitin sulfate using dimethylmethylene blue; bovine chondroitin sulfate was used as standard [25]. The hydroxyproline content was determined spectrophotometrically after acid hydrolysis and reaction with p-dimethylamino-benzaldehyde and chloramin-T [26]. The amount of total collagen was calculated using a hydroxyproline to collagen ratio of 1:10 [27].

The other part of the construct was prepared as a histological sample by fixing in 2 % glutaraldehyde in PBS for 30 min and then storing in 10 % formaldehyde. The formalin-fixed samples were embedded in paraffin and cross-sectioned into 7 µm sections; deparaffinized sections were stained with hematoxylin, fast green and safranin-O.

Statistical analysis

Biochemical data are expressed as means ± standard deviation. Statistical significance was assessed by one-way analysis of variance ANOVA in conjunction with multiple comparison Tukey's studentized range test at a level of $p < 0.01$ for Fig. 6 and with comparison versus control Dunnett's test at a level of $p < 0.01$ for Fig. 2, Fig. 4 and Fig. 7.

Results

Cells in fibrin gel

Primary bovine chondrocytes suspended in fibrin gel were homogenously distributed throughout the whole gel and had a round and differentiated phenotype directly after preparation as well as within culture time, as shown in Fig. 1A and 1B. During culture in medium containing 10 % FBS, cells produced extracellular matrix, indicated by distinct areas around single cells and lacuna (Fig. 1B). After histological staining, these areas were strongly stained red with safranin-O for glycosaminoglycans (Fig. 1C – 1F). With regard to biochemical analysis, data showed an increase in cell number, and GAG and collagen within the 5 weeks of culture time (Fig. 2). However, reducing of FBS content to 5 % resulted in a significant smaller cell number and decreased amounts of GAG and collagen per wet weight, compared to cultivation with 10 % FBS (Fig. 2). Reduced development of cartilaginous tissue was also confirmed by histological cross-sections, showing less intensive safranin-O stain after 5 weeks of culture (Fig. 1C – 1F).

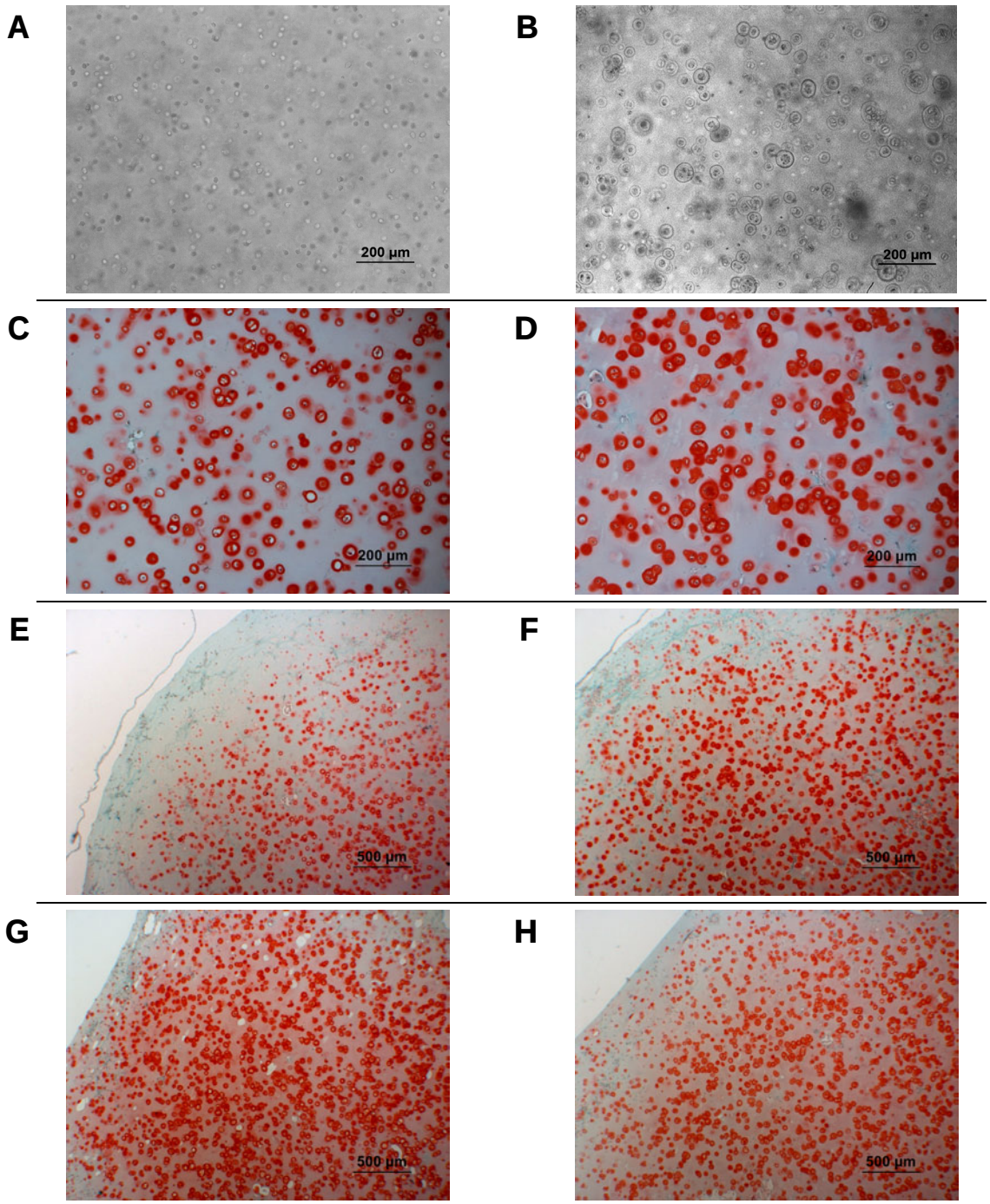


Fig. 1: Chondrocytes suspended in fibrin gel, directly after preparation (A) and after 4 weeks in culture (B). Microscopic pictures demonstrated three-dimensional cell distribution throughout the whole gel, as well as development of ECM, indicated by distinct area around cells. Histological cross-sections of constructs prepared with chondrocytes in fibrin gel, cultured for 5 weeks in medium containing 5 % FBS (C, E) and 10 % FBS (D, F) without insulin, as well as 5 % FBS with 2.5 µg/ml insulin (G) and insulin lipid microparticles (LMP) (H). GAG was stained red with safranin-O. For results of insulin application, please see also p. 51.

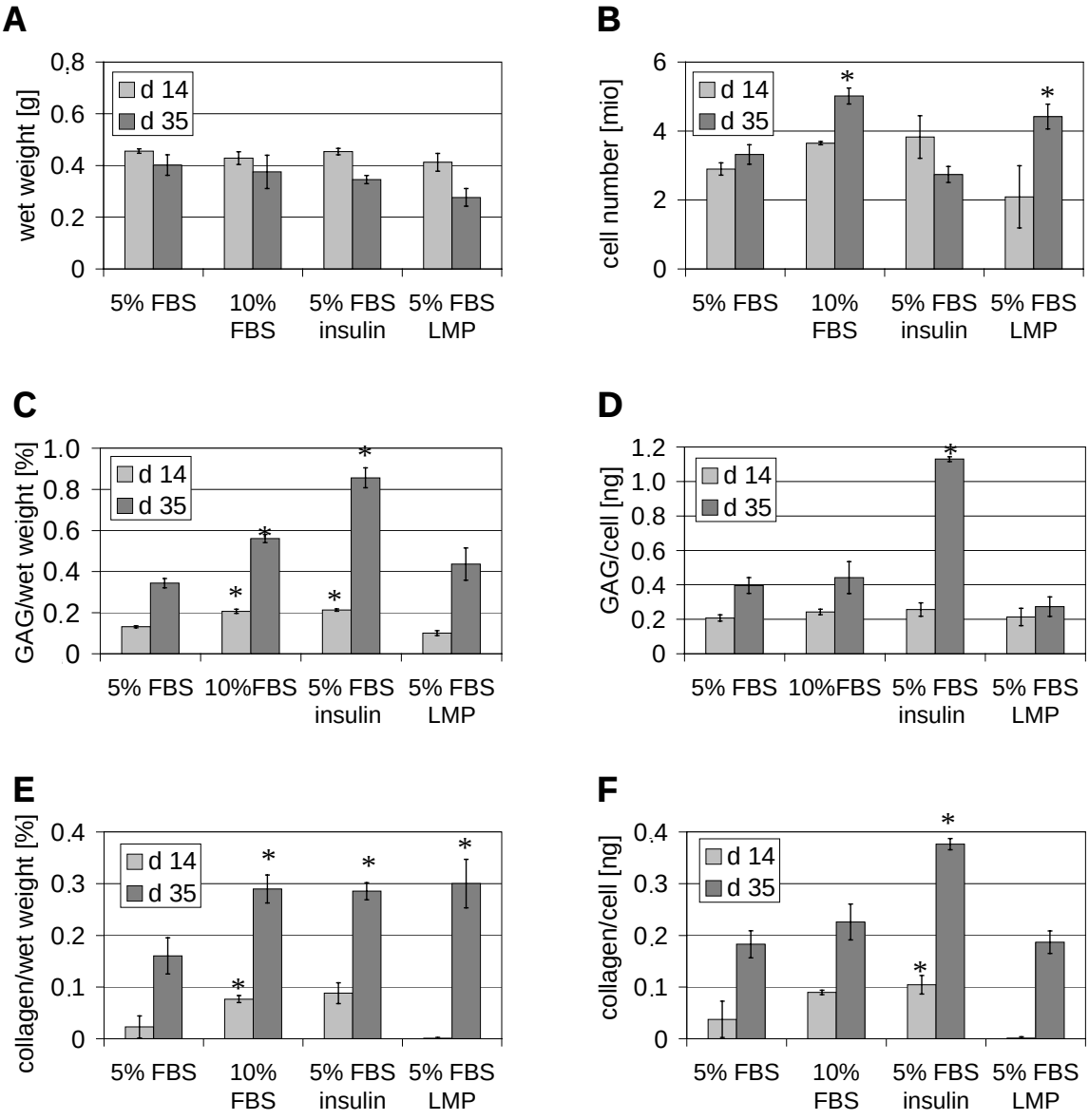


*Fig. 2: Wet weight (A), cell number (B), and extracellular matrix components GAG (C, D) and collagen (E, F) per wet weight (C, E) and per cell (D, F) of constructs cultivated at different culture conditions. Constructs were harvested after 2 and 5 weeks in culture. Data represents the average ± S.D. of three independent measurements. Statistically significant differences of experimental groups compared to control (medium containing 5 % FBS) are denoted by * ($p < 0.01$). For results of insulin application, please see also p. 51.*

Effect of dynamic cultivation

Histological cross-sections of constructs dynamically cultured for 5 weeks showed less intensive staining of the areas around cells with safranin-O for glycosaminoglycans (Fig. 3). Biochemical analysis demonstrated only little differences after 2 weeks of dynamic cultivation on an orbital shaker compared to static cultivation (Fig. 4). In contrast, after 5 weeks, only 73 % of construct wet weight were found in constructs dynamically cultured, compared to no shaking. Furthermore, only 80 % of extracellular matrix components GAG and 72 % of collagen per wet weight as well as 48 % and 43 % per cell, respectively, were detected, as displayed in Fig. 4. Thus, dynamical cultivation of bovine chondrocytes in a long-term stable fibrin gel seems to not improve development of cartilaginous tissue.

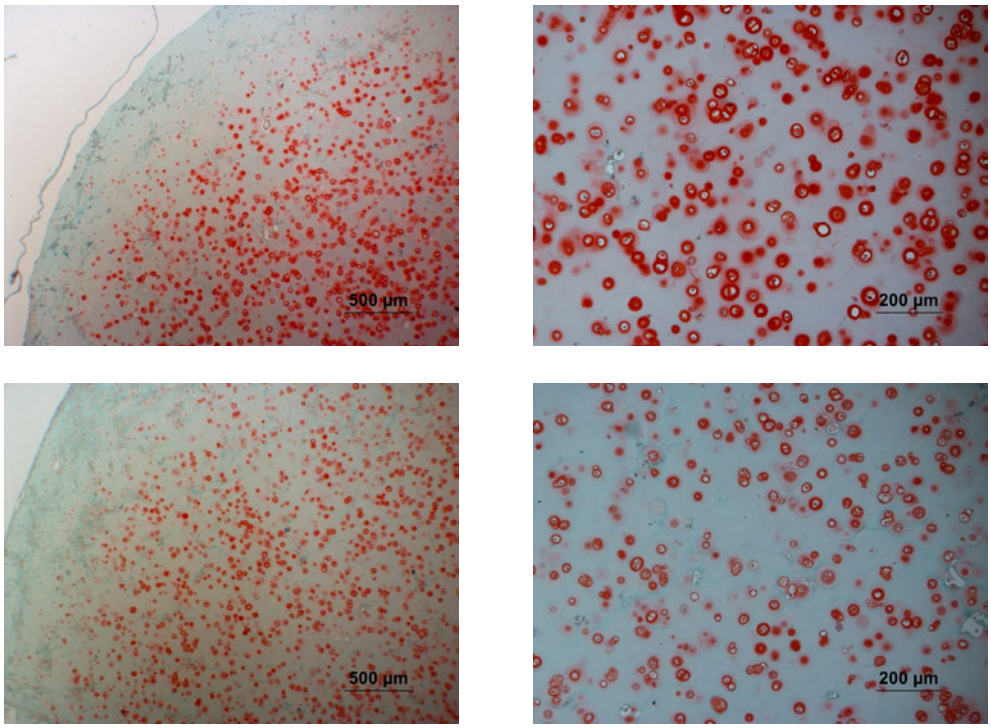


Fig. 3: Histological cross-sections of constructs prepared with primary bovine chondrocytes in fibrin gel (100 mg/ml fibrinogen), statically (upper) and dynamically (lower) cultured for 5 weeks in medium containing 5 % FBS. Cells are encircled by an area containing extracellular matrix, stained red with safranin-O for GAG.

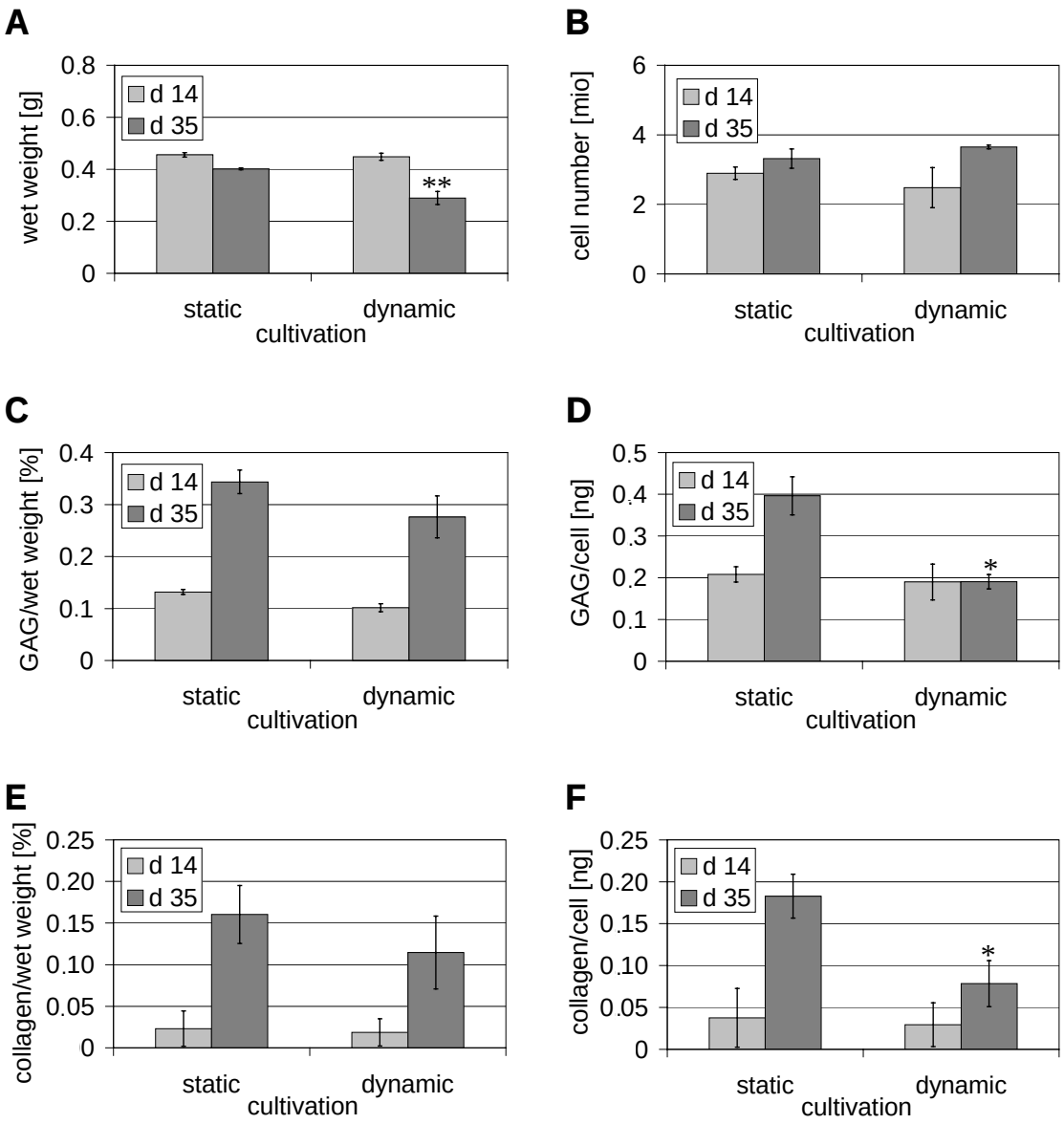


Fig. 4: Wet weight (A), cell number (B), and extracellular matrix components GAG (C, D) and collagen (E, F) per wet weight (C, E) and per cell (D, F) of constructs prepared with primary bovine chondrocytes in fibrin gel (100 mg/ml fibrinogen). Constructs were cultured for 2 or 5 weeks, either statically or dynamically on an orbital shaker. Data represents the average ± S.D. of three independent measurements. Statistically significant differences of statical cultivation compared to dynamical cultivation are denoted by asterisks ($p < 0.01$, ** $p < 0.05$).*

Effect of fibrinogen concentration

When gels were prepared with different concentrations of fibrinogen, strong differences were observed in cell shape as well as development and distribution of newly produced extracellular matrix (Fig. 5). Using 12.5 mg/ml fibrinogen resulted in small, more elongated cells, a typical indicator for dedifferentiated cells (Fig. 5). However, histological staining demonstrated the development of homogenously distributed extracellular matrix, usually produced by differentiated chondrocytes (Fig. 5). Generally, results of quantitative analysis of extracellular matrix composition suggested that chondrocytes suspended in a lower fibrinogen concentration produced as much extracellular matrix as cells in the presence of higher fibrinogen concentration (Fig. 6). However, histology of constructs prepared with higher fibrinogen concentration showed more round cells located in defined lacunae. The more fibrinogen, i.e., the firmer the gel, the more strongly restricted the new matrix was found in a small area around cells. In contrast, the lower the fibrinogen concentration, the more the construct shrunk and degraded within 5 weeks in culture, indicated by an decrease of 75 % in wet weight of constructs prepared with 12.5 mg/ml fibrinogen compared to 100 mg/ml (Fig. 6). This resulted in higher amounts of GAG and collagen per wet weight, however, amounts per cell remained similar.

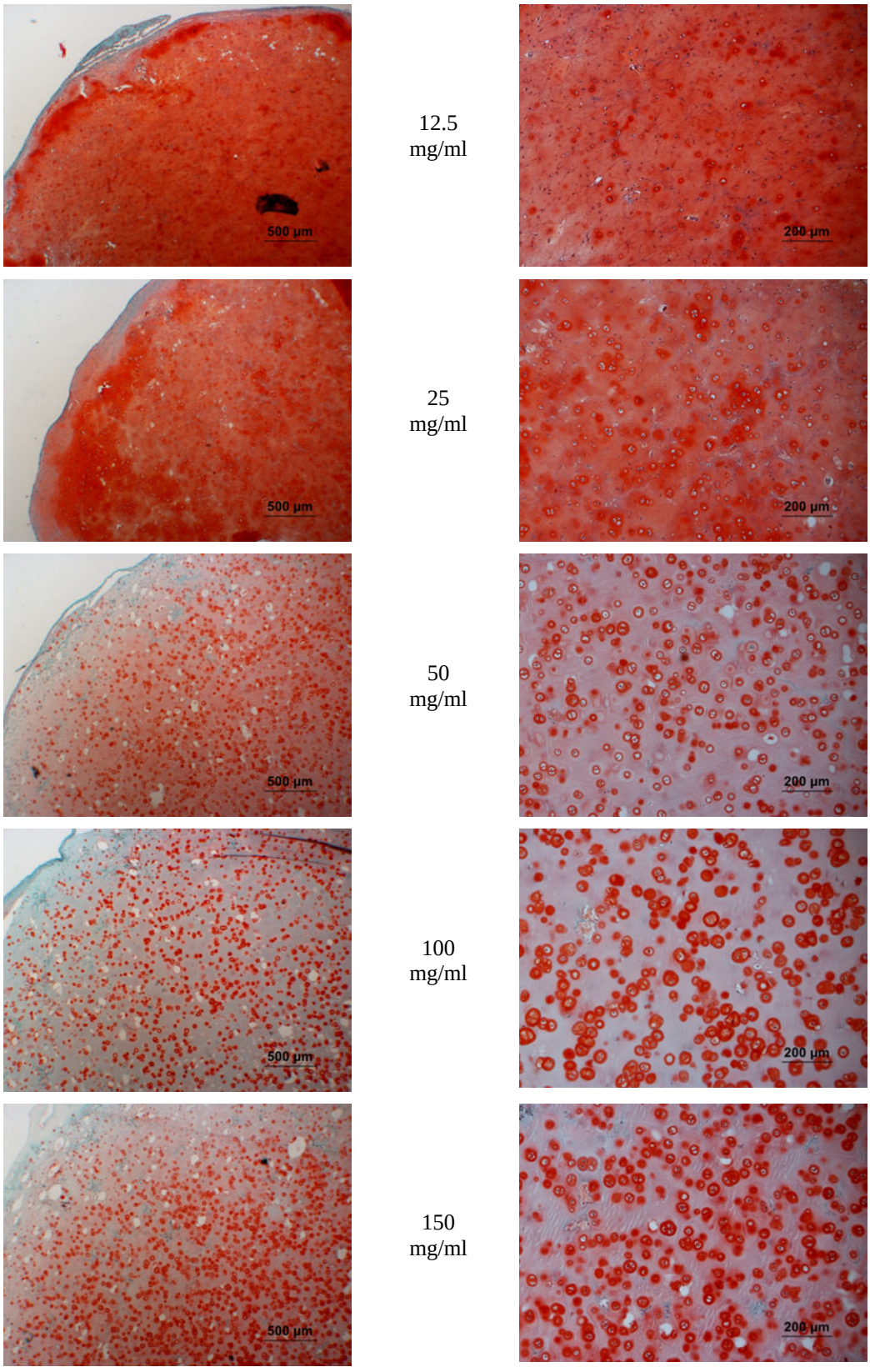


Fig. 5: Histological cross-sections of cell-fibrin constructs prepared with different concentrations of fibrinogen, cultured for 5 weeks (safranin-O stain).

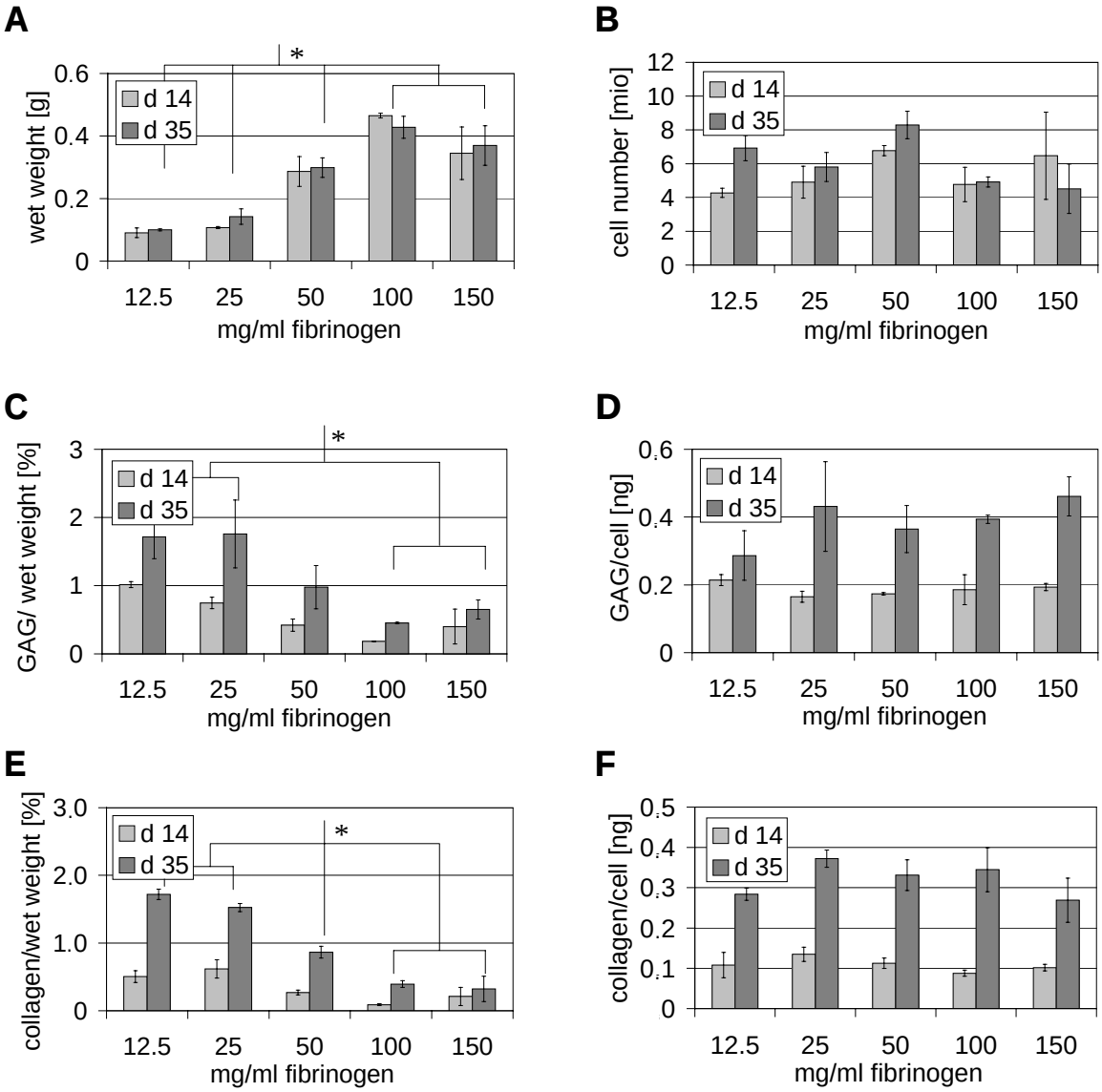


*Fig. 6: Wet weight (A), cell number (B), and extracellular matrix components GAG (C, D) and collagen (E, F) per wet weight (C, E) and per cell number (D, F) of constructs prepared with different concentrations of fibrinogen. Constructs were cultured for 2 or 5 weeks in medium containing 10 % FBS. Data represents the average ± S.D. of three independent measurements. Statistically significant differences between two groups are indicated by * ($p < 0.01$).*

Effect of cell density

Histological cross-sections of bovine chondrocytes in long-term stable fibrin gels cultured for 5 weeks showed homogenously distributed cells throughout the whole constructs. However, constructs prepared with various initial cell densities demonstrated strong differences regarding matrix development and distribution (Fig. 7 and Fig. 8). Using $5*10^6$ and $10*10^6$ cells per construct resulted in matrix development exclusively in the immediate vicinity of the cells within the whole gel. In contrast, constructs prepared with a higher initial cell density of $20*10^6$ cells showed a more homogenous matrix distribution within the whole construct, i.e., a more coherent cartilaginous tissue, within 5 weeks in culture. However, the higher initial cell density, the stronger the differences between center and periphery of the construct. Using cell densities higher than $20*10^6$ per construct resulted in more intensive stain of the periphery of the construct, however, less intensive stain in the center of the gel, indicating less matrix development. Therefore, cell density seems to be a limiting factor in fibrin construct in the size of 500 µl.

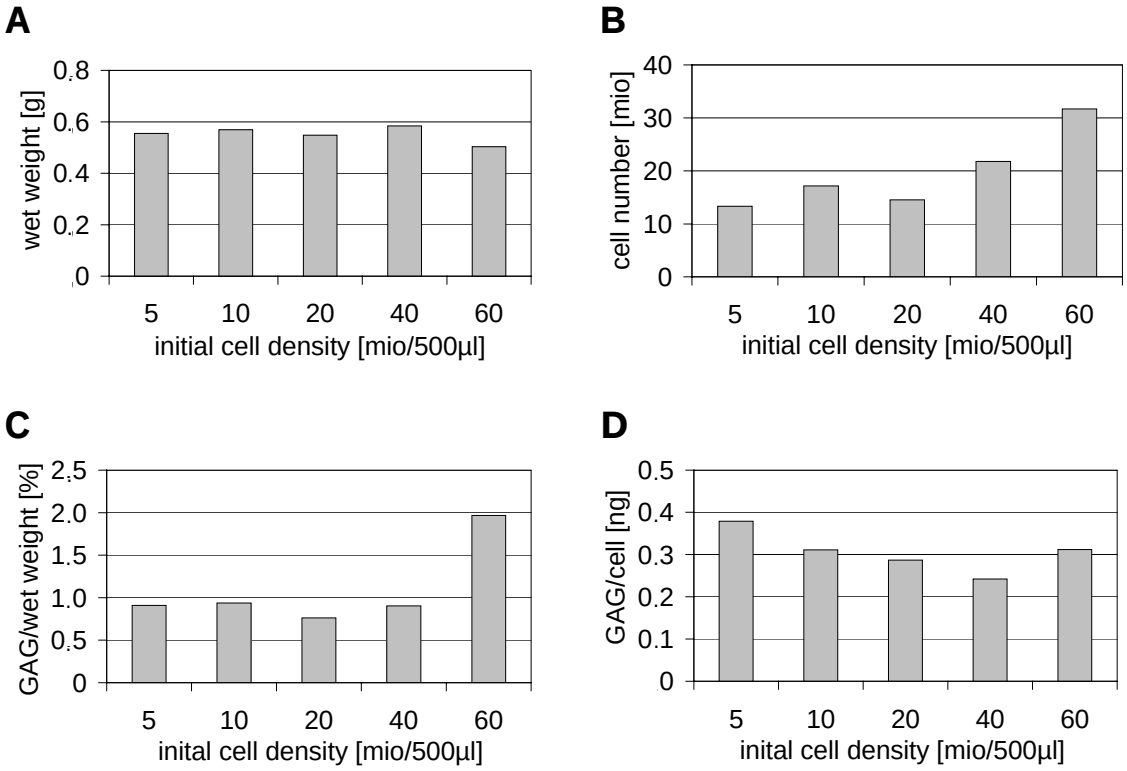
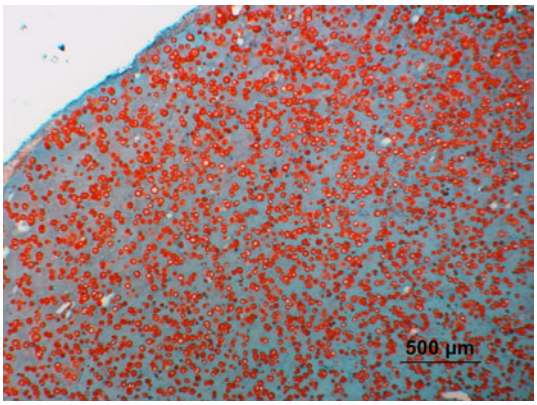
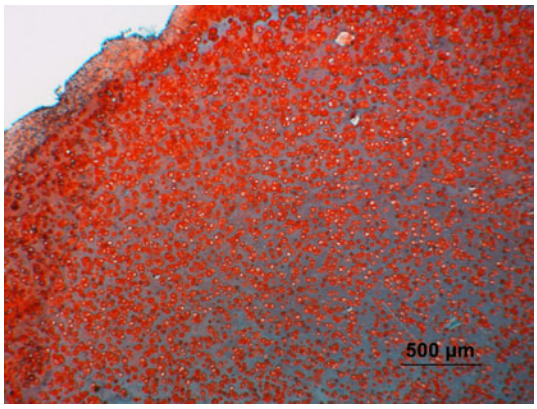


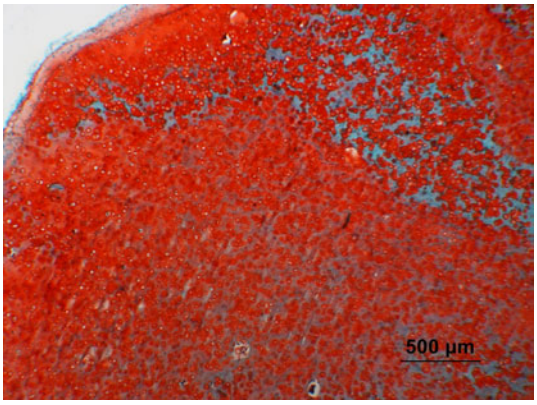
Fig. 7: Wet weight (A), cell number (B), and GAG per wet weight (C) and per cell (D) of constructs prepared with different initial cell densities (n = 1), after 5 weeks in culture.



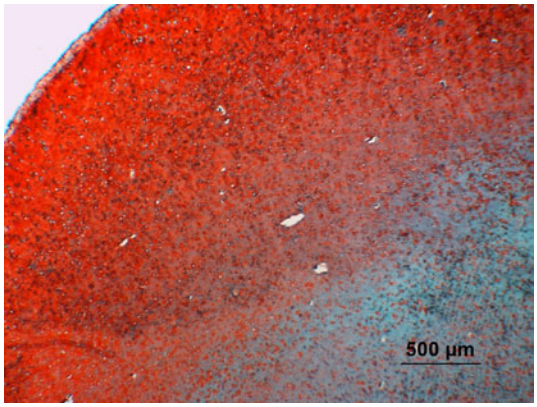
$5*10^6$ cells/gel



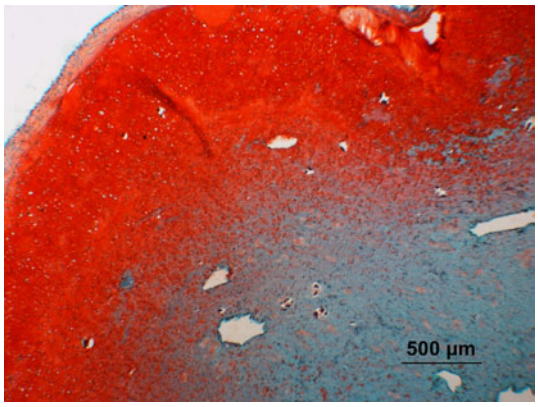
$10*10^6$ cells/gel



$20*10^6$ cells/gel



$40*10^6$ cells/gel



$60*10^6$ cells/gel

Fig. 8: Histological cross-sections of constructs prepared with different initial cell densities, culture for 5 weeks in vitro. Newly developed extracellular matrix was intensively stained red with safranin-O.

Effect of insulin

As already mentioned above, using medium with 5 % FBS resulted in decreased cell number and development of extracellular matrix, compared to cultivation with 10 % FBS, except for GAG per cell. However, adding 2.5 µg/ml insulin to medium containing 5 % FBS showed a significant 2.7-fold increase in GAG per wet weight and 2.8-fold increase per cell after 5 weeks of culture (Fig. 2). Interestingly, increase was 1.7-fold and 2.6-fold, respectively, compared to cultivation in medium with 10 % FBS without insulin. With regard to total collagen, addition of insulin resulted in a 1.8-fold higher amount per wet weight and 2-fold higher amount per cell, compared to cultivation without insulin. The collagen amounts expressed per wet weight were similar and expressed per cell were 1.7-fold higher, compared to those in constructs cultured in medium containing 10 % FBS. Histological cross-sections clearly confirmed these results, demonstrating a more intensive stain with safranin-O located around cells of constructs cultured with 2.5 µg/ml insulin, compared to cultivation without insulin (Fig. 1). In conclusion, supplementing insulin to the medium containing 5 % FBS not only compensates for the lower concentration of serum, but has an improving effect on matrix development.

