Bone Tissue Engineering from Marrow Stromal Cells

Effects of Growth Factors and Biomaterials

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften
(Dr. rer. nat.)
der Fakultät Chemie und Pharmazie der Universität Regensburg

vorgelegt von
Esther Lieb
aus Leverkusen
2003

Promotionsgesuch eingereicht am:	02.07.03
Die Arbeit wurde angeleitet von:	Prof. Dr. A. Göpferich
Mündliche Prüfung am:	28.07.03
Prüfungsausschuss:	Prof. Dr. S. Elz
	Prof. Dr. A. Göpferich
	Prof. Dr. A. Buschauer
	Prof. Dr. G. Franz

Meinen Eltern
in Liebe und Dankbarkeit gewidmet.

Table of Contents

Chapter 1

Introduction

1 Tissue Engineering

The loss of a tissue or its function due to congenital defects, disease or trauma is one of the most difficult, frequent and costly problems in human medicine [1]. Current treatment modalities include organ and tissue transplantation from one individual to another, tissue transfer from a healthy body site to the affected site in the same individual, and replacement of tissue function with mechanical devices, such as prosthetic valves and joints [2]. Although these strategies have made great progress in the field of medicine, they have a number of inherent limitations, which include shortage of donor tissue, immune rejection, pathogen transfer or limited service life [1,2]. For example, in 2001 74,105 patients were on the US organ transplant waiting list versus 6,081 donors (Texas Organ Sharing Alliance, National Transplant Waiting List), which reveals the limitation of organ transplantation by the number of available donors. Following an organ transplantation, transplant recipients must follow lifelong immunosuppression regimens, which come with increased risks of infection, tumor development and other unwanted side effects [3]. Additionally, transplantation of donor organs and tissues involves the risk of virus infection such as hepatitis and HIV [4]. Mechanical devices for tissue replacement are limited by a finite durability and non-physiological performance, as well as an increased risk of infection or thromboembolism [3]. Due to these shortcomings and the clinical need for tissue replacement, the field of tissue engineering was born. Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences to the development of biological substitutes that restore, maintain, or improve tissue function [5]. It serves as a means to replace diseased tissue with living tissue that is designed and constructed to meet the needs of each individual patient [3]. Two general strategies have been adopted for the creation of new tissue: the in vitro cultivation of three-dimensional matrices loaded with cells for in vivo implantation and the direct in vivo implantation of isolated cells and/or three-dimensional matrices of biomaterials [1]. The utilized matrices provide an architecture on which cells can attach, organize and develop into the desired tissue.

Since the 1990s, tissue engineering has evolved tremendously; scientists have attempted to engineer tissues and organs of nearly every part of the body, including the cornea, liver, pancreas, blood vessels, hart valves, bone, cartilage and skin [6]. Thus far, however, only a few products, such as cartilage for the repair of joint defects and incompetent urethral sphincters (Bio Seed®-C, Carticel®), bone for non-load-bearing use in the jaw (BioSeed® Oral Bone) and skin (BioSeed®-S, Apligraf®), have entered clinical trials or received Food and Drug Administration (FDA) approval for clinical application.

2 Bone Tissue Engineering

2.1 The Need for Bone Tissue Engineering

The loss of bony tissue can occur through infection, loss of blood supply, disease such as osteoporosis, as a complication of a fracture or genetic disorders, e.g. osteogenesis imperfecta. Current management of bony defects includes tissue replacement with transplanted autografts or allografts or synthetic devices. However, each of these therapies has its own serious risks and constraints. Harvesting autografts, typically from the iliac crest, is constrained by anatomical limitations and associated with donor-site morbidity [7]. The problems and risks associated with the use of allografts include not only disease transmission, but also the risk of tissue rejection. In addition, the loss of osteoinductive factors during allograft processing may impair the tissue quality. Synthetic prosthesis such as bone cements and metals, e.g. titanium and its alloys or stainless steel, often result in insufficient osseous integration and stress-shielding of the surrounding bone or fatigue failure of the implant [7]. Hence, the above shortcomings and the number of clinical applications emphasize the need for tissue engineered bone.

2.2 Bone

Successful bone tissue engineering requires an understanding of the structural and functional basics of bone. Therefore, a short review will provide the necessary information.

The bones of the adult skeleton consist of 80% compact (or cortical bone) and 20% trabecular (or cancellous, or spongy) bone (Fig. 1). Compact bone is distinguished from trabecular bone by the spatial orientation of their common substructures, the lamellae, which consist of 65% mineral (hydroxyapatite) and 35% organic matrix elements (90% collagen type I). The features of bone especially depend on the characteristics of the mineralized bone matrix, providing compressive strength, tensile strength and elasticity. The substructure of compact bone is the osteon or Haversian system consisting of concentrically orientated lamellae wrapping longitudinal canals, known as Haversian canals. These canals contain capillaries and nerve fibers. A second system of canals, Volkmann´s canals, penetrates the bone more or less perpendicular to its surface and to the Haversian canals. Vessels in Volkmann´s canals are connected to vessels in the Haversian canals and are responsible for the nutrient supply of cells in compact bone. Trabecular bone, which is less dense than compact bone, is comprised of an array of plates and rods of bone tissue that form an open-celled foam. The unvascularized plates and rods of trabecular bone reach a maximum thickness of 0.2 mm. The cavities formed by the sponge-like structure of trabecular bone are filled with bone marrow. Furthermore, compact bone is distinguished from trabecular bone by its characteristic locations in the skeleton. Long bones such as limb bones are divided into three physiological sections, i.e. a compact shaft (diaphysis), an intermediate area (metaphysis), and a terminal portion (epiphysis). The diaphysis is a hollow cylinder of compact bone which contains a medullary cavity. In contrast, the epiphysis and metaphysis consist of trabecular bone, surrounded by a thin eggshell of compact bone. Flat bones, which are predominantly found in the skull, comprise two layers of compact bone separated by a layer of trabecular bone. Short bones, such as carpal and tarsal bones, consist primarily of a core of trabecular bone bounded by a cortex of compact bone of variable thickness [8].

Mature bone is lamellar bone, consisting of both trabecular and compact bone. New bone, whether formed at the physis, during fracture repair, in neoplasia, in embryonic life, or as a result of bone graft incorporation, is woven bone. Woven bone does not contain lamellae, but has a relatively disorganized array of collagen and irregular mineralization pattern. Woven bone becomes lamellar bone through the process of remodelling. The randomly orientated collagen fibers of woven bone become parallel fibers in lamellar bone [9].

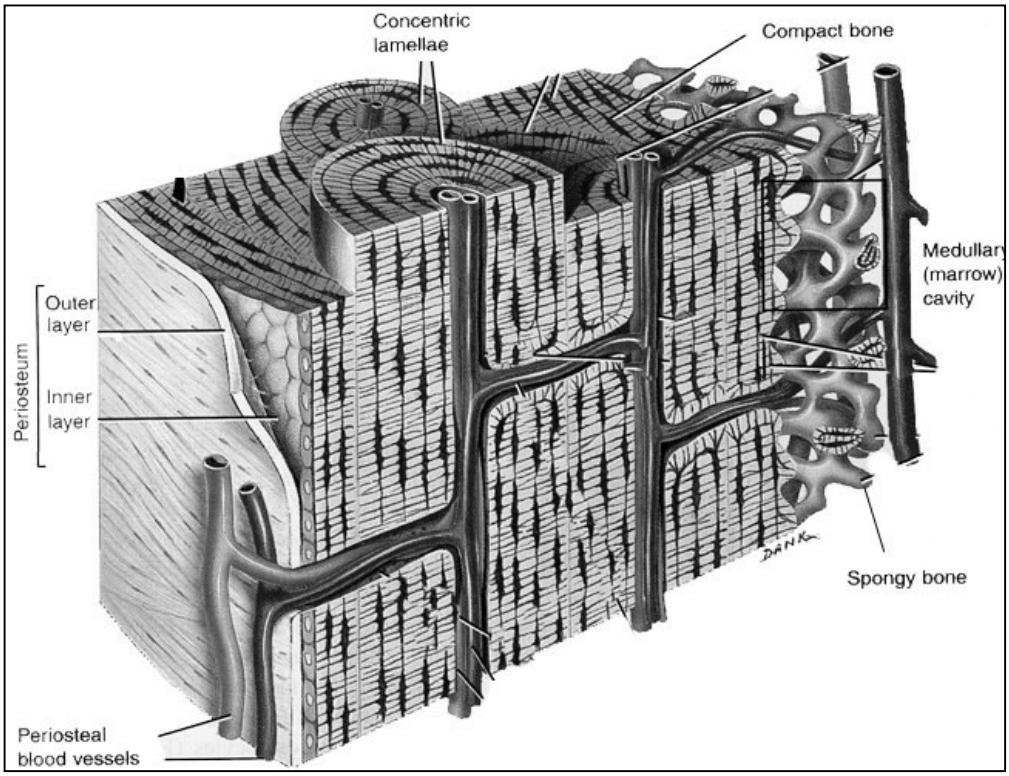


Figure 1: Compact and trabecular (spongy) bone, [10]

Four different cell types, osteoblasts, osteocytes, bone lining cells, and osteoclasts, may be found in bone (Fig. 2). Osteoblasts are responsible for the formation and organization of the extracellular matrix of bone and its subsequent mineralization. A major product of the bone-forming osteoblast is collagen type I. Further matrix proteins are bone sialoprotein, osteocalcin, osteonectin, and osteopontin. Osteoblasts express relatively high amounts of alkaline phosphatase, which plays a role in bone mineralization. Some osteoblasts become trapped in lacunae within the matrix of bone and differentiate into osteocytes [11]. The embedded osteocytes are connected by a system of canaliculi in order to communicate with each other and to ensure nutrient supply. The function of osteocytes includes the mechanotransduction of mechanical into biochemical signals, probably through different pathways, which orchestrates bone tissue repair by modelling and remodelling [12]. Bone

lining cells are flat, elongated, inactive cells that cover bone surfaces and undergo neither bone formation nor resorption. Osteoclasts, which are large (50 to 100 µm in diameter), multinucleated cells cause bone resorption [11].

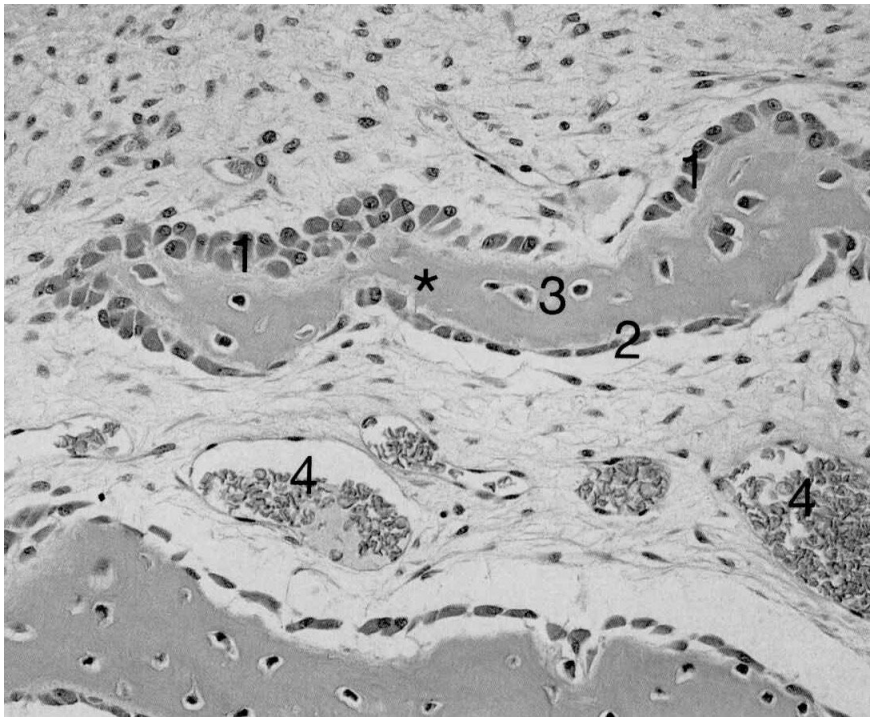


Figure 2,[8]**:** trabecula (*), active osteoblasts (1), little active osteoblasts (2), osteocytes (3), vessels (4)

2.3 The Concept of Bone Tissue Engineering

Bone engineering concepts have focused on two approaches: the use of three-dimensional matrices (I) as cell-free conduits to guide bone ingrowth from the defect surrounding bone or (II) as carriers for seeded cells for in vitro or in vivo bone formation. Cell sources for bone tissue engineering include bone marrow or periosteum [2].

When attempting to regenerate bone via the conduction of bone into biomaterials [13], the conduit material is implanted adjacent to bone tissue. Cells from the tissue begin to invade and populate the material, lay down new matrix, and eventually form new bone. Additionally, conduit materials can be made osteoinductive by combining them with growth factors.

Endobon® is a commercially available bovine bone conduit, which induces bone ingrowth after implantation. For the second approach, a tissue biopsy is taken, bone forming cells are isolated and multiplied to obtain a sufficient cell number for the tissue engineering approach and seeded onto a cell carrier, which serves as a framework for the forming bone tissue. The cells attach to the cell carrier, receive stimuli for the osteoblastic differentiation and start to build their tissue. At a suitable time point, the cell-loaded carrier is implanted and undertakes in vivo reconstruction according to the needs of the defect site, eventually assuming normal bone function. Ideally the cell carrier should be degraded by the time a coherent bone tissue is fully formed. The tissue engineering strategy applied in this thesis follows this second approach, as depicted in Fig. 3. To be precise marrow stromal cells were isolated from bone marrow, expanded in two-dimensional cell culture and dynamically seeded on polymer scaffolds using spinner flasks. For the cell seeding procedure, a cell suspension was prepared and added to the spinner flask containing the scaffolds, which hung on needles that are pinned to the stopper of the flask. Then, the cell suspension was stirred for a period of 24 h to achieve homogeneous cell attachment to the scaffolds. Following cell seeding, the cell-polymer constructs were cultivated in vitro under treatment with osteogenic agents in order to engineer the bone-like tissue (Fig. 4). Previous studies cited in the literature describe various culture periods before implantation, ranging from implantation immediately following cell seeding without the induction of differentiation to various lengths of in vitro cultivation in osteogenic medium from 1 day up to several weeks [14,15,16,17]. Long culture times may favor the slowly forming bone tissue that has to compete in vivo with the rapid intrusion of fibrous tissue. Furthermore, a long culture period, which we utilized, may be applied to obtain bone-like tissue in vitro, which could serve as a three-dimensional in vitro test system for drugs. However, bone is a vascularized tissue and consequently the size of the in vitro engineered bone-like tissue is limited by a lack of nutrition supply. Very recent approaches to overcome this problem include perfusion chambers to improve the nutrient supply [18] and co-cultures to form a capillary network in the engineered bone. However, at least the second task still demands much more research before such co-cultures may be optimized and used reliably.

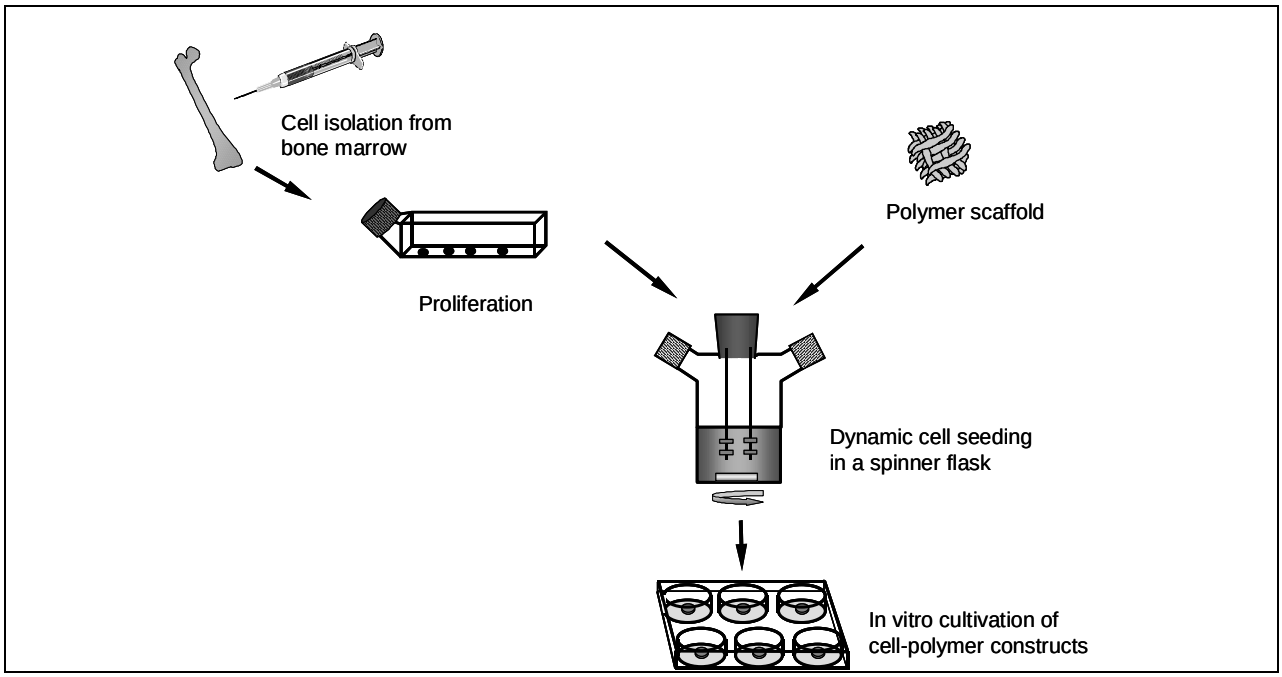


Figure 3: An example of a bone tissue engineering strategy. Preparation of the implants includes cell isolation from bone marrow, proliferation, dynamic cell seeding on scaffolds using spinner flasks and cultivation of cell-polymer constructs up to implantation.

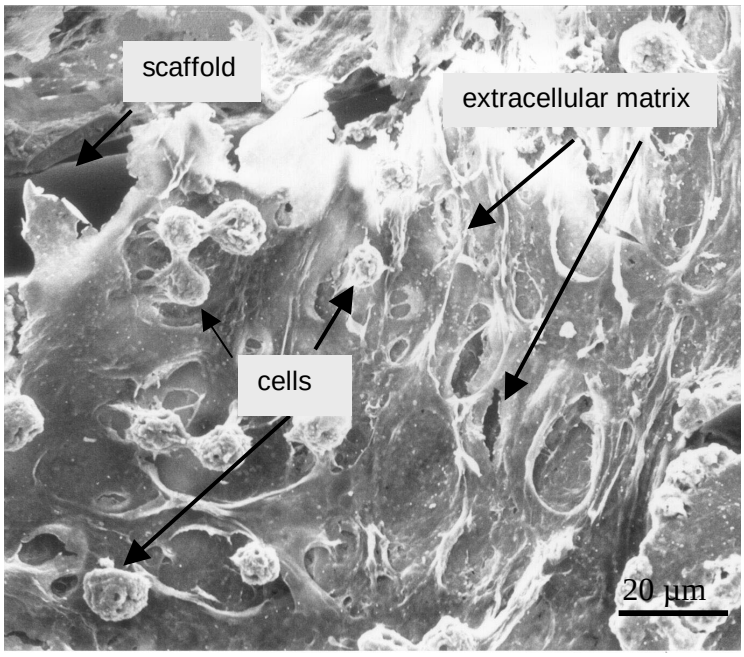


Figure 4: Scanning electron microscopy (SEM) picture of marrow stromal cells on a poly(*L*-lactic-co-glycolic acid) (PLLGA) fiber mesh after 18 days of cultivation in osteogenic medium.

2.4 Key Factors Influencing Tissue Engineering

When using the cell-seeded scaffold, as described in 2.3 (Fig. 3), four key components may influence bone tissue formation: the cell source, the scaffold, the osteogenic agents and the mechanical forces during in vitro cultivation on scaffolds.

2.4.1 The Cell Source

Cells used for tissue engineering purposes may be allogeneic or autologous. Ideally, the cells would be acceptable to the recipient´s immune system without the need for immunosuppressive therapy. Most tissue engineering approaches focus therefore on autologous cells. In addition, the ideal cells should be easily accessible and highly proliferative in vitro without being mutagenic in vivo [19]. Suitable cell types for this approach to bone tissue engineering, as described in literature, are periosteal cells, which consist of osteoprogenitor cells [20,21] and marrow stromal cells. These cells are preferred to osteoblasts because the use of autologous osteoblasts is constrained by the creation of new defects isolating osteoblasts. Moreover, osteoblasts are not capable of extensive proliferation to gain sufficient cells for tissue engineering purposes [22]. We harvested undifferentiated bone marrow stromal cells for use in our scaffolds, because they are easily accessible through bone marrow aspiration, expandable in 2-dimensional culture and have the potential to differentiate into a large variety of tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma [23]. This cell type is described in detail in the following:

Bone Marrow Stromal Cells

Bone marrow stroma provides a unique cellular reservoir for the engineering of bone tissue. Friedenstein et al. [24] first showed that when marrow stromal cells were transplanted into a closed system, i.e., a diffusion chamber, these cells formed bone tissue. Subsequently, various groups demonstrated that marrow stromal cells are capable of multi-lineage differentiation in vitro, including differentiation to bone and cartilage, tendon, muscle, fat, and a marrow stromal cell connective tissue, which supports the differentiation of hematopoietic cells [25]. Other kinds of experiments, however, are needed to address whether marrow stroma contains a definitive stem cell, as defined by a capacity for self-renewal and the ability

to reconstitute all the appropriate differentiated lineages [26]. To this end, experiments using cell clones were performed. It was recently shown that of 185 non-immortalized human marrow stromal cell clones, approximately half expressed osteo-chondrogenic potential and only 17% were capable of differentiating into bone, fat, and cartilage. Additionally, the multi-potential clones progressively lost their adipogenic and chondrogenic potential with increasing passage numbers [27]. Pittenger et al. [23], who cultivated cells in pre-screened sera lots having used a battery of tests, reported a higher ratio of multi-potential clones. One-third of the initially adherent bone-marrow-derived stromal colonies were found to be capable of differentiation into the osteogenic, chondrogenic, and adipogenic lineage as demonstrated by lineage specific in vitro assays. All of these findings indicate that stromal cells are a mixture of cells with varying developmental potentials, including possibly stem cells and various other progenitor cells. Unfortunately, no specific antigens have been described that can associate the developmental potential of marrow stromal cells with a specific phenotypic trait. In the absence of such an antigen, it is difficult to determine the proportion of stem cells, multi-potential progenitors and determined precursors in cultured stromal cell populations. Since the discovery of the potential of marrow stromal cells by Friedenstein et al. [24], these cells have been denoted in the literature by a variety of terms, including fibroblast colony-forming cells, colony-forming unit-fibroblast, mesenchymal progenitor cells, mesenchymal stem cells and marrow stromal cells. In this dissertation they are referred to marrow stromal cells, because of the aforementioned lack of characterisation.

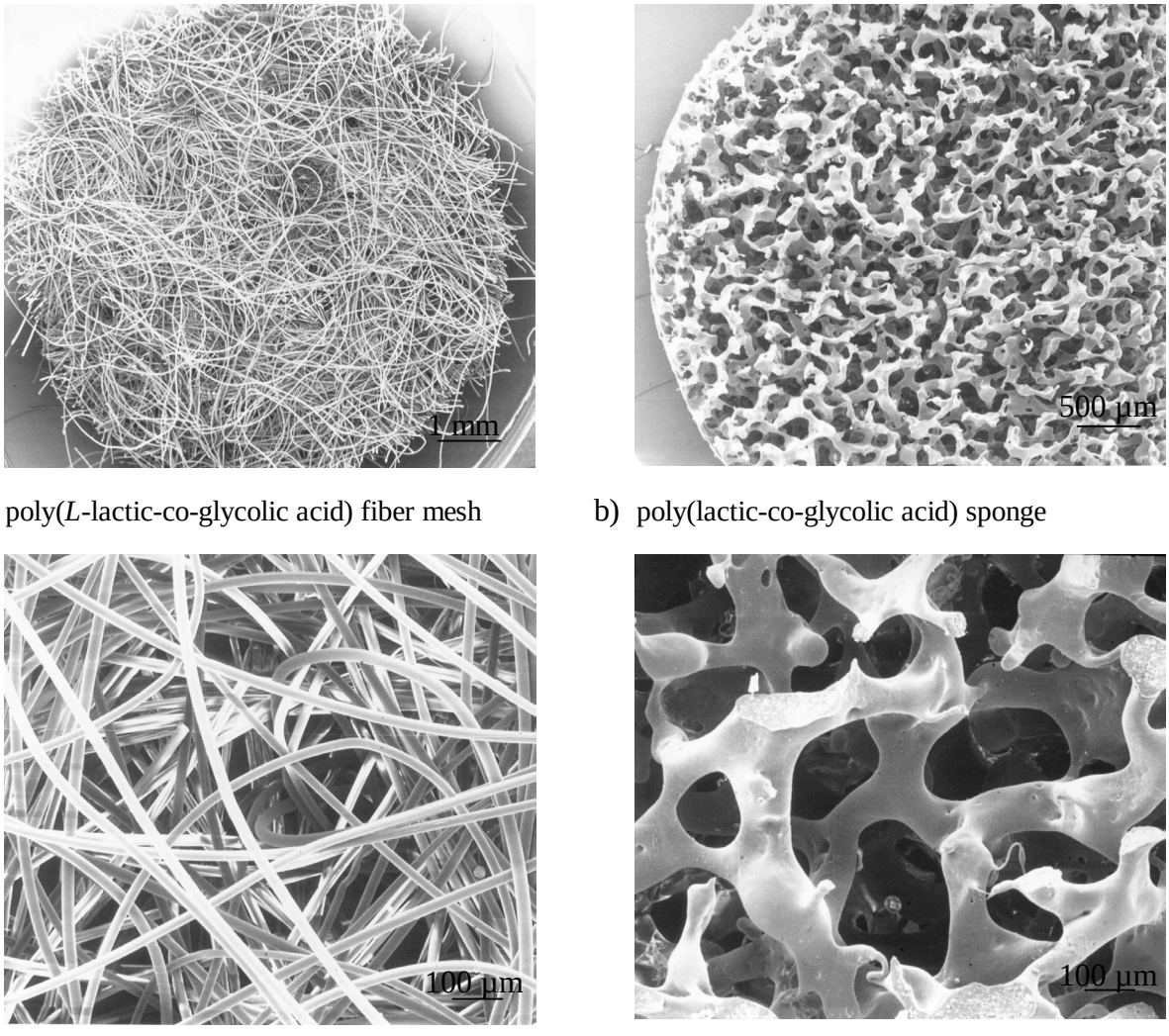
Marrow stromal cells (MSCs) can be encouraged to follow the osteoblastic lineage by the addition of diverse induction factors to their growth medium. It is well established that dexamethasone stimulates the osteogenic differentiation of human and rat marrow stromal cells [28,29]. In addition to dexamethasone, other supplements have an effect on differentiation to the osteoblastic phenotype. L-ascorbic acid is necessary for the production of the bone matrix protein collagen I [30]. The addition of β-glycerophosphate is required to provide a potential source of phosphate ions for matrix mineralization [30].

In conclusion, though MSCs are not fully characterized yet, they have been demonstrated to be an appropriate cell source for bone tissue engineering, because they possess the ability to

be expanded to gain a high cell number for tissue engineering purposes and the capacity to differentiate into bone under treatment with osteogenic agents.

2.4.2 The Scaffold

The scaffold, which modulates and coordinates tissue development, is an important key component in tissue formation. The cell behavior is modulated by the structure of the cell carrier, i.e., sponge-based or fiber-based polymer scaffolds (Fig. 5), gel or ceramic cell carriers, as well as by the material the carrier consists of.



a) poly(*L*-lactic-co-glycolic acid) fiber mesh

b) poly(lactic-co-glycolic acid) sponge

c) poly(*L*-lactic-co-glycolic acid) fiber mesh

d) poly(lactic-co-glycolic acid) sponge

Figure 5: SEM pictures of fiber-based and sponge-based polymer scaffolds.

Both natural and synthetic materials have been used to form cell carriers. Scaffolds made of natural materials may closely mimic the native cellular environment as they often consist of extracellular matrix components, including collagen and hydroxyapatite, among others [19] [13]. Collagen sponges, for example, have been successful in supporting osteoblast growth and function in vitro, but the low mechanical strength of these materials excludes them from use in load-bearing sites [13]. Hydroxyapatite, providing higher mechanical strength, also supports osteogenesis in vivo, however its remodelling is limited by a very slow degradation rate [13]. Synthetic materials, however, have the advantage that they may be tailored to define specific properties such as mechanical strength, degradation time, porosity and microstructure [19]. The reproducibility and charge conformity of the material also lends a great advantage to the synthetic materials. Additionally, defined shapes and sizes can be fabricated reproducibly from synthetic materials. The most widely used synthetic polymers in bone tissue engineering include poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), which are already approved by the FDA as suture materials [13]. Further synthetic polymers in bone tissue engineering involve poly(propylene fumarate) and poly(-ε-caprolactone-co-lactide) [13]. Moreover, the influence of combinations of different materials e.g. poly(*L*-lactic acid) combined with the osteoconductive hydroxyapatite were investigated [31]. All of these outlined materials diversely influence cell behavior. However, when lipophilic biomaterials come into contact with peptides and proteins from the body or cell culture fluids, the adsorbed molecules mask the specific surface properties of these biomaterials. Therefore, a step towards controlled cell/biomaterial interaction is the reduction of the unspecific protein adsorption. In this way a recent study on a poly(*D,L*-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymer (Me.PEG-PLA) demonstrated the effect of reduced protein adsorption, caused by the presence of poly(ethylene glycol) (PEG) [32,33], on cell adhesion and cell differentiation compared to unmodified PLA [34]. In another approach to selectively influence cell behavior, subtype-specific RGD (Arg-Gly-Asp)-peptides were coated [35] on diverse scaffold materials or covalently anchored to respectively modified biomaterials [36]. The RGD-sequence is the cell attachment site of a large number of adhesive extracellular matrix (ECM), blood, and cell surface proteins [37]. Because cells contain cell adhesion receptors (integrins) that recognize

only certain ECM molecules, the use of an appropriate cell-binding sequence leads to cell-selective surfaces [38]. The advantage of using small peptides compared to the whole extracellular matrix proteins is the ease of their synthesis and handling, not to mention their low immunogenic activity [39]. Nevertheless, successful bone tissue regeneration cannot be achieved by the scaffold and the cells alone. There is the necessity of the supplementation with appropriate osteogenic agents.

2.4.3 The Osteogenic Agents

When bone marrow stromal cells are utilized for bone formation, the supplementation with osteoinductive agents, including dexamethasone, ascorbic acid and β-glycerophosphate, is required. Additionally, a plethora of growth factors has been implicated in osteogenesis [7], which are introduced in the following:

Growth Factors

Bone is a storehouse for growth factors that are capable of stimulating both cell proliferation and osteoblastic differentiation [40]. Classes of growth factors produced by osteoblasts include transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), and fibroblast growth factors (FGFs) [40].

Of the three transforming growth factors known to be produced by various mammalian tissues, TGF-β1 seems to be the major member produced by human osteoblasts and stored in bone [40]. TGF-β1 is known to be produced locally during bone development and regeneration [41,42]. Additionally, various studies on the effects of TGF-β1 in different cell cultures have proven TGF-β1 to be effective at stimulating the formation of collagen I [41,43,20], the main matrix protein of bone [44]. The effects of TGF-β1 on matrix mineralization and differentiation of osteoblastic progenitor cells, however, reported a large number of conflicting results. TGF-β1 has been described as both inhibiting, as well as increasing, mineralization [45,46,47] and osteoblastic differentiation [40,48]. These divergent effects of TGF-β1 in in vitro cell culture seem to depend strongly on the cell type used in the study, their state of maturation when

TGF-β1 is added, the dosages and the dosing regimen of TGF-β1 and the chosen culture conditions.

The discovery of the BMPs stems from the findings that the implantation of demineralized bone at ectopic sites caused an induction of extraskeletal bone development [49]. To date, more than 15 BMPs have been identified and all of these BMPs, except for BMP-1, belong to the TGF-β superfamily and share significant sequence homology in the carboxy-terminal region with a conserved pattern of seven cysteine residues [40]. Considering in vitro studies, BMP-2, for example, has been shown to increase the osteoblastic differentiation of bone marrow stromal cells [50,40]. Thereby, it has been reported that BMP-2 not only up-regulates differentiation in preexisting osteoblasts, but also induces commitment of mesenchymal cells to the osteoblastic pathway [50].

IGFs represent the most abundant growth factors produced by osteoblasts and stored in bone [40]. A major problem, however, is that IGF treatment not only increased bone formation, but also bone resorption [40].

FGFs play key roles during physiological and pathological conditions, such as wound healing, skeletal repair, neovascularization, and tumor growth [40]. Both FGF1 (acidic FGF) and FGF2 (basic FGF) stimulate osteoblast proliferation and promote bone growth [40]. Most studies have been performed with FGF2, because FGF2 is more potent than FGF1 [40].

Hence, the addition of growth factors seems to be a promising tool to improve bone formation in tissue engineering.

2.4.4 Mechanical Forces

Mechanical forces profoundly affect the development, maintenance, and remodeling of bone in vivo and therefore may be important determinants of the quality of engineered bone-like tissue grown in vivo or in vitro. In vivo, forces applied on bone during movement result in changes of the hydrostatic pressure, direct cell strain, or fluid-flow-induced shear stress [51]. Flow of the interstitial fluid occurs because the application of mechanical strain causes the volume of some pores to decrease slightly and the volume of other pores to increase slightly, creating differences in bone fluid pressure, which results in fluid flow [51]. Following the

physical forces, the primary or secondary physical stimulus must be converted into an electrical or biochemical response. This step is called mechanotransduction and followed by signal transduction pathways, which result in a final cell response [52]. To date several signalling pathways have been thought to be induced by fluid flow. For example, cultured osteoblasts exposed to shear stress exhibited increased cyclic adenosine monophosphate (cAMP) production [51]. Furthermore, fluid shear stress increased NO release rate in osteoblasts from rat calvaria and human primary bone cell cultures [51]. In vitro studies assessing the effect of mechanical forces on the osteoblastic differentiation showed an increased expression of osteoblastic markers as opposed to static cultures [53,51]. In conclusion, mechanical stimulation of the osteoprogenitor cells appears to be an important part of the optimization of the process of osteoblastic differentiation.

Collectively, the key factors in tissue engineering, including the cell source, the scaffold, the osteoinductive agents and the mechanical forces during cultivation may be powerful tools in order to improve tissue formation in bone tissue engineering.

3 Goals of the Thesis

The field of bone tissue engineering is still a great challenge. The focus of this thesis was to improve bone tissue engineering by optimizing the osteogenic agents and the scaffold material, two of the key factors that influence tissue engineering. As a cell source, bone marrow stromal cells from Sprague-Dawley rats (rMSCs) were utilized.

The first objective was to optimize culture conditions, including cell seeding density at bone marrow isolation, the choice of the basal medium and the time point for osteogenic agent supplementation **(chapter 2)**.

In the next part of this work, the supplementation of the growth factor TGF-β1, in addition to the standard osteogenic agents (dexamethasone, β-glycerophosphat, ascorbic acid), was evaluated in order to assess whether or not the problem of limited matrix formation and mineralization in three-dimensional bone cell culture from rMSCs, could be solved by the impact of TGF-β1 **(chapter 3)**. In a follow-up study, the influence of TGF-β1 on osteoblastic

differentiation was investigated in order to determine a dosing regimen for TGF-β1 in three-dimensional bone cell culture, such that matrix formation is improved and osteoblastic differentiation is concomitantly preserved or even increased **(chapter 4)**.

Furthermore, we hoped to enhance osteoblastic differentiation versus TGF-β1 alone by administering the growth factor BMP-2 in combination with TGF-β1 **(chapter 5)**.

The next objective was to explore the effects of different scaffold materials on serum protein adsorption, cell adhesion, cell shape, cell proliferation and osteoblastic differentiation, in order to improve the key factor biomaterial for tissue engineering applications. To this end, the cell behavior on Me.PEG-PLA diblock copolymers was compared with the cell behavior on PLA and PLGA, which are commonly used in tissue engineering. In a previous study [54], it was demonstrated that a Me.PEG5-PLA20 diblock copolymer, which suppressed protein adsorption, increased osteoblastic differentiation compared to unmodified PLA. However, due to the strongly reduced cell adhesion on the tested diblock copolymer, we conducted a study in order to look for other compositions of Me.PEG-PLA diblock copolymers. We investigated if cell attachment could be improved to a reasonable percentage by incrementally decreasing the Me.PEG content and its chain length, while we aimed to preserve the polymer's differentiating properties **(chapter 6)**.

A further challenge was to employ a suitable representative out of the recently developed class of amine-reactive polymers ST-NH-PEG-PLA [55] – synthesized from mono amine derivatives (H_2N-PEG-PLA) of the Me.PEG-PLA diblock copolymers by covalent attachment of dissucinimidyl tartrate – for the covalent anchoring of a cyclic RGD-peptide [c(RGDfK)] [39], in order to selectively influence cell adhesion with regard to implant technology and tissue engineering applications **(chapter 7)**.

4 References

1. Freed, L. E. and Vunjak-Novakovic, G. (1998). Culture of organized cell communities. *Adv.Drug Deliv.Rev.* **33**, 15-39.

2. Marler J.J., Upton J., Langer, R., and Vacanti, J. P. (1998). Transplantation of cells in matrices for tissue regeneration. *Adv.Drug Deliv.Rev.* **33**, 165-182.

3. Fuchs, J. R., Nasseri, B. A., and Vacanti, J. P. (2001). Tissue engineering: a 21st century solution to surgical reconstruction. *Ann.Thorac.Surg.* **72**, 577-591.

4. Laurencin, C. T., Ambrosio, A. M., Borden, M. D., and Cooper, J. (1999). Tissue engineering: orthopedic applications. *Annu.Rev.Biomed.Eng.* **1**, 19-46.

5. Langer, R. and Vacanti, J. P. (1993). Tissue engineering. *Science* **260**, 920-926.

6. Langer, R. (2000). Tissue engineering. *MOL THER* **1**, 12-15.

7. Rose, F. R. A. J. and Oreffo, R. O. C. (2002). Bone tissue engineering: hope vs hype. *Biochem.Biophys.Res.Commun.* **292**, 1-7.

8. Welsch, U. Sobotta, Lehrbuch Histologie. 2002. Urban & Fischer.

9. Yaszemski, M. J., Payne, R. G., Hayes, W. C., Langer, R., and Mikos, A. G. (1996). Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone. *Biomaterials* **17**, 175-185.

10. http://www.bmb.psu.edu/courses/bisci004a/bone/bone/htm . 2003.

11. Manolagas, S. C. (2000). Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr.Rev.* **21**, 115-137.

12. Frank, J. D., Ryan, M., Kalscheur, V. L., Ruaux-Mason, C. P., Hozak, R. R., and Muir, P. (2002). Aging and accumulation of microdamage in canine bone. *Bone* **30**, 201-206.

13. Crane, G. M., Ishaug, S. L., and Mikos, A. G. (1995). Bone tissue engineering. *Nature Medicine* **1**, 1322-1324.

14. Van den Dolder, J., Vehof, J. W. M., Spauwen, P. H. M., and Jansen, J. A. (2002). Bone formation by rat bone marrow cells cultured on titanium fiber mesh: Effect of in vitro culture time. *J.Biomed.Mater.Res.* **62**, 350-358.

15. Holy, C. E., Shoichet, M. S., and Davies, J. E. (2000). Engineering three-dimensional bone tissue in vitro using biodegradable scaffolds: Investigating initial cell-seeding density and culture period. *J.Biomed.Mater.Res.* **51**, 376-382.

16. Dong, J., Kojima, H., Uemura, T., Kikuchi, M., Tateishi, T., and Tanaka, J. (2001). In vivo evaluation of a novel porous hydroxyapatite to sustain osteogenesis of transplanted bone marrow-derived osteoblastic cells. *J.Biomed.Mater.Res.* **57**, 208-216.

17. Van den Dolder, J., Farber, E., Spauwen, P. H. M., and Jansen, J. A. (2003). Bone tissue reconstruction using titanium fiber mesh combined with rat bone marrow stromal cells. *Biomaterials* **24**, 1745-1750.

18. Bancroft, G. N., Sikavitsas, V. I., Van den Dolder, J., Sheffield, T. L., Ambrose, C. G., Jansen, J. A., and Mikos, A. G. (2002). Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. *Proc.Natl.Acad.Sci.U.S.A* **99**, 12600-12605.

19. Nasseri, B. A., Ogawa, K., and Vacanti, J. P. (2001). Tissue engineering: an evolving 21st-century science to provide biologic replacement for reconstruction and transplantation. *Surgery* **130**, 781-784.

20. Arnold, U., Lindenhayn, K., and Perka, C. (2002). In vitro-cultivation of human periosteum derived cells in bioresorbable polymer-TCP-composites. *Biomaterials* **23**, 2303-2310.

21. Perka, C., Schultz, O., Spitzer, R. S., Lindenhayn, K., Burmester, G. R., and Sittinger, M. (2000). Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. *Biomaterials* **21**, 1145-1153.

22. Hardouin, P., Anselme, K., Flautre, B., Bianchi, F., Bascoulenguet, G., and Bouxin, B. (2000). Tissue engineering and skeletal diseases. *Joint Bone Spine* **67**, 419-424.

23. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147.

24. Friedenstein, A. J., Piatetzky-Shapiro, I. I., and Petrakova, K. V. (1966). Osteogenesis in transplants of bone marrow cells. *Journal Of Embryology And Experimental Morphology* **16**, 381-390.

25. Bruder, S. P., Jaiswal, N., and Haynesworth, S. E. (1997). Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J.Cell Biochem.* **64**, 278-294.

26. Aubin, J. E. (1998). Bone stem cells. *J Cell Biochem* **30-31**, 73-82.

27. Phinney, D. G. (2002). Building a consensus regarding the nature and origin of mesenchymal stem cells. *J.Cell Biochem.* **85 Supplement 38**, 7-12.

28. Scutt, A., Bertram, P., and Brautigam, M. (1996). The role of glucocorticoids and prostaglandin E2 in the recruitment of bone marrow mesenchymal cells to the osteoblastic lineage: positive and negative effects. *Calcif.Tissue Int.* **59**, 154-162.

29. Aubin, J. E. (1999). Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. *J.Cell Biochem.* **72**, 396-410.

30. Maniatopoulos, C., Sodek, J., and Melcher, A. H. (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res.* **254**, 317-330.

31. Ma, P. X., Zhang, R., Xiao, G., and Franceschi, R. (2001). Engineering new bone tissue in vitro on highly porous poly(alpha-hydroxyl acids)/hydroxyapatite composite scaffolds. *J.Biomed.Mater.Res.* **54**, 284-293.

32. Altankov, G., Thom, V., Groth, T., Jankova, K., Jonsson, G., and Ulbricht, M. (2000). Modulating the biocompatibility of polymer surfaces with poly(ethylene glycol): Effect of fibronectin. *J.Biomed.Mater.Res.* **52**, 219-230.

33. Amiji, M. and Park, K. (1992). Prevention of protein adsorption and platelet adhesion on surfaces by PEO/PPO/PEO triblock copolymers. *Biomaterials* **13**, 682-692.

34. Göpferich, A., Peter, S. J., Lucke, A., Lu, L., and Mikos, A. G. (1999). Modulation of marrow stromal cell function using poly(D,L-lactic acid)-block-poly(ethylene glycol)-monomethyl ether surfaces. *J.Biomed.Mater.Res.* **46**, 390-398.

35. Eid, K., Chen, E., Griffith, L., and Glowacki, J. (2001). Effect of RGD coating on osteocompatibility of PLGA-polymer disks in a rat tibial wound. *J.Biomed.Mater.Res.* **57**, 224-231.

36. Shin, H., Jo, S., and Mikos, A. G. (2002). Modulation of marrow stromal osteoblast adhesion on biomimetic oligo[poly(ethylene glycol) fumarate] hydrogels modified with Arg-Gly-Asp peptides and a poly(ethyleneglycol) spacer. *J.Biomed.Mater.Res.* **61**, 169-179.

37. Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. *Annu.Rev.Cell Dev.Biol.* **12**, 697-715.

38. Giancotti, F. G. and Ruoslahti, E. (1999). Integrin signaling. *Science* **285**, 1028-1032.

39. Kantlehner, M., Schaffner, P., Finsinger, D., Meyer, J., Jonczyk, A., Diefenbach, B., Nies, B., Holzemann, G., Goodman, S. L., and Kessler, H. (2000). Surface Coating with Cyclic RGD Peptides Stimulates Osteoblast Adhesion and Proliferation as well as Bone Formation. *Chembiochem.Europ.J.Chem.Biol.* **1**, 107-114.

40. Qin, X., Gysin, R., Mohan, S., and Baylink, D. J. (2001). Bone growth factors. *In* "Osteoporosis " (Marcus R., Feldman D., and Kelsey J., Eds.), Academic Press, San Diego CA.

41. Lilli, C., Marinucci, L., Stabellini, G., Belcastro, S., Becchetti, E., Balducci, C., Staffolani, N., and Locci, P. (2002). Biomembranes enriched with TGFbeta1 favor bone matrix protein expression by human osteoblasts in vitro. *J.Biomed.Mater.Res.* **63**, 577-582.

42. Robey, P. G., Young, M. F., Flanders, K. C., Roche, N. S., Kondaiah, P., Reddi, A. H., Termine, J. D., Sporn, M. B., and Roberts, A. B. (1987). Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-beta) in vitro. *J.Cell Biol.* **105**, 457-463.

43. Hock, J. M., Canalis, E., and Centrella, M. (1990). Transforming growth factor-beta stimulates bone matrix apposition and bone cell replication in cultured fetal rat calvariae. *Endocrinology* **126**, 421-426.

44. Anselme, K. (2000). Osteoblast adhesion on biomaterials. *Biomaterials* **21**, 667-681.

45. Antosz, M. E., Bellows, C. G., and Aubin, J. E. (1989). Effects of transforming growth factor beta and epidermal growth factor on cell proliferation and the formation of bone nodules in isolated fetal rat calvaria cells. *J.Cell Physiol* **140**, 386-395.

46. Faucheux, C., Ulysse, F., Bareille, R., Reddi, A. H., and Amedee, J. (1997). Opposing actions of BMP3 and TGF beta 1 in human bone marrow stromal cell growth and differentiation. *Biochem.Biophys.Res.Commun.* **241**, 787-793.

47. Kale, S., Biermann, S., Edwards, C., Tarnowski, C., Morris, M., and Long, M. W. (2000). Three-dimensional cellular development is essential for ex vivo formation of human bone. *Nat.Biotechnol.* **18**, 954-958.

48. Breen, E. C., Ignotz, R. A., McCabe, L., Stein, J. L., Stein, G. S., and Lian, J. B. (1994). TGF beta alters growth and differentiation related gene expression in proliferating

osteoblasts in vitro, preventing development of the mature bone phenotype. *J.Cell Physiol* **160**, 323-335.

49. Urist, M. R. (2002). Bone: formation by autoinduction. 1965. *Clin.Orthop.* 4-10.

50. Rickard, D. J., Sullivan, T. A., Shenker, B. J., Leboy, P. S., and Kazhdan, I. (1994). Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev.Biol.* **161**, 218-228.

51. Sikavitsas, V. I., Temenoff, J. S., and Mikos, A. G. (2001). Biomaterials and bone mechanotransduction. *Biomaterials* **22**, 2581-2593.

52. Gooch, K. J., Blunk, T., Vunjak-Novakovic, G., Langer, R., and Freed, L. E. (1998). Mechanical Forces and Growth Factors Utilized In Tissue Engineering. *In* " Frontiers in Tissue Engineering" (C. W. Patrick, Jr., A. G. Mikos, and L. V. McIntire, Eds.), Pergamon; Oxford.

53. Hillsley, M. V. and Frangos, J. A. (1994). Bone tissue engineering: the role of interstitial fluid flow. *Biotechnol.Bioeng.* **43**, 573-581.

54. Göpferich, A., Peter, S. J., Lucke, A., Lu, L., and Mikos, A. G. (1999). Modulation of marrow stromal cell function using poly(D,L-lactic acid)-block-poly(ethylene glycol)-monomethyl ether surfaces. *J.Biomed.Mater.Res.* **46**, 390-398.

55. Tessmar, J. K., Mikos, A. G., and Göpferich, A. (2002). Amine-reactive biodegradable diblock copolymers. *Biomacromolecules.* **3**, 194-200.

Chapter 2

Optimization of culture conditions for bone cell culture of marrow stromal cells:

Cell seeding density, basal medium and culture time
with differentiation supplements

E. Lieb[1], A. Göpferich [1], M.B. Schulz [1]

[1] Department of Pharmaceutical Technology, University of Regensburg, Germany

1 Introduction

Studies reporting basic cell culture techniques, as well as various combinations of media and differentiation factors, for bone cell culture with marrow stromal cells have been previously published [1,2]. Maniatopoulos et al. [1], for example, described a procedure for the isolation of rat bone marrow, while Friedenstein et al. [3] developed a method to separate marrow stromal cells from nonadherent cells, such as blood cells, by the attachment to tissue culture surfaces, a method which is still widely employed due to its effectiveness [4]. To date, however, there is no published data regarding the optimal initial cell seeding density for bone marrow isolation, despite the fact that cell density is known to have an effect on cell proliferation [5]. Hence, we examined different cell seeding densities in tissue culture polystyrene (TCPS) flasks after bone marrow isolation in order to optimize the yield of rat marrow stromal cells (rMSCs). Moreover, with regard to the literature, despite many years of research into matching particular media to specific cell types, the choice of the basal medium for bone cell culture from rMSCs is still not obvious and is often empirical. As the expression of the phenotype depends on the culture conditions with regard to both the basal culture medium and the time point at which osteogenic agents are administered [2,6], we performed experiments examining the effects of both variables. At first, we focused on the effect of different basal media on rMSC proliferation and differentiation to the osteoblastic phenotype. After choosing the optimal culture medium, we investigated the influence of the time point of differentiation factor addition (dexamethasone, β-glycerophosphate and ascorbic acid) on cell proliferation and osteoblastic differentiation. Hence, this study intended to optimize culture conditions for bone cell culture with rMSCs, investigating three variables: cell seeding density at bone marrow isolation, the choice of culture media and, finally, the time point of osteogenic supplement addition.

In detail, the optimum cell seeding density with regard to a maximum cell count per flask, and concomitantly the minimum number of sacrificed rats, was examined by seeding the pooled bone marrow from the femurs and tibias of multiple rats onto tissue culture plastic, such that the cell density corresponded to 2, 3, 4, 5, 6, 7 or 8, 75-cm^2 flasks per rat. The cell count was

determined after 13 days of cultivation. The ascertained optimum cell seeding density was used for the examination of the effects of various basal media.