Effect of insulin lipid microparticles for controlled release

Lipid microparticles (LPM) loaded with 2 % insulin and suspended within cell-fibrin construct resulted in round and vital cells within 5 weeks of culture period and an 1.4-fold increase in cell number, similar to cultivation with 10 % FBS (Fig. 1 and Fig. 2). Biochemical analysis demonstrated no increase in GAG per wet weight, but a distinct 1.9-fold increase in collagen per wet weight, similar to cultivation with 10 % FBS (Fig. 2). However, extracellular matrix content per cell was not influenced.

Discussion

Within this study, a newly developed long-term stable fibrin gel was demonstrated to be suitable as a 3-D scaffold system for chondrocyte cultivation. Several factors including fibrinogen content, initial cell density, static versus dynamic culture, and FBS and insulin supplementation were found to influence chondrocyte behavior within the gel.

Itay et al. made first attempts in generation of cartilage with the use of fibrin in injecting chick chondrocytes in fibrin glue into defects in the surface of condylar articular cartilage of the joint of a rooster. However, this method showed only limited ability to repair cartilage lesions, which was attributed to cytotoxic effects of the fibrin glue [28]. This was disproved by Homminga et al. investigating in detail chondrocyte behavior in fibrin glue in vitro [10]. It was shown, that rabbit chondrocytes in fibrin glue proliferated, retained their morphology, and produced new extracellular matrix, stained with alcian blue. However, intense fibrin disintegration and dissolution was found starting from day 2, especially using higher cell numbers of up to $2*10^6$ cells per ml. This rapid degradation resulted in increasing dedifferentiation of cells and failure of new cartilaginous tissue generation. Therefore, fibrin glue was evaluated to be unsuitable for long-time culture of chondrocytes in vitro. In contrast, Isoda et al. found fibrin disintegration to occur within a longer time of 3 months when fibrochondrocytes were suspended in unstable fibrin glue and cultured in vitro, resulting in an increase in more branched cells compared to cultivation in agarose [29]. Additionally, rapidly dissolved fibrin glue was demonstrated to be replaced by fibrous tissue [30].

Fast degradation of fibrin glue could be an advantage in wound sealing or other surgical applications in which dissolution is desired after closure of the defect [6, 9], however, this can represent a major problem for use as a shape-specific scaffold in tissue engineering [18, 31]. Here, long-term stability is necessary to provide enough time for cell proliferation and matrix production. Therefore, fibrinolysis inhibitors, mostly protease inhibitors, e.g. aprotinin, ε-amino acid or tranexamic acid, are used within the fibrin gel and/or as a supplement to the cell culture medium and can help slow down degradation and, thus, stabilize the fibrin gel shape [32]. Meinhart et al. also found that fibrin constructs containing up to $1*10^6$ human chondrocytes per ml were dissolved too early, before cartilaginous tissue formation could occur [20]. However, it could be demonstrated that high concentration of aprotinin and tranexamic acid for stabilization slowed degradation to an extent that provided longer time for matrix production preventing construct dissolution.

However, this was only effective in using low cell densities. Constructs with higher cell densities in the range of $4*10^6$ to $20*10^6$ cells per ml had disintegrated completely within 4 weeks, and only remnants of fibrin were visible. In the same study, a mixture containing fibrin glue and rabbit chondrocytes were injected into the cartilage defect in the contralateral ear of rabbits [19]. For decelerating fibrin degradation, high concentrations of aprotinin and tranexamic acid were added to the mixture, resulting in formation of cartilaginous tissue within 6 month in vivo, stained with Alcian blue and immunohistochemically for collagen type II. However, only 35 % to 90 % of the defect area was filled with new cartilaginous matrix, which was attributed to an uneven fibrin-cell distribution or continuing fibrin degradation, thus, resulting in decrease in implant size. These studies clearly demonstrate that fibrin degradation still represents a major problem for the use in cartilage tissue engineering. Therefore, in order to obtain a fibrin gel that is more stable in cell culture, we modified specific fibrin gel parameters resulting in gels that are transparent and stable in culture medium for at least one year (chapter 4). Within this chapter, we demonstrated that these optimized gels are generally a suitable material for the long-term culture of chondrocytes. Primary bovine chondrocytes suspended in these fibrin gels were homogeneously distributed and maintained round and vital throughout the culture period. The cells proliferated and produced extracellular matrix components GAG and total collagen within 5 weeks in culture, at the same time not compromising shape stability of the gel.

It has also been shown in literature that degradation of a fibrin glue can be slowed by a higher fibrinogen concentration [33]. An in vitro study demonstrated that constructs prepared with higher fibrinogen concentration and addition of high amounts of fibrinolysis inhibitors into the culture medium were more stable, compared to constructs with lower concentration of fibrinogen or addition of low amounts of antifibrinolysis inhibitors. However, matrix production was reduced compared to constructs prepared with lower concentration of fibrinogen. These results were contrary to our results. In the study presented here, a gel containing a low concentration of fibrinogen was demonstrated to shrink and degrade, compared to fibrin prepared with high concentration of fibrinogen, however, the amounts of newly produced extracellular matrix components per cell were very similar. Nevertheless, with the objective of formation of a connected new cartilaginous tissue, the use of a fibrin gel prepared with a lower concentration of fibrinogen is not suitable for a shape-specific application in tissue engineering. However, uniformity of the new matrix was reduced in constructs containing high amounts of

fibrinogen, likely due to the stiffness of the gel that prevent homogenous distribution of matrix molecules.

An alternative approach to enhance generation of new cartilage includes an optimized cultivation method. In other studies, constructs prepared with polymeric scaffolds were cultured in a Petri dish on an orbital shaker at 75 rpm or in spinner flasks and resulted in thicker constructs with more spatially uniform distribution of cells and new extracellular matrix compared to static culture [34-36]. Furthermore, it has been found that shear stress can have a significant impact on the morphology and the mechanical properties of newly generated cartilage [37-43]. In contrast, Hunter et al. found that a static compression of chondrocytes seeded in fibrin gels had little effect on the mechanical stiffness or biochemical content of the construct compared to free-swelling conditions. Furthermore, an oscillatory compression resulted in softer gels, inhibited accumulation of extracellular matrix components and enhanced diffusion of molecules out of the construct into culture media [44]. In our study, dynamical culture of cells suspended in fibrin gel resulted in decrease in construct size and reduced production of cartilaginous extracellular matrix components. The differences in results reported in the literature and also in this study were attributed to differences in the effect of mechanical stimuli on development of cartilaginous tissue in various scaffold systems, in particular in soft hydrogels compared to solid polymeric scaffolds.

With the objective to obtain a homogenous cartilaginous tissue, we further investigated the influence of cell density on matrix distribution. It was shown in the literature that cells seeded too sparsely resulted in incomplete filling of the scaffold, loss of structural construct integrity, at the same time enabling ingrowth of fibroblast in vivo which in turn would adversely affect the properties of the newly generated tissue [45, 46]. Common approaches use a high cell number of $10*10^6$ to $120*10^6$ bovine chondrocytes per cm^3. However, only limited studies investigated the optimal cell density in order to obtain an adequate new tissue (discussed in detail in chapter 4) [33, 45, 47, 48]. Results in our study showed an enhancement of matrix distribution when increasing initially seeded cell number. The higher the cell density, the more coherent the newly developed extracellular matrix resulting in a uniform cartilaginous tissue. However, histological cross-sections demonstrated the failure of tissue formation in the center of the fibrin construct, when seeding a high cell number of more than $20*10^6$ cells per construct. This may be attributed to the large construct (500 µl gel) becoming less permeable, resulting in reduced diffusion of nutrients, oxygen and/or waste, and increased cell death [47, 49, 50]. Alternatively,

increased consumption of nutrients and oxygen may be an explanation for the observed effects [50]. Thus, cell density in larger gels seems to be a limiting factor for formation of cartilaginous tissue in fibrin gel. Consequently, a smaller gel size of 40 µl was used for all following experiments involving high cell densities to allow adequate diffusion (chapter 4). However, in this study, as a cell number of $20*10^6$ resulted in uniform development of new extracellular matrix within the whole large-size construct during 5 weeks, a longer cultivation in vitro of constructs with this lower cell density may result in formation of a more coherent tissue without limiting diffusion. As an alternative, spinner flasks or rotating vessels may be used [51, 52], which were demonstrated to enhance mass transport through the construct and improve formation of cartilaginous matrix. However, as discussed above, in our study, dynamical cultivation was shown to have no enhancing effect on development of cartilaginous extracellular matrix, therefore, the effect of spinner flasks or rotating vessels would have to be carefully tested in our culture system.

Growth factors can stimulate or inhibit cell division, differentiation and migration and consequently influence tissue development, thus, are powerful tools for many tissue engineering approaches [23, 43, 53-55]. Insulin has been previously shown to have strong anabolic effects on engineered cartilaginous constructs in vitro, similar to the effects of the growth factor IGF-I [22]. Therefore, within this study, exogenous insulin added to the medium containing 5 % FBS was tested regarding development of extracellular matrix components GAG and collagen of cells suspended in fibrin gel. Analysis and histology clearly showed an increase in content of GAG and total collagen after 2 and 5 weeks of in vitro culture. The effect was even higher compared to constructs cultured in medium containing 10 % FBS. However, addition of insulin did also not lead to generation of a coherent cartilaginous tissue in these experiments employing 500 µl fibrin gels and a comparatively low cell number. Nevertheless, these results clearly demonstrate that growth factors or other bioactive molecules added to the culture medium may improve the performance of chondrocytes suspended and cultured in long-term stable fibrin gels. In addition, amount of FBS in culture medium may be reduced, which represents a step towards clinical application, where autologous supplements are preferred.

Furthermore, this chondrocyte culture system can be used as a test system for delivery devices for bioactive molecules. Approaches in application of proteins still face several problems due to the very short biological half-life of the polypeptides as well as rapid diffusion and cellular uptake [54, 55]. Therefore, sustained release devices of proteins may enhance protein stability and, thus, improve protein application. In our study, crystalline

insulin was encapsulated in and released from lipid microparticles which were suspended within the fibrin gel; this released insulin was shown to enhance development of cartilaginous tissue, indicating release of still active molecules from the particles within the construct. In future studies, release of insulin or other growth factors from microparticles suspended in the gel may also improve cartilage development in vivo.

To conclude, long-term stable fibrin gels were shown to be a powerful material for three-dimensional culture of bovine chondrocytes, enabling diffusion of nutrients and bioactive molecules and formation of cartilaginous tissue, in the same time providing shape stability. Construct size and cell density was shown to strongly influence development of homogenous extracellular matrix. However, a large patient-specific defect may necessitate formation of a larger engineered tissue. Therefore, further investigations have to be conducted regarding optimal cell density in a defined construct size as well as most favorable cultivation time (chapter 4). Furthermore, as insulin was shown to improve development of extracellular matrix of chondrocytes in fibrin gel, other bioactive factors may have to be tested for an advantageous effect on formation of cartilaginous tissue within the fibrin gels. Finally, towards a clinical application, human chondrocytes isolated from small biopsies have to be evaluated in the optimized fibrin gel, which is supposed to be more complex compared to the use of bovine chondrocytes [56-58] (chapter 6).

Acknowledgement

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Chapter 4

Long-Term Stable Fibrin Gels for Cartilage Engineering

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Abstract

Long-term shape stability and mechanical integrity of hydrogel scaffold systems is essential for many applications in tissue engineering. Within this study, we aimed at the optimization of a commercially available fibrin gel in order to develop a long-term stable fibrin gel and, subsequently, investigated the suitability of the optimized gel for in vitro cartilage engineering. Only fibrin gels prepared with a fibrinogen concentration of 50 mg/ml or higher, a Ca^{2+} concentration of 40 mM and a pH between 6.8 and 9 were transparent and stable for three weeks, the duration of the experiment. In contrast, when preparing fibrin gels with concentrations out of these ranges, turbid gels were obtained that shrunk and completely dissolved within a few weeks. In rheological experiments, the optimized gels showed a broad linear viscoelastic region and withstood mechanical loadings up to 10,000 Pa. Bovine chondrocytes suspended in the optimized fibrin gels proliferated well and produced high amounts of GAG and collagen type II. When initially seeding 3 million cells or more per construct (5 mm diameter, 2 mm thick), after 5 weeks of culture, a coherent cartilaginous extracellular matrix was obtained that was homogenously distributed throughout the whole construct. The developed fibrin gels are suggested also for other tissue engineering applications in which long-term stable hydrogels appear desirable.

Introduction

Among the many requirements a scaffold has to fulfill in tissue engineering in order to promote tissue growth, it acts as space filling agent and three-dimensional structure to organize the expanded cells, to maintain a specific shape and structural integrity.

Fibrin glue is a commonly used surgical haemostatic agent and has been commercially available for over 20 years in surgery and clinical practice. In recent years, fibrin has been utilized for different applications in the field of tissue engineering, especially in cartilage tissue engineering, with specific physical and biological requirements [1]. The hydrogel is a polypeptide consisting of the plasma components fibrinogen and thrombin. Physiologically, fibrin formation occurs as the final step in the natural blood coagulation cascade, producing a clot that assists wound healing [2, 3]. Due to the fact, that fibrin is a physiological blood component, it is considered to be biocompatible and biodegradable.

An important fibrin characteristic is an increasing instability and solubility over time in vitro and in vivo, due to fibrinolysis. A fast degradation could be an advantage in wound sealing or other surgical applications as well as for cell and growth factor delivery. However, this can represent a problem for the use as a shape-specific scaffold in tissue engineering [4-7]. Long-term stability and mechanical integrity may be essential for cells that require a certain time and sufficient stiffness to produce their tissue-specific matrix. Also for an earlier implantation after cell-fibrin construct preparation, i.e., to shorten the in vitro culture period, mechanically stronger gels appear desirable. However, the problems of shrinkage, fast dissolution and, thus, lack of shape stability often still exist in tissue engineering approaches. Therefore optimizing fibrin composition would be a fundamental approach to obtain a scaffold system providing optimal shape stability and integrity for specific applications in tissue engineering. It is known that variation of fibrin parameters, such as fibrinogen concentration, thrombin concentration, and ionic strength, can generate gels with different appearance, mechanical properties and stability [8-10], although the exact contributions of each parameter are not fully understood.

Fibrin is widely used in approaches to tissue engineering of cartilage [1]. One major goal is the development of a coherent cartilaginous extracellular matrix. However, scaffold properties alone do not guarantee adequate new tissue development. In particular, cells often require minimum cell contact for optimal cell morphology and behavior. Using a mechanically strong fibrin gel may immure the single chondrocyte inhibiting cell proliferation and migration, and subsequently preventing extracellular matrix development.

It is known that modifying the concentration of fibrinogen and thrombin could affect fibroblast proliferation and migration as well as gene expression in fibrin gels [11]. Therefore, a fibrin system which is optimized with regard to mechanical stability has to be tested for the specific application in cartilage engineering. In particular, cell density has to be adapted to obtain an adequate newly developed tissue. Common cell concentrations vary from 10 to 120 million cells per cubic centimeter [4, 12-14], using different chondrocyte sources and scaffold systems. However, only a few studies investigated the effect of different cell densities on cartilaginous tissue development [15-19].

In this study, we developed long-term stable fibrin gels and investigated them with regard to their suitability for cartilage engineering. First, we investigated specific fibrin gel parameters determining gel stability. Therefore, we modified the concentration of fibrinogen and Ca^{2+} as well as the pH and tested the appearance and the stability of the resultant fibrin gels in chondrocyte culture medium for 3 weeks. Additionally, mechanical properties of the resulting fibrin gels were determined and related to the gel preparation parameters. The optimized fibrin gels were tested as three-dimensional carriers for freshly isolated bovine chondrocytes. In order to obtain an adequate coherent extracellular matrix within the newly developed fibrin gels, we investigated the effects of different initial cell densities on extracellular matrix production and distribution.

Materials and Methods

Materials

Aprotinin solution (Trasylol®) was bought from Bayer (Leverkusen, Germany). Thrombin (as a part of Tissucol®), thrombin dilution buffer and the commercially available fibrin glue Tissucol® was kindly provided from Baxter (Unterschleißheim, Germany). Bovine fibrinogen and calcium chloride were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Knee joints from three-months-old bovine calves were obtained from a local abattoir within 12-18 hours of slaughter. Type II collagenase and papainase were purchased from Worthington (CellSystem, St. Katharinen, Germany). Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, fetal bovine serum (FBS), MEM non-essential amino acid solution, penicillin, streptomycin, N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES buffer) and phosphate buffer solution (PBS buffer) were obtained from Gibco (Karlsruhe, Germany). 149 µm pore size polypropylene filters were purchased from Spectrum (Rancho Dominguez, CA, USA). All cell culture plastics were purchased from Corning Costar (Bodenheim, Germany).

Ascorbic acid, deoxyribonucleic acid, diaminobenzidine, dimethylmethylene blue, glutaraldehyde, glycine, hematoxylin, proline, safranin-O, Tween® 80 and pepsin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Chloramin-T, formalin 37%, and p-dimethylaminobenzaldehyde (p-DAB) were from Merck (Darmstadt, Germany).

Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA) and L-hydroxyproline from Fluka (Neu-Ulm, Germany). Chondroitin sulfate was from ICN (Aurora, Ohio, USA) and Tissue Tek was from Sakura Finetek (Torrane, CA, USA). Vectastain ABC-kit and DAB-kit, and normal horse serum were obtained from Vector Laboratories Inc. (Burlingame, CA, USA). Type II collagen monoclonal antibody was obtained from the development studies Hybridoma Bank and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, USA). Type I collagen antibody (monoclonal anti-collagen type I col-1) mouse ascites fluid was obtained from Sigma-Aldrich (Saint Louis, Missouri, USA).

Preparation of fibrin gels

For improvement of fibrin gel appearance and stability, the concentration of fibrinogen and Ca^{2+} and the pH were modified. To study the influence of fibrinogen concentration, a range of 12.5 to 100 mg/ml purified fibrinogen containing approximately 60 % protein was dissolved in 10,000 KIE/ml aprotinin solution. Fibrinogen solutions were mixed with the same volume of thrombin 5 U/ml in dilution buffer (500 U/ml thrombin in 40 mM $CaCl_2$ diluted 1:100 in dilution buffer containing 40 mM $CaCl_2$) and allowed to gel in a glass ring with an inner diameter of 5 mm for 45 min at 37 °C; the pH of the resultant gels was 7.0. For modification of the Ca^{2+} concentration, fibrinogen solution with a fixed concentration of 100 mg/ml was mixed with the same volume of thrombin 5 U/ml in dilution buffer containing 5 to 40 mM $CaCl_2$ and allowed to gel as described above. For variation of the pH, fibrinogen solution with a fixed concentration of 100 mg/ml was mixed with the same volume of thrombin 5 U/ml at a pH between 6 and 9.5 in dilution buffer containing 40 mM $CaCl_2$. The pH in the thrombin solutions was adjusted using 0.01 M HCl and 0.01 M NaOH. All fibrin gels were covered with 4 ml of chondrocyte culture medium and incubated at 37 °C for 3 weeks. Tissucol® was prepared according to the protocol provided by the supplier. The resultant gels were visually examined to assess turbidity and contraction in size directly after gelling. For determination of shrinkage, fibrin gels were photographed at different points of time and size was digitally measured. A stable gel showed no change in size and shape, whereas an instable gel resulted in decrease in size (partial dissolution) or complete dissolution with no remnants.

Rheological characterization of fibrin gels

For rheological experiments, an AR 2000 rheometer (TA Instruments, Alzenau, Germany) with 20 mm steel plate geometry was used. For fibrin gel preparation, the two liquid components were mixed and then transferred to the lower plate of the rheometer. Solutions were allowed to gel in the measuring gap (1000 µm) at 37 °C for 45 min. To prevent dehydration, the measuring gap was covered with a solvent trap filled with distilled water. For dynamic measurements, an increasing oscillatory stress ranging from 0.1 to 10,000 Pa at a fixed oscillatory frequency of 1 Hz at 37 °C was applied, and the storage (G´) and loss (G´´) moduli were recorded. For static measurements, creep tests were performed applying a constant stress of 100 Pa for 5 min and recording the resulting deformation over time, followed by creep recovery.

Cell culture

Primary chondrocytes were isolated from the surface of the femoral patellar groove using sterile technique. The cartilage was cut into small pieces and enzymatically digested over night in DMEM containing 4.5 g/l glucose, 10 % FBS, 584 mg/l glutamine, 0.1 mM MEM non-essential amino acids, 0.4 mM proline, 50 µg/ml ascorbic acid, 50 U/ml penicillin, 50 µg/ml streptomycin, and 470 U/ml of type II collagenase. The digest was re-pipetted, filtered through a 149 µm filter and washed three times with PBS. The cell number was determined by cell counting using a hemocytometer and an inverted phase-contrast microscope. Based on the results from gel development, fibrin gels prepared with 100 mg/ml fibrinogen in 10,000 KIE/ml aprotinin solution and 5 U/ml thrombin in dilution buffer containing 40 mM $CaCl_2$ were used. 0.5, 1, 2, 3, 4 or $5*10^6$ freshly isolated chondrocytes were resuspended in fibrinogen solution; subsequently gels were prepared adding the same volume of thrombin in a stabilizing glass ring to obtain a fibrin disc of 5 mm diameter and 2 mm thickness (40µl gel). The fibrin gels were allowed to gel for 45 min at 37 °C before removing the glass ring and covering the cell-fibrin constructs with 4 ml culture medium. Complete chondrocyte medium consisted of DMEM containing 4.5 g/l glucose, 5 % FBS, 584 mg/l glutamine, 0.1 mM MEM non-essential amino acids, 0.4 mM proline, 50 µg/ml ascorbic acid, 50 U/ml penicillin, 50 µg/ml streptomycin, and was replaced three times per week. The constructs were statically cultured in 6-well-plates in an incubator at 37 °C, 5 % CO_2 and 95 % humidity, and harvested after 2 and 5 weeks, respectively.

Analysis of cartilaginous constructs

The cell-fibrin constructs were analyzed as previously described [13, 20]. The constructs were weighed (= wet weight) and cut in half.

In brief, one part of the construct was lyophilized, then digested with 1 ml of a papainase solution (3.2 U/ml in buffer) for 18 h at 60 °C and used for the determination of cell number, collagen content, and glycosaminoglycan content. The number of cells per cell-fibrin construct was assessed from the DNA content using Hoechst 33258 dye and a conversion factor of 7.7 pg DNA per chondrocyte [21]. The amount of sulfated glycosaminoglycans was determined spectrophotometrically at 525 nm as chondroitin sulfate using dimethylmethylene blue; bovine chondroitin sulfate was used as

standard [22]. The hydroxyproline content was determined spectrophotometrically after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramin-T [23]. The amount of total collagen was calculated using a hydroxyproline to collagen ratio of 1:10 [24].

A part from the cell-fibrin construct was prepared as a histological sample by fixing in 2 % glutaraldehyde in PBS for 30 min and then storing in 10 % formaldehyde in PBS. The formalin-fixed samples were embedded in paraffin and cross-sectioned into 5 µm sections; deparaffinized sections were stained with hematoxylin, fast green and safranin-O [25].

A part of the cell-fibrin constructs were prepared for immunohistochemical staining by fixing in methanol-formalin mixture, and then successively dehydrated in different concentrations (10 % - 40 %) of a sucrose solution followed by embedding into Tissue Tek. Frozen samples were cryosectioned at 7 µm and stained with antibodies for type I collagen (monoclonal anti-collagen type I col-1) mouse ascites fluid, dilution (1:2000), and type II collagen (DSHB), dilution (1:6). To prevent non-specific antibody binding samples were incubated with 5 % normal horse serum in buffer. The sections were then incubated overnight at room temperature with the primary antibodies; PBS without antibodies was used for control sections. On the next day, sections incubated with biotinylated secondary antibody, anti-mouse/rabbit IgG (Vector Laboratories; Inc.; Burlingame, CA, USE), for 30 min at room temperature. Finally, the samples were stained using a Vectastain ABC-kit and DAB-kit according to the protocol for avidin-biotin-peroxidase complex formation.

Statistical analysis

Biochemical data are expressed as means ± standard deviation. Statistical significance was assessed by one-way analysis of variance ANOVA in conjunction with multiple comparison Tukey’s studentized range test at a level of $p < 0.01$.

Results

Fibrin gel appearance and stability

Variation of fibrin gel parameters resulted in gels of distinctly different appearance. With regard to fibrinogen, only gels prepared with fibrinogen concentration of 50 mg/ml or higher had a transparent appearance (Fig. 1A; Tab. 1A) and were stable in chondrocyte culture medium for the 3 weeks of experimental time (Fig. 1B; Tab. 1A). Gels prepared with fibrinogen concentration lower than 50 mg/ml appeared turbid directly after gelling, shrunk and almost completely dissolved within three weeks (Fig. 1A, Tab. 1A). With regard to Ca^{2+}, only a concentration of 40 mM $CaCl_2$ resulted in transparent and stable gels (Tab. 1B). Compared to these gels, fibrin prepared with $CaCl_2$ concentration lower than 40 mM appeared turbid and unstable (Tab. 1B). Finally, only fibrin gels prepared with a thrombin solution of a pH between 6.8 and 9 had the described transparent appearance and were stable in culture medium for 3 weeks (Tab. 1C). Using a thrombin solution with pH lower than 6.8 or higher than 9 resulted in turbid fibrin gels that shrunk and almost completely dissolved within three weeks.

Besides the gels with varied fibrinogen and Ca^{2+} concentration and pH, commercially available fibrin glue was assessed. The gel also had a turbid appearance, shrunk, and dissolved over time (Fig. 1B, Tab. 1A).

In general, all gels that were turbid after preparation shrunk during the first few days and large parts dissolved within the three weeks of experimental time. In contrast, all transparent gels appeared stable and maintained their specific shape during the experiment.

Rheological characterization of fibrin gels

Fig. 2 plots the storage modulus (G´) and the loss modulus (G´´), representing an elastic and viscous behavior of the system, respectively, of fibrin gels prepared with different concentrations of fibrinogen versus the applied oscillatory stress (0.1-10,000 Pa). In general, G´ of all gels were distinctly higher compared to G´´, which indicated a highly elastic dominated behavior of the gel system. With regard to single gels, moduli of fibrin gels prepared with a fibrinogen concentration of 100 mg/ml remained constant up to an oscillatory stress of 300 Pa, indicating a broad linear viscoelastic region. Applying higher stresses, the moduli increased until the structure broke down. Gels prepared with decreasing fibrinogen concentrations exhibited a narrower linear viscoelastic region. In

gels prepared with 12.5 mg/ml fibrinogen, G´ and G´´ already increased at approx. 2 Pa, thus, at 400 Pa the structure broke down. Additionally, G´ and G´´ are much lower in gels of 12.5 mg/ml fibrinogen (60 Pa and 4 Pa, respectively) compared to 100 mg/ml fibrinogen (3000 Pa and 80 Pa, respectively). Furthermore, the optimized fibrin gel (100 mg/ml fibrinogen, 40 mM Ca^{2+}, pH 7) was compared to gels prepared with a Ca^{2+} concentration of 5 mM or a pH of 6. As illustrated in Fig. 3, a stress sweep of all the gels showed a similar viscoelastic behavior (Fig. 3A). While the absolute values of G´ differed only slightly, G´´ seemed to be increased for gels prepared with Ca^{2+} concentration of 5 mM and pH of 6, which indicated a slightly higher irreversible behavior of these gels. Creep experiments of these turbid gels (Ca^{2+} 5 mM or pH 6) showed an increase in strain, whereas the optimized transparent fibrin gel remained relatively constant over the 5 min of stress time (Fig. 3B). Additionally, creep recovery tests revealed an almost complete reversible deformation of the optimized fibrin gels, whereas gels with 5 mM Ca^{2+} or pH 6 recovered only in parts, thus, the recovery of its original state was not reached.

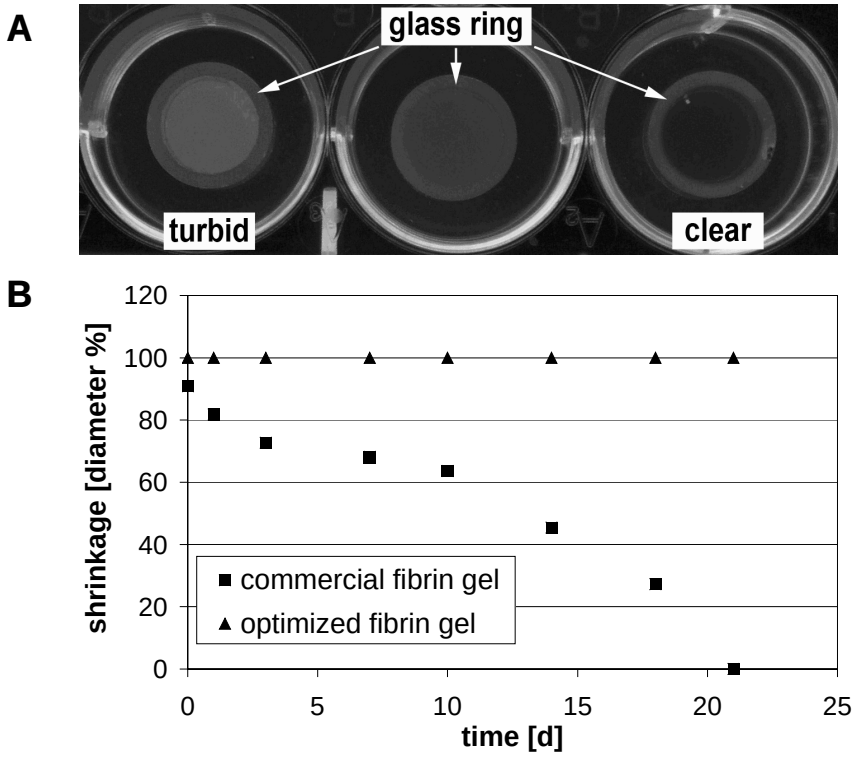

Fig. 1: (A) Turbidity of fibrin gels prepared with 12.5 (left), 25 (middle) and 100 (right) mg/ml fibrinogen in 10,000 KIE/ml aprotinin and 5 U/ml thrombin in dilution buffer containing 40 mM $CaCl_2$, in a stabilizing glass ring. (B) Shrinkage of optimized fibrin gel (▲) compared to commercial fibrin glue (■) in cell culture medium.

A

Components	Variation Fibrinogen [mg/ml]	Appearance		
		after gelling	after 1 week	after 3 weeks
Fibrinogen as specified Thrombin 5U/ml $CaCl_2$ 40mM pH 7.0	12.5	turbid, some contraction	turbid, partially dissolved	dissolved
	25	turbid, some contraction	turbid, partially dissolved	almost completely dissolved
	50	transparent	stable, transparent	stable, transparent
	100	transparent	stable, transparent	stable, transparent
Commercial fibrin glue $CaCl_2$ 40mM pH 7.4	70-110	turbid, some contraction	turbid, some dissolution	dissolved

B

Components	Variation $CaCl_2$ [mM]	Appearance		
		after gelling	after 1 week	after 3 weeks
Fibrinogen 100 mg/ml Thrombin 5U/ml $CaCl_2$ as specified pH 7.0	5	turbid, some contraction	turbid, partially dissolved	dissolved
	20	turbid	turbid, partially dissolved	almost completely dissolved
	40	transparent	stable, transparent	stable, transparent

C

Components	Variation pH	Appearance		
		after gelling	after 1 week	after 3 weeks
Fibrinogen 100 mg/ml Thrombin 5U/ml $CaCl_2$ 40mM pH as specified	6	extremely turbid, some contraction	turbid, partially dissolved	dissolved
	6.5	turbid, some contraction	turbid, partially dissolved	dissolved
	6.8	transparent	stable, transparent	stable, transparent
	7	transparent	stable, transparent	stable, transparent
	7.4	transparent	stable, transparent	stable, transparent
	8.25	transparent	stable, transparent	stable, transparent
	9	transparent	stable, transparent	stable, transparent
	9.5	turbid, some contraction	turbid, partially dissolved	dissolved

Tab. 1: Gel appearance and stability in cell culture medium; effect of fibrinogen concentration (A), Ca^{2+} (B) and pH of fibrin gel (C).

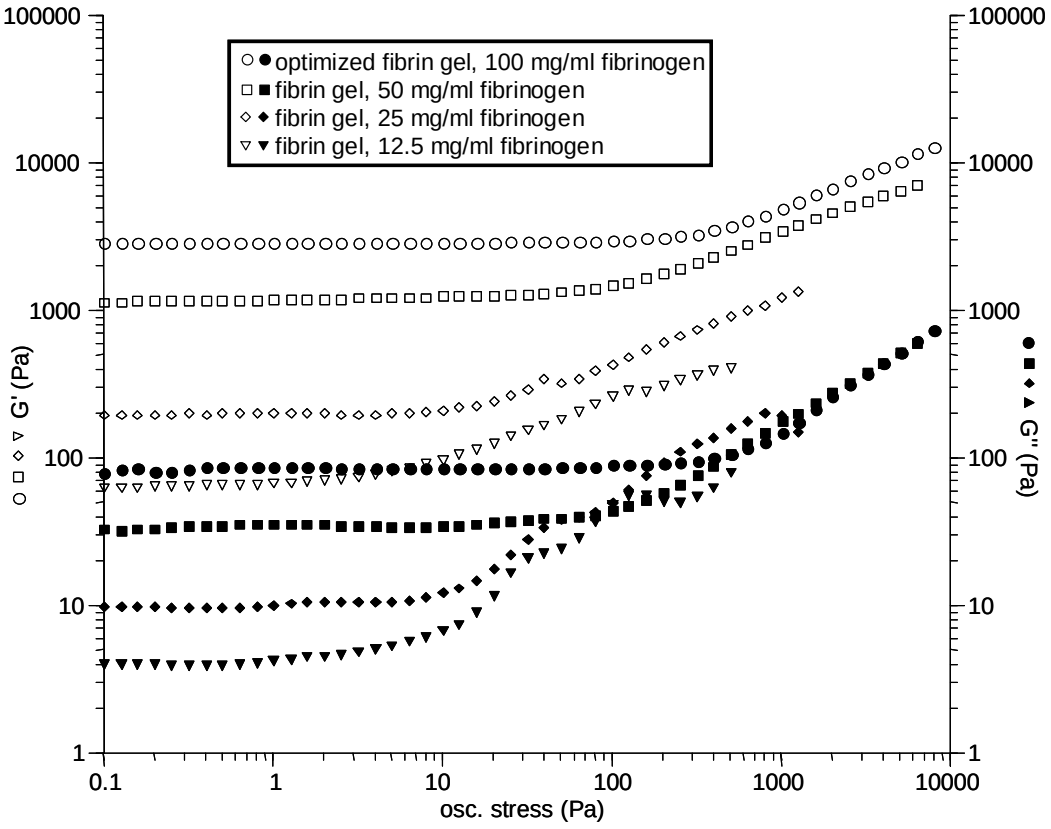

Fig. 2: Storage (G´) and loss (G´´) modulus of fibrin gels prepared with different fibrinogen concentrations versus oscillatory stress.