To evaluate different basal media, rMSCs were cultured in three widely used media: Dulbecco´s Modified Eagle´s Medium (DMEM), with low and high glucose content, and in Minimum Essential Medium Eagle, alpha-modification (α-MEM). These three media have different characteristics; namely, DMEM has a narrower range of aminoacids and vitamins, but a higher total nutrient concentration in comparison to α-MEM, which contains a higher variety of aminoacids and vitamins. The DMEM low glucose medium has a glucose content of 1000 mg/l, whereas DMEM high glucose contains 4500 mg glucose per liter. To assess the effect of the different basal media on osteoblastic differentiation, we investigated the alkaline phosphatase (ALP) activity per cell, which is an important early osteogenic marker [7,8,9]. Furthermore, the cell number was determined as a measure of proliferation and for the normalization of ALP activity measurements.

The third part of this study dealt with the effects of the time point at which the differentiating supplements were added on proliferation and on the expression of the osteoblastic phenotype. To this end, cells were exposed to medium supplemented with osteogenic agents either immediately upon harvest, i.e. from day 0 or three days (day 3) after marrow stromal cell isolation. The cell count and ALP activity were compared to the results obtained [7,8,9] when cells were proliferated for up to two weeks and then differentiated to the osteoblastic phenotype.

2 Materials and Methods

2.1 Evaluation of seeding densities at bone marrow isolation

2.1.1 Marrow stromal cell isolation and proliferation

Marrow stromal cells were obtained from 6-week old male Sprague-Dawley rats (weight: 170 – 180 g, Charles River Laboratories, Sulzfeld, Germany). Cell isolation from the femur and tibia was performed in accordance with a protocol published by Ishaug et al. [10].

Following marrow isolation and dispersion, cells were centrifuged at 1200 rpm (259 x g) for 7 min. The resulting cell pellet was resuspended in primary medium, which consisted of DMEM high glucose (Life Technologies GmbH, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products Inc., Calabasas, California, USA) and 1% penicillin/streptomycin (Sigma, Taufkirchen, Germany). To optimize cell seeding densities the cells from multiple rats were pooled and seeded onto tissue culture plastic (TCPS), such that the cell density corresponded to 2, 3, 4, 5, 6, 7 or 8, 75-cm^2 flasks (T-75 flask) per rat. Three flasks were cultivated for each cell density.

On the third day of expansion, the flasks were rinsed twice with phosphate buffer (PBS, Life Technologies GmbH) to remove the nonadherent cells. Thereafter, the 12 ml of primary medium were changed every 2 - 3 days. On day 13, cells were trypsinized (0.25 % trypsin in ethylenediaminetetraacetic acid (EDTA), Life Technologies) and cell numbers were determined by a Coulter Counter measurements.

Couter Counter

The cell counts obtained after 13 days of proliferation were determined by Coulter Counter (Coulter Mastersizer II, Beckmann, Krefeld, Germany) measurements. For each condition, the cells from 3 T-75 flasks were pooled and an aliquot was dispersed in Isoton II, the electrolyte solution for Coulter Counter measurements (Beckmann). A capillary of 100 µm orifice size was used, while a cell diameter of 8 µm was assumed. Each dispersion was measured two times.

2.2 Evaluation of basal media

2.2.1 Marrow stromal cell isolation and proliferation

RMSCs were isolated as described in 2.1.1. Basal media α-MEM (Sigma, M 0894), DMEM low glucose (Biochrom, T041, Berlin, Germany) and DMEM high glucose (Life Technologies, 52100), were all supplemented with 10% FBS and 1% penicillin/streptomycin before use. The cells from multiple rats were pooled and cells were seeded, so that the cell seeding density corresponded to 4 T-75 flasks per rat.

On the third day of expansion, the flasks were rinsed twice with PBS to remove the nonadherent cells. Thereafter, the 12 ml of primary medium were changed every 2 - 3 days. Upon reaching 80 % confluence, cells were trypsinized and cell numbers were determined with a hemacytometer in preparation for subculture.

2.2.2 Cell differentiation

For investigation of osteoblastic differentiation, 53,000 cells per cm^2 were seeded into twelve-well plates.

Primary medium was exchanged for complete medium (the appropriate basal medium supplemented with 10 % FBS, dexamethasone 10^{-8} M (Sigma), ascorbic acid 50 mg/l (Sigma) and β-glycerophosphate 7 mM (Sigma)) 24 hours after subculturing.

Cell proliferation after subculturing was determined by a DNA assay instead of Coulter Counter measurements or counting with a hemacytometer due to matrix formation in complete medium, which hinders cell dispersement. The osteoblastic differentiation was examined by the determination of alkaline phosphatase activity. The total alkaline phosphatase activity per sample was normalized to the determined cell number.

DNA Assay

A fluorimetric assay was performed to ascertain the total amount of DNA, and subsequently the cell number, per well [11]. The assay was performed on cells after 5, 8, 11, 14 and 17 days (day 1 = 24 hours after passage) in culture in the twelve-well plates. After rinsing with PBS, the well plates were kept frozen at –20 °C until the DNA assay was performed. For the assay, cells were scraped off the culture surface with disposable cell scrapers (Biochrom) and dispersed in 1.4 ml of EDTA-solution (10 mM, pH 12.3). The assay followed a protocol described by Ishaug et al. [12]. 1.5 ml of Hoechst 33258 dye [200 ng/ml dye (Polysciences, Warrington, PA, USA) in 100 mM NaCl and 10 mM tris(hydroxymethyl)aminomethane, pH 7.0] were added to 1 ml of the cell suspension. Cell standards of 30,000 to 300,000 cells/ml and DNA standards (calf thymus, Sigma) were prepared and examined in parallel to determine the average DNA content of a single cell.

Alkaline phosphatase assay

For determination of alkaline phosphatase activity (ALP activity), an enzyme activity kit purchased from Sigma was used (Diagnostic Kit 104, Sigma). Cell samples were taken at 5, 8, 11, 14 and 17 days, as for cell number determination. To perform the assay, cells were scraped off the culture surface with disposable cell scrapers (Biochrom) and dispersed in tris(hydroxymethyl)aminomethane buffer (1 M, pH 8.0) to lyse the cells. Collagen matrix material was fragmented via sonification in an ice bath over a period of ten minutes. The assay was performed as described in the protocol provided by Sigma and the extent of the hydrolysis of p-nitrophenyl phosphate in the presence of ALP was measured with a photometer (Specord 40, Analytik Jena AG, Jena, Germany) at 405 nm.

2.3 Cell differentiation in response to the time schedule of osteogenic agent supplementation

2.3.1 Marrow stromal cell isolation and proliferation

RMSCs were isolated as described in 2.1.1. The cells from multiple rats were pooled and cells were seeded, so that the cell seeding density corresponded to two T-75 flasks, because it has been documented that proliferation is decreased following the addition of differentiation factors [13]. Cells were cultured in complete medium (α-MEM, 10 % FBS, dexamethasone 10^{-8} mol/l, ascorbic acid 50 mg/l and β-glycerophosphate 7 mM) either from day 0, initiating differentiation immediately upon bone marrow isolation, or starting on day 3, following initial cultivation in primary medium. On the third day of expansion, the flasks were rinsed twice with PBS to remove the nonadherent cells. Thereafter, 12 ml of complete medium were added to all flasks and changed every two to three days. On day 8, cells had to be subcultured, because the beginning formation of collagen I, characteristic for the osteoblastic phenotype [2], complicated lifting cells. Cell numbers were determined with a hemacytometer.

2.3.2 Cell differentiation

For the investigation of osteoblastic differentiation, 53,000 cells per cm^2 were seeded into twelve-well plates.

Cell proliferation following subculture was determined by a DNA assay and the osteoblastic differentiation was examined by the determination of alkaline phosphatase activity. The alkaline phosphatase activity was normalized to the determined cell number.

DNA Assay

The DNA assay was performed as described in 2.2.2. Cell samples were taken after 5, 7 and 9 days (day 1 = 24 hours after passage) of cultivation.

Alkaline phosphatase assay

The alkaline phosphatase assay was performed as described in 2.2.2. Cell samples were taken after 5, 7 and 9 days of cultivation.

2.4 Statistical analysis

Measurements for the DNA and the ALP assays were collected (n = 3) and expressed as the mean ± standard deviation (SD). Single factor analysis of variance (ANOVA) was used in conjunction with a multiple comparison test (Tukey test) to assess the statistical significance.

3 Results

3.1 Evaluation of seeding densities at bone marrow isolation

3.1.1 Marrow stromal cell isolation and proliferation

To optimize cell seeding densities the cells from multiple rats were pooled and seeded onto TCPS, such that the cell density corresponded to 2, 3, 4, 5, 6, 7 or 8, T-75 flask per rat. The cell number per flask after 13 days of proliferation was determined by Coulter Counter measurement. We calculated between 1.7 million and 2.1 million cells per T-75 flask when the bone marrow was seeded into 2 to 4 flasks and between 500,000 and 800,000 cells per T-75 flask when bone marrow had been divided into 5 to 8 flasks (Fig. 1). Consequently, the total

cell numbers obtained per rat was higher using a seeding density of 4 flasks per rat as opposed to 2 flasks per rat or 7 flasks instead of 5 flasks per rat within the lower seeding density of 5 to 8 T-75 flasks, (Fig. 2).

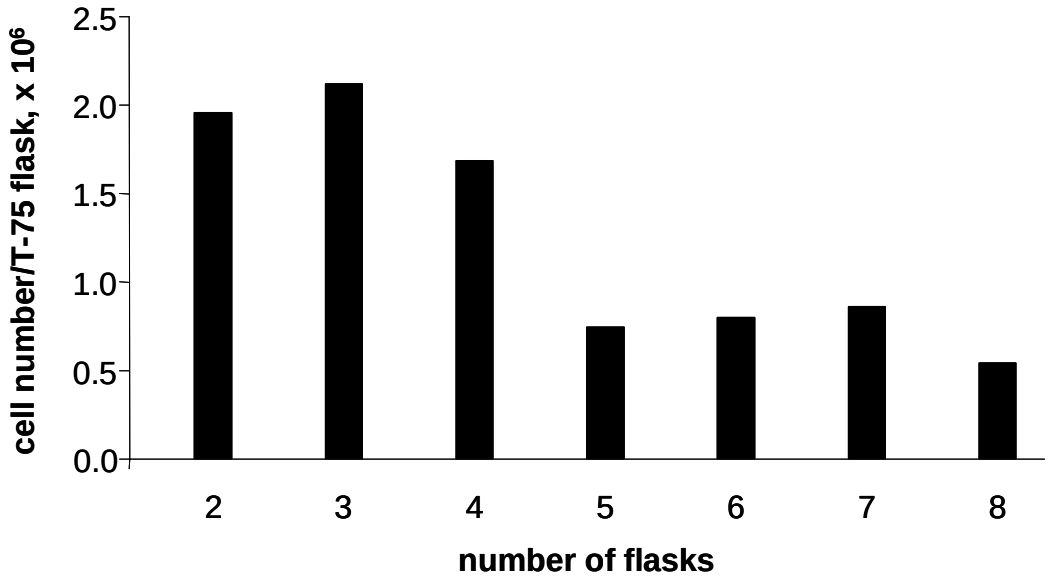


Figure 1: Cell number per T-75 flask, determined by Coulter Counter measurements after 13 days of cultivation, having investigated cell seeding densities of bone marrow from one rat for 2 T-75 flasks up to 8 T-75 flasks. Each column represents 3 T-75 flasks pooled for measurement.

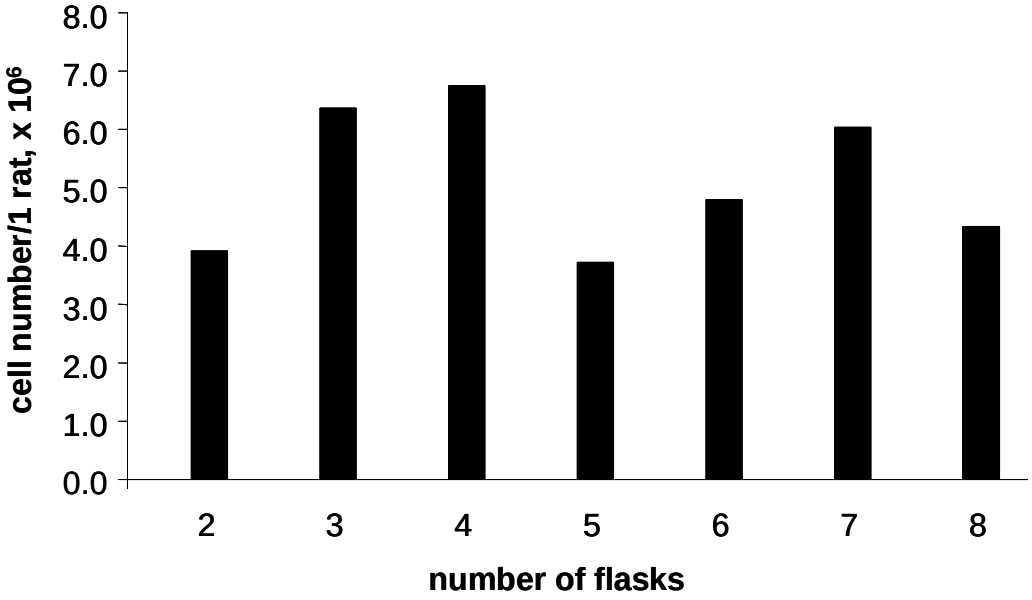


Figure 2: Cell number per rat determined by Coulter Counter measurement after 13 days of cultivation with cell densities corresponding to 2 to 8 T-75 flasks per rat. Each column represents 3 T-75 flasks pooled for measurement.

3.2 Evaluation of basal media

3.2.1 Marrow stromal cell isolation and proliferation

A cell count of 4 million cells per T-75 flask, determined with a hemacytometer, was obtained after 10 days of cultivation in α-MEM (Table 1). In DMEM low glucose, however, we found only 2 million cells per T-75 flask after 13 days of proliferation. Similar results were obtained in DMEM high glucose, where 2.2 million cells per flask were determined.

3.2.2 Cell differentiation

DNA Assay

The cell number after passage was determined by a DNA assay. The measurements showed a significantly higher ($p < 0.01$) cell number per cm^2 after cultivation in α-MEM than in DMEM, high or low glucose, throughout the period (Fig. 3).

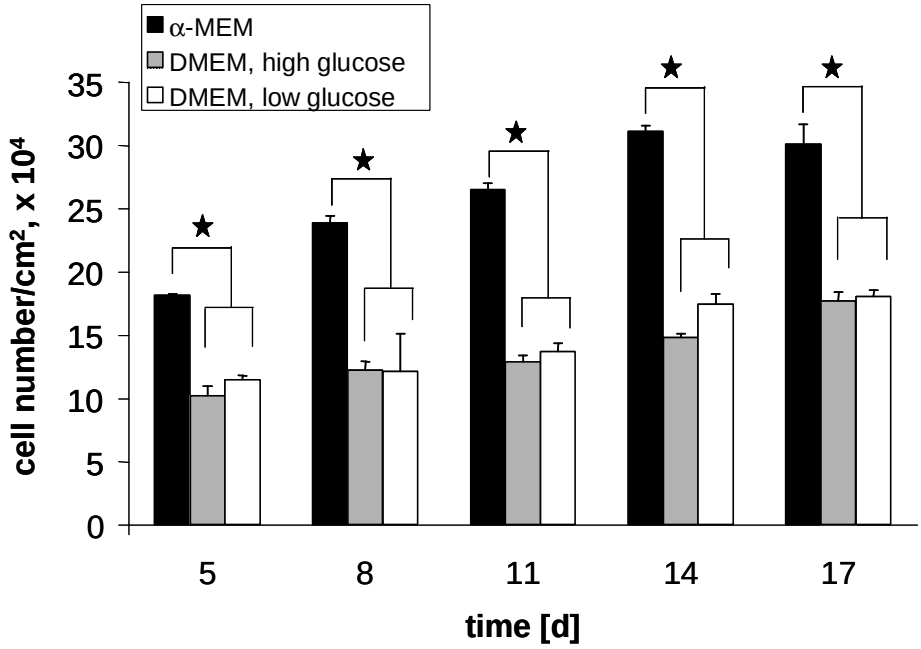


Figure 3: Proliferation kinetics of rMSCs in three different media. Cells were treated with differentiation factors for 17 days. Columns and error bars represent the mean ± SD for n = 3. The statistical significance ($p < 0.01$) was assessed by Tukey-Test. Significance is indicated by a ★.

Alkaline phosphatase assay

The determination of ALP activity for the comparison of the effect of different basal media on the expression of the osteoblastic marker showed that ALP activity per cell increased to a maximum on days 14 and 17 using α-MEM medium (Fig. 4). During cultivation in α-MEM, the ALP activity per cell was significantly ($p < 0.01$) higher than the ALP activity per cell after cultivation in DMEM.

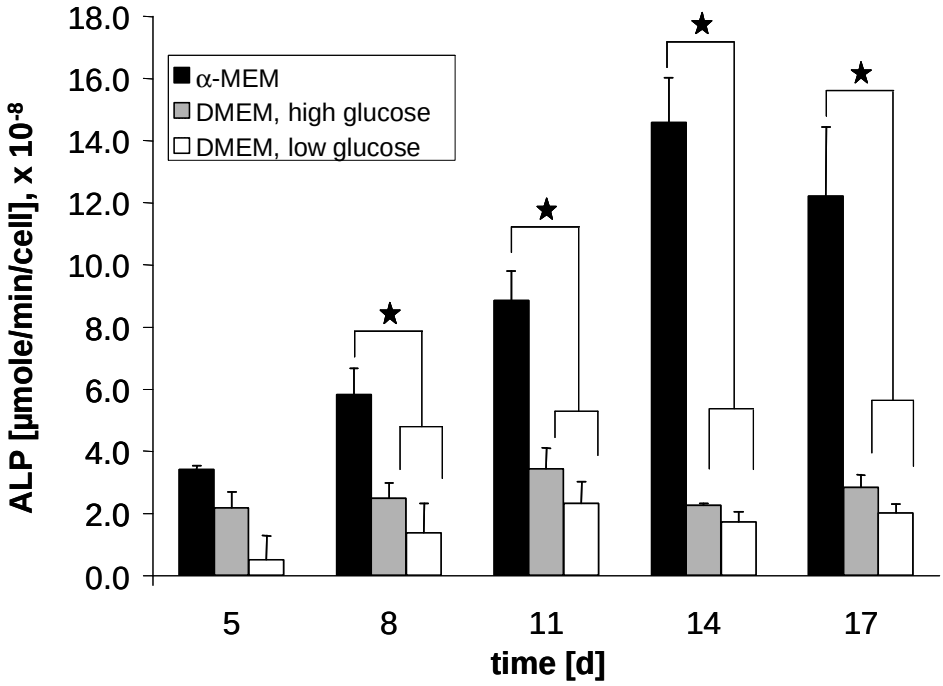


Figure 4: Alkaline phosphatase activity per cell after 5, 8, 11, 14 and 17 days of cultivation in three different basal media. Columns and error bars represent the mean ± SD for n = 3. The statistical significance was assessed by Tukey-Test. Significance ($p < 0.01$) is indicated by a ★.

3.3 Cell differentiation in response to the time schedule of osteogenic agent supplementation

3.3.1 Marrow stromal cell isolation and proliferation

A cell count of 1 million cells per T-75 flask was obtained on the eighth day following the initiation of differentiation on either day 0 or day 3 after rMSC isolation, meaning that only 2 million cells were obtained per rat (Table 1).

3.3.2 Cell differentiation

DNA Assay

The cell number after passage was determined utilizing a DNA assay. An increase in cell number was observed between days 5 and 7, whereas no change was found from day 7 to

day 9 (Fig. 5). Furthermore, no difference in the cell number between cells cultured in complete medium starting on day 0 or day 3 was noted, except for on day 5, when a slightly higher cell number was found for the cells, which were treated with differentiation factors from day 0.

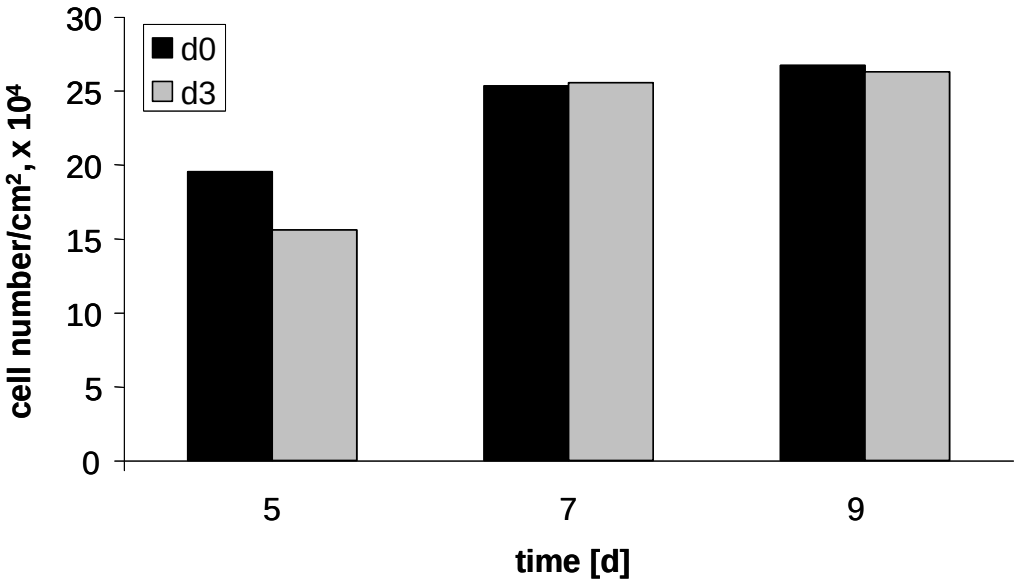


Figure 5: Proliferation kinetics of rMSCs cultured from day 0 or day 3 in complete medium. Columns represent the means for n = 2.

Alkaline phosphatase assay

The determination of the activity of the osteoblastic marker ALP for the evaluation of the effect of the time point at which osteogenic supplements are added showed a constant ALP activity per cell over the investigated period, regardless of whether complete medium was added on day 0 or on day 3 after marrow stromal isolation (Fig. 6). The ALP activity of the cells that were differentiated immediately upon bone marrow isolation or on day 3 after marrow stromal isolation (Fig. 6) was up to four times higher than the ALP activity of cells proliferated in primary medium (α-MEM) for two weeks before exposing to differentiating medium (Fig. 4, Table 1).

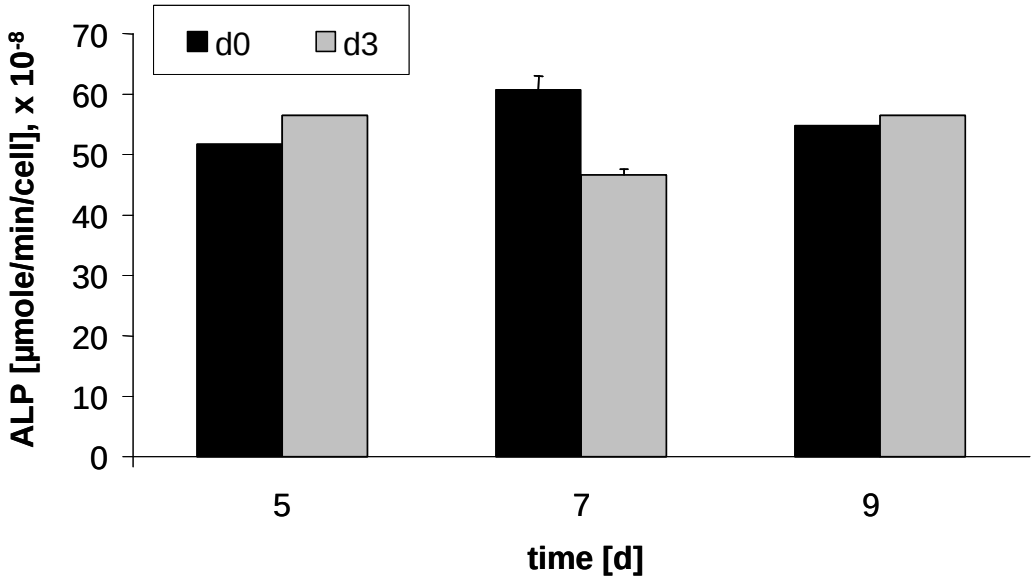


Figure 6: Alkaline phosphatase activity per cell on days 5, 7 and 9, with cultivation from day 0 or day 3 after rMSC isolation in complete medium. Columns and error bars represent the mean ± SD for n = 3 on day 7, while on day 5 and 9 columns represent the means for n = 2.

Table 1: Comparison of the characteristics for cultures differentiated upon harvest or upon the 1st passage, using α-MEM as basal medium.

	Differentiation after the 1st passage	**Differentiation directly upon harvest**
Cell number obtained per rat at passage	16 million	2 million
Maximum ALP activity after passage [µmol/min/cell]	1.46×10^{-7}	6.07×10^{-7}

4 Discussion

The objective of this study was to optimize the cell seeding density at bone marrow isolation and the subsequent culture conditions, specifically the choice of basal media and the time point for osteogenic supplement addition. With regard to the examination of cell seeding densities, we expressed the seeding density for marrow isolation in culture flasks per rat, as the determination of the adherent cell number at harvest is very difficult, because many cellular systems constitute the stroma, i.e. hematopoietic, endothelial and stromal cells [14]. Thus, counting the cells after marrow isolation is only a rough approach to determining the number of stromal cells, even when the red blood cells are lysed with acetic acid, a technique which is applied by some authors [15]. The optimization of rMSC isolation showed that high cell seeding densities from 2 to 4 T-75 flasks per rat resulted in nearly the same cell number per flask, i.e. between 1.7 and 2.1 million cells per flask, whereas lower seeding densities from 5 to 8 flasks per rat resulted uniformly in about one third of this cell count with 500,000 to 800,000 cells per flask. Thus, lower initial seeding densities did not improve the cell yield per rat. The flasks with lower seeding densities were furthermore suboptimal, because we aimed at 80% confluence [16] per flask after a proliferation phase of about two weeks [15], but only 20% confluence was reached after 13 days of proliferation. Several explanations may be given for the non-linear relationship between the density of plated cells and the cell number achieved before passage. On the one hand, there is the possibility that not all the available adherent cells attach to the bottom of the culture flask due to the cell-cell interactions with nonadherent cells or due to multilayering in the highly concentrated cell suspension. On the other hand, cells have to be seeded within a definite proximity to one another in order to proliferate. This may depend on paracrine interactions between the attached cells, as well as on the necessary cell-cell contacts. To minimize the number of rats, we suggest seeding 4 T-75 flasks per rat, which results in about 7 million cells per rat when cultured with DMEM as basal medium instead of 4 million cells per rat with a seeding density of 2 T-75 flasks.

To determine the optimal environment for rat marrow stromal cell expansion and osteoblastic differentiation, bone marrow stromal cells were cultured in α-MEM and in

DMEM, with low and high glucose concentrations. The influence of the different media on the cell growth and differentiation to the osteoblastic phenotype was compared.

α-MEM was shown to enhance rMSC proliferation before passage in primary medium and after passage in complete medium before the onset of differentiation, as compared to culture in DMEM. Thus, a lower demand for rats at marrow isolation results from the use of α-MEM. The most important result of the medium comparison, however, was the highly significant increase of the osteogenic marker alkaline phosphatase when cells were cultivated in α-MEM compared to a cultivation in DMEM. No significant differences between DMEM low glucose and DMEM high glucose were observed. Similar results concerning the ALP activity were reported by Coelho et al. [6], who examined the effect of different basal media on human bone marrow stromal cells. Hence, we suggest the use of α-MEM as basal medium for bone cell culture from rMSCs. In summary, the results of the medium test showed that different nutrient containing media such as α-MEM and DMEM affect both the proliferation of undifferentiated stromal cells and the expression of the osteoblastic phenotype.

The experiments performed to investigate cell differentiation in response to the time point of osteogenic supplement addition showed that osteogenic agents in the early culture period inhibited proliferation when administered before the first passage. Proliferation in complete medium resulted in 2 million cells per rat compared to 16 million cells per rat after proliferation in primary medium (Table 1). However, the ALP activity was strongly enhanced, elevated four-fold, when cultivated with osteogenic supplements before the passage compared to cells maintained in primary medium for about two weeks before the osteoblastic differentiation (Table 1). Similar results concerning osteoblastic differentiation have been reported by Peter et al. [2], whose results suggest adding osteogenic agents immediately upon harvest. However, our results concerning suppressed proliferation, confirmed several times, are in contrast to the observations of Peter et al. [2], who reported no influence on proliferation when osteogenic agents were added immediately upon harvest. These differences in the rate of proliferation remained unchanged even when the same lots of FBS were used. However, there is a necessity for cell proliferation in order to gain sufficient cell numbers as prerequisite for their use in tissue engineering. Although the effect of the differentiation factors on cell

proliferation may be delayed under special culture conditions, the addition of these supplements will constrain an extensive proliferation in the long-term, because of the inverse correlation between cell proliferation and differentiation. Hence, we suggest utilizing primary medium until passage and then administering complete medium later to induce differentiation.

In conclusion, the optimized cell culture conditions for bone cell culture from bone marrow stromal cells are a seeding density of 4 T-75 flasks per rat at harvest, α-MEM as basal medium and the supplementation with differentiating components 24 hours after the first passage, following the proliferation of cells for about two weeks in primary medium.

5 References

1. Maniatopoulos, C., Sodek, J., and Melcher, A. H. (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res.* **254**, 317-330.

2. Peter, S. J., Liang, C. R., Kim, D. J., Widmer, M. S., and Mikos, A. G. (1998). Osteoblastic phenotype of rat marrow stromal cells cultured in the presence of dexamethasone, beta-glycerolphosphate, and L-ascorbic acid. *J.Cell Biochem.* **71**, 55-62.

3. Friedenstein, A. J., Chailakhjan, R. K., and Lalykina, K. S. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell And Tissue Kinetics* **3**, 393-403.

4. Phinney, D. G. (2002). Building a consensus regarding the nature and origin of mesenchymal stem cells. *J.Cell Biochem.* **85 Supplement 38**, 7-12.

5. Colter, D. C., Class, R., DiGirolamo, C. M., and Prockop, D. J. (2000). Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc.Natl.Acad.Sci.U.S.A* **97**, 3213-3218.

6. Coelho, M. J., Cabral, A. T., and Fernande, M. H. (2000). Human bone cell cultures in biocompatibility testing. Part I: osteoblastic differentiation of serially passaged human bone marrow cells cultured in alpha-MEM and in DMEM. *Biomaterials* **21**, 1087-1094.

7. Rickard, D. J., Sullivan, T. A., Shenker, B. J., Leboy, P. S., and Kazhdan, I. (1994). Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev.Biol.* **161**, 218-228.

8. Aubin, J. E. and Heersche, J. N. M. (2000). Osteoprogenitor cell differentiation to mature bone-forming osteoblasts. *Drug Development Research* **49**, 206-215.

9. Malaval, L., Modrowski, D., Gupta, A. K., and Aubin, J. E. (1994). Cellular expression of bone-related proteins during in vitro osteogenesis in rat bone marrow stromal cell cultures. *J.Cell Physiol* **158**, 555-572.

10. Ishaug, S. L., Crane, G. M., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **36**, 17-28.

11. West, D. C., Sattar, A., and Kumar, S. (1985). A simplified in situ solubilization procedure for the determination of DNA and cell number in tissue cultured mammalian cells. *Anal.Biochem.* **147**, 289-295.

12. Ishaug, R. S., Crane, K. G., Yaszemski, M. J., and Mikos, A. G. (1998). Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* **19**, 1405-1412.

13. Scutt, A., Bertram, P., and Brautigam, M. (1996). The role of glucocorticoids and prostaglandin E2 in the recruitment of bone marrow mesenchymal cells to the osteoblastic lineage: positive and negative effects. *Calcif.Tissue Int.* **59**, 154-162.

14. Deans, R. J. and Moseley, A. B. (2000). Mesenchymal stem cells: Biology and potential clinical uses. *Exp.Hematol.* **28**, 875-884.

15. Lennon, D. P., Haynesworth, S. E., Young, R. G., Dennis, J. E., and Caplan, A. I. (1995). A chemically defined medium supports in vitro proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. *Exp.Cell Res.* **219**, 211-222.

16. Göpferich, A., Peter, S. J., Lucke, A., Lu, L., and Mikos, A. G. (1999). Modulation of marrow stromal cell function using poly(D,L-lactic acid)-block-poly(ethylene glycol)-monomethyl ether surfaces. *J.Biomed.Mater.Res.* **46**, 390-398.

Chapter 3

Effects of TGF-β1 on Bone-Like Tissue Formation in Three-Dimensional Cell Culture

Part I: Culture Conditions and Tissue Formation

E. Lieb [1], S. Milz [2], M. Hacker [1], M. Dauner [3], A. Göpferich [1], M.B. Schulz [1, 4]

[1] Department of Pharmaceutical Technology, University of Regensburg, Germany

[2] Anatomische Anstalt, LMU, Munich, Germany

[3] Institute of Textile Technology and Process Engineering (ITV), Denkendorf, Germany

[4] Department of Pharmaceutical Technology, University of Graz, Austria

submitted to Tissue Engineeering

1 Abstract

Bone tissue engineering from bone marrow stromal cells on poly(*L*-lactic-co-glycolic acid) fiber meshes suffers from limited matrix production and mineralization when cultured with the standard differentiation supplements (dexamethasone, β-glycerophosphate and ascorbic acid). To overcome this problem we included transforming growth factor-β1 (TGF-β1), which is described as playing a key role in collagen type I formation, although its effect on mineralization is controversially discussed. Initial experiments in two-dimensional cell culture confirmed that mineralization was not suppressed due to TGF-β1 treatment. Further investigations focused on establishing culture conditions for the application of TGF-β1 in three-dimensional cell culture and on the effects of different doses of TGF-β1 on bone-like extracellular matrix formation. Immunohistochemical staining showed that TGF-β1 enhanced the formation of procollagen type I, collagen type I and collagen type V dose-dependently, especially under dynamic culture conditions (orbital shaker). Mineralization, evaluated by the determination of the Ca^{2+} content per scaffold and by von Kossa staining, was enhanced by TGF-β1 compared to the control. Additionally, matrix mineralization was confirmed by SEM in combination with energy dispersive X-ray analysis. In conclusion, TGF-β1 seems to be an effective growth factor to improve extracellular bone-like matrix formation in vitro.

2 Introduction

Bone marrow stromal cells are suitable cells for bone tissue engineering, because stromal cells are both easily accessible and can be differentiated to the osteoblastic phenotype with the addition of supplements such as dexamethasone, β-glycerophosphate and ascorbic acid [1,2]. Biodegradable and biocompatible poly(*L*-lactic-co-glycolic acid) (PLLGA) fiber meshes are favorable vehicles for cells in bone tissue engineering [2,3,4], because, due to their structure, they provide a maximum degree of permeability. Previous studies performed in our laboratory, however, have shown limited matrix production and only thin layers of mineralized tissue on PLLGA fiber meshes seeded with marrow stromal cells when cultured with the standard differentiation supplements (dexamethasone, β-glycerophosphate, ascorbic acid). These supplements are typically used in two-dimensional cell culture for stromal cell differentiation to the osteoblastic phenotype. Thus, the cells seeded on the PLLGA meshes expressed the markers characteristic for the osteoblastic phenotype, but did not form a coherent tissue connected by extracellular matrix. This formation of extracellular matrix is crucial for the development of bone tissue, because it provides the mechanical stability of bone after deposition of hydroxyapatite [5]. Interaction with the surrounding extracellular matrix also influences cell metabolism, gene expression and differentiation [6]. We therefore considered this approach to tissue engineering of bone, which utilizes three-dimensional cell polymer constructs and the standard differentiation supplements, as limited by a lack of matrix formation. One possible reason for this limited matrix formation is a lack of essential osteogenic growth factors. Hence, we chose a growth factor capable of enhancing the formation of bone-like extracellular matrix. Transforming growth factor-β1 (TGF-β1) was regarded as a suitable growth factor, because TGF-β1 is known to be produced locally during bone development and regeneration [7,8]. Furthermore, this factor is secreted by both bone marrow stromal cells and osteoblasts and is stored in the bone matrix, from which it is released and activated upon bone resorption [9,8]. In addition, various studies on the effects of TGF-β1 in different cell cultures proved TGF-β1 to effectively stimulate the formation of collagen I [7,10,11], the main matrix protein of bone [12]. Contradicting reports on the in vitro effects of

TGF-β1 in two-dimensional cell culture on matrix mineralization, however, have raised doubts on the suitability of TGF-β1 for tissue engineering of bone [13,14,15,16,17]. On the one hand, TGF-β1 has been described as inhibiting bone nodule formation [13,15], whereas, on the other hand, it has been shown that TGF-β1 caused the formation of three-dimensional mineralized bone spheroids, in which hydroxyapatite was detected [16]. As the variety of experimental conditions used in the described studies provided widely divergent results, the action of TGF-β1 seems to strongly depend upon culture conditions, dosages, the type of osteoblastic cells employed and their stage of maturation [15] [14,17]. Consequently, the aim of our study was to establish the culture conditions and dosing regimen for TGF-β1 that enhance matrix formation and mineralization in three-dimensional cultures of rat marrow stromal cells.

The study started with the investigation of TGF-β1 effects in two-dimensional cultures in order to clarify at an early stage of the study whether or not matrix mineralization was indeed suppressed. The following experiments focused on effects in three-dimensional cell culture, based on the experiences in two-dimensional cell culture. To optimize the culture conditions, we investigated the influence of static and dynamic cultivation with TGF-β1 on collagenous bone matrix proteins such as collagen type I, procollagen type I and collagen type V. With regard to the reported dose-dependent inhibition of bone-nodule formation [13], we tested both single doses and a low concentration multiple dose regimen of TGF-β1 to determine an effective dosage and dosing regimen for the application of TGF-β1. We examined the influence of the various dosages on matrix formation, as well as on matrix mineralization, using immunohistochemistry and a colorimetric assay for the determination of calcium deposition during mineralization. Moreover, scanning electron microscopy combined with energy dispersive X-ray analysis was performed to further evaluate matrix mineralization.

3 Materials and Methods

3.1 Transforming growth factor-β1 (TGF-β1)

Recombinant human transforming growth factor-β1 (TGF-β1) was purchased from Pepro Tech Inc., Rocky Hill, NJ, USA. For the investigations, TGF-β1, lyophilized from sodium citrate buffer (pH 3.5), was reconstituted in water and diluted to a stock solution of 500 ng/ml TGF-β1 with phosphate buffer (PBS, Life Technologies GmbH, Karlsruhe, Germany) containing 2 mg/ml bovine serum albumin (BSA) (Sigma, Taufkirchen, Germany).

3.2 Marrow stromal cell isolation

Marrow stromal cells were obtained from 6-week old male Sprague-Dawley rats (weight: 170 – 180 g , Charles River Laboratories, Sulzfeld, Germany). Cell isolation from the femur and tibia followed a protocol published by Ishaug et al. [2]. Following marrow isolation and dispersion, cells were centrifuged at 1200 rpm (259 x g) for 7 min. The resulting cell pellet was resuspended in primary medium [α-MEM (Sigma); 10% fetal bovine serum (FBS, Gemini Bio-Products Inc., Calabasas, California, USA); 1% penicillin/streptomycin (Sigma); 0.5% L-glutamine 200 mM (Sigma)]. The cells from multiple rats were pooled and seeded onto tissue culture plastic, such that the cell density corresponded to two 150-cm^2 flasks per rat. On the third day of expansion, the flasks were rinsed twice with PBS to remove the nonadherent cells. Thereafter, the 12 ml of primary medium were changed every 2 - 3 days and cells were trypsinized (0.25% trypsin in ethylenediaminetetraacetic acid, Life Technologies) for subculture when 80% confluence was reached.

3.3 Two-dimensional cell culture

3.3.1 Cell seeding and cell differentiation

In two-dimensional cell culture, the effect of different doses of TGF-β1 on cell distribution (microscope: Leica DM IRB, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany; camera: DYNAX, 600si Classic, Minolta GmbH, Ahrensburg, Germany) and mineralization (von Kossa silver nitrate staining) was investigated.

Proliferated cells were subcultured in primary medium into well-plates at a density of 53,000 cells / cm^2. 24 hours after passage, the medium was changed to complete medium [α-MEM, 10 % FBS, 1% penicillin/streptomycin, 0.5% L-glutamine 200 mM, dexamethasone 10^{-8} M (Sigma), ascorbic acid 50 mg/l (Sigma) and β-glycerophosphate 7 mM (Sigma)] supplemented with TGF-β1 in the following concentrations and dosing regimens: 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 20 ng/ml, added once; 1 ng/ml added with every 4^{th} medium change (once a week) and 1 ng/ml supplemented with every medium change (every 2 - 3 days). As control, complete medium supplemented with the dilution buffer for TGF-β1 (2 mg BSA per 1 ml PBS) was administered. Medium was changed every 2 – 3 days and cells were cultivated for up to 31 days.

3.3.2 Mineralization

Matrix mineralization was assessed by von Kossa silver nitrate staining. Samples were fixed with 10% neutral buffered (PBS) formalin for 12 h. After rinsing with water, cells were incubated with a 5% aqueous silver nitrate solution and exposed to natural light. Images of stained cells were acquired from a Kodak EDAS 290 camera (Fisher Scientific, Schwerte, Germany).

3.4 Three-dimensional cell culture

First, we compared the matrix formation of cell-polymer constructs in medium containing TGF-β1 in static versus dynamic culture conditions.

Further investigations, which were conducted under dynamic culture conditions, as dynamic cultivation enhanced the formation of collagenous proteins, dealt with the examination of various dosages and dosing regimens of TGF-β1. Thus, the influence of various doses of TGF-β1 on matrix formation and matrix mineralization was investigated.

3.4.1 Polymer scaffolds

Non-woven meshes of extruded poly(*L*-lactic-co-glycolic acid) (PLLGA) (90% *L*-lactic acid : 10% glycolic acid) fibers were kindly provided by the Institute of Textile Technology and Process Engineering (ITV) (Denkendorf, Germany) (Table 1). The scaffolds were disinfected in 70% ethanol. After rinsing thoroughly with PBS, the scaffolds were incubated in

primary medium for 24 hours in a humidified incubator (37°C / 5% CO_2) before the cell seeding procedure.

Table 1: PLLGA fiber meshes [18]

Diameter	Thickness	Porosity	Average pore size	Filament diameter
8 mm	1.4 mm	96%	90 µm	18.1 µm

3.4.2 Cell seeding and cell differentiation

Spinner flasks were used for cell seeding. Scaffolds were threaded onto needles (length: 10 cm, diameter: 0.5 mm) and fixed with short segments of silicone tubing. Four needles were inserted into a silicone stopper, which was in turn placed into the mouth of a spinner flask. The spinner flasks were filled with 100 ml primary medium each and placed in a humidified incubator (37°C / 5% CO_2) to allow for serum protein adsorption. After 24 hours, the primary medium was replaced with a cell suspension consisting of either 2 x 10^6 (for the study on static and dynamic culture) or 4 x 10^6 cells per scaffold in 100 ml primary medium. The spinner flasks were returned to the incubator and placed on a magnetic stir plate (Bellco Glas, Vineland, NJ, USA) at 80 rpm for cell attachment to the polymer fibers. After 24 hours, each cell-polymer construct was placed in 5 ml complete medium in a 6-well plate (Corning, Corning Costar, Bodenheim, Germany) and cultivated statically or on an orbital shaker (Stuart Scientific, Surrey, United Kingdom) set at 60 rpm for dynamic cultivation. At this time, transforming growth factor-β1 was added according to the following dosing regimen:
Single doses of TGF-β1: 1, 10 and 20 ng/ml
Multiple dose of TGF-β1: 1 ng/ml, added with every 4th medium change (once a week)
Control: complete medium supplemented with the dilution buffer for TGF-β1 (dilution buffer: 2 mg BSA per 1 ml PBS)
Complete medium was changed every 2 - 3 days.

3.4.3 Immunohistochemistry

Samples were taken after 14, 16 and 21 days of cultivation. The tissues were fixed in methanol (Merck, Darmstadt, Germany) and before cryosectioning incubated overnight in a

solution of 5% sucrose (Merck, Darmstadt, Germany) and 10% Tissue-Tek (Sakura Finetek, Torrance, CA, USA) in PBS. Then, the scaffolds were embedded in Tissue-Tek, cryosectioned at 12 µm (Microm Microtome, HM500 OMV, Microm, Walldorf, Germany) and finally incubated with a panel of antibodies against collagenous proteins. The source and specificity of each antibody are given in Table 2. The staining procedure was performed as follows: To prevent non-specific antibody binding, sections were incubated with 5% normal horse serum (Vector Laboratories Inc., Burlingame, CA, USA) in PBS. Thereafter, the sections were incubated with the primary antibodies for 30 min at room temperature (RT). In control sections, PBS was used instead of the primary antibody. After washing with PBS, further incubation with a biotinylated secondary antibody, anti-mouse/rabbit IgG (Table 2), was performed for 30 min at RT. Formation of the avidin-biotin-peroxidase complexes was conducted using the Vectastain Elite ABC-kit (Vector Laboratories, Inc., Burlingame, USA) and the DAB Substrate kit for peroxidase (Vector Laboratories, Inc., Burlingame, USA). Sections were counterstained with Mayer´s haematoxylin (Sigma, Taufkirchen, Germany) followed by mounting with DPX mountant (Fluka, Taufkirchen, Germany).

Table 2: Primary antibodies and secondary antibody used for immunohistochemistry.

Antibody description	**Antibody source**
collagen type I (monoclonal anti-collagen type I col-1), mouse ascites fluid, dilution: 1 : 2000	Sigma
collagen type II (monoclonal antibody, mouse CIIC1), supernatant, dilution: 1 : 6	DSHB
procollagen type I (monoclonal antibody, mouse M-38), supernatant, dilution: 1 : 5	DSHB
collagen type V (monoclonal antibody, mouse V-3C9), supernatant, dilution: 1 : 500	ICN
secondary antibody, biotinylated `` universal `` anti-mouse/rabbit IgG (H+L), horse, 1 : 100	Vector Laboratories

3.4.4 Mineralization

Matrix mineralization was assessed by von Kossa silver nitrate staining. Samples were taken after 21 days of dynamic cultivation and fixed and cryosectioned as described in 2.4.3. Then, the sections were washed thoroughly with water to remove residuals of PBS and von Kossa silver nitrate staining was conducted as described in 2.3.2.

3.4.5 Scanning electron microscopy and energy dispersive X-ray analysis

After 18 days of dynamic cultivation, cell-polymer constructs (control, single dose of 10 ng/ml TGF-β1) were rinsed with PBS and fixed with glutaraldehyde (2.5% in PBS) for 15 min. After repeated rinsing steps, cells were further fixed with an aqueous solution of OsO_4 (1%) (Carl Roth GmbH & Co., Karlsruhe, Germany) on ice for 30 min. The excess OsO_4 was removed with water. Then, the cells on the polymer films were frozen at -80 °C and freeze-dried (Christ Beta 2-16, Martin Christ Gefriertrocknungsanlagen, GmbH, Osterode am Harz, Germany). For scanning electron microscopy (SEM), samples were mounted on aluminium stubs using conductive carbon tape and coated with gold - palladium (Polaron SC515, Fisons surface systems, Grinstead, UK). Photomicrographs were acquired at 10 kV on a DSM 950 (Zeiss, Oberkochen, Germany).

Energy dispersive X-ray analysis (EDX) was carried out with a Link Analytical QX 2000 (Link Analytical Ltd., High Wycombe, Bucks, England). Samples were examined with an acceleration voltage of 20 kV.

3.4.6 Calcium assay

For the determination of Ca^{2+} content per scaffold, the reaction of the cation with the chromogenic agent o-cresolphthalein complexone in alkaline medium to a red complex with an absorbance maximum at 575 nm was employed (Diagnostic Kit 587, Sigma) (Specord 40, Analytik Jena AG, Jena, Germany). The Ca^{2+} content was measured in samples after treatment with the following doses of TGF-β1: 1 ng/ml, added once, 1 ng/ml, added once a week, 10 ng/ml and 20 ng/ml, added once versus control. Samples were taken after 21 days of dynamic cultivation. For analysis the scaffolds were cut on dry ice, sonicated for matrix fragmentation

(Sonifier W-250/W-450, Branson, Danbury, Connecticut, USA) and incubated in HCl (0.5 N) on a shaker for 12 hours to allow dissolution of Ca^{2+}. Then the assay followed the protocol provided by Sigma.

3.4.7 Statistical analysis

Measurements from the calcium assay were collected (n = 3) and expressed as the mean ± standard deviation (SD). Single factor analysis of variance (ANOVA) was used in conjunction with a multiple comparison test (Tukey test) to assess the statistical significance.

4 Results

4.1 Two-dimensional cell culture

The addition of TGF-β1 caused visible changes in the appearance of two-dimensional rat marrow stromal cell cultures. Doses of 1 ng/ml TGF-β1 or more caused a dose-dependent partial detachment of cell sheets from the bottom of the wells, which contracted to dense brown tissue layers (Fig. 1b). This contraction continued upon further cultivation and led finally to the formation of mineralizing spheroidal structures as revealed by von Kossa silver nitrate staining on day 31 (Fig. 1e - h). No such structures were observed in cultures treated with high TGF-β1 doses, such as 20 ng/ml, where most of the cells had detached before spheroids formed. We did not find any sheet detachment and aggregation in the control or in cultures treated with only 0.1 ng/ml TGF-β1; they showed the usual bone nodule formation (Fig. 1c, d).

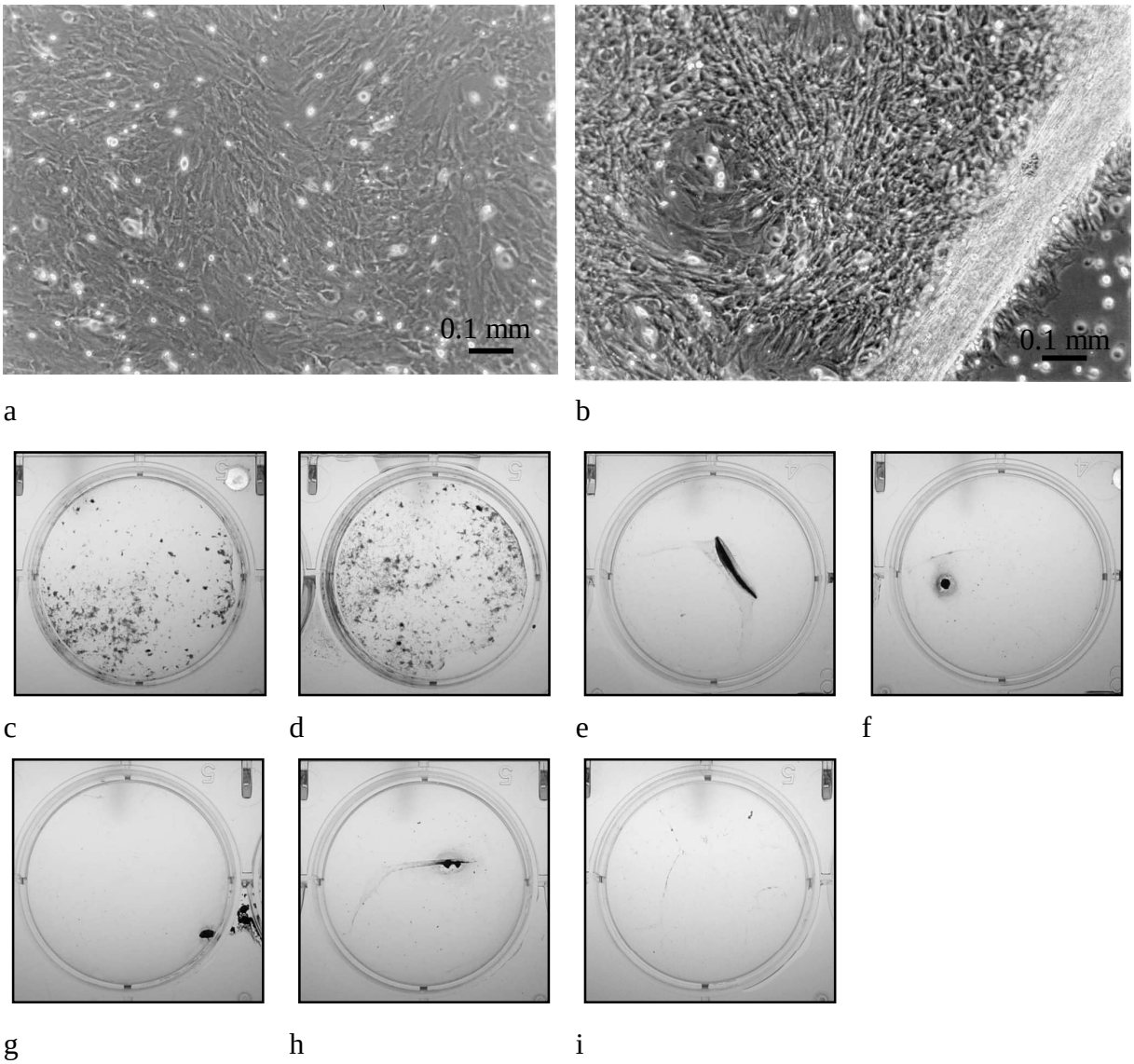


Figure 1: Two-dimensional cell culture:

- 3 days after the addition of TGF-β1: (a) control (b) 10 ng/ml (1x)

- 31 days after the addition of TGF-β1 (von Kossa silver nitrate staining): (c) control (d) 0.1 ng/ml (1x) (e) 1 ng/ml (1x) (f) 1 ng/ml (once a week) (g) 1 ng/ml (with every medium change) (h) 10 ng/ml (1x) (i) 20 ng/ml (1x)

4.2 Three-dimensional cell culture

4.2.1 Immunohistochemistry

Effects of culture conditions on matrix formation upon TGF-β1 supplementation

The addition of TGF-β1 to three-dimensional rat marrow stromal cell cultures dose-dependently stimulated the formation of collagenous proteins, i.e. procollagen type I, collagen type I and type V. This stimulating effect was modulated by the culture conditions. All three collagenous proteins were generally enhanced in dynamically cultured constructs, as compared to static culture conditions (Fig 2, 3, and 4). As a consequence of these experiments, all further investigations on TGF-β1 effects were performed under dynamic culture conditions.

Investigation of dose patterns under dynamic culture conditions

This part of the study dealt with the investigation of different TGF-β1 dosing regimens. Immunohistochemical labeling for collagen type I showed that TGF-β1 enhanced the formation of collagen type I compared to the control. Moreover, with increasing doses from 1 ng/ml to 20 ng/ml, collagen type I formation was dose-dependently raised (Fig. 5).

Collagen type II, a marker for chondrogenic differentiation, was not detectable in any of the cultures (data not shown).

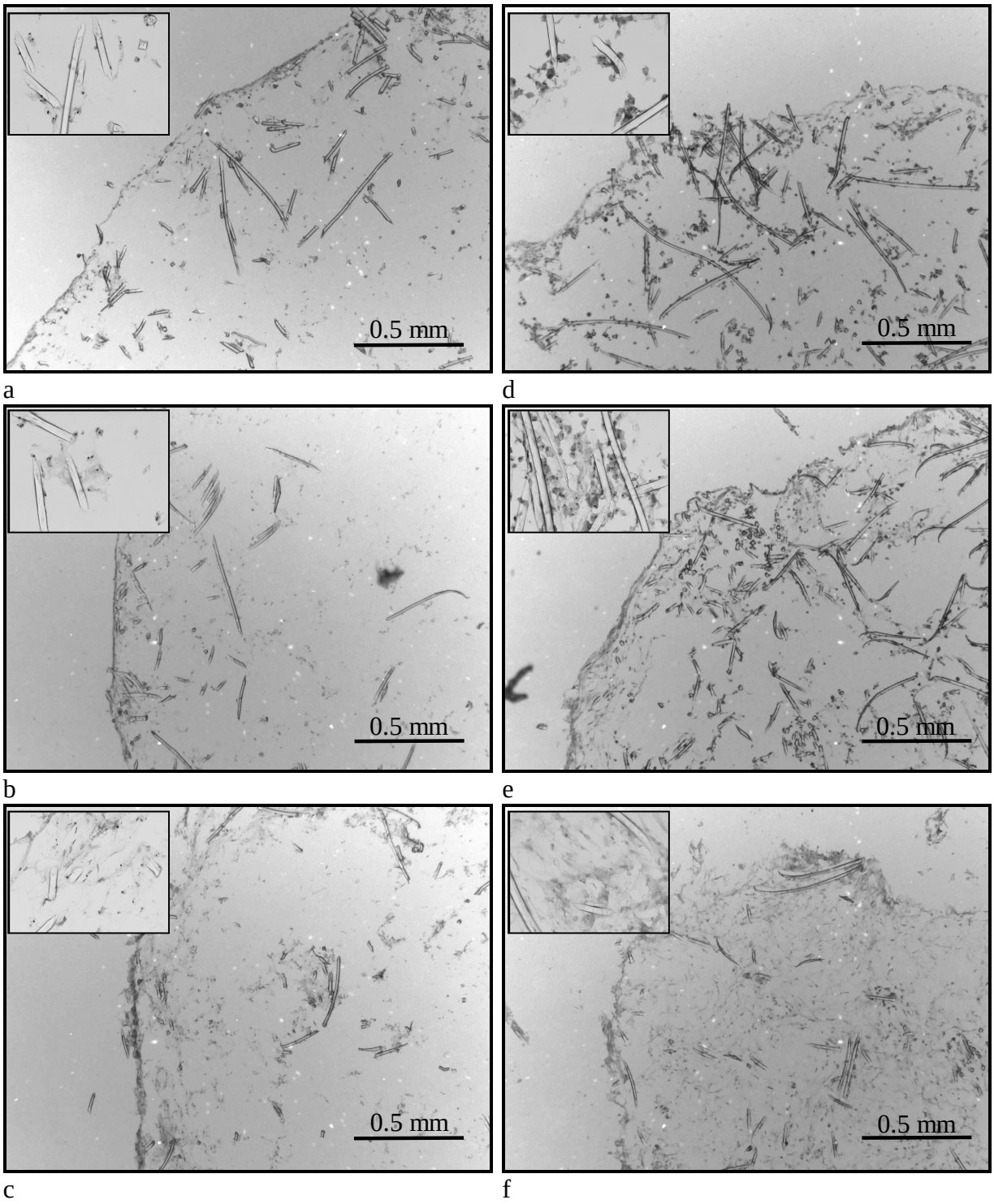


Figure 2: Immunohistochemical staining for procollagen type I after 16 days of cultivation (seeded with 2 million cells per construct). Static cultivation: (a) control, (b) TGF-β1: 1 ng/ml (1x), (c) TGF-β1: 10 ng/ml (1x). Dynamic cultivation: (d) control, (e) TGF-β1: 1 ng/ml (1x), (f) TGF-β1: 10 ng/ml (1x). Insets show a higher magnification (▬ 50 µm).

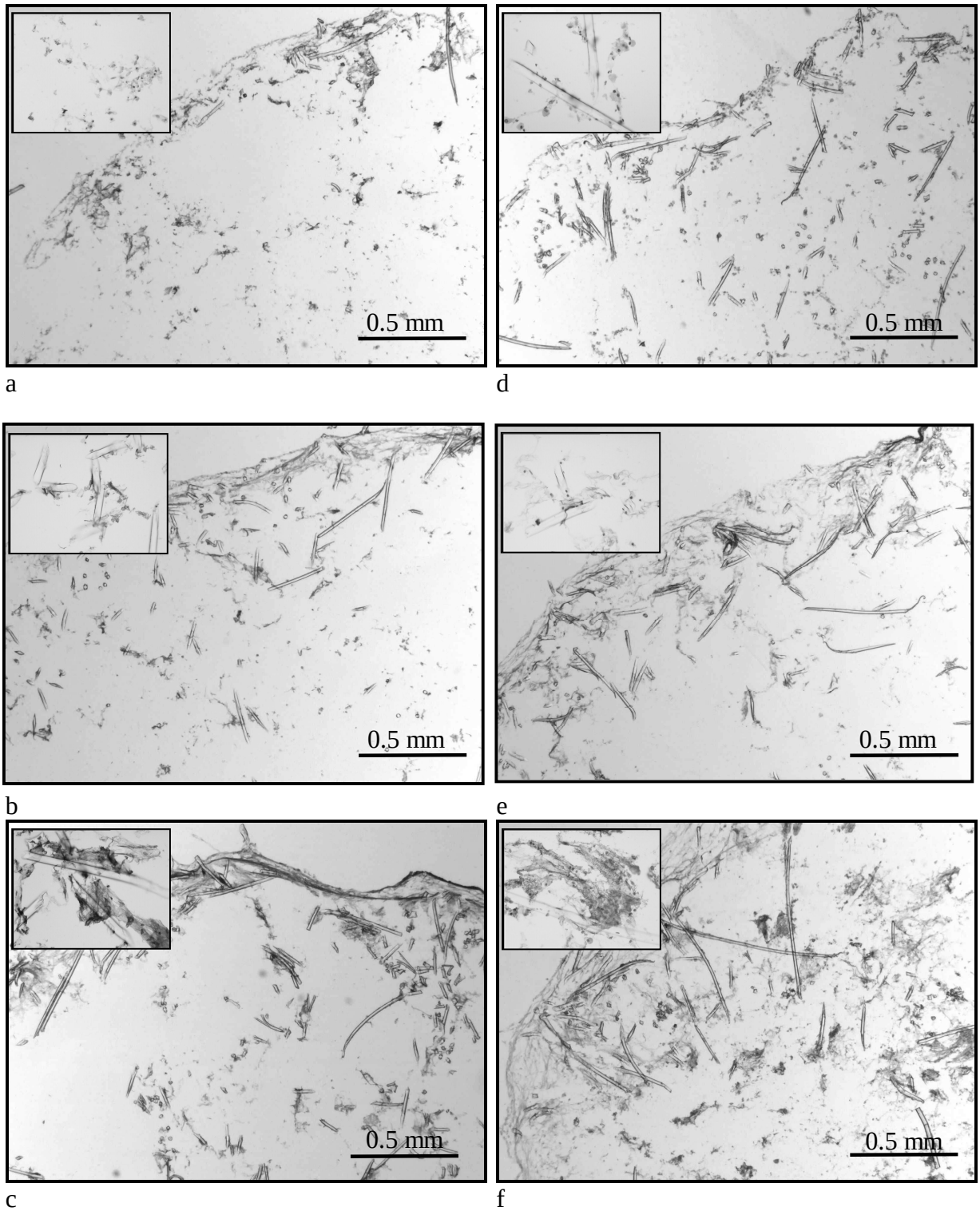


Figure 3: Immunohistochemical staining for collagen type I after 16 days of static or dynamic cultivation (seeded with 2 million cells per construct). a – f as in Fig. 2. Insets show a higher maginification (▬ 50 µm).

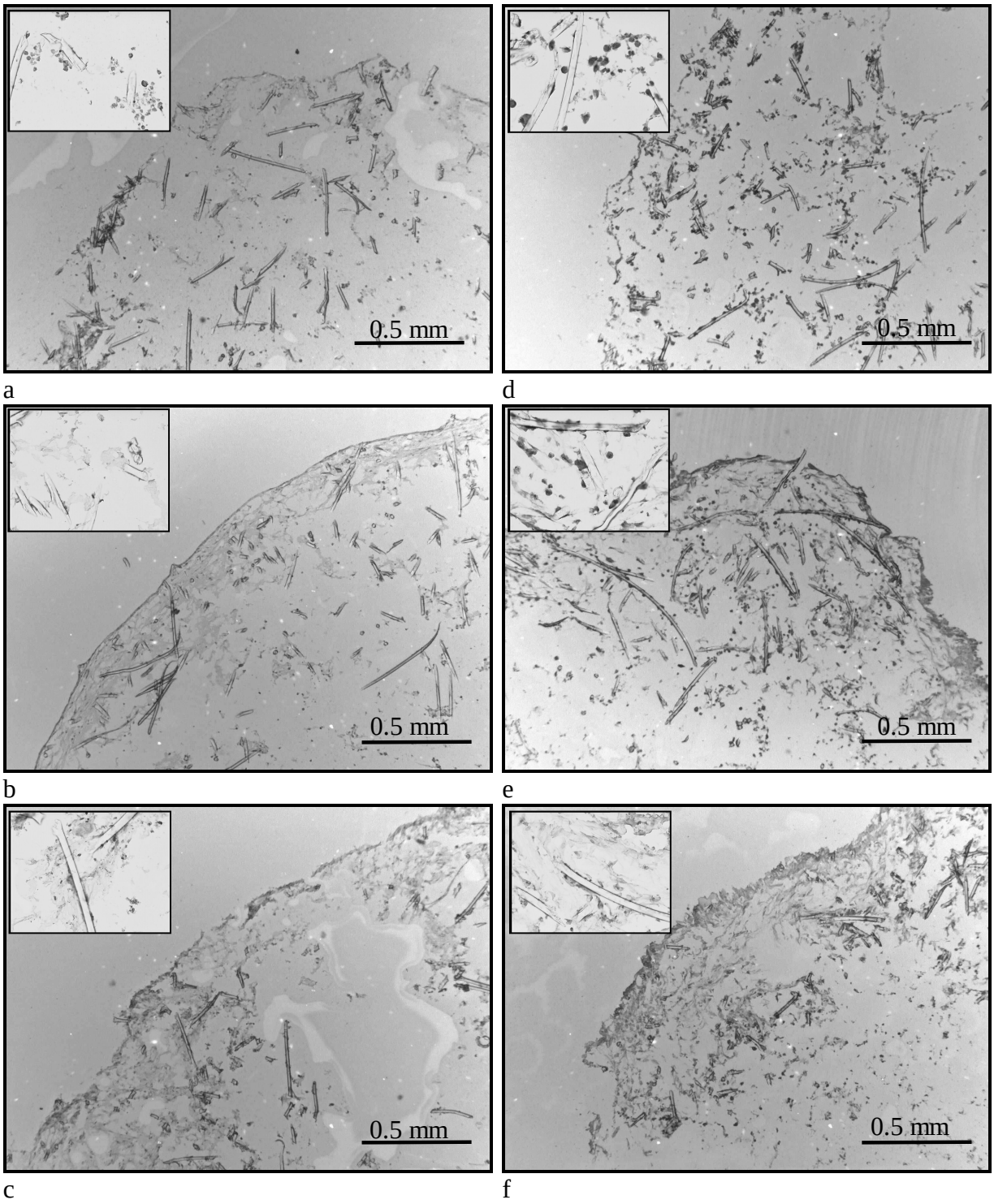


Figure 4: Immunohistochemical staining for collagen type V after 16 days of static or dynamic cultivation (seeded with 2 million cells per construct). a – f as in Fig. 2. Insets show a higher magnification (— 50 µm).

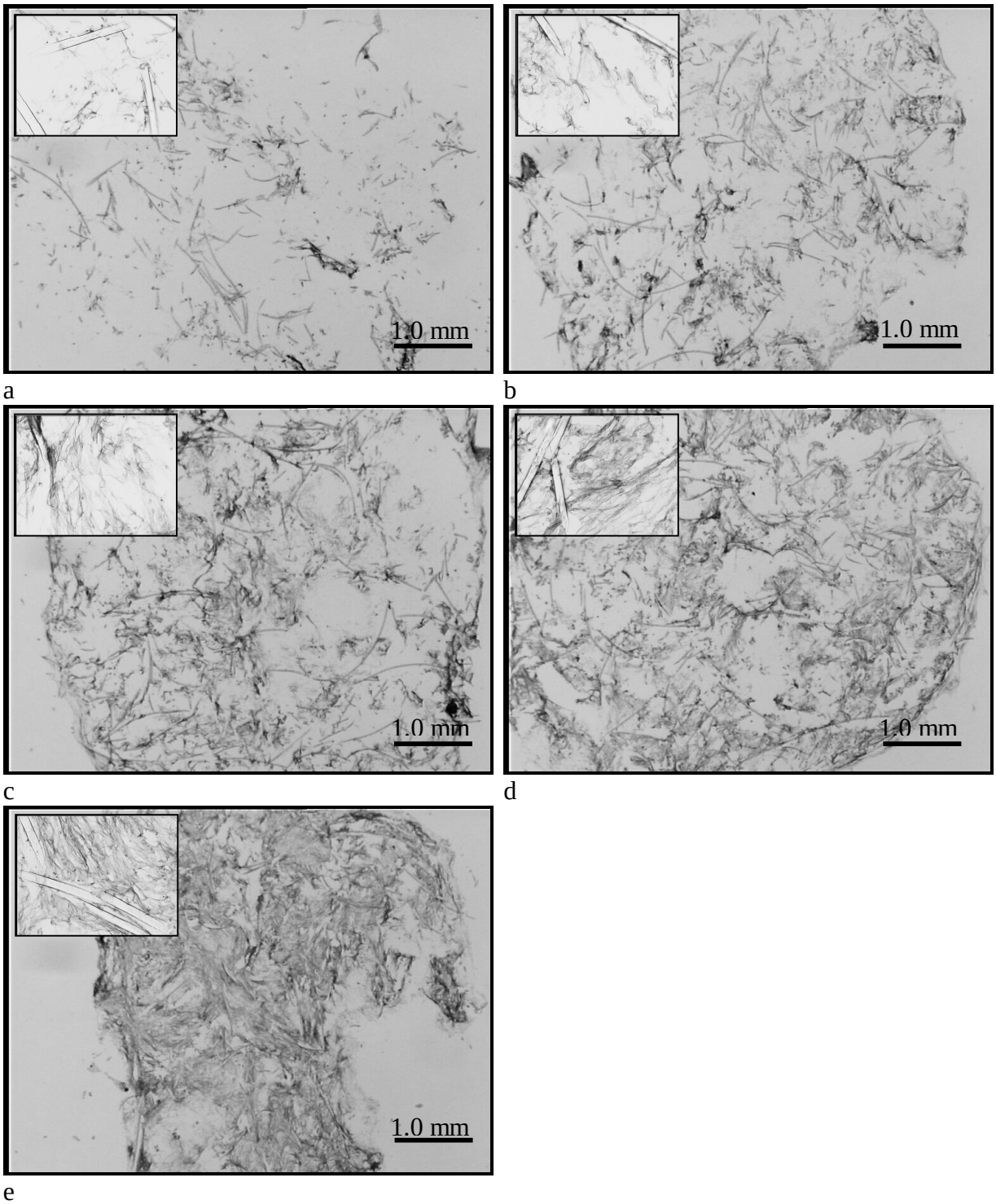


Figure 5: Immunohistochemical staining for collagen type I after 14 days of dynamic cultivation (seeded with 4 million cells per construct). (a) control (b) TGF-β1: 1 ng/ml (1x) (c) TGF-β1: 1 ng/ml (once a week) (d) TGF-β1: 10 ng/ml (1x) (e) TGF-β: 20 ng/ml (1x). Insets show a higher magnification (▬ 50 µm).

4.2.2 Mineralization

After 21 days of dynamic cultivation, von Kossa staining revealed increased Ca^{2+} displayed in the TGF-β1 treated cell-polymer constructs (Fig. 6), as compared to the control. The intensity of the stain correlated with the applied doses of TGF-β1. This increase in staining might be enhanced by the shrinking of the cell-polymer constructs that was observed especially after the addition of 20 ng/ml TGF-β1, which is caused by forces developing upon tissue formation.

4.2.3 Scanning electron microscopy and energy dispersive X-ray analysis

SEM pictures show matrix formation on the constructs´ edges with and without the addition of TGF-β1 (Fig. 7). The matrix found on control scaffolds had a more sheet-like appearance than the matrix on scaffolds treated with 10 ng/ml TGF-β1. Matrix was detectable on the whole of the scaffold, including the inner regions, when TGF-β1 was supplemented, whereas matrix was found primarily on the edges of the control construct. Survey pictures from the inner part of the scaffold suffered from low conductivity (data not shown). With regard to matrix mineralization, more calcified globular accretions were visible on scaffolds treated with TGF-β1. Figure 8 shows accretions in control and in TGF-β1 treated constructs.

The globular accretions were investigated with EDX. We were able to demonstrate the presence of calcium, oxygen and phosphorus signals in the globular accretions. The ratio of Ca/P in the globular accretions on constructs treated with TGF-β1 was 1.63, which is similar to the ratio of Ca/P in hydroxyapatite of natural bone (1.67) [19].

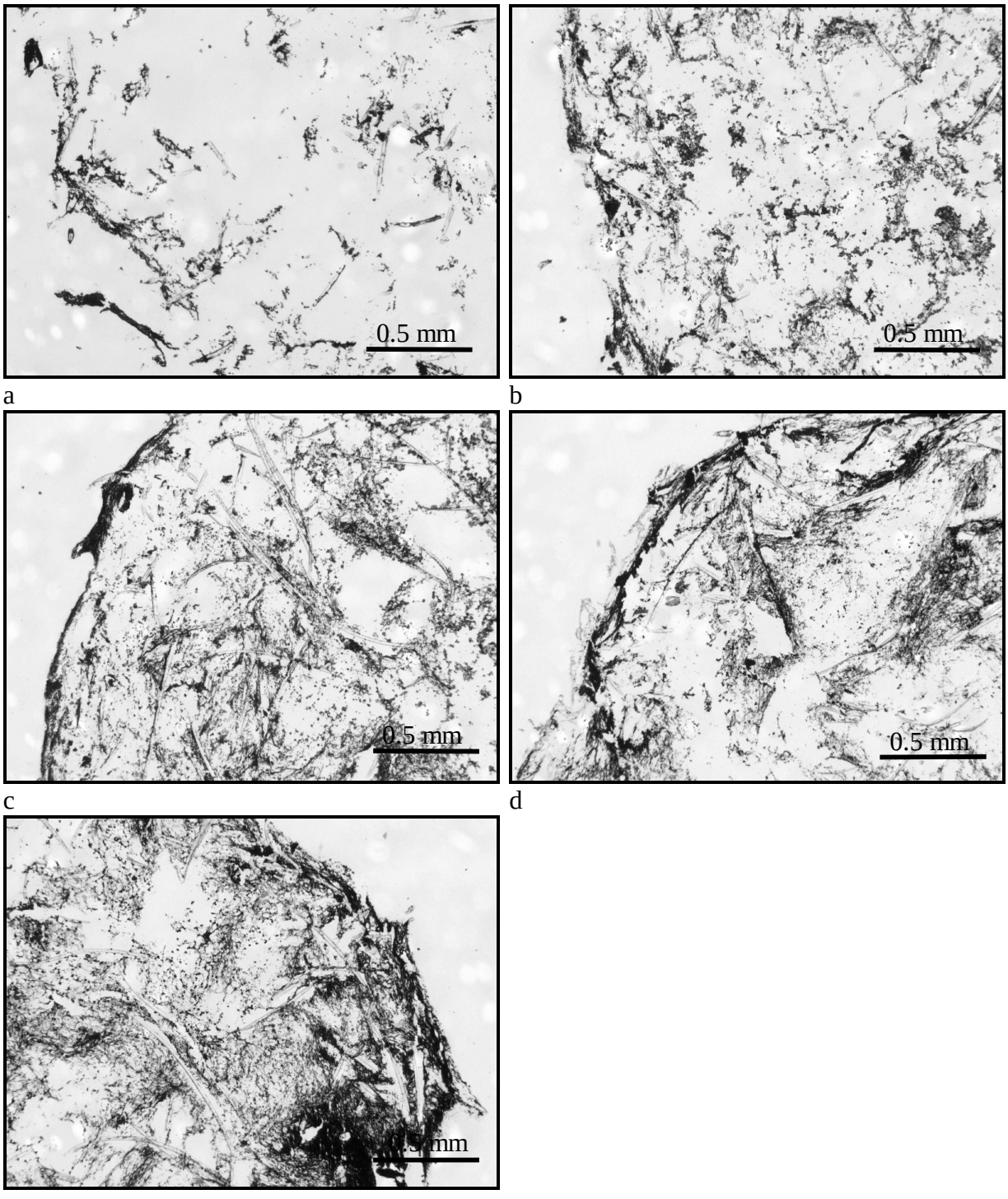
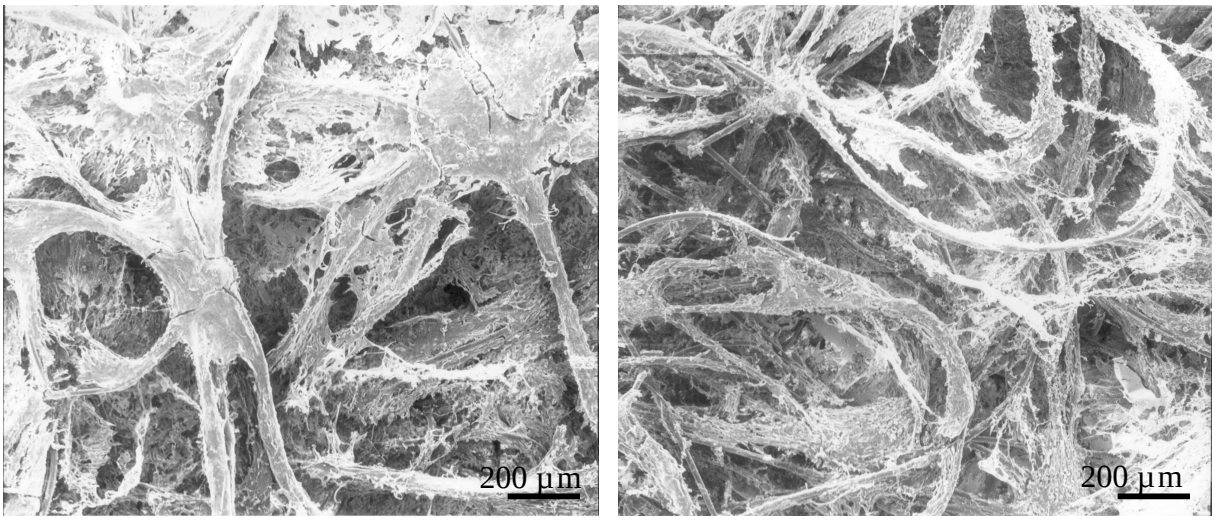


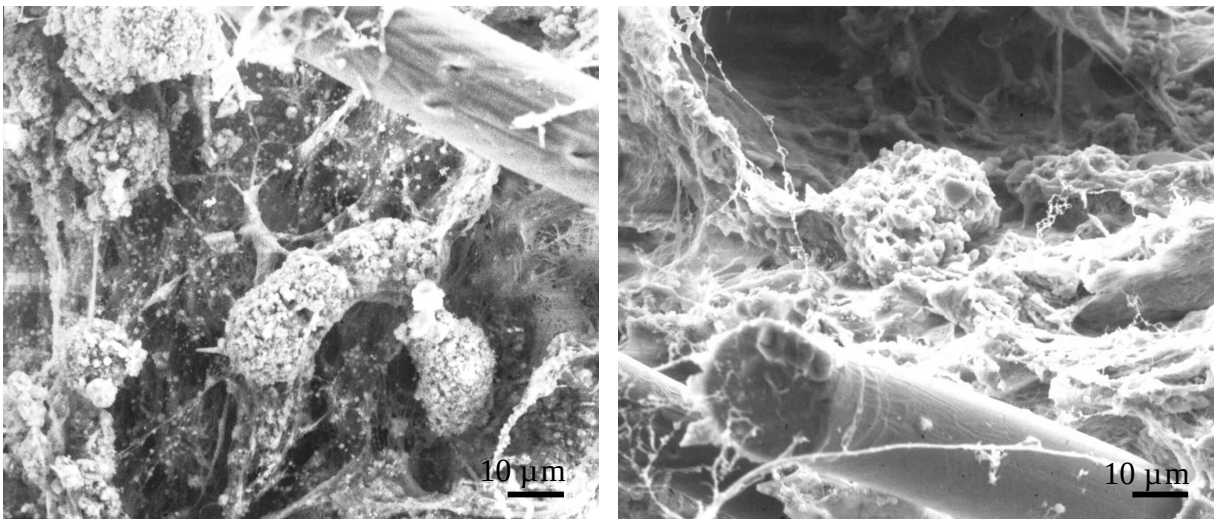
Figure 6: Von Kossa staining after 21 days of dynamic cultivation.

(a) control (b) TGF-β1: 1 ng/ml (1x) (c) TGF-β1: 1 ng/ml (once a week) (d) TGF-β1: 10 ng/ml (1x) (e) TGF-β1: 20 ng/ml (1x).



a b

Figure 7: SEM pictures of cell-polymer constructs after 18 days of dynamic cultivation, overview. (a) control (original magnification x 50) (b) TGF-β1: 10 ng/ml (1x) (original magnification x 50).



a b

Figure 8: SEM pictures of cell-polymer constructs after 18 days of dynamic cultivation.

(a) cross section of the control cell-polymer construct, calcified globular accretions (original magnification x 1000) (b) cross section of the cell-polymer construct treated with TGF-β1: 10 ng/ml (1x), calcified globular accretions (original magnification x 1000).

4.2.4 Calcium assay

The determination of Ca^{2+} content per scaffold showed that the addition of TGF-β1 led to a significantly increased calcium content per scaffold compared to the control ($p < 0.05$) (Fig. 9). However, there were no significant differences between the groups treated with different doses of TGF-β1.

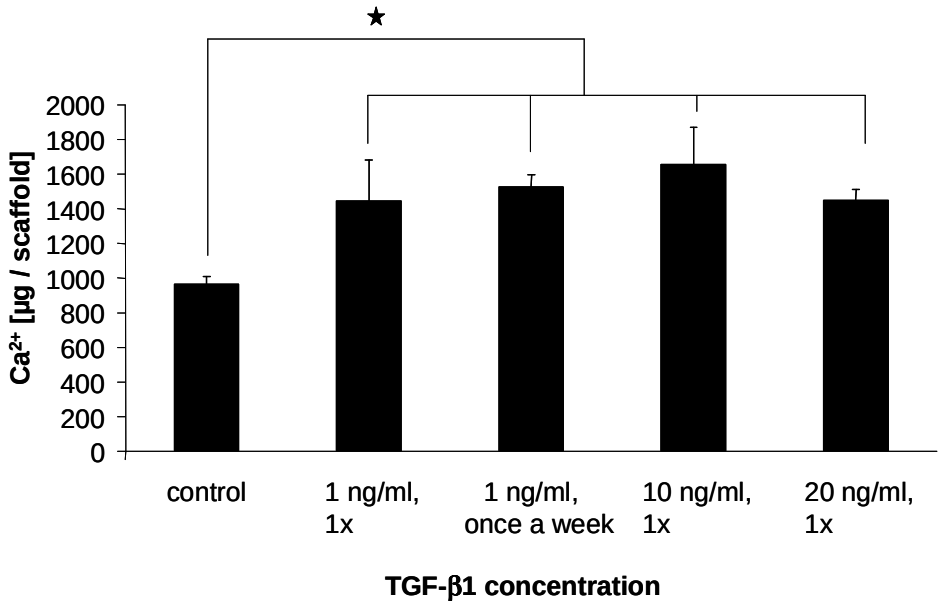


Figure 9: Ca^{2+} content per scaffold after 21 days of dynamic cultivation. Columns and error bars represent means ± SD for n = 3. The statistical significance was assessed by Tukey-Test. Significance is indicated by a ★.

5 Discussion

Bone tissue engineering on polymer fiber meshes with rat bone marrow stromal cells suffers from low matrix formation when the standard differentiation supplements, usually tested in two-dimensional cell cultures, are added without any further growth factor supplementation. Therefore, the objective of this study was to establish culture conditions and a dosing regimen for the application of the growth factor TGF-β1 in combination with the

standard differentiation supplements. We investigated the effects of TGF-β1 on collagenous bone matrix proteins such as collagen type I, its procollagen and collagen type V [20,21] and on matrix mineralization. The first part of the study was performed in two-dimensional cell culture. We hoped to determine at an early stage whether or not mineralization was suppressed under any of the applied doses. While cultures treated with 0.1 ng/ml TGF-β1 showed no difference in their appearance to control cultures, cells and cell sheets partly detached and formed mineralizing spheroids after the addition of TGF-β1 in doses of 1 ng/ml and 10 ng/ml. The described spheroid formation was also observed by Kale et al. [16], who added 5 ng/ml TGF-β1 to human marrow stromal cells. They reported osteoblastic differentiation and matrix formation in the core of the formed spheroids, which is in agreement with our findings on the mineralization of the spheroids. Kale et al. suggested that the formation of spheroids resulted from an increased expression of collagen I and collagen I binding integrins upon the addition of TGF-β1. Our investigations showed that even higher doses of TGF-β1 up to 10 ng/ml did not inhibit matrix mineralization.

In view of these findings, we chose to evaluate doses starting with 1 ng/ml TGF-β1 in three-dimensional cell culture, where tissue formation was assessed immunohistochemically. In general, TGF-β1 stimulated the formation of collagenous proteins, i.e. procollagen type I, collagen type I and collagen type V. We investigated the effects of TGF-β1 on matrix formation under dynamic culture conditions (on an orbital shaker) in comparison to standard static conditions. Dynamic cultivation further enhanced the formation of the investigated collagenous proteins in the presence of TGF-β1. Different mechanisms may be responsible for this phenomenon. One certainly involves the improved supply of growth factor and nutrients to the cells due to the enhanced convection under dynamic culture conditions [22,23]. Further possible mechanisms involve the mechanical stimulation provided by fluid flow and the activation of responsive signaling pathways [24], e.g. the phosphorylation of extracellular signal-regulated kinase (ERK) [25]. ERK is described as being essential for growth, differentiation, integrin expression and cell function in human osteoblastic cells [26]. As a consequence of the improved formation of collagenous proteins after TGF-β1 application in dynamic cell culture, all further investigations were conducted under dynamic culture conditions.

In three-dimensional cell culture, we showed a dose-dependent enhancement of collagen type I formation with increasing doses of TGF-β1. The observed enhancement of collagen type I formation was consistent with most other reports on TGF-β1 effects on different osteogenic cell types. Lilli et al. [7], for example, described that TGF-β1 enriched biomembranes led to increased collagen type I production in two-dimensional human bone cell culture, while Locklin et al. [27] reported increased collagen type I formation in two-dimensional rat marrow stromal cell culture after treatment with TGF-β1. Arnold et al. [11] applied TGF-β1 in three-dimensional periosteal cell culture and described an enhanced collagen type I formation after TGF-β1 application, but no dose-dependence was depicted.

With regard to procollagen type I and collagen type V, we also showed a dose-dependent stimulation of these matrix proteins by TGF-β1 in the cross sections.

As TGF-β1 is often applied in cartilage tissue engineering, we tested to see if the formation of collagen type II, typically expressed in cartilage, was prevented by treatment with TGF-β1 under the conditions applied in our study, which included supplementation with dexamethasone, β-glycerophosphate and ascorbic acid. Immunohistochemical labelling for collagen type II showed that no collagen II was formed in our cultivated constructs. However, collagen type II has been reported to be induced by TGF-β1 in three-dimensional cultures of bone marrow stromal cells, when TGF-β1 was combined with dexamethasone and insulin for cartilage engineering [28].

The calcium assay served as a measure for matrix mineralization. Furthermore, von Kossa silver nitrate was used to stain the tissue sections for Ca^{2+} and we checked for calcium accretions in tissue constructs, utilizing SEM combined with EDX. All three methods showed increased mineralization upon the supplementation with TGF-β1 compared to the dexamethasone treated controls. Furthermore, EDX showed that the globular accretions, detected especially in TGF-β1 treated constructs, consist of calcium and phosphorus in a ratio of 1.63 close to the theoretical value of 1.67 in hydroxyapatite [19]. While the measurement of Ca^{2+} content showed no significant dose dependence, von Kossa staining revealed a dose-dependent increase of stained area with increasing doses of TGF-β1. This increase may be enhanced by the shrinking of the cell-polymer constructs that was observed particularly after addition of 20 ng/ml TGF-β1, caused by forces developing upon tissue formation. Hence, the

chosen culture conditions seemed to lead to an increased Ca^{2+} content and hydroxyapatite formation in the TGF-β1 treated constructs. This is in contrast to some other published reports on TGF-β1 effects on matrix mineralization. TGF-β1 has been shown to inhibit as well as to enhance mineralization [13,16,15]. Different reasons may be responsible for the divergent published observations concerning the mineralization of the matrix after TGF-β1 treatment. One may involve the investigation of different cell types [15]; the effects of TGF-β1 are known to strongly depend on the cell type and their state of maturation. TGF-β1 seems to have positive effects on more mature osteoblastic cells [29]. Along these lines, Breen et al. described negative effects of TGF-β1 on matrix mineralization in cultures of preosteoblastic cells [14]. However, the negative effects were lessened when the cells had been pretreated with a dexamethasone containing osteogenic supplement. In our study, however, mineralization was actually stimulated in constructs after co-treatment with TGF-β1 and dexamethasone under dynamic culture conditions. One factor is probably the different culture conditions, such as dynamic three-dimensional cell culture versus two-dimensional static cell culture. Besides, the enhanced formation of collagenous proteins under TGF-β1 treatment is thought to initiate mineralization [30]. This effect may be enhanced by the superior conditions for matrix accumulation in three-dimensional cultures.

In summary, we successfully improved matrix formation and mineralization in three-dimensional cultures of rat marrow stromal cells on PLLGA fiber meshes by the addition of TGF-β1, especially under dynamic culture conditions. This study therefore implies that TGF-β1 is a potent growth factor for the stimulation of matrix formation and mineralization in three-dimensional cultures of rat marrow stromal cells on PLLGA fiber meshes.

Though matrix mineralization is an important marker for bone development [30], there are many further bone markers, which are decisive for bone formation. Hence, we have performed a second study (Part II) to determine the effects of TGF-β1 on cell proliferation and differentiation in order to develop a suitable dosing regimen for the application of TGF-β1.

6 Conclusion

The objective of this study was to improve matrix formation and mineralization of rat marrow stromal cells in culture, focusing on three-dimensional cell culture as an approach to tissue engineering of bone. We investigated the effects of different TGF-β1 dosages and dosing regimens on the formation of collagenous matrix proteins and matrix mineralization. A preliminary experiment in two-dimensional cell culture showed that matrix mineralization was not suppressed by TGF-β1 doses between 0.1 and 10 ng/ml when coadministered with osteogenic supplements containing dexamethasone. In the main part of the study, which was conducted in three-dimensional cell culture, it was revealed that the formation of procollagen type I, collagen type I and collagen type V was increased with increasing doses of TGF-β1. Furthermore, the formation of these collagenous matrix proteins was stimulated under dynamic culture conditions, especially after TGF-β1 treatment, probably by improved nutrient supply to the cells and an induction of signal transduction pathways responsive to fluid flow mechanical stimulation. Matrix mineralization, investigated by a calcium assay, by von Kossa staining of cross sections and SEM-EDX investigations, was also improved upon TGF-β1 treatment. In contrast to the dose-dependent formation of the collagenous matrix, no significant dose-dependent increase of mineralization was found.

This study implies that TGF-β1 is a potent growth factor for the stimulation of matrix formation and mineralization in three-dimensional cultures of rat marrow stromal cells on PLLGA fiber meshes. A second study was performed to investigate the effects of the selected TGF-β1 doses on proliferation and non-collagenous bone-related proteins.

7 Acknowledgements

Special thanks are due to Florian Truksa, Department of Anorganic Chemistry, University of Regensburg, Germany for EDX measurements, Prof. Dr. Norbert Lehn, Institute for Medical Microbiology and Hygiene, University of Regensburg, Germany for providing facilities and Allison Dennis, Department of Pharmaceutical Technology, University of

Regensburg, Germany for proof-reading. The monoclonal antibodies CIIC1 developed by Rikard Holmdahl / Kristofer Rubin and M38 by John A. McDonald were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

8 References

1. Peter, S. J., Liang, C. R., Kim, D. J., Widmer, M. S., and Mikos, A. G. (1998). Osteoblastic phenotype of rat marrow stromal cells cultured in the presence of dexamethasone, beta-glycerolphosphate, and L-ascorbic acid. *J.Cell Biochem.* **71**, 55-62.

2. Ishaug, S. L., Crane, G. M., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **36**, 17-28.

3. Peter, S. J., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1998). Polymer concepts in tissue engineering. *J.Biomed.Mater.Res.* **43**, 422-427.

4. Puelacher, W. C., Vacanti, J. P., Ferraro, N. F., Schloo, B., and Vacanti, C. A. (1996). Femoral shaft reconstruction using tissue-engineered growth of bone. *Int J Oral Maxillofac Surg* **25**, 223-228.

5. Buckwalter, J. A. and Cooper, R. R. (1987). Bone structure and function. *Instructional Course Lectures* **36**, 27-48.

6. Ferrera, D., Poggi, S., Biassoni, C., Dickson, G. R., Astigiano, S., Barbieri, O., Favre, A., Franzi, A. T., Strangio, A., Federici, A., and Manduca, P. (2002). Three-dimensional cultures of normal human osteoblasts: proliferation and differentiation potential in vitro and upon ectopic implantation in nude mice. *Bone* **30**, 718-725.

7. Lilli, C., Marinucci, L., Stabellini, G., Belcastro, S., Becchetti, E., Balducci, C., Staffolani, N., and Locci, P. (2002). Biomembranes enriched with TGFbeta1 favor bone matrix protein expression by human osteoblasts in vitro. *J.Biomed.Mater.Res.* **63**, 577-582.

8. Robey, P. G., Young, M. F., Flanders, K. C., Roche, N. S., Kondaiah, P., Reddi, A. H., Termine, J. D., Sporn, M. B., and Roberts, A. B. (1987). Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-beta) in vitro. *J.Cell Biol.* **105**, 457-463.

9. Liu, P., Oyajobi, B. O., Russell, R. G., and Scutt, A. (1999). Regulation of osteogenic differentiation of human bone marrow stromal cells: interaction between transforming growth factor-beta and 1,25(OH)(2) vitamin D(3) In vitro. *Calcif.Tissue Int.* **65**, 173-180.

10. Hock, J. M., Canalis, E., and Centrella, M. (1990). Transforming growth factor-beta stimulates bone matrix apposition and bone cell replication in cultured fetal rat calvariae. *Endocrinology* **126**, 421-426.

11. Arnold, U., Lindenhayn, K., and Perka, C. (2002). In vitro-cultivation of human periosteum derived cells in bioresorbable polymer-TCP-composites. *Biomaterials* **23**, 2303-2310.

12. Anselme, K. (2000). Osteoblast adhesion on biomaterials. *Biomaterials* **21**, 667-681.

13. Antosz, M. E., Bellows, C. G., and Aubin, J. E. (1989). Effects of transforming growth factor beta and epidermal growth factor on cell proliferation and the formation of bone nodules in isolated fetal rat calvaria cells. *J.Cell Physiol* **140**, 386-395.

14. Breen, E. C., Ignotz, R. A., McCabe, L., Stein, J. L., Stein, G. S., and Lian, J. B. (1994). TGF beta alters growth and differentiation related gene expression in proliferating osteoblasts in vitro, preventing development of the mature bone phenotype. *J.Cell Physiol* **160**, 323-335.

15. Faucheux, C., Ulysse, F., Bareille, R., Reddi, A. H., and Amedee, J. (1997). Opposing actions of BMP3 and TGF beta 1 in human bone marrow stromal cell growth and differentiation. *Biochem.Biophys.Res.Commun.* **241**, 787-793.

16. Kale, S., Biermann, S., Edwards, C., Tarnowski, C., Morris, M., and Long, M. W. (2000). Three-dimensional cellular development is essential for ex vivo formation of human bone. *Nat.Biotechnol.* **18**, 954-958.

17. Lian, J. B. and Stein, G. S. (1993). The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGF beta 1) and hormones (vitamin D and glucocorticoids). *J.Oral Implantol.* **19**, 95-105.

18. Dauner, M. Eigenschaften von Spinnfaservliesstoffen für das Tissue Engineering. International Textile Bulletin Vliesstoffe - Technische Textilien 48(2), 45-48. 2002.

19. Joschek, S., Nies, B., and Krotz, R. G. A. (2000). Chemical and physicochemical characterization of porous hydroxyapatite ceramics made of natural bone. *Biomaterials* **21**, 1645-1658.

20. Sato, I., Sunohara, M., and Sato, T. (1999). Quantitative analysis of extracellular matrix proteins in hypertrophic layers of the mandibular condyle and temporal bone during human fetal development. *Cells Tissues Organs* **165**, 81-90.

21. Anselme, K. (2000). Osteoblast adhesion on biomaterials. *Biomaterials* **21**, 667-681.

22. Kellner, K., Liebsch, G., Klimant, I., Wolfbeis, O. S., Blunk, T., Schulz, M. B., and Gopferich, A. (2002). Determination of oxygen gradients in engineered tissue using a fluorescent sensor. *Biotechnol.Bioeng.* **80**, 73-83.

23. Freed, L. E., Vunjak-Novakovic, G., and Langer, R. (1993). Cultivation of cell-polymer cartilage implants in bioreactors. *J.Cell Biochem.* **51**, 257-264.

24. Ogata, T. (2000). Fluid flow-induced tyrosine phosphorylation and participation of growth factor signaling pathway in osteoblast-like cells. *J.Cell Biochem.* **76**, 529-538.

25. Weyts, F. A., Li, Y. S., Van Leeuwen, J., Weinans, H., and Chien, S. (2002). ERK activation and alphavbeta3 integrin signaling through Shc recruitment in response to mechanical stimulation in human osteoblasts. *J.Cell Biochem.* **87**, 85-92.

26. Lai, C. F., Chaudhary, L., Fausto, A., Halstead, L. R., Ory, D. S., Avioli, L. V., and Cheng, S. L. (2001). Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. *J.Biol.Chem.* **276**, 14443-14450.

27. Locklin, R. M., Williamson, M. C., Beresford, J. N., Triffitt, J. T., and Owen, M. E. (1995). In vitro effects of growth factors and dexamethasone on rat marrow stromal cells. *Clin.Orthop.* 27-35.

28. Martin, I., Shastri, V. P., Padera, R. F., Yang, J., Mackay, A. J., Langer, R., Vunjak-Novakovic, G., and Freed, L. E. (2001). Selective differentiation of mammalian bone marrow stromal cells cultured on three-dimensional polymer foams. *J.Biomed.Mater.Res.* **55**, 229-235.

29. Kassem, M., Kveiborg, M., and Eriksen, E. F. (2000). Production and action of transforming growth factor-beta in human osteoblast cultures: dependence on cell differentiation and modulation by calcitriol. *Eur.J.Clin.Invest* **30**, 429-437.

30. Boskey, A. L. (1998). Biomineralization: conflicts, challenges, and opportunities. *J Cell Biochem* **30-31**, 83-91.

Chapter 4

Effects of TGF-β1 on Bone-like Tissue Formation in Three-Dimensional Cell Culture

Part II: Osteoblastic Differentiation

E. Lieb[1], S. Milz[2], M. Dauner[3], A. Göpferich[1], M.B. Schulz[1,4]

[1] Department of Pharmaceutical Technology, University of Regensburg, Germany

[2] Anatomische Anstalt, LMU, Munich, Germany

[3] Institute of Textile Technology and Process Engineering (ITV), Denkendorf, Germany

[4] Department of Pharmaceutical Technology, University of Graz, Austria

submitted to Tissue Engineering

1 Abstract

We supplemented rat marrow stromal cells (rMSCs) seeded on poly(*L*-lactic-co-glycolic acid) fiber meshes with transforming growth factor-β1 (TGF-β1) to improve bone tissue formation for tissue engineering. While our first study [1] investigated the effects of TGF-β1 on matrix formation and mineralization, this second study focused on the differentiation of rMSC to the osteoblastic phenotype in dynamic cell culture (orbital shaker). We assessed a series of bone markers, in order to determine a dosing regimen for TGF-β1 that enhances collagenous matrix formation and preserves or increases osteoblastic differentiation. Alkaline phosphatase activity (ALP), bone sialoprotein, osteonectin, and osteocalcin were examined, utilizing RT-PCR, immunohistochemistry and, for ALP, an enzyme assay. We showed that while bone sialoprotein was dose-dependently increased after supplementation with TGF-β1, osteonectin was unchanged and both ALP activity and osteocalcin were suppressed by high doses of TGF-β1. Considering the effects of TGF-β1 both on differentiation and on matrix formation and mineralization, 1 ng/ml TGF-β1, added once a week, was selected as an effective dose to improve bone-like tissue formation.

2 Introduction

In vitro tissue engineering of bone with rat marrow stromal cells (rMSCs) on poly(*L*-lactic-co-glycolic acid) (PLLGA) fiber meshes often leads to minor matrix production and only thin layers of mineralized tissue [1]. To overcome this problem, we supplemented cell cultures with the growth factor, transforming growth factor-β1 (TGF-β1). In the first study [1], we aimed at finding dosages and dosing regimens for this growth factor suitable for the improvement of matrix formation. We showed that TGF-β1 enhances the formation of collagenous proteins and improves matrix mineralization in three-dimensional cultures of rMSCs, especially under dynamic culture conditions [1]. The objective of this second study was to investigate the effect of matrix stimulating TGF-β1 dosages [1] on osteoblastic differentiation, in order to elucidate the applicability of TGF-β1 for in vitro bone tissue formation. Published in vitro studies on the effects of TGF-β1 on the differentiation of osteoblastic progenitor cells, however, reported a large number of conflicting results [2]. TGF-β1 has been described as both inhibiting, as well as increasing, osteoblastic differentiation [2]. These divergent effects of TGF-β1 seem to depend strongly on the cell type used in the study, their state of maturation when TGF-β1 is added, the dosages and dosing regimen and the chosen culture conditions [3,4,5]. We chose dynamic culture conditions and effective matrix stimulating doses, as determined in the first study, to find dosages and dosing regimens that on the one hand enhance matrix formation and on the other hand preserve or even increase osteoblastic differentiation. Additionally, we were interested in the effect of the growth factor on cell proliferation. In order to examine the osteoblastic differentiation of rMSCs under TGF-β1 treatment, we performed an alkaline phosphatase (ALP) activity assay and immunohistochemistry for non-collagenous bone-markers such as bone sialoprotein and osteonectin. Moreover, we investigated the gene expression of these proteins as well as of osteocalcin, a late marker for osteoblastic differentiation using reverse transcription polymerase chain reaction (RT-PCR).

3 Materials and Methods

3.1 Transforming growth factor-β1 (TGF-β1)

Recombinant human transforming growth factor-β1 (TGF-β1) was purchased from Pepro Tech Inc., Rocky Hill, NJ, USA. For the investigations, TGF-β1, lyophilized from sodium citrate buffer (pH 3.5), was reconstituted in water and diluted to a stock solution of 500 ng/ml TGF-β1 with phosphate buffer (PBS, Life Technologies GmbH, Karlsruhe, Germany) containing 2 mg/ml bovine serum albumin (BSA) (Sigma, Taufkirchen, Germany).