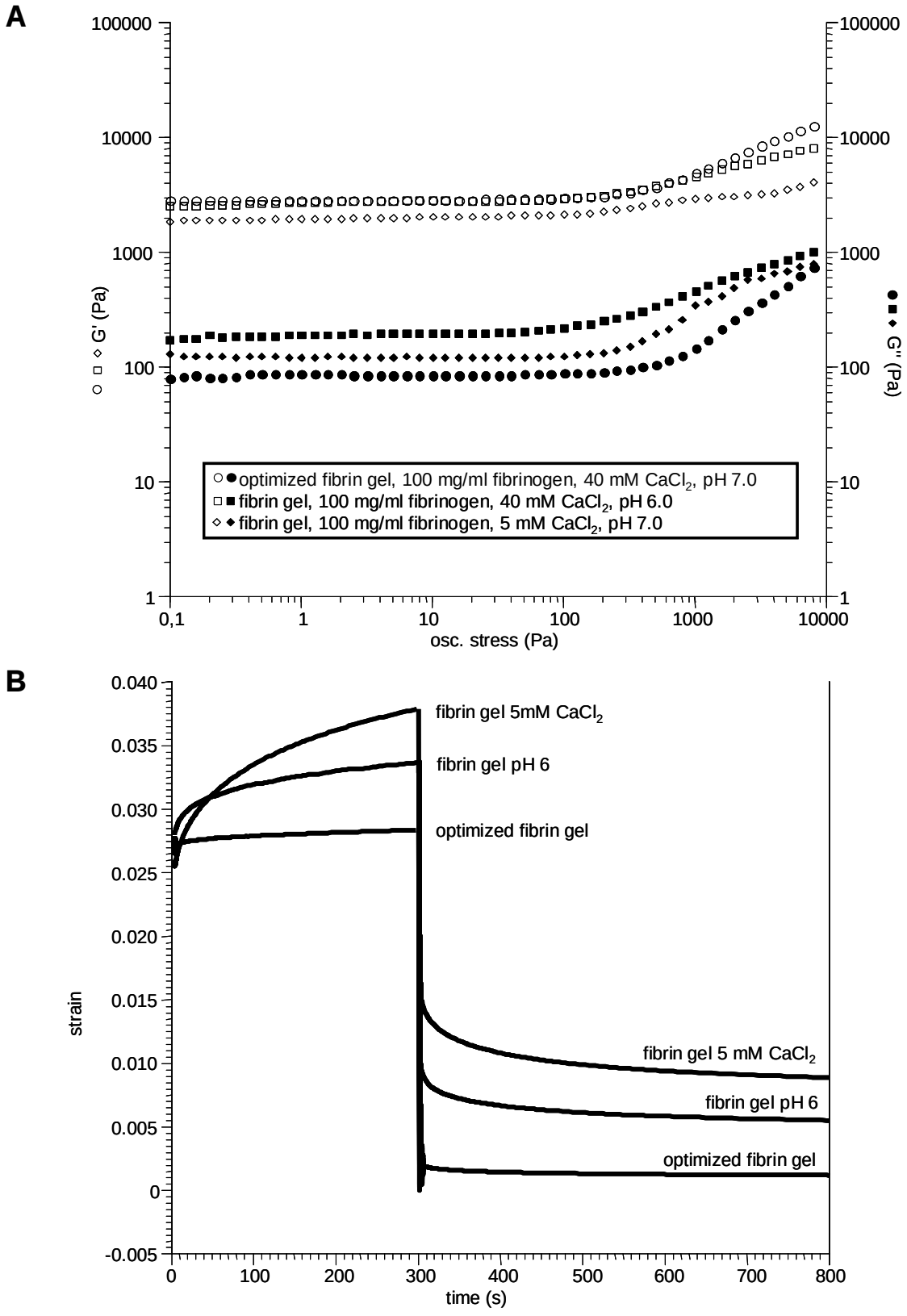

Fig. 3: (A) Storage (G´) and loss (G´´) modulus of optimized fibrin gel compared to fibrin gel prepared at pH 6 and fibrin gel prepared with 5 mM Ca^{2+} versus oscillatory stress. (B) Deformation and recovery of optimized fibrin gels compared to fibrin gels prepared at pH 6 and fibrin gel prepared with 5 mM Ca^{2+} versus experimental time.

Investigations toward a coherent cartilaginous tissue within the optimized fibrin gel

Primary chondrocytes in the long-term stable fibrin gel cultured in medium with 5 % FBS maintained a round shape during the study and produced high amounts of extracellular matrix containing GAG and collagen (Fig. 4). However, variation of initial cell density resulted in cartilaginous tissues of different appearance. In general, increasing initial cell number resulted in an increase in wet weight of the constructs within the culture period. Already after two weeks, wet weight of the constructs with 5 million seeded cells were 1.8-fold larger compared to 0.5 million constructs; the increase was 4.9-fold after 5 weeks (Fig. 4A).

The cell number after 2 and 5 weeks of culture increased with increasing initial cell seeding density (Fig. 4B). After 5 weeks, using an initial high seeding density (5 million cells) resulted in a 4.7-fold higher absolute cell number compared to the lowest seeding density (0.5 million cells) (Fig. 4B), though the cell number relative to the number of seeded cells remained lower (1.8-fold) compared to the value at the lowest seeding density (3.8-fold).

Biochemical analysis showed an increase in GAG and total collagen content per wet weight with increasing cell number after 2 weeks of culture (Fig. 4C and 4D). After 5 weeks of cultivation, fractions of ECM components approached constant maximum values when 3 million cells or more were seeded, i.e., approx. 4.5 % GAG and 6 % total collagen per wet weight.

Histology showed the development of cartilaginous tissue of different quality and homogeneity. The different GAG fractions were reflected in histological cross-sections of the cell-fibrin constructs stained red with safranin-O (Fig. 5). After 2 weeks of culture, cells were encircled by distinct areas containing ECM stained red with safranin-O. However, constructs with an initially seeded cell number of 4 million and higher resulted in a more homogenous GAG distribution already after 2 weeks (Fig. 5). When cultivated for 5 weeks, a cell number of 2 million or lower still resulted in cells encircled by unconnected areas containing ECM; thus, a coherent cartilaginous tissue was not obtained within these groups. In contrast, an initial cell number of 3 million and higher resulted in an adequate coherent GAG distribution throughout the whole construct (Fig. 5).

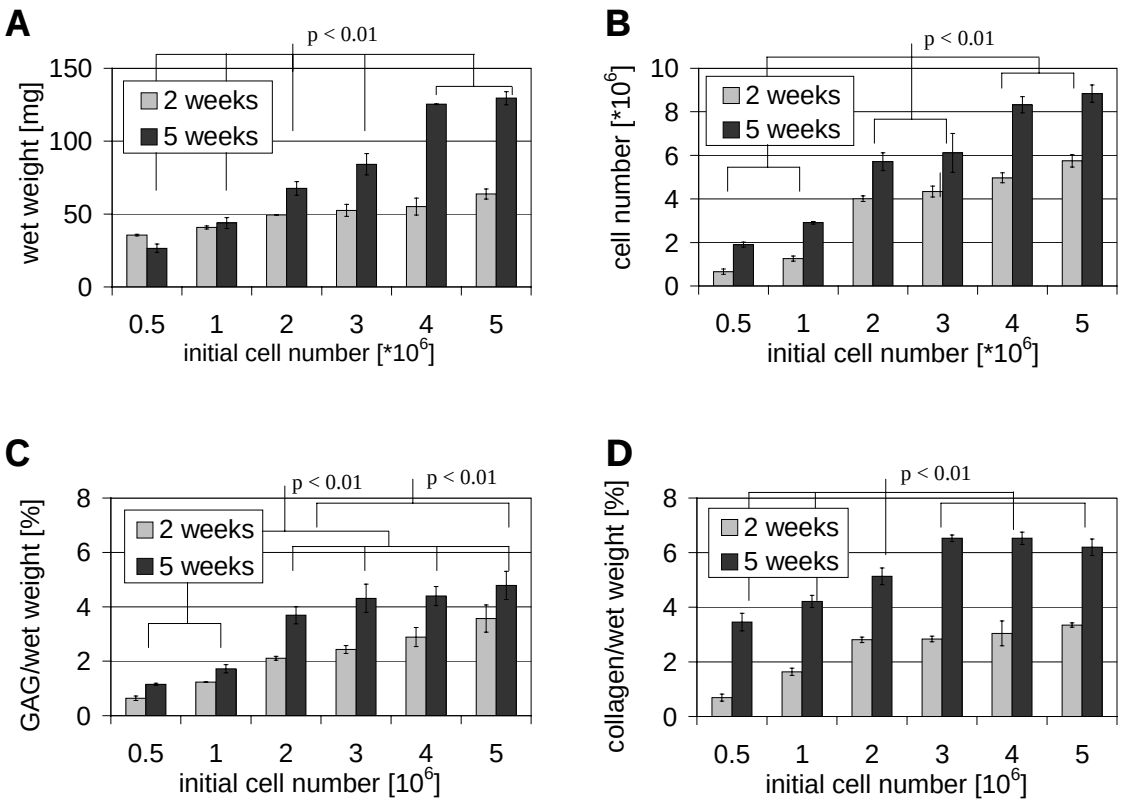

Fig. 4: Effect of initial cell number on wet weight (A), cell number (B) as well as GAG (C) and collagen (D) fraction per wet weight of gels cultured for 2 and 5 weeks. Data represent the average ± standard deviation of three independent measurements. Statistically significant differences between gels prepared with different initial cell numbers are denoted with $p < 0.01$.

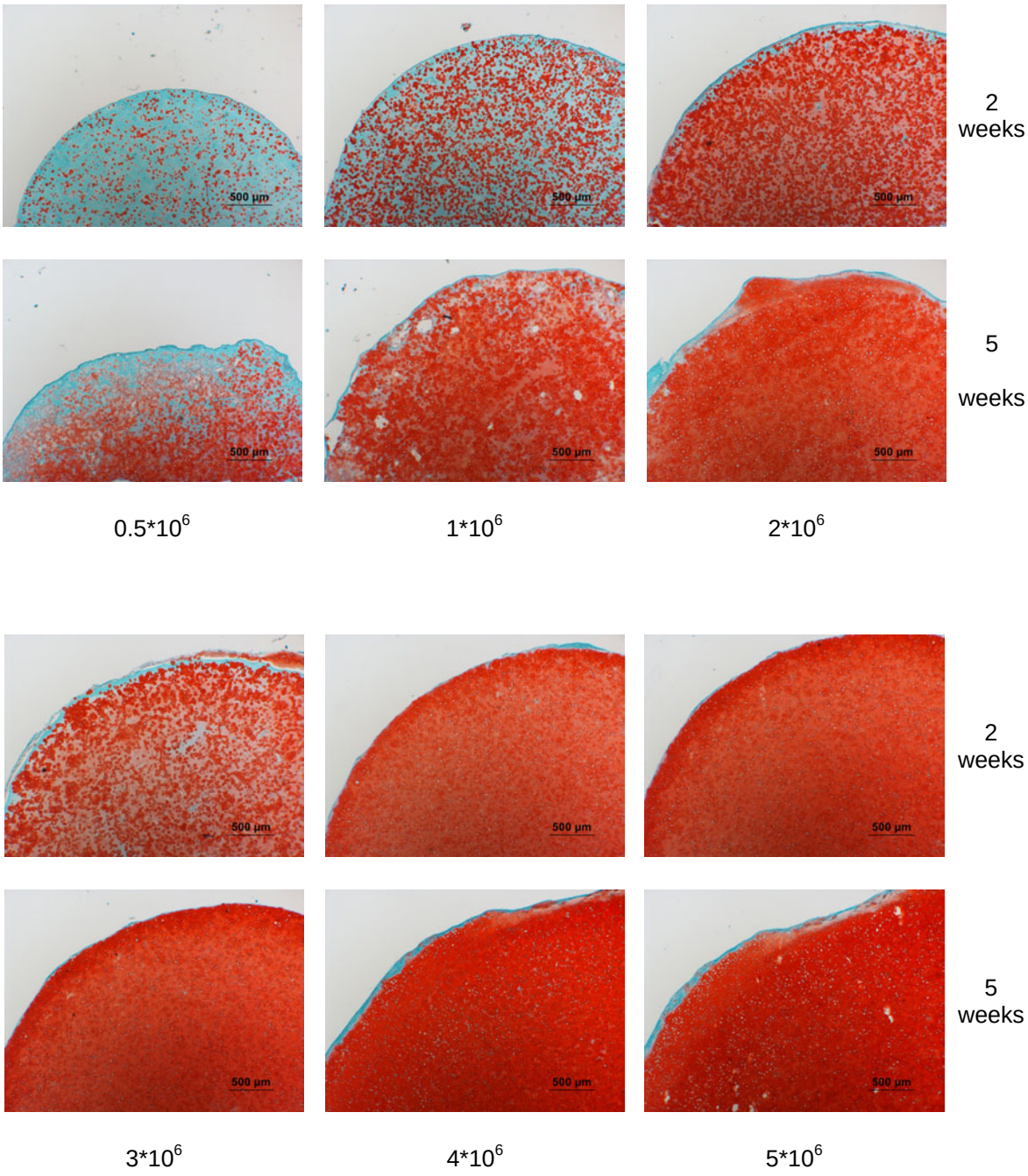

Fig. 5: Histological cross-sections of 2- and 5-week gels prepared with different initial cell numbers ($0.5*10^6$ – $5*10^6$), stained red for glycosaminoglycans (GAG) with safranin-O.

Immunohistochemical cross-sections of constructs with an initial cell number of 3 million or higher were strongly stained for cartilage-specific collagen type II throughout the construct (Fig. 6). Undesired collagen type I was found mainly in a thin area at the edge of the construct. In contrast, similar staining for collagen type I and type II were found throughout the whole cross-sections of constructs prepared with an initial cell number of 2 million.

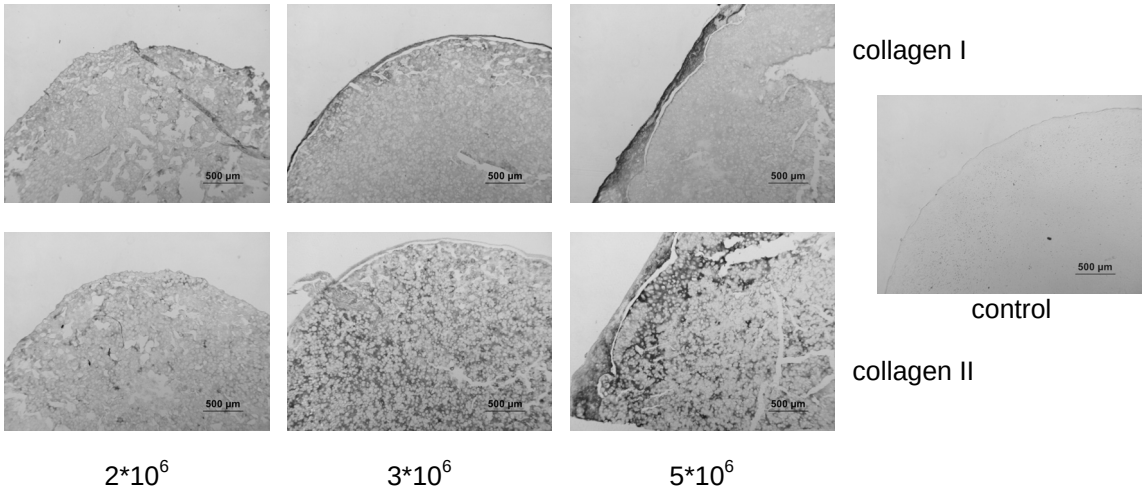

Fig. 6: Immunohistochemical cross-sections of 5 week gels with an initial cell number of $2*10^6$ (left), $3*10^6$ (middle) and $5*10^6$ (right).

Discussion

Fibrin gels are potentially attractive scaffolds for use in tissue engineering, however, their use is often impaired by fast dissolution and lack of shape stability. In this study, we determined specific fibrin gel preparation parameters that influence gel stability and shape integrity. Variation of fibrinogen concentration, Ca^{2+} concentration and pH resulted in gels of distinctly different appearance. In general, gels prepared with fibrinogen concentration lower than 50 mg/ml, Ca^{2+} concentration lower than 40 mM and pH lower than 6.8 or higher than 9 appeared turbid directly after preparation, shrunk during the first few days and almost completely dissolved within a few weeks. In contrast, gels prepared with fibrinogen concentration of 50 mg/ml or higher, Ca^{2+} of 40 mM as well as pH between 6.8 and 9 had a transparent and stable appearance and maintained their shape during the experimental time. Based on these results, for application in cartilage tissue engineering, a preparation of fibrinogen solution containing 100 mg/ml in 10,000 KIE/ml aprotinin and 5 U/ml thrombin in dilution buffer containing 40 mM $CaCl_2$ (pH 7.0) was used.

Development of long-term stable fibrin gels

A material that is intended for the use as a scaffold in tissue engineering has to provide an environment enabling adequate cellular function, e.g., cell migration, proliferation, and differentiation, and must allow for tissue development. Additionally, shape stability is an important factor for specific clinical applications, e.g., for regeneration of the auricle. Here, long-term scaffold stability is necessary to provide enough time for cells to proliferate and to produce their specific matrix, thus, enabling the maintenance of the specific shape. Fibrin is a versatile material that is used in various fields in surgery and clinical practice as well as in the field of tissue engineering [1]. However, commercially available fibrin sealants tend to shrink and disintegrate in vitro and in vivo after a few days and almost completely dissolve within 3 to 4 weeks [2, 8, 10]. Therefore, their use is prevented in many applications in tissue engineering.

One major approach is the use of fibrinolysis inhibitors, mostly protease inhibitors such as aprotinin or tranexamic acid that are added to the fibrin gel and/or as a supplement to the cell culture medium. They can help slow down degradation and, thus, stabilize fibrin gel shape [4-7]. An alternative is the combination of fibrin gels with polymeric scaffolds for improved biomechanical strength and volume stability [26-28]. However, these approaches cannot completely resolve the problem of shrinkage, fast dissolution and lack of shape

stability of the gel. On the other hand, it is known that a variation of fibrin parameters such as concentration of fibrinogen and thrombin, and ionic strength, enormously influence gel appearance, mechanical properties and stability [8-10]. Much work has been done to investigate the effects of various aspects of fibrinogen and fibrin in the past 30 years, e.g., modifications and additives on the clotting process in vitro [2, 29-33]. However, many important questions remain still unsolved.

Ferry et al. first reported about two different types of fibrin gels, coarse and fine clots [34, 35]. It is known that fine gels consist of a high number of thin and branched fibrin fibers built of protofibrils, resulting in transparent and rigid gels with small pores. In contrast, coarse gels are made of thicker fibers due to lateral aggregation of protofibrils, which result in turbid and loose gels with larger pore size. This lateral aggregation can be influenced by several parameters including enzyme and substrate concentration as well as pH and ionic strength of the solutions [2, 29].

At very low fibrinogen concentrations, increasing concentrations in the range of 0.5 to 1.5 mg/ml have been reported to result in increase in turbidity, which was attributed to large fiber diameter and pore size [29, 36]. In contrast, it has also been reported that at higher fibrinogen concentration in the range of 1.5 mg/ml to 23.4 mg/ml the opposite effect occurred; increasing fibrinogen concentration resulted in finer and more rigid gels [34]. We also found, within a concentration range of 12.5 to 100 mg/ml, transparent and long-term stable gels at high concentrations and turbid and unstable gels at low concentrations. With regard to variation in thrombin, it is known that only low concentrations in the range of 0.001 to 1 U/ml influences gel structure [29]; concentrations in the order of magnitude of the one we used in our experiment have no effect on gel appearance, only on speed of polymerization.

Repeatedly, differences between turbid and transparent fibrin gels have been interpreted in terms of ionic strength effects and electrostatic forces [34, 37], in particular influenced by calcium ions that were suggested to increase gel turbidity (5 mM Ca^{2+} compared to no Ca^{2+}) [38]. In our study, increasing Ca^{2+} from 5 to 40 mM resulted in more transparent, i.e., in increasingly rigid and stable gels.

With regard to the effects of pH, in our experiments, fine and stable gels were obtained between pH 6.8 and 9; gels outside of this range were turbid and became unstable. Well in agreement with our results, Ferry et al. showed an increase in fibrin turbidity when decreasing the pH from 7.4 to 6.3, using fibrinogen concentrations in the range of 1.5 - 23.4 mg/ml, however, pH higher than 7.4 was not analyzed [34].

When comparing the investigated parameters fibrinogen concentration, Ca^{2+} concentration, and pH of the stable gels in our study (50-100 mg/ml fibrinogen, 40 mM Ca^{2+}, pH 6.8-9) with those of the commercially available fibrin glue (70-110 mg/ml fibrinogen, 40 mM Ca^{2+}, pH 7.4), no differences were observed. It can be concluded that at least one other parameter must contribute to the distinct differences in appearance and stability. One possible explanation may be a difference in chloride ion concentrations. Whereas in both types of gels 40 mM Cl^- resulted from the supplementation of the thrombin solution, all stable gels received additional chloride ions at concentrations of 107.5 mM (50 mg/ml fibrinogen) or of 215 mM (100 mg/ml fibrinogen), resulting from the use of purified fibrinogen stabilized with 25 % sodium chloride. This would be in agreement with a report by Di Stasio et al. [39]; binding of chloride ions were shown to oppose the lateral aggregation of protofibrils and resulted in thinner fibers and, thus, more rigid and transparent gels. An almost complete inhibition of lateral aggregation was found around the concentration of 200 mM. Other possible explanations for the differences in gel appearance in our study include additives within the commercially available fibrin glue that are unknown to the authors, e.g., originating from plasma preparation.

Taking together the review of the literature and the results of our study, a single preparation parameter obviously is not responsible for gel turbidity and stability, rather a combination of different parameters. However, the interaction of the preparation parameters is still far from being fully elucidated and, thus, many investigations have to be done to fully understand fibrin clot formation. At least, as a result of the current study, ranges for certain parameters could be identified in which the production of long-term stable fibrin gels is possible.

All transparent and rigid fibrin gels developed in this study were stable in cell culture medium in vitro for at least three weeks (duration of the experiment). Furthermore, optimized gels were observed to be stable for at least 12 months, even without fibrinolysis inhibitors in medium (data not shown). Collet et al. found out that fine fibrin gels are dissolved at a slower rate than coarse gels though thin fibers are cleaved at a faster rate than thick ones [40-42]. This was attributed to the enormously higher number of thin fibers in fine fibrin gels compared to the lower number of thick fibers in coarse gels. Additionally, Collet et al. reported about a broader recombinant tissue plasminogen activator (rt-PA) binding front in coarse fibrin gels that moved more rapidly compared to the binding front in fine fibrin gels. Therefore, besides the thickness of the fibers, fibrin network architecture appears to plays an important role in fibrinolysis.

The mechanical properties of fibrin have been measured for many different types of clots under various conditions [37, 43, 44]. Fibrin gels have remarkable and unique viscoelastic properties, though the origin of these properties is still unknown. Weisel et al. investigated viscoelastic properties of different types of fibrin gels in dependence of the oscillatory frequency. At low frequencies, fibrin gels showed an elastic dominated behavior, while at high frequencies, the viscous response dominated [45]. These data are well in agreement with the results obtained in our experiments applying an oscillatory stress. Moduli of fibrin gels prepared with 100 mg/ml fibrinogen showed a broad linear viscoelastic region up to an oscillatory stress of approx. 300 Pa. However, both moduli increased as the strain increased to 300 Pa and higher, indicating some sort of 'stress hardening' of the structure. This fibrin behavior is known but still not fully understood [45]. Decreasing the fibrinogen concentration also decreased the absolute values of G´ and G´´ indicating a reduced stiffness of the gels. Structure of these gels broke down already at lower mechanical stress, which likely prevents them from use in many applications in tissue engineering. Interestingly, fibrin gels prepared with high fibrinogen concentration of 100 mg/ml, but in contrast to the optimized gels at pH 6 or a Ca^{2+} concentration of 5 mM resulting in the described turbid gels, showed similar viscoelastic properties in dynamic experiments, compared to the optimized fibrin gels. However, statical creep tests indicated distinct differences between these gels. Turbid gels showed a more viscous dominated behavior, which is likely due to the existence of thick, weakly cross-linked protofibrils. These fibers tended to flow under applied forces and show more irreversible deformation than transparent gels, attributed to slippage of protofibrils past each other [45]. In contrast, transparent fibrin gels showed an almost ideal elastic behavior, which is likely due to thinner fibers resulting in a more densely cross-linked network withstanding higher mechanical loading.

Utilization of the optimized fibrin gel for cartilage engineering

A common application of fibrin is tissue engineering of cartilage [1]. However, using a stable fibrin gel that maintains its shape throughout a specific time does not necessarily permit the development of a homogenous cartilaginous tissue of high quality. A rather strong gel may immure the single chondrocytes preventing cell migration and proliferation as well as extracellular matrix production and distribution [11, 46, 47]. One goal of this study was to investigate the production and distribution of cartilaginous ECM within the

newly developed optimized stable fibrin gel; the quality of cell-fibrin constructs were assessed in dependence of the initially seeded number of cells. The use of the fibrin gels as scaffold material resulted in cells that remained round and vital within 5 weeks in vitro. In all groups distinct cell proliferation was detected disproving the possibility that a strong fibrin gel would immure the single chondrocyte. This was also demonstrated by the production of high amounts of extracellular matrix. However, histology of the engineered constructs demonstrated enormous differences in quality of the tissues depending on initial cell density. Safranin-O stain revealed the need of at least 3 million cells per 40 µl construct to obtain an engineered tissue with adequate glycosaminoglycan production and homogenous matrix distribution throughout the whole construct after the 5 weeks of experimental time. Furthermore, constructs prepared with a cell density of 3 million cells per construct or higher were strongly stained for collagen type II throughout the whole construct, which is a major cartilage-specific extracellular matrix protein and indicates adequate cartilaginous tissue development. Undesired collagen type I was mainly found at the edge of the cell-fibrin constructs. This is a phenomenon well documented also in other tissue engineering studies, which indicates a limited number of dedifferentiated chondrocytes in a small area at the surface of the constructs [13, 20]. In contrast, for constructs with low initial cell number (< 3 million cells/construct) isolated cells were detected which were encircled by small amounts of unconnected extracellular matrix. In such constructs, staining for collagen type II was distinctly weaker than in the constructs with high cell density; similar staining for collagen type I and type II throughout the whole construct indicated a higher number of dedifferentiated chondrocytes likely due to low cell contact or impaired cell-cell signaling. Such a tissue is supposed not to tolerate strong mechanical forces, which occur during the process of implantation as well as in the patient's defect after implantation. Quantitative analysis of glycosaminoglycans and total collagen was well in agreement with the histological differences between the cell-fibrin constructs. Using an initial high cell density (3 million cell per construct or more) resulted in strong tissue growth reflected by wet weight and high fractions of GAG (approx. 4.5 %) and total collagen (approx. 6 %).

It is well known that cell density is an important parameter in the development of an appropriate engineered cartilaginous tissue. Mauck et al. cultured primary bovine chondrocytes in agarose hydrogels and found an enhanced tissue growth using higher seeding densities (60 million cells/ml) compared to lower densities (10 million cells/ml) in vitro [15]. The effective densities in our experiments were well in agreement with that

study; in our study a cell density of 75 million cells/ml or more (3 million cells per constructs) led to appropriate ECM development and to strongly improved constructs compared to those with an initial density of 12.5 million cells/ml (0.5 million cells per constructs). As an alternative, several studies have investigated the effect of cell concentration on matrix development after injection of a cell suspension or implantation of constructs subcutaneously in vivo. Analyzing histology and extracellular matrix components, Silverman et al. determined best cartilage tissue development using 40 million/ml porcine articular chondrocytes suspended in fibrin glue and injected subcutaneously into nude mice, compared to using lower cell densities of 10 and 25 million/ml [48]. Furthermore, Paige et al. proposed the use of at least 5 million primary bovine chondrocytes/ml in 1.5 % alginate for implantation in vivo [18]. Finally, Puelacher et al. reported about a correlation between initial cell number and the final thickness and weight of the engineered tissue [17]. They proposed variation of cell density before in vivo implantation to control the desired shape, thickness and quality of the developed cartilage.

Within this study, we demonstrated in principle the suitability of the newly developed fibrin gels for cartilage engineering. Cultivation of primary bovine chondrocytes within these gels resulted in development of an adequate cartilaginous tissue. It still has to be proven that these results can be transferred to harder-to-culture human adult chondrocytes, which need a longer time to proliferate and to synthesize ECM components, and for which special care has to be taken that an adequate cartilaginous matrix is produced. However, the presented fibrin gels feature necessary long-term stability that may provide sufficient time and may facilitate formation of cartilaginous tissue also with human chondrocytes. Furthermore, these fibrin gels, which are regarded as an improvement over currently available and widely used fibrin preparations, are suggested also for other applications in tissue engineering in which biocompatible, long-term stable hydrogels are desired.

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Chapter 5

Proliferation on a Fibrin Surface Enhances the Potential of Expanded Chondrocytes to Generate Engineered Cartilage

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Abstract

Small biopsies from human cartilage result in only a limited number of cells, therefore, in vitro expansion is often necessary to obtain a sufficient number of cells for a feasible application in the field of tissue engineering. However, during proliferation chondrocytes in monolayer rapidly dedifferentiate from cartilaginous phenotype towards fibroblast-like phenotype accompanied by production of inadequate extracellular matrix molecules. Therefore, in this study, a long-term stable fibrin gel was tested for the use in the proliferation of primary bovine chondrocytes, with specific regard to its potential to retain the ability of the thus expanded chondrocytes to form engineered cartilage. Cells were three-dimensionally seeded into these gels and cultured for 4 weeks in vitro in medium containing 10 % FBS, which resulted in formation of adequate cartilaginous tissue. Interestingly, some of the cells were found to migrate out of the fibrin gels onto the surface of the gel, proliferate, and form a thick and compact three-dimensional cell sheet within 4 weeks in culture. Histological staining demonstrated the presence of high amounts of newly developed homogenously distributed extracellular matrix within the new cell sheet, stained red with safranin-O. When re-isolated from the cell sheet and re-seeded into fibrin gels, these chondrocytes were found to produce dramatically higher amounts of extracellular matrix components glycosaminoglycan and total collagen, compared to cells expanded in monolayer on tissue-culture plates, determined by histological staining and biochemical analysis. These results indicate a reduced dedifferentiation of the cells and/or an enhanced redifferentiation of dedifferentiated cells, that had proliferated on fibrin gels. Therefore, long-term stable fibrin may represent a promising culture system to improve proliferation of chondrocytes with regard to the retention of their potential to generate engineered cartilage.

Introduction

Common cartilage tissue engineering approaches use high numbers of chondrocytes seeded onto biomaterials to enable adequate production of extracellular matrix homogenously distributed within a three-dimensional scaffold system [1-3]. For a feasible clinical application, the use of autologous chondrocytes would be beneficial to avoid disease transmission or immune rejection, as well as ethical discussions. However, small biopsies result in only a limited number of fully differentiated chondrocytes [4]. Therefore, these isolated cells are commonly expanded in two-dimensional monolayers on a conventional plastic surface to obtain a sufficient number for seeding onto scaffolds. However, during monolayer culture, chondrocytes lose their differentiated phenotype as the culture process progresses, and stop synthesizing cartilage-specific collagen type II and aggrecan, but increase producing molecules characteristic for a more fibroblast-like phenotype, e.g. collagen type I [5-8]. The matrix thus synthesized will have inferior mechanical properties and, therefore, cannot fulfill its physiological function.

It is known that the culture material surface has enormous influence on adhesion and migration of cells. Coating of culture surfaces with extracellular matrix proteins, e.g. collagen I, collagen VI, fibrinogen or aggrecan, support cell attachment via specific integrins located on the cell membrane, compared to conventional culture surfaces [9-13]. In preliminary experiments, coating of cell culture plastics with fibrin gel was found to enormously enhance adhesion (Fig. 1) and proliferation of primary bovine chondrocytes, compared to seeding onto conventional cell culture surface. Furthermore, various studies have been conducted to multiply chondrocytes in monolayer cultures and then transfer them into a long-term, high density, three-dimensional culture system to regain their phenotype. Three-dimensional scaffold systems like alginate, agarose, collagen I, collagen II, or hyaluronic acid have been reported to enhance redifferentiation capacity of these dedifferentiated chondrocytes back to a chondrocyte phenotype [14-18].

In recent years, fibrin glue has been utilized for different applications in the field of tissue engineering with specific physical and biological requirements [19]. Due to the fact that fibrin is a physiological blood component, it is considered to be biocompatible and biodegradable [20]. However, an important fibrin characteristic is an increasing instability and solubility over time in vitro and in vivo, which could be a problem for the application in tissue engineering [21-23]. Therefore, in modifying specific fibrin gel components our

group has previously developed a long-term stable fibrin gel that is suitable for cartilage engineering and applications where long-term shape stability is necessary (chapter 4).
In preliminary experiments, cells seeded into these three-dimensional fibrin gels were found to migrate out of and to proliferate on top of the gels, and the formation of a cell sheet onto these gels was observed containing high amounts of newly developed cartilaginous extracellular matrix. Based on these findings, in this study fibrin was tested with regard to its potential to retain the ability of the thus expanded chondrocytes to form engineered cartilage. Primary bovine chondrocytes were three-dimensionally seeded into the fibrin gels and cultured for 4 weeks in vitro to evaluate the stepwise development of the cell sheet and production of new matrix within this sheet. In order to test the potency of the cells expanded within the sheet, the cells were then re-isolated and re-seeded into the three-dimensional fibrin gels. After 5 weeks of culture, the amount of newly produced extracellular matrix as well as formation of a connected cartilaginous tissue was compared to that of cells expanded in monolayer on conventional tissue culture polystyrene (TCPS) and also re-seeded into the gel system.

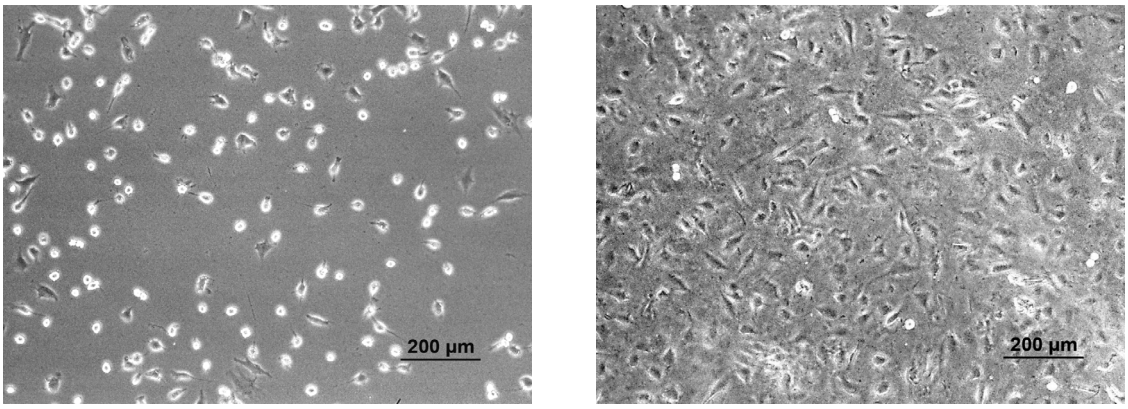


Fig. 1: Adhesion of primary bovine chondrocytes after seeding 15,000 cells per cm^2 onto TCPS-well (left) and onto fibrin-coated well (right,) after incubation at 37 °C for 24 h.

Materials and Methods

Materials

Aprotinin solution (Trasylol®) was bought from Bayer (Leverkusen, Germany). Thrombin (as a part of Tissucol®), thrombin dilution buffer and the commercially available fibrin glue kit Tissucol® was kindly provided by Baxter (Unterschleißheim, Germany). Bovine fibrinogen was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Knee joints from three-months-old bovine calves were obtained from a local abattoir within 12-18 hours of slaughter. Type II collagenase and papainase were purchased from Worthington (CellSystem, St. Katharinen, Germany). Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, fetal bovine serum (FBS), MEM non-essential amino acid solution, penicillin, streptomycin, N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES buffer), phosphate buffer solution (PBS buffer) and trypsin EDTA were obtained from Gibco (Karlsruhe, Germany). 149 µm pore size polypropylene filters were purchased from Spectrum (Rancho Dominguez, CA, USA).

Ascorbic acid, deoxyribonucleic acid, diaminobenzidine, dimethylmethylene blue, glutaraldehyde, glycine, hematoxylin, proline and safranin-O were purchased from Sigma-Aldrich (Taufkirchen, Germany). Bovine insulin from bovine pancreas, chloramin-T, formalin 37%, and p-dimethylaminobenzaldehyde (p-DAB) were from Merck (Darmstadt, Germany). Cell culture plastics were purchased from Corning (Bodenheim, Germany).

Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA) and L-hydroxyproline from Fluka (Neu-Ulm, Germany). Chondroitin sulfate was from ICN (Aurora, Ohio, USA).

Cell isolation

Primary chondrocytes were isolated from the surface of the femoral patellar groove using sterile technique. The cartilage was cut into small pieces and enzymatically digested over night in complete chondrocyte medium, DMEM containing 4.5 g/l glucose, 584 mg/l glutamine, 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.1 mM MEM non-essential amino acids, 0.4 mM proline and 50 µg/ml ascorbic acid, with addition of 470 U/ml of type II collagenase. The digest was re-pipetted, filtered through a 149 µm filter and washed three times with PBS. The cell number was determined by cell counting using a hemocytometer and an inverted phase-contrast microscope.

Cell seeding and culture

Based on previous results from gel development (chapter 4), fibrin gels were prepared with 100 mg/ml fibrinogen in 10,000 KIE/ml aprotinin solution and 5 U/ml thrombin in 40 mM $CaCl_2$ dilution buffer.

For investigation of cell migration out of the fibrin and formation of a cell sheet onto the gel, $5*10^6$ primary bovine chondrocytes were resuspended in 250 µl fibrinogen solution; subsequently gels were prepared adding the same volume of thrombin in a stabilizing silanized glass ring to obtain a fibrin disc of 10 mm diameter and 6.4 mm thickness (500 µl gel). The fibrin gels were allowed to gel for 45 min at 37 °C before removing the glass ring and covering the cell-fibrin constructs with 4 ml complete chondrocyte culture medium. Medium contained 10 % FBS with 2.5 µg/ml bovine insulin based on previous results [24], and was replaced three times per week. The constructs were statically cultured in 6-well-plates in an incubator at 37 °C, 5 % CO_2 and 95 % humidity, harvested after 4, 7, 14, 21, and 28 days, and histochemically analyzed.

For investigation of the potential to form engineered cartilage of chondrocytes migrated out of the fibrin gel and proliferated on the gel, $5*10^6$ primary bovine chondrocytes were suspended in fibrin gel and cultured for 4 weeks as described above. As control, $1*10^6$ cells were seeded in T-150 culture flasks and cultured in monolayer. After 4 weeks, the newly developed cell sheet were carefully removed from fibrin gels and digested over night using type II collagenase as described above. The next day, $5*10^6$ cells were resuspended in 20 µl fibrinogen solution and mixed with the same volume of thrombin solution in a stabilizing silanized glass ring to obtain a fibrin disc of 5 mm diameter and 2 mm thickness (40 µl gel). Constructs were covered with 4 ml complete chondrocyte medium containing 10 % FBS with and without 2.5 µg/ml insulin based on previous results [24]; medium was replaced three times per week. For controls, cells proliferated in monolayer were detached using trypsin (0.25 %) and counted. $5*10^6$ cells were also resuspended in 20 µl fibrinogen solution and mixed with 20 µl of thrombin solution. The glass rings were removed after 45 min, and fibrin discs were covered with 4 ml complete chondrocyte medium containing 10 % FBS with and without 2.5 µg/ml insulin. After 5 weeks of culture, constructs were harvested, and biochemically and histochemically analyzed.

Histological and biochemical analysis

Fibrin constructs with proliferated cells were analyzed as previously described [1, 24]. Briefly, the constructs were weighed (= wet weight) and cut in half. One part of the construct was lyophilized and digested with 1 ml of a papainase solution (3.2 U/ml in buffer) for 18 h at 60 °C for determination of cell number and content of glycosaminoglycan and total collagen.

The number of cells per cell-fibrin construct was assessed from the DNA content using Hoechst 33258 dye and a conversion factor of 7.7 pg DNA per chondrocyte [25]. The amount of sulfated glycosaminoglycans was determined spectrophotometrically at 525 nm as chondroitin sulfate using dimethylmethylene blue; bovine chondroitin sulfate was used as standard [26]. The hydroxyproline content was determined spectrophotometrically after acid hydrolysis and reaction with chloramin-T and p-dimethylaminobenzaldehyde [27]. The amount of total collagen was calculated using a hydroxyproline to collagen ratio of 1:10 [28].

The other part of the construct as well as constructs for determination of cell migration were prepared as a histological sample by fixing in 2 % glutaraldehyde in PBS for 30 min and then storing in 10 % formaldehyde in PBS. The formalin-fixed samples were embedded in paraffin and cross-sectioned into 5 µm sections; deparaffinized sections were stained with hematoxylin, fast green and safranin-O.

Statistical analysis

Biochemical data are expressed as means ± standard deviation. Statistical significance was assessed by one-way analysis of variance ANOVA in conjunction with multiple comparison Tukey's studentized range test at a level of $p < 0.01$.

Results

Chondrocyte migration and formation of cell sheet

Histological cross-sections of constructs consisting of $5*10^6$ primary bovine chondrocytes and cultivated for up to 4 weeks clearly demonstrate the migration of cells out of the fibrin gel and the stepwise development of a cartilaginous tissue onto the surface of the fibrin gel within 4 weeks of culture time (Fig. 2). Already after 1 week in culture, cells within the gel

were encircled by an area containing high amounts of extracellular matrix, strongly stained red with safranin-O. However, not before two weeks of culture, cells were found on the surface of the fibrin gel, slightly stained red for glycosaminoglycans. As the culture process progressed, these cells formed a thick and compact cell sheet containing high amounts of homogenously distributed extracellular matrix. However, the original surface of the fast green-stained fibrin gel was still visible due to differences in matrix distribution.

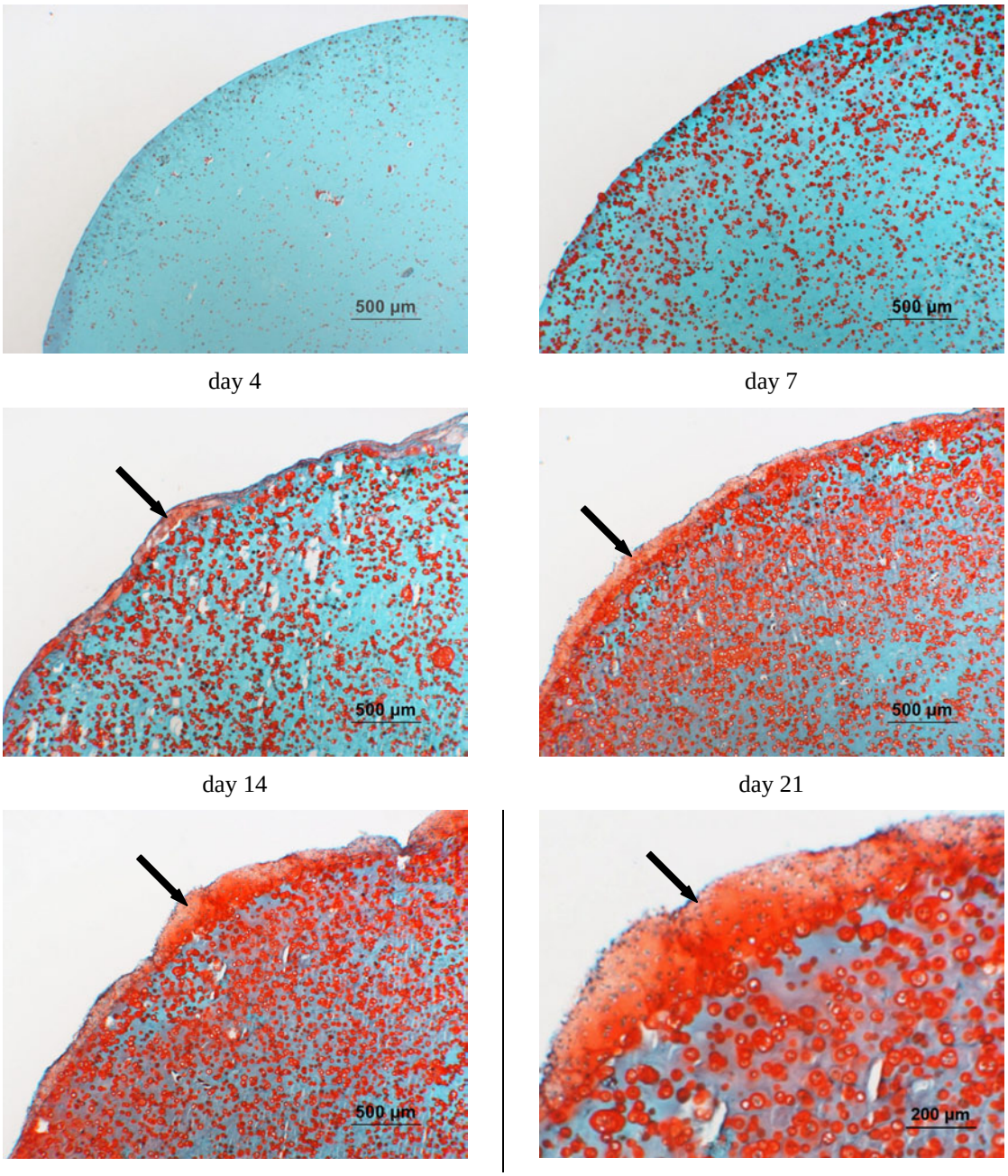
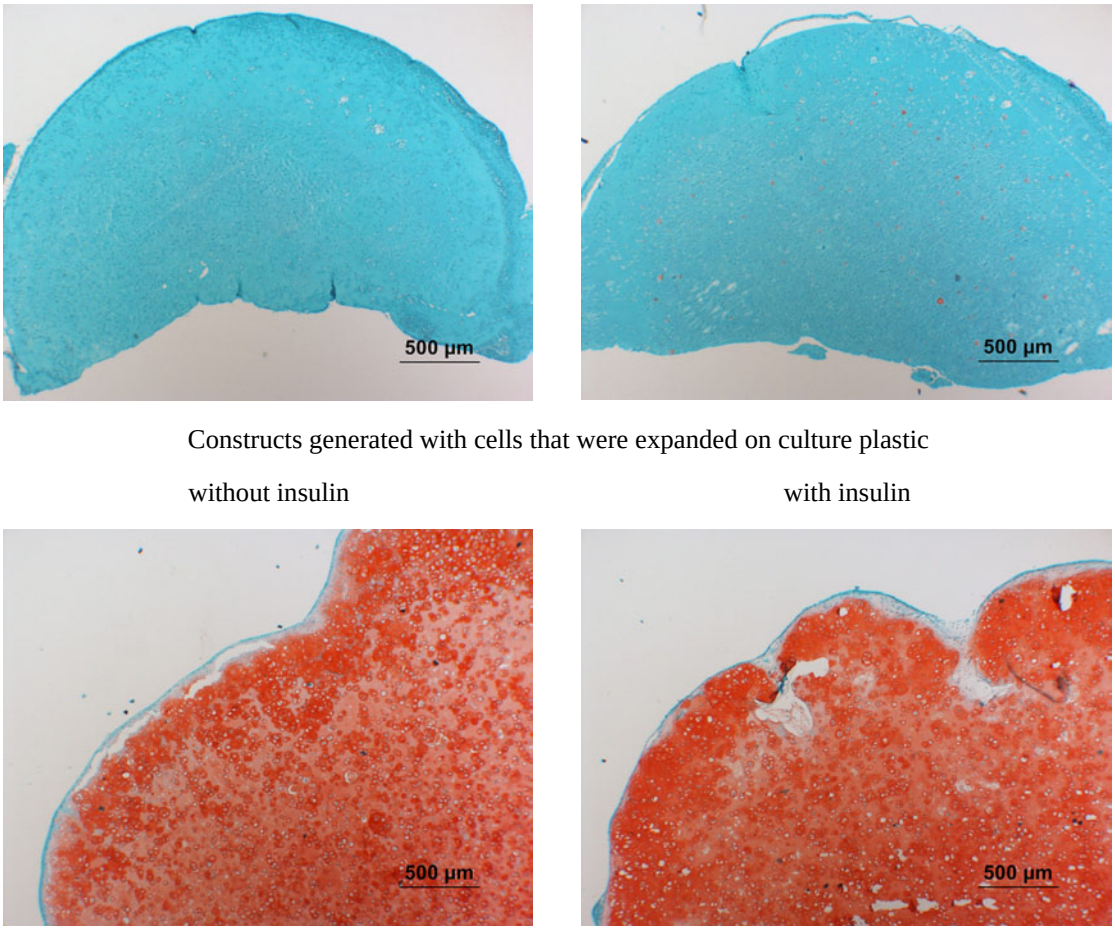


Fig. 2: Histological cross-sections of constructs cultivated for up to 28 days, gradually building a cell sheet on the gel surface (displayed by arrow); stained red with safranin-O.

Differentiation of chondrocytes proliferated on fibrin gel

Biochemical analysis demonstrated the formation of a cell sheet of about 250 mg per construct containing about $4.2*10^6$ cells, within 4 weeks in culture. In contrast, cultivation of cells on conventional cell culture plates resulted in $15*10^6$ cells, which represents a 15-fold increase within the cultivation time. Cells of both proliferation processes were re-seeded in fibrin gels in a cell density of $5*10^6$ cells per constructs and cultured for 5 weeks in medium with and without insulin in medium.



Constructs generated with cells that were expanded on culture plastic

without insulin with insulin

Constructs generated with cells that were expanded within sheets on fibrin gels

without insulin with insulin

Fig. 3: Histological cross-sections of constructs prepared with cells that were expanded on culture plastic (above) or within cell sheets on fibrin gels (below), after 5 weeks in culture with (right) and without (left) insulin; stained red with safranin-O for GAG.

Histological cross-sections of proliferated chondrocytes in long-term stable fibrin gels cultured for 5 weeks showed homogenously distributed cells throughout the whole constructs (Fig. 3). However, constructs prepared with cells proliferated in monolayer or within the sheets on fibrin gels demonstrated tremendous differences. Cell that had migrated out of the fibrin gel, proliferated on the gel and were re-seeded in three-dimensional fibrin gels produced distinctly higher amounts of extracellular matrix, stained red with safranin-O for glycosaminoglycans, compared to cells expanded in monolayer on TCPS (Fig. 3). Constructs containing cells expanded in monolayer were solely stained with fast green and not with safranin-O, indicating the development of a more fibroblastic tissue with no cartilage-specific extracellular matrix (Fig. 3). Adding of 2.5 µg/ml insulin to constructs generated with the sheet-expanded cells resulted in construct that demonstrated an even slightly stronger staining with safranin-O, thus, the development of slightly higher amounts of extracellular matrix (Fig. 3). However, constructs prepared with cells expanded in monolayer showed only very small isolated areas within the constructs that were stained red for glycosaminoglycans.

These results were corroborated by biochemical analysis (Fig. 4). A 2.9-fold increase in wet weight and 1.9-fold increase in cell number were observed for constructs prepared with cells proliferated on fibrin gels and cultured in medium without insulin, compared to constructs prepared with cells in monolayer (Fig. 4A and 4B). Addition of insulin to the medium showed a slight, but not significant increase in wet weight and cell number. Regarding extracellular matrix components, seeding with cells migrated out of the fibrin gel and proliferated on the gel resulted in 5.5-fold higher amount of glycosaminoglycans per wet weight and 6.9-fold higher amount per single cell (Fig. 4C and 4D). In addition, total collagen per wet weight was 8.1-fold increased and collagen per cell was 10.1-fold increased, compared to cells expanded in tissue culture flasks (Fig. 4E and 4F). However, for the constructs with sheet-expanded cells, addition of insulin to the medium resulted in only slight, but not significant increases in extracellular matrix components GAG and total collagen.

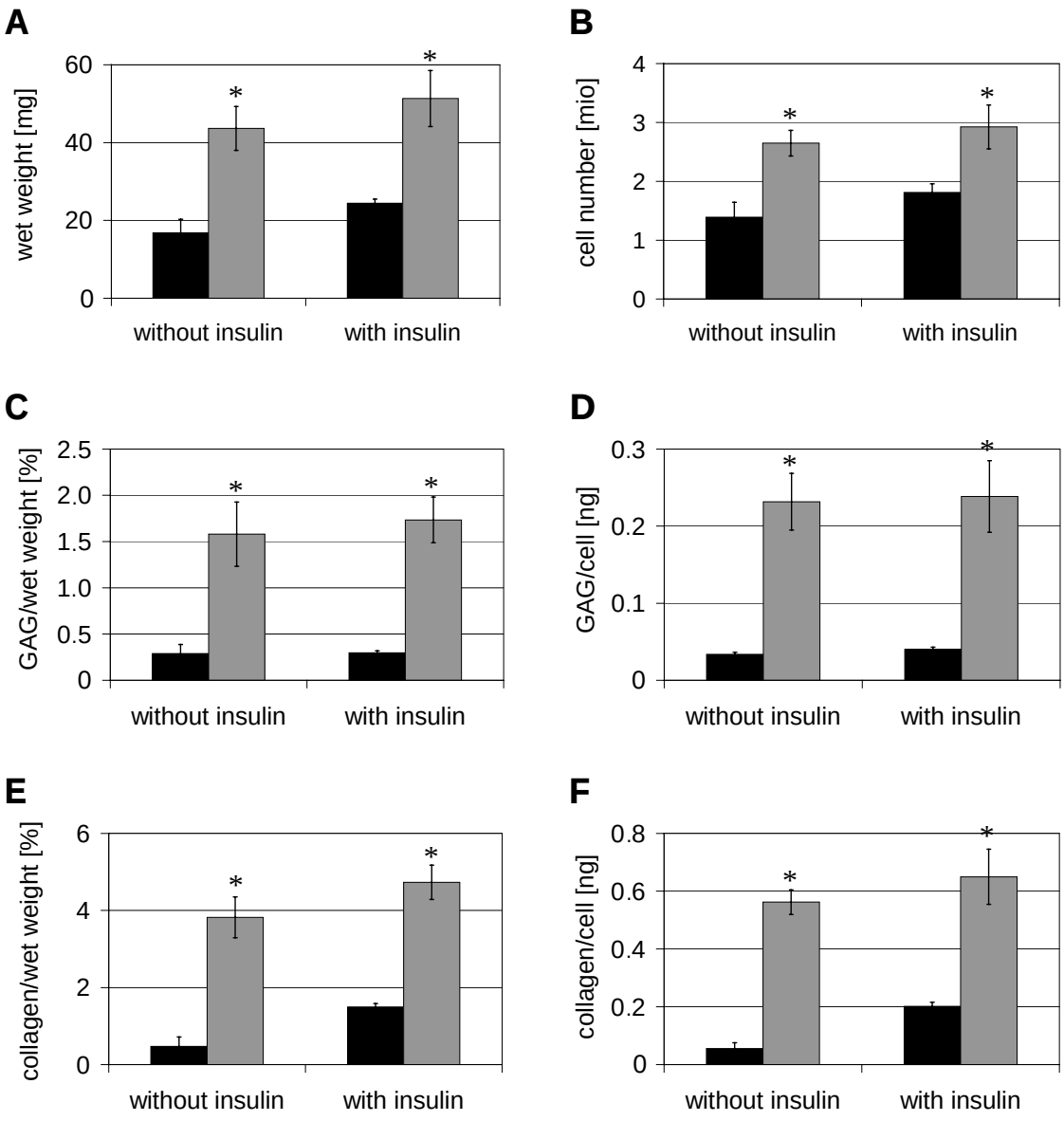


Fig. 4: Wet weight (A), cell number (B) and amount of GAG (C, D) and collagen (E, F) per wet weight and per cell of constructs prepared with cells that had been expanded on culture plastic (black ■) or within sheets on fibrin gels (gray ■), after 5 weeks in culture. Data represents the average ± S.D. for three independent measurements. Asterisks denote a statistically significant increase compared to control (culture plastic) ($p < 0.01$).

Discussion

Within this study, we demonstrated the potential of a long-term stable fibrin gel to enable the proliferation of chondrocytes and at the same time facilitate the retention of the ability of the thus expanded chondrocytes to form engineered cartilage. A fraction of cells seeded in these gels were found to migrate out of the fibrin onto the surface of the gel and, at first, form a thin two-dimensional cell sheet on the gel. As the culture process progressed, a thick and compact three-dimensional cell sheet was built on the gel containing high amounts of newly developed homogenously distributed extracellular matrix, stained red with safranin-O. Most remarkably, chondrocytes isolated from these sheets were found to produce dramatically higher amounts of extracellular matrix components GAG and total collagen, as confirmed by histology and biochemical analysis, when re-seeded into three-dimensional fibrin gels, compared to cells expanded on tissue-culture plates. These results indicate a reduced dedifferentiation of chondrocytes proliferated onto fibrin gels and/or an enhanced redifferentiation capacity when re-seeded into a three-dimensional matrix.

In order to generate a functional autologous cartilaginous tissue, primary chondrocytes isolated from a small biopsy need to be expanded to obtain a cell number adequate for high-density three-dimensional cell seeding. However, chondrocytes dedifferentiate during in vitro expansion in monolayer culture, indicated by a more fibroblast-like morphology and expression of molecules typical of pre-chondrogenic mesenchymal cells or fibroblasts. Dramatic changes in gene expression of cartilage extracellular matrix proteins of chondrocytes isolated from the goat were observed already during the first passage [5]. Regarding delicate human chondrocytes, Schnabel et al. demonstrated unfavorable differences in gene expression of proliferated human articular chondrocytes compared to cells freshly isolated, investigated by immunohistology, Northern blot analysis, RT-PCR and cDNA array [29]. Goessler et al. found specific types of collagen, e.g. collagen type I, III, IV and XI, and collagen-related proteins like biglycan, fibromodulin and lumican, to be markers for dedifferentiation of human septal chondrocytes, analyzed by microarray gene expression and immunohistochemical staining [30]. Furthermore, they demonstrated changes in expression of specific growth factor, e.g. IGF-I, during in vitro expansion of chondrocytes [31]. Taken together, even one expansion in monolayer might have a major detrimental effect on the chondrocytic pheno- and genotype, resulting in inadequate cartilaginous tissue that may fail to fulfill its physiological function after implantation.

Several approaches have been evaluated to solve this key problem of cartilage tissue engineering. One approach to enhance proliferation without gradual dedifferentiation is the use of optimized culture surfaces. It is known that conventional tissue-culture surfaces initiate dedifferentiation already after first seeding in monolayer [12]. However, some studies showed the effect of surfaces on the morphology and distribution of specific receptors on the cell surface [9-13, 32]. Thus, it is obvious, that variation of culture surfaces may influence cell behavior and, therefore, differentiation of delicate cells like chondrocytes. Kino-Oka et al. cultured primary rabbit chondrocytes on a collagen type I-coated surface which resulted in more round cells that produced significantly more collagen type II, compared to cells expanded on polystyrene plastic surface [11]. This study supports the view that cell morphology strongly influences differentiation of chondrocytes [33]. In another study, passaged dedifferentiated chondrocytes were seeded on an aggrecan surface, which is a typical chondrocyte extracellular matrix component, resulting in dramatic changes in gene expression from fibroblastic to chondrocytic phenotype [12]. With regard to fibrinogen and fibrin, though many efforts have been undertaken to investigate the influence on platelet morphology, activation and aggregation and, thus, blood coagulation [34-37], little is known about the influence on chondrocytes, in particular on prevention of dedifferentiation or enhancement of redifferentiation. In our study, chondrocytes not only migrate out of the fibrin gel, but also seem to adhere more strongly to the fibrin surface compared to conventional cell-culture plates, even in long-term culture, resulting in new three-dimensional cartilaginous tissue containing high amounts of cartilage specific extracellular matrix.

As mentioned above, the shape of cells can play a major role in gene expression [33]. If cells spread out, they will rather proliferate, but if cells are rounded, they may express genes for synthesis of proteins instead. This round morphology of cells could be forced by suspension in three-dimensional hydrogels, where cells are closely surrounded by the gel. In particular, alginate was frequently shown to prevent dedifferentiation and to enhance redifferentiation of expanded human chondrocytes after several passages, indicated by development of extracellular matrix components as well as chondrocytic gene expression [14, 15, 38]. As an alternative, typical chondrocytic extracellular components like hyaluronic acid or different types of collagen, as well as agarose have been used as three-dimensional matrix in order to retain and regain chondrocytic phenotype [16-18, 39].

Another possible method for improved expansion of chondrocytes is the use of growth factors. For example, adding TGF-β2 to the culture medium of chondrocytes in monolayer

maintains the proliferation ability, and expression of collagen type II is lost not before the third passage [40]. Jakob et al. found out that human articular chondrocytes expanded in the presence of FGF-2 and TGF-β1 showed not only the highest proliferation rate and the most enhanced dedifferentiation in monolayer, but also the highest capacity for redifferentiation and generation of a cartilaginous tissue, when re-seeded in three-dimensional pellet cultures and cultivated with medium containing TGF-β1 and dexamethasone [4, 41]. In a following study, capacity to redifferentiate was further enhanced by cyclooxygenase-2-dependent prostaglandins PGD(2) and PGF(2) added to the pellet culture medium containing TGF-β1 and dexamethasone [42]. In a further approach, a replacement of fetal calf serum by a combination of IGF-I and TGF-β2 was shown to enhance reversion of dedifferentiation of rabbit as well as human chondrocytes when re-seeded into alginate beads, indicated by increased numbers of collagen type II-producing cells [43-45]. To conclude, a variety of promising growth factor studies have been conducted to improve the ability of chondrocytes to form engineered cartilage after they have been expanded, in particular of human chondrocytes, when re-seeded into three-dimensional scaffold systems.

In summary, the results of the presented study indicate a reduced dedifferentiation and/or an enhanced redifferentiation capacity of chondrocytes proliferated on fibrin gels and may be used for multiplication of small numbers of chondrocytes isolated from small biopsies. Further investigations have to be done with regard to the expression of specific chondrocytic marker genes to fully characterize the cells that migrated and proliferated in the cell sheet. As a next step, cells may be seeded directly onto fibrin gels and cultured for a comparable time, to analyze a sheet formation without the need of cell migration out of the gel. In this study, a high number of primary cells were seeded into fibrin gels to assure sufficient cell migration out of the gel. As the exact number of migrated cells cannot be determined, it seems to be too complex to compare the expansion efficiency of this kind of cell proliferation with conventional two-dimensional cell expansion onto plastic surfaces. However, seeding cells directly onto the fibrin gel would be a suitable method without loss of cells inside the gel. Furthermore, it has to be tested if this proliferation method is also efficient for delicate human autologous chondrocytes as it was shown here for bovine cells. Finally, it appears worthwhile to evaluate if the additional application of growth factors such as bFGF in combination with the fibrin gel leads to additional improvement. Taken together, a reliable method to generate expanded chondrocytes capable of forming high

quality cartilage tissue would enormously benefit the field of cartilage engineering. The here presented approach utilizing fibrin surfaces may contribute to such a development.

Acknowledgement

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Chapter 6

Cartilage Tissue Engineering Using Human Chondrocytes in a Fibrin Matrix

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Abstract

In order to generate tissue-engineered autologous cartilage, there is tremendous interest in using human chondrocytes isolated from small biopsies. In this study, we investigated the potential of freshly isolated human chondrocytes cultivated in long-term stable fibrin gels in vitro for the use in cartilage tissue engineering. Insulin exogenously added to the culture medium was found to dramatically enhance production and distribution of extracellular matrix components GAG and total collagen using human nasal as well as articular chondrocytes, compared to culture without insulin. This effect was even higher compared to the commonly used insulin-like growth factor-I (IGF-I), though various concentrations of IGF-I were tested. Additionally, the effects of insulin were also observed in a scaffold-free pellet system, i.e., independent of the fibrin system. In order to generate a coherent cartilaginous tissue, a high initial number of primary human chondrocytes was seeded in the fibrin gel ($5*10^6$ per 40 µl gel), which resulted in development of a homogenously distributed matrix intensively stained red with safranin-O for glycosaminoglycans. Though several investigations have to be done to further improve and analyze the generated constructs, cultivating human nasal and articular chondrocytes in a long-term stable fibrin gel with addition of insulin in the medium may be an important contribution towards clinical application of tissue-engineered cartilage.

Introduction

Already in the late nineties, the ability of xenogenic chondrocytes isolated from articular bovine [1], equine [2], porcine [3], and rabbit [4] cartilage to generate tissue-engineered cartilage in vitro and in vivo has been successfully demonstrated. In particular, primary juvenile bovine chondrocytes were seeded onto three-dimensional polymeric scaffolds or into biocompatible hydrogels, and dynamically cultured in optimized culture medium, resulting in newly developed extracellular matrix-rich tissue similar to native cartilage [1, 5-8]. For a feasible clinical application, though, the use of autologous chondrocytes would be beneficial to avoid disease transmission or immune rejection, as well as ethical discussions. However, the use of human chondrocytes is much more complicated compared to juvenile xenogenic chondrocytes, due to limited cell availability and intense donor age-dependent changes in cell proliferation, differentiation and redifferentiation capacity, which enormously influences tissue development [9-11].

Growth factors are known to stimulate or inhibit cell division, differentiation and influence cartilage tissue development [12], e.g. IGF-I [2, 6]. This could be beneficial in using human chondrocytes from aged patients or for multiplication of cells in order to obtain a cell number sufficient for three-dimensional cell seeding. Insulin is homologous to parts of the IGF-I molecule and because of the great similarity, the IGF-I receptor binds both IGF-I and, with lower affinity, insulin. It has previously been shown that insulin increases the tissue growth rate and improves the ECM composition of tissue-engineered cartilage in vitro, similar to the effects of IGF-I, using bovine chondrocytes in a three-dimensional PGA scaffold [7]. Therefore, insulin may also be a suitable protein to improve cartilage tissue engineering using human autologous cells.

In recent years, fibrin has been utilized as scaffold system in the field of tissue engineering, especially in cartilage tissue engineering, with specific physical and biological requirements [13] (chapter 2). However, an important fibrin glue characteristic is an increasing instability and solubility over time in vitro and in vivo, which could be a problem for the application in tissue engineering [14-16]. Therefore, by modifying specific fibrin gel components our group has previously developed a long-term stable fibrin gel that was demonstrated to be suitable for cartilage engineering using juvenile bovine chondrocytes (chapter 4).

Within this study, with regard to a future clinical application of engineered autologous cartilage, we investigated the potential of human adult chondrocytes suspended in

long-term stable fibrin gels for cartilage tissue engineering. Therefore, freshly isolated human nasal chondrocytes were suspended in fibrin gel, cultured in vitro and analyzed after 5 weeks. Please note, that the experiments of this study were of preliminary nature and are thought to be the base for further investigations. Therefore, not all constructs were analyzed in detail and not all results were statistically proved. With regard to improvement of ECM development, in particular, the effects of exogenous insulin added to the culture medium were evaluated and compared to those of commonly used IGF-I. In order to corroborate our results, the enhancing effect of insulin on formation of cartilaginous tissue was also tested using articular chondrocytes isolated from human joint, at the same time investigating the effect of different concentrations of IGF-I. In order to investigate whether the insulin effects were independent of the use of the fibrin gel, the effect of insulin on human chondrocytes was further evaluated in a scaffold-free pellet system. Finally, in order to generate a coherent human cartilaginous tissue, constructs were seeded with a high cell number for enhanced matrix distribution, based on results from chapter 4.

Materials and Methods

Materials

Aprotinin solution (Trasylol®) was bought from Bayer (Leverkusen, Germany). Thrombin (as a part of Tissucol®), thrombin dilution buffer and the commercially available fibrin glue kit Tissucol® was kindly provided by Baxter (Unterschleißheim, Germany). Bovine fibrinogen was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Human nasal septal cartilage was collected from patients undergoing septorhinoplasties at the University Hospital of Regensburg (Regensburg, Germany), after informed consent was given. Human articular cartilage was obtained post mortem from the surface of the femoral patellar groove of knee joints within 48 h after death (Institute of Forensic Medicine, Munich, Germany). Type II collagenase and papainase were purchased from Worthington (CellSystem, St. Katharinen, Germany). Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, fetal bovine serum (FBS), MEM non-essential amino acid solution, penicillin, streptomycin, N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES buffer) and phosphate buffer solution (PBS buffer) were obtained from Gibco (Karlsruhe, Germany). 149 µm pore size polypropylene filters were purchased from Spectrum (Rancho Dominguez, CA, USA).

Ascorbic acid, deoxyribonucleic acid, diaminobenzidine, dimethylmethylene blue, glutaraldehyde, glycine, hematoxylin, proline, safranin-O, Tween® 80 and pepsin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Bovine insulin from bovine pancreas, chloramin-T, formalin 37%, and p-dimethylaminobenzaldehyde (p-DAB) were from Merck (Darmstadt, Germany). Human insulin was provided by Sanofi-Aventis (Frankfurt, Germany). Culture plastic was from Corning Costar (Bodenheim, Germany).

Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA) and L-hydroxyproline from Fluka (Neu-Ulm, Germany). Chondroitin sulfate was from ICN (Aurora, Ohio, USA) and Tissue Tek was from Sakura Finetek (Torrane, CA, USA). Vectastain ABC-kit and DAB-kit, and normal horse serum were obtained from Vector Laboratories Inc. (Burlingame, CA, USA). Type II collagen monoclonal antibody was obtained from the development studies Hybridoma Bank and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, USA). Type I collagen antibody (monoclonal anti-collagen type I col-1) mouse ascites fluid was bought from Sigma-Aldrich (Saint Louis, USA).

Cell isolation

Primary human nasal (age 20 to 40 years old) and articular (age 20 years old) chondrocytes were isolated using sterile technique. The cartilage was cut into small pieces, washed three times with PBS containing 50 U/ml penicillin and 50 U/ml streptomycin, and enzymatically digested overnight in complete chondrocyte medium, DMEM containing 4.5 g/l glucose, 584 mg/l glutamine, 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.1 mM MEM non-essential amino acids, 0.4 mM proline, 50 µg/ml ascorbic acid and 470 U/ml of type II collagenase. The digest was re-pipetted, filtered through a 149 µm filter and washed three times with PBS. The cell number was determined by cell counting using a hemocytometer and an inverted phase-contrast microscope.

Cell seeding and construct culture

Effect of insulin and IGF on nasal chondrocytes

Based on previous results from gel development (chapter 4), fibrin gels as three-dimensional scaffolds were prepared with 100 mg/ml fibrinogen in 10,000 KIE/ml aprotinin solution and 5 U/ml thrombin in dilution buffer. For standard culture, $2*10^6$ freshly isolated chondrocytes (age 20 to 40 years old, pooled) were re-suspended in fibrinogen solution; subsequently gels were prepared adding the same volume of thrombin in a stabilizing silanized glass ring to obtain a fibrin disc of 5 mm diameter and 2 mm thickness (40 µl gel). The fibrin gels were allowed to gel for 45 min at 37 °C before removing the glass ring and covering the cell-fibrin constructs with 4 ml complete chondrocyte culture medium. Medium contained 10 % FBS with or without 2.5 µg/ml insulin or 50 ng/ml IGF-I based on previous results [7], and was replaced three times per week. The constructs were statically cultured in 6-well-plates in an incubator at 37 °C, 5 % CO_2 and 95 % humidity, harvested after 5 weeks, and biochemically and histochemically analyzed.

Effect of insulin and IGF-I on articular chondrocytes

$2*10^6$ freshly isolated articular chondrocytes were seeded into 40 µl fibrin gels as described above. Constructs were then covered with 4 ml chomplete chondrocyte medium containing 10 % FBS with or without 2.5 µg/ml insulin and cultured for 5 weeks. For determination of the effect of various concentrations of IGF-I, protein in the range of 25 ng/ml to 150 ng/ml was freshly added to the culture medium three times per week.

Pellet culture

For pellet culture, $0.5*10^6$ nasal chondrocytes (age 29 years old) were centrifuged for 5 min at 1200 rpm to form a pellet, and covered with 3 ml medium containing 10 % FBS with or without 2.5 µg/ml bovine insulin. As control, $1*10^6$ cells were suspended in 40 µl fibrin gel as described above, and cultured in complete chondrocyte medium with or without 2.5 µg/ml bovine insulin.

Seeding a higher cell number

For constructs containing a higher cell density than in the initial experiments employing fibrin gels, $5*10^6$ nasal chondrocytes (age 20 years old) were distributed in a fibrin disc of 5 mm diameter and 2 mm thickness (40 µl fibrin gel) as described above and cultured for 5 weeks. Complete chondrocyte medium contained 10 % FBS with and without 2.5 µg/ml bovine insulin and was exchanged three times per week.

Histological and biochemical analysis

The constructs were analyzed as previously described [6, 7]. The three-dimensional fibrin constructs were weighed (= wet weight) and cut in half.