3.2 Polymer scaffolds

Non-woven meshes of extruded poly(*L*-lactic-co-glycolic acid) (PLLGA) (90% *L*-lactic acid : 10% glycolic acid) fibers were kindly provided by the Institute of Textile Technology and Process Engineering (ITV, Denkendorf, Germany) [6]. Scaffolds 8 mm in diameter and 1.4 mm thick were disinfected with 70% ethanol. After rinsing thoroughly with PBS, the scaffolds were incubated in culture medium [α-MEM (Sigma); 10% fetal bovine serum (FBS, Gemini Bio-Products Inc., Calabasas, California, USA); 1% penicillin/streptomycin (Sigma); 0.5% L-glutamine 200 mM (Sigma)] for 24 h in a humified incubator (37°C / 5% CO_2) before the cell seeding procedure.

3.3 Marrow stromal cell isolation

Marrow stromal cells were obtained from 6-week old male Sprague-Dawley rats (weight: 170 – 180 g, Charles River Laboratories, Sulzfeld, Germany). Cell isolation from the femur and tibia followed a protocol published by Ishaug et al. [7]. Following marrow isolation and dispersion, cells were centrifuged at 1200 rpm (259 x g) for 7 min. The resulting cell pellet was resuspended in primary medium [α-MEM (Sigma); 10% fetal bovine serum (FBS, Gemini Bio-Products Inc.); 1% penicillin/streptomycin (Sigma); 0.5% L-glutamine 200 mM (Sigma)]. The cells from multiple rats were pooled and seeded onto tissue culture plastic, such that the cell density corresponded to two 150-cm^2 flasks per rat. On the third day of expansion, the flasks were rinsed twice with PBS to remove the nonadherent cells. Thereafter, the 12 ml of

primary medium were changed every 2 - 3 days until 80% confluence was reached. After 10 to 13 days, cells were trypsinized (0.25% trypsin in ethylenediaminetetraacetic acid, Life Technologies) for subculture. At 80 % confluence, 6 – 8 million cells were obtained per flask.

3.4 Cell seeding and cell differentiation

Spinner flasks were used for cell seeding. Scaffolds were threaded onto needles (length:10 cm, diameter: 0.5 mm) and fixed with short segments of silicone tubing. Four needles were inserted into a silicone stopper, which was in turn placed into the mouth of a spinner flask. The spinner flasks were then filled with 100 ml primary medium each and placed in a humified incubator (37°C / 5% CO_2) to allow for serum protein adsorption. After 24 hours, the primary medium was replaced with a cell suspension containing 4 x 10^6 cells per scaffold in 100 ml primary medium. The spinner flasks were then returned to the incubator, placed on a magnetic stir plate (Bellco Glas, Vineland, NJ, USA) set at 80 rpm for cell attachment to the polymer fibers. After 24 hours, each cell-polymer construct was placed in 5 ml complete medium [α-MEM, 10 % FBS, 1% penicillin/streptomycin, 0.5% L-glutamine 200 mM, dexamethasone 10^{-8} M (Sigma), ascorbic acid 50 mg/l (Sigma) and β-glycerolphosphate 7 mM (Sigma)] in 6-well plates (Corning, Corning Costar, Bodenheim, Germany) on an orbital shaker (Stuart Scientific, Surrey, United Kingdom) set at 60 rpm. At this time, transforming growth factor-β1 was added according to the following dosing regimen:

Single doses of TGF-β1: 1, 10 and 20 ng/ml

Multiple dose of TGF-β1: 1 ng/ml, added with every 4th medium change (once a week)

Control: complete medium supplemented with the dilution buffer for TGF-β1 (dilution buffer: 2mg BSA in 1 ml PBS)

Complete medium was changed every 2 or 3 days. Cell proliferation was determined by a DNA assay. Cell differentiation was determined by measuring the enzymatic activity of alkaline phosphatase, by immunohistochemistry and by reverse transcription polymerase chain reaction (RT-PCR) analysis for bone markers such as osteonectin, bone sialoprotein, and osteocalcin.

3.4.1 DNA Assay

A fluorimetric assay was performed to measure the total amount of DNA on the cell-polymer constructs and to subsequently determine the cell number. For determination of the influence of TGF-β1 on the cell number per scaffold, the following doses of TGF-β1 were chosen versus control: 1 ng/ml, added once a week, 10 ng/ml, added once. Samples were taken 24 hours after cell seeding and after 5, 10 and 14 days of cultivation. The assay followed a protocol described by Kim et al. [8]. Cell standards and DNA standards (calf thymus, Sigma, Taufkirchen, Germany) were prepared and examined in parallel to determine the average DNA content of a single cell. A conversion factor of 8 pg DNA per cell was calculated.

3.4.2 Weights of cell constructs

After 5, 10 and 14 days of cultivation, the wet weights of the cell-polymer constructs cultivated with 1 ng/ml TGF-β1, added once a week, and 10 ng/ml, added once, versus control were determined and the average weight of a fiber mesh was subtracted in order to determine the weight of the engineered tissue.

3.4.3 Alkaline phosphatase assay

For determination of alkaline phosphatase (ALP) activity, an enzyme activity kit purchased from Sigma was used (Diagnostic Kit 104, Sigma). To determine the influence of TGF-β1 on alkaline phosphatase activity per cell, the following doses of TGF-β1 were investigated versus the control: 1 ng/ml, added once a week, 10 ng/ml, added once. Samples were taken after 5, 10 and 14 days. To perform the assay, each cell-polymer construct was immersed in 4 ml tris(hydroxymethyl)aminomethane buffer (1 M, pH 8.0) to lyse the cells. Collagen matrix material was fragmented via sonification with a sonifier (Sonifier W-250/W-450, Branson, Danbury, Connecticut,USA). The assay was performed as described in the protocol provided by Sigma; the extent of the hydrolysis of p-nitrophenyl phosphate in the presence of ALP was measured with a photometer (Specord 40, Analytik Jena AG, Jena, Germany) at 405 nm.

3.4.4 Immunohistochemistry

For immunohistochemistry, samples were taken after 14 days of cultivation. The tissues were fixed in methanol (Merck, Darmstadt, Germany) and before cryosectioning incubated overnight with a solution of 5% sucrose (Merck) and 10% Tissue-Tek (Sakura Finetek, Torrance, CA, USA) in PBS. Then, the scaffolds were embedded in Tissue-Tek, cryosectioned at 12 µm (Microm Microtome, HM500 OMV, Microm, Walldorf, Germany) and finally incubated with antibodies against the bone-markers. The source and specificity of each antibody are given in Table 1. The staining procedure was performed as follows: To prevent non-specific antibody binding, sections were incubated with 5% normal horse serum (Vector Laboratories Inc., Burlingame, CA, USA) in PBS. Thereafter, the sections were incubated with the primary antibodies for 30 min at room temperature (RT). In control sections, PBS was used instead of the primary antibody. After washing with PBS, further incubation with biotinylated secondary antibody, anti-mouse/rabbit IgG (Table 1), was performed for 30 min at RT. Formation of avidin-biotin-peroxidase complexes was conducted using the Vectastain Elite ABC-kit (Vector Laboratories, Inc.) and the DAB Substrate kit for peroxidase (Vector Laboratories, Inc.). Sections were counterstained with Mayer´s haematoxylin (Sigma) followed by mounting with DPX mountant (Fluka, Taufkirchen, Germany).

Table 1: Primary antibodies and secondary antibody used for immunohistochemistry.

Antibody description	**Antibody source**
bone sialoprotein (monoclonal antibody, mouse, WVID1(9C5)), supernatant, dilution: 1 : 5	DSHB
osteonectin (monoclonal antibody, mouse, AON-1), supernatant, dilution: 1 : 5	DSHB
collagen type I (monoclonal anti-collagen type I col-1), mouse ascites fluid, dilution: 1 : 2000	Sigma
secondary antibody, biotinylated `` universal `` anti-mouse/rabbit IgG (H+L), horse, dilution: 1 : 100	Vector Laboratories

3.4.5 Reverse transcription polymerase chain reaction

To collect the data concerning gene expression, total RNA was harvested from the cells with TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) and isolated according to the protocol provided by Invitrogen. First-strand cDNA was synthesized from total RNA by using oligo$(dT)_{12\text{-}18}$ primer (Invitrogen) and M-MLV Reverse Transcriptase (Life Technologies). After an initial heating at 65°C (5 min) and cooling on ice for enzyme addition, samples were incubated at 37°C for 1 hour and heated afterwards for 2 min at 95°C to inactivate the enzyme. Subsequently, polymerase chain reaction (PCR) was performed with Taq-DNA-Polymerase (1 U/µl) (Roche Diagnostics GmbH, Mannheim, Germany). After a hot start, initial denaturation occurred at 95°C for 1 min, primer annealing, using specific primers for each gene (Table 2), at 60°C for 1 min and extension at 72°C for 1 min. A definite number of cycles (Table 2) was followed by further incubation at 72°C for 10 min for complete DNA synthesis and holding at 4°C. β-actin served as an internal control. Reverse transcription and PCR were performed in a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany).

The PCR products were separated by electrophoresis on a 2% agarose gel with ethidium bromide staining. Finally, the gels were subjected to imaging and densitometric scanning of the resulting bands under UV light (λ = 312 nm) using the Kodak EDAS 290 (Fisher Scientific, Schwerte, Germany).

Table 2: Denaturation occurred at 95°C for 1 min, primer annealing at 60 °C for 1 min and extension at 72°C for 1 min. β-actin served as internal control. All primers were synthesized by MWG-Biotech GmbH, Ebersberg, Germany.

Gene	**Sense and antisense primer sequences**	**Cycles**
osteonectin	5´CGGGATCCAACTGTCGAGGAGGTG3´ 5´GGAATTCTGCGCAGAGGGAATTC3´	36
bone sialoprotein	5´CGGGATCCAGGCAGCGACTCTTCGGA3´ 5´GGAATTCTCAGTGACGCTTGCCT3´	36
osteocalcin	5´CGGGATCCAGACTCCGGCGCTACCT3´ 5´GGAATTCGGGTCGAGTCCTGGA3´	36
housekeeping-gene: β-actin	5´CGGGATCCCCGACCTAGGCACCAGGGTG3´ 5´GGAATTCGGCTGGGGTGTTGAAGGTCTCAAA3´	28

3.5 Long-term culture

The dosage of 1 ng/ml TGF-β1, added once a week, was regarded as the most effective dose for the improvement of bone-like tissue formation. Hence, cell-polymer constructs, which were treated with the dose of 1 ng/ml TGF-β1 once a week, were cultivated for 32 days and compared to control cell-polymer constructs, not receiving TGF-β1. We aimed at testing the penetration depth of the bone-like tissue formation. Tissue formation was investigated by immunohistochemistry for collagen I, bone sialoprotein and osteonectin, as described in 3.4.4. The source and specifity of each antibody are given in Table 1.

3.6 Statistical analysis

Measurements for wet weight determination (n=6), DNA assay (n=3) and ALP assay (n=3) were collected and expressed as a mean ± standard deviation (SD). Single factor analysis of variance (ANOVA) was used in conjunction with a multiple comparison test (Tukey test) to assess the statistical significance.

4 Results

4.1 DNA Assay

Immediately following the 24-hour seeding period, about 2 Mio. cells were found per fiber mesh, demonstrating that 50% of the seeded cells had attached to the fiber meshes. The DNA assay was repeated on other scaffold samples after 5, 10, and 14 days in order to determine what effect TGF-β1 has on proliferation (Fig. 1). The cell number on the control cell-polymer constructs increased until day 10 ($p < 0.05$); no further proliferation was found thereafter. Low doses of TGF-β1, such as 1 ng/ml, supplemented once a week, led to a similar profile ($p < 0.05$), while after treatment with a single dose of 10 ng/ml no proliferation was observed. Both investigated TGF-β1 doses caused a significant decrease in the cell number between day 10 and 14 ($p < 0.05$).

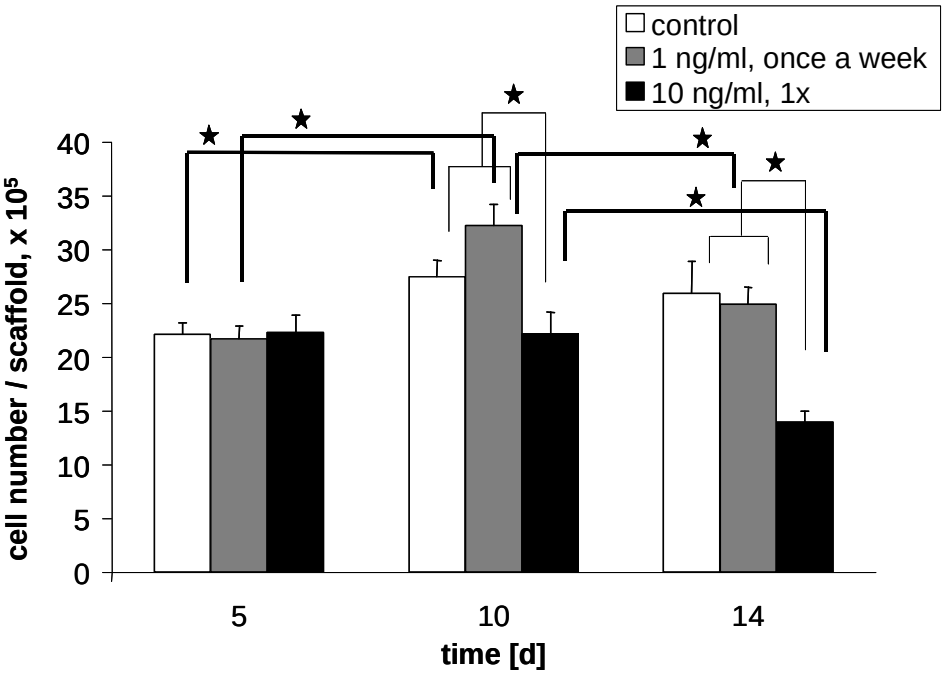


Figure 1: Proliferation kinetics of marrow stromal cells differentiated to the osteoblastic phenotype on PLLGA fiber meshes for 14 days. Columns and error bars represent means ± SD for n = 3. The statistical significance was assessed by Tukey-Test. Significance is indicated by a ★.

4.2 Weights of cell constructs

Wet weights of the controls and of the constructs treated with 1 ng/ml TGF-β1 once a week, significantly increased between day 5 and 10 ($p < 0.01$) and then remained constant (Fig. 2a). No significant increase in wet weight was detectable after addition of 10 ng/ml TGF-β1. Normalization to the cell number, however, revealed a significant increase in the normalized wet weight ($p < 0.01$) from day 5 and 10 to day 14, when a single dose of 10 ng/ml TGF-β1 was administered (Fig. 2 b). On day 14, the normalized wet weight of these constructs was significantly increased compared to the control ($p < 0.01$) (Fig. 2 b).

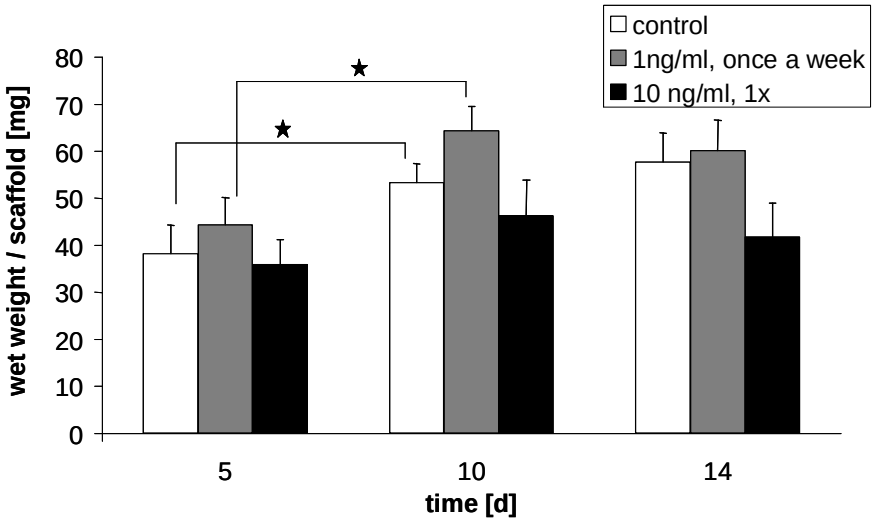


Figure 2 a Wet weight of engineered tissue after cultivation for 5, 10 and 14 days.

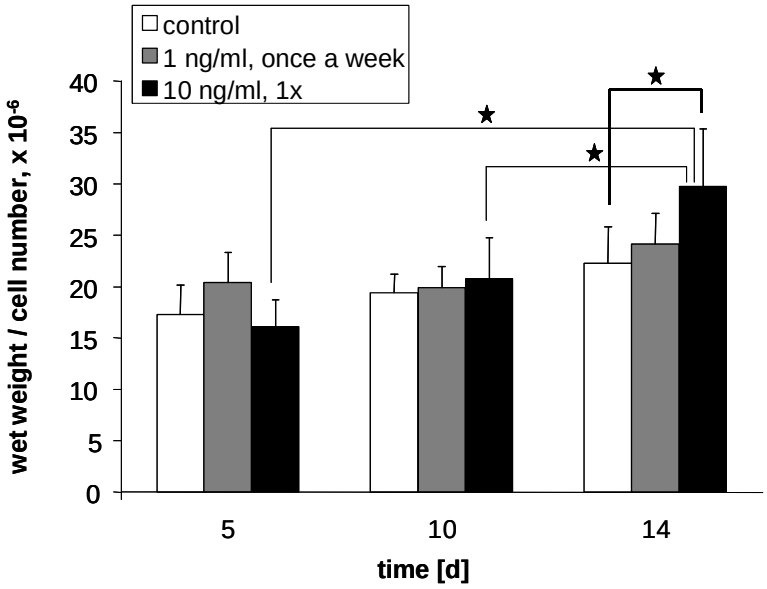


Figure 2 b Wet weight normalized to cell number.

Columns and error bars represent ± SD for n = 6. The statistical significance was assessed by Tukey-Test. Significance is indicated by a ★.

4.3 Alkaline phosphatase assay

ALP activity was determined after 5, 10 and 14 days of cultivation and expressed as activity per cell (Fig. 3). ALP activity of control cultures increased from day 5 to 10 ($p < 0.01$), but decreased from day 10 to 14. This profile reflecting osteoblastic differentiation remained unchanged when 1 ng/ml TGF-β1 was supplemented once a week ($p < 0.01$).

However, this characteristic increase in ALP activity failed to occur in the investigated time scale when the constructs were treated with a dose of 10 ng/ml TGF-β1 ($p < 0.01$).

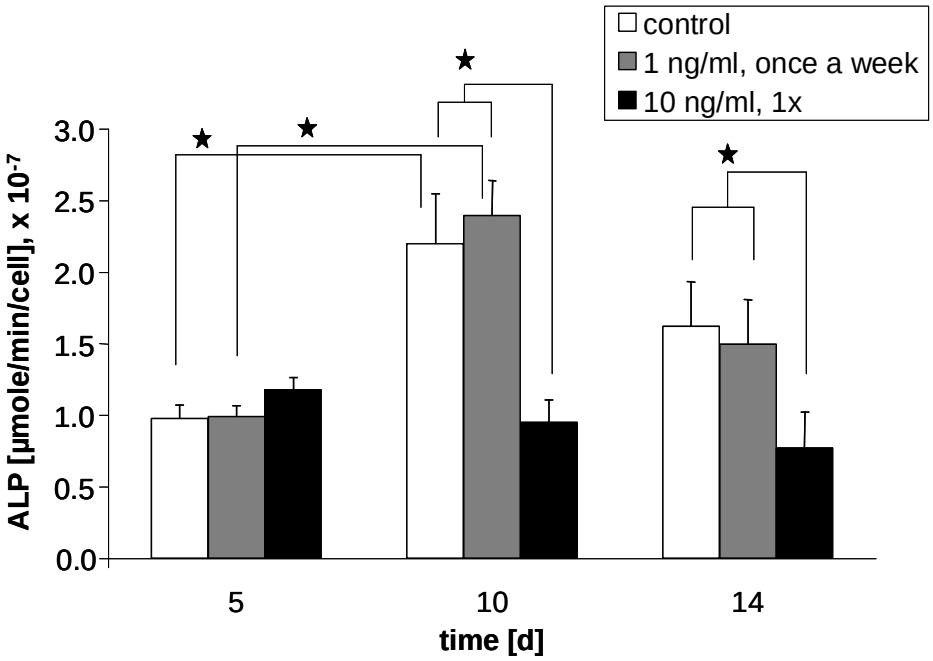


Figure 3: Alkaline phosphatase activity per cell after 5, 10 and 14 days of cultivation. Columns and error bars represent means ± SD for n = 3. The statistical significance was assessed by Tukey-Test. Significance is indicated by a ★.

4.4 Immunohistochemistry

The bone marker osteonectin was detectable both after TGF-β1 addition and in the sections of control constructs (Fig. 4 a - e). Higher amounts of positively stained matrix were found in constructs that received TGF-β1. The formation of the osteoblastic marker bone sialoprotein increased corresponding to increasing doses of TGF-β1 (Fig. 5 a - e). Bone sialoprotein was detectable both in the matrix and in the cells, especially at the edges of the scaffold.

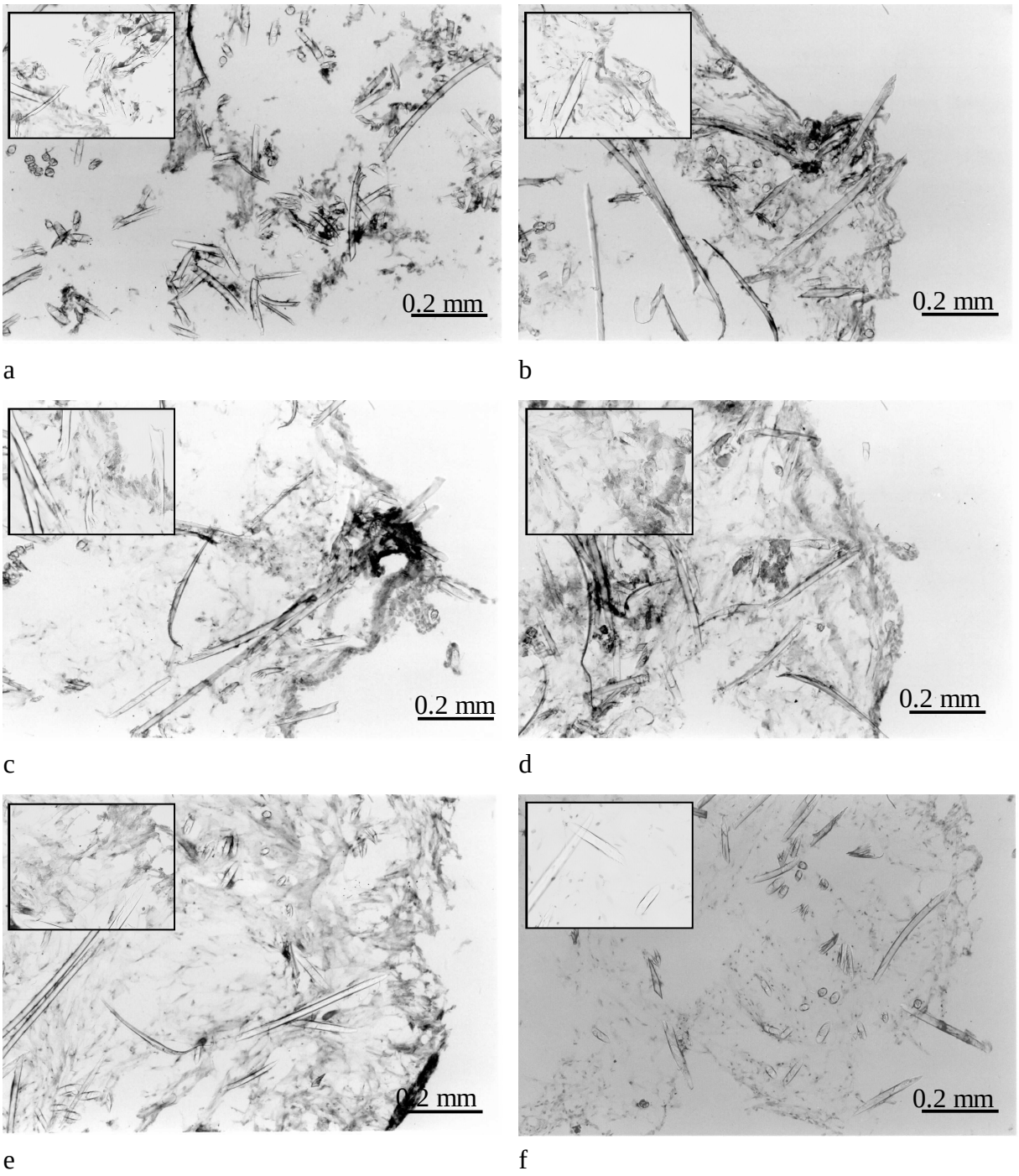


Figure 4: Immunohistochemical staining for osteonectin of cross sections of cell-polymer constructs after 14 days of cultivation. (a) control, (b) TGF-β1: 1 ng/ml, 1x, (c) TGF-β1: 1 ng/ml, once a week, (d) TGF-β1: 10 ng/ml, 1x, (e) TGF-β1: 20 ng/ml, 1x, (f) PBS instead of the primary antibody, TGF-β1: 20 ng/ml, 1x. Insets show a higher magnification (▬ 50 µm).

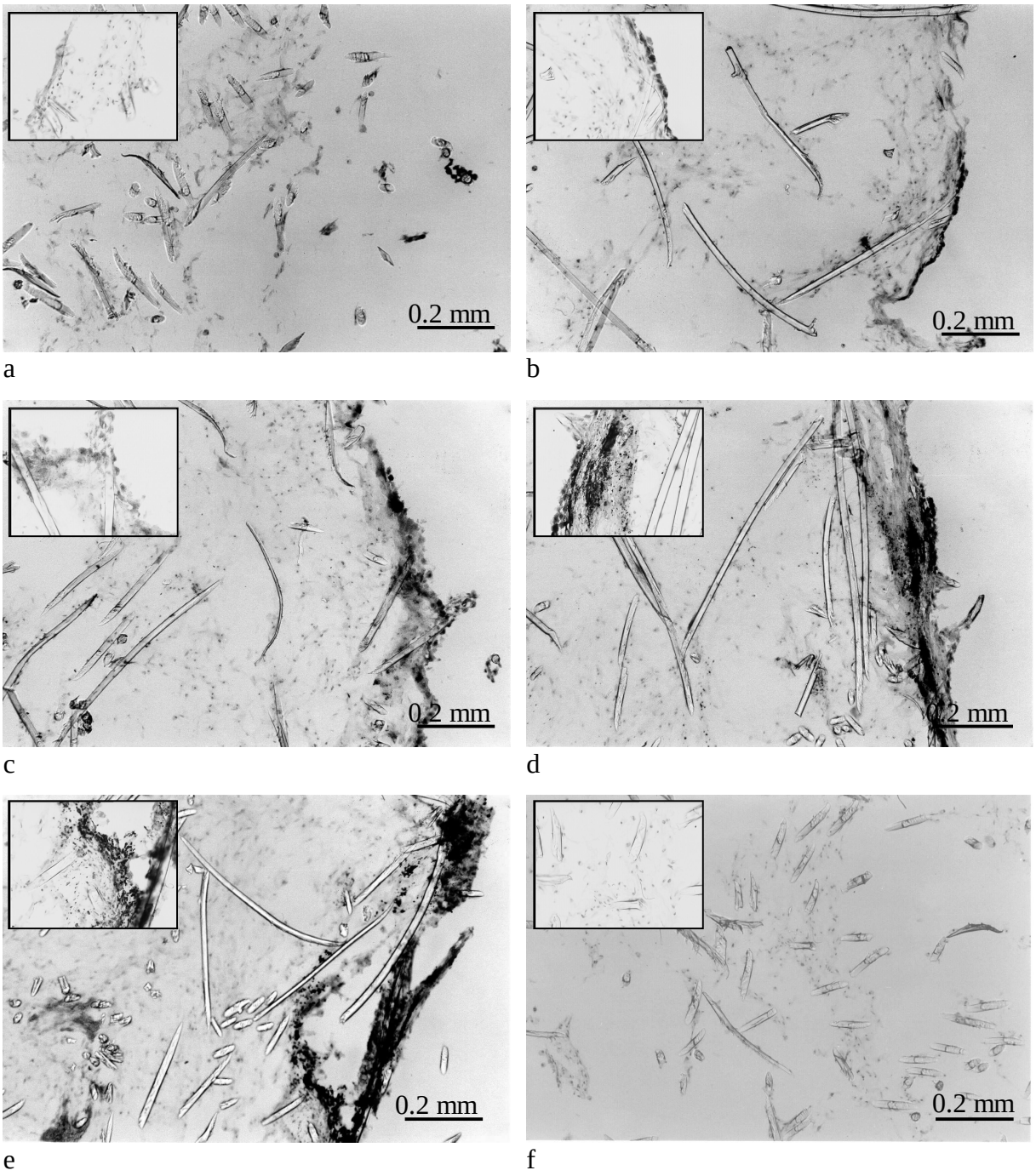
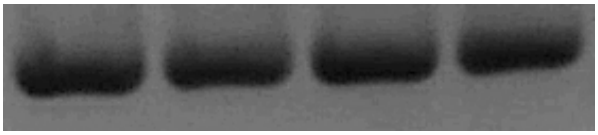


Figure 5: Immunohistochemical staining for bone sialoprotein of cross sections of cell-polymer constructs after 14 days of cultivation. (a) control, (b) TGF-β1: 1 ng/ml, 1x, (c) TGF-β1: 1 ng/ml, once a week, (d) TGF-β1: 10 ng/ml, 1x, (e) TGF-β1: 20 ng/ml, 1x, (f) PBS instead of the primary antibody, control. Insets show a higher magnification (— 50 µm).

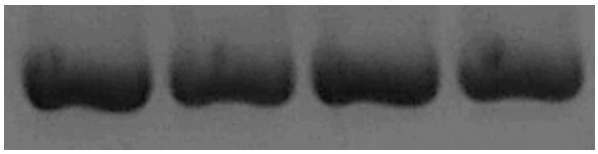
4.5 RT-PCR

We investigated the influence of TGF-β1 on the expression of mRNA of different bone marker proteins on day 5 after the application of the first TGF-β1 dose. No influence of TGF-β1 was observed on mRNA expression of osteonectin and bone sialoprotein (Fig. 6). mRNA expression of the late bone marker protein osteocalcin was determined on day 16 (Fig. 7). No difference between the control and the different TGF-β1 doses was detectable except for TGF-β1 treatment with 20 ng/ml, added once, which decreased mRNA expression for osteocalcin (Fig. 7, 8).



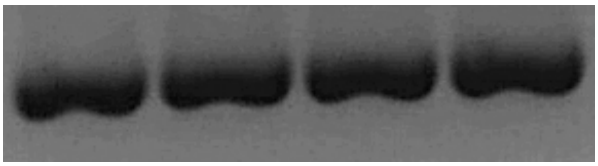
a b c d

osteonectin



a b c d

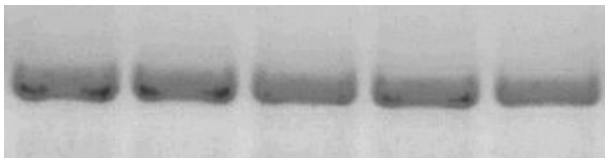
bone sialoprotein



a b c d

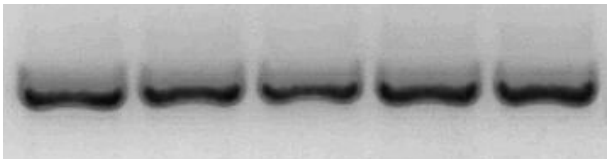
β-actin

Figure 6: TGF-β1 effect on mRNA expression of osteonectin and bone sialoprotein after 5 days of cultivation. 2 cell-polymer constructs per group were used for RNA isolation. (a) control, (b) TGF-β1: 1 ng/ml, 1x, (c) TGF-β1: 10 ng/ml, 1x, (d) TGF-β1: 20 ng/ml, 1x.



a b c d e

osteocalcin



a b c d e

β-actin

Figure 7: TGF-β1 effect on mRNA expression of osteocalcin after 16 days of cultivation. 2 cell-polymer constructs per group were used for RNA isolation. (a) control, (b) TGF-β1: 1 ng/ml, 1x, (c) TGF-β1: 1 ng/ml, once a week, (d) TGF-β1: 10 ng/ml, 1x, (e) TGF-β1: 20 ng/ml, 1x.

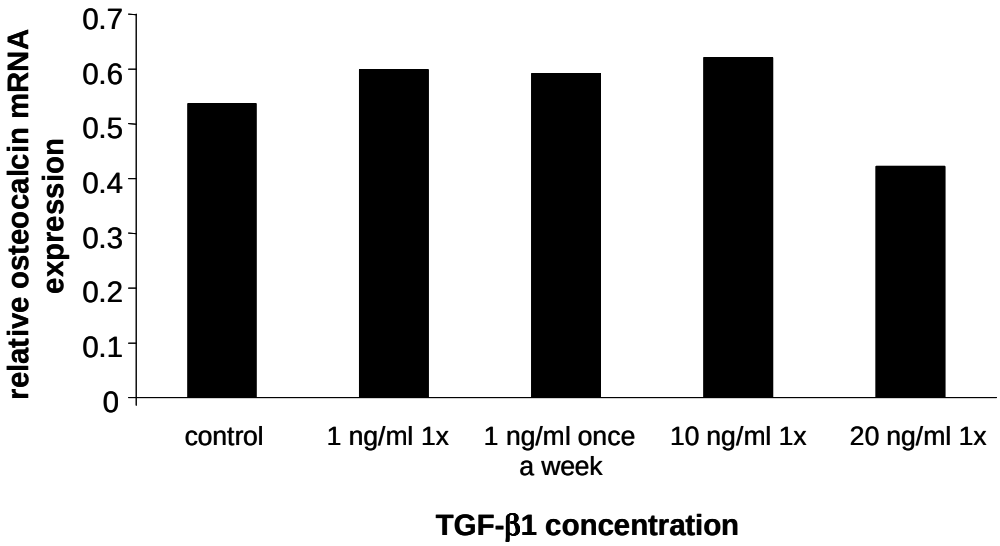


Figure 8: Relative osteocalcin mRNA expression, normalized to β-actin expression.

4.6 Long-term culture

The immunohistochemical investigations of constructs treated with 1 ng/ml TGF-β1 (Fig. 9) showed an almost coherent tissue to a penetration depth of approximately 250 µm - 300

µm. Collagen type I, bone sialoprotein and osteonectin were strongly expressed in these tissue sections. In the sections of control constructs (Fig. 9 h, i) no coherent tissue was detectable.

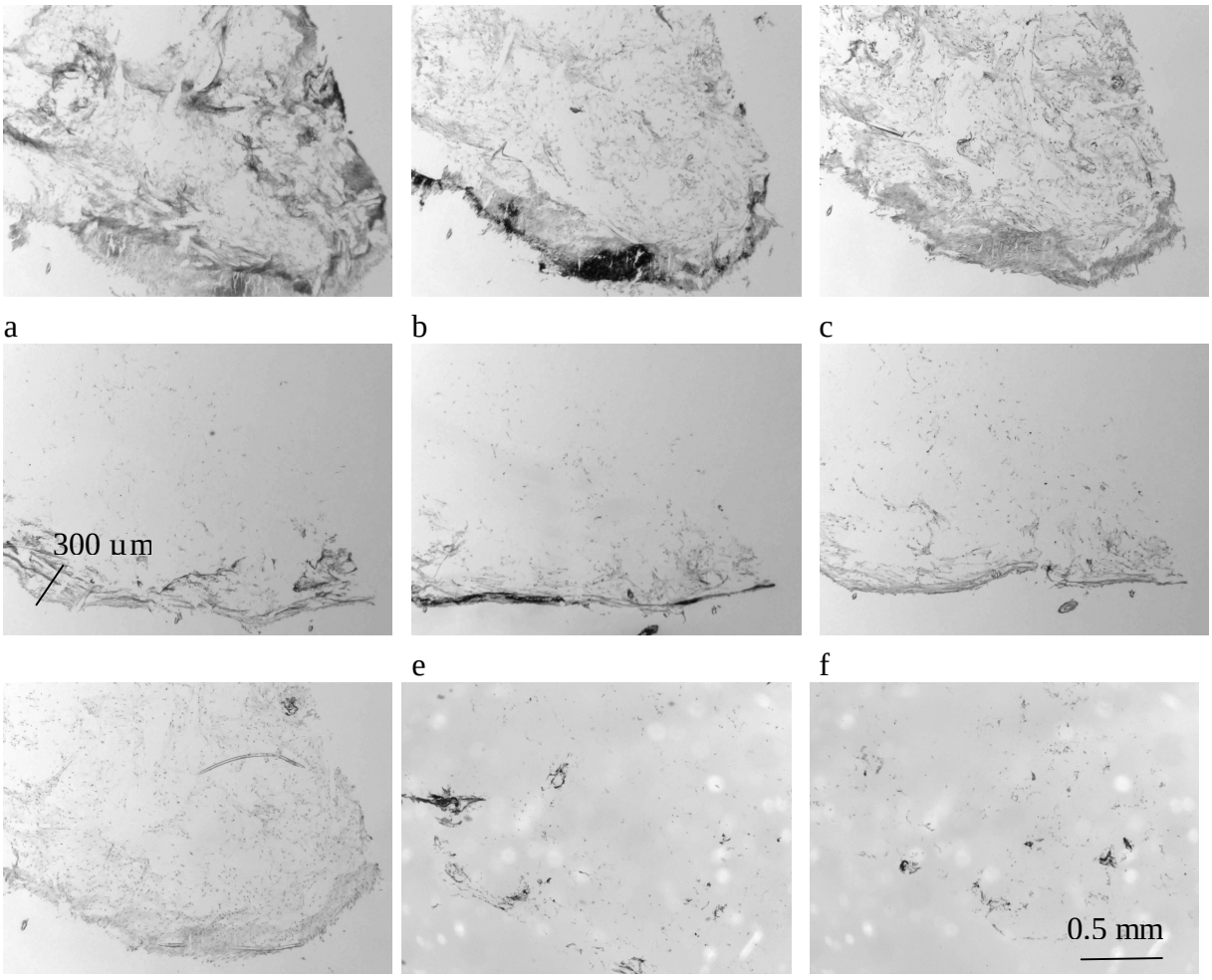


Figure 9: Long-term culture for 32 days of constructs treated with 1 ng/ml TGF-β1, once a week and of control constructs. Cross-sections of a half of the scaffolds. Immunohistochemical staining for (a) collagen type I, (b) bone sialoprotein, (c) osteonectin in sections of the outer part of with TGF-β1 treated constructs. Immunohistochemical staining for (d) collagen type I, (e) bone sialoprotein, (f) osteonectin in sections of the inner part (middle) of with TGF-β1 treated constructs. (g) PBS control by using PBS instead of the primary antibody in sections of the outer part of with TGF-β1 treated constructs. Immunohistochemical staining for (h) collagen type I in sections of the outer part of control constructs, (i) collagen type I in sections of the inner part of control constructs.

5 Discussion

Low matrix formation on in vitro cultivated cell-polymer constructs is a main problem in bone tissue engineering. Therefore, we applied the growth factor TGF-β1 to overcome this lack of matrix formation [1]. TGF-β1 was added in combination with the standard osteoblastic differentiation supplements, containing dexamethasone in order to enable the differentiation of rMSCs to the osteoblastic phenotype. Having shown the dose-dependent stimulation of TGF-β1 on matrix formation and mineralization in part I [1], this second study was conducted to examine the effects of TGF-β1 on the proliferation and differentiation of rMSCs in three-dimensional culture. We intended to answer the question of whether osteoblastic differentiation and collagenous matrix formation could be concomitantly increased with increasing doses of TGF-β1, or which dose both effectively enhances matrix formation and preserves osteoblastic differentiation. To this end, the experiments focused on the application of effectively matrix stimulating doses as determined in the first study, i.e. 1 ng/ml, added once a week and a single application of 10 ng/ml, while the total spectrum of doses from 1 ng/ml to 20 ng/ml TGF-β1 was examined immunohistochemically and by RT-PCR.

In a first step, cell proliferation under the influence of TGF-β1 was determined. Cells on control constructs and constructs receiving 1 ng/ml TGF-β1 once a week proliferated until the 10^{th} day. Following treatment with a dose of 10 ng/ml, however, no proliferation was observed. From day 10 to 14, the cell numbers on both TGF-β1 treated groups decreased, while no decrease was found on control constructs. The wet weights, however, which comprise both the cell number and the amount of the formed extracellular matrix per scaffold, remained unchanged in all groups between day 10 and 14. Thus, the amount of matrix on the TGF-β1 treated constructs seemed to be increased. Normalizing the wet weight to the determined cell number then revealed a significantly higher wet weight/cell on the constructs treated with 10 ng/ml TGF-β1 than on the control constructs on day 14. The normalization further showed that the matrix accumulation on these constructs, treated with TGF-β1, accelerated significantly between days 10 and 14. Thus, the normalization of the wet weight to the cell number reflected the positive effect of TGF-β1 on matrix formation.

The main topic of the second study, however, dealt with the effects of TGF-β1 on osteoblastic differentiation. The activity of the osteoblastic marker ALP becomes transiently increased during osteoblastic differentiation [9,10]. With regard to the effect of TGF-β1 on the ALP activity, 1 ng/ml, added once a week, had no influence on this typical ALP activity profile, as observed on control constructs, while 10 ng/ml TGF-β1 suppressed the development of such a peak. The examination of the mRNA expression of osteocalcin that occurs with the onset of mineralization [11,9], showed that the expression of this bone marker was preserved even up to doses of 10 ng/ml TGF-β1. Thus, a decrease of osteocalcin expression became only apparent after a single dose of 20 ng/ml TGF-β1. Similar effects concerning ALP activity and osteocalcin have been observed by other groups before, who investigated TGF-β1 in two-dimensional cell culture and described a decrease of both differentiation markers, ALP and osteocalcin after the addition of high doses of TGF-β1 [12,13,3]. In addition to the outlined dose response, the effects of TGF-β1 seem also to depend on the state of maturation of the investigated cells. Thus, TGF-β1 increased the ALP activity in highly differentiated cells for example the rat osteoblast cell line ROS 17/2.8, while this marker was suppressed in less mature cells [14,15]. Correspondingly, cells that were pre-treated with dexamethasone were also less responsive towards the dedifferentiating effects of TGF-β1 [16,4] and showed increased ALP activity and osteocalcin when low doses (1 ng/ml once) were applied [12]. In contrast to these authors, we added TGF-β1 simultaneously with the osteogenic supplements and dealt thus with less mature cells, which were treated with higher TGF-β1 doses. Nonetheless, we also achieved an ALP activity and osteocalcin expression as high as on control constructs within a definite range of doses. We did not pursue a further reduction of the applied TGF-β1 doses, because a dose of 1 ng/ml, once a week, was shown to be necessary in order to solve the problem of insufficient matrix formation. The differentiation suppressing effects of high TGF- β1 doses, however, were restricted to the bone markers ALP and osteocalcin. Osteonectin, another bone marker, which is described as an abundant non-collagenous protein in the bone matrix [17], was present independent of the supplementation with TGF-β1, as investigated by RT-PCR and immunohistochemistry. Immunohistochemical investigations even showed that the amount of osteonectin positive matrix increased when TGF-β1 was added to the cultures. RT-PCR for the bone marker bone sialoprotein, which is

effective in promoting nucleation for mineralization [18], showed unchanged mRNA formation for bone sialoprotein after the addition of TGF-β1. Immunohistochemical labelling for bone sialoprotein, however, actually revealed increased amounts of this protein with increasing doses of TGF-ß1 that may be provided by posttranscriptional up- or downregulation or due to a later increase in expression of bone sialoprotein mRNA.

On the whole, we observed remarkable effects of TGF-β1 on collagenous as well as on non-collagenous bone-related proteins such as collagen type I, collagen type V, osteonectin and bone sialoprotein. Furthermore, matrix mineralization was improved under TGF-β1 treatment. Even the bone markers ALP and osteocalcin, often described to be inhibited by TGF-β1, were preserved compared to the control up to high doses, with regard to osteocalcin even up to 10 ng/ml. We assume that the assessed positive effects of TGF-β1 to depend on the dynamic culture conditions, which enhanced the effect of TGF-β1 on the formation of collagenous proteins. In addition, the supplementation of TGF-β1 with the differentiation supplements containing dexamethasone was effective in the preservation of ALP activity and osteocalcin expression within the outlined range of doses. Consequently, we recommend a dosage of TGF- β1, which on the one hand considerably increases matrix formation, but on the other hand preserves ALP activity and osteocalcin expression. The dose of 1 ng/ml TGF-β1, added once a week, was proved in a long-term culture of 32 days to induce the formation of mostly coherent bone-like tissue to a maximum penetration depth of 250 µm - 300 µm. Limitations in nutrient supply probably prevented further tissue formation [19], which may be addressed by perfusion culture [20]. In conclusion, TGF-β1, applied with the dose pattern of 1 ng/ml, added once a week, may be used as a potent growth factor to improve in vitro bone-like tissue formation for the use in bone tissue engineering.

6 Conclusion

The first study [1] had shown that TGF-β1 increased the formation of collagenous matrix proteins dose-dependently and enhanced matrix mineralization, especially under dynamic culture conditions. This follow-up study dealt with the influence of TGF-β1 on osteoblastic cell

differentiation. Having investigated the bone markers, ALP, osteonectin, bone sialoprotein, and osteocalcin, we showed that differentiation was similar to control in a definite range of doses or even enhanced with increasing doses of TGF-β1 with regard to bone sialoprotein. Considering the effects of TGF-β1 on matrix formation and mineralization and on differentiation, the dosing regimen of 1 ng/ml TGF-β1, added once a week is suggested as an effective dose to improve bone-like tissue formation for bone tissue engineering.

7 Acknowledgements

Special thanks are due to Dr. Peter Sandner, former scientist of the Department of Physiology, University of Regensburg, Germany and PD Dr. Gerhard Gross, GBF, Braunschweig, Germany for the generous support in establishing RT-PCR methods, Prof. Dr. Norbert Lehn, Department Medical Microbiology and Hygiene, University of Regensburg, Germany for providing facilities, and Allison Dennis, Department of Pharmaceutical Technology, University of Regensburg, Germany for proof-reading. The monoclonal antibodies WVID1(9C5) developed by Michael Solursh / Ahnders Franzen and AON-1 by John D. Termin were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

8 References

1. Lieb, E., Milz S., Hacker, M., Dauner, M., Göpferich, A., and Schulz, M. B. (2003). Effects of TGF-ß1 on Bone-Like Tissue Formation in Three-Dimensional Cell Culture Part I: Culture Conditions and Tissue Formation. *Tissue Eng.(submitted)*.

2. Qin, X., Gysin, R., Mohan, S., and Baylink, D. J. (2001). Bone growth factors. *In* "Osteoporosis " (Marcus R., Feldman D., and Kelsey J., Eds.), Academic Press, San Diego CA.

3. Faucheux, C., Ulysse, F., Bareille, R., Reddi, A. H., and Amedee, J. (1997). Opposing actions of BMP3 and TGF beta 1 in human bone marrow stromal cell growth and differentiation. *Biochem.Biophys.Res.Commun.* **241**, 787-793.

4. Iba, K., Sawada, N., Nuka, S., Chiba, H., Obata, H., Isomura, H., Satoh, M., Ishii, S., and Mori, M. (1996). Phase-Dependent effects of transforming growth factor beta 1 on osteoblastic markers of human osteoblastic cell line sV-HFO during mineralization. *Bone* **19**, 363-369.

5. Arnold, U., Lindenhayn, K., and Perka, C. (2002). In vitro-cultivation of human periosteum derived cells in bioresorbable polymer-TCP-composites. *Biomaterials* **23**, 2303-2310.

6. Dauner, M. Eigenschaften von Spinnfaservliesstoffen für das Tissue Engineering. International Textile Bulletin Vliesstoffe - Technische Textilien 48(2), 45-48. 2002.

7. Ishaug, S. L., Crane, G. M., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **36**, 17-28.

8. Kim, Y. J., Sah, R. L., Doong, J. Y., and Grodzinsky, A. J. (1988). Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal.Biochem.* **174**, 168-176.

9. Malaval, L., Modrowski, D., Gupta, A. K., and Aubin, J. E. (1994). Cellular expression of bone-related proteins during in vitro osteogenesis in rat bone marrow stromal cell cultures. *J.Cell Physiol* **158**, 555-572.

10. Lieb, E., Tessmar, J., Hacker, M., Fischbach, C., Rose, D., Blunk, T., Mikos, A. G., Göpferich, A., and Schulz, M. B. (2003). Poly(D,L-lactic acid)-poly(ethylene glycol)-monomethyl ether Diblock Copolymers Control Adhesion and Osteoblastic Differentiation of Marrow Stromal Cells. *Tissue Eng.* **9**, 71-84.

11. Aubin, J. E. and Heersche, J. N. M. (2000). Osteoprogenitor cell differentiation to mature bone-forming osteoblasts. *Drug Development Research* **49**, 206-215.

12. Peter, S. J., Lu, L., Kim, D. J., Stamatas, G. N., Miller, M. J., Yaszemski, M. J., and Mikos, A. G. (2000). Effects of transforming growth factor beta1 released from biodegradable polymer microparticles on marrow stromal osteoblasts cultured on poly(propylene fumarate) substrates. *J.Biomed.Mater.Res.* **50**, 452-462.

13. Yamada, T., Kamiya, N., Harada, D., and Takagi, M. (1999). Effects of transforming growth factor-beta1 on the gene expression of decorin, biglycan, and alkaline phosphatase in osteoblast precursor cells and more differentiated osteoblast cells. *Histochem.J.* **31**, 687-694.

14. Kassem, M., Kveiborg, M., and Eriksen, E. F. (2000). Production and action of transforming growth factor-beta in human osteoblast cultures: dependence on cell differentiation and modulation by calcitriol. *Eur.J.Clin.Invest* **30**, 429-437.

15. Noda, M. and Rodan, G. A. (1986). Type-beta transforming growth factor inhibits proliferation and expression of alkaline phosphatase in murine osteoblast-like cells. *Biochem.Biophys.Res.Commun.* **140**, 56-65.

16. Breen, E. C., Ignotz, R. A., McCabe, L., Stein, J. L., Stein, G. S., and Lian, J. B. (1994). TGF beta alters growth and differentiation related gene expression in proliferating osteoblasts in vitro, preventing development of the mature bone phenotype. *J.Cell Physiol* **160**, 323-335.

17. Robey, P. G. (1996). Vertebrate mineralized matrix proteins: structure and function. *Connect.Tissue Res.* **35**, 131-136.

18. Ogata, Y., Niisato, N., Furuyama, S., Cheifetz, S., Kim, R. H., Sugiya, H., and Sodek, J. (1997). Transforming growth factor-beta 1 regulation of bone sialoprotein gene transcription: identification of a TGF-beta activation element in the rat BSP gene promoter. *J.Cell Biochem.* **65**, 501-512.

19. Kellner, K., Liebsch, G., Klimant, I., Wolfbeis, O. S., Blunk, T., Schulz, M. B., and Gopferich, A. (2002). Determination of oxygen gradients in engineered tissue using a fluorescent sensor. *Biotechnol.Bioeng.* **80**, 73-83.

20. Bancroft, G. N., Sikavitsas, V. I., Van den Dolder, J., Sheffield, T. L., Ambrose, C. G., Jansen, J. A., and Mikos, A. G. (2002). Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. *Proc.Natl.Acad.Sci.U.S.A* **99**, 12600-12605.

Chapter 5

Combined application of BMP-2 and TGF-β1 for bone-like tissue formation of bone marrow stromal cells

E. Lieb[1], A. Göpferich [1], M.B. Schulz [1]

[1] Department of Pharmaceutical Technology, University of Regensburg, Germany

1 Introduction

Growth factors are powerful tools for many tissue engineering approaches [1]. Transforming growth factor-β1 (TGF-β1), for example, has been shown to be effective in improving the formation of collagenous bone matrix proteins and matrix mineralization in three-dimensional bone cell culture, elements which are necessary for obtaining a coherent mineralized bone-like matrix for tissue engineering purposes [2]. However, with regard to osteoblastic differentiation the application of TGF-β1 is critical, because the effect of TGF-β1 on differentiation strongly depends on the used dosages and culture conditions [3]. Nevertheless, the osteoblastic differentiation was shown to be preserved under treatment with defined dosages of TGF-β1, while the expression of the bone marker bone sialoprotein even increased following TGF-β1 supplementation, as compared to standard differentiation conditions (dexamethasone, β-glycerophosphate and ascorbic acid) [4]. There is a need for further growth factor supplementation, however, in order to not only preserve, but to enhance the expression of all osteoblastic bone markers, while the application of TGF-β1 is sufficient to deal with the problem of low matrix formation. This intended increase of bone marker expression can be achieved, on the one hand via the commitment of a higher percentage of marrow stromal cells to the osteoblastic lineage and on the other hand through the differentiation to a more mature type of osteoblastic cells. Bone morphogenetic protein-2 (BMP-2) is regarded as a useful growth factor to increase osteoblastic differentiation of rat marrow stromal cells (rMSCs), because BMP-2 is a strong osteoinductive agent both in vivo and in vitro. For example, BMP-2 was reported to be effective in bone regeneration in segmental fracture and spinal fusion models in animal studies [3]. The induced bone formation in vivo is supposed to be caused by stimulating differentiation of mesenchymal stem cells toward an osteoblastic lineage [5]. Considering in vitro studies, BMP-2 has been described as increasing osteoblastic differentiation of bone marrow stromal cells and osteoblasts, including alkaline phosphatase activity, osteopontin, osteocalcin, and bone sialoprotein [6,7,8]. Thereby, it has been reported that BMP-2 not only up-regulates differentiation in preexisting osteoblasts, but also induces commitment of mesenchymal cells to the osteoblastic pathway [6]. Doses between 25 and 1000 ng/ml were reported to be

effective in increasing osteoblastic differentiation in vitro. This broad range of doses is due to the different bioactivities of BMP-2 purchased from different companies or even different lots of the same company. A higher dosage of BMP-2 is thought to be necessary to enhance collagen I formation as compared to the dosage, which is required to effectively stimulate osteoblastic cell differentiation [7,8]. Our intention to combine the both growth factors TGF-β1 and BMP-2 was encouraged by an in vivo study, which reported that bone formation was more potently increased by the combination of TGF-β1 and BMP-2 than by BMP-2 alone in ceramic bovine bone implanted into the thigh muscle of mice [9]. This synergistic effect was suggested to be due to the different preferential effects of BMP-2 and TGF-β1 on cells at different stages of maturation [9]. A further advantage of this growth factor combination would be a reduction of the dosage of BMP-2, because matrix formation was demonstrated to be enhanced by TGF-β1 [2], while the dose of BMP-2 should only be sufficiently high in order to increase osteoblastic differentiation. Thus, such a dose reduction would be advantageous with regard to cost saving and a reduction of systemic side effects.

In this first study we investigated the effect of a single dose of 1 ng/ml or 10 ng/ml TGF-β1 in combination with 100 ng/ml BMP-2, administered with every medium change, on rMSCs seeded on poly(*L*-lactic-co-glycolic acid) (PLLGA) fiber meshes in order to achieve both a high osteoblastic differentiation and a coherent tissue. The growth factor combination was applied in addition to the standard osteogenic supplements (dexamethasone, β-glycerophosphate, ascorbic acid). We investigated immunohistochemically the formation of collagen I, osteonectin and bone sialoprotein under treatment with BMP-2 or TGF-β1, alone or in combination to get a first impression of the effect of the outlined growth factor combination. With regard to BMP-2, the effect of a single dose of 100 ng/ml BMP-2 on rMSCs was also examined in order to clarify if a single dose of BMP-2 may be sufficient to increase osteoblastic differentiation.

2 Materials and Methods

2.1 Growth factors

Recombinant human transforming growth factor-β1 (TGF-β1) was purchased from Pepro Tech Inc., Rocky Hill, NJ, USA. For investigations, TGF-β1, lyophilized from sodium citrate buffer (pH 3.5), was reconstituted in water and diluted to a stock solution of 500 ng/ml with phosphate buffer (PBS, Life Technologies GmbH, Karlsruhe, Germany) containing 2 mg/ml bovine serum albumin (BSA) (Sigma, Taufkirchen, Germany).

Recombinant human bone morphogenetic protein-2 (BMP-2) [10] was kindly provided by Roche Pharma Research Penzberg, Germany. Lyophilized BMP-2 was diluted to a stock solution of 10 µg/ml with medium, containing 10% BSA before use.

2.2 Polymer scaffolds

Non-woven meshes of extruded poly(*L*-lactic-co-glycolic acid) (PLLGA) (90% *L*-lactic acid : 10% glycolic acid) fibers were kindly provided by the Institute of Textile Technology and Process Engineering (ITV, Denkendorf, Germany). Scaffolds, 8 mm in diameter and with a thickness of 1.4 mm were disinfected in 70% ethanol. After rinsing thoroughly with PBS, the scaffolds were incubated in primary medium [α-MEM (Sigma); 10% fetal bovine serum (FBS, Gemini Bio-Products Inc., Calabasas, California, USA); 1% penicillin/streptomycin (Sigma); 0.5% L-glutamine 200 mM (Sigma)] in a humidified incubator (37°C / 5% CO_2) for 24 hours before the cell seeding procedure.

2.3 Cell Culture

2.3.1 Marrow stromal cell isolation

Marrow stromal cells were obtained from 6-week old male Sprague-Dawley rats (weight: 170 – 180 g , Charles River Laboratories, Sulzfeld, Germany). Cell isolation from the femur and tibia followed a protocol published by Ishaug et al. [11]. Following marrow isolation and dispersion, cells were centrifuged at 1200 rpm (259 x g) for 7 min. The resulting cell pellet was resuspended in primary medium. The cells from multiple rats were pooled and

seeded onto tissue culture plastic, such that the cell density corresponded to two 150-cm^2 flasks per rat. On the third day of expansion, the flasks were rinsed twice with PBS to remove the nonadherent cells. Thereafter, primary medium was changed every 2 - 3 days and cells were trypsinized (0.25% trypsin in ethylenediaminetetraacetic acid, Life Technologies) for subculture when 80% confluence was reached.

2.3.2 Cell seeding and cell differentiation

Spinner flasks were used for cell seeding. Scaffolds were threaded onto needles (length:10 cm, diameter: 0.5 mm) and fixed with short segments of silicone tubing. Four needles were inserted into a silicone stopper, which was in turn placed into the mouth of a spinner flask. The spinner flasks were filled with 100 ml primary medium each and placed in a humified incubator (37°C / 5% CO_2) to allow for serum protein adsorption. After 24 hours, the primary medium was replaced with a cell suspension consisting of 4 x 10^6 cells per scaffold in 100 ml medium. The spinner flasks were returned to the incubator and placed on a magnetic stir plate (Bellco Glas, Vineland, NJ, USA) at 80 rpm for cell attachment to the polymer fibers. After 24 hours, each cell-polymer construct was placed in 5 ml complete medium [α-MEM, 10 % FBS, 1% penicillin/streptomycin, 0.5% L-glutamine 200 mM, dexamethasone 10^{-8} M (Sigma), ascorbic acid 50 mg/l (Sigma) and β-glycerophosphate 7 mM (Sigma)] in a 6-well plate (Corning, Corning Costar, Bodenheim, Germany) on an orbital shaker (Stuart Scientific, Surrey, United Kingdom) set at 60 rpm for dynamic cultivation. At this time, growth factors were added according to the following dosing regimen:

Single doses of TGF-β1: 1 or 10 ng/ml

Single dose of BMP-2: 100 ng/ml

Multiple dose of BMP-2: 100 ng/ml with every medium change, i.e. every 2 – 3 days

Combined application of TGF-β1 and BMP-2: single doses of 1 ng/ml or 10 ng/ml TGF-β1 and 100 ng/ml BMP-2, with every medium change

Control: complete medium without further growth factor supplementation

Medium was changed every 2 - 3 days.

2.3.3 Immunohistochemistry

Samples were taken after 21 days of cultivation. The tissues were fixed in methanol (Merck, Darmstadt, Germany) and before cryosectioning incubated overnight with a solution of 5% sucrose (Merck) and 10% Tissue-Tek (Sakura Finetek, Torrance, CA, USA) in PBS. Then the scaffolds were embedded in Tissue-Tek, cryosectioned at 12 µm (Microm Microtome, HM500 OMV, Microm, Walldorf, Germany) and finally incubated with a panel of primary antibodies. The source and specificity of each antibody are given in Table 1. The staining procedure was performed as follows: To prevent non-specific antibody binding, sections were incubated with 5% normal horse serum (Vector Laboratories Inc.; Burlingame, CA, USA) in PBS. Thereafter, the sections were incubated with the primary antibodies for 30 min at room temperature (RT), while in control sections, PBS was used instead of the primary antibody. Further incubation with a biotinylated secondary antibody, anti-mouse/rabbit IgG (Table 1), was performed for 30 min at RT after washing with PBS. Formation of avidin-biotin-peroxidase complexes was conducted using the Vectastain Elite ABC-kit (Vector Laboratories, Inc.) and the DAB Substrate kit for peroxidase (Vector Laboratories Inc.). Sections were counterstained with Mayer´s haematoxylin (Sigma) followed by mounting with DPX mountant (Fluka, Taufkirchen, Germany).

Table 1: Primary antibodies and secondary antibody used for immunohistochemistry.

Antibody description	**Antibody source**
bone sialoprotein (monoclonal antibody, mouse, WVID1(9C5)), supernatant, dilution: 1 : 5	*DSHB
osteonectin (monoclonal antibody, mouse, AON-1), supernatant, dilution: 1 : 5	*DSHB
collagen type I (monoclonal anti-collagen type I col-1), mouse ascites fluid, dilution: 1 : 2000	Sigma
secondary antibody, biotinylated `` universal `` anti-mouse/rabbit IgG (H+L), horse, dilution: 1 : 100	Vector Laboratories

**The monoclonal antibodies WVID1(9C5) developed by Michael Solursh / Ahnders Franzen and AON-1 by John D. Termin were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.*

3 Results

3.1 Collagen type I

Immunohistochemical investigations after 21 days of cultivation showed that the application of 10 ng/ml TGF-β1 led to tissue containing large amounts of collagen type I (Fig. 1b), strongly enhanced compared to the control (Fig. 1a). The application of BMP-2 100 ng/ml, added once or with every medium change, only slightly affected collagen type I formation (Fig. 1c, d). The application of BMP-2 combined with 1 ng/ml or 10 ng/ml TGF-β1, however, led to a coherent tissue containing collagen type I (Fig. 1e, f), though the intensity of staining was decreased when rMSCs were treated with the combination of TGF-β1 and BMP-2 instead of TGF-β1 alone. In order to check for possible background staining, sections of all samples were incubated with PBS instead of with the primary antibody. The sections treated in this way are depicted in Figure 2.

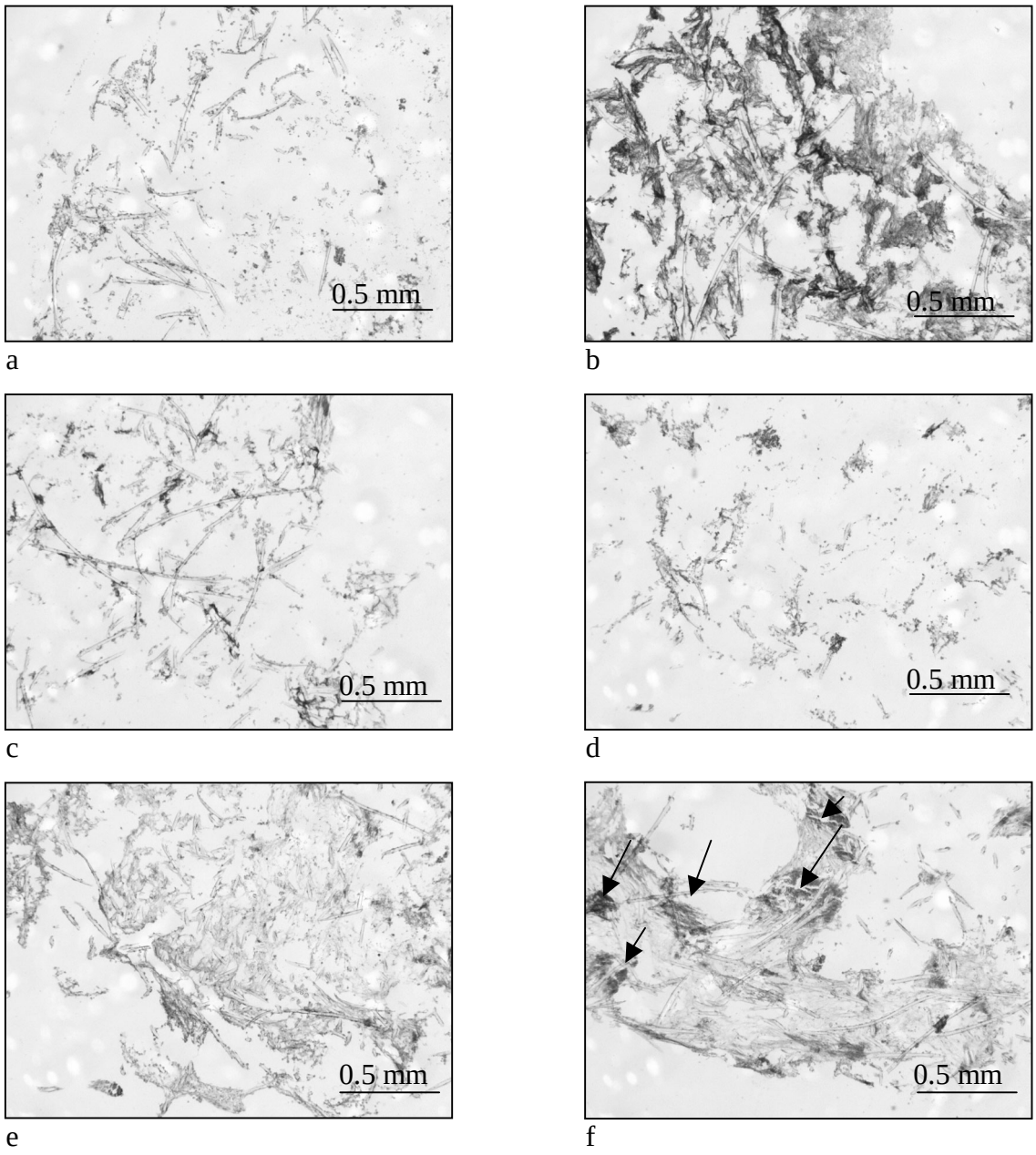


Figure 1: Cross sections of cell-polymer constructs after 21 days of cultivation. Immunohistochemical labeling for collagen type I: (a) control, (b) TGF-β1: 10 ng/ml (1x), (c) BMP-2: 100 ng/ml (1x), (d) BMP-2: 100 ng/ml (with every medium change), (e) TGF-β1: 1 ng/ml (1x) and BMP-2: 100 ng/ml (with every medium change), (f) TGF-β1: 10 ng/ml (1x) and BMP-2: 100 ng/ml (with every medium change). The arrows in (f) indicate mineralization.

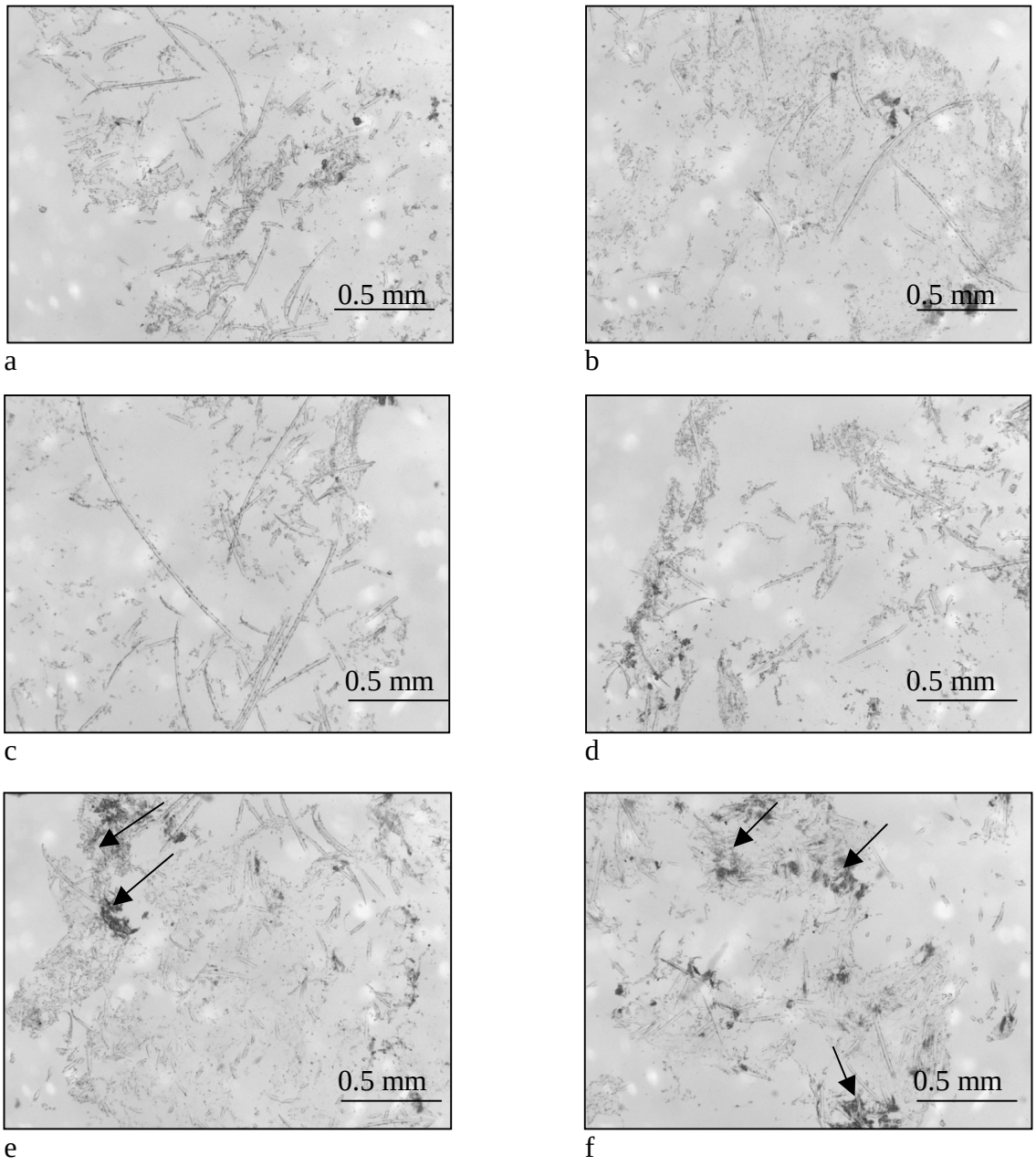


Figure 2: Cross sections of cell-polymer constructs after 21 days of cultivation. Incubation with PBS instead of primary antibody: (a) control, (b) TGF-β1: 10 ng/ml (1x), (c) BMP-2: 100 ng/ml (1x), (d) BMP-2: 100 ng/ml (with every medium change), (e) TGF-β1: 1 ng/ml (1x) and BMP-2: 100 ng/ml (with every medium change), (f) TGF-β1: 10 ng/ml (1x) and BMP-2: 100 ng/ml (with every medium change). The arrows in (e) and (f) indicate mineralization.

3.2 Bone sialoprotein

Immunohistochemical labeling for the characteristic bone marker bone sialoprotein showed the following: The application of 10 ng/ml TGF-β1 led to an enhanced formation of bone sialoprotein compared to control (Fig. 3a, b). Here, increasing doses of TGF-β1 enhance the amount of bone sialoprotein as described by Lieb et al. [4]. After the addition of a single dose of 100 ng/ml BMP-2, only a few small specks of staining for bone sialoprotein were formed (Fig. 3c), while after permanent supplementation with 100 ng/ml BMP-2, many medium sized specs of bone sialoprotein were detectable throughout the scaffold (Fig. 3d). This is in contrast to treatment with TGF-β1 alone, where large areas of bone sialoprotein were especially detectable at the edges of the scaffold, while only small amounts of bone sialoprotein were found in the middle of the construct. With regard to the combined application of 1 ng/ml TGF-β1, added once, and 100 ng/ml BMP-2, added with every medium change (Fig. 3e) the formation of bone sialoprotein was only slightly increased as compared to adding a high dose of TGF-β1, such as 10 ng/ml, alone. This combination also induced large positive areas, not only at the edges of the construct but also in the middle. However, only small amounts of bone sialoprotein were detectable following treatment with BMP-2 and 10 ng/ml TGF-β1 (Fig. 3f).

3.3 Osteonectin

With regard to the bone marker osteonectin we observed an increased formation of osteonectin following the treatment with BMP-2 (Fig. 4c, d) versus TGF-β1 treatment (Fig. 4b). Repeated addition of 100 ng/ml BMP-2 led to positive staining for osteonectin both in the cells and in the formed matrix (4d), whereas after a single supplementation with BMP-2, only the cells were strongly stained (4c). The combination of BMP-2 with 1 ng/ml TGF-β1 led to an increased osteonectin expression (Fig. 4e), compared to the application of TGF-β1 and BMP-2 alone, while after combination of BMP-2 with 10 ng/ml TGF-β1 osteonectin formation was slightly decreased relative to the combination with 1 ng/ml TGF-β1 (Fig. 4f).

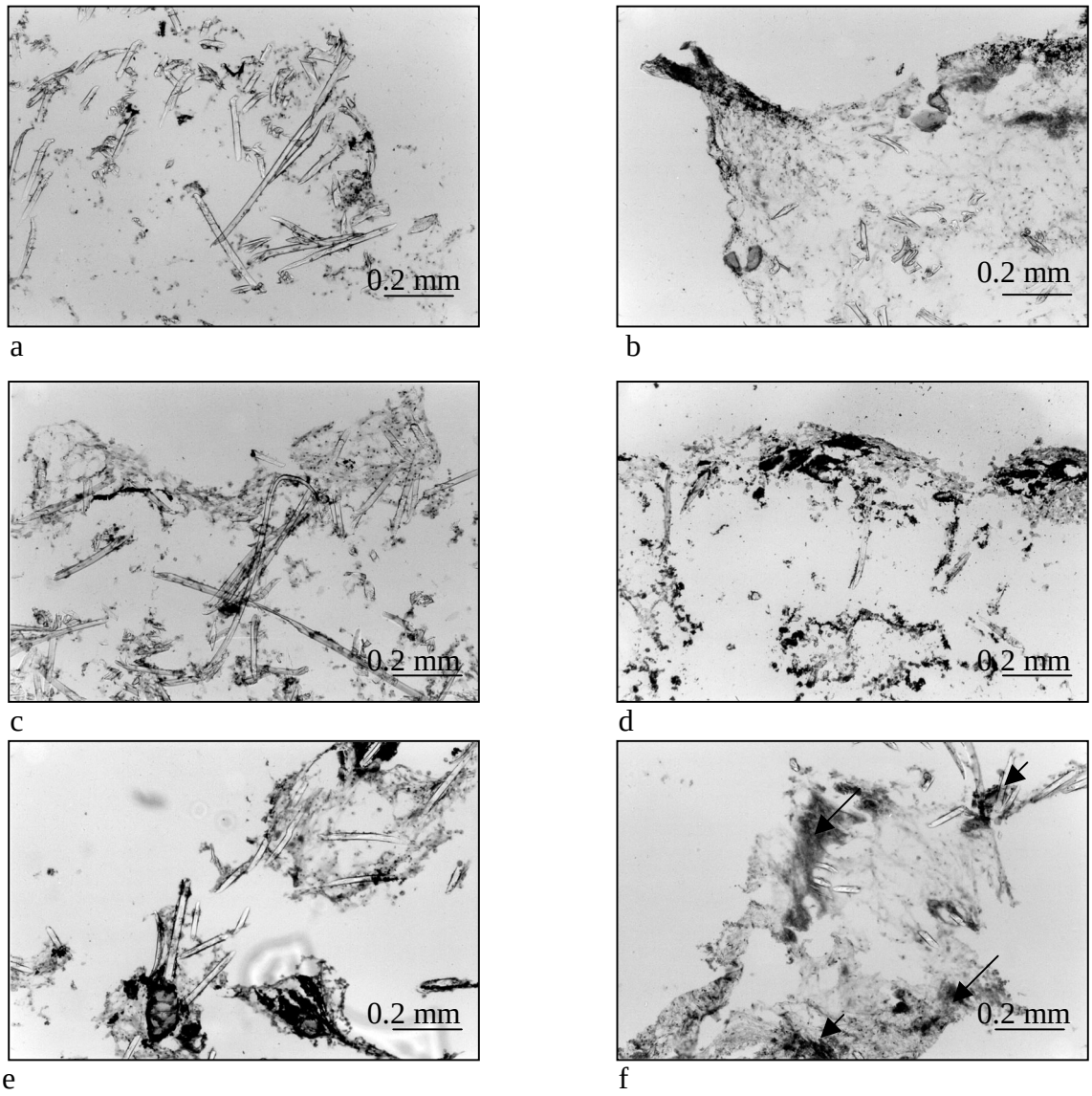


Figure 3: Cross sections of cell-polymer constructs after 21 days of cultivation. Immunohistochemical labeling for bone sialoprotein: (a) control, (b) TGF-β1: 10 ng/ml (1x), (c) BMP-2: 100 ng/ml (1x), (d) BMP-2: 100 ng/ml (with every medium change), (e) TGF-β1: 1 ng/ml (1x) and BMP-2: 100 ng/ml (with every medium change), (f) TGF-β1: 10 ng/ml (1x) and BMP-2: 100 ng/ml (with every medium change). The arrows in (f) indicate mineralization.

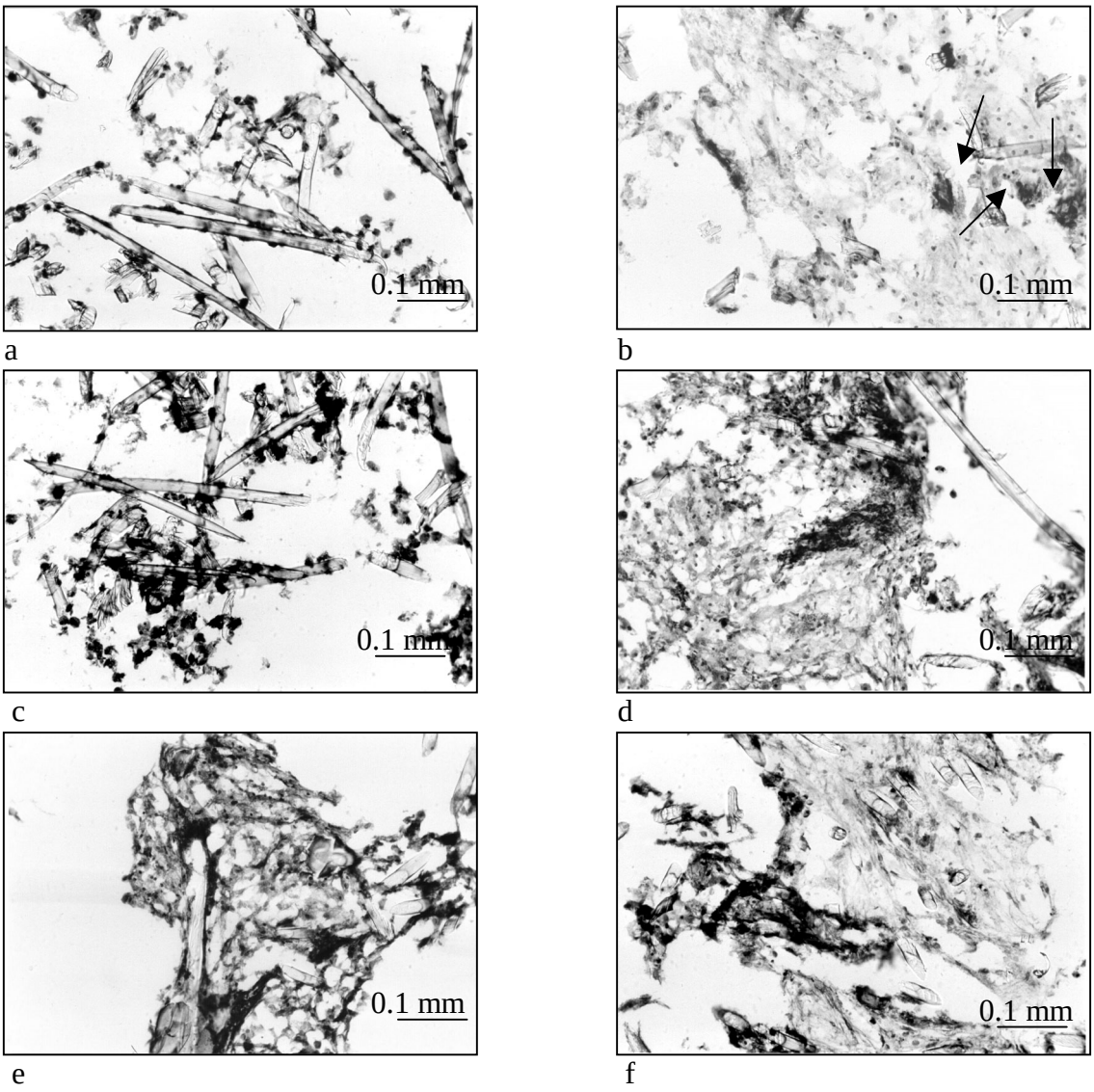


Figure 4: Cross sections of cell-polymer constructs after 21 days of cultivation. Immunohistochemical labeling for osteonectin: (a) control, (b) TGF-β1: 10 ng/ml (1x), (c) BMP-2: 100 ng/ml (1x), (d) BMP-2: 100 ng/ml (with every medium change), (e) TGF-β1: 1 ng/ml (1x) and BMP-2: 100 ng/ml (with every medium change) (f) TGF-β1: 10 ng/ml (1x) and BMP-2: 100 ng/ml (with every medium change). The arrows in (b) indicate mineralization.

4 Discussion

The application of TGF-β1 in combination with BMP-2 was performed in order to increase the osteoblastic differentiation of marrow stromal cells in three-dimensional cell culture, as compared to the treatment with TGF-β1 alone. Furthermore, it was hypothesized that the necessary dose of BMP-2 to effectively induce bone formation could be reduced by this combination. In order to investigate the described hypotheses, we applied two different single doses of TGF-β1 (1 ng/ml, 10 ng/ml) in combination with a repeatedly added dose of 100 ng/ml BMP-2. The two growth factors were administered in addition to the standard osteogenic supplements (dexamethasone, β-glycerophosphate, ascorbic acid). To evaluate the growth factor effects, we investigated collagen type I, osteonectin and bone sialoprotein formation after 21 days of cultivation. A coherent tissue containing collagen type I was obtained after 21 days, following a supplementation with either combination of TGF-β1 and BMP-2, although the amount of collagen type I was decreased compared to TGF-β1 alone. The dose of 100 ng/ml BMP-2 itself only slightly affected collagen type I formation. In contrast to these results, Lecanda et al. [7] described BMP-2 doses of 100 ng/ml as effectively stimulating collagen type I formation in cell culture of undifferentiated human bone marrow stromal cells. There are several explanations for these divergent results. First, one has to take into account that the bioactivity of BMP-2 from different companies and even different lots strongly differs. Thus, the dose of 100 ng/ml BMP-2 administered in our study, was probably at the lower edge of the effective BMP-2 doses and insufficient to enhance collagen type I formation. A further possible explanation for the differing results may be the additional supplementation with dexamethasone in our study. Dexamethasone has been described as decreasing the effect of BMP-2 on collagen type I synthesis [12], so that no increase of collagen type I formation under treatment with BMP-2 could be observed.

The expression of the bone markers bone sialoprotein and osteonectin was increased after the application of BMP-2 compared to administering TGF-β1. This is in agreement with Lecanda et al., who reported that BMP-2 strongly enhanced the expression of bone sialoprotein in bone marrow stromal cell culture. Applying BMP-2 in our study also changed the immunohistochemical staining pattern, especially for bone sialoprotein. Cells all over the scaffold strongly expressed bone sialoprotein in contrast to following TGF-β1 treatment,

where only cells at the edges of the scaffold expressed bone sialoprotein. Positive and even additive effects of the two growth factors on bone sialoprotein and osteonectin were achieved by the combination with the low TGF-β1 dose. This became apparent by an increased amount and the more homogenous distribution of bone sialoprotein over the cross section and a strong staining of the entire matrix for osteonectin. However, the combination of 10 ng/ml TGF-β1 with 100 ng/ml BMP-2 seemed to exceed the range of concentrations in which TGF-β1 and BMP-2 act additively, possibly because of signaling interference between the growth factors. For example, though TGF-β1 and BMP-2 signal through different receptors and Smads, Co-Smads are shared components in TGF-β1 and BMP-2 signal transduction [13] and may lead to the described inhibition of differentiation. A further explanation may be that the differentiation under treatment with BMP-2 and 10 ng/ml TGF-β1 was accelerated, so that the bone marker bone sialoprotein and osteonectin were already decreased on day 21. Consequently, earlier time points would be necessary to answer the question if bone sialoprotein and osteonectin are already downregulated on day 21 or generally suppressed. However, the combination of BMP-2 with 1 ng/ml TGF-β1 seemed to be advantageous with regard to osteoblastic differentiation. Thus, even with a low dose of BMP-2, which alone did not enhance collagen type I, positive effects on the formation of bone sialoprotein and osteonectin were observed. Furthermore, our study revealed that the application of single doses of BMP-2 in contrast to single doses of TGF-β1 had only slight effects on bone-like tissue formation, which is in accordance with reports from Puleo et al. [14]. These authors described that the longer the cells were exposed to rh-BMP-2, the more fully they expressed and sustained osteoblastic traits, i.e., they exhibited exposure-dependent higher levels of alkaline phosphatase and osteocalcin and larger amounts of mineral deposition for the duration of their culture in BMP-2. Thus, a repeated application of BMP-2 seems to be required to achieve a maximum effect.

Summarizing our results, it was shown that with the administered low dosage of BMP-2, the supplementation with TGF-β1 was also necessary in order to obtain sufficient matrix formation, which is crucial for tissue engineering purposes. The investigation of the combined application of TGF-β1 with BMP-2 was pursued to increase osteoblastic differentiation compared to TGF-β1 alone. We showed that on the one hand a coherent collagen I containing tissue was formed and on the other hand the formation of bone sialoprotein and osteonectin

was increased compared to either TGF-β1 or both growth factors, respectively. Since the dosage of 100 ng/ml BMP-2 alone was too low to induce collagen type I formation, while in combination with TGF-β1 a coherent collagen I containing tissue was achieved, we assume that the effective dosage of BMP-2 on bone formation can be considerably decreased by the combination with low dosages of TGF-β1. This is a great advantage considering costs and systemic side effects. However, this study only referred to one dosage of BMP-2, either once or repeatedly applied. In order to investigate the effects of this growth factor combination more deeply, a dose-dependent study will be necessary. Additionally, a study dealing with the investigation of the bone markers alkaline phosphatase and osteocalcin under treatment with TGF-β1 and BMP-2 should be conducted aiming at increasing these bone markers compared to control and TGF-β1 treatment.

5 References

1. Babensee, J. E. (2000). Growth factor delivery for tissue engineering. *Pharm.Res.* **17**, 297-504.

2. Lieb, E., Milz S., Hacker, M., Dauner, M., Göpferich, A., and Schulz, M. B. (2003). Effects of TGF-ß1 on Bone-Like Tissue Formation in Three-Dimensional Cell Culture Part I: Culture Conditions and Tissue Formation. *Tissue Eng.(submitted)*.

3. Qin, X., Gysin, R., Mohan, S., and Baylink, D. J. (2001). Bone growth factors. *In* "Osteoporosis " (Marcus R., Feldman D., and Kelsey J., Eds.), Academic Press, San Diego CA.

4. Lieb, E., Milz, S., Dauner, M., Göpferich, A., and Schulz, M. B. (2003). Effects of TGF-ß1 on Bone-like Tissue Formation in Three-Dimensional Cell Culture Part II: Osteoblastic Differentiation. *Tissue Eng.(submitted)*.

5. Woo, B. H., Fink, B. F., Page, R., Schrier, J. A., Jo, Y. W., Jiang, G., DeLuca, M., Vasconez, H. C., and DeLuca, P. P. (2001). Enhancement of bone growth by sustained delivery of recombinant human bone morphogenetic protein-2 in a polymeric matrix. *Pharm.Res.* **18**, 1747-1753.

6. Rickard, D. J., Sullivan, T. A., Shenker, B. J., Leboy, P. S., and Kazhdan, I. (1994). Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev.Biol.* **161**, 218-228.

7. Lecanda, F., Avioli, L. V., and Cheng, S. L. (1997). Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *J.Cell Biochem.* **67**, 386-396.

8. Fromigue, O., Marie, P. J., and Lomri, A. (1998). Bone morphogenetic protein-2 and transforming growth factor-beta2 interact to modulate human bone marrow stromal cell proliferation and differentiation. *J.Cell Biochem.* **68**, 411-426.

9. Si, X., Jin, Y., and Yang, L. (1998). Induction of new bone by ceramic bovine bone with recombinant human bone morphogenetic protein 2 and transforming growth factor beta. *Int J Oral Maxillofac Surg* **27**, 310-314.

10. Ruppert, R., Hoffmann, E., and Sebald, W. (1996). Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. *Eur.J.Biochem.* **237**, 295-302.

11. Ishaug, S. L., Crane, G. M., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **36**, 17-28.

12. Centrella, M., Rosen, V., Wozney, J. M., Casinghino, S. R., and McCarthy, T. L. (1997). Opposing effects by glucocorticoid and bone morphogenetic protein-2 in fetal rat bone cell cultures. *J.Cell Biochem.* **67**, 528-540.

13. Attisano, L. and Wrana, J. L. (2002). Signal transduction by the TGF-[Beta;] superfamily. *Science* **296**, 1646-1647.

14. Puleo, D. A. (1997). Dependence of mesenchymal cell responses on duration of exposure to bone morphogenetic protein-2 in vitro. *J.Cell Physiol* **173**, 93-101.

Chapter 6

Poly(*D,L*-lactic acid)-Poly(ethylene glycol)-Monomethyl Ether Diblock Copolymers Control Adhesion and Osteoblastic Differentiation of Marrow Stromal Cells

E. Lieb[1], J. Tessmar[1], M. Hacker[1], C. Fischbach[1], D. Rose[2], T. Blunk[1], A.G. Mikos[3], A. Göpferich[1], M.B. Schulz[1]

[1] Department of Pharmaceutical Technology, University of Regensburg, Germany

[2] Working unit Material Investigation, University of Regensburg, Germany

[3] Department of Engineering, Rice University, Houston, Texas, USA

Tissue Engineering (2003), Volume 9, Number 1, 71 - 84

1 Abstract

Biodegradable polymers, such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), are attractive materials for tissue engineering because of their degradative and mechanical properties, which permit scaffolds to be tailored to the individual requirements of different tissues. Although these materials support tissue development, their chemical properties offer no control of cell adhesion or function since their surfaces become immediately masked by adsorbing serum proteins when the materials come into contact with body fluids. Furthermore, adhesion proteins undergo conformational changes and a decrease in bioactivity when adsorbed to hydrophobic materials, such as PLA. To overcome these limitations, we modified the properties of PLA by synthesizing a diblock copolymer with poly(ethylene glycol) (PEG), which is known to reduce the amount of adsorbed proteins and to modify their conformation. By altering the PEG content of these diblock copolymers we were able to control the adsorption of adhesion proteins and, because cell adhesion takes place only in the presence of serum proteins, to control cell adhesion and cell shape. Marrow stromal cell differentiation to the osteoblastic phenotype was strongly improved on PEG-PLA compared to PLA, PLGA and tissue culture polystyrene and led to a 2-fold increase in alkaline phosphatase activity and mineralization.

2 Introduction

Use of biodegradable poly(α-hydroxy acid) polymers is widespread in tissue engineering due to their flexibility in scaffold preparation, degradation times, and mechanical properties. Moreover, poly(α-hydroxy acids), such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA), are known to support adhesion and proliferation of most cell types involved in development and regeneration of musculo-skeletal tissue [1,2]. Cell adhesion and cell function are expected to be influenced by the chemical composition of the polymer. However, when a biomaterial comes into contact with body fluids, serum protein adsorption takes place immediately, masking its specific surface properties. The adsorbed protein layer includes ligands for cell adhesion and thus acts as a mediator between the cells and the biomaterial [3,4]. However, protein adsorption to hydrophobic materials, such as PLA, leads to a high adsorption strength and changes in protein conformation counteracting cellular matrix reorganization [3,5]. To control cell-biomaterial interactions, the polymer surface must be modified to control serum protein adsorption. We therefore attempted to find a material that limits serum protein adsorption and stabilizes the bioactive conformation of adsorbed proteins to positively influence cell function.

Poly(ethylene glycol) (PEG) has gained wide recognition as a biocompatible polymer that is able to modify surface properties due to its noninteraction with proteins and cells [3,6,7]. Moreover, low to intermediate PEG contents in a biomaterial surface are able to modify protein adsorption rather than prevent it [7,8,9]. To control protein adsorption, we synthesized a diblock copolymer [poly(*D,L*-lactic acid)-poly(ethylene glycol)-monomethyl ether] (Me.PEG-PLA) consisting of Me.PEG and PLA, thus preserving the advantages of polyesters and allowing for relevant surface modification.

In an earlier study, cell adhesion and cell function on films of the diblock copolymer Me.PEG5-PLA20 with a Me.PEG content of 20% were examined [10]. The effectively diminished serum protein adsorption on the film surfaces, determined by X-ray photoelectron spectroscopy (XPS), strongly reduced adhesion of rat marrow stromal cells. After addition of differentiating medium (see Cell Culture, below), alkaline phosphatase, an early marker for

osteoblastic function, was highly expressed when calculated on a per cell basis [10]. However, because of the low cell attachment to Me.PEG5-PLA20 we were looking for other compositions of Me.PEG-PLA diblock copolymers that maintain sufficient cell adhesion for bone tissue engineering purposes.

The following four questions led to this study:

Can we improve cell attachment to a reasonable percentage by incrementally decreasing the Me.PEG content and its chain length?

Does cell attachment correlate with the amount of serum proteins adsorbed to the film surfaces?

Will we be able to preserve the differentiating properties of the polymer when we reduce the Me.PEG content to achieve improved cell adhesion?

Is there a correlation between these favorable properties and the Me.PEG content?

The PEG content was decreased by roughly doubling the PLA chain length and decreasing the Me.PEG chain length considerably from 5 to 2 kDa. Cell attachment, cell shape, proliferation and differentiation of rat marrow stromal cells were investigated on smooth, transparent Me.PEG-PLA films prepared by a spin casting technique. PLA, PLGA, and tissue culture polystyrene (TCPS) served as controls.

3 Materials and Methods

3.1 Materials

The biodegradable polymers used in the experiments were P(*D*,*L*)LA, PLGA (Resomer RG 756; kindly provided by Boehringer Ingelheim, Ingelheim, Germany) with a molar ratio of 75:25 (P(*D*,*L*)LA:PGA), and four different Me.PEGx-PLAy. PLA and Me.PEGx-PLAy were synthesized in our laboratory. The x and y in Me.PEGx-PLAy represent the molecular weight of the Me.PEG and the PLA block, respectively, in

kilodaltons. Molecular weight and polydispersity index of these polymers are listed in Table 1.

3.2 Methods

3.2.1 Polymer synthesis

The diblock copolymers were synthesized by a ring-opening reaction from dioxandione (*D,L*-dilactide) and poly(ethylene glycol)-monomethyl ether (Me.PEG) using stannous 2-ethylhexanoate as a catalyst [11] as described previously [12]. PLA was synthesized according to the same reaction scheme [12], using ethyl(S)-(-)-lactate instead of Me.PEG.

3.2.2 Polymer characterization

Determination of polymer molecular weight by gel permeation chromatography

A combination of a precolumn (Phenogel 5 µm, 50 x 7.8 mm) with two identical analytical columns (Phenogel 1000 Å, 5 µm, 300 x 7.8 mm, from Phenomenex, Torrance, CA) was used. Chloroform served as the mobile phase (flux, 0.9 ml/min) in a 10AVP high-perfomance liquid chromatography (HPLC) system (Shimadzu, Duisburg, Germany) thermostated to 35 °C. The polymer was detected with an RID 10A refractive index detector (Shimadzu, Duisburg, Germany). The molecular weights were calculated from the retention time of poly(styrene) standards (Phenomenex, Torrance, CA, and Merck, Darmstadt, Germany) using the Class VP GPC software package included in Class VP 5.03 Software (Shimadzu).

Determination of polymer molecular weight by ^{1}H-NMR

Although gel permeation chromatography (GPC) provides an indication of the molecular weight distribution, the actual molecular weights determined by GPC are questionable whenever the material of the standard differs from the sample material. For this reason, the number average molecular weight was also measured by ^{1}H nuclear magnetic resonance (NMR). A mass of 10 to 20 mg of polymer was dissolved in $CDCl_3$. ^{1}H-NMR spectra were taken at 250.13 MHz with a Bruker AC250 spectrometer (Bruker, Rheinstetten,

Germany) provided with a dual sample head and an autosampler. Tetramethylsilane (TMS) served as internal reference standard. The molecular weights from ^{1}H-NMR were calculated based on a standard integral, which was provided by the three chemically identical hydrogen atoms of the methyl end group of the diblock copolymers.

3.2.3 Spin casting

For film fabrication, solutions with 0.4 g or 0.5 g polymer in 10 ml dichlormethane (Merck) were prepared, depending on the viscosity of the polymer solution (Table 2) (Ubbelohde viscometer [Lauda, Lauda-Koenigshofen]; capillary type I [Schott, Mainz, Germany]). Viscosities between 1.5 and 2.5 mPa·s were chosen to ensure smooth film surfaces. Polypropylene (PP) disks (13 mm in diameter) (Targor Application Technology, Ludwigshafen, Germany) instead of glass cover slips served as carriers in order to improve adhesion of the polymer film to the carrier surface [13]. For film casting, 100 µl of the polymer solution were dropped on top of each PP disk and then accelerated to 1900 rpm to produce thin polymer films with smooth surfaces. The films were then air dried for 12 h and subsequently vacuum dried for 48 h. Finally, the films were exposed to UV light for 1 h.

For XPS investigations, V2A steel disks served as carriers. Spin casting was carried out under laminar air flow to avoid any surface contamination.

3.2.4 Contact angle measurements

The wettability of films was measured with the sessile drop method with an Erma G1 goniometer (Erma, Tokyo, Japan) 1 and 5 min after the deposition of droplets (1 µl). The contact angles were determined on two areas of each polymer film (n=3).

3.2.5 Adsorption of fibronectin on polymer films

Protein adsorption to Me.PEG-PLAs and PLGA as standard material was investigated by incubation of the polymer films with an aqueous solution of fibronectin (300 µg/ml) (bovine plasma fibronectin, 440,000 Da, lyophilized; Life Technologies, Karlsruhe, Germany). After 4 h, the films were rinsed three times with water to remove nonadsorbed protein, frozen at –80°C, and freeze-dried for 24 h in a desiccator on dry ice. The surface composition of polymer films was determined by X-ray photoelectron spectroscopy (XPS) on

a Phi 5700 XPS system (Physical Electronics, Ismaning, Germany) using an $Al_{K\alpha}$ source. Since the polymers themselves contain no nitrogen, we considered the nitrogen content of the surface as a measure for adsorbed protein. For further details please refer to [13].

3.2.6 Cell culture

Marrow stromal cell isolation, seeding, and culture

Marrow stromal cells were obtained from 6-week old male Sprague Dawley rats (weight, 170 – 180 g , Charles River Laboratories, Sulzfeld, Germany). Cell isolation from the femur and tibia was performed in accordance with a protocol published by Ishaug et al. [1]. After marrow isolation and dispersion cells were centrifuged at 1200 rpm (259 x g) for 7 min. The resulting cell pellet was resuspended in primary medium (Dulbecco´s Modified Eagle´s Medium [DMEM; Life Technologies], 10 % fetal bovine serum [FBS, Gemini Bio-Products, Calabasas, CA, USA], 1% penicillin/streptomycin [Sigma, Deisenhofen, Germany]) and seeded in T-75 flasks (Corning, Corning Costar, Bodenheim, Germany). The cells from one rat were plated in two flasks.

On the third day of expansion, the flasks were rinsed twice with phosphate-buffered saline (PBS, Life Technologies GmbH, Karlsruhe, Germany) to remove the non adherent cells. Primary medium (12 ml) was then exchanged every 2-3 days until 80 % confluence was reached. After 13 to 15 days, cells were subcultured (0.25 % trypsin in ethylenediaminetetraacetic acid (EDTA); Life Technologies). Cell numbers were determined with a hemacytometer at a concentration of about 400,000 cells/ml. 1.5 to 2 million cells at 80 % confluence were obtained per flask.

Cell Adhesion study

The polymer coated PP disks or the tissue culture polystyrene disks, respectively, were placed in 24-well plates (Corning, Corning Costar, Bodenheim, Germany). A volume of 150 µl of the cell suspension (5,000 cells/cm^2, i.e., 6,650 cells/film) was seeded onto each disk and allowed to attach for 3 or 7 h. Previous studies of comparable materials have demonstrated that cell attachment levels off during this period [14]. Nonattached cells were then removed by rinsing with PBS. The attached cells were fixed with 10% formalin in PBS

for 10 min, and finally rinsed twice with water. Cells were then stained with aqueous safranin O (0.5% Sigma) to improve the contrast for counting. To remove the surplus stain, disks were rinsed twice with water. Finally, photographs (DYNAX, 600si Classic; Minolta, Ahrensburg, Germany) were taken of four randomly chosen places of the films at a magnification of x100 (Leica DM IRB; Leica Microsystems Wetzlar, Wetzlar, Germany). The attached cells were counted and extrapolated to the area of one film (1.33 cm^2). The low seeding density of 5000/cm^2 cells was chosen to facilitate cell counting and to avoid artifacts from cell aggregation. Four or five films of each polymer were examined.