Briefly, for the determination of cell number, collagen content, and glycosaminoglycans (GAG) content, one part of the construct was lyophilized and digested with 1 ml of a papainase solution (3.2 U/ml in buffer) for 18 h at 60 °C. The number of cells per cell-fibrin construct was assessed from the DNA content using Hoechst 33258 dye and a conversion factor of 7.7 pg DNA per chondrocyte [17]. The amount of sulfated glycosaminoglycans was determined spectrophotometrically at 525 nm as chondroitin sulfate using dimethylmethylene blue; bovine chondroitin sulfate was used as standard [18]. The hydroxyproline content was determined spectrophotometrically after acid

hydrolysis and reaction with chloramin-T and p-dimethylaminobenzaldehyde [19]. The amount of total collagen was calculated using a hydroxyproline to collagen ratio of 1:10 [20].

A part of the construct as well as cell pellets were prepared as a histological sample by fixing in 2 % glutaraldehyde in PBS for 30 min and then storing in 10 % formaldehyde in PBS. The formalin-fixed samples were embedded in paraffin and cross-sectioned into 5 µm sections; deparaffinized sections were stained with hematoxylin, fast green and safranin-O.

For immunohistochemical analysis, a part of the constructs were prepared by fixing in methanol-formalin mixture, and then successively dehydrated in different concentrations (10 % - 40 %) of a sucrose solution followed by embedding into Tissue Tek. Frozen samples were cryosectioned at 7 µm and stained with antibodies for type I collagen (monoclonal anti-collagen type I col-1) mouse ascites fluid, dilution (1:1000), and type II collagen (DSHB), dilution (1:6). To prevent non-specific antibody binding samples were incubated with 5 % normal horse serum in buffer. The sections were incubated overnight at room temperature with primary antibodies; PBS without antibodies was used for control sections. On the next day, sections were incubated with biotinylated secondary antibody, anti-mouse/rabbit IgG (Vector Laboratories; Inc.; Burlingame, CA, USA), for 30 min at room temperature. Finally, the samples were stained using a Vectastain ABC-kit and DAB-kit according to the protocol for avidin-biotin-peroxidase complexes formation.

Statistical analysis

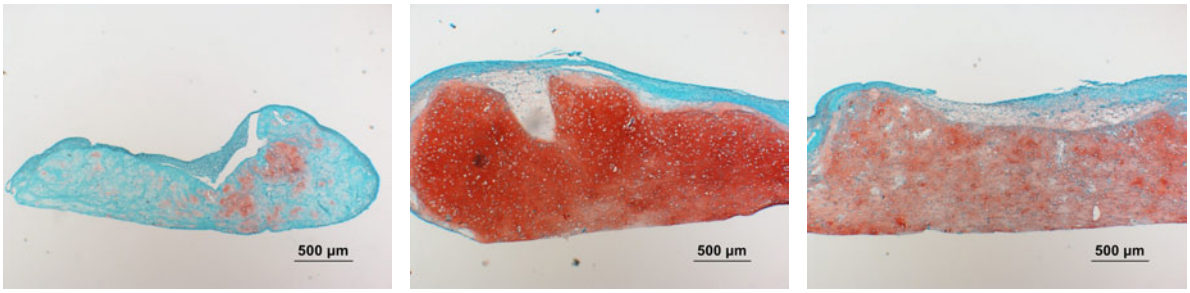
Biochemical data are expressed as means ± standard deviation. Statistical significance was assessed by one-way analysis of variance ANOVA in conjunction with multiple comparison test (Tukey's test) at a level of $p < 0.05$ for Fig. 2.

Results

Effect of insulin and IGF-I on nasal chondrocytes

Primary human nasal chondrocytes in long-term stable fibrin gels were homogenously distributed throughout the whole gel and maintained vital during the culture period. Cells cultured in medium with 10 % FBS with addition of 2.5 µg/ml insulin or 50 ng/ml IGF-I maintained a round shape during the study and produced extracellular matrix containing GAG and collagen (Fig. 1). However, histological cross-sections of constructs cultivated in medium containing 2.5 µg/ml insulin were more intensively stained red with safranin-O compared to the IGF-I group, indicating distinctly higher amounts of GAG within the gel. In contrast, control groups showed only slight staining with safranin-O, which demonstrated the development of only low amounts of extracellular matrix (Fig. 1).

These results were also reflected in biochemical analysis (Fig. 2). Wet weight and cell number of constructs cultured with insulin were significantly higher compared to controls (2.8-fold and 2.4-fold). Values were slightly higher compared to constructs cultivated with IGF-I, though there were no significant differences. Regarding extracellular matrix components, constructs cultured with insulin in medium showed a distinct 2.0-fold increase in GAG per wet weight and 2.2-fold increase in GAG per single cell, compared to control. Compared to the IGF-I group, GAG per wet weight as well as per cell was 1.6-fold higher when cultivated with insulin in medium. These results were paralleled by collagen content per wet weight and per cell, showing a distinct increase compared to control; no collagen was measured in the control group. Compared to IGF-I, though results showed high variations within this group, analysis demonstrated the development of 3.9-fold significantly higher amounts of collagen per wet weight and 3.4-fold significantly higher amounts of collagen per cell in constructs cultivated with insulin.



control insulin IGF-I

Fig. 1: Effect of insulin and IGF-I on GAG production and distribution compared to control. Histological cross-sections of gels after 5 weeks in vitro, stained with safranin-O.

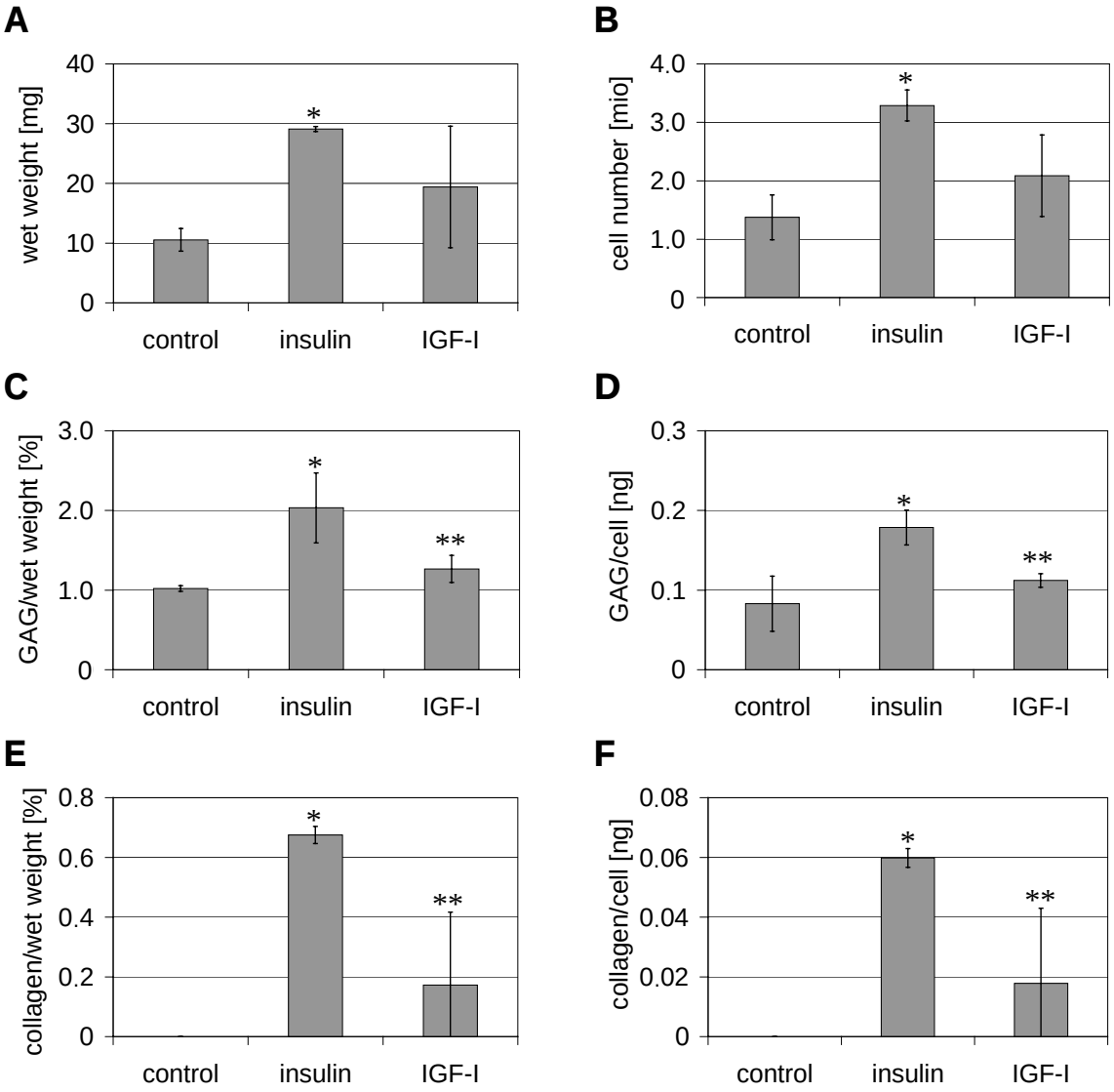
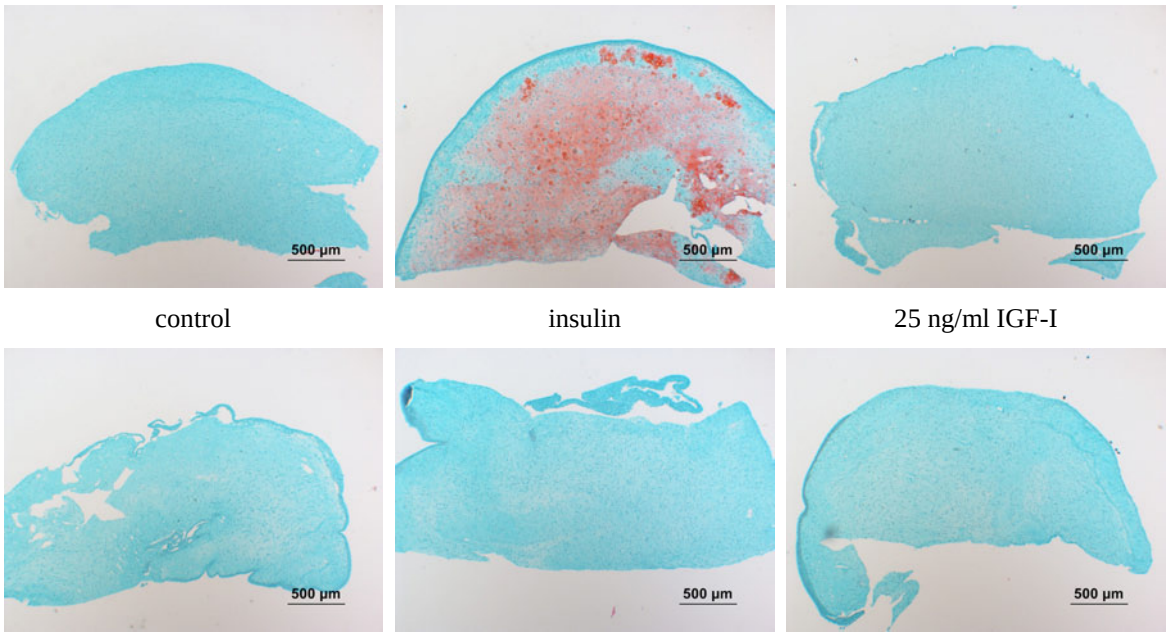


*Fig. 2: Effect of insulin and IGF-I on wet weight (A), cell number(B), and GAG (C, D) and collagen (E, F) content per wet weight (C, E) as well as per cell (D, F), compared to control, after 5 weeks of culture. Data represents the average ± S.D. of three independent measurements. Statistically significant differences between the insulin group and control are denoted by * ($p < 0.05$), between the IGF-I group and the insulin group by ** ($p < 0.05$).*

Effect of insulin and IGF-I on articular chondrocytes

Histological cross-sections of constructs containing human articular chondrocytes and cultured in medium containing different concentrations of IGF-I in the range of 25 ng/ml to 150 ng/ml showed no development of cartilaginous extracellular matrix, similar to the controls (Fig. 3). Constructs were intensively stained green with fast green indicating dedifferentiation of the primary chondrocytes, and showed no staining with safranin-O for glycosaminoglycans. In contrast, cultivation with medium containing 2.5 µg/ml insulin resulted in less staining with fast green and distinctly more staining with safranin-O for glycosaminoglycans, which demonstrates an improved development of cartilaginous extracellular matrix, compared to control and groups supplemented with IGF-I in medium (Fig. 3).



control | insulin | 25 ng/ml IGF-I

50 ng/ml IGF-I | 100 ng/ml IGF-I | 150 ng/ml IGF-I

Fig. 3: Effect of insulin and different concentrations of IGF-I on GAG production and distribution compared to control. Histological cross-sections of constructs after 5 weeks in culture, stained red with safranin-O.

Regarding biochemical analysis (Fig. 4), using medium with 2.5 µg/ml insulin showed a 2.0-fold increase in wet weight and 1.6-fold increase in cell number compared to cultivation without insulin, after 5 weeks of culture. Additionally, development of extracellular matrix component GAG showed a 2.9-fold and a 2.8-fold increase per wet

weight and per cell, respectively, compared to control. Development of collagen was found to be 2-fold increased per wet weight and 2.1-fold per cell, compared to cultivation without insulin. With regard to addition of IGF-I, GAG production was a lot lower than in the insulin group and hardly improved compared to control. For wet weight, cell number, and collagen content, results in the range of the insulin group were obtained.

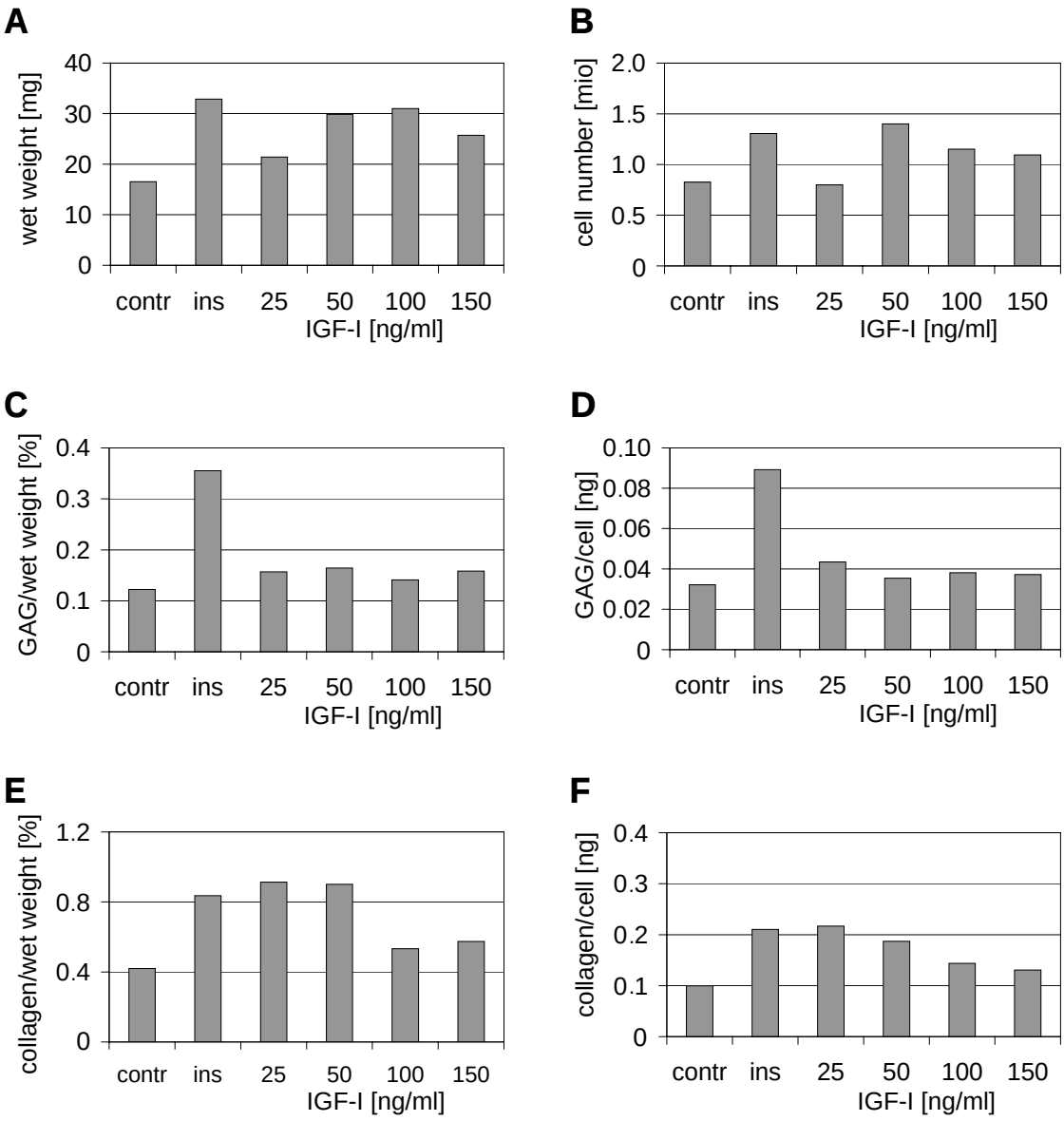


Fig. 4: Effect of insulin and different concentrations of IGF-I on wet weight (A), cell number (B), and GAG (C, D) and collagen (E, F) content per wet weight (C, E) and per cell (D, F) of human primary chondrocytes compared to control (n = 2).

Effect of insulin on cells in a pellet system

Primary human nasal chondrocytes cultivated in a scaffold-free three-dimensional pellet system maintained vital during the culture period. Addition of 2.5 µg/ml insulin to the culture medium resulted in the development of distinctly higher amounts of extracellular matrix, stained red with safranin-O, compared to cultivation without insulin (Fig. 5). Control constructs with cells in fibrin gel showed similar results, however, extracellular matrix was less intensively stained red compared to the pellet group, likely due to a lower cell density.

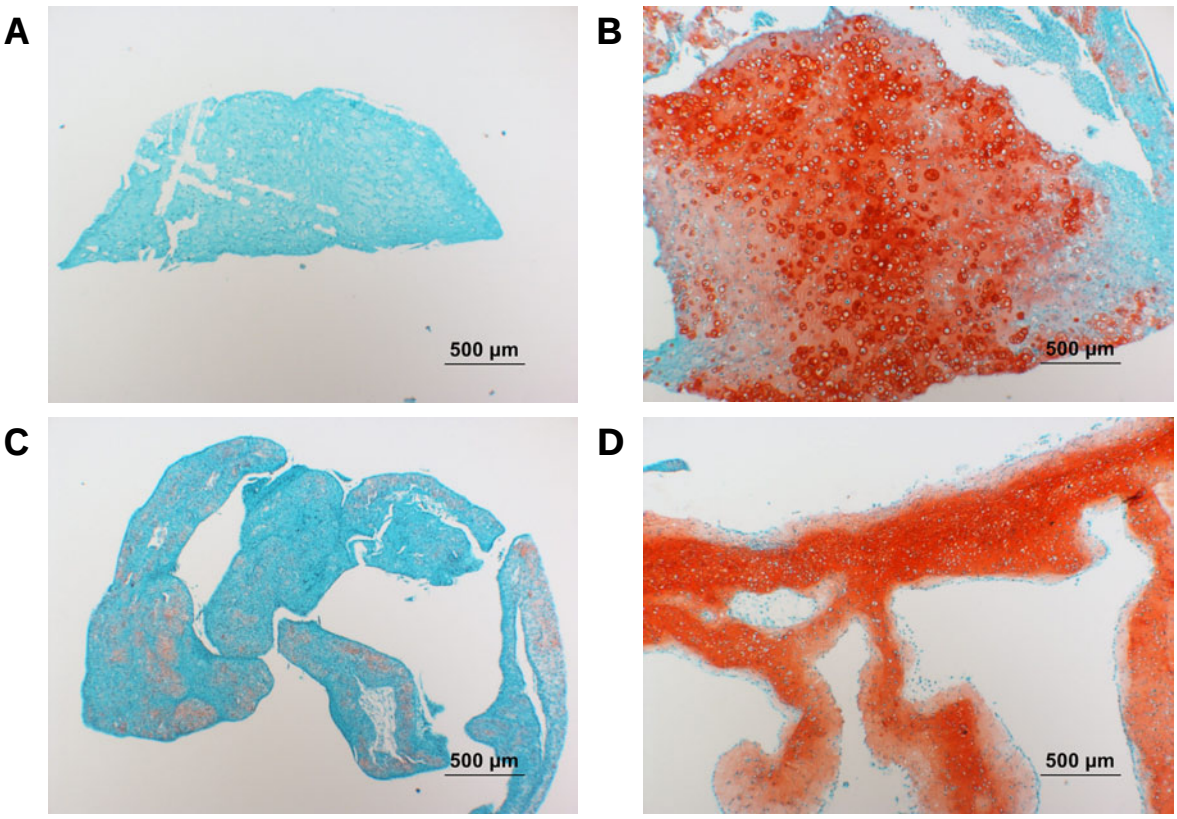


Fig. 5: Effect of insulin on human chondrocytes in a scaffold-free pellet system (C, D) compared to cells in fibrin gel (A, B), with (B, D) and without (A, C) insulin in medium. Histological cross-sections of constructs after 5 weeks in culture, stained red with safranin-O. The irregular configuration seen in C and D is an artifact of centrifugation at the time of pellet formation.

Seeding a higher cell number

Histological cross-sections of constructs seeded with a high cell number and cultured in medium containing 2.5 µg/ml insulin demonstrated the development of a coherent cartilaginous tissue exhibiting homogenous GAG distribution, strongly stained red with safranin-O (Fig. 6B). In contrast, cultivation in medium without insulin showed production of distinctly lower amounts of extracellular matrix, indicated by less intensive staining with safranin-O, but more intensive with fast green (Fig. 6A).

Immunohistological cross-section of constructs seeded with high cell density and cultured in medium with insulin was distinctly stained for cartilage specific collagen type II throughout the whole construct (Fig. 6C – Fig. 6E). Undesired collagen type I was found particularly in a thin area at the edge and a larger area in the middle of the construct.

Biochemical results were well in agreement with the histological findings (Tab. 1). Seeding with higher cell density and culture in medium containing insulin resulted in 2-fold higher wet weight and 1.5-fold higher cell number after 5 weeks in vitro. GAG per wet weight was 4.2-fold and GAG per cell was 6-fold increased compared to culture without insulin. With regard to collagen, data showed only a slight increase in collagen per cell, but no differences in collagen per wet weight.

	control	insulin		
	(n = 1)	mean (n = 3)	±	sdv
wet weight [mg]	24.00	48.83	±	9.58
cell number [mio]	1.24	1.83	±	0.40
GAG/wet weight [%]	0.753	3.216	±	0.317
GAG/cell [ng]	0.145	0.858	±	0.057
collagen/wet weight [%]	0.639	0.599	±	0.126
collagen/cell [ng]	0.123	0.160	±	0.036

Tab. 1: Wet weight, cell number and amount of extracellular matrix components per wet weight and per cell of constructs prepared with high cell density and cultivated in medium with 2.5 µg/ml insulin compared to control without insulin.

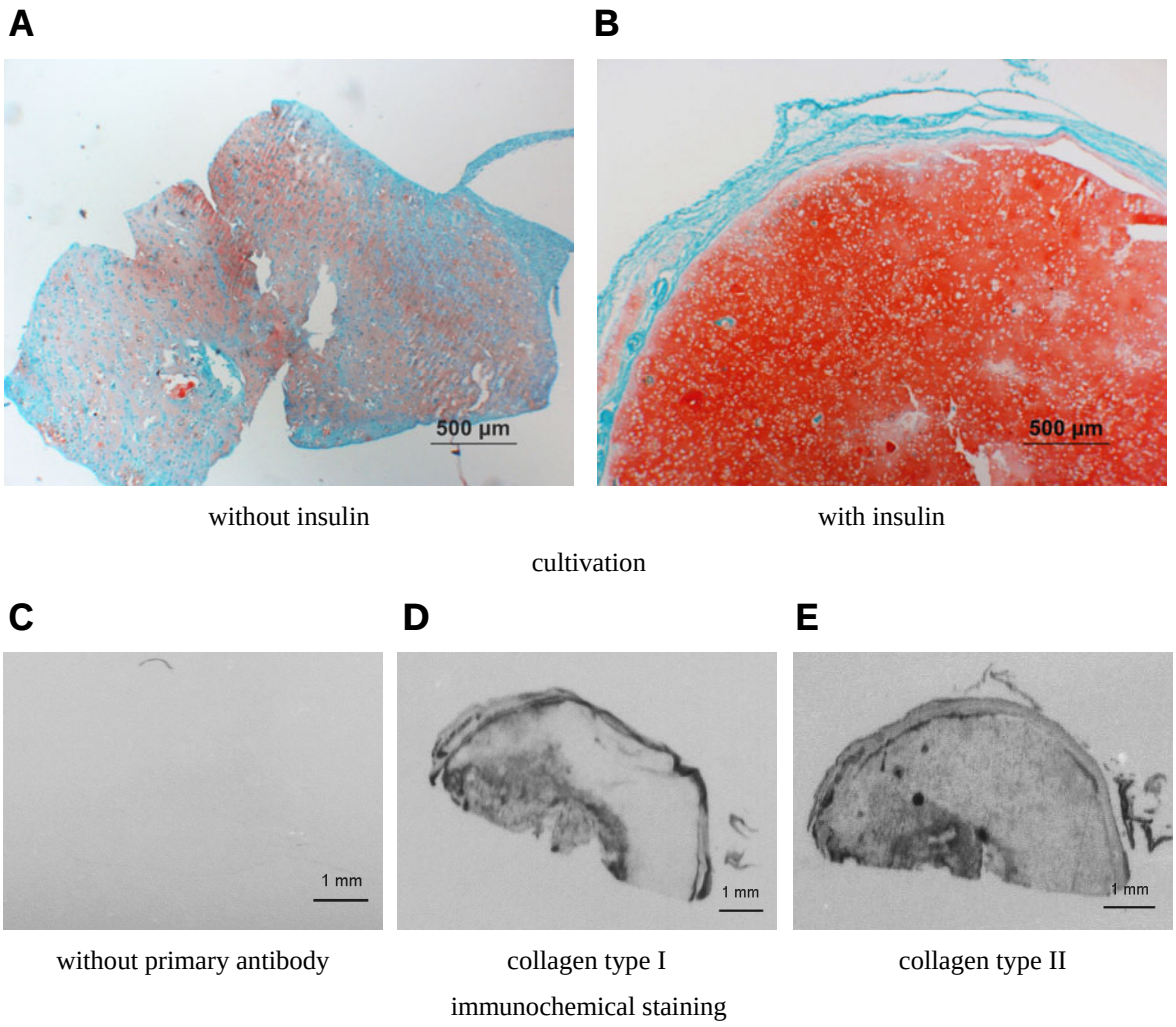


Fig. 6: Histological cross-sections of constructs prepared with high cell density and cultured for 5 weeks with (B) and without (A) 2.5 µg/ml insulin in medium, stained red with safranin-O. Immunohistochemical cross-sections of constructs cultured in medium containing 2.5 µg/ml insulin for 5 weeks; control without primary antibody (C), collagen type I (D) and collagen type II (E).

Discussion

In this study, we successfully investigated the potential of primary human chondrocytes cultured in long-term stable fibrin gels for the application in cartilage tissue engineering. Insulin exogenously added to the culture medium was demonstrated to dramatically enhance the development of cartilaginous extracellular matrix using human nasal as well as articular chondrocytes, even more than commonly used IGF-I. Using a high initial cell number, a coherent human cartilaginous tissue was obtained containing GAG in high amounts and well distributed, when cultured in medium supplemented with insulin.

Our group recently developed a fibrin gel that is stable in vitro and in vivo over several weeks and was demonstrated to be a suitable scaffold for the use in cartilage engineering using primary bovine chondrocytes, in detail discussed in chapter 4. Though several approaches using juvenile chondrocytes isolated from different animals have succeeded in the engineering of three-dimensional cartilage [1-3, 6], only few investigations resulted in generation of human cartilaginous tissue using cells isolated from patients. Three-dimensional human constructs containing noticeable amounts of new cartilage specific extracellular matrix molecules were developed by culturing cells in a small cell pellet [21-23]. Primary human cells were isolated from the hip, knee, ear, nose or rib of adult patients, expanded in medium with addition of several supplements and, subsequently, cultured in three-dimensional cell pellets for up to 2 weeks. Pellets were distinctly stained red for glycosaminoglycans with safranin-O and immunohistochemically for collagen type II. However, these studies resulted in adequate cartilaginous constructs of only a small size, i.e., insufficient for a clinical application. Alternatively, chondrocytes were isolated from pediatric patients (5 – 17 years old) [24, 25] or fetus (17 - 20 weeks of gestation) [26], resulting in development of high amounts of extracellular matrix components within three-dimensional constructs using PGA meshes or when directly injected into the back of nude mice. However, this represents a cell source which does not reflect the mean age of patient suffering from cartilage defects and, as an autologous transplantation would be beneficial, is not fully representative for application in clinical practice. In our study, primary human adult chondrocytes were seeded into three-dimensional discs of 5 mm diameter and 2 mm thickness, which is a distinctly larger construct size compared to small pellets. A coherent human cartilaginous tissue containing high amounts of GAG homogenously distributed within this gel system was obtained. Collagen type II was observed throughout the construct, however, relatively low contents of total collagen were

measured, as compared to those achieved with bovine juvenile chondrocytes (e.g., see chapter 4). Furthermore, a coherent tissue was only obtained when using primary cells at a relatively high cell density. Therefore, future investigations with regard to chondrocyte proliferation are needed to generate a high number of differentiated / re-differentiable cells capable of forming engineered cartilage (see also chapter 5). Nevertheless, the results presented here may represent a valuable contribution towards clinical application.

IGF-I is a well-known growth factor in cartilage tissue engineering that affects chondrocyte proliferation, differentiation and synthesis of extracellular matrix, via binding to IGF-I receptors located in cartilage [6, 27, 28]. As an alternative mechanism, it has been shown that IGF-I induces interleukin-1 receptor II (IL-1RII) that subsequently binds catabolic IL-1 and, thus, prevents matrix degradation [29, 30]. Beside IGF-I receptors, insulin receptors are also found in cartilage [31]. Insulin itself is homologous to parts of the IGF-I molecule and because of the great similarity, the IGF-I receptor binds both IGF-I and, with lower affinity, insulin [32]. Thus, insulin was shown to induce similar effects on chondrocytes as IGF-I [7, 33, 34]. In contrast, Olney et al. found an antagonistic effect when combining both molecules in culture medium [35]. With regard to specific effects on chondrocytes, insulin was demonstrated to enhance proliferation and synthesis of proteoglycans [7, 33, 34, 36-38], and to modulate the production of collagen type II and X, and matrilin-1 [39]. Additionally, in combination with thyroid hormone and glucocorticoids, hypertrophy of chondrocytes was found to be induced [40], a physiological step towards bone formation, characterized by high expression of collagen type X and Ch21 protein. In contrast, reduced apoptosis of mature chondrocytes was shown when low concentrations of insulin were added to the culture medium [37, 41].

In our group, insulin has been shown to have strong anabolic effects on engineered cartilaginous constructs in vitro, consisting of bovine chondrocytes seeded on a PGA-mesh [7] or in fibrin gels (chapter 4). However, little is known about the effect on human chondrocytes in three-dimensional matrices, though, insulin seems to be a suitable candidate to improve human autologous tissue engineering. Based on these findings, within this study, exogenously given insulin to the culture medium was tested regarding extracellular matrix development of human chondrocytes in fibrin gels, and compared to the effect of IGF-I, which is a commonly used growth factor in cartilage tissue engineering [6, 28, 29]. Analysis and histology of the insulin group clearly showed a tremendous increase in GAG and total collagen content and a more homogenous matrix distribution after five weeks of in vitro culture compared to cultivation without insulin. These results

were successfully confirmed using primary human articular cells, indicating similar effect on chondrocytes isolated from different regions of the body. Interestingly, this increase was significantly higher compared to constructs cultivated with a concentration of IGF-I in culture medium that was previously shown to have similar effects compared to insulin [7]. Even a variation of IGF-I concentration showed no improvement of extracellular matrix development of human cells in fibrin; the reason for this different effect is still unknown. Additionally, the effects of insulin on human chondrocytes were also found in a scaffold-free pellet system, which clearly indicated that the effects are independent of the fibrin gel system.

Though tissue engineering strategies using xenogenic chondrocytes demonstrated promising results during the last years, the use of autologous human chondrocytes faces still a lot of problems. Availability of human autologous chondrocytes is very limited due to the relatively small size of a biopsy. Common cell sources are cartilage from the ear, nose and rib, and articular cartilage from the knee, thus, resulting in severe donor morbidity. Chondrocytes isolated from different sites of the body show large differences regarding cell yield, proliferation capacity and suitability to generate autologous newly engineered cartilage [21, 22]. Additionally, it has been shown that human chondrocytes isolated from older donors, the major part of patients suffering from cartilage defects, have enormously lower cell viability and need a longer time to proliferate than chondrocytes isolated from younger donors [11, 21, 42, 43]. The problem of donor age is also reflected in higher dedifferentiation activity during in vitro multiplication, resulting in more fibroblast-like cells that produce less cartilaginous extracellular matrix components GAG and collagen type II, but more undesired collagen type I [44-47].

In conclusion, in this study we demonstrated the suitability of primary human chondrocytes for the use in cartilage engineering in optimized three-dimensional fibrin gels. A tremendous effect of insulin added to the culture medium on cartilage tissue development was shown. A homogenously distributed new extracellular matrix was obtained within 5 weeks in vitro when sufficient cells were initially seeded in fibrin gel. However, further studies are required to extend our work towards the goal of clinical application. Though the effect of insulin is well demonstrated, the optimal concentration in medium for adequate matrix formation in fibrin gel has to be found and further investigations towards molecular mechanisms have to be done. Furthermore, it is known that growth factors like TGF-β, FGF-2 and PDGF-bb and their combinations can enhance proliferation and redifferentiation capacity of human articular and non-articular

chondrocytes [48]. Based on these findings, it may be worthwhile to elucidate the potential of insulin on multiplication and redifferentiation of human chondrocytes to reduce biopsy size and, thus, patient morbidity, as a further step towards clinical practice.

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Chapter 7

Combination of Long-Term Stable Fibrin Gels and Polymeric Scaffolds For Cartilage Engineering

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Abstract

Within this study, the combination of long-term stable fibrin gels and polymeric scaffolds was tested for the use in cartilage engineering. Suspension of freshly isolated bovine chondrocytes in fibrin gel and injection into a gas-foamed polycaprolactone-based polyurethane scaffold resulted in homogenously distributed gel and cells within the composite construct, and the development of high amounts of cartilaginous extracellular matrix components GAG and collagen, as indicated by histological and biochemical analysis. No differences in matrix formation were found using this composite system compared to injection of cell-fibrin suspension into commercially available PGA fiber mesh as well as custom-made PLGA scaffolds, produced by solid lipid templating technique, except for differences in polymer degradation. Furthermore, four different types of polycaprolactone-based scaffolds were in detail investigated with the objective to select the most suitable ones for a future in vivo study. The scaffolds were evaluated regarding interconnective pore size, porosity, and robustness, as well as cell compatibility, cell seeding, proliferation, and subsequent matrix production within the material. As a proof of principle-experiment towards auricle tissue engineering, a polycaprolactone-based scaffold casted in a silicon mold in the shape of the cartilage part of an adult human ear was seeded with a high number of bovine chondrocytes. Dynamic cultivation for 4 weeks in vitro resulted in adequate formation of new cartilaginous tissue within the construct. The shape of the ear was not compromised by cell migration or subsequent matrix development, consequently, a construct was generated in the original human ear shape. Thus, this system represents a first step towards the generation of implants in an individually customized shape for healing of patient-specific defects.