For investigation of cell adhesion under serum-free conditions, cells were seeded onto films of Me.PEG2-PLA40 for three 3 h (5,000 cells/cm^2, i.e. 6,650 cells/film) (n = 3). PLA served as the control. Cell attachment was determined as described for the cell adhesion study.

Study of cell shape

For this part of the study, 50,000 cells/cm^2, that is, 66,500 cells/film were plated onto polymer films. After 3 h of cell attachment and after 8 days of cultivation, cells were rinsed with PBS and fixed with glutaraldehyde (2.5 % in PBS). After repeated rinsing steps, cells were further fixed with an aqueous solution of OsO_4 (1%; Roth, Karlsruhe, Germany) for 30 min, and cooled on ice. Remnants of OsO_4 were removed with water. The cells on the polymer films were then frozen at -80 °C and freeze-dried for scanning electron microscopy (SEM). Photomicrographs were acquired at 10 kV (JEOL JSM-840; Jeol Ltd. Japan).

Cell proliferation and cell differentiation

For the long-term studies, polymer-coated PP disks were affixed inside low attachment 24-well plates (low-attachment 24-well plates were kindly provided by Costar Corning) with silicone glue (Elastosil; Wacker-Chemie, Munich, Germany). Low-attachment tissue culture well plates were used to minimize cell adhesion and migration to surfaces surrounding the investigated polymer films. Cell proliferation on TCPS, which served as a control material, was established in standard 24-well plates (Costar, Corning Costar). A density of 53,000 cells/cm^2, that is, 70,500 cells/film, that is, 101,200 cells/well (~ 100 % confluence) was

plated on top of each polymer film or seeded in standard 24-well plates, respectively. After 24 h (day 1), primary medium was replaced by complete medium (DMEM; 10% FBS; dexamethasone [10^{-8} M]; ascorbic acid [50 mg/l]; β-glycerophosphate, disodium salt, hydrate [2.16 g/l]). Thereafter, complete medium was exchanged every 2 or 3 days. Cell proliferation was determined by a DNA assay. Cell differentiation was determined by alkaline phosphatase activity and by von Kossa silver nitrate staining.

DNA Assay

A fluorimetric assay was performed to measure the total amount of DNA on a polymer and subsequently determine the cell number [15]. Samples were taken after 10, 13 and 16 days. The wells carrying the polymer covered disks or standard TCPS 24-wells, respectively, were harvested after rinsing with PBS. The wells were kept frozen at –20 °C until the DNA assay was performed. For the assay cells were scraped off the surface with disposable cell scrapers (Biochrom, Berlin, Germany) and dispersed in 1.4 ml of EDTA-solution (10 mM, pH 12.3). The assay was performed according to the protocol described by Ishaug-Riley et al. [16]. For individual DNA measurements, 1.5 ml of Hoechst 33258 dye (Polysciences, Warrington, PA, USA) (dye at 200 ng/ml in 100 mM NaCl and 10 mM Tris, pH 7.0) were added to 1 ml of sample. Cell standards of 30,000 to 300,000 cells/ml and DNA standards (calf thymus, Sigma) were prepared and examined in parallel to determine the average DNA content of one cell.

Alkaline phosphatase assay: ALPase activity

For determination of alkaline phosphatase activity, an enzyme assay kit was used (Diagnostic kit 245; Sigma). Samples were taken as described for the DNA assay. To perform the assay, cells were scraped off the surface of the films with a disposable cell scraper and dispersed in 1 ml of Tris buffer (1 M, pH 8.0). The collected cell suspensions were sonicated for 10 min in an ice bath and stored on ice until analysis. The assay was performed in accordance with the protocol provided by Sigma, reading the kinetics of p-nitrophenyl phosphate hydrolysis in the presence of alkaline phosphatase (ALPase) with a photometer (Uvikon 941, Kontron Instruments, Munich, Germany). ALPase activity was calculated from the slope of absorbance versus time.

Mineralization

Matrix mineralization was assessed by von Kossa silver nitrate staining after 20 days. Samples were fixed with 10% neutral buffered (PBS) formalin for 12 h. After rinsing with water, films were incubated with a 5% aqueous silver nitrate solution and exposed to natural light. Images of films were acquired from a video system (color video camera CCD-100; Mitsubishi CCD, Ratingen, Germany) with a stereo microscope (Leitz Laborlux-S and Leica Wild M10; Wetzlar, Germany) and the black-stained mineralized area of the polymer surfaces was measured with NIH Image analysis software (PICEd Cora, Metzger EDV, Munich, Germany).

3.2.7 Statistical analysis

All measurements were collected (n = 3 to 7) and expressed as means ± standard deviation (SD). Single factor analysis of variance (ANOVA) was used in conjunction with a multiple comparison test (Tukey test) to assess the statistical significance.

4 Results

4.1 Polymer characterization

GPC and ^{1}H-NMR

The synthesized polymers were characterized by ^{1}H-NMR and GPC. In Table 1, the molecular weights of the prepared diblock copolymers obtained by both methods are listed. The data gained by ^{1}H-NMR confirmed the declared block lengths of the synthesized polymer. Moreover, the polydispersity indices, determined by GPC, indicate a comparatively narrow molecular weight distribution. These findings were prerequisites for the subsequent investigations of this study.

Table 1: Weight average molecular weight (Mw), number average molecular weight (Mn) and polydispersity indices (PI=Mw/Mn) of the polymers as determined by gel permeation chromatography [a].

Polymer	**Mw[b]**	**Mn[b]**	**PI[b]**	**Mn(PEG)[c]**	**Mn(PLA)[c]**	**PEG:PLA**
Me.PEG2-PLA40	51,900	28,400	1.8	2,000	38,200	5 %:95 %
Me.PEG2-PLA20	47,600	23,000	2.1	2,000	20,900	9 %:91 %
Me.PEG5-PLA45	62,900	45,300	1.4	5,100	47,500	10 %:90 %
Me.PEG5-PLA20	49,500	35,900	1.4	5,000	19,500	20 %:80 %
PLGA	84,100	48,300	1.7	-.-	-.-	-.-
PLA	83,300	55,400	1.6	-.-	-.-	-.-

[a] The number average molecular weight of the PEG and PLA blocks of each polymer was calculated from ^{1}H-NMR data. All molecular weight data were rounded to the nearest 100. The last column shows the actual PEG/PLA ratio of the polymers determined from ^{1}H-NMR data.

[b] Data obtained by GPC

[c] Data obtained by ^{1}H-NMR

Table 2: Viscosities of polymer solutions used for spin casting

Polymer solution in dichlormethane	**Dynamic viscosity [mPa·s]**
Me.PEG2-PLA40 (5%)	1.884
Me.PEG2-PLA20 (5%)	1.556
Me.PEG5-PLA20 (5%)	1.869
PLGA (4%)	2.421

4.2 Contact angle measurements

Contact angles for the different polymer films measured 1 and 5 min after droplet deposition are shown in Table 3. Hardly any difference in contact angles between water and the different polymers was found after 1 min, with the only exception being Me.PEG5-PLA20, which showed a significantly lower contact angle. After 5 min clear differences between the hydrophobic PLA and PLGA, on the one hand, and the diblock copolymers, on the other hand, became apparent. Moreover, we also found differences between Me.PEG2 and Me.PEG5 containing polymers, but no direct correlation with the PEG content.

Table 3: Contact angles of two areas of polymer film after droplet deposition [a]

Polymer film	**Contact angle after 1 min**	**Contact angle after 5 min**
Me.PEG2-PLA40	64.7 ± 2.5	44.0 ± 2.6
Me.PEG2-PLA20	64.2 ± 1.0	37.5 ± 1.0
Me.PEG5-PLA45	66.2 ± 1.2	28.7 ± 3.2
Me.PEG5-PLA20	57.5 ± 2.8	30.7 ± 1.5
PLGA	63.3 ± 0.8	48.5 ± 1.8
PLA	65.8 ± 2.8	49.8 ± 1.6

[a] Contact angles were determined by the sessile drop method on two areas of each polymer film 1 and 5 min after droplet deposition (n=3).

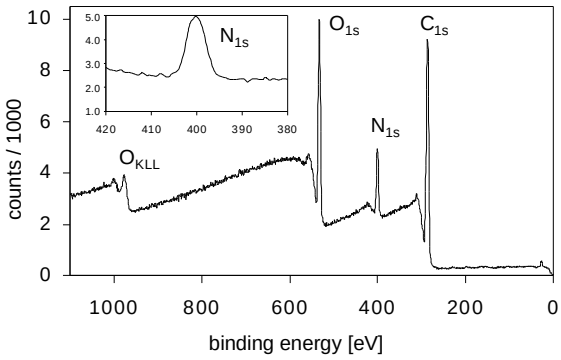
4.3 Adsorption of fibronectin to polymer films

To determine the adsorption of fibronectin to Me.PEG-PLA, polymer films were incubated with a fibronectin solution. The amount of adsorbed fibronectin was determined with XPS analysis via the nitrogen content of the surface (Table 4 and Fig. 1a-e).

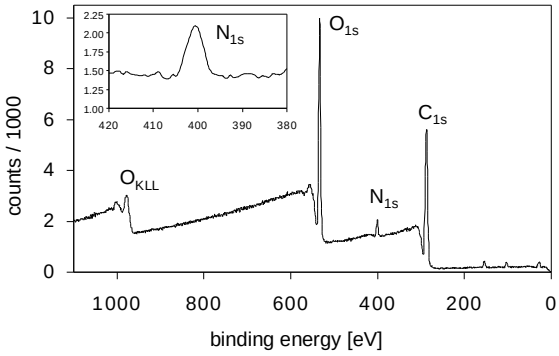
While we found a considerable nitrogen peak on films of PLGA, the percentage of nitrogen detected on the surface of the diblock copolymers decreased with increasing Me.PEG content. The percentage of nitrogen on surfaces of Me.PEG2-PLA40 films was approximately one-third of the nitrogen content detected on PLGA films. Less protein was adsorbed to Me.PEG2-PLA20 and Me.PEG5-PLA45, the diblock copolymers with a Me.PEG content of about 10%. On these films we determined 6 and 7% of the nitrogen content found on PLGA films. No fibronectin adsorption could be detected on Me.PEG5-PLA20 films.

Table 4: Carbon, oxygen, and nitrogen content of polymer films as determined from XPS survey scans after incubation with fibronectin solution.

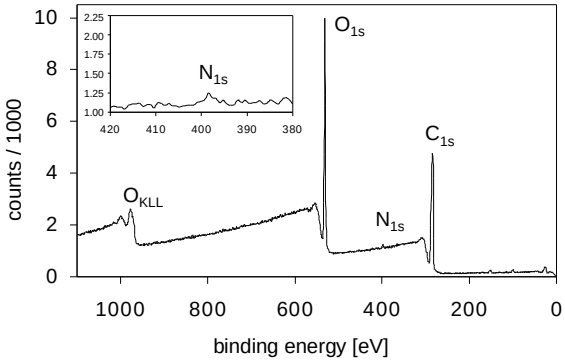
Polymer	**Carbon [%]**	**Oxygen [%]**	**Nitrogen [%]**
PLGA	67.12	22.34	10.54
Me.PEG2-PLA40	62.15	34.40	3.45
	61.91	34.28	3.81
Mean:	62.03	34.34	3.63
Me.PEG2-PLA20	61.59	37.79	0.62
	61.77	37.59	0.64
Mean:	61.68	37.69	0.63
Me.PEG5-PLA45	62.04	37.23	0.73
	61.12	38.11	0.77
Mean:	61.58	37.67	0.75
Me.PEG5-PLA20	62.05	37.94	0.01



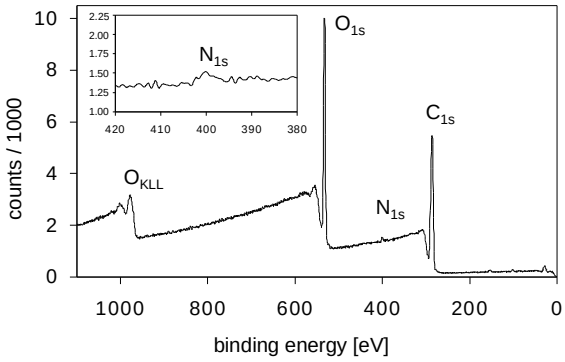
a



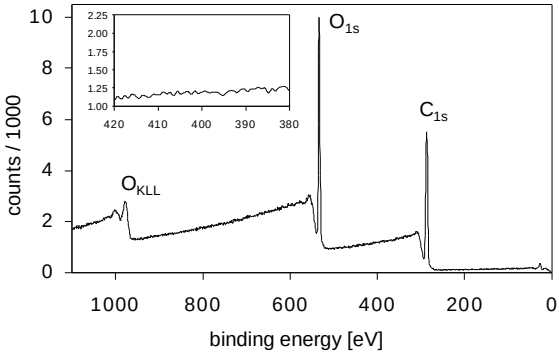
b



c



d



e

Figure 1: XPS spectra of polymer films after incubation with fibronectin solution:
(a) PLGA, (b) Me.PEG2-PLA40,
(c) Me.PEG2-PLA20, (d) Me.PEG5-PLA45,
(e) Me.PEG5-PLA20.
Inserted spectra show the nitrogen signal at high resolution.

4.4 Cell adhesion

Cell number

Cell attachment to four different Me.PEGx-PLAy films, Me.PEG5-PLA20, Me.PEG5-PLA45, Me.PEG2-PLA20 and Me.PEG2-PLA40, was investigated and compared with PLA, PLGA and TCPS (Fig. 2). A high percentage of the seeded cells attached to the three hydrophobic materials PLA, PLGA and TCPS. Cell attachment was generally decreased on films made of the diblock copolymers. Comparison of the four diblock copolymers revealed significant differences in cell adhesion between Me.PEG2-PLA40, Me.PEG5-PLA20 and Me.PEG5-PLA45 ($p < 0.05$). Cell attachment was inversely related to the Me.PEG content of the polymer. In reference to the diblock copolymers, the highest number of cells attached to Me.PEG2-PLA40, the diblock copolymer with the lowest Me.PEG content. About 30% of the seeded cells attached to this material within 7 h. Almost no cell adhered to the most hydrophilic polymer, Me.PEG5-PLA20.

The number of attached cells increased with incubation time. This effect was most pronounced on the hydrophobic materials, while the increase in cell attachment on the diblock copolymers was only moderate in the 3- to 7-h interval.

Cell adhesion under serum-free conditions

Cell attachment to Me.PEG2-PLA40 and PLA films was investigated under serum-free conditions. Less than 1 % of the seeded cells attached to Me.PEG2-PLA40 films after 3 h, whereas 16 % attached to PLA films.

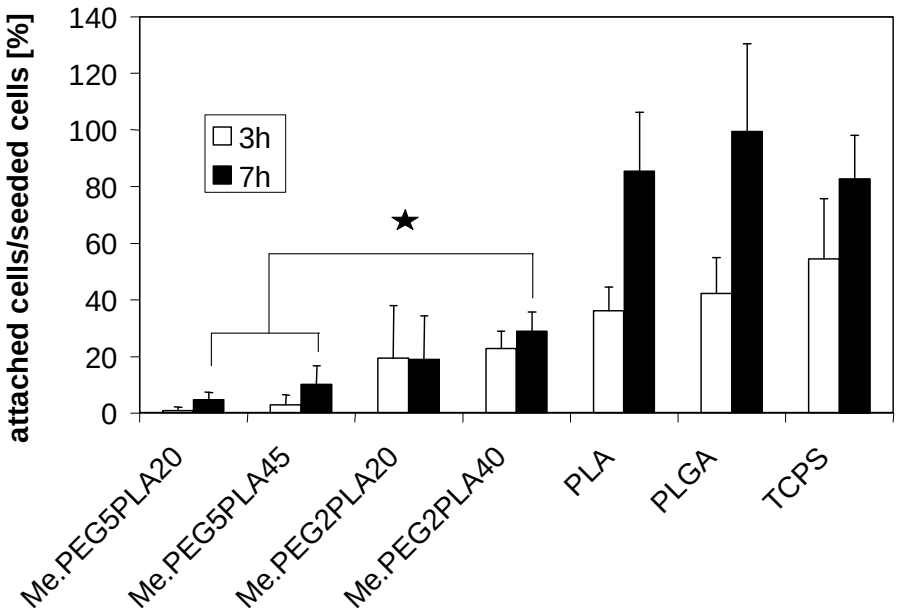
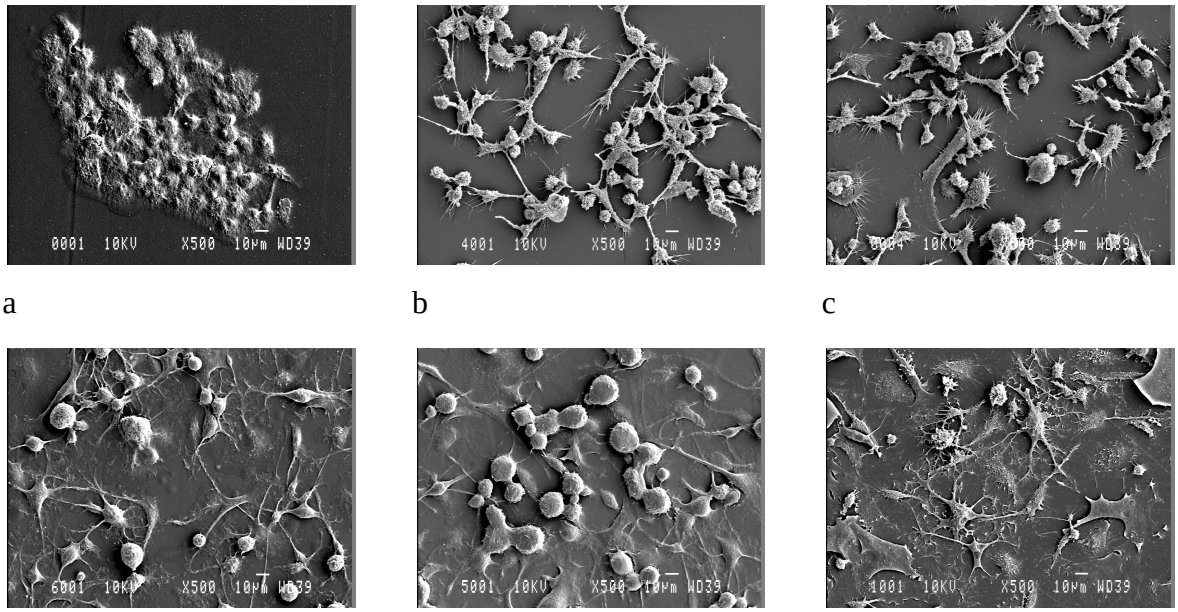


Figure 2: Percentage of plated marrow stromal cells that attached to the different polymers with a seeding density of 5,000 cells/cm^2 after 3 and 7 h. Error bars represent means ± SD for n = 4 except for PLGA, Me.PEG5-PLA20, Me.PEG2-PLA20 and Me.PEG2-PLA40 after 7 h attachment with n = 5. The statistical significance between the PEG-PLA diblock copolymers was assessed by Tukey-Test.

Cell shape

After 3 h of incubation, cell shape strongly depended on the material to which the cells attached (Fig. 3a-f). Cells were well spread on the three hydrophobic materials (Fig. 3d-f), while they remained round on the Me.PEG-containing polymers (Fig. 3a-c). Cells appeared elongated and stretched on films with increasing Me.PEG2 content. Cells on Me.PEG5-PLA45 films were clustered and irregularly distributed. No cells were found on films made of Me.PEG5-PLA20 after sample preparation for the SEM procedure.

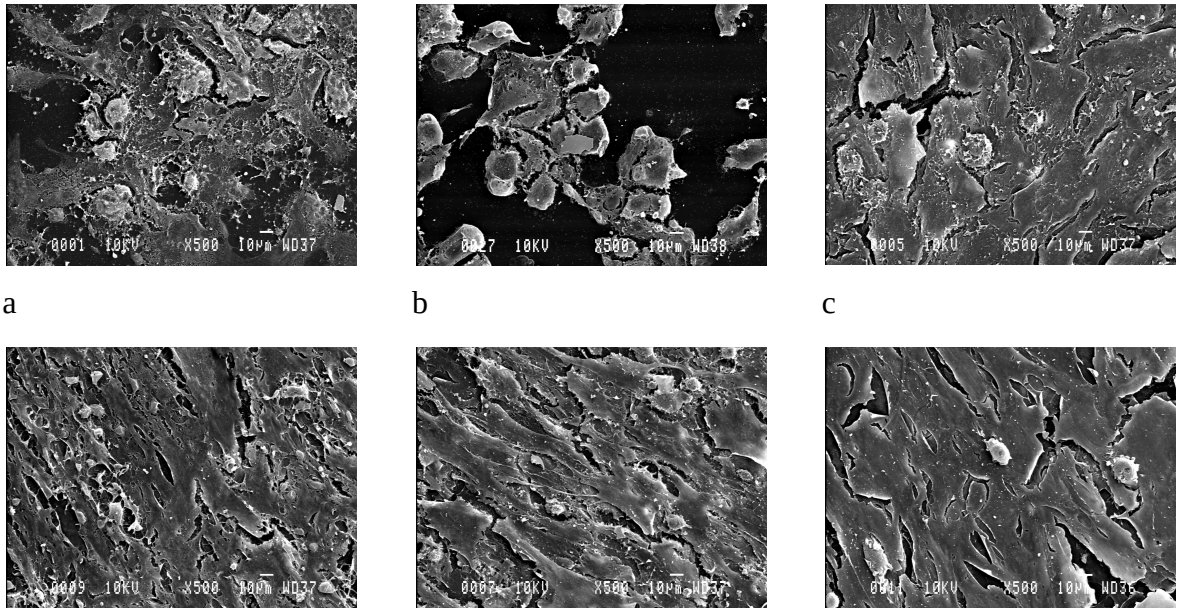
After 8 days, cells on TCPS, PLA, PLGA, and Me.PEG2-PLA40 (Fig. 4c-f) formed a confluent cell sheet, while cells on Me.PEG5-PLA45 and Me.PEG2-PLA20 (Fig. 4a and b) remained distinct.



a b c

d e f

Figure 3: SEM pictures of cells on different polymer surfaces after 3 h of incubation. (a) Me.PEG5-PLA45, (b) Me.PEG2-PLA20, (c) Me.PEG2-PLA40, (d) PLA, (e) PLGA, (f) TCPS.



a b c

d e f

Figure 4: SEM pictures of cells on different polymer surfaces 8 days after seeding. (a-f) as in Fig. 3.

4.5 Cell differentiation study

Proliferation

During the first 10 days cell number on PLA, PLGA and TCPS reached 60,000 to 110,000 (Fig. 5). In this period, the cell number on the diblock copolymers with a Me.PEG content below 20% remained near the lower margin of 60,000 cells. Since almost no cells attached to Me. PEG5-PLA20, this polymer was excluded from further investigation. In the following 3 days, the cell number on the diblock copolymers doubled. An even larger increase in cell number was observed on PLA, PLGA, and TCPS. No significant change in cell numbers took place between day 13 and day 16.

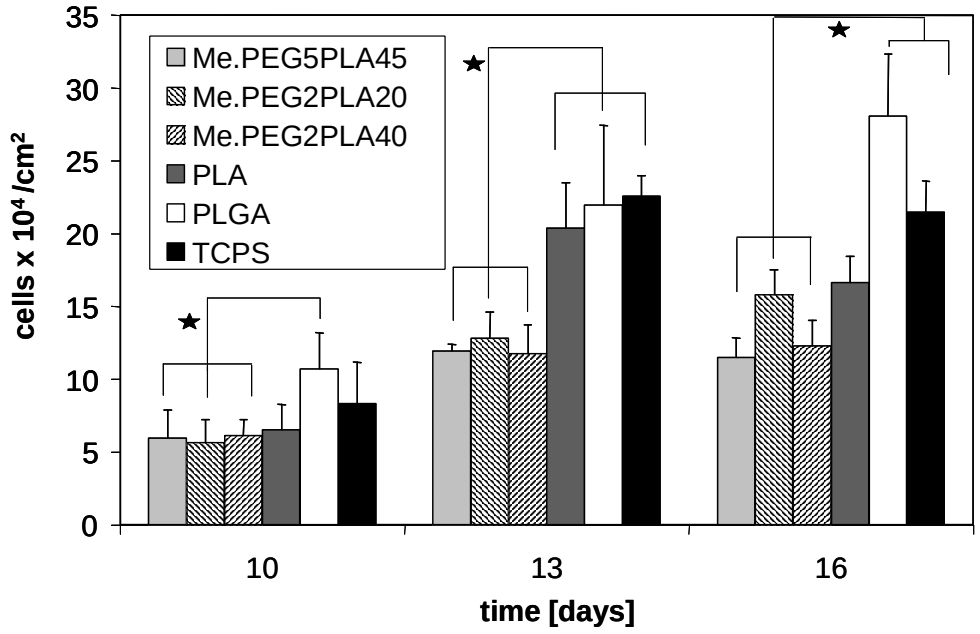


Figure 5: Proliferation kinetics of marrow stromal cells cultured in vitro with an initial seeding density of 53,000 cells/cm^2 on the various polymers. Columns and error bars represent means ± SD for n = 4 except for TCPS after 10 days cultivation (n = 3). The statistical significance between the different polymers was assessed by Tukey-Test.

Alkaline phosphatase activity

ALPase is a marker for early osteoblastic differentiation that develops its transitory maximum early during differentiation. To determine the kinetics of ALPase activity under the

chosen culture conditions, a control study was conducted first on TCPS. The peak of ALPase activity developed between the days 10 and 15, with a maximum on day 13 (Fig. 6). Thus, in the following study on differentiation on the different diblock copolymers, ALPase activity was determined on days 10, 13, 16.

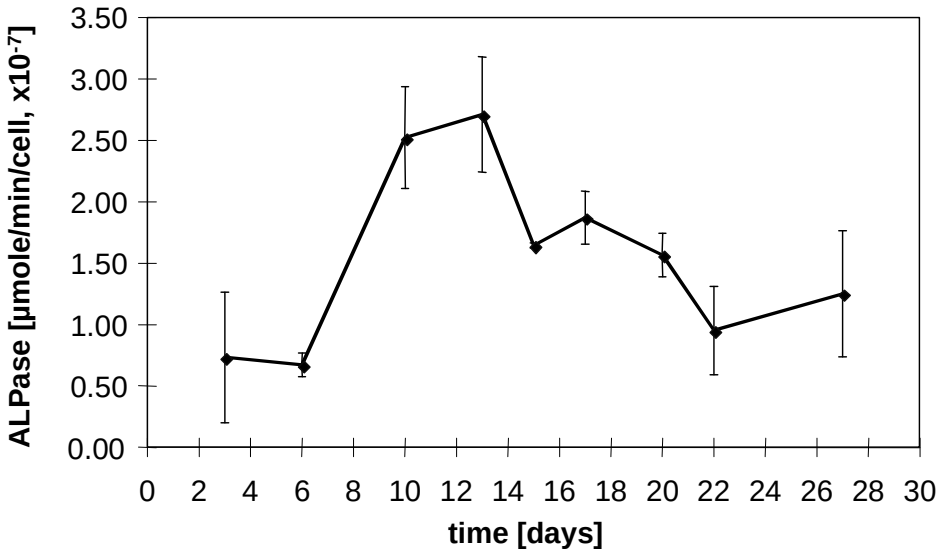


Figure 6: Kinetic of alkaline phosphatase activity per cell of marrow stromal cells on TCPS. Data points and error bars represent means ± SD for n = 3.

For PLA and TCPS, maximum values in ALPase activity were found after 10 days. However, cells on the diblock copolymers expressed the highest ALPase activity on day 16 (Fig. 7). Significantly higher activities ($p < 0.05$) were found on films of Me.PEGx-PLAy after 16 days than on PLA after 10 days.

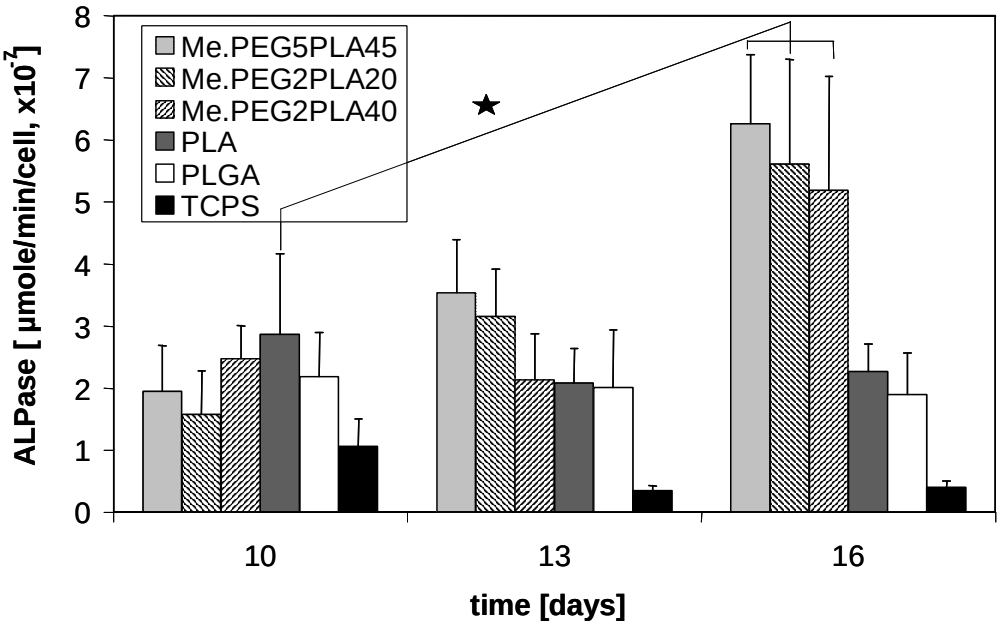


Figure 7: Alkaline phosphatase activity per cell after 10, 13 and 16 days of cultivation. Columns and error bars represent means ± SD for n = 5 to n = 7. The statistical significance between PLA and the Me.PEG-PLA diblock copolymers was assessed by Tukey-Test.

Mineralization

The mineralized area on the films was determined twenty days after addition of the differentiating medium. Major differences in the amount of mineralization on the films were found (Fig. 8 and 9a-f). Cells on Me.PEG2-PLA40 produced a significantly larger area of mineralization than cells on any other material ($p < 0.05$). About 40% of the film surface was covered with mineralized extracellular matrix. Mineralization on TCPS was the lowest, with less than 5%. Medium areas of mineralized matrix, between 10 and 21%, were found on Me.PEG2-PLA20, Me.PEG5-PLA45 as well as PLA and PLGA.

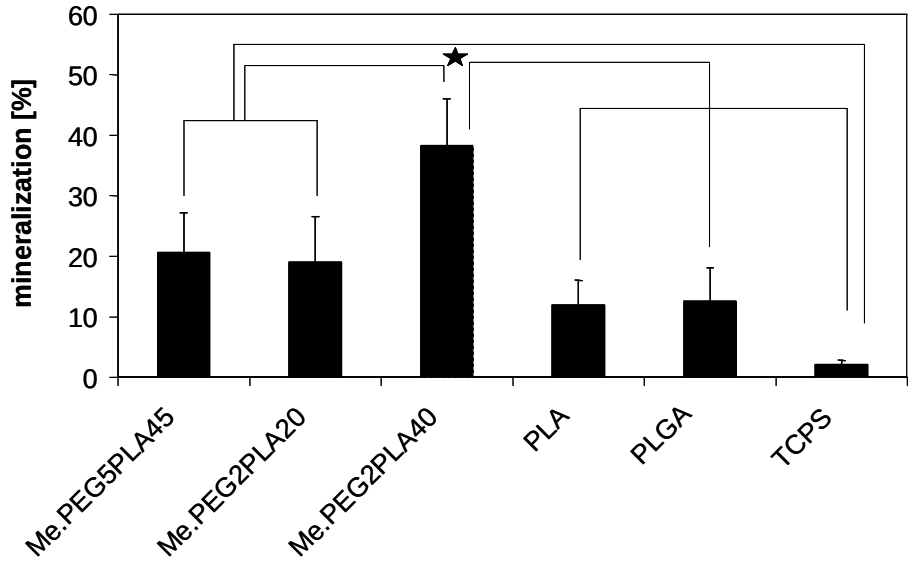


Figure 8: Von Kossa silver nitrate staining: Percentage of mineralized area after 20 days of cultivation. Columns and error bars represent means ± SD for n = 4 except for TCPS (n = 3). The statistical significance between the different polymers was assessed by Tukey-Test.

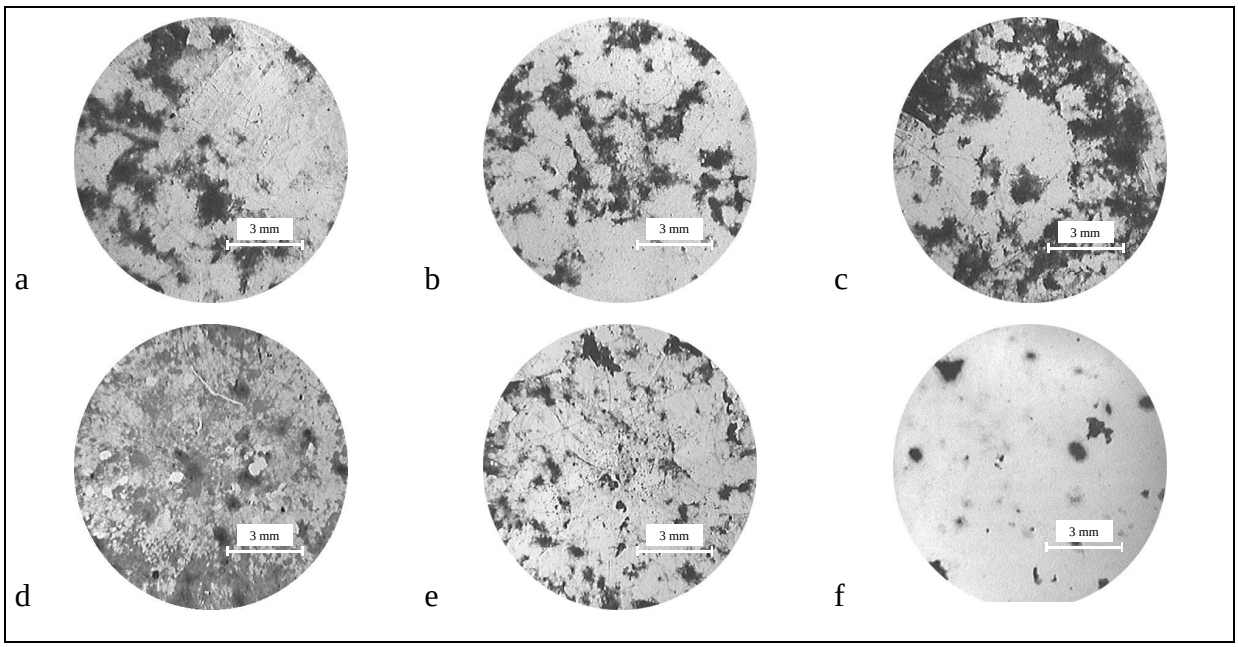


Figure 9: Von Kossa silver nitrate staining: Overall view of the stained mineralized area on the different polymers (original magnification x 5). (a-f) as in Fig. 3.

5 Discussion

Preliminary experiments preceding this study on cell adhesion and differentiation of rat marrow stromal cells revealed Me.PEG5-PLA20 to be unsuitable for tissue engineering purposes because of low cell attachment. The first part of this study thus dealt with the effect of the composition of Me.PEG-PLA diblock copolymers on cell adhesion. While we confirmed negligible cell attachment on Me.PEG5-PLA20, cell adhesion was improved by incremental reduction of the Me.PEG content from 20 to 5%.

Further, we aimed at distinguishing between the effects of Me.PEG content and its chain length. Keeping the PLA block constant and varying the Me.PEG chain length, such as in Me.PEG5-PLA20 and Me.PEG2-PLA20, and approximately in Me.PEG5-PLA45 and Me.PEG2-PLA40, allowed us to deal with the influence of chain length. In both examples, contact angles increased considerably and cell attachment was improved by decreasing the Me.PEG chain length from 5 to 2 kDa. However, no difference in cell adhesion was found between diblock copolymers with the same ratio of the polymer blocks Me.PEG and PLA, such as in Me.PEG2-PLA20 and Me.PEG5-PLA45, both with approximately 10% Me.PEG. These results suggest Me.PEG content is the major influence and the chain length as being less important for cell attachment. However, our conclusions concerning the Me.PEG content were drawn from only one pair of diblock copolymers, since Me.PEG2-PLA10, having the same PEG content as Me.PEG5-PLA20, and Me.PEG5-PLA100, comparable to Me.PEG2-PLA40, were not available for this study. Me.PEG2-PLA10 has a glass transition below room temperature and Me.PEG5-PLA100 could not be synthesized by the method applied in this study.

The change in cell attachment was accompanied by a change in cell shape reflecting modified interactions of cells with the film surface. An increase in the Me.PEG content thus caused reduced cell spreading and, consequently, a decrease in cell proliferation.

The second question of this study aimed at correlating cell attachment to the investigated diblock copolymers with protein adsorption. We chose fibronectin as a relevant adhesion protein [17] to check for protein adsorption to the investigated materials. XPS

analysis revealed decreasing amounts of adsorbed fibronectin with increasing Me.PEG content of the diblock copolymers. While fibronectin was already strongly decreased on Me.PEG2-PLA40 (5% Me.PEG), we did not find any protein on films of Me.PEG5-PLA20 (20% Me.PEG). Comparable results on protein adsorption were obtained for serum proteins adsorbed from a solution with 10% serum to Me.PEG5-PLA20 films [10] and to Me.PEG2-PLA20 films [13]. Taken together with the results of the cell adhesion experiments this study revealed a correlation between protein adsorption and cell adhesion to the diblock copolymers. Moreover, under the serum-free conditions, cell adhesion to even Me.PEG2-PLA40 was very low, suggesting the proteins mediate cell attachment to Me.PEG-PLA.

The third question aimed at the differentiating properties of the diblock copolymers: Can we preserve these favorable properties if we decrease the Me.PEG content?

We found remarkable differences in both the expression of alkaline phosphatase and the degree of mineralization between the diblock copolymers and the standard materials. However, there was no direct correlation between the Me.PEG content and the expression of osteoblastic markers. While we could not find significant differences in alkaline phosphatase activity in the group of the diblock copolymers, the amount of mineralization on Me.PEG2-PLA40 films was significantly enhanced over the other types of Me.PEG-PLA. This suggests that a reasonable number of attaching cells is necessary for matrix formation and its mineralization. Thus, in the investigated range of 5 to 20% Me.PEG, the presence of Me.PEG, and only to a minor degree its content, seems to be important for the improved differentiation of the cells on a per-cell basis. However, to enhance matrix formation and its mineralization a low Me.PEG content that allows for improved cell adhesion might be advantageous.

Numerous studies have been published on the subject of protein adsorption to PEG-grafted polymers and its correlation with cell adhesion [18,3,19]. These studies showed that protein adsorption and cell adhesion to polymer surfaces can be diminished or even prevented by high-density grafting with PEG. This effect was more pronounced with increasing molecular weight of PEG and depended on the investigated protein and the cell type [18]. A study on the adsorption of fibronectin [19] to PEG grafted surfaces revealed a linearly decreasing amount of adsorbed protein with increasing PEG content. However, studies on cell behavior on PEG-grafted surfaces after adsorption of serum proteins [3,19, 20, 21] revealed a non linear relation

to the PEG density. Compared to nongrafted or densely grafted surfaces, low PEG-grafting favored focal contact formation [3] and migration [19]. Tziampazis et al. [19] tried to elucidate this phenomenon, utilizing an ELISA of fibronectin bioactivity after adsorption. Total fibronectin bioactivity remained unchanged with increasing PEG content while normalization to the amount of adsorbed fibronectin revealed an increasing specific bioactivity with increasing PEG contents until a limiting PEG content was reached. A further increase in PEG content caused a sharp drop in the bioactivity of the protein. The authors suggested that the increased specific bioactivity resulted from a protein configuration which might have changed from multiple site coil-substrate interactions at hydrophobic surfaces to fewer interactions on PEG-grafted substrates, which in turn might have enhanced the availability of integrin-binding domains to attaching cells [19]. Similarly Altankov et al. postulated that the conformational changes of adsorbed fibronectin were due to a combination of hydrophobic interactions and opposing lateral forces of the grafted PEG chains [3]. Thus, PEG variant materials were considered to regulate the conformation of adsorbed cell adhesion proteins that might affect cell function [19].

Several other changes in surface properties may also be considered responsible for the improved cell differentiation on Me.PEG-PLA: One additional influence on cell function could be the changed mechanical properties of the surface which is supposed to be gel-like under cell culture conditions when PEG is present. Additionally, AFM (atomic force microscopy) investigations proved the surface of Me.PEG-PLA films to be considerably rough [13]. Roughness of surfaces is known to improve cell differentiation of osteoblasts [22, 23].

Each of the mentioned changes of surface properties might have caused modified cell/biomaterial interactions which are known to induce changes in cytoskeletal filament assembly and, thus, changes in gene expression and cell function [22,24,25,26]. We conclude that changes in cell/biomaterial interactions led to reduced cell adhesion and cell spreading but may have also caused the remarkable increase in cell differentiation.

In summary, we have shown that diblock copolymers consisting of Me.PEG and a relevant polyester allow for control of cell adhesion and positively influence cell differentiation. In examining a range of diblock copolymers with different PEG contents, we

found materials that allowed for sufficient cell attachment and are considered promising for tissue-engineering purposes.

6 Conclusion

The content of PEG in the diblock copolymer apparently controlled protein adsorption and consequently cell adhesion mediated by adsorbed serum proteins. We found reduced proliferation of rat marrow stromal cells attached to the Me.PEG-PLA films, probably because of reduced cell spreading relative to PLA, PLGA and TCPS. However, differentiation to the osteoblastic phenotype as determined by ALPase activity and the degree of mineralization of the extracellular matrix were significantly improved over all the other materials. Enhanced bioactivity of adsorbed adhesion proteins due to conformational changes might be responsible for this phenomenon. Diblock copolymers made of PEG and PLA are thus attractive materials for bone tissue engineering, as they allow for control of protein adsorption and improve osteoblastic cell differentiation.

7 Acknowledgements

This work was supported in part by a grant from the National Institutes of Health (R01 DE13031). Special thanks are due to Dr. Johann Vancea (Department of Physics, University of Regensburg, Germany) for the XPS measurements, Prof. Dr. Norbert Lehn (Department of Medizinische Mikrobiologie and Hygiene, University Clinic, Regensburg) and Dr. Otto Gleich, (ENT-Department, University Clinic, Regensburg) for providing facilities.

8 References

1. Ishaug, S. L., Crane, G. M., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **36**, 17 - 28.

2. Freed, L. E., Grande, D. A., Lingbin, Z., Emmanual, J., Marquis, J. C., and Langer, R. (1994). Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **28**, 891 - 899.

3. Altankov, G., Thom, V., Groth, T., Jankova, K., Jonsson, G., and Ulbricht, M. (2000). Modulating the biocompatibility of polymer surfaces with poly(ethylene glycol): Effect of fibronectin. *J.Biomed.Mater.Res.* **52**, 219 - 230.

4. Webb, K., Hlady, V., and Tresco, P. A. (2000). Relationships among cell attachment, spreading, cytoskeletal organization, and migration rate for anchorage-dependent cells on model surfaces. *J.Biomed.Mater.Res.* **49**, 362 - 368.

5. Altankov, G., Grinnell, F., and Groth, T. (1996). Studies on the biocompatibility of materials: fibroblast reorganization of substratum-bound fibronectin on surfaces varying in wettability. *J.Biomed.Mater.Res.* **30**, 385 - 391.

6. Amiji, M. and Park, K. (1992). Prevention of protein adsorption and platelet adhesion on surfaces by PEO/PPO/PEO triblock copolymers. *Biomaterials* **13**, 682 - 692.

7. Hubbell, J. A. (1995). Biomaterials in tissue engineering. *Biotechnology N.Y.* **13**, 565 - 567.

8. Gref, R., Luck, M., Quellec, P., Marchand, M., Dellacherie, E., Harnisch, S., Blunk, T., and Muller, R. H. (2000). 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids Surf., B* **18**, 301 - 313.

9. Sofia, S. J., Premnath, V., and Merrill, E. W. (1998). Poly(ethylene oxide) Grafted to Silicon Surfaces: Grafting Density and Protein Adsorption. *Macromolecules* **31**, 5059 - 5070.

10. Göpferich, A., Peter, S. J., Lucke, A., Lu, L., and Mikos, A. G. (1999). Modulation of marrow stromal cell function using poly(D,L-lactic acid)-block-poly(ethylene glycol)-monomethyl ether surfaces. *J.Biomed.Mater.Res.* **46**, 390 - 598.

11. Bazile, D., Prud'homme, C., Bassoullet, M. T., Marlard, M., Spenlehauer, G., and Veillard, M. (1995). Stealth Me.PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system. *J.Pharm.Sci.* **84**, 493 - 498.

12. Lucke, A., Tessmar, J., Schnell, E., Schmeer, G., and Goepferich, A. (2000). Biodegradable poly(D,L-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymers: Structures and surface properties relevant to their use as biomaterials. *Biomaterials* **21**, 2361 - 2370.

13. Fischbach, C., Tessmar, J., Lucke, A., Schnell, E., Schmeer, G., Blunk, T., and Goepferich, A. (2001). Does UV irradiation affect polymer properties relevant to tissue engineering? *Surface Science* **491**, 333 - 345.

14. Ishaug, S. L., Yaszemski, M. J., Bizios, R., and Mikos, A. G. (1994). Osteoblast function on synthetic biodegradable polymers. *J.Biomed.Mater.Res.* **28**, 1445 - 1453.

15. West, D. C., Sattar, A., and Kumar, S. (1985). A simplified in situ solubilization procedure for the determination of DNA and cell number in tissue cultured mammalian cells. *Anal.Biochem.* **147**, 289 - 295.

16. Ishaug-Riley S.L., Crane-Kruger G.M., Yaszemski, M. J., and Mikos, A. G. (1998). Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* **19**, 1405 - 1412.

17. Moursi, A. M., Damsky, C. H., Lull, J., Zimmerman, D., Doty, S. B., Aota, S., and Globus, R. K. (1996). Fibronectin regulates calvarial osteoblast differentiation. *J.Cell Sci.* **109**, 1369 - 1380.

18. Du, H., Chandaroy, P., and Hui, S. W. (1997). Grafted poly-(ethylene glycol) on lipid surfaces inhibits protein adsorption and cell adhesion. *Biochim. Biophys.Acta* **1326**, 236 - 248.

19. Tziampazis, E., Kohn, J., and Moghe, P. (2000). V. PEG-variant biomaterials as selectively adhesive protein templates: model surfaces for controlled cell adhesion and migration. *Biomaterials* **21**, 511 - 520.

20. Steele, J. G., Johnson, G., Griesser, H. J., and Underwood, P. A. (1997). Mechanism of initial attachment of corneal epithelial cells to polymeric surfaces. *Biomaterials* **18**, 1541 - 1551.

21. Thomas, C. H., McFarland, C. D., Jenkins, M. L., Rezania, A., Steele, J. G., and Healy, K. E. (1997). The role of vitronectin in the attachment and spatial distribution of bone-derived cells on materials with patterned surface chemistry. *J.Biomed.Mater.Res.* **37**, 81 - 93.

22. Boyan, B. D., Sylvia, V. L., Liu, Y., Sagun, R., Cochran, D. L., Lohmann, C. H., Dean, D. D., and Schwartz, Z. (1999). Surface roughness mediates its effects on osteoblasts via protein kinase A and phospholipase A2. *Biomaterials* **20**, 2305 - 2310.

23. Kieswetter, K., Schwartz, Z., Hummert, T. W., Cochran, D. L., Simpson, J., Dean, D. D., and Boyan, B. D. (1996). Surface roughness modulates the local production of growth factors and cytokines by osteoblast-like MG-63 cells. *J.Biomed.Mater.Res.* **32**, 55 - 63.

24. Mooney, D. J., Langer, R., and Ingber, D. E. (1995). Cytoskeletal filament assembly and the control of cell spreading and function by extracellular matrix. *J.Cell Sci.* **108**, 2311 - 2320.

25. Ireland, G. W., Dopping, Hepenstal P., Jordan, P., and O'Neill, C. (1987). Effect of patterned surfaces of adhesive islands on the shape, cytoskeleton, adhesion and behaviour of Swiss mouse 3T3 fibroblasts. *J.Cell Sci.Suppl.* **8**, 19 - 33.

26. Martin, J. Y., Dean, D. D., Cochran, D. L., Simpson, J., Boyan, B. D., and Schwartz, Z. (1996). Proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63) cultured on previously used titanium surfaces. *Clin.Oral Implants.Res.* **7**, 27 - 37.

Chapter 7

Mediating Cell-Biomaterial Interactions:

Instant modification of `stealth` surfaces with a cyclic αvβ3/αvβ5 integrin subtype specific RGD-peptide

E. Lieb [1*], M. Hacker [1*], J. Tessmar [2], L.A. Kunz-Schughart [3], J. Fiedler [4], C. Dahmen [5], U. Hersel [5], H. Kessler [5], M.B. Schulz [1, 6], A. Göpferich [1]

∗ These authors equally contributed to the paper.

[1] Department of Pharmaceutical Technology, University of Regensburg, Universitaetsstr. 31, 93040 Regensburg, Germany

[2] Department of Bioengineering, Rice University, 6100 Main, Houston, Texas 77005-1892, USA

[3] Institute of Pathology, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

[4] Department of Orthopaedics, Div. for Biochem. of Joint Diseases, University of Ulm, Oberer Eselsberg 45, 89081 Ulm, Germany

[5] Department of Organic Chemistry / Biochemistry, Technical University Munich, Lichtenbergstr. 4, 85747 Garching, Germany

[6] Department of Pharmaceutical Technology, University of Graz, Schubertstr. 6, 8010 Graz, Austria

to be submitted to Biomaterials

1 Abstract

One key interest in biomedical materials research, specifically in the fields of tissue engineering and implant technology, is the control of the interaction between the biomaterial surfaces and attaching cells. One promising strategy to approach this aim involves the covalent grafting of adhesion peptides containing the integrin binding tripeptide RGD to polymers on which protein adsorption – mediating unspecific cell adhesion - is basically suppressed. This study demonstrated the grafting of an amine-reactive diblock copolymer, N-succinimidyl tartrate monoamine poly(ethylene glycol)-*block*-poly(*D,L*-lactic acid) (ST-NH-PEG-PLA), with an RGD-peptide in an instant procedure. To this end, films of these recently developed derivatives of 'stealth' poly(*D,L*-lactic acid)-poly(ethylene glycol)-monomethyl ether (Me.PEG-PLA) copolymers were modified by incubation with a buffered solution of an αvβ3/αvβ5 integrin subtype specific cyclic RGD-peptide cyclo(-Arg-Gly-Asp-*D*-Phe-Lys-) [c(-RGDfK-)]. A cell adhesion study with human osteoblasts revealed significantly increased cell spreading and cell count on these RGD-modified surfaces compared to the controls. Furthermore, the cell adhesion of rat bone marrow stromal cells on such RGD-modified polymer films and on RGD-modified glass surfaces providing a higher peptide density was investigated. Stromal cell adhesion was only enhanced on RGD-modified glass. To illuminate the divergence between the two investigated cell types, the αvβ5 integrin expression was determined by immunofluorescence microscopy.

2 Introduction

Whenever biomaterials are applied in implant technology or tissue engineering, precise control over cell adhesion on the material surface is necessary to ensure physiological integration into the surrounding tissue or the development of functional tissue, respectively [1-5]. In general, cell adhesion is mediated by integrins, heterodimeric transmembrane cell receptors comprised of non-covalently bound α- and β-subunits, which selectively bind to different proteins of the extracellular matrix (ECM) and are often cell type specific in their composition [6,7]. Since the identification of the tripeptide sequence Arg-Gly-Asp (RGD) as an integrin binding site of such ECM proteins, a plethora of small adhesion peptides (RGD-peptides) has been synthesized. Flanking the RGD motif with different amino acids, as well as varying the backbone conformation of the peptides, are strategies to obtain integrin receptor subtype specific peptides [8-10]. Covalently linking such adhesion peptides to biomaterials is a widely accepted approach to improve a material's biocompatibility, biological activity and its interactions with cells [11-14]. Biomaterials used in such attempts are preferably hydrophilic to suppress protein adsorption on their surfaces, a process which is accompanied by unspecific cell adhesion and other adverse effects [15]. Therefore, various hydrogel forming polymers, which provide the required functional groups, have been modified with RGD-peptides and used as three-dimensional cell carriers in tissue engineering applications [13,16-18]. Hydrogels, however, inherently have a low mechanical strength and limited macroporous structure, which limits the permeability of the structures, and thus also medium convection. These characteristics make hydrogels suboptimal for use in implant technology and the engineering of hard tissue. As an alternative, non-swelling, lipophilic polymers have been investigated as materials for the fabrication of implants or scaffolds in the engineering of hard tissue [19,20]. Covalently linking RGD-peptides to lipophilic polymers, however, is often a laborious procedure. Since most of these materials lack functional groups on their surface, these must be introduced either physically or chemically [21], for example by blending or coating with poly(L-lysine) [22] or poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) [23], plasma deposition [24], track-etching [25], co-polymerization [16], oxidation or alkaline treatment [26,27]. Subsequently, one has to employ a suitable activation

or chemoselective ligation strategy [28,29] to anchor a peptide sequence to the functionalized polymer surface [21]. By examining the properties of both hydrogels and lipophilic polymers, it is possible to determine the three critical regions of the ideal implant or scaffold material: a lipophilic part to provide water insolubility for processing, a hydrophilic region to suppress unspecific protein adsorption and at least one functional group for modification with adhesion peptides. Diblock copolymers, consisting of a hydrophilic and lipophilic chain, such as poly(*D,L*-lactic acid)-poly(ethylene glycol)-monomethyl ether (Me.PEG-PLA), which are non-conductive for protein and peptide adsorption and therefore applied as 'stealth' biomaterials in numerous applications [30-32], contain two of the three desired regions. Moreover, we have recently shown that protein adsorption and the adhesion of rat marrow stromal cells (rMSC) derived from bone marrow can be controlled via the length and content of the Me.PEG block in Me.PEG-PLA diblock copolymers [32]. Adhesion and differentiation experiments with rMSCs proved the potential of these materials for tissue engineering applications. To enable a comfortable modification with adhesion peptides, mono amine derivatives (H_2N-PEG-PLA) of the Me.PEG-PLA diblock copolymers were made amine-reactive by covalent attachment of disuccinimidyl tartrate [33]. These N-succinimidyl tartrate monoamine poly(ethylene glycol)-*block*-poly(*D,L*-lactic acid) (ST-NH-PEG-PLA) copolymers (Fig. 1) are designed to covalently bind peptides from sterile aqueous solutions to preformed polymer surfaces with moderate conditions. In the present study, we bound the αvβ5/αvβ3 integrin subtype specific cyclic RGD-peptide cyclo(-Arg-Gly-Asp-*D*-Phe-Lys-) [c(-RGDfK-)] (Fig. 2) to the activated polymer [34,35] using an incubation procedure adjusted to the needs of the polymer and solid phase modification. Covalent modification of the polymer films with the RGD-peptide was investigated by the adhesion of human osteoblasts, which are known to strongly express the αvβ5 integrin subtype [36], mediating cell adhesion to vitronectin. We determined the number and spreading of adhering human osteoblasts as a measure of the cell biomaterial interactions. Additionally, we investigated cell adhesion on various controls, in order to distinguish the contributions of unspecific and charge-dependent cell adhesion from specific interactions with the RGD-modified surfaces. Mature cells, such as osteoblasts, however, may not be an ideal source for tissue engineering applications because of their limited proliferative capacity. Most tissue engineering applications require cells, which are easily accessible and expandable in culture, for example

bone marrow stromal cells (MSCs). Since the integrin composition on rMSCs is not completely characterized yet, we studied their αvβ5-integrin expression using fluorescence microscopy and investigated the adhesion of rMSCs on the c(-RGDfK-) grafted polymer, as well as on glass surfaces, the latter providing a higher density of bound adhesion peptides. Moreover, we attempted to enhance the expression of the αvβ5-integrin on rMSCs by supplementing dexamethasone [37] or differentiating medium.

The overall objective of this study was to specifically influence the attachment of osteoblasts and rMSC by amine-reactive diblock copolymer surfaces via modification with a cyclic RGD-peptide in an instant procedure in view of implant technology and tissue engineering applications.

3 Materials and Methods

3.1 Polymer synthesis and characterization

The amine-reactive copolymer ST-NH-PEG2-PLA20 (Fig. 1), consisting of a 2 kDa poly(ethylene glycol)-monoamine (H_2N-PEG) block and a 20 kDa poly(*D,L*-lactic acid) (PLA) block, was synthesized as described by Tessmar et al. [33]. In brief, 2.0 g of poly(ethylene glycol)-mono amine were reacted with 20.0 g of *D,L*-dilactide in a ring-opening polymerization using stannous 2-ethylhexanoate as catalyst. The resulting H_2N-PEG2-PLA20 was isolated and coupled with disuccinimidyl tartrate to obtain ST-NH-PEG2-PLA20, which was stored under vacuum until further use.

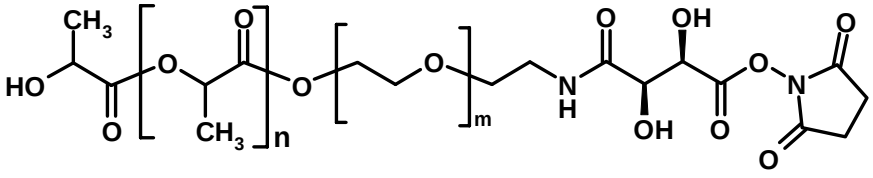


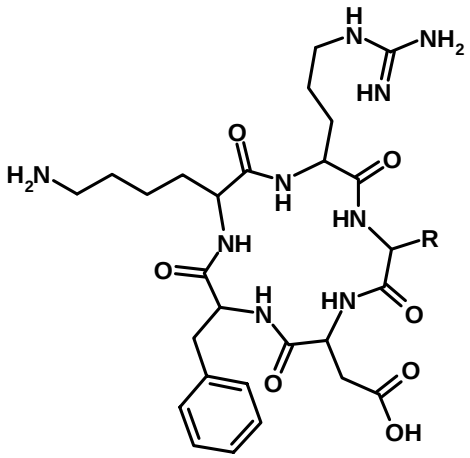
Figure 1: Chemical structure of ST-NH-PEG2-PLA20.

The molecular weights of H_2N-PEG2-PLA20 and ST-NH-PEG2-PLA20 were confirmed by gel permeation chromatography (GPC) as previously described [33]. Prior to every cell

adhesion study, the polymer was incubated with the amine-reactive fluorescent dye 5-aminoeosin and GPC was performed to assess the reactivity of the ST-NH-PEG2-PLA20 [33].

3.2 Peptide synthesis

The peptides cyclo(-Arg-Gly-Asp-*D*-Phe-Lys-) [c(-RGDfK-)] and cyclo(-Arg-Ala-Asp-*D*-Phe-Lys-) [c(-RADfK-)] (Fig. 2) were synthesized as described by Haubner, et. al.[34].



R = H *cyclo*(-Arg-Gly-Asp-*D*-Phe-Lys-)
c(-RGDfK-)

R = CH_3 *cyclo*(-Arg-Ala-Asp-*D*-Phe-Lys-)
c(-RADfK-)

Figure 2: Chemical structure of *cyclo*(-Arg-Gly-Asp-*D*-Phe-Lys-) [c(-RGDfK-)] and of *cyclo*(-Arg-Ala-Asp-*D*-Phe-Lys-) [c(-RADfK-)].

3.3 Cell culture

3.3.1 Human osteoblasts

Osteoblasts were harvested from cancellous human bone fragments extracted during routine hip and knee replacements following a protocol published by Mayr-Wohlfart et al. [38]. The fragments were dissected, minced, and rinsed several times with physiological saline solution to remove blood components. For collagenase digestion, the bone chips were incubated in serum-free DMEM (Biochrom, Berlin, Germany) containing 1% collagenase

(type IV; Sigma Chemical Company, St Louis, Missouri) at 37°C for 2 hours. The bone chips were then washed with serum-containing medium to inhibit further collagenase digestion and plated in six-well plates. The cells were incubated with calcium free DMEM, which was supplemented with 10% heat-inactivated fetal calf serum (FCS, Biochrom), 2 mM L-glutamine (Biochrom), 1% penicillin/streptomycin (Biochrom), 1% amphotericin B (Biochrom) and 0.8% certomycin 50 (Essex Pharma GmbH, Munich, Germany). The cell cultures were incubated at 37° in saturated humidity with 5% CO_2. The culture medium was changed twice a week. After about four to five weeks, a confluent monolayer of primary human osteoblasts was obtained. Cells were passaged with 0.25% trypsin in ethylenediaminetetraacetic acid (Life Technologies, Karlsruhe, Germany). Further cultivation was performed in DMEM with 10% fetal bovine serum (FBS, Gemini Bio-Products Inc., Calabasas, California, USA), 1% penicillin/streptomycin (Sigma, Taufkirchen, Germany) and ascorbic acid 50 mg/l (Sigma). For cell adhesion experiments, cells were used following the third passage.

Apart from the aforesaid culture conditions, human osteoblasts were exposed to culture medium supplemented with dexamethasone (10^{-7} M) (Sigma) for 48 hours in order to investigate if the expression of the αvβ5 integrin can be stimulated by dexamethasone, as described by Cheng et al. [37]. Since the protocol of Cheng et al. [37] only involved human cells, this experiment was conducted to serve as a positive control for the same experiment performed with rMSCs (**3.3.2**). The expression of the αvβ5 integrin was visualized by immunofluorescence following incubation with the fluorescein-labeled antibody. Human osteoblasts, which had been passaged six times, were utilized for these experiments.

3.3.2 Rat marrow stromal cells

Rat marrow stromal cells (rMSCs) were obtained from 6-week old male Sprague-Dawley rats (weight: 170 – 180 g , Charles River Laboratories, Sulzfeld, Germany). Cell isolation from the femur and tibia followed a protocol published by Ishaug et al. [19]. Following marrow isolation and dispersion, cells were centrifuged at 1200 rpm (259 x g) for 7 min. The resulting cell pellet was resuspended in primary medium [α-MEM (Sigma); 10% FBS (HyClone, Perbio Science, Germany); 1% penicillin/streptomycin (Sigma); 0.5% L-glutamine 200 mM (Sigma)] and seeded into tissue culture polystyrene (TCPS) flasks. Plated

cells were incubated at 37°C in saturated humidity with 5% CO_2. On the third day of expansion, the flasks were rinsed twice with phosphate buffer (PBS, Life Technologies) to remove the nonadherent cells. Medium was changed every 2 - 3 days and cells were subcultured (0.25% trypsin in ethylenediaminetetraacetic acid, Life Technologies) for cell adhesion experiments when 80% confluence was reached.

In a further experiment, rMSCs were treated in two different ways in order to stimulate αvβ5 integrin expression, which is less prominent on rMSCs compared to human osteoblasts. Firstly, dexamethasone (10^{-7} M) was administered to primary medium for a duration of 48 hours before the first subculturing according to the protocol of Cheng et al. [37]. For the second attempt to stimulate the expression of the αvβ5 integrin, we differentiated the rMSCs towards the osteoblastic phenotype by administering complete medium, which was supplemented with dexamethasone [10^{-8} M], ascorbic acid [50 mg/l] (Sigma) and β-glycerophosphate [7 mM] (Sigma). Cells were exposed to this differentiating medium from day 3 after marrow isolation to day 11 and then subcultured for investigations of the integrin expression. The expression of the αvβ5 integrin was visualized by immunofluorescence.

3.4 Experimental setup

Glass object slides (Super-Frost Plus, Menzel-Gläser, Braunschweig, Germany) were used as carriers for the cell adhesion experiments. To create a definite area for the cell adhesion experiments, two rings (diameter: 1.5 cm) were engraved on the glass object slides using a diamond drill. After amination of the glass surface **(3.4.1)** this area was either coated with polymer films **(3.4.2)** or activated by gradual reaction with succinic anhydride and N-hydroxysuccinimide **(3.4.3)**. Glass object slides, covered with polymer films, were dried for the first 5 min after film casting under a petri dish - decelerating solvent evaporation - to ensure the formation of a smooth polymer film surface. Afterwards, the surrounding groove on the slides was covered with a lipophilic barrier (Dako-Pen, Dako, Glostrup, Denmark) so that fluids remained in the engraved area during the incubation and cell adhesion study. Finally, glass object slides with polymer films were vacuum-dried for 12 hours. The surface modification is described in **3.5,** the cell adhesion study in **3.6** and the investigation of cell shape in **3.7**. The experimental set-up is illustrated in Figure 3.

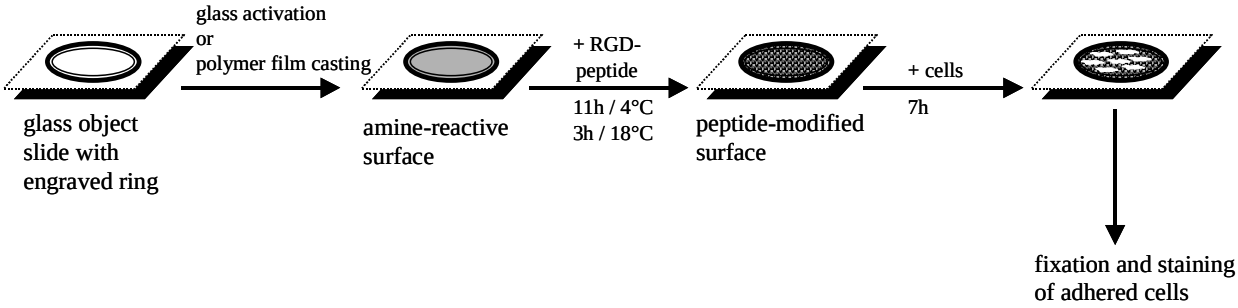


Figure 3: Illustration of the experimental set-up.

3.4.1 Amination of glass object slides

Having engraved the two rings, the glass object slides were carefully washed using a detergent followed by intensive rinsing with water. Finally, the slides were washed with acetone to remove any lipids. All slides were autoclaved. For amination, the slides were submerged in a stirred solution of 2% (v/v) 3-aminopropyltriethoxysilane (ABCR GmbH & Co. KG, Karlsruhe, Germany) in absolute ethanol for 30 min. After careful rinsing with absolute ethanol, the slides were annealed in an oven for another 30 min at 90°C. The aminated slides were stored in a desiccator under vacuum.

3.4.2 Polymer film casting

For film casting, solutions of 50 mg ST-NH-PEG2-PLA20 diblock copolymer in 1 ml acetone (Merck, Darmstadt, Germany) were prepared and subsequently 20 µl of the polymer solution, which corresponds to a mass of 1 mg polymer, were dropped and distributed on each marked area of 1.77 cm^2 on aminated glass.

3.4.3 Glass activation

To create amine-reactive groups on the glass surface similar to those present on the polymers, the aminated glass object slides were first reacted with succinic anhydride (Fluka Chemicals, Buchs, Switzerland). In a second step, the reactive N-hydroxysuccinimide ester group was generated by reaction with N-hydroxysuccinimide (Fluka Chemicals) in the presence of N,N'-dicyclohexylcarbodiimide (Fluka Chemicals). In a typical preparation, 30 slides were submerged in 600 ml dry 1,4-dioxane. After the addition of 5 g of succinic

anhydride, the system was allowed to react for 16 hours at room temperature. The slides were rinsed with dry 1,4-dioxane and transferred into another 600 ml of dry 1,4-dioxane and 4 g of N-hydroxysuccinimide were added. Cooling the system on an ice-bath, 5 g of N,N'-dicyclohexylcarbodiimide were added. The system was reacted for 18 hours under constant stirring. Finally, the slides were rinsed with acetone to remove the reaction mixture and precipitated dicylohexyl urea. The amine-reactive slides were dried and stored under vacuum.

3.5 Surface modification with RGD-peptides

For surface modification (Table 1), both activated polymer and glass surfaces were incubated with 250 µl of an aqueous solution of 2 mg/ml RGD-peptide c(-RGDfK-) (2.4 mM), in order to achieve a covalent anchoring of the peptides to the N-hydroxysuccinimide ester of the polymer or the activated glass. Incubation with 0.5 mg RGD-peptide per 1 mg polymer – the amount of polymer coating the enclosed area of 1.77 cm^2 - corresponds to a five-fold surplus versus the calculated amine-reactive groups on the polymer surface. The activated polymer and glass surfaces were variously treated to form controls: by incubation with the non-binding c(-RADfK-), with the reaction buffer (0.15 M $NaHCO_3$, pH=8) and with the passivating reagent ethanol amine (stop). Ethanol amine was used for surface modification in order to favor aminolysis and to avoid the formation of charged groups on the surface caused by the hydrolysis of the N-hydroxysuccinimide ester. Each procedure was performed for 11 hours at 4°C to favor aminolysis, and subsequently for 3 hours at 18°C to complete the reaction, during which the object slides were kept in petri-dishes. Additionally, we investigated the effect of non-covalently attached RGD-peptide. To this end, surfaces were hydrolyzed by incubation with $NaHCO_3$ buffer for 11 hours (18°C) and subsequently brought in contact with 200 µl solution of c(-RGDfK-) for 3 hours (4°C).

Additionally, we aimed at inhibiting cell adhesion to c(-RGDfK-) grafted surfaces by blocking the corresponding cell surface receptor - αvβ3/αvβ5 integrin - with dissolved c(-RGDfK-) peptide. For that reason, cells were incubated with c(-RGDfK-) containing basal medium before cell seeding. Afterwards, the pretreated cells were seeded onto surfaces, which had been modified with c(-RGDfK-), as described above. This experiment was conducted in

order to investigate if cell adhesion on c(-RGDfK-) modified surfaces is mediated by the corresponding αvβ3/αvβ5 integrin.

A stepwise washing procedure followed the incubation step. First, the incubation solutions were removed and the surfaces were covered with 250 µl $NaHCO_3$ buffer (0.15 M, pH=8) for 15 min. Then the object slides were twice submerged in phosphate buffer (PBS, Life Technologies) and in distilled water to remove remnants of buffer salts from the surfaces.

Table 1: Description of the different surface modifications applied in the cell adhesion study and in the study on cell shape.

Abbreviation	**Reagent/ concentration**	**Incubation time / temperature**	**Material**
Binding RGD-peptide			
c(-RGDfK-)	2.4 mM c(-RGDfK-) *(reaction buffer*)*	11 h at 4°C, 3 h at 18°C	polymer, glass
Control reagents			
c(-RADfK-)	2.4 mM c(-RADfK-) *(reaction buffer*)*	11 h at 4°C, 3 h at 18°C	polymer, glass
hydrolysis	0.15 M $NaHCO_3$ buffer; pH 8.0	11 h at 4°C, 3 h at 18°C	polymer
stop	0.1 M ethanol amine, pH 8.0 *(reaction buffer*)*	11 h at 4°C, 3 h at 18°C	polymer, glass
hydrolysis / c(-RGDfK-)	1.) reaction buffer 2.) 2.4 mM c(-RGDfK-) *(reaction buffer*)*	1.) 11 h at 4°C 2.) 3 h at 18°C	polymer
c(-RGDfK-) / blocked cells	1.) 2.4 mM c(-RGDfK-) *(reaction buffer*)* 2.) 0.24 mM c(-RGDfK-) *(reaction buffer*/medium)*	1.) 11 h at 4°C, 3 h at 18°C 2.) cells + c(-RGDfK-) (30 min, 37°C)	polymer

* reaction buffer: 0.15 M $NaHCO_3$ buffer; pH: 8.0

3.6 Cell adhesion study

In order to investigate cell adhesion on the modified polymer and glass surfaces, 200 µl of a cell suspension containing human osteoblasts or rMSCs at a concentration of 44250

cells/ml was seeded onto each marked area (5000 cells/cm^2, i.e., 8850 cells/marked area) and cells were allowed to attach for 7 hours – a period which allows for complete cell adhesion to the standard cell culture material, TCPS [39,32]. The low seeding density of 5000 cells/cm^2 was chosen to avoid artifacts from cell aggregation. Non-attached cells were then removed by rinsing twice with PBS. The attached cells were fixed with 10% formalin in PBS and cells were stained with an aqueous solution of safranin O (0.5%) (Sigma) before counting. A representative section of each seeded surface was counted and the cell count was extrapolated to the total surface area. All cell adhesion studies were performed under serum-free conditions.

In one control group the human osteoblasts were incubated with serum-free basal medium containing c(-RGDfK-) at a concentration of 0.24 mM c(-RGDfK-) in an incubator (30 min) before seeding on c(-RGDfK-) modified films, in order to block the αvβ3/αvβ5 receptor on the cell surface, as described in **3.5**. During incubation, the cell suspension was gently mixed every 10 min to minimize cell adhesion to the tube wall. Cell seeding was performed as described above. Additionally, the pretreated cells were seeded onto TCPS in order to ensure that cells could undergo non-integrin mediated cell attachment.

3.7 Study on cell shape

The shape of the attached cells was evaluated under a light microscope (Leica DM IRB, Leica Microsystems Wetzlar, Wetzlar, Germany) and documented photographically (Panasonic System Camera, Japan; DYNAX, 600si Classic, Minolta, Ahrensburg, Germany).

To assess the shape of the attached cells by scanning electron microscopy (SEM), a further cell adhesion experiment with human osteoblasts on modified polymer films was performed. For this study, 5000 cells/cm^2 (8850 cells/film) were plated onto ST-PEG2PLA20 films, modified with c(-RGDfK-) and c(-RADfK). After 7 hours adhesion-time in an incubator (37°C, 95% humidity, 5% CO_2), the cells were rinsed with PBS and fixed with glutaraldehyde (2.5 % in PBS) for 15 min. Following repeated rinsing steps, cells were further fixed with an aqueous solution of OsO_4 (1 %) (Roth, Karlsruhe, Germany) for 30 min under ice-cooling. Excess OsO_4 was removed with water. Then, the object-slides were frozen at -80 °C and freeze-dried (Christ Beta 2-16, Martin Christ Gefriertrocknungsanlagen; Osterode am

Harz, Germany). For SEM, samples were mounted on aluminium stubs using conductive carbon tape and coated with gold - palladium (Polaron SC515, Fisons Surface Systems; Grinstead, UK). Photomicrographs were acquired at 10 kV on a DSM 950 (Carl Zeiss; Oberkochen, Germany).

3.8 Fluorescence microscopy (integrin expression)

Immunofluorescence was used in order to investigate the αvβ5 integrin expression on human osteoblasts and rMSCs. Additionally, rMSCs, displaying low amounts of αvβ5 integrin, were treated with dexamethasone, as described in **3.3.2**, in order to increase the expression of the αvβ5 integrin according to Cheng et al. [37]. These authors described that the αvβ5 integrin receptor on human osteoblastic cells is increased by treatment with dexamethasone. Therefore, human osteoblasts were also exposed to medium supplemented with dexamethasone (**3.3.1**) and investigated with regard to a stimulated integrin expression, which served as positive control. In another attempt to increase the expression of the αvβ5 integrin receptor on rMSCs, we administered differentiating medium from day 3 after marrow isolation in order to differentiate the rMSCs to the osteoblastic phenotype (**3.3.2**).

For the immunofluorescence experiments, 10,000 cells per cm^2 were seeded onto 4-well chamber slides (Nalge Nunc International, Lab-Tek II Chamber Slide™ System, Merck Eurolab, Bruchsal, Germany). After 7 hours of adhesion-time, cells were washed with PBS (Life Technologies) and fixed with cold acetone (Merck) for 15 min. Before immunolabelling, cells were incubated with 20% bovine serum albumin in PBS (37°C, 20 min), in order to prevent non-specific antibody binding. Then, cells were incubated with fluorescein-conjugated anti-αvβ5 (fluorescein-conjugated monoclonal antibody, clone: P1F6, dilution: 1 : 50; Chemicon, California, USA) at room temperature for 2h. Mounting was performed with Vectashield (Vector Laboratories, Texas, USA). Images were taken with an Axiovert S1000 microscope (Carl Zeiss, Goettingen, Germany).

3.9 Statistical Analysis

Cell numbers were determined (n = 4) and expressed as means ± standard deviation (SD). Single factor analysis of variance (ANOVA) was used in conjunction with a multiple comparison test (Tukey test) to assess the statistical significance.

4 Results

4.1 Cell adhesion study

The cell adhesion study was conducted on RGD-modified polymer films versus various control groups. Thereby, the cell attachment of two different cell types, i.e. human osteoblasts and rMSCs, was investigated. In addition to polymer films, the cell attachment of rMSCs was examined on RGD-modified glass surfaces versus different controls.

The percentages of cell attachment described in the following sections were normalized to the initial cell seeding density.

4.1.1 Human Osteoblasts

Modified polymer films (Fig. 4):

7 hours after seeding, 70% of the seeded human osteoblasts had attached to c(-RGDfK-) modified ST-NH-PEG2-PLA20 polymer films. The linkage of the non-binding c(-RADfK-) to ST-NH-PEG2-PLA20, however, led to a cell attachment of only 30%. About 10% of the seeded cells attached to the polymer films incubated with reaction buffer and the stop reagent ethanol amine. Hydrolysis of the N-hydroxysuccinimide ester of ST-NH-PEG2-PLA20 using the bicarbonate buffer, followed by incubation with c(-RGDfK-), led to the same low cell attachment as observed on surfaces incubated with buffer and stop reagent. The number of cells attached to ST-NH-PEG2-PLA20 surfaces grafted with c(-RGDfK-) was significantly increased compared to all control groups ($p < 0.01$).

An almost complete suppression of cell attachment was observed ($p < 0.01$) when cells were pretreated with dissolved c(-RGDfK-) before cell seeding. Hence, by blocking the $\alpha v\beta 3/\alpha v\beta 5$ integrin with the c(-RGDfK-) peptide, cell adhesion to c(-RGDfK-) modified

surfaces was prevented. This demonstrated that cell adhesion to these surfaces was mediated by the corresponding αvβ3/αvβ5 integrin.

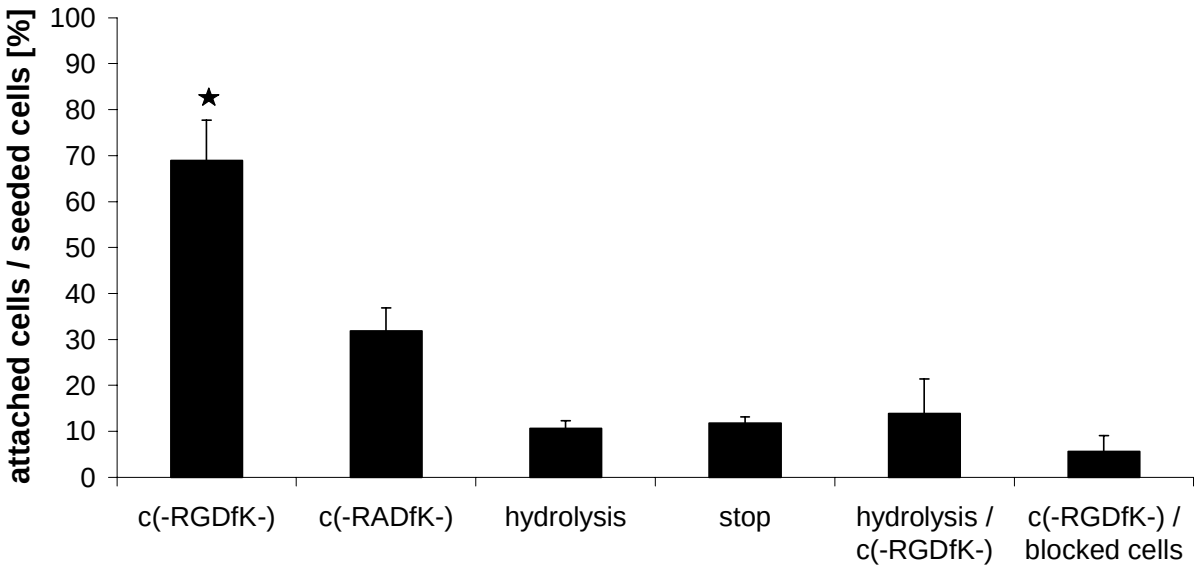


Figure 4: Percentage of cell attachment of human osteoblasts normalized to the initial cell seeding density on modified ST-NH-PEG2-PLA20 surfaces 7 hours after cell seeding. Columns and error bars represent means ± SD for n = 4. The statistical significance was assessed by Tukey-Test. Significance is indicated by a ★.

4.1.2 Rat marrow stromal cells

Modified polymer films (Fig. 5):

No significant influence of the RGD-modified polymer films on rMSC adhesion was observed. RMSC attachment ranging between 40% and 50% was observed on c(-RGDfK-) grafted polymer films as well as on polymer films modified with the control reagents c(-RADfK-) and stop.

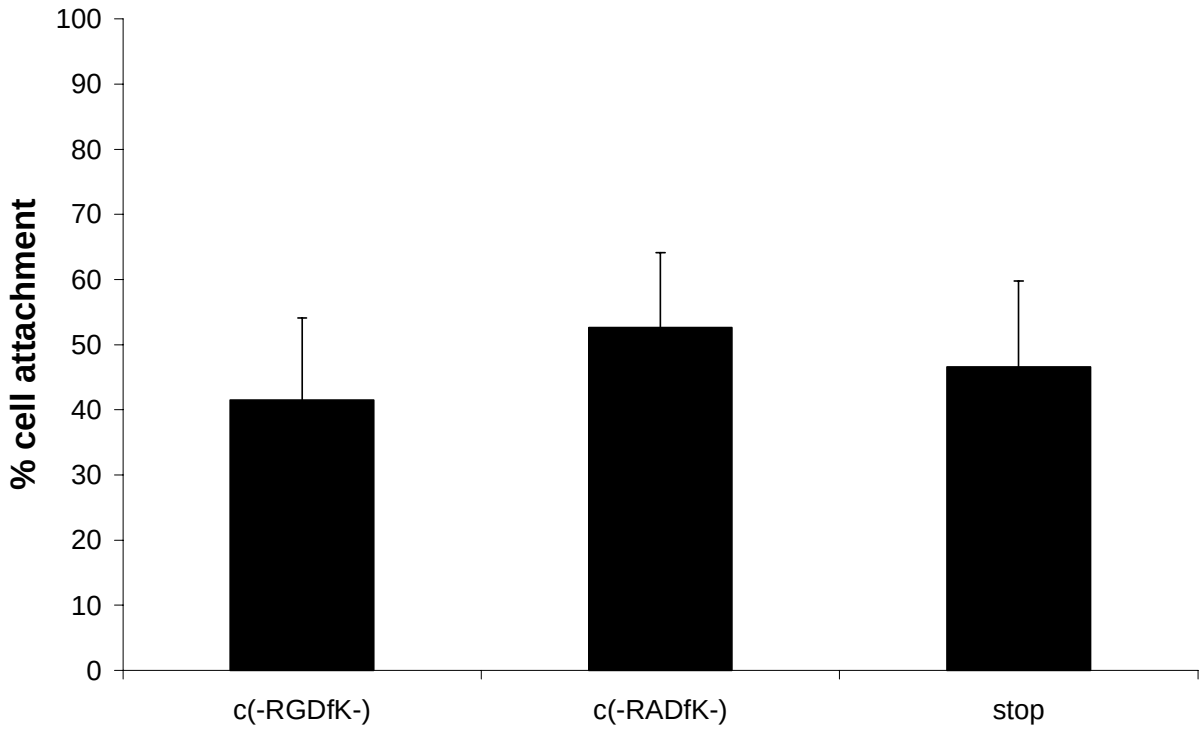


Figure 5: Percentage of cell attachment of rMSCs normalized to the initial cell seeding density on modified ST-NH-PEG2-PLA20 surfaces 7 hours after cell seeding. Columns and error bars represent means ± SD for n = 4.

Modified glass surfaces (Fig. 6):

On c(-RGDfK-) grafted glass surfaces, we found a significantly enhanced attachment of rMSCs (100% cell attachment) compared to surfaces modified with the non-binding c(-RADfK-) (54% cell attachment) or incubated with the stop reagent ethanol amine (46% cell attachment) ($p < 0.05$).

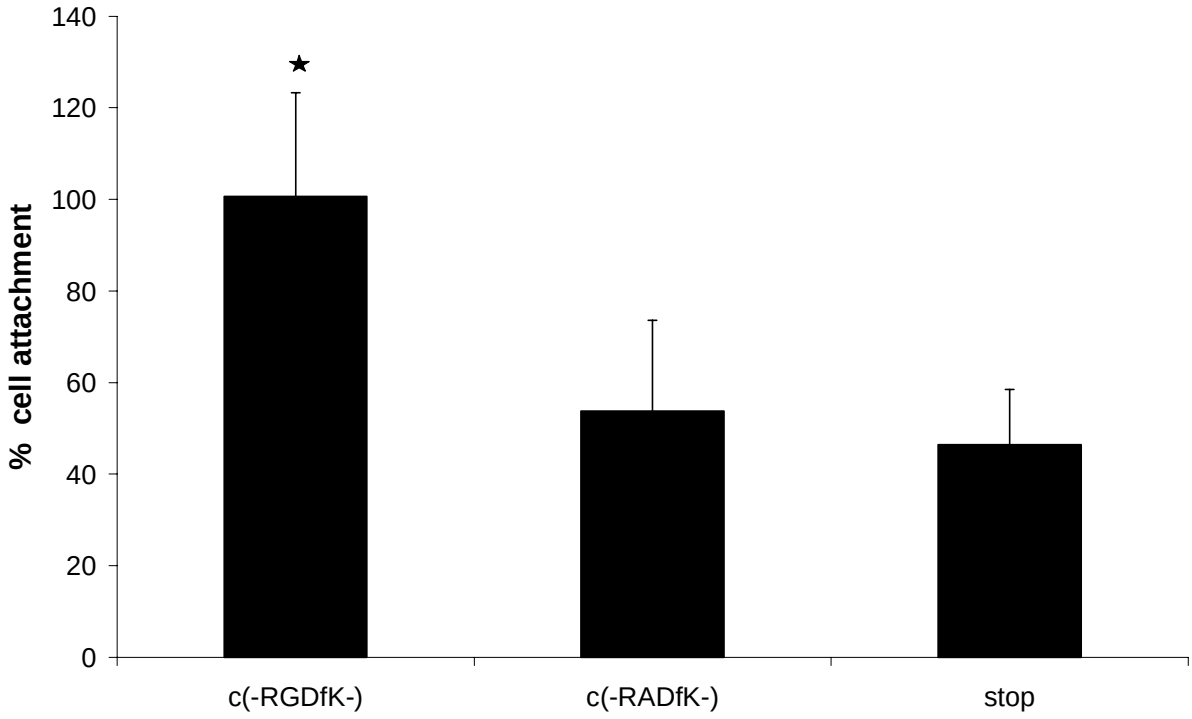


Figure 6: Percentage of cell attachment of rMSCs normalized to the initial cell seeding density on modified aminated glass surfaces 7 hours after cell seeding. Columns and error bars represent means ± SD for n = 4. The statistical significance was assessed by Tukey-Test. Significance is indicated by a ★.

4.2 Study on cell shape

4.2.1 Human osteoblasts

Modified polymer films (Fig. 7; 8 a, b, c; 9):

In good accordance with the determined cell numbers in the cell adhesion study, we observed well-spread cells on the c(-RGDfK-) modified polymer surfaces, indicating a strong cell-biomaterial interaction. Human osteoblasts seeded on surfaces incubated with c(-RADfK-), with the reaction buffer, with ethanol amine or with c(-RGDfK-) after hydrolysis of the reactive N-hydroxysuccinimide ester, however, remained rounded, indicating low cell-biomaterial interaction. Human osteoblasts pretreated with c(-RGDfK-) (0.24 mM), in order to block the integrin receptors, also showed a round cell shape on c(-RGDfK-) modified surfaces, whereas pretreated cells seeded on TCPS spread well. Thus, cell

interaction with c(-RGDfK-) grafted surfaces was inhibited by blocking the corresponding surface receptor, while cell adhesion to TCPS, where cell adhesion was non-integrin mediated, remained unaffected (data not shown).

4.2.2 Rat marrow stromal cells

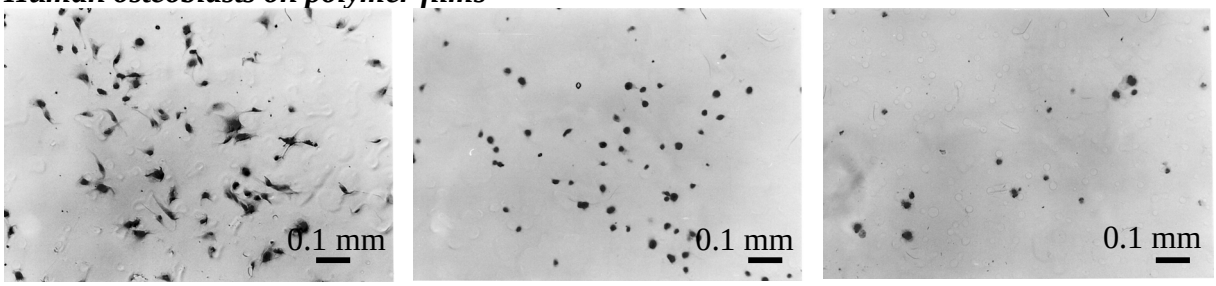
Modified polymer films (Fig. 8 d, e, f):

RMSCs were poorly spread on the RGD-modified polymer films, as well as on the controls. The poor spreading corresponded to the low cell count on these surfaces.

Modified glass surfaces (Fig. 8 g, h, i):

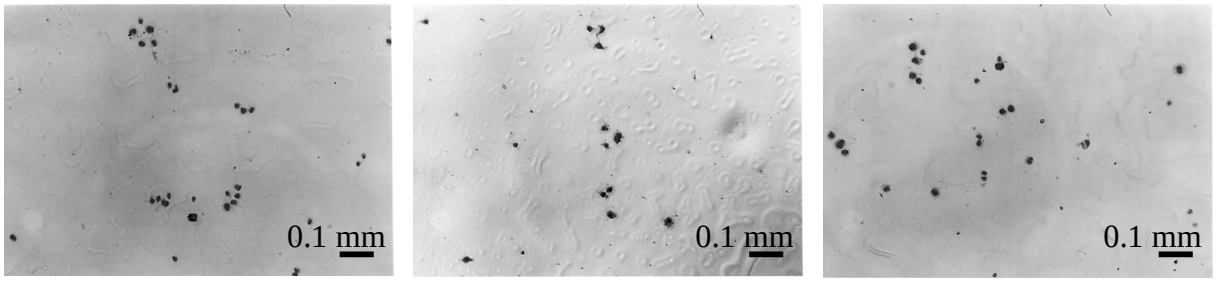
RMSCs were well spread on c(-RGDfK-) modified glass surfaces, which is indicative of a strong cell-surface interaction and corresponded to the determined high cell count on those surfaces. On surfaces incubated with c(-RADfK-) and ethanol amine, rMSCs were poorly spread, according to the low cell count.

Human osteoblasts on polymer films



a) c(-RGDfK-) b) c(-RADfK-) c) hydrolysis

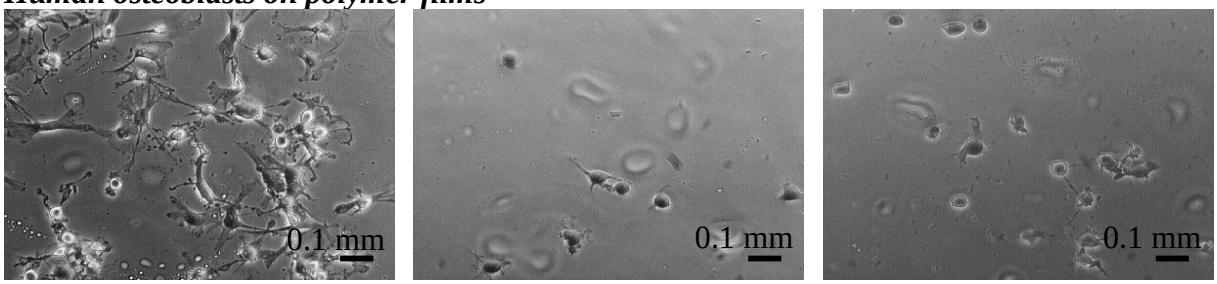
Human osteoblasts on polymer films



d) stop e) hydrolysis / c(-RGDfK-) f) c(-RGDfK-)/blocked cells

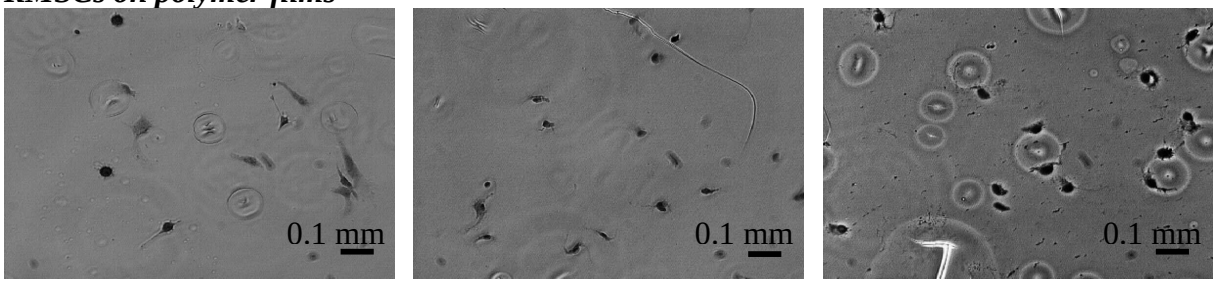
Figure 7: Depiction of the cell shape of human osteoblasts on ST-NH-PEG2-PLA20 surfaces incubated with a) c(-RGDfK-) b) c(-RADfK-) c) reaction buffer d) stop e) reaction buffer / c(-RGDfK-) f) c(-RGDfK-) / blocked cells.

Human osteoblasts on polymer films



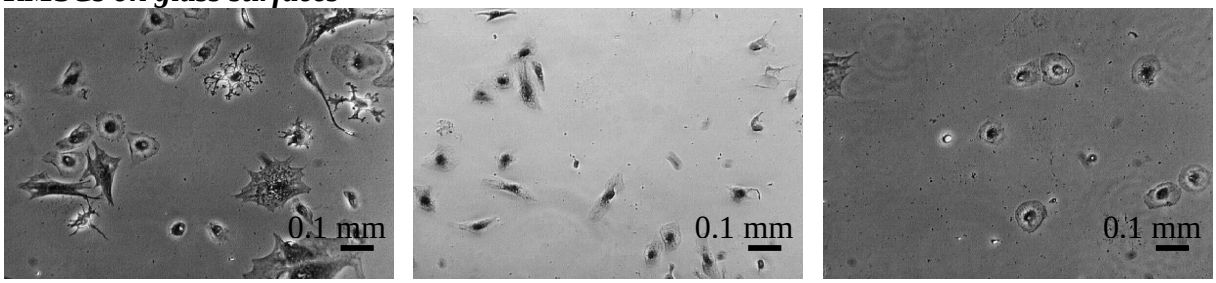
a) c(-RGDfK-) b) c(-RADfK-) c) stop

RMSCs on polymer films



d) c(-RGDfK-) e) c(-RADfK-) f) stop

RMSCs on glass surfaces



g) c(-RGDfK-) h) c(-RADfK-) i) stop

Figure 8: Depiction of the cell shape of human osteoblasts and rMSCs on modified surfaces.

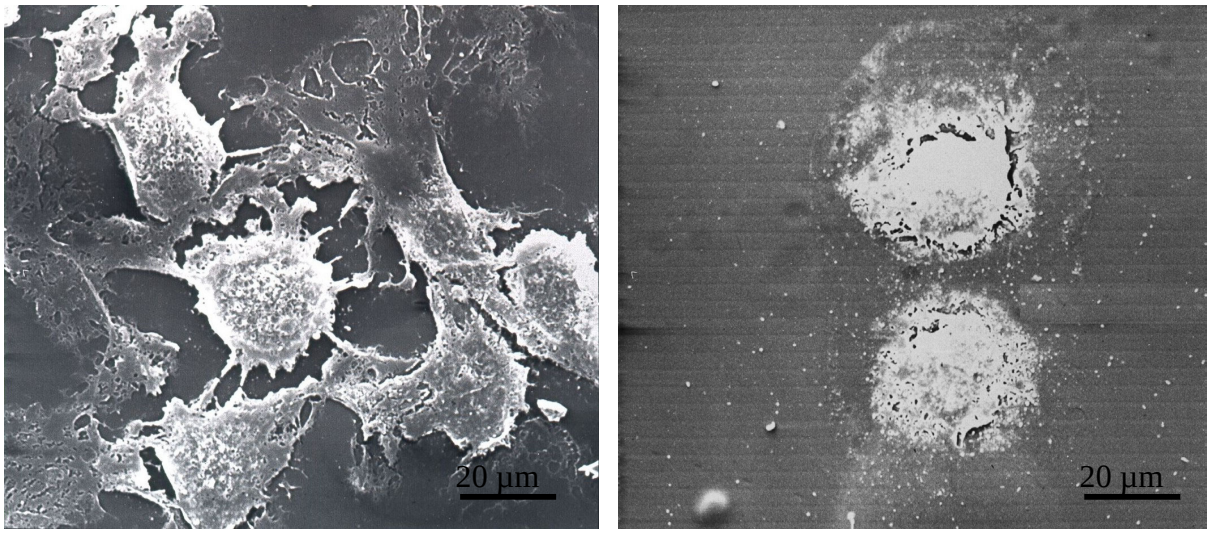
Human osteoblasts on ST-NH-PEG2-PLA20 polymer films incubated with:
a) c(-RGDfK-) b) c(-RADfK-) c) stop

RMSCs on ST-NH-PEG2-PLA20 polymer films incubated with:

d) c(-RGDfK-) e) c(-RADfK-) f) stop

RMSCs on activated glass surfaces incubated with:

g) c(-RGDfK-) h) c(-RADfK-) i) stop

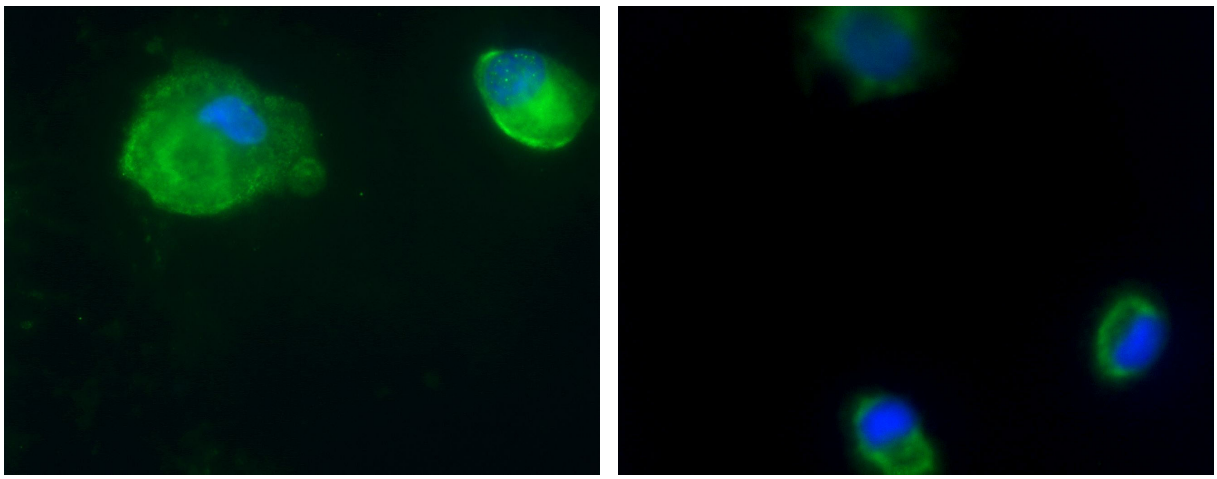


a) c(-RGDfK-) b) c(-RADfK-)

Figure 9: SEM investigations of the cell shape of human osteoblasts on ST-NH-PEG2-PLA20 polymer films modified with a) c(-RGDfK-) b) c(-RADfK-).