Introduction

Common tissue engineering approaches use biocompatible, biodegradable polymeric materials to act as three-dimensional structure to organize the cells, to maintain a specific shape and structural integrity. Within these attempts, the scaffold plays a crucial role in cell seeding, proliferation, and new tissue formation in three-dimensions [1-3]. Depending on the mechanical properties of the tissue to be engineered and the extent of cell-material interaction desired, a suitable polymer has to be selected with regard to its degradation kinetic, composition and molecular weight. With regard to the prepared scaffold, critical properties and demands are pore size and shape, porosity and pore interconnectivity, as they have a direct impact on cell seeding, migration, growth, transport of oxygen, nutrients and wastes, and new tissue formation [2, 4]. Though several promising scaffolds have been developed generated by salt leaching techniques [5, 6], fibrous fabric processing [7], gas foaming [8], and several solid freeform fabrication techniques [9], further improvements for specific demands are still necessary.

Though polymeric scaffolds can provide sufficient load-bearing capacity and mechanical integrity, they often lack adequate cell seeding efficiency leading to inadequate cell distribution, and may exhibit increasing cell dedifferentiation over time due to two-dimensional cell spreading onto the surface of the polymer, and poor retention of newly developed extracellular matrix due to diffusion of the molecules into the culture medium [10, 11]. Therefore, the use of a hydrogel for efficient and homogenous cell delivery into the polymeric scaffold as well as improved three-dimensional matrix development and retention has been proposed [12-14]. Fibrin is a commonly used surgical haemostatic agent and due to the fact, that it is a physiological blood component, it is considered to be biocompatible and biodegradable [15-19]. Our group recently developed a long-term stable fibrin gel that was shown to be suitable for cartilage engineering (chapter 4). Based on these result, this optimized fibrin gel may be used as a cell delivery gel system within polymeric scaffolds for cartilage engineering.

Biodegradable poly(α-hydroxy acids), namely poly(D,L-lactic acid) (PLA) and copolymers (PLGA) with poly(glycolic acid) (PGA), are attractive and widely used commercially available materials to generate fibrous scaffolds with high porosity and permeability, with advantages over other materials like ceramics or metals, because they degrade as the new tissue is formed [7, 20]. PLGA scaffolds were produced in our group by solid lipid templating technique providing an interconnected pore structure [21, 22]. Though a rapid

biodegradation, as provided by PGA and PLGA, facilitates coherence of newly developed extracellular matrix in many applications in tissue engineering, long-term shape stability would be beneficial for application in the treatment of several patient-specific defects physiologically exposed to high mechanical loadings. As an alternative, polyurethanes may be used for application in tissue engineering providing an extended duration of biodegradation and added long-term mechanical stability. Elastomeric polyurethanes have been extensively used in various implantable devices in clinical practice [23-25], though only few investigations were conducted with regard to the use in cartilage tissue engineering. This material class provides the possibility to vary the properties in a broad range; using well known biocompatible and bioresorbable building blocks like polyesters, a sterilizable scaffold can be obtained exhibiting adequate mechanical stability, high porosity and adjustable pore size enabling efficient cell seeding, proliferation and subsequently extracellular matrix production. Within the ForTEPro research collaboration, highly porous polycaprolactone-based polyurethane scaffolds were developed by simple casting into a silicon mold using a two-component system with liquid precursors [26] (to be submitted). Applying this method, a scaffold may be produced in an individually customized shape for patient-specific defects.

Within this study, we investigated the potential of a combination of long-term stable fibrin gels and polymeric scaffolds for cartilage engineering. Therefore, primary bovine chondrocytes were suspended in fibrin gel and injected into a newly developed gas-foamed polycaprolactone-based polyurethane scaffold. This system was compared to injecting a cell-fibrin gel suspension into commercially available fibrous PGA meshes, as well as PLGA scaffolds, prepared in our group using the solid lipid templating technique. As a next step, four different types of polycaprolactone-based scaffolds were in detail tested with regard to material properties, in particular interconnective pore size, porosity, cytocompatibility, and gel filling, as well as chondrocyte proliferation and cartilaginous extracellular matrix production and distribution, with the objective of selecting the most suitable ones for a future in vivo study. Finally, as a proof of principle-experiment, a polycaprolactone-based scaffold in the shape of the cartilage part of a human external ear was seeded with primary bovine chondrocytes in fibrin gel, and development of cartilaginous tissue within this constructs as well as shape stability in vitro was evaluated.

Materials and Methods

Materials

Poly(caprolactone)diol (M_n = 1250), poly(caprolactone)triol (M_n = 900), methylal and dextrose were purchased from Sigma-Aldrich (Taufkirchen, Germany), DABCO 3242 was bought from Air products (Hattingen, Germany) and diazabicycloundecen was bought from Acros (St-Augustin, Germany), for fabricating the PU scaffolds (polyMaterials AG, Kaufbeuren, Germany). The silicon negative mold in the shape of an ear was made from the positive model generated by stereo lithography (KL-Technik, Gauting, Germany).

Resomer® RG 756, synthesized from 75 % lactic acid and 25 % glycolic acid (M_w = 90 Da, T_g = 50.7 °C), were kindly provided by Boehringer Ingelheim (Ingelheim, Germany). Solid lipids used for porogen production, namely Witepsol® H42, were kindly provided by SASOL Germany GmbH (Witten, Germany). Methylethyl ketone, tetrahydro furane, ethyl acetate and n-hexane were purchased in analytical grade from Merck (Darmstadt, Germany).

Polyglycolic acid (PGA) non-woven meshes (12-14 µm fiber diameter; 96 % porosity; 62 mg/cm^3 bulk density) with an initial molecular weight of PGA of approximately 70 kDa [7] were obtained from Albany International (Mansfield, MA, USA).

Aprotinin solution (Trasylol®) was bought from Bayer (Leverkusen, Germany). Thrombin (as a part of Tissucol®), thrombin dilution buffer and the commercially available fibrin glue kit Tissucol® was kindly provided by Baxter (Unterschleißheim, Germany). Bovine fibrinogen was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Knee joints from three-months-old bovine calves were obtained from a local abattoir within 12-18 hours of slaughter. Human septal cartilage was collected from patients undergoing septorhinoplasties at the University Hospital of Regensburg (Regensburg, Germany), after informed consent was given. Type II collagenase and papainase were purchased from Worthington (CellSystem, St. Katharinen, Germany). Dulbeccos's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, fetal bovine serum (FBS), MEM non-essential amino acid solution, penicillin, streptomycin, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES buffer) and phosphate buffer solution (PBS buffer) were obtained from Gibco (Karlsruhe, Germany).

149 µm pore size polypropylene filters were purchased from Spectrum (Rancho Dominguez, CA, USA). Cell culture plastics were purchased from Corning Costar

(Bodenheim, Germany). Spinner flask was custom-made (250 ml volume, 6 cm bottom diameter, side arms for gas exchange).

Ascorbic acid, deoxyribonucleic acid, diaminobenzidine, dimethylmethylene blue, fluorescein diacetate, glutaraldehyde, glycine, hematoxylin, proline, propidium iodide, safranin-O, Tween® 80 and pepsin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Chloramin-T, formalin 37 %, and p-dimethylaminobenzaldehyde (p-DAB) were from Merck (Darmstadt, Germany).

Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA) and L-hydroxyproline from Fluka (Neu-Ulm, Germany). Chondroitin sulfate was from ICN (Aurora, Ohio, USA) and Tissue Tek was from Sakura Finetek (Torrane, CA, USA). Vectastain ABC-kit and DAB-kit, and normal horse serum were obtained from Vector Laboratories Inc. (Burlingame, CA, USA). Type II collagen monoclonal antibody was obtained from the development studies Hybridoma Bank and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, USA). Type I collagen antibody (monoclonal anti-collagen type I col-1) mouse ascites fluid was bought from Sigma-Aldrich (Saint Louis, Missouri, USA).

Scaffold and fibrin glue preparation

Polycaprolactone-based scaffolds were manufactured by polyMaterials AG (Kaufbeuren, Germany). Briefly, poly(caprolactone)diol, poly(caprolactone)triol, DABCO 3042, dextrose, methylal and diazabicycloundecene were premixed and the mixture was given into a two chamber syringe with a static mixer. The other chamber was filled with isophorone diisocyanate and methylal, and the syringe was heated to 37 °C. The mixture was slowly injected into a silicon mold and heated to 67 °C for two hours. Afterwards, constructs were washed in boiling distilled water for one hour to remove substances adherent to the surface and dried in vacuo for 24 hours. Scaffolds were prepared by die-punching into discs 5 mm in diameter and 2 mm thick, pre-wetted with 70 % EtOH, rinsed extensively with PBS and autoclaved in a flask containing excess of PBS. For detailed investigations of the polycaprolactone based scaffolds, four different scaffold types were tested after they had been pre-selected out of more than 200 on the basis of cell adhesion and biocompatibility. Type A primarily consisted of PCL-Triol 900 resulting in a high cross-linking density of about ten building blocks. For type B and type C, PCL-Diol 2000 was added for a lower cross-linking density, to accelerate the expected long duration of

biodegradation. Additionally, type B contained PEG 600 to increase hydrophilicty, i.e., to facilitate gel filling. Finally, for type D, PCL-Diol 4000 was added to PCL-Triol 900, which resulted in longer polyurethane segments, compared to the other types, i.e., in higher polymer crystallinity and lowest cross-linking density of about 50 building blocks. For preparation of the polycaprolactone-based ear scaffold, the mixture of the polymer solution of type C was injected into a silicon negative mold in the shape of the cartilaginous part of an external ear and prepared for cell seeding as described above.

Electron microscopic investigation of the polyurethane scaffold were performed using S-3599-N Hitachi High-Technologies Europe GmbH (Krefeld, Germany), and size of interconnective pores and porosity were evaluated with AutoPore VI 9500 mercury porosimeter from Micromeritics Instrument Corporation (Mönchengladbach, Germany), both by the Central Institute for Medical Technology (Garching, Germany).

To fabricate macroporous PLGA scaffolds, our group recently developed an anhydrous technique that uses solid lipid microparticles as porogens [21, 22]. Briefly, solid lipid microparticles, prepared by melt dispersion technique, were mixed with polymer solution in a polymer to porogen ratio of 1:2 (w/w). The homogenous dispersion was transferred into Teflon® molds with a cylindrical cavity 0.8 cm in diameter. Subsequently, molds were submerged in warm n-hexane to induce solvent extraction followed by the precipitation of the polymer and extraction of the lipid porogen. After cooling and drying under vacuum for 48 h, the constructs were cut into 5 mm in diameter and 2 mm thick discs, pre-wetted in 70 % EtOH and rinsed extensively with PBS, prior to cell seeding.

Commercially available PGA-mesh (Albany International) was die-punched into discs 5 mm in diameter and 2 mm thick, pre-wetted with 70 % EtOH and rinsed extensively with PBS, prior to cell seeding.

Based on previous results (chapter 4), the gel was prepared by mixing 100 mg/ml fibrinogen in 10,000 KIE/ml aprotinin with the same volume of 5 U/ml thrombin in dilution buffer and allowed to gel for 45 min.

Cell isolation

Primary bovine chondrocytes were isolated from the surface of the femoral patellar groove using sterile technique. The cartilage was cut into small pieces, washed three times with PBS and enzymatically digested over night in complete chondrocyte medium, DMEM containing 4.5 g/l glucose, 584 mg/l glutamine, 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.4 mM proline, 0.1 mM MEM non-essential amino acids, 50 µg/ml ascorbic acid and 470 U/ml of type II collagenase. The digest was re-pipetted, filtered through a 149 µm filter and washed three times with PBS. Cell number was determined using a hemocytometer and an inverted phase-contrast microscope.
Human chondrocytes were cut into small pieces and 0.1 g was enzymatically digested for 6 hours using 1108 U/ml collagenase type II dissolved in complete chondrocyte medium as described above. The digest was re-pipetted and seeded into T-75 culture flasks. Cells were then expanded in monolayer at 37 °C, 5 % CO_2 and 95 % humidity for two passages.

Vitality testing of polyurethane scaffolds

Vitality testing was performed by the Department of Otolaryngology of the University Hospital of Regensburg. Human nasal chondrocytes (passage 2) were detached from T-75 culture flask by trypsinization and $2*10^6$ cells were pipetted onto a polymer sample of 1 cm^3. Cells were allowed to attach onto the sample for 45 to 60 min at 37 °C, 5 % CO_2 and 95 % humidity, before carefully covering the constructs with complete chondrocyte medium.
After 4 weeks, constructs were covered with a mixture of 25 mg/ml fluorescein diacetate (FDA) dissolved in acetone and the same volume of 1 mg/ml propidium iodide (PI) in water for 30 sec. After removing the dye, cell vitality was analyzed using an inverted microscope.

In vivo compatibility testing of polyurethane scaffolds

In vivo testings were performed by the Department of Otolaryngology of the University Hospital of Regensburg. For in vivo study, 8- to 12-weeks-old female immunocompetent NMRI mice were used, according to the institutional guidance of the central animal laboratories of the University of Regensburg. Mice were randomly bred in the nude mouse laboratory at the University of Regensburg. After anesthetization with a combination of

100 mg/kg ketamine and 4-6 mg/kg xylazine, the animals underwent random subcutaneous implantation into the back, one disc per mouse (n = 4). The wound was closed with sterile Michel suture surgical clips, and the animals were returned to the housing facility, where they had free access to food and water. Weighing was performed twice a week to check the sanitary constitution of the mice. After 1 and 3 weeks post-operation, mice were sacrificed by cervical dislocation and constructs were excised with the adjacent tissue (n = 1 after 1 week, n = 3 after 3 weeks), rinsed in PBS, and fixed for histological analysis.

Composite cell seeding and construct culture

For cell-fibrin-polymer composite constructs, $5*10^6$ cells were suspended in 20 µl fibrinogen solution and mixed with the same volume of thrombin solution. Subsequently, cell-fibrin suspension was injected into the polymeric discs, consisting of polycaprolactone-based polyurethane, PLGA, or commercially available PGA and was allowed to gel for 45 min at 37 °C. Afterwards, constructs were covered with 4 ml of complete chondrocyte culture medium containing 10 % FBS, and medium was replaced three times per week. The constructs were statically cultured in 6-well-plates in an incubator at 37 °C, 5 % CO_2 and 95 % humidity for 4 weeks. After harvesting, constructs were histologically and biochemically analyzed.

For preparation of the ear construct, $500*10^6$ freshly isolated cells were suspended in 2 ml fibrinogen solution and mixed with the same volume of thrombin solution. The cell-fibrin suspension was immediately injected into the polycaprolactone-based ear-shaped scaffold by multiple injections into both sides of the scaffold (front and back), to ensure complete filling. The fibrin gel was allowed to gel for 45 min at 37 °C in a cell culture petri dish. The scaffold was then transferred in a spinner flask and covered with 120 ml of complete chondrocyte culture medium containing 10 % FBS that was replaced three times per week. The construct was dynamically cultured on an orbital shaker in an incubator at 37 °C, 5 % CO_2 and 95 % humidity. After 4 weeks, the ear construct was harvested and analyzed.

Histological and biochemical analysis

The constructs were analyzed as previously described [27, 28]. Composite constructs were weighed (= wet weight) and cut in half. For analysis of the ear construct, small samples from different areas of the ear were cut out and were biochemically and histochemically analyzed.

Briefly, for the determination of cell number, collagen content, and glycosaminoglycan content, one part of the construct was lyophilized and digested with 1 ml of a papainase solution (3.2 U/ml in buffer) for 18 h at 60 °C. After digest time, complete polycaprolactone scaffold as well as parts of PGA and PLGA remained undigested in papainase solution, but were supposed to not influence biochemical analysis of the liquid digest, based on preliminary experiments. The number of cells per cell-fibrin construct was assessed from the DNA content using Hoechst 33258 dye and a conversion factor of 7.7 pg DNA per chondrocyte [29]. The amount of sulfated glycosaminoglycans was determined spectrophotometrically at 525 nm as chondroitin sulfate using dimethylmethylene blue; bovine chondroitin sulfate was used as standard [30]. The hydroxyproline content was determined spectrophotometrically after acid hydrolysis and reaction with p-dimethylamino-benzaldehyde and chloramin-T [31]. The amount of total collagen was calculated using a hydroxyproline to collagen ratio of 1:10 [32].

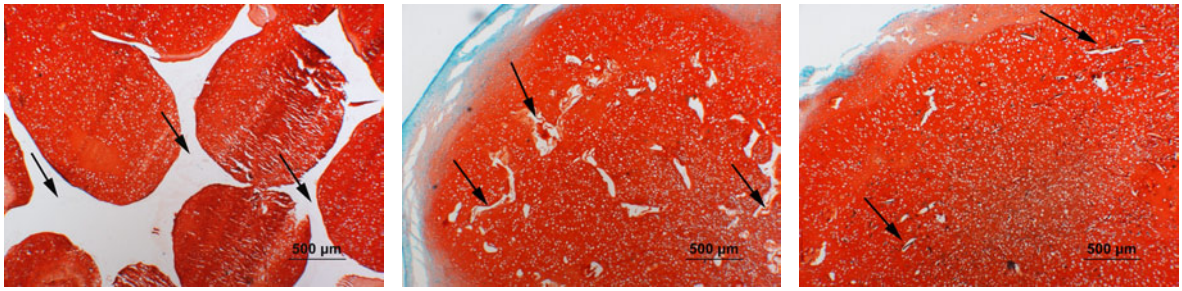
A part from the construct as well as in vivo constructs were prepared as a histological sample by fixing in 2 % glutaraldehyde in PBS for 30 min and then storing in 10 % formaldehyde in PBS. The formalin-fixed samples were embedded in paraffin and cross-sectioned into thick sections of 10 µm due to polymer elasticity; deparaffinized sections of composite constructs were stained with hematoxylin, fast green and safranin-O. In vivo constructs were only stained with hematoxylin and eosin by the Department of Otolaryngology, University of Regensburg (Regensburg, Germany).

For immunohistochemical analysis, a part of the constructs were prepared by fixing in methanol-formalin mixture, and then successively dehydrated in different concentrations (10 % - 40 %) of a sucrose solution followed by embedding into Tissue Tek. Frozen samples were cryosectioned at 7 µm and stained with antibodies for type I collagen (monoclonal anti-collagen type I col-1) mouse ascites fluid, dilution (1:1000), and type II collagen (DSHB), dilution (1:6). To prevent non-specific antibody binding samples were incubated with 5 % normal horse serum in buffer. The sections were then incubated overnight at room temperature with primary antibodies; PBS without antibodies was used for control sections. On the next day, sections were incubated with biotinylated secondary antibody, anti-mouse/rabbit IgG (Vector Laboratories; Inc.; Burlingame, USA), for 30 min at room temperature. Finally, the samples were stained using a Vectastain ABC-kit and DAB-kit according to the protocol for avidin-biotin-peroxidase complex formation.

Results

Comparison of three different types of polymer scaffolds

Suspension of freshly isolated bovine chondrocytes in fibrin gel and injection into polycaprolactone-based scaffolds, PLGA scaffolds, or commercially available PGA meshes resulted in homogenously distributed fibrin gel and round cells within the constructs. Histological cross-sections of all constructs were intensively stained red with safranin-O for glycosaminoglycans, indicating the development of high amounts of extracellular matrix components (Fig. 1). These findings were also reflected in wet weight, cell number and amounts of GAG and total collagen (Fig. 2). Biochemical analysis showed no differences in amount of extracellular matrix of the three types of polymeric scaffold. However, in histological cross-sections of PGA and PLGA scaffolds, only few residues of polymer were found within the construct due to biodegradation, therefore these tissues seem to be more coherent compared to constructs prepared with long-term stable polyurethane. However, except for small gaps within the newly developed tissue due to limited space, PU scaffold did not negatively influence cartilaginous tissue development.



PU PLGA PGA

Fig. 1: Histological cross-sections of composite constructs prepared with chondrocytes suspended in fibrin gel and injected into polyurethane (PU), PLGA and PGA scaffold, after 4 weeks in culture; stained red with safranin-O. Arrows indicate scaffold residues.

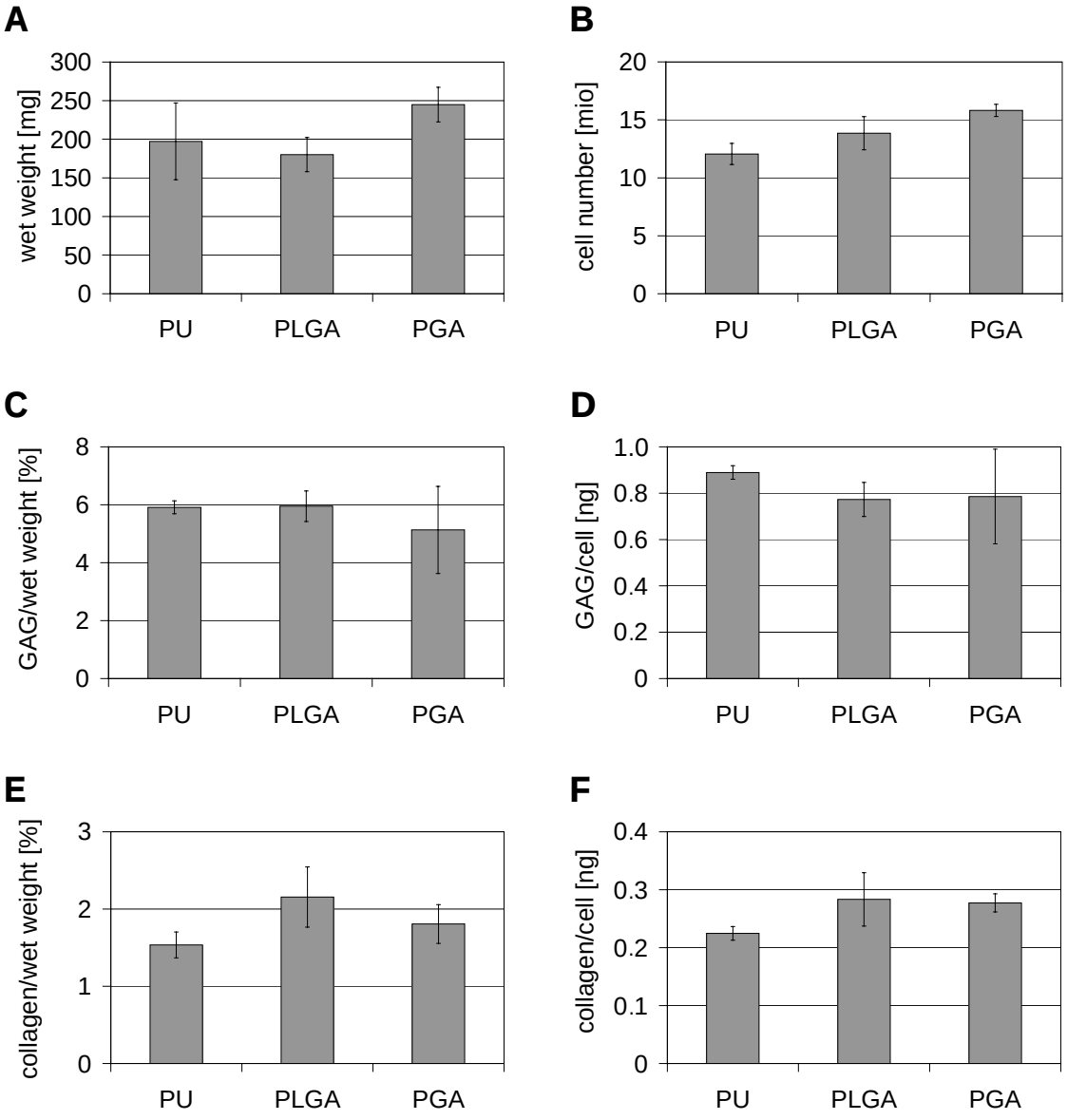
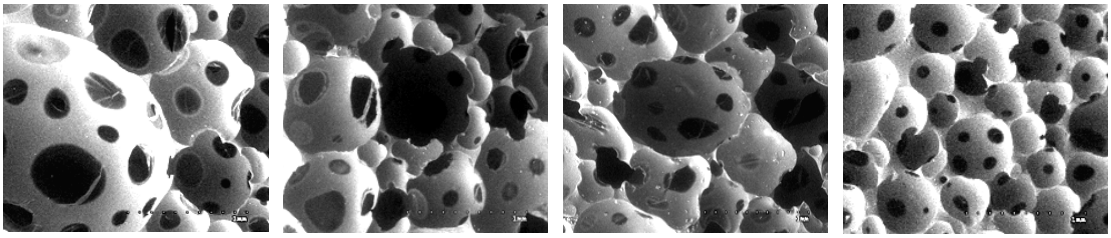


Fig. 2: Wet weight (A), cell number (B) and amount of GAG (C, D) and collagen (E, F) per wet weight (C, E) and per cell (D, F) of composite constructs with fibrin injected into different types of scaffold, after 4 weeks in culture. Data represents the average ± S.D. of four independent measurements.

Comparison of four different types of polycaprolactone-based scaffolds

Electron microscopic pictures and investigation of porosity of the four types of scaffold, indicating capacity of cell and gel infiltration, showed distinct differences in interconnective pore size and porosity (performed by the Central Institute for Medical Technology) (Fig. 3 and Fig. 4). Type B and C had a mean size of interconnective pores of 200 µm, whereas type A had a slightly larger mean size, and type D a distinctly smaller mean size of about 130 µm (Fig. 4). Furthermore, porosity of polymer A and B was higher (81% and 80%, respectively) compared to polymer C and D (70% and 68%, respectively) (data not shown).



A B C D

Fig. 3: Electron microscopic pictures of four different types of polycaprolactone-based polyurethane scaffolds (x 150).

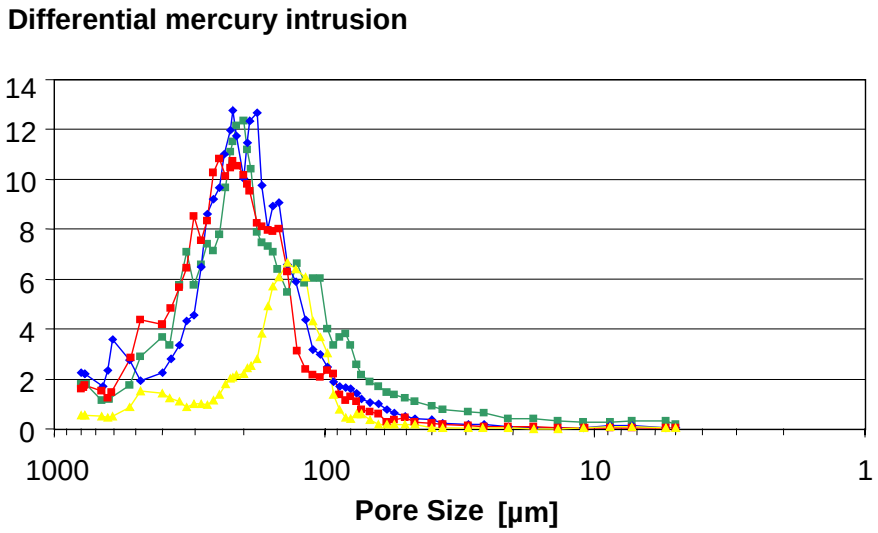
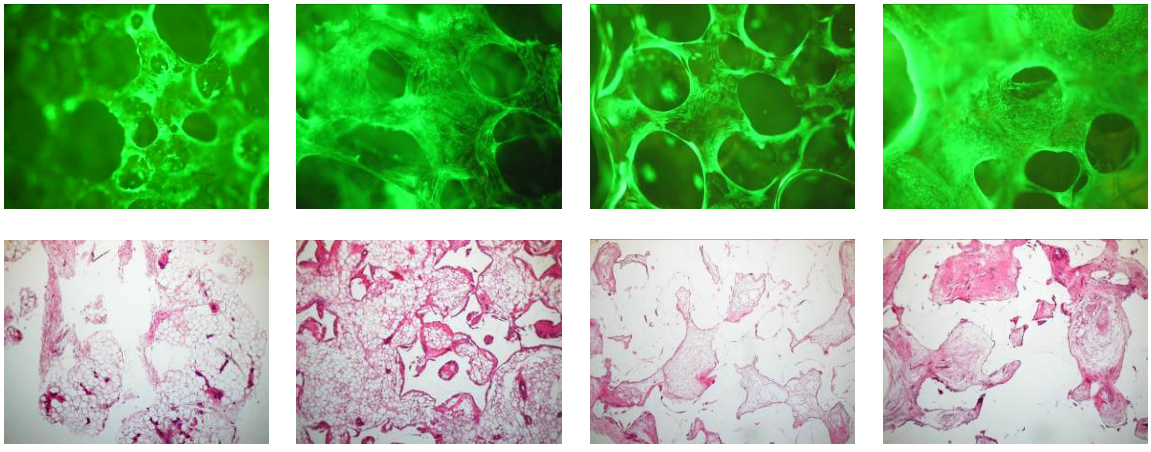


Fig. 4: Distribution of interconnective pore sizes of four different types of polyurethane scaffolds (red = type A, blue = type B, green = type C and yellow = type D), measured with mercury intrusion method.

In other preliminary investigations performed by the Department of Otolaryngology of the University Hospital of Regensburg and by the Department of ENT, Head and Neck Surgery of the Technical University of Munich, all types of polycaprolactone-based scaffolds were shown to be cell compatible, indicated by live-dead staining and in vivo testing. Human chondrocytes (passage 2) directly seeded onto scaffolds showed a bright green fluorescence staining after 4 weeks in vitro, resulting from FDA within the intact cell membrane metabolized by esterases (Fig. 5). No red fluorescence PI was found within the cells, indicating no penetration of PI through damaged cell membrane, i.e., no dead cells. With regard to the number of vital cells, cell counting demonstrated 98 % vitality of cells seeded onto polymer type A and type D, 95 % vitality onto polymer type B and 99 % vitality onto polymer type C (data not shown).

Additionally, implantation of these four types of scaffold into the back of immunocompetent mice resulted in infiltration of fibroblast and adipocytes, but no toxic reactions caused by the scaffold, indicated by staining with hematoxylin and eosin (Fig. 5).

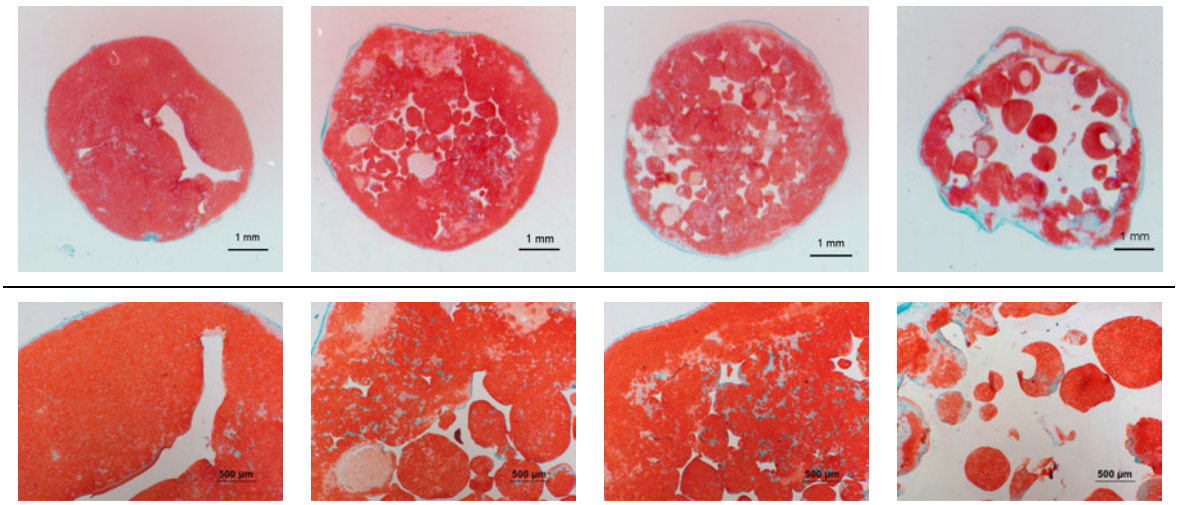


A B C D

Fig. 5: Vitality staining of four different types of polyurethane scaffold seeded with human chondrocytes (passage 2) after 4 weeks of culture, stained with FDA and PI (upper, original magnification x 200). Histological cross-sections of four different types of polyurethane scaffolds after 3 weeks in vivo, stained with hematoxylin & eosin (lower, original magnification x 100).

With regard to the investigations performed in our lab, primary bovine chondrocytes suspended in fibrin gel and injected into the four different types of polycaprolactone-based scaffolds maintained round and vital within the four weeks of experimental time, and produced high amounts of cartilaginous extracellular matrix. However, using scaffolds with various interconnective pore sizes resulted in constructs with different appearance, as displayed in Fig. 6. Injection into scaffold type B and C resulted in development of cartilaginous tissue within 4 weeks in culture, homogenously distributed within the pores of the scaffold and stained red with safranin-O for glycosaminoglycans. Histological cross-section of polymer type A with larger pore size showed less compartmentalization. In contrast, polymer type D with smaller pore size resulted in a less homogenous matrix distribution. The majority of cells and newly developed extracellular matrix were found at the periphery of the construct, likely due to inadequate cell seeding due to the small interconnective pores.

In Tab. 1, results from scaffold characterization as well as testings in cell culture were summarized. Based on these findings regarding cytocompatibility, robustness, porosity, and a interconnective pore size that enable cell seeding, proliferation and subsequently production of extracellular matrix, the two best scaffold types B and C were chosen for more detailed in vitro and in vivo experiments (chapter 8).



A B C D

Fig. 6: Histological cross-sections of constructs prepared with primary bovine chondrocytes suspended in fibrin gel and injected into four different types of polyurethane, after 4 weeks in culture; stained red with safranin-O for glycosaminoglycans.

type	components	mean pore size	robustness	cell compatibility	gel filling	matrix development	matrix distribution	summary
A	PCL-Triol 900	220µm	- fragile	+++	+++	+++	+++	+
B	PCL-Triol 900, PCL-Diol 2000, PEG 600	200µm	+++	+++	+++	+++	+++	+++
C	PCL-Triol 900, PCL-Diol 2000	200µm	+++	+++	+++	+++	+++	+++
D	PCL-Triol 900, PCL-Diol 4000	130µm	+++	++	++	++	+	+

Tab. 1: Summary of investigations of the four different types of polycaprolactone-based polyurethane scaffolds, as a pre-selection for a future in vivo study (+++ best, ++ adequate, + fair, - not suitable).

Polycaprolactone-based scaffold in the shape of an ear

In a proof of principle-experiment, suspending primary bovine chondrocytes in optimized fibrin gel and injection into a polycaprolactone-based scaffold in the shape of the cartilage part of a human ear resulted in phenotypically adequate formation of cartilaginous tissue throughout the whole construct. No deformation of the original ear size was found within the 4 weeks of culture time (Fig. 7). In addition, biochemical analysis of random samples (n = 7) from the construct showed the development of high amounts of extracellular matrix components GAG and total collagen per wet weight as well as per cell (Tab. 2). However, as demonstrated by histological cross-sections, cell distribution was not homogenous. In Fig. 8, representative sections of tissues developed within 4 weeks in vitro are displayed showing, on the one hand, areas with adequate cell and matrix distribution (left) and, on the other hand, areas with less cell and extracellular matrix, thus, an incoherent cartilaginous tissue (right).

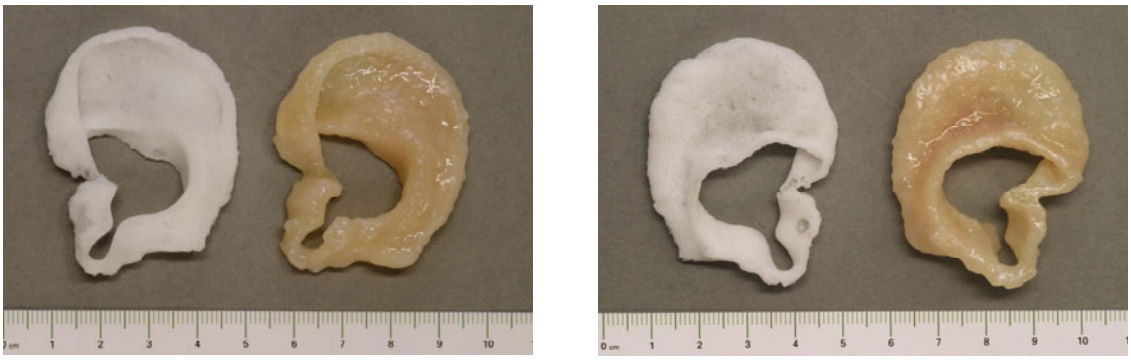


Fig. 7. Polyurethane scaffold in the shape of the cartilage part of a human ear (white), seeded with primary bovine chondrocytes after 5 weeks in culture (beige), front side (left) and back side (right).

	mean	sdv
wet weight [mg]	47.32	20.85
cell number/wet weight [1000/mg]	54.39	13.54
GAG/wet weight [%]	1.09	0.51
GAG/cell [ng]	0.21	0.07
collagen/wet weight [%]	3.94	0.88
collagen/cell [ng]	0.74	0.17

Tab. 2: Wet weight, cell number and amount of extracellular matrix components per wet weight and per cell of parts of the ear constructs (n = 7), after 5 weeks in culture.

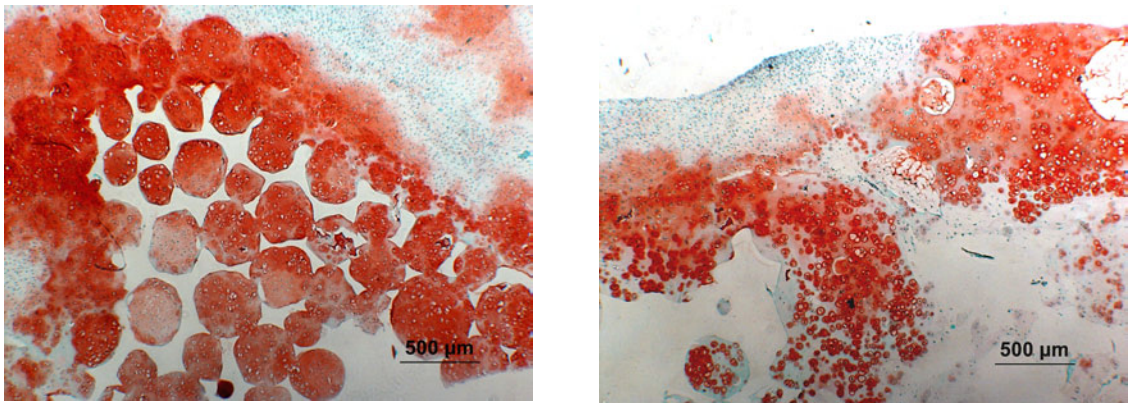


Fig. 8. Histological cross-sections of parts of the ear construct after 5 weeks in culture, with homogenously (left) and inhomogeneously (right) distributed cells and extracellular matrix, stained red with safranin-O for glycosaminoglycans.

Discussion

Within this study, we demonstrated the potential of a combination of long-term stable fibrin gels and polymeric scaffolds for the use in cartilage engineering. Primary bovine chondrocytes suspended in fibrin gel and injected into newly developed polycaprolactone-based scaffolds resulted in adequate cell and extracellular matrix distribution within 4 weeks in culture. Biochemical analysis demonstrated the development of high amounts of extracellular matrix components GAG and total collagen. No differences were found in using this composite system compared to injection into PLGA scaffolds or commercially available fibrous PGA meshes. As a next step, in evaluating four different types of polycaprolactone-based polyurethane scaffolds, the two best scaffolds were chosen for future more detailed in vitro and in vivo testings. Finally, with regard to a clinical application of this composite system, a polycaprolactone-based scaffold in the shape of an adult human ear was casted and, as a prove of principle, successfully seeded with primary bovine chondrocytes. In vitro culture of this construct resulted in adequate cartilaginous matrix development within the construct and excellent shape stability within the 4 weeks of culture time.

Though solid polymeric scaffolds are often used in the field of tissue engineering, researchers often face several problems, e.g., low seeding efficiency, inhomogenous cell distribution, increasing chondrocyte dedifferentiation over time due to two-dimensional cell spreading onto the pore walls, and poor retention of newly developed extracellular matrix due to diffusion of the molecules into the culture medium [10, 11]. Therefore, a number of groups recently developed strategies by entrapping cells in different types of soft hydrogels such as fibrin, alginate or collagen before injection into various polymeric scaffolds, in detail discussed in chapter 8 [12, 13, 33]. In particular, fibrin is often used in cartilage tissue engineering, as it is easily prepared by mixing the individual fibrinogen and thrombin components and allows for injection of cell-gel suspension prior to gelation [34, 35]. In this study, the combination of freshly isolated chondrocytes in long-term stable fibrin gels injected in different types of polymer resulted in a high seeding efficiency, a homogenous cell distribution and the development of adequate extracellular matrix throughout the whole construct, indicated by histological cross-sections and biochemical analysis. At the same time an added stability is provided based on the scaffold material. In particular, using polycaprolactone-based scaffolds, this composite construct is supposed to

withstand the load-bearing process of implantation and strong mechanical loadings in the patient after implantation.

In cell carrier-based tissue engineering approaches, the scaffold plays a pivotal role in the formation of the new tissue. On the basis of the hydrophilicity and mechanical properties, the scaffold materials can be divided into materials that swell significantly in water forming a hydrogel [1, 36, 37] and materials that only dissolve in organic solvents and are used for the fabrication of macroporous solids [2, 3, 38]. Biodegradable materials like PGA, PLLA and polycaprolactone have been focused on for the use in tissue engineering due to approval from the Food and Drug Administration [7, 39]. Fibers produced by textile technology have been used to fabricate non-woven meshes from PGA [40]. These fiber meshes still represent the standard scaffolds for many applications in the field of tissue engineering likely due to the high porosity and permeability enabling high cell density and adequate diffusion of nutrient, oxygen and waste. However they often lack necessary structural stability, rapidly degrade up to 50 % in its mechanical strength over 2 weeks and are completely absorbed in about 4 weeks [4]. Within our study, suspension of chondrocytes in fibrin gel and injection into a commercially available PGA mesh resulted in a homogenous cartilaginous tissue containing high amounts of GAG and collagen. Only few fiber residues could be found, indicating an almost complete biodegradation within the 4 weeks of culture time. However, these types of constructs are supposed to be not able to withstand extreme mechanical loadings and are not suitable for generation of cartilaginous constructs in a delicate shape like a human ear.

To improve the mechanical properties of the meshes, PGA may be blended with solutions of other polymers, for example PLLA, to obtain PLGA. PLLA scaffolds itself slowly degrade over 2 years, and a combination with PLLA could prolong rapid biodegradation of PGA [39]. PLGA scaffolds were produced in our group by solid lipid templating technique with lipid microparticles as porogens and non-halogenated polymer solvents; the biodegradable polymer was processed into macroporous scaffolds, providing an interconnected pore structure [21, 22]. Histological cross-sections of constructs prepared with chondrocytes suspended in fibrin gel and injected into PLGA scaffolds showed the development of an adequate cartilaginous tissue containing high amounts of extracellular matrix, within 4 weeks in vitro, similar to the use of commercial PGA-mesh. Small amounts of polymer residues, but higher amounts compared to the PGA-group, remained within the constructs after 4 weeks in vitro, which demonstrated a slightly slower biodegradation of the employed PLGA compared to PGA.

With regard to polyurethane, only few investigations have been conducted in the field of tissue engineering, especially with regard to cultivation of chondrocytes. Within the ForTEPro research collaboration, a variety of polycaprolactone-based polyurethane scaffolds were recently developed with different cross-linking densities, hydrophilicites and crystallinities. The decision on a polyurethane foam as scaffold material was made, because this material class gives the possibility to vary the properties in a broad range. Cells seeded into these scaffolds via injection of fibrin resulted in development of cartilaginous tissue that contained high amounts of GAG and collagen, similar to PGA mesh and PLGA scaffolds. However, polycaprolactone-based scaffolds were not degraded within the 4 weeks of culture time; therefore, the newly developed tissue was located in the round pores of the scaffold, and small gaps could be found within the tissue caused by the scaffold material. This scaffold material will degrade within 1 to 2 years in vivo, providing an added mechanical stability and shape integrity of the implant. During degradation, the gaps within the new tissue are supposed to be refilled with chondrocytes producing new extracellular matrix, and subsequently result in a connected cartilaginous tissue. With regard to a possible clinical application, different types of polyurethane scaffolds were in detail tested regarding cytocompatibility, porosity and interconnective pore size as well as cell seeding, proliferation and subsequent development of cartilaginous tissue (Tab. 2). Comparing all results obtained within the ForTEPro collaboration, polymer types B and C were chosen for more detailed testings and implantation into nude mice to investigate tissue development in vivo (Chapter 8).

Total external ear reconstruction still remains one of the most challenging problems in the field of plastic and reconstructive surgery. An approach using autologous costal cartilage placed in a subcutaneous pocket for ear reconstruction was first described by Gillies in 1920 [41]. Refined by Tanzer [42] and Brent [43], this complicated procedure remained the standard method for this kind of surgery. However, this technique has several limitations including lack of tissue flexibility, long-lasting surgical procedure and donor-site morbidity [44, 45]. The use of alloplastic materials exhibits the obvious advantages of a decreased operation time and the possibility of determination of construct size and shape. However, they include the risk of infections and immune reactions, and often lack adequate material flexibility [46, 47]. Therefore, tissue engineering provides a promising alternative in using autologous chondrocytes seeded onto a pre-shaped scaffold that is replaced by newly developed cartilaginous tissue. First spectacular attempts were made in 1997 by Cao et al., who demonstrated the development of cartilaginous tissue in the shape of a 3-year-old

child's auricle. Primary bovine chondrocytes were seeded onto a PGA polymer in the shape of a juvenile ear that was coated with poly(L-lactic acid), pre-cultured in vitro for 1 week und subsequently implanted into the back of a nude mouse [48]. However, the mechanical properties of the neo-cartilage after 12 weeks in vivo was insufficient to maintain the original contours and structural features of the delicate human construct, and the subsequent temporary placement of an external stent was required. Kamil et al. evaluated auricular cartilage regeneration using pig and sheep auricular chondrocytes in various soft and solid biomaterials after autogenous implantation directly after construct preparation. However, they faced several problems, e.g. inflammatory reactions, cell loss into the surrounding tissue, and subsequent construct resorption [49]. Furthermore, Isogai et al. reported about long-term retention of regenerated auricular cartilage in vivo using bovine chondrocytes and a biodegradable poly(L-lactic acid - caprolactone) copolymer scaffold in the shape of a human ear. However, deformation of the ear shape could not be prevented during the complete 40 weeks in vivo [39, 50]. Although these preliminary results have been promising, problems of shape stability and long-term durability still exist, and the long-term fate of these constructs in vivo is still unknown.

Within this study, a long-term stable polycaprolactone-based scaffold was prepared in the shape of the cartilage part of an adult human external ear. Data obtained by high resolution computer tomography were analyzed using the software 'Mimics' (Materialise, Munich, Germany). Based on gray values of the resulting digital image correlating with native tissue density, it was possible to separate the data of the cartilaginous parts of the ear from those from the soft tissue. Subsequently, using the software 'Magics', a three-dimensional positive ear model was generated by rapid prototyping methods, which provided a basis for casting a negative silicon mold. Using this negative mold, a highly porous ear-shaped polyurethane foam was produced and seeded with a high number of primary bovine chondrocytes in fibrin gel. Analysis demonstrated the development of cartilaginous tissue containing high amounts of GAG and collagen within 4 weeks in vitro. Mechanical stability of the scaffold was strong enough to withstand forces resulting from cells proliferation and newly developed matrix, in the same time enabling adequate construct flexibility, as determined by gross examination. However, it must be noted, that porosity and interconnectivity of the ear-shaped scaffold was not uniform throughout the whole construct, indicated by visual examination. These differences finally resulted in areas with low matrix distribution within the construct, which was attributed to insufficient initial cell seeding. Therefore, scaffold processing has to be optimized to obtain a uniform scaffold

with high and homogenous porosity. Nevertheless, this study demonstrated the potential of these newly developed polycaprolactone-based scaffolds to be casted in a delicate patient-specific shape and seeded with chondrocytes, to subsequently obtain a construct for implantation that likely withstands high mechanical loadings, e.g. after implantation in vivo.

It should be noted that this study has been conducted using a large number of chondrocytes obtained from juvenile bovine articular cartilage. Applying human autologous chondrocytes is much more complicated (in detail discussed in chapter 6). Small biopsies result in only a limited number of primary chondrocytes that need to be expanded in monolayer to obtain a cell number sufficient for three-dimensional cell seeding [51]. However, during two-dimensional proliferation, chondrocytes often dedifferentiate towards a more fibroblastic appearance and produce non-chondrocytic collagen type I [52-54]. Therefore, a method for proliferation of human cells must be developed that prevents cell dedifferentiation or enhances cell redifferentiation when re-seeded into a three-dimensional culture system. Subsequently, the development of new cartilaginous tissue may be investigated using thus obtained human chondrocytes suspended in fibrin glue and injected into a polymeric scaffold, e.g. the newly developed ear-shaped polycaprolactone-based scaffold.

Acknowledgement

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Chapter 8

In Vitro and in Vivo Cartilage Engineering Using a Combination of Long-Term Stable Fibrin Gels and Polycaprolactone-Based Scaffolds

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Abstract

The use of either hydrogel or solid polymeric scaffold alone is often associated with distinct drawbacks in many tissue engineering applications. Therefore, in this study, we investigated the potential of a combination of long-term stable fibrin gels and polyurethane scaffolds for cartilage tissue engineering. These composite constructs were implanted into the back of nude mice and examined regarding cartilaginous tissue development. In particular, the effect of pre-cultivation in vitro prior to implantation on in vivo tissue development was examined.

Primary bovine chondrocytes were suspended in an optimized fibrin gel and subsequently injected into a polycaprolactone-based polyurethane scaffold. Cells were homogenously distributed within this composite system, maintained round and vital within 4 weeks in vitro and produced high amounts of cartilage-specific extracellular matrix components GAG and collagen type II. In contrast, cells seeded directly onto the scaffold without fibrin resulted in a lower seeding efficiency, and less homogenous matrix distribution containing large areas without cartilaginous tissue after 4 weeks in vitro. Fibrin-scaffold constructs implanted into the back of nude mice showed the development of excellent cartilaginous tissue within the polymer after 1, 3, and 6 months in vivo, containing high amounts of GAG and collagen type II. However, even after 6 months in vivo, histology of constructs seeded without fibrin showed still an inhomogeneous and, thus, not adequate extracellular matrix distribution compared to seeding with fibrin. Fibrin-scaffold constructs that were pre-cultured prior to implantation contained distinctly more extracellular matrix components GAG and collagen more homogenously distributed throughout the explant compared to constructs implanted directly after preparation. Interestingly, a pre-cultivation for 1 week in vitro elicited similar results compared to pre-cultivation for 4 weeks, i.e., a pre-cultivation for longer than 1 week did not enhance tissue development. The presented composite system is suggested as a promising step towards clinical application of engineered cartilaginous tissue for plastic and reconstructive surgery.

Introduction

A scaffold in cartilage tissue engineering must fulfill many requirements to promote tissue growth, e.g., act as space filling agent and three-dimensional structure to accommodate the cells, to maintain a specific shape and structural integrity. Common cartilage tissue engineering approaches either use microporous hydrogels or macroporous sponge-like solid polymeric scaffolds. Hydrogels (e.g. alginate, collagen, fibrin or agarose) on the one hand can be easily prepared and injected directly into the specific defect. The isolated single cells can be easily suspended during the gelation process, which result in adequate three-dimensional cell distribution throughout the whole construct, and newly produced extracellular matrix molecules are retained within the construct due to a narrow network. However soft hydrogels often lack adequate mechanical strength, which would be beneficial for the process of implantation into the patient and especially for resistance against mechanical loading in vivo after implantation [1-5]. Solid polymeric scaffolds (e.g., made from polyglycolide, polylactide or polyurethane), on the other hand, can provide this required load-bearing capacity and mechanical integrity. They can be manufactured with high porosity and elasticity, a specific hydrophilicity required for cell adhesion, and an appropriate degradation time depending on application [6-8]. However, large pore sizes often result in low cell seeding efficiency, inadequate cell distribution, increasing cell dedifferentiation over time due to two-dimensional cell spreading onto the pore walls, and poor retention of newly developed extracellular matrix due to diffusion of the molecules into the culture medium [9, 10]. Taken together, though each scaffold system possesses useful properties, both hydrogels and solid polymeric scaffolds are still associated with several problems in many tissue engineering applications.

As an example for hydrogels, fibrin has been utilized for different applications in the field of tissue engineering in recent years, especially in cartilage engineering [11]. Fibrin glue is a commonly used surgical haemostatic agent and has been commercially available for over 20 years in surgery and clinical practice [12-14]. The hydrogel is a polypeptide consisting of the plasma components fibrinogen and thrombin. Physiologically, fibrin formation occurs as the final step in the natural blood coagulation cascade, producing a clot that assists wound healing [15]. Due to the fact that fibrin is a physiological blood component, it is considered to be biocompatible and biodegradable. However, an important fibrin glue characteristic is an increasing instability and solubility over time in vitro and in vivo, which could be a problem for the application in tissue engineering [16-19]. Therefore, in

modifying specific fibrin gel preparation parameters, our group has previously developed a long-term stable fibrin gel that is suitable for cartilage engineering (chapter 4).

Regarding polymeric scaffolds, elastomeric polyurethanes have been extensively used in various implantable devices in clinical practice [20-22]. However, only few investigations have been conducted using polyurethane for tissue engineering applications, especially with regard to cultivation of chondrocytes. Only recently, a variety of new polycaprolactone-based scaffolds with different cross-linking densities, hydrophilicities, and crystallinities were developed [23]. In order to enable the future manufacture of highly porous scaffolds by simple casting into a patient-specific silicon mold, a two-component polyurethane system with liquid precursors was used. Based on preliminary studies evaluating more than 200 scaffolds, the two most promising scaffold types (see chapter 7) were chosen for more detailed in vitro and in vivo experiments within this study.

We investigated the potential of a combination of long-term stable fibrin gels and polycaprolactone-based scaffolds for cartilage engineering regarding cartilaginous matrix development and distribution. In particular, we examined if cells suspended in fibrin gel and injected into the scaffold resulted in a better cell distribution and, thus, in a more homogenous matrix development, compared to cells seeded directly into the polymeric scaffold. These constructs were subcutaneously implanted into the back of nude mice to study in vivo growth of cartilaginous tissue within 1, 3, and 6 months. Furthermore, we investigated if a pre-cultivation for 1 or 4 weeks in vitro improved tissue development in vivo, compared to implantation directly after construct preparation.

Materials and Methods

Materials

Poly(caprolactone)diol (M_n = 1250), poly(caprolactone)triol (M_n = 900), methylal and dextrose were purchased from Sigma-Aldrich (Taufkirchen, Germany), DABCO 3042 was bought from Air Products (Hattingen, Germany) and diazabicycloundecen was bought from Acros (St-Augustin, Germany).

Aprotinin solution (Trasylol®) was bought from Bayer (Leverkusen, Germany). Thrombin (as a part of Tissucol®), thrombin dilution buffer and the commercially available fibrin glue kit Tissucol® was kindly provided by Baxter (Unterschleißheim, Germany). Bovine fibrinogen was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Knee joints from three-months-old bovine calves were obtained from a local abattoir within 12-18 hours of slaughter. Type II collagenase and papainase were purchased from Worthington (CellSystem, St. Katharinen, Germany). Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, fetal bovine serum (FBS), MEM non-essential amino acid solution, penicillin, streptomycin, N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES buffer) and phosphate buffer solution (PBS buffer) were obtained from Gibco (Karlsruhe, Germany). 149 µm pore size polypropylene filters were purchased from Spectrum (Rancho Dominguez, CA, USA).

Ascorbic acid, deoxyribonucleic acid, diaminobenzidine, dimethylmethylene blue, glutaraldehyde, glycine, hematoxylin, proline, safranin-O, Tween® 80 and pepsin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Bovine insulin from bovine pancreas, chloramin-T, formalin 37%, and p-dimethylaminobenzaldehyde (p-DAB) were from Merck (Darmstadt, Germany).

Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA) and L-hydroxyproline from Fluka (Neu-Ulm, Germany). Chondroitin sulfate was from ICN (Aurora, Ohio, USA) and Tissue Tek was from Sakura Finetek (Torrane, CA, USA). Vectastain ABC-kit and DAB-kit, and normal horse serum were obtained from Vector Laboratories Inc. (Burlingame, CA, USA). Type II collagen monoclonal antibody was obtained from the development studies Hybridoma Bank and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, USA). Type I collagen antibody (monoclonal anti-collagen type I col-1) mouse ascites fluid was bought from Sigma-Aldrich (Saint Louis, Missouri, USA).

Scaffold and fibrin glue preparation

Polycaprolactone-based scaffolds were manufactured as previously described [23]. Briefly, poly(caprolactone)diol, poly(caprolactone)triol, DABCO 3042, dextrose, methylal and diazabicycloundecene were premixed and given into a two chamber syringe with a static mixer. The other chamber was filled with isophorone diisocyanate and methylal, and the syringe was heated to 37 °C. The mixture was slowly injected into a silicon mold and heated to 67 °C for two hours. Afterwards, constructs were washed in boiling distilled water for one hour to remove substances adherent to the surface and dried in vacuo for 24 h. Scaffolds were prepared by die-punching into discs 5 mm in diameter and 2 mm thick, pre-wetted with 70 % EtOH, rinsed extensively with PBS and autoclaved in a flask containing excess of PBS. Designation of polymers as polymer B and C is based on a previous study (chapter 7), in which four different types of polymer were compared regarding scaffold porosity and hydrophilicity, gel infiltration, as well as cell compatibility and distribution. Based on these results, polymer B and C were chosen for the in vivo study presented here. Compared to polymer C, polymer B contains poly(ethylene glycol) units to increase the hydrophilicity. In contrast, polymer C has a lower crosslinking density to accelerate biodegradation, as compared to the supposedly slow degradation of polymer B. Pore sizes of both scaffold types were mainly between 100 µm to 300 µm and porosities were 80 % and 70 %, respectively.

Based on previous results (chapter 4), fibrin gel was prepared by mixing 100 mg/ml fibrinogen dissolved in 10,000 KIE/ml aprotinin with the same volume of 5 U/ml thrombin in dilution buffer.

Cell isolation

Primary chondrocytes were isolated from the surface of the femoral patellar groove using sterile technique. The cartilage was cut into small pieces and enzymatically digested over night in DMEM containing 4.5 g/l glucose, 584 mg/l glutamine, 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.4 mM proline, 0.1 mM MEM non-essential amino acids, 50 µg/ml ascorbic acid and 470 U/ml of type II collagenase. The digest was re-pipetted, filtered through a 149 µm filter and washed three times with PBS. The cell number was determined by cell counting using a hemocytometer and an inverted phase-contrast microscope.

Cell seeding and construct culture

For cell-fibrin-scaffold composite constructs, $5*10^6$ cells were suspended in 20 µl fibrinogen solution and mixed with the same volume of thrombin solution. Subsequently, cell-fibrin suspension was injected into the polymeric disc and was allowed to gel for 45 min at 37 °C. For cell-fibrin constructs, serving as control, 40 µl cell-fibrin suspension was pipetted into a stabilizing 5 mm diameter silanized glass ring and also allowed to gel for 45 min at 37 °C before removing the glass ring. For cell-scaffold constructs, the polymeric disc was placed into a 5 mm diameter glass ring and $5*10^6$ cells suspended in 40 µl culture medium were pipetted onto the scaffold. Cells were allowed to attach onto the scaffold for 2 hours at 37 °C before removing the glass ring. For all groups, after the seeding procedure, constructs were either stored in PBS buffer until direct implantation into nude mice within 2 h of preparation, or covered with 4 ml of complete chondrocyte culture medium and incubated at 37 °C for in vitro pre-cultivation. Medium contained 4.5 g/l glucose, 584 mg/l glutamine, 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.4 mM proline, 0.01 mM MEM non-essential amino acids and 50 µg/ml ascorbic acid, and was replaced three times per week. The constructs were statically cultured in 6-well-plates in an incubator at 37 °C, 5 % CO_2 and 95 % humidity, and implanted after 1 and 4 weeks of pre-culture. For each group, co-constructs were not implanted; they were harvested as in vitro controls after 1 and 4 weeks in vitro, and were biochemically and histochemically analyzed. As in vivo controls, scaffolds B and C alone as well as scaffolds B and C with fibrin were treated the same way as constructs containing cells, and stored in PBS buffer until implantation within 6 h of autoclaving and 2 h of fibrin injection, respectively.

Construct implantation

For in vivo study, 10- to 12-weeks-old female immunodeficient NMRI (nu/nu) mice were employed, according to the institutional guidance of the central animal laboratories of the University of Regensburg. Mice were randomly bred in the nude mouse laboratory at the University of Regensburg under pathogen-free conditions at 26 °C, 70 % relative humidity, and a 12 h light/dark cycle. After anesthetization with a combination of 100 mg/kg ketamine and 4-6 mg/kg xylazine, each animal underwent random subcutaneous implantation of three constructs of different groups into the back (n = 18 per group). One

construct containing cells was implanted into the left front flank and another one into the right front flank. Constructs containing no cells were placed into the left back flank, therefore possible influences of the implantation on the back site on cartilaginous tissue development might be prevented. The wounds were closed with sterile Michel suture surgical clips, and the animals were returned to the housing facility, where they had free access to food and water. Weighing was performed twice a week to check the sanitary constitution of the mice. After 1, 3, and 6 months post-operation, mice were sacrificed by cervical dislocation and constructs were excised with the adjacent tissue (n = 6 per group and time point), rinsed in PBS, and fixed for histochemical analysis.

Histological and biochemical analysis

The constructs were analyzed as previously described [24, 25]. The constructs were weighed (= wet weight) and cut in half.

Briefly, for the determination of cell number, collagen content, and glycosaminoglycan content, one part of the construct was lyophilized and digested with 1 ml of a papainase solution (3.2 U/ml in buffer) for 18 h at 60 °C. The number of cells per construct was assessed from the DNA content using Hoechst 33258 dye and a conversion factor of 7.7 pg DNA per chondrocyte [26]. The amount of sulfated glycosaminoglycans was determined spectrophotometrically at 525 nm as chondroitin sulfate using dimethylmethylene blue; bovine chondroitin sulfate was used as standard [27]. The hydroxyproline content was determined spectrophotometrically after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramin-T [28]. The amount of total collagen was calculated using a hydroxyproline to collagen ratio of 1:10 [29].

A part of the construct was prepared as a histological sample by fixing in 2 % glutaraldehyde in PBS for 30 min and then storing in 10 % formaldeyde in PBS. The formalin-fixed samples were embedded in paraffin and cross-sectioned into 7 µm sections; deparaffinized sections were stained with hematoxylin, fast green and safranin-O.

For immunohistochemical analysis, a part of the construct was prepared by fixing in methanol-formalin mixture, and then successively dehydrated in different concentrations (10 % - 40 %) of a sucrose solution followed by embedding into Tissue Tek. Frozen samples were cryosectioned at 7 µm and stained with antibodies for type I collagen (monoclonal anti-collagen type I col-1) mouse ascites fluid, dilution (1:1000), and type II collagen (DSHB), dilution (1:6). To prevent non-specific antibody binding samples were

incubated with 5 % normal horse serum in buffer. The sections were then incubated overnight at room temperature with primary antibodies; PBS without antibodies was used for control sections. On the next day, sections were incubated with biotinylated secondary antibody, anti-mouse/rabbit IgG (Vector Laboratories; Inc.; Burlingame, CA, USA), for 30 min at room temperature. Finally, the samples were stained using a Vectastain ABC-kit and DAB-kit according to the protocol from the provider for avidin-biotin-peroxidase complex formation.

Statistical analysis

Biochemical data are expressed as means ± standard deviation. Statistical significance was assessed by one-way analysis of variance ANOVA in conjunction with multiple comparison test (Tukey's test) for Fig. 2.

Results

Pre-cultivation in vitro

Primary chondrocytes distributed in fibrin, in polymeric scaffolds as well as in composite fibrin-scaffold constructs maintained a round shape during the four weeks in vitro and produced high amounts of extracellular matrix containing GAG and collagen (Fig. 1 and Fig. 2). Biochemical analysis showed only slight increases in wet weight and cell number, but distinct increases in extracellular matrix components glycosaminoglycans (GAG) and total collagen after four weeks in vitro compared to values after one week (Fig. 1). With regard to wet weight, similar values were obtained within all groups after four weeks, only fibrin-polymer B constructs showed slightly higher values (Fig. 1A). Regarding cell number, data after 1 week showed no change or only slightly lower values as initially seeded cells. In contrast, after 4 weeks in vitro, cell numbers increased to approx. $6*10^6$ cells per construct. Compared to seeding with fibrin, seeding directly into the scaffold resulted in significantly lower cell numbers after 4 weeks in vitro (Fig. 1B).
Biochemical analysis showed the development of high amounts of GAG and total collagen per wet weight as well as per cell after 4 weeks in vitro (Fig. 1C-1F). Regarding extracellular matrix components per wet weight, similar results of about 4.5 % GAG per wet weight and 7 % total collagen per wet weight were obtained for the fibrin-scaffold constructs (Fig. 1C and 1E). Using fibrin alone, analysis showed slightly higher values

(5 % GAG and 8.5 % total collagen per wet weight) compared to composite constructs, which is attributed to the volumetric part of the scaffold (porosity 80 %). In contrast, using scaffold without fibrin resulted in lower amounts of GAG and collagen (3.5 % GAG and 6 % total collagen per wet weight). With regard to extracellular matrix components per cell, analysis demonstrated the development of similar amounts of about 0.6 ng GAG per cell and 1.0-1.2 ng total collagen per cell in all groups (Fig. 1D and 1F). Only fibrin-polymer B constructs showed slightly higher values of GAG per cell (0.9 ng per cell).

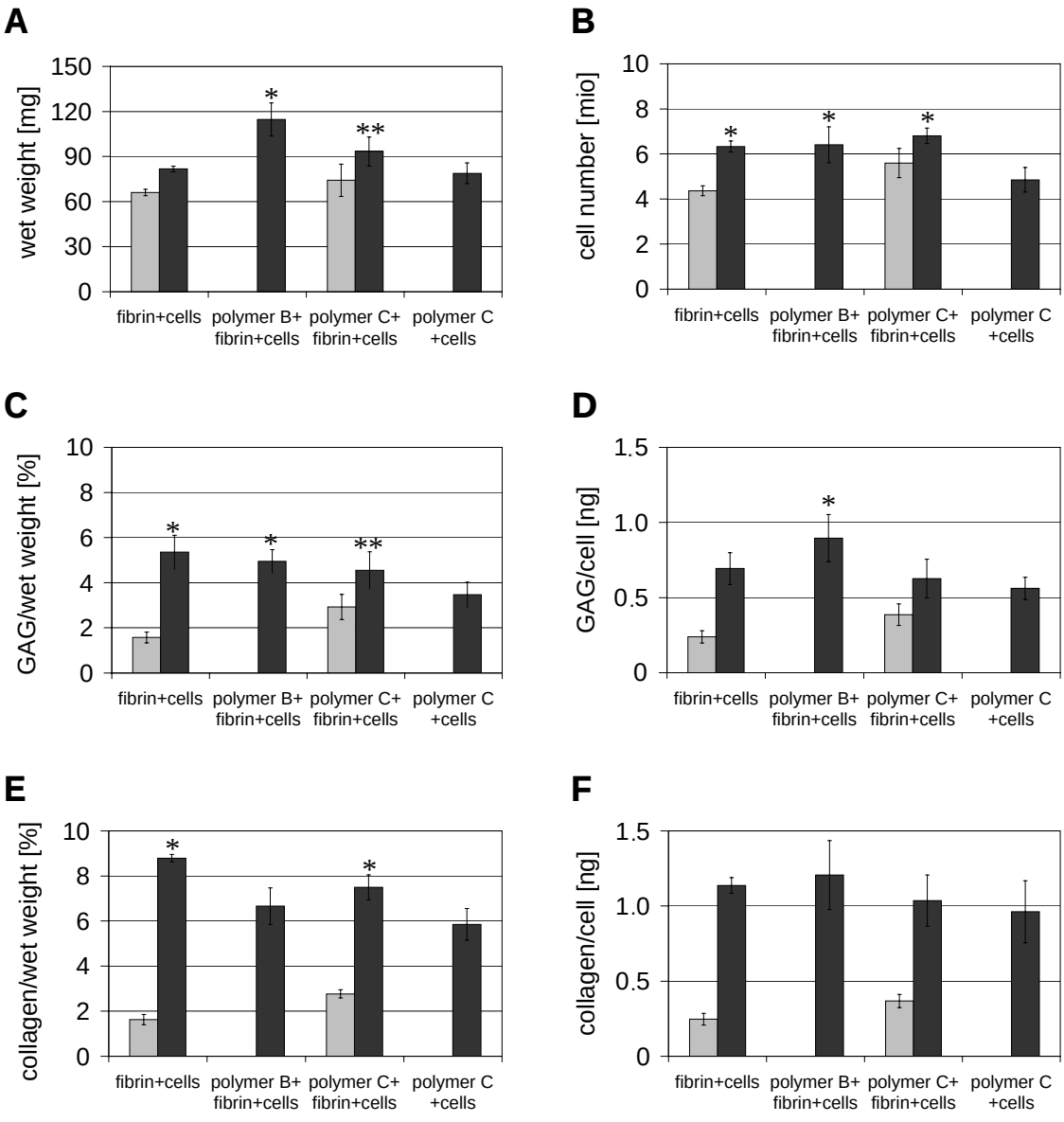

*Fig. 1: Wet weight (A), cell number (B) and amount of GAG (C, D) and collagen (E, F) per wet weight and per cell of constructs after 1 week (■ gray) and after 4 weeks (■ black) in vitro. Data represents the average ± S.D. of seven independent measurements. Statistically significant differences between constructs including fibrin and the group with cells seeded directly onto polymer C (far right) are denoted by * $p < 0.01$ and ** $p < 0.05$.*

The ECM fractions were also reflected in histological cross-sections of the constructs stained red with safranin-O (Fig. 2). Fibrin gel was homogenously spread in the pores of the polymeric scaffolds, and round and vital chondrocytes were homogenously distributed throughout the whole construct. The development of engineered cartilaginous tissue of high quality and homogeneity was observed. Already within the first week, cells produced glycosaminoglycans stained red with safranin-O (Fig. 2, left). Cultivation for 4 weeks resulted in an even denser and more intensive safranin-O stain, which indicated the development of high amounts of GAG. Using polymeric scaffold with fibrin gel resulted in only small areas without cartilaginous tissue caused by the polymer. In contrast, compared to the combination, using scaffolds alone without fibrin showed a less homogenous matrix distribution containing extensive areas without cartilaginous tissue. The majority of cells and newly developed extracellular matrix were found at the periphery of the construct.

Regarding immunohistochemistry, all constructs were intensively stained for cartilage specific collagen type II homogenously distributed throughout the whole construct (Fig. 2, right). In contrast, only a thin area at the edge were intensively stained for undesired collagen type I, the major part of the construct was only slightly stained.