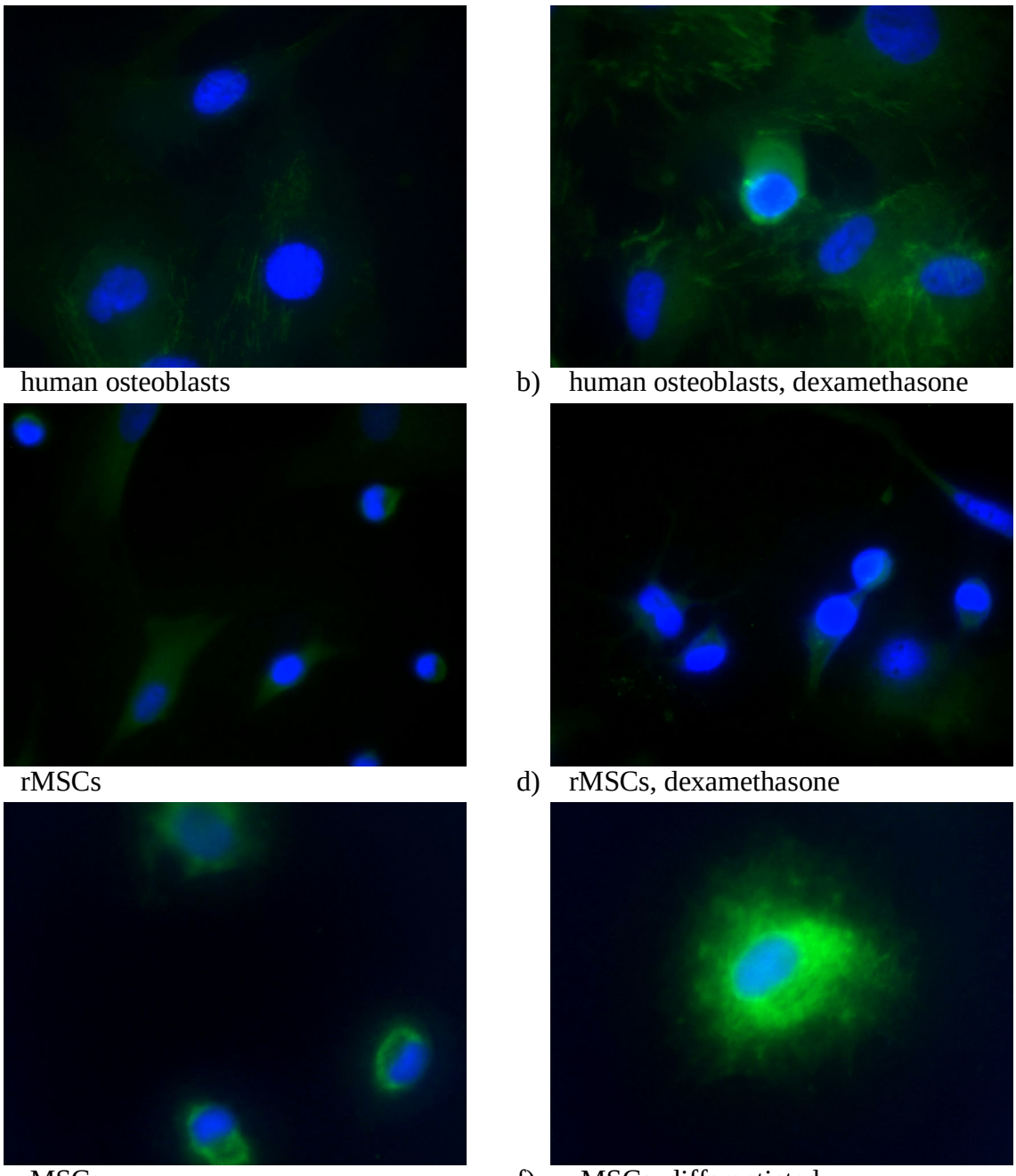
4.3 Fluorescence microscopy (integrin expression)

The presence of the αvβ5 integrin was visualized on human osteoblasts and rMSCs using immunofluorescence with a fluorescein-conjugated αvβ5 specific antibody (P1F6). We could demonstrate that both cell types expressed the αvβ5 integrin receptor, although a higher integrin expression was observed on human osteoblasts (Fig. 10). With regard to the investigation of the αvβ5 integrin expression on rMSCs and human osteoblasts after the 48h exposure to dexamethasone, we found that the rMSCs displayed no enhancement of the αvβ5 integrin, while this integrin was stimulated on human osteoblasts (Fig. 11 a - d). However, exposing rMSCs to complete medium for 9 days, inducing differentiation towards the osteoblastic phenotype, did lead to an increased expression of the αvβ5 integrin (Fig. 11 e, f).



a) human osteoblasts b) rMSCs

Figure 10: Investigation of αvβ5 integrin expression using immunofluorescence a) human osteoblasts b) rMSCs. Green staining: integrins stained with the fluorescein-conjugated αvβ5 specific antibody, blue staining: cell nucleus stained with 4',6-diamidino-2-phenylindole (DAPI).



a) human osteoblasts b) human osteoblasts, dexamethasone

c) rMSCs d) rMSCs, dexamethasone

e) rMSCs f) rMSCs, differentiated

Figure 11: Investigation of αvβ5 integrin expression using immunofluorescence after integrin stimulation. Green staining: integrins stained with the fluorescein-conjugated αvβ5 specific antibody, blue staining: cell nucleus stained with DAPI.

a) human osteoblasts b) human osteoblasts, stimulated with dexamethasone (10^{-7} M)
c) rMSCs d) rMSCs, stimulated with dexamethasone (10^{-7} M)
e) rMSCs f) rMSCs, differentiated to the osteoblastic phenotype

5 Discussion

The main objective of this study was to employ a suitable representative of the recently developed class of amine-reactive polymers ST-NH-PEG-PLA [33] for the covalent anchoring of an αvβ3/αvβ5 subtype specific RGD-peptide [c(-RGDfK-)] [35] to study the integrin-mediated cell biomaterial interactions with regard to implant technology and tissue engineering applications. A covalent modification of these off-the-shelf amine-reactive polymers with the cyclic RGD-peptide was achieved by utilizing an incubation procedure adjusted to the needs of the functional group of the polymer and to solid phase modification.

The amine-reactive ST-NH-PEG-PLA copolymers are composed of a lipophilic biodegradable PLA chain, which enables the processing of the polymer into scaffolds for tissue engineering or implant coatings, and a hydrophilic H_2N-PEG chain that limits unspecific protein adsorption. Finally, the copolymers are made amine-reactive by covalent attachment of disuccinimidyl tartrate [33] for further modification. For our investigations, the particular composition of a 20 kDa PLA chain and a 2 kDa PEG chain was chosen, because it may be processed into tissue engineering scaffolds [40] and the content of hydrophilic PEG was sufficient to limit unspecific protein adsorption and cell adhesion on this material [32]. On the non-reactive 'stealth' Me.PEG-PLA derivative, Me.PEG2-PLA20, about 20% cell attachment was found after a 7 hour cell adhesion period under serum-containing conditions compared to about 85% cell attachment on PLA surfaces [32], while an accompanying control experiment performed under serum-free conditions showed even less cell adhesion to Me.PEG2-PLA20 (data not shown).

In this study, films made from ST-NH-PEG2-PLA20, were modified with an αvβ3/αvβ5 integrin subtype specific cyclic RGD-peptide [c(-RGDfK-)] in a simple incubation procedure enabled by the amine-reactive N-hydroxysuccinimide group of the polymer. The high αv selectivity of c(-RGDfK-) is associated with the insertion of *D*-phenylalanin (=f) in the cycle and the fixed conformation of the peptide side chains [34,35]. The impact of these modified surfaces on the amount and shape of the attaching human osteoblast, known to express the αvβ5/αvβ3 integrin, was examined. For that purpose, we developed an experimental setup, which is easy to handle, allows for direct observation of attaching cells, is applicable to various polymeric biomaterials and requires only low amounts of RGD-peptides.

This set-up could also be used to test the effect of other covalently bound molecules, for example growth factors, on cells. More specifically, two rings were engraved onto aminated glass slides. Afterwards, the enclosed areas were coated with polymer and the surrounding groove was covered with a lipophilic barrier. In this way, only small amounts of peptide-containing buffer were required to cover the polymer surface for modification. Following different washing steps, cells were seeded onto these areas for 7 hours. Thereafter, the attached cells were fixed, stained and their number and shape could be directly observed under the microscope.

Since we intended to covalently bind the RGD-peptide to the solid polymer surface from a buffered aqueous solution, reaction conditions had to favor aminolysis of the N-hydroxysuccinimide ester of the polymer instead of hydrolysis. Therefore, the peptide sequences were dissolved in a sodium bicarbonate buffer of pH 8 to ensure that the neutral ε-amino-group of the lysine side was present for coupling with the N-hydroxysuccinimide ester of the polymer, whereas, due to protonation at pH 8, the nucleophilicity of the arginine side chain in the cyclic RGD-peptide was nearly abolished [41,42]. Typical reaction protocols of peptides or proteins with N-hydroxysuccinimide esters propose reaction times up to a few hours and an, at least initially, low reaction temperature to favor aminolysis. Since we attempted a reaction on a solid surface and the RGD-peptide migrated only by passive diffusion to the reaction sites inside the 250 µl droplet - any mixing or shaking would have removed the sessile drop from the marked area - we increased the coupling time to 11 h at 4°C and another 3 h at 18°C.

The conducted cell adhesion studies with human osteoblasts showed that cell adhesion and spreading on c(-RGDfK-) modified ST-NH-PEG2-PLA20 surfaces were significantly increased compared to the control surfaces. These results demonstrated that an effective covalent surface modification has been achieved by incubating the amine-reactive polymer surface with an RGD-peptide under the selected conditions. On polymer films incubated with buffer, which led to hydrolysis of the amine-reactive N-hydroxysuccinimide group and the formation of negatively charged surfaces, cell adhesion was indistinguishable from cell adhesion on non-charged surfaces, generated by aminolysis with ethanol amine. Thus, under the applied conditions, we observed no effect of the surface charge on cell adhesion and cell spreading, which is described in literature as having a considerable impact on cell adhesion

[15]. Even on surfaces modified with the non-binding c(-RADfK-) peptide, presenting the positively charged arginine side chain and the negatively charged aspartate side chain in a similar configuration as in the c(-RGDfK-) peptide, comparably low amounts of attached cells were found. The integrin receptor blocking experiment, in which cells were incubated with dissolved c(-RGDfK-) before cell seeding on c(-RGDfK-) grafted surfaces, confirmed these findings. The method for this experiment was derived from Shin et. al. [13]. Thereby, cell attachment on the c(-RGDfK-) modified surfaces was reduced from 70% to 5%. These findings indicate that adhesion of non-blocked cells on c(-RGDfK-) modified surfaces was specifically mediated by interactions between the RGD-peptide and the integrins on the cell surface. In order to test for the impact of any non-covalently attached, adsorbed c(-RGDfK-), on cell adhesion and spreading on the polymer film, we treated ST-NH-PEG2-PLA20 surfaces with buffer to achieve hydrolysis of the N-hydroxysuccinimide ester. Afterwards, we incubated these polymer films with the c(-RGDfK-) peptide. Following cell adhesion we found hardly any cells attached on these surfaces. To complete the depicted cell adhesion study, we also tested purchased primary human osteoblasts (Cell-Lining / Oligene Berlin, Germany) for cell attachment on these surfaces. This experiment confirmed our previous results and consequently the applicability of the c(-RGDfK-) grafted ST-NH-PEG2-PLA20 polymer to selectively mediate osteoblast adhesion, independent of the donor. The cell shape, which was observed on surfaces modified with RGD, as well as on the various control surfaces, corresponded to the determined cell count. A high number of attached cells correlated with a strong cell spreading, while low cell attachment corresponded to a round cell shape. Many binding sites on the biomaterial are required to induce a strong cell spreading, so that the cells attach, begin to flatten and their plasma membranes spread over the substratum [1,43]. To check for the αvβ5 integrin expression on human osteoblasts we conducted immunofluorescence investigations, which confirmed the described expression of this integrin [6,36].

We were successful in modifying amine-reactive ST-NH-PEG2-PLA20 polymer films with a cyclic RGD-peptide. Studies on cell adhesion and cell shape showed that human osteoblast adhesion is mediated by interactions between the peptide and the corresponding integrin receptors of the cells. In implant technology, coatings with RGD-peptides would be a possible application for this RGD-modified ST-NH-PEG2-PLA20 polymer, in order to promote

physiological integration into the surrounding tissue. For tissue engineering applications, however, cells much be easily accessible and expandable in culture, meaning that mature osteoblasts may not provide the necessary proliferative capacity for tissue engineering purposes. Therefore, we investigated the adhesion of a different type of cells, rMSCs, which are easily accessible as well as expandable. Following proliferation, we aimed at attaching the undifferentiated rMSCs to the RGD-modified ST-NH-PEG2-PLA20 polymer. The cell adhesion study showed that c(-RGDfK-) modified ST-NH-PEG2-PLA20 surfaces did not affect rMSC attachment compared to the control groups. Correspondingly, cells on all three surfaces were poorly spread. In order to explore the reason for this behavior on the RGD-modified polymer, the expression of the αvβ5 integrin on rMSCs was investigated using immunofluorescence, because to our knowledge no information about αvβ5 integrin expression on rMSCs has been published to date. Human marrow stromal cells, however, have been reported to display αvβ3/αvβ5 integrins [44,45]. Our investigations showed that rMSCs express the αvβ5 integrin, although the expression appeared to be lower compared to human osteoblasts. It must also be taken into account that cells of the two different species compared may differ in their interaction with the antibody. Nevertheless, we hypothesized that a higher grafting density may allow for an αvβ3/αvβ5 integrin mediated rMSCs adhesion, despite the reduced expression of the integrin αvβ5. Unfortunately, a higher density of active groups on the ST-NH-PEG-PLA polymer surface would be correlated with an increased PEG density, which would negatively impact the mechanical strength of the polymer, such that the processing to scaffolds would be limited. To test whether or not rMSCs attach to surfaces grafted with a higher peptide density, activated glass surfaces, grafted with the same amine-reactive functional group, were modified with c(-RGDfK-) instead of polymer films. These glass surfaces have already been applied in various studies on surface modifications [46,47] and provide a higher grafting density compared to ST-NH-PEG2-PLA20. Indeed, 100% of the seeded rMSCs attached on c(-RGDfK-) grafted glass surfaces, compared to about 50% attachment on control surfaces, indicating a c(-RGDfK-)-mediated cell adhesion. Correspondingly, fully spread rMSCs were visible on c(-RGDfK-) modified glass surfaces. Hence, a critical minimum density of RGD seems to be necessary for cell response, while it is known from the literature that a direct dose-dependent increase of cell attachment is followed by an asymptotic approximation to a maximum number of attached cells, which is individual

for different surface materials and cell types [21]. Therefore, in order to employ the ST-NH-PEG2-PLA20 for tissue engineering applications and with regard to possibly insufficient integrin expression on rMSCs, we intended to stimulate the αvβ5 integrin expression by supplementing dexamethasone (10^{-7} M) for 48 hours [37] or by differentiating rMSCs to the osteoblastic phenotype. We could not confirm increased amounts of αvβ5 integrin on rMSCs after the 48h exposure to dexamethasone, even though the expression of the αvβ5 integrin on the human osteoblasts was increased following dexamethasone treatment, as described for human stromal cells and human osteoblasts by Cheng et al. [37]. The unfeasibility of this protocol is possibly based on the species differences between human and rat marrow stromal cells. However, employing a standard differentiation protocol for rMSCs to the osteoblastic phenotype did enhance the expression of αvβ5 integrin. Nevertheless, further investigations for example with fluorescence-activated cell sorting (FACS) should be performed to confirm the results. Moreover, another strategy would involve screening for other selective RGD-sequences in order to favor attachment of undifferentiated stromal cells to the modified ST-NH-PEG2-PLA20 polymer.

In conclusion, the successful grafting of the amine-reactive ST-NH-PEG2-PLA20 with an adhesion peptide in an instant procedure following polymer processing was demonstrated by a receptor-ligand mediated cell adhesion of human osteoblasts. As the used adhesion peptide c(-RGDfK-) favors the attachment of osteoblasts, the c(-RGDfK-) modified ST-NH-PEG2-PLA20 is a promising approach for controlling tissue interactions at the implant site. With regard to rMSCs, further investigations may show the applicability of modified ST-NH-PEG2-PLA20 as scaffold material in three-dimensional cultures of rMSCs for tissue engineering applications.

6 Acknowledgements

Special thanks are due to Allison Dennis, Department of Pharmaceutical Technology, University of Regensburg, Germany for proof-reading.

7 References

1. LeBaron, R. G. and Athanasiou, K. A. (2000). Extracellular matrix cell adhesion peptides: functional applications in orthopedic materials. *Tissue Eng.* **6**, 85-103.

2. Shakesheff, K. M., Cannizzaro, S. M., and Langer, R. (1998). Creating biomimetic micro-environments with synthetic polymer-peptide hybrid molecules. *J.Biomater.Sci., Polym.Ed.* **9**, 507-518.

3. Hu, Y., Winn, S. R., Krajbich, I., and Hollinger, J. O. (2003). Porous polymer scaffolds surface-modified with arginine-glycine-aspartic acid enhance bone cell attachment and differentiation in vitro. *J.Biomed.Mater.Res.* **64A**, 583-590.

4. Ferris, D. M., Moodie, G. D., Dimond, P. M., Gioranni, C. W., Ehrlich, M. G., and Valentini, R. F. (1999). RGD-coated titanium implants stimulate increased bone formation in vivo. *Biomaterials* **20**, 2323-2331.

5. Schaffner, P., Meyer, J., Dard, M., Wenz, R., Nies, B., Verrier, S., Kessler, H., and Kantlehner, M. (1999). Induced tissue integration of bone implants by coating with bone selective RGD-peptides in vitro and in vivo studies. *J.Mater.Sci.: Mater.Med.* **10**, 837-839.

6. Gronthos, S., Stewart, K., Graves, S. E., Hay, S., and Simmons, P. J. (1997). Integrin expression and function on human osteoblast-like cells. *J.Bone Miner.Res.* **12**, 1189-1197.

7. Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. *Annu.Rev.Cell.Dev.Biol.* **12**, 697-715.

8. Schaffner, P. and Dard, M. M. (2003). Structure and function of RGD peptides involved in bone biology. *Cell.Mol.Life Sci.* **60**, 119-132.

9. Pfaff, M., Tangemann, K., Muller, B., Gurrath, M., Muller, G., Kessler, H., Timpl, R., and Engel, J. Selective recognition of cyclic RGD peptides of NMR defined

conformation by alpha IIb beta 3, alpha V beta 3, and alpha 5 beta 1 integrins. *J.Biol.Chem.* **269**, 20233-20238.

10. Aumailley, M., Gurrath, M., Muller, G., Calvete, J., Timpl, R., and Kessler, H. (1991). Arg-Gly-Asp constrained within cyclic pentapeptides. Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1. *FEBS Letters* **291**, 50-54.

11. Massia, S. P. and Stark, J. (2001). Immobilized RGD peptides on surface-grafted dextran promote biospecific cell attachment. *J.Biomed.Mater.Res.* **56**, 390-399.

12. Hern, D. L. and Hubbell, J. A. Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing. *J.Biomed.Mater.Res.* **39**, 266-276.

13. Shin, H., Jo, S., and Mikos, A. G. (2002). Modulation of marrow stromal osteoblast adhesion on biomimetic oligo[poly(ethylene glycol) fumarate] hydrogels modified with Arg-Gly-Asp peptides and a poly(ethyleneglycol) spacer. *J.Biomed.Mater.Res.* **61**, 169-179.

14. Cannizzaro, S. M., Padera, R. F., Langer, R., Rogers, R. A., Black, F. E., Davies, M. C., Tendler, S. J., and Shakesheff, K. M. (1998). A novel biotinylated degradable polymer for cell-interactive applications. *Biotechnol.Bioeng.* **58**, 529-535.

15. Anselme, K. (2000). Osteoblast adhesion on biomaterials. *Biomaterials* **21**, 667-681.

16. Stile, R. A., Burghardt, W. R., and Healy, K. E. (1999). Synthesis and characterization of injectable poly(N-isopropylacrylamide)-based hydrogels that support tissue formation in vitro. *Macromolecules* **32**, 7370-7379.

17. Rowley, J. A., Madlambayan, G., and Mooney, D. J. (1999). Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* **20**, 45-53.

18. Burdick, J. A. and Anseth, K. S. (2002). Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. *Biomaterials* **23**, 4315-4323.

19. Ishaug, S. L., Crane, G. M., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **36**, 17-28.

20. Schaefer, D., Martin, I., Shastri, P., Padera, R. F., Langer, R., Freed, L. E., and Vunjak-Novakovic, G. (2000). In vitro generation of osteochondral composites. *Biomaterials* **21**, 2599-2606.

21. Hersel, U., Dahmen, C., and Kessler, H. (2003). RGD Modified Polymers: Biomaterials for Stimulated Cell Adhesion. *Biomaterials* **(in press)**.

22. Yang, X. B., Roach, H. I., Clarke, N. M. P., Howdle, S. M., Quirk, R., Shakesheff, K. M., and Oreffo, R. O. C. (2001). Human osteoprogenitor growth and differentiation on synthetic biodegradable structures after surface modification. *Bone* **29**, 523-531.

23. Neff, J. A., Tresco, P. A., and Caldwell, K. D. (1999). Surface modification for controlled studies of cell-ligand interactions. *Biomaterials* **20**, 2377-2393.

24. Carlisle, E. S., Mariappan, M. R., Nelson, K. D., Thomes, B. E., Timmons, R. B., Constantinescu, A., Eberhart, R. C., and Bankey, P. E. Enhancing hepatocyte adhesion by pulsed plasma deposition and polyethylene glycol coupling. *Tissue Eng.* **6**, 45-52.

25. Marchand-Brynaert, J. (1998). Surface modifications and reactivity assays of polymer films and membranes by selective wet chemistry. *Recent Research Developments in Polymer Science* **2**, 335-362.

26. Lin, H. B., Sun, W., Mosher, D. F., Garcia-Echeverria, C., Schaufelberger, K., Lelkes, P., I, and Cooper, S. L. Synthesis, surface, and cell-adhesion properties of polyurethanes containing covalently grafted RGD-peptides. *J.Biomed.Mater.Res.* **28**, 329-342.

27. Lin, H. B., Zhao, Z. C., Garcia-Echeverria, C., Rich, D. H., and Cooper, S. L. Synthesis of a novel polyurethane co-polymer containing covalently attached RGD peptide. *J.Biomater.Sci., Polym.Ed.* **3**, 217-227.

28. Tam, J. P., Yu, Q., and Miao, Z. (1999). Orthogonal ligation strategies for peptide and protein. *Biopolymers - Peptide Science Section* **51**, 311-332.

29. Tuchscherer G. (1993). Template Assembled Synthetic Proteins: Condensation of a Multifunctional Peptide to a Topological Template via Chemoselective Ligation. *Tetrahedron Lett.* **34**, 8410-8422.

30. Molina, J., Urbina, J., Gref, R., Brener, Z., and Rodriguez, J. M. (2001). Cure of experimental Chagas' disease by the bis-triazole D0870 incorporated into 'stealth' polyethyleneglycol-polyactide nanospheres. *J.Antimicrob.Chemother.* **47**, 101-104.

31. Gref, R., Luck, M., Quellec, P., Marchand, M., Dellacherie, E., Harnisch, S., Blunk, T., and Muller, R. H. (2000). 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): Influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids and Surfaces B: Biointerfaces* **18**, 301-313.

32. Lieb, E., Tessmar, J., Hacker, M., Fischbach, C., Rose, D., Blunk, T., Mikos, A. G., Göpferich, A., and Schulz, M. B. (2003). Poly(D,L-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymers control adhesion and osteoblastic differentiation of marrow stromal cells. *Tissue Eng.* **9**, 71-84.

33. Tessmar, J. K., Mikos, A. G., and Göpferich, A. (2002). Amine-reactive biodegradable diblock copolymers. *Biomacromolecules.* **3**, 194-200.

34. Haubner, R., Gratias, R., Diefenbach, B., Goodman, S. L., Jonczyk, A., and Kessler, H. (1996). Structural and Functional Aspects of RGD-Containing Cyclic Pentapeptides as Highly Potent and Selective Integrin aVb3 Antagonists. *J.Am.Chem.Soc.* **118**, 7461-7472.

35. Kantlehner, M., Schaffner, P., Finsinger, D., Meyer, J., Jonczyk, A., Diefenbach, B., Nies, B., Holzemann, G., Goodman, S. L., and Kessler, H. (2000). Surface coating with cyclic RGD peptides stimulates osteoblast adhesion and proliferation as well as bone formation. *ChemBioChem* **1**, 107-114.

36. Kantlehner, M., Finsinger, D., Meyer, J., Schaffner, P., Jonczyk, A., Diefenbach, B., Nies, B., and Kessler, H. (1999). Selective RGD-mediated adhesion of osteoblasts at surfaces of implants. *Angew.Chem.Int.Ed.Engl.* **38**, 560-562.

37. Cheng, S. L., Lai, C. F., Fausto, A., Chellaiah, M., Feng, X., McHugh, K. P., Teitelbaum, S. L., Citivelli, R., Hruska, K. A., Ross, F. P., and Avioli, L. V. (2000).

Regulation of alphavbeta3 and alphavbeta5 integrins by dexamethasone in normal human osteoblastic cells. *Journal of Cellular Biochemistry* **77**, 265-276.

38. Mayr-Wohlfart, U., Kessler, S., Puhl, W., Gunther, K. P., and Knochel, W. (2001). BMP-4 of Xenopus laevis stimulates differentiation of human primary osteoblast-like cells. *J.Bone Joint Surg.Br* **83**, 144-147.

39. Ishaug, S. L., Yaszemski, M. J., Bizios, R., and Mikos, A. G. (1994). Osteoblast function on synthetic biodegradable polymers. *J.Biomed.Mater.Res.* **28**, 1445-1453.

40. Hacker, M., Tessmar, J., Neubauer, M., Blunk, T., Göpferich, A., and Schulz, M. B. (2003). Towards biomimetic scaffolds: Anhydrous scaffold fabrication from biodegradable amine-reactive diblock copolymers. *Biomaterials* **(in press)**.

41. Besselink, G. A. J., Beugeling, T., and Bantjes, A. (1993). N-Hydroxysuccinimide-Activated Glycine-Sepharose - Hydrolysis of Activated Groups and Coupling of Amino-Compounds. *App Biochem Biotech.* **43**, 227-246.

42. Hern, D. L. and Hubbell, J. A. Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing. *J.Biomed.Mater.Res.* **39**, 266-276.

43. Rezania, A., Thomas, C. H., Branger, A. B., Waters, C. M., and Healy, K. E. (1997). The detachment strength and morphology of bone cells contacting materials modified with a peptide sequence found within bone sialoprotein. *J.Biomed.Mater.Res.* **37**, 9-19.

44. Gronthos, S., Simmons, P. J., Graves, S. E., and Robey, P. G. (2001). Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. *Bone* **28**, 174-181.

45. Verrier, S., Pallu, S., Bareille, R., Jonczyk, A., Meyer, J., Dard, M., and Amedee, J. (2002). Function of linear and cyclic RGD-containing peptides in osteoprogenitor cells adhesion process. *Biomaterials* **23**, 585-596.

46. Olbrich, K. C., Andersen, T. T., Blumenstock, F. A., and Bizios, R. (1996). Surfaces modified with covalently-immobilized adhesive peptides affect fibroblast population motility. *Biomaterials* **17**, 759-764.

47. El Ghannam, A., Ducheyne, P., Shapiro, I., and Composto, R. (2002). Engineered RGD peptide surface regulates adhesion, spreading and cytoskeletal organization of osteoblast-like cells. *Key Engineering Materials* **218-220**, 245-248.

Chapter 8

Summary and Conclusions

Summary and Conclusions

The tissue engineering of bone is a promising new field of research to address the problems arising from the loss of bony tissue occurring through infection, loss of blood supply or disease, such as osteoporosis, or as a complication of a fracture or genetic disorders. However, due to the complex composition of bone and many critical parameters (osteogenic factors, scaffold material, mechanical forces, cell source) influencing bone formation, there are still many obstacles that need to be surmounted to obtain in vitro engineered bone-like tissue. The goal of this thesis was to optimize two of the key factors that influence tissue engineering, the osteogenic agents and the scaffold material, in order to improve the formation of bone-like tissue. The strategy that we applied to engineer bone-like tissue involved aspiration of rMSCs from the bone marrow, cell expansion in two-dimensional cell culture to obtain a high cell number, dynamic cell seeding on PLLGA fiber meshes in spinner flasks, and finally in vitro cultivation of the cell-polymer constructs under treatment with osteogenic agents **(chapter 1)**.

At first, the optimization of the culture conditions for the bone cell culture of rMSCs was a necessary prerequisite for a successful approach to bone tissue engineering. To this end, we investigated the optimum cell seeding density at rMSC isolation, the influence of basal media with different nutrient concentrations and the time point, at which the osteogenic supplements may most advantageously be added. In view of the seeding density, four 75-cm^2 flasks per rat were selected as the optimal density with respect to a maximum cell count per flask at the first subculture with a minimum number of sacrificed rats. During cultivation with the basal medium α-MEM, the proliferation before the onset of the osteoblastic differentiation and the expression of the osteoblastic marker alkaline phosphatase were strongly enhanced opposed to the culture in DMEM. Hence, the subsequent studies were performed in α-MEM. Thereby, we observed an increased osteoblastic differentiation, if the osteogenic supplements were added immediately after harvest, rather than following a two week proliferation phase. As cell proliferation was crucially reduced when cells were exposed to differentiating medium immediately after harvest, however, it made no sense to embark on this strategy, because a high number of cells is required for tissue engineering purposes **(chapter 2)**.

To improve bone tissue engineering, it is essential to overcome the problem of limited extracellular matrix formation in cell-polymer constructs of rMSCs when cultured with the standard osteogenic agents (dexamethasone, β-glycerophosphate and ascorbic acid). Thus, we included TGF-β1 in order to solve the outlined problem. TGF-β1 was considered to be a promising growth factor to improve the matrix formation in our three-dimensional cell culture system, because TGF-β1 has been described as playing a key role in collagen type I formation, the main matrix protein of bone [1,2,3]. Its effect on matrix mineralization, however, has been controversially discussed in the literature [4,5,6,7,8]. By investigating various dosages of TGF-β1, we could demonstrate a dose-dependent increase of collagen type I formation with increasing dosages of TGF-β1. Moreover, procollagen type I and collagen type V, further bone matrix proteins, were increased under TGF-β1 treatment. The effects of TGF-β1 on the formation of these extracellular bone matrix proteins were found to be significantly enhanced under dynamic culture conditions (orbital shaker) compared to static culture. Thus, the importance of dynamic cultivation for improved nutrient supply [9,10] and possibly affecting bone formation by fluid flow mechanical stimulation [11,12] was revealed. Furthermore, our investigations showed that matrix mineralization was enhanced following TGF-β1 treatment compared to the control. Energy dispersive X-ray analysis revealed a Ca/P ratio of 1.63 in the calcified globular accretions of the cell-polymer constructs treated with TGF-β1, which is close to the theoretical value of 1.67 in hydroxyapatite, the natural bone matrix mineral [13]. Hence, the supplementation with TGF-β1 enhanced bone-like extracellular matrix formation abolishing the problem of limited matrix formation in three-dimensional cell culture **(chapter 3)**.

A follow-up study focused on the investigation of osteoblastic differentiation markers under treatment with TGF-β1 in dynamic cell culture. Thereby, the bone marker bone sialoprotein was shown to be enhanced with increasing doses of TGF-β1, while osteonectin was expressed independent of the addition of TGF-β1. Even the bone markers ALP activity and osteocalcin, often described to be inhibited by TGF-β1 [14,15], were preserved up to high doses of TGF-β1 compared to the control. We assumed that the application of low dosages of TGF-β1, three-dimensional versus two-dimensional cell culture, dynamic culture conditions, and the presence of dexamethasone favor the impact of TGF-β1 on osteoblastic differentiation and matrix mineralization. On the whole, we established dosages and optimal culture

conditions for the application of TGF-β1 to three-dimensional bone cell cultures of rMSCs for tissue engineering purposes. The dosing regimen of 1 ng/ml TGF-β1, administered once a week, was selected as an effective dosage, on the one hand to increase matrix formation and mineralization and on the other hand to preserve or even increase osteoblastic differentiation **(chapter 4)**.

The bone growth factor BMP-2 was regarded as a useful tool to increase osteoblastic differentiation, due to various published in vitro studies, which have described an increase of osteoblastic markers following BMP-2 treatment of bone marrow stromal cell culture [16,17,18]. Therefore, a combined application of BMP-2 and TGF-β1 was employed in three-dimensional rMSC culture to investigate if this combination had additional positive effects on osteoblastic differentiation versus TGF-β1 alone. Immunohistochemical investigations showed that the formation of the bone markers bone sialoprotein and osteonectin was increased with the combined supplementation compared to either TGF-β1 treatment alone and to either BMP-2 or TGF-β1, respectively. Thus, in three-dimensional cell culture, the growth factor combination of TGF-β1 and BMP-2 provides the ability to enhance osteoblastic differentiation. Moreover, since the administered dosage of 100 ng/ml BMP-2 alone was too low to induce collagen type I formation, while in combination with a low single dose of TGF-β1 a coherent tissue containing collagen I was achieved, we assume that the effective dosage of BMP-2 on bone formation can be considerably decreased by the combination with TGF-β1, a great advantage considering costs and systemic side effects **(chapter 5)**.

In order to improve the second key factor, the scaffold material for tissue engineering applications, the effect of different biomaterials on cell adhesion, cell shape, cell proliferation and osteoblastic differentiation was examined. Poly(α-hydroxy acids), such as PLA, PGA and their copolymers, are widely used biomaterials in tissue engineering [19,20,21], due to FDA approval as suture material and the suitability to be tailored to definite properties, such as mechanical strength and degradation time, according to the special needs of the tissue. However, these materials offer no control of cell adhesion and function since protein adsorption from the body fluids or cell culture medium leads to uncontrolled changes in the surface composition of the material. Moreover, protein adsorption to hydrophobic surfaces, such as PLA, has been described as resulting in a high adsorption strength and consequently

denaturing changes in protein conformation [22]. Thus, the polymer has to be modified in order to control the amount, and probably the conformation, of adsorbed proteins mediating cell adhesion. A recent study on a Me.PEG-PLA diblock copolymer, i.e. Me.PEG5-PLA20, showed that reducing protein adsorption via the modification with PEG, known for its non-interaction with proteins [23], cell adhesion and differentiation can be influenced [24]. However, because of the low cell attachment to Me.PEG5-PLA20, we were looking for other compositions of Me.PEG-PLA diblock copolymers that maintain sufficient cell adhesion for bone tissue engineering purposes, while preserving the polymer's differentiating properties. We could demonstrate that the content of poly(ethylene glycol) (PEG) in the diblock copolymers controlled protein adsorption and consequently rMSC adhesion. The differentiation to the osteoblastic phenotype as determined by ALP activity and the degree of mineralization of the extracellular matrix, was significantly improved relative to culturing on the standard materials such as PLA and PLGA. The enhanced bioactivity of adsorbed adhesion proteins due to a stabilized native conformation might be responsible for this phenomenon [22]. Thus, by reducing the PEG content, the differentiating properties of the Me.PEG-PLA diblock copolymers were preserved, while the initial cell attachment was increased. Consequently, diblock copolymers made of PEG and PLA that lead to a sufficient cell attachment at cell seeding, such as Me.PEG2-PLA40, are attractive materials for the processing into scaffolds [25] and for use in bone tissue engineering **(chapter 6)**.

In our department, amine-reactive polymers [ST-NH-PEG-PLA] [26] of the ´stealth´ Me.PEG-PLA diblock copolymers were developed to covalently anchor bioactive molecules for the use in tissue engineering or implant technology. In the present study, we demonstrated the grafting of a cell adhesion peptide – the αvβ3/αvβ5 integrin subtype specific cyclic RGD-peptide [c(-RGDfK-)] [27] – to ST-NH-PEG2-PLA20 surfaces using an incubation procedure adjusted to the needs of the polymer and solid phase modification in order to control cell adhesion. The covalent binding of the RGD-peptide to the ST-NH-PEG2-PLA20 films was achieved by utilizing a sodium bicarbonate buffer of pH 8 as a reaction buffer and coupling times of 11 hours at 4°C and another 3 hours at 18°C. A cell adhesion study with human osteoblasts, which are known to strongly express the αvβ5 integrin [28], revealed a significant increase of the cell number and spreading on RGD-modified ST-NH-PEG2-PLA20 films

compared to the controls such as the non-binding c(-RADfK-) peptide, buffer, non-covalently anchored c(-RGDfK-) or ethanol amine (passivating reagent). Additionally, we incubated the human osteoblasts with c(-RGDfK-) containing medium before cell seeding to c(-RGDfK-) modified ST-NH-PEG2-PLA20 films. Thereby, cell attachment was almost completely prevented as observed for the other controls, confirming that adhesion of non-blocked cells on these surfaces was specifically mediated by interactions between the covalently anchored RGD-peptide and the integrins on the cell surface. A possible application for this RGD-modified ST-NH-PEG2-PLA20 polymer may be the use for coatings in implant technology in order to promote physiological integration into the surrounding tissue. In tissue engineering, however, osteoblasts are not the ideal cell type due to their insufficient proliferative capacity [29]. Therefore, we also investigated the cell adhesion of the easily accessible and expandable rMSCs on c(-RGDfK-) grafted ST-NH-PEG2-PLA20 films. However, rMSC attachment was not increased on the modified polymer surfaces, while it was enhanced on glass surfaces, which were grafted with the same amine-reactive functional group at a higher grafting density compared to ST-NH-PEG2-PLA20 films. These glass surfaces served as an available means to investigate the influence of a higher grafting density on the attachment of rMSC that apparently express lower levels of the integrin αvβ5, as visualized by immunofluorescence microscopy **(chapter 7)**.

In conclusion, this thesis contributed to the improvement of bone tissue engineering by the optimization of the two key factors, the osteogenic agents and the scaffold material. In bone tissue engineering, the application of TGF-β1 in addition to the standard osteogenic agents is indispensible to obtain a coherent bone-like extracellular matrix. Furthermore, culture conditions and dosages were established, such that TGF-β1 preserved or even increased osteoblastic cell differentiation. The combined application of BMP-2 and TGF-β1 offers a chance to further enhance the osteoblastic differentiation of rMSCs in three-dimensional cell culture. Concerning scaffold materials, the examination of various biomaterials revealed that Me.PEG-PLA diblock copolymers, especially Me.PEG2-PLA40, are promising candidates for the processing into scaffolds and the use in bone tissue engineering, because they provide controlled protein adsorption and positively influenced osteoblastic differentiation. At last, the successful anchoring of a cyclic RGD-peptide in an instant procedure to an amine-reactive

derivative [ST-NH-PEG2-PLA20] of the `stealth` Me.PEG-PLA diblock copolymers allowed for selective cell adhesion. This bears a great potential to control cell adhesion in tissue engineering and implant technology.

References

1. Lilli, C., Marinucci, L., Stabellini, G., Belcastro, S., Becchetti, E., Balducci, C., Staffolani, N., and Locci, P. (2002). Biomembranes enriched with TGFbeta1 favor bone matrix protein expression by human osteoblasts in vitro. *J.Biomed.Mater.Res.* **63**, 577-582.

2. Hock, J. M., Canalis, E., and Centrella, M. (1990). Transforming growth factor-beta stimulates bone matrix apposition and bone cell replication in cultured fetal rat calvariae. *Endocrinology* **126**, 421-426.

3. Arnold, U., Lindenhayn, K., and Perka, C. (2002). In vitro-cultivation of human periosteum derived cells in bioresorbable polymer-TCP-composites. *Biomaterials* **23**, 2303-2310.

4. Antosz, M. E., Bellows, C. G., and Aubin, J. E. (1989). Effects of transforming growth factor beta and epidermal growth factor on cell proliferation and the formation of bone nodules in isolated fetal rat calvaria cells. *J.Cell Physiol* **140**, 386-395.

5. Faucheux, C., Ulysse, F., Bareille, R., Reddi, A. H., and Amedee, J. (1997). Opposing actions of BMP3 and TGF beta 1 in human bone marrow stromal cell growth and differentiation. *Biochem.Biophys.Res.Commun.* **241**, 787-793.

6. Breen, E. C., Ignotz, R. A., McCabe, L., Stein, J. L., Stein, G. S., and Lian, J. B. (1994). TGF beta alters growth and differentiation related gene expression in proliferating osteoblasts in vitro, preventing development of the mature bone phenotype. *J.Cell Physiol* **160**, 323-335.

7. Kale, S., Biermann, S., Edwards, C., Tarnowski, C., Morris, M., and Long, M. W. (2000). Three-dimensional cellular development is essential for ex vivo formation of human bone. *Nat.Biotechnol.* **18**, 954-958.

8. Lian, J. B. and Stein, G. S. (1993). The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGF beta 1) and hormones (vitamin D and glucocorticoids). *J.Oral Implantol.* **19**, 95-105.

9. Kellner, K., Liebsch, G., Klimant, I., Wolfbeis, O. S., Blunk, T., Schulz, M. B., and Göpferich, A. (2002). Determination of oxygen gradients in engineered tissue using a fluorescent sensor. *Biotechnol.Bioeng.* **80**, 73-83.

10. Freed, L. E., Vunjak-Novakovic, G., and Langer, R. (1993). Cultivation of cell-polymer cartilage implants in bioreactors. *J.Cell Biochem.* **51**, 257-264.

11. Ogata, T. (2000). Fluid flow-induced tyrosine phosphorylation and participation of growth factor signaling pathway in osteoblast-like cells. *J.Cell Biochem.* **76**, 529-538.

12. Weyts, F. A., Li, Y. S., Van Leeuwen, J., Weinans, H., and Chien, S. (2002). ERK activation and alphavbeta3 integrin signaling through Shc recruitment in response to mechanical stimulation in human osteoblasts. *J.Cell Biochem.* **87**, 85-92.

13. Joschek, S., Nies, B., and Krotz, R. G. A. (2000). Chemical and physicochemical characterization of porous hydroxyapatite ceramics made of natural bone. *Biomaterials* **21**, 1645-1658.

14. Kassem, M., Kveiborg, M., and Eriksen, E. F. (2000). Production and action of transforming growth factor-beta in human osteoblast cultures: dependence on cell differentiation and modulation by calcitriol. *Eur.J.Clin.Invest* **30**, 429-437.

15. Breen, E. C., Ignotz, R. A., McCabe, L., Stein, J. L., Stein, G. S., and Lian, J. B. (1994). TGF beta alters growth and differentiation related gene expression in proliferating osteoblasts in vitro, preventing development of the mature bone phenotype. *J.Cell Physiol* **160**, 323-335.

16. Rickard, D. J., Sullivan, T. A., Shenker, B. J., Leboy, P. S., and Kazhdan, I. (1994). Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev.Biol.* **161**, 218-228.

17. Lecanda, F., Avioli, L. V., and Cheng, S. L. (1997). Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *J.Cell Biochem.* **67**, 386-396.

18. Puleo, D. A. (1997). Dependence of mesenchymal cell responses on duration of exposure to bone morphogenetic protein-2 in vitro. *J.Cell Physiol* **173**, 93-101.

19. Freed, L. E., Grande, D. A., Lingbin, Z., Emmanual, J., Marquis, J. C., and Langer, R. (1994). Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **28**, -899.

20. Ishaug, S. L., Crane, G. M., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J.Biomed.Mater.Res.* **36**, 17-28.

21. Peter, S. J., Miller, M. J., Yasko, A. W., Yaszemski, M. J., and Mikos, A. G. (1998). Polymer concepts in tissue engineering. *J.Biomed.Mater.Res.* **43**, 422-427.

22. Altankov, G., Thom, V., Groth, T., Jankova, K., Jonsson, G., and Ulbricht, M. (2000). Modulating the biocompatibility of polymer surfaces with poly(ethylene glycol): Effect of fibronectin. *J.Biomed.Mater.Res.* **52**, 219-230.

23. Amiji, M. and Park, K. (1992). Prevention of protein adsorption and platelet adhesion on surfaces by PEO/PPO/PEO triblock copolymers. *Biomaterials* **13**, 682-692.

24. Göpferich, A., Peter, S. J., Lucke, A., Lu, L., and Mikos, A. G. (1999). Modulation of marrow stromal cell function using poly(D,L-lactic acid)-block-poly(ethylene glycol)-monomethyl ether surfaces. *J.Biomed.Mater.Res.* **46**, 390-398.

25. Hacker, M., Tessmar, J., Neubauer, M., Blaimer, A., Blunk, T., Göpferich, A., and Schulz, M. B. (2002). Towards biomimetic scaffolds - Anhydrous scaffold fabrication from biodegradable amine reactive diblock copolymers. *Biomaterials* **(in press)**.

26. Tessmar, J. K., Mikos, A. G., and Göpferich, A. (2002). Amine-reactive biodegradable diblock copolymers. *Biomacromolecules.* **3**, 194-200.

27. Kantlehner, M., Schaffner, P., Finsinger, D., Meyer, J., Jonczyk, A., Diefenbach, B., Nies, B., Holzemann, G., Goodman, S. L., and Kessler, H. (2000). Surface Coating with Cyclic RGD Peptides Stimulates Osteoblast Adhesion and Proliferation as well as Bone Formation. *Chembiochem.Europ.J.Chem.Biol.* **1**, 107-114.

28. Gronthos, S., Stewart, K., Graves, S. E., Hay, S., and Simmons, P. J. (1997). Integrin expression and function on human osteoblast-like cells. *J.Bone Miner.Res.* **12**, 1189-1197.

29. Hardouin, P., Anselme, K., Flautre, B., Bianchi, F., Bascoulenguet, G., and Bouxin, B. (2000). Tissue engineering and skeletal diseases. *Joint Bone Spine* **67**, 419-424.

Appendices

Abbreviations

α-MEM	Minimum Essential Medium Eagle, alpha-modification
AFM	atomic force microscopy
ALP activity	alkaline phosphatase activity
ANOVA	analysis of variance
BMP	bone morphogenetic protein
BSA	bovine serum albumin
c(-RADfK-)	peptide sequence: cyclo (-Arg-Ala-Asp-*D*-Phe-Lys-)
c(-RGDfK-)	peptide sequence: cyclo (-Arg-Gly-Asp-*D*-Phe-Lys-)
cDNA	complementary DNA
Da	Dalton
DAB	3, 3`-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco´s Modified Eagle´s Medium
DNA	deoxyribonucleic acid
DSHB	Developmental studies hybridoma bank
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EDX	energy dispersive X-ray analysis
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FCS	fetal calf serum

FDA	Food and Drug Administration
FGF	fibroblast growth factor
GPC	gel permeation chromatography
^{1}H-NMR	^{1}H nuclear magnetic resonance spectroscopy
H_2N-PEG-PLA	mono amine poly(ethylene glycolic)-*block*-poly(*D,L*-lactic acid)
HPLC	high performance liquid chromatography
IGF	insulin-like growth factor
Me.PEG-PLA	poly(*D,L*-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymer
Me.PEGx-PLAy	poly(*D,L*-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymer with PLA block of y kDa and PEG block of x kDa
M-MLV Reverse Transcriptase	Moloney Murine Leukemia Virus Reverse Transcriptase
Mn	number average molecular weight
mRNA	messenger ribonucleic acid
MSCs	marrow stromal cells
Mw	weight average molecular weight
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	poly(ethylene glycol)
PGA	poly(glycolic acid)
PI	polydispersity index (=Mw/Mn)
PLA	poly(lactic acid)

PLGA	poly(lactic-co-glycolic acid)
PLLGA	poly(*L*-lactic-co-glycolic acid)
PP	polypropylene
RGD	peptide sequence: Arg-Gly-Asp
rMSCs	rat marrow stromal cells
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SEM	scanning electron microscopy
ST-NH-PEG2-PLA20	N-succinimidyl tartrate monoamine poly(ethylene glycol)-*block*-poly(*D*,*L*-lactic acid) consisting of 2 kDa poly(ethylene glycol)-monoamine (H_2N-PEG) block and a 20 kDa poly(*D*,*L*-lactic acid) (PLA) block
ST-NH-PEG-PLA	N-succinimidyl tartrate monoamine poly(ethylene glycol)-*block*-poly(*D*,*L*-lactic acid)
T-75 flask	75-cm^2 flasks
TCPS	tissue culture polystyrene
TGF-β1	transforming growth factor-β1
TMS	tetramethylsilane
Tris buffer	tris(hydroxymethyl)aminomethane buffer
UV	ultraviolet light
XPS	X-ray photoelectron spectroscopy

Curriculum vitae

Name:	Esther Lieb
Beruf:	Apothekerin
Geburtsdatum:	07.08.1973
Geburtsort:	Leverkusen
Staatsangehörigkeit:	deutsch
Familienstand:	ledig

Ausbildung / Praktika

1980 - 1984:	Gemeinschaftsgrundschule Morsbroicher Straße in Leverkusen
1984 - 1993:	Freiherr-vom-Stein Gymnasium in Leverkusen, Abitur im Juni 1993
09/93 - 09/97:	Studium der Pharmazie an der Rheinischen Friedrich-Wilhelms-Universität in Bonn
03/1996:	1. Staatsexamen
09/1997:	2. Staatsexamen
11/97 - 04/98:	Pharmaziepraktikum in der St. Hubertus Apotheke in Bonn
05/98 - 10/98:	Industriepraktikum in der galenischen Entwicklung bei der Firma

	Bayer in Leverkusen
12/1998:	3. Staatsexamen Approbation zur Apothekerin
ab 02/1999:	Promotion bei Herrn Prof. Dr. A. Göpferich, Lehrstuhl für Pharmazeutische Technologie in Regensburg - Bone Tissue Engineering from Marrow Stromal Cells: Effects of Growth Factors and Biomaterials -

List of Publications

Publications

Lieb, E., Milz S., Hacker, M., Dauner, M., Göpferich, A., Schulz, M.B.; Effects of TGF-ß1 on Bone-Like Tissue Formation in Three-Dimensional Cell Culture Part I: Culture Conditions and Tissue Formation. *Tissue Engineering, submitted* (chapter 3)

Lieb, E., Milz, S., Dauner, M., Göpferich, A., Schulz, M.B.; Effects of TGF-ß1 on Bone-like Tissue Formation in Three-Dimensional Cell Culture Part II: Osteoblastic Differentiation. *Tissue Engineering, submitted* (chapter 4)

Lieb, E., Teßmar, J., Hacker, M., Fischbach, C., Rose, D., Blunk, T., Mikos, A.G., Göpferich, A., Schulz, M.B.; Poly(*D,L*-lactic acid)-Poly(ethylene glycol)-Monomethyl Ether Diblock Copolymers Control Adhesion and Osteoblastic Differentiation of Marrow Stromal Cells. *Tissue Engineering Volume 9, Number 1, 71 – 84, 2003* (chapter 6)

Lieb, E., Hacker, M., Teßmar, J., Kunz-Schughart, L.A., Fiedler, J., Dahmen, C., Hersel, U., Kessler, H., Schulz M.B., Göpferich A.; Mediating Cell-Biomaterial Interactions: Instant modification of `stealth` surfaces with a cyclic αvβ3/αvβ5 integrin subtype specific RGD-peptide. *to be submitted* to *Biomaterials* (chapter 7)

Conference Abstracts

Lieb, E., Milz, S. Hacker, M., Dauner, M., Göpferich, A., Schulz, M.B.; In Vitro Effects of TGF-β1 on Rat Marrow Stromal Cells for Bone Tissue Engineering. 5th Annual Meeting of the Tissue Engineering Society international, Kobe, Japan, 2002.

Hacker, M., Neubauer, M., Untch, J., Teßmar, J., Lieb, E., Blunk, T., Göpferich, A., Schulz, M.B.; Solid lipid templating: Macroporous scaffold fabrication from biodegradable polymers. 5th Annual Meeting of the Tissue Engineering Society, Kobe, Japan, 2002.

Lieb, E., Milz, S., Dauner, M., Göpferich, A., Schulz, M.B.; The Effects of TGF-β1 on 3-D Bone Cell Culture An In Vitro Test System for Drugs. APV, Florenz, Italy, 2002.

Lieb, E., Milz, S., Hacker, M., Dauner, M., Göpferich, A., Schulz, M.B.; 3-D cell culture for the evaluation of growth factor effects on bone formation with TGF-β1 as model protein. DPhG, Berlin, Germany, 2002.

Hacker, M., Lieb, E., Teßmar, J., Hersel, U., Dahmen, C., Kessler, H., Göpferich, A., Schulz, M.B.; Controlling cell-biomaterial interactions: Instant modification of "stealth" surfaces with RGD-peptides. DPhG, Berlin