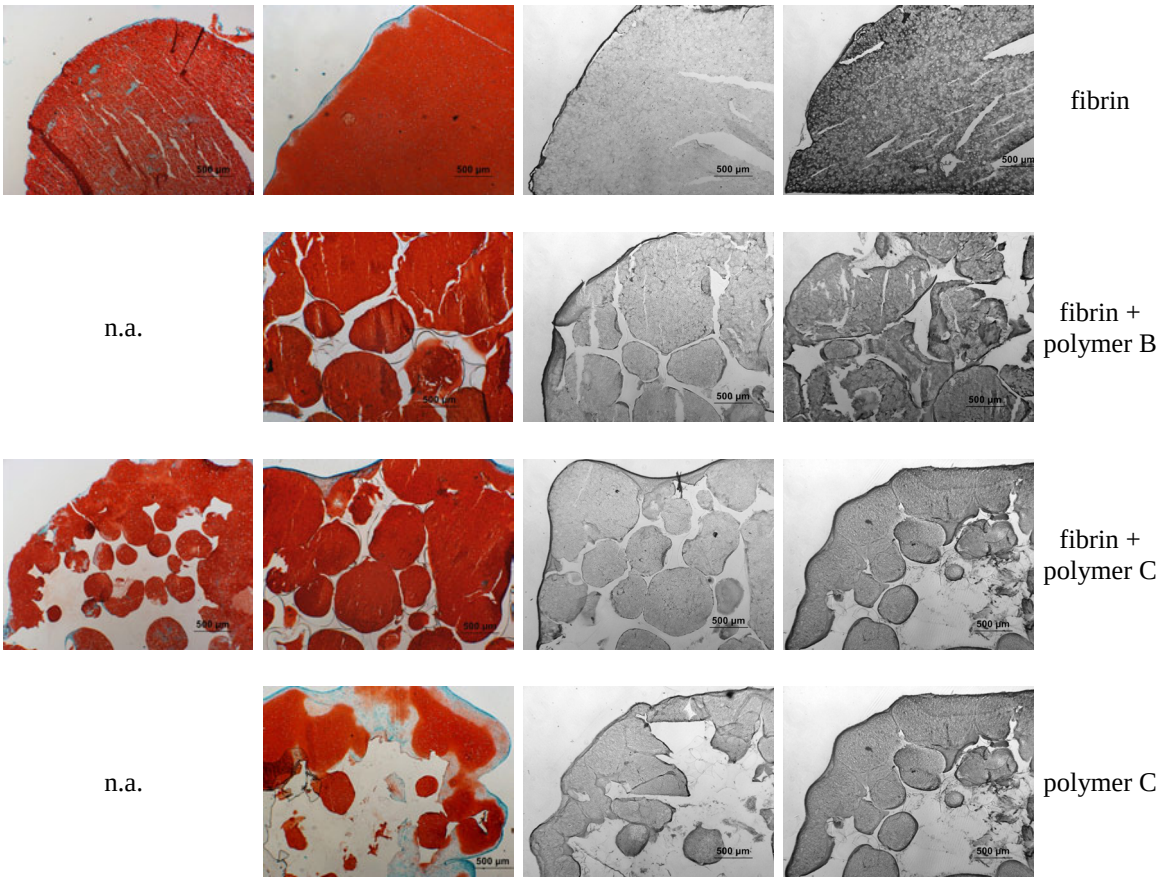

Fig. 2: Cartilaginous extracellular matrix development of cells in fibrin (first line), cells in fibrin-polymer B (second line), cells in fibrin-polymer C (third line) and cells in polymer C (last line) after 1 week (left column) and 4 weeks in vitro (safranin-O stain and immunohistochemical cross-sections).

In vivo study

After 1, 3, or 6 months in vivo, chondrocytes in all groups, pre-cultivated for 4 weeks in vitro, had a round and vital appearance and were still homogenously distributed (Fig. 3-5). No elongated, fibroblastic cell morphology was found within these constructs. A few explants were surrounded by a thin, capsule-like fibrous tissue layer.

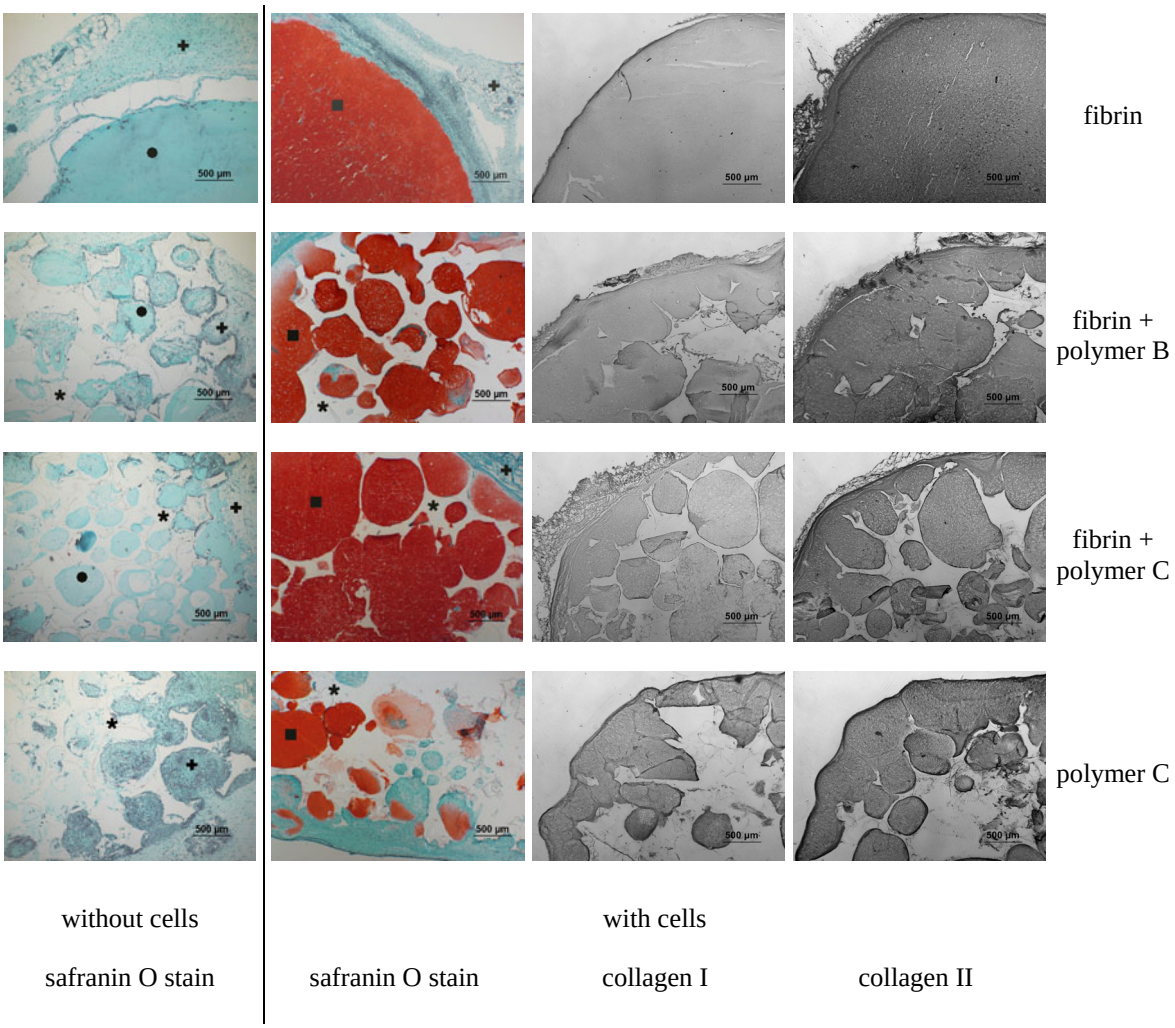

Fig. 3: Cartilaginous extracellular matrix development of cells in fibrin (first line), cells in fibrin-polymer B (second line), cells in fibrin-polymer C (third line) and cells in polymer C (last line) after 4 weeks in vitro followed by 4 weeks in vivo, compared to controls without cells (left column) (safranin-O stain; ✱ polymer, ● fibrin, ✚ infiltrating mouse cells, ■ cartilaginous tissue).

Histological safranin-O stain of constructs after 1 month in vivo demonstrated the presence of high amounts of GAG. Immunohistological cross-sections were intensively stained for collagen type II and less stained for collagen type I, only at the periphery of the construct similar to in vitro controls (Fig. 3, right). However, cell and extracellular matrix distribution of cell-polymer constructs without fibrin were still more inhomogeneous than in constructs seeded with fibrin. Compared to seeding with cells, controls without cells showed infiltration of mouse cells in all groups (Fig. 3, left). Fibrin-polymer controls showed the presence of remaining fibrin gel within the scaffold pores. Histology of fibrin controls alone showed no degradation and only low infiltration of mouse cells at the edge of the gel (Fig. 3).

In Fig. 4, representative sections of tissues developed in vitro for 4 weeks and afterwards in vivo for 3 months (Fig. 4, left) and 6 months (Fig. 4, right) are displayed. All explants were similarly stained with safranin-O for glycosaminoglycans compared to constructs after 1 month in vivo (Fig. 3). No degradation of polymeric scaffold or vascularization was observed in any of the explants. Small areas without matrix within the newly developed cartilaginous tissue caused by the scaffold were still observed after 3 and 6 months. However, histology of constructs seeded without fibrin showed still a distinctly more inhomogeneous extracellular matrix distribution compared to seeding with fibrin.

Regarding pre-cultivation in vitro, constructs that were pre-cultured for four weeks contained distinctly higher amounts of extracellular matrix components GAG and collagen that were also more homogenously distributed throughout the explant compared to constructs with no pre-cultivation (Fig. 5, first, second and third line). Interestingly, constructs pre-cultured for only one week showed results that were indistinguishable from those of constructs cultivated for four weeks (Fig. 5). Constructs were intensively stained for collagen type II and less intensively stained for undesired collagen type I, the latter mainly in a small area at the periphery of the explant. These results were also reflected in histological sections of explants after 3 and 6 months in vivo. There was no enhancement or equalization of extracellular matrix distribution in constructs not pre-cultivated within 6 months in vivo (Fig. 5).

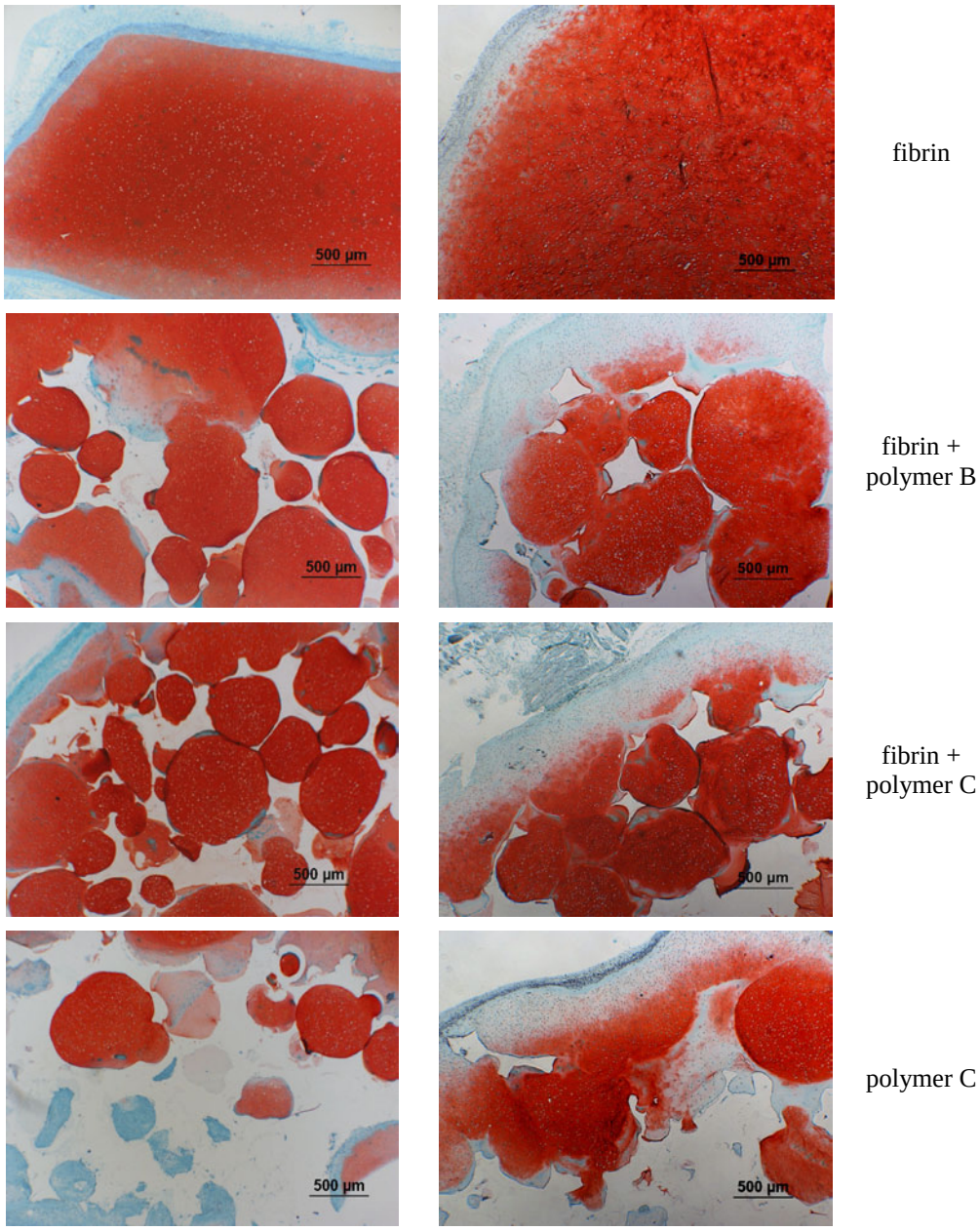

Fig. 4: Staining for GAG in constructs: fibrin (first line), cells in fibrin-polymer B (second line), cells in fibrin-polymer C (third line) and cells in polymer C (last line) after 4 weeks in vitro followed by 3 months (left) and 6 months (right) in vivo (safranin-O stain).

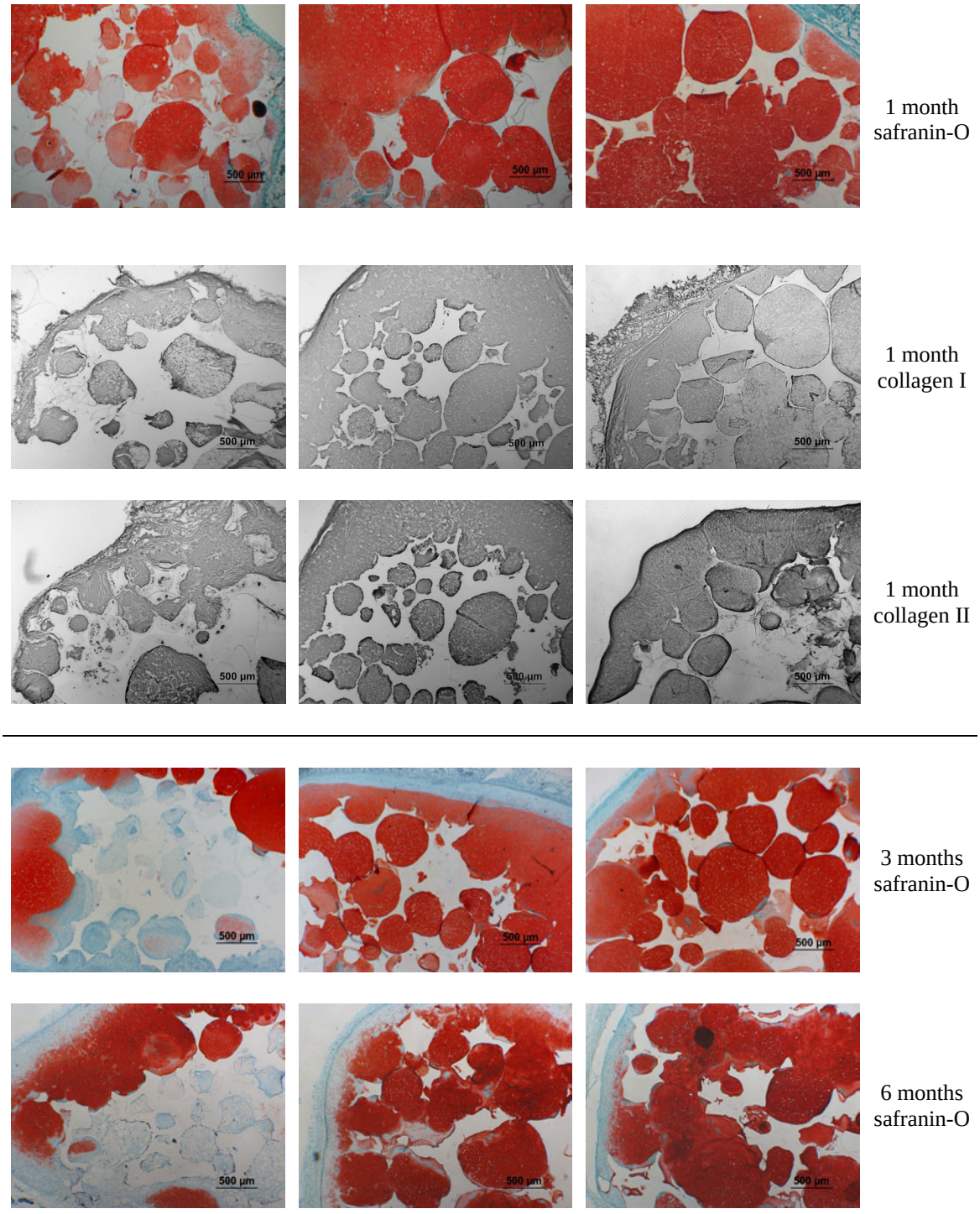

Fig. 5: Effect of 0, 1, or 4 weeks of pre-cultivation in vitro on cartilaginous extracellular matrix development of fibrin-polymer C composite constructs. Histological cross-sections of constructs after 1 month (first line), 3 months (fourth line) and 6 months (last line) in vivo (safranin-O stain). Immunohistological cross-sections of constructs after 1 month for collagen I (second line) and collagen II (third line).

Discussion

Within this study, we demonstrated the potential of a combination of optimized long-term stable fibrin gels and newly developed polycaprolactone-based polyurethane scaffolds for the use in cartilage engineering. Primary bovine chondrocytes distributed within this composite system maintained round and vital within 4 weeks in vitro and produced high amounts of cartilage-specific extracellular matrix components GAG and collagen. The composite system resulted in higher seeding efficiency and a more homogenous cell and matrix distribution compared to cells seeded onto scaffolds alone. Histology of constructs implanted into the back of nude mice demonstrated excellent engineered cartilaginous tissue within the polymer after 1, 3, and 6 months in vivo. Furthermore, it was shown that a short pre-cultivation of constructs in vitro enhances further cartilage tissue development in vivo compared to no pre-cultivation.

Current practical approaches in cartilage engineering still face problems regarding three-dimensional cell seeding and cell distribution, which is a distinctly critical step for development of new extracellular matrix and consequently of the newly generated tissue [9, 10, 30-32]. Especially for engineering of autologous tissue, where cell availability is limited, a high seeding efficiency would be beneficial. Furthermore, it is described that an inhomogeneous cell distribution result in inhomogeneous extracellular matrix development, often located near the periphery of the construct [4, 5]. Besides conventional pipetting of cells directly onto scaffolds, current seeding approaches recommend dynamic cell seeding onto polymeric scaffolds using spinner flasks or rotating bioreactor vessels [24, 30, 33]. As an alternative, hydrogels are used, which generally incorporate all of the applied cells and enable a good cell distribution providing the requirements for a coherent tissue development [1]. Therefore, a combination of a hydrogel system and a polymeric scaffold seems to be beneficial. In our study, seeding efficiency directly after preparation was 2.4-fold higher using fibrin as delivery system compared to seeding directly onto the scaffold (data not shown). And most notably, histology of constructs seeded with fibrin showed distinctly more homogenous cell and matrix distribution throughout the constructs after 4 weeks in vitro, resulting in a more coherent cartilaginous tissue, compared to cells seeded directly onto the scaffold.

An increasing dedifferentiation of chondrocytes could be an important problem of polymeric scaffolds, due to two-dimensional cell spreading along the pore walls [10, 32]. In contrast, a variety of hydrogels are known to be favorable with regard to chondrocyte

phenotype and genotype, in particular fibrin, which is thought to modulate the attachment, migration, and proliferation of chondrocytes [5, 11, 34, 35]. Biocompatibility of fibrin and the use of fibrin gels for cartilage engineering have been well established [17-19, 36]. However, an important disadvantage of fibrin for the use in tissue engineering is an increasing instability and solubility over time. Therefore, we recently developed a long-term stable fibrin gel in modifying specific preparation parameters (chapter 4). This optimized fibrin gel was demonstrated to be suitable for cartilage engineering. Primary bovine chondrocytes cultured within these gels remained round and vital, and produced an adequate cartilaginous tissue containing high amounts of GAG and collagen type II.

Highly porous polymeric scaffolds are sometimes associated with problems regarding diffusion of newly synthesized extracellular matrix molecules into the culture medium, for example when dynamic compression is applied [5, 10, 32]. This is likely due to large pore size and open scaffold structure, which allows adequate nutrient diffusion within the construct, but also diffusion of newly synthesized matrix components out of the constructs. Therefore, the use of a hydrogel system like fibrin with smaller pore size seems reasonable to prevent diffusion of matrix molecules until an initial stable matrix is formed by the cells [37].

On the other hand, soft hydrogels often lack adequate biomechanical strength and volume stability. During development of a new extracellular matrix, cells are able to deform mechanically unstable gel or scaffold architecture with contraction forces by both migration and proliferation [38]. Additionally, hydrogels may degrade within days to weeks, i.e., too fast for cells to produce an adequate extracellular matrix stable in shape. However, load-bearing capacity would be desirable for the process of implantation and especially to resist mechanical loading in the patient after implantation.

Therefore, a number of groups recently developed strategies by entrapping cells in different types of soft hydrogels such as fibrin, alginate or collagen and subsequent injection into various polymeric scaffolds [30, 35, 39-41]. In particular, fibrin is often used in cartilage tissue engineering, as it is easily prepared by mixing the individual fibrinogen and thrombin components and allows injection of cell-gel suspension prior to gelation. It has been reported that a high number of swine chondrocytes suspended in fibrin gel and added to a PGA mesh already resulted in mechanically more stable constructs directly after cell seeding, i.e., the gel injected into the scaffold was more stable than the scaffold alone [42]. After 4 weeks of cultivation in vitro, the combination of fibrin and polymeric scaffold resulted in a higher amount of glycosaminoglycans, an effect that was partially attributed

to increased matrix retention in the fibrin gel, and advanced mechanical stability of constructs for implantation compared to polymeric scaffold alone. Histological staining showed the development of high amounts of extracellular matrix, though concentrated near the periphery of the construct. However, histology of the composite constructs was similar compared to constructs seeded without fibrin. Using human septal chondrocytes, Haisch et al. presented a system incorporating cells by fibrin within bioresorbable polyglycolide-polylactide copolymer scaffolds. Histological cross-sections of these constructs cultivated for 5 weeks in vitro showed the development of cartilage-specific extracellular matrix components GAG and collagen type II and a stable tissue for at least 5 weeks in vitro, which was attributed to the combination of fibrin with polymer compared to fibrin alone [43]. In a further approach, similar to our study, primary bovine chondrocytes were suspended in commercially available fibrin glue and injected in newly developed polyurethane scaffolds fabricated with an interconnected pore structure [5]. Using a commercial fibrin gel as a cell delivery system resulted in a higher cell-seeding efficiency, a more even cell distribution and an increase in retention of newly synthesized GAG. However, cells as well as extracellular matrix in all groups were only found near the edge of the constructs after 4 weeks of cultivation, even with addition of protease inhibitor aprotinin to prevent fibrin degradation. This was attributed to either cell migration, increased cell proliferation at the edge, or increased cell death in the middle of the construct. Compared to these findings, in our study, only seeding without fibrin resulted in constructs containing less cells and extracellular matrix in the middle of construct. For fibrin-scaffold composite constructs, an optimized fibrin gel was applied that is stable in cell culture for several months and was shown to be suitable for cartilage tissue engineering providing shape stability (chapter 4), in contrast to the commercially available fibrin gel that tend to disintegrate and dissolve within a few days [16, 17, 19]. Seeding with our optimized fibrin gel resulted in homogenous cell and matrix distribution after 4 weeks in vitro even without addition of protease inhibitor aprotinin to the culture medium, which may be attributed to absence of fibrin degradation likely leading to reduced cell migration to the periphery of the construct and a reduced diffusion of matrix molecules out of the construct.

Only a few studies have been published showing tissue engineering of cartilage in vivo using hydrogel-polymer composite constructs. Haisch et al. prepared constructs in the shape of a human ear using human chondrocytes in fibrin glue and bioresorbable PGLA-PLLA scaffolds [44]. After an in vitro pre-cultivation of 6 weeks, fragments of the

construct were implanted subcutaneously in the back of nude mice. After up to 12 weeks in vivo, histology showed the development of cartilaginous tissue consisting of round-shaped clusters of chondrocytes within a matrix containing collagen. However, a slight decrease in construct size was found within 12 weeks in vivo. In a study of Rotter et al., human chondrocytes were suspended in agarose and transferred into different types of resorbable non-woven scaffolds with different degradation characteristics based on polylactic acid; constructs were cultured in vitro for 1 week and subsequently implanted into the back of athymic mice [45]. It was found that a polymer scaffold with a short degradation time of about 3 weeks seems to be a more suitable material for development of cartilage extracellular matrix in vivo compared to scaffolds with shape stability of longer than 24 weeks, thus, no degradation within the experimental time.

However, in our study, using long-term stable polycaprolactone-based scaffolds, both types of fibrin-polymer composites pre-cultured in vitro for 4 weeks and implanted into nude mice showed an adequate cartilaginous tissue with homogenous cell distribution even after 6 months in vivo. Histology demonstrated the presence of high amounts of extracellular matrix components GAG and collagen type II. Only small areas without matrix were found within the newly developed tissue caused by the polymeric scaffold. The applied polycaprolactone-based scaffold is supposed to degrade after 1 to 2 years based on in vitro studies (data not shown). Therefore, this polyurethane system is suggested to be used for application in tissue engineering where long-term shape stability after implantation is desired, e.g. total ear reconstruction.

Only few studies have been conducted regarding the effect of pre-cultivation on in vivo tissue development. It was shown that in vitro pre-incubated constructs had greater retention of donor cells after implantation into an osteochondroal defect compared to non-incubated controls when seeding perichondrial cells into a polylactic acid scaffold [46]. Ibusuki et al. found less hydrogel leakage and implant deformation of 2-week pre-incubated constructs consisting of chondrocytes and modified gelatin, compared to non-incubated control after 5 weeks in a chondral defect in rabbits [47]. Rotter et al. observed only slight differences in in vivo development of human chondrocytes seeded onto PLA/PGA scaffolds when pre-cultured for 3 weeks compared to direct implantation [48]. However, Moretti et al. prepared constructs consisting of human chondrocytes and hyalograft C discs, and investigated the effect of pre-cultivation with different cell densities and medium supplements on in vivo development of cartilaginous tissue [49]. It was found that constructs that had been pre-cultured for 2 weeks in differentiation medium

contained slightly higher amounts of GAG and collagen compared to constructs pre-cultured in proliferation medium, and distinctly higher amounts of extracellular matrix components compared to constructs implanted directly after preparation. Results in our study showed that an in vitro pre-cultivation of 1 week enormously enhanced further development and homogeneity of new cartilaginous tissue in vivo indicated by histology and immunohistochemistry. A pre-cultivation for 4 weeks demonstrated similar results compared to pre-cultivation for 1 week. Thus, the study suggests that an in vitro cultivation of longer than 1 week is not necessary for the in vivo development of bovine chondrocytes in a fibrin-polyurethane scaffold subcutaneously implanted into nude mice. Interestingly, though constructs pre-cultured for 1 and 4 weeks showed similar results in cartilaginous tissue development after 4 weeks in vivo, histological cross-sections demonstrated strong differences prior to implantation. Therefore, though constructs showed still an inadequate tissue development after 1 week in vitro compared to 4 weeks, the short pre-cultivation time is sufficient for an adequate further tissue development in vivo, similar to 4 weeks pre-cultivation. It is clear, that subcutaneous implantation into the back of nude mice does not mimic the complex nutrient and mechanical situation in the patient. However, well in agreement with the results of Moretti et al., our results indicate that a short in vitro pre-cultivation has an enormous effect on in vivo development of newly generated tissue. In future studies, it may be worthwhile investigating the effects of pre-culture times even shorter than 1 week.

To conclude, the current study demonstrates the potential of a combination of long-term stable fibrin gels and newly developed polycaprolactone-based polyurethane scaffolds for the use in cartilage tissue engineering. However, towards a clinical application, further investigations have to be done using more delicate human autologous chondrocytes. Furthermore, the performance of the composite constructs has to be assessed in a clinically relevant implantation site associated with a specific nutrient and mechanical situation. Nevertheless, this combination of hydrogel and scaffold appears as a promising step towards regeneration of cartilaginous tissue in plastic and reconstructive surgery.

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Chapter 9

Summary and Conclusions

Fibrin glue is a commonly used hydrogel for different applications in the field of tissue engineering [1, 2]. Besides the use as cell matrix alone, fibrin glue is combined as a cell matrix material with a polymeric scaffold, and can function as a delivery system for growth factors or other therapeutic agents (**chapter 2**). The gel is a biocompatible and biodegradable material that provides an environment enabling adequate cellular function, e.g. cell migration, proliferation and differentiation, and allows for formation of cartilaginous tissue [3-6]. Disintegration and solubility over time are taken as advantageous specific properties of fibrin glue when used as a sealant or for delivery of molecules [7-9], but for many tissue engineering based applications they represent a major problem [6, 10, 11], in particular, in applications in which shape stability is required and for generation of implants that are physiologically exposed to high mechanical loadings.

As a major step within the ForTEPro project ("Bavarian Research Cooperation for Tissue Engineering and Rapid Prototyping"), specific fibrin parameters influencing gel appearance and stability are determined and a fibrin gel was developed that has a transparent appearance and is stable in size and shape under common cell culture conditions (**chapter 4**). These optimized fibrin gels showed a predominantly linear viscoelastic behavior and withstood high mechanical loadings investigated by rheological measurements. In contrast, preparing fibrin gels with concentrations out of the defined ranges, turbid gels were obtained that shrunk during the first few days and completely dissolved within a few weeks in culture medium. This achievement in gel stability may have a considerable impact for the use of fibrin in the field of tissue engineering.

The general suitability of the optimized fibrin gel for the use in cartilage engineering was demonstrated using primary bovine chondrocytes (**chapters 3, 4**). Cells cultured within the three-dimensional gel were round and vital, proliferated well and produced high amounts of extracellular matrix components GAG and collagen type II. Addition of insulin to the culture medium, a factor known to have anabolic effects on engineered bovine cartilage using commercially available PGA mesh [12] resulted in a strong increase in development of extracellular matrix components within the new fibrin gels. In contrast, though it is known that dynamic cultivation as well as shear stress can have a significant impact on the morphology and, thus, the mechanical properties of newly generated cartilage [13-16], a dynamical culture on an orbital shaker showed no improvement of cartilage formation within the fibrin gels, compared to conventional static cultivation. Concentration of fibrinogen within the gel was found to have a strong influence on distribution of newly developed extracellular matrix, at the same time not influencing the amount of

extracellular matrix production, however, diminishing construct stability. Additionally, this chondrocyte-fibrin system is suggested as a suitable test system for evaluation of controlled release devices for morphogens and other bioactive molecules, as demonstrated for lipid microparticles loaded with insulin. To conclude fibrin gel testings, a minimum initial cell density was found that was required for the formation of a coherent extracellular matrix within 5 weeks of culture time using primary bovine chondrocytes (**chapter 4**).

The optimized fibrin gels were further evaluated for the suitability as surface material for culture and expansion of cells. In **chapter 5**, primary bovine chondrocytes were shown to migrate out of the fibrin gels, resulting in formation of a three-dimensional cell sheet on the gel. Remarkably, these cells demonstrated a distinctly higher potency to form engineered cartilage when re-seeded into three-dimensional fibrin gels compared to cells conventionally expanded in cell culture flasks. Therefore, fibrin surface may have a considerable advantageous effect on expansion of differentiated chondrocytes, in particular for using more delicate human chondrocytes in future studies.

In **chapter 6**, the method of culturing bovine chondrocytes within the optimized fibrin gel was successfully transferred to the use of human chondrocytes. However, generating human cartilaginous tissue still represents a major challenge, indicated by a limited cell number isolated from small biopsies, and development of distinctly smaller amounts of extracellular matrix components within fibrin gels, compared to the use of bovine cells. Nevertheless, insulin was found to have a tremendous enhancing effect on the development of extracellular matrix components GAG and collagen, thus, on formation of new human cartilaginous tissue, when seeding adult human chondrocytes into fibrin gels. This may have a strong impact on future approaches in the engineering of human cartilage.

Within this thesis, furthermore, the combination of the long-term stable fibrin gels and polymeric scaffolds was investigated (**chapter 7**). Injection of a cell-fibrin suspension into newly developed polycaprolactone-based polyurethane scaffolds, commonly used PGA meshes as well as PLGA scaffolds resulted in formation of adequate cartilaginous tissue, at the same time providing an added stability for the delicate process of implantation and for mechanical loading in vivo. Furthermore, as the overall goal within the ForTEPro project, a polycaprolactone-based scaffold in the shape of the cartilage part of an adult human ear was successfully seeded with chondrocytes suspended in fibrin gel. Previous studies attempting the generation of the external part of the human ear reported problems with construct shrinkage and deformation [17-19]. Remarkably, in our study, construct size and shape was highly preserved within the culture period, indicating the potential of these

composite constructs for the generation of individually customized implants for the treatment of cartilage defects.

In the next study, the potential of the composite constructs made from fibrin and polycaprolactone-based scaffolds for generating new cartilage was further investigated in a detailed in vivo experiment (**chapter 8**). Constructs (5 mm diameter, 2 mm thick) implanted into the back of nude mice for up to 6 months showed formation of adequate new cartilaginous tissue; the superiority of composite constructs to polymeric scaffolds alone was demonstrated. Remarkably, in vitro pre-culture of these constructs was demonstrated to have strong enhancing effects on further in vivo cartilaginous tissue development. Interestingly, a short in vitro pre-culture period of 1 week elicited the same results as a longer period of four weeks. This observation may have advantageous implications for future applications in clinical practice.

In conclusion, within this thesis a long-term stable fibrin gel was developed. The potential of the fibrin gel for application in cartilage engineering was demonstrated, using either bovine or delicate human chondrocytes. Insulin was found to strongly improve the development of extracellular matrix within the fibrin gel, thus, formation of new cartilaginous tissue, in particular when using human chondrocytes. Furthermore, this fibrin gel has an advantageous effect on expansion of chondrocytes, with specific regard to the retention of their potential to form engineered cartilage. Finally, in combination with a solid polycaprolactone-based scaffold, a prototype of an individually customized cartilaginous construct in the shape of the external part of a human ear was generated in vitro. Disc-shaped composite constructs showed satisfying long-term development and maintenance (6 months) of engineered cartilage after subcutaneous implantation in nude mice. In future, performing further studies using human cells, this system may result in fully functional individually shaped implants for cartilage regeneration.

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Appendices

List of Abbreviations

2-D	two-dimensional
3-D	three-dimensional
ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
DMEM	Dulbeccos's Modified Eagle's Medium
DNA	deoxyribonucleic acid
ECGF	endothelial cell growth factor
ECM	extracellular matrix
EDTA	ethylenediaminetetraacedic acid
EGF	epidermal growth factor
EtOH	ethanol
FBS	fetal bovine serum
FDA	food and drug administration
FDA	fluorescein diacetate
FG	fast green
G´	storage/elastic modulus
G´´	loss/viscous modulus
GAG	glycosaminoglycane
GDNF	glial cell line-derived neurotrophic factor
H&E	hematoxylin and eosin
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
IGF-I	insulin-like growth factor I
IL-1	interleucin-1
IL-1RII	interleukin-1 receptor II
Da	Dalton
LPM	lipid microparticle
mio	million
NEAA	non-essential amino acids
NGF	nerve growth factor
NTF	neurotrophic factor
PBS	phosphate-buffered saline
PCL	poly(ε-caprolactone)

p-DAB	p-dimethylaminobenzaldehyde
PEG	poly(ethylene glycol)
PGA	poly(glycolic acid)
PI	propidium iodide
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PS	polystyrene
PU	polyurethane
rpm	rounds per minute
rt-PA	recombinant tissue plasminogen activator
saf-O	safranin-O
S.D.	standard deviation
SEM	scanning electron microscopy
T-75 flask	75 cm^2 cell culture flask
T-150 flask	150 cm^2 cell culture flask
TCPS	tissue culture polystyrene
TE	tissue engineering
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

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List of Publications

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Eyrich D, Goepferich A, Blunk T. 'Fibrin in Tissue Engineering'. in 'Tissue Engineering', Series: *Adv Exp Med Biol*, Fisher J P (ed.) **585** (2006); in press as a review **(Chapter 2)**

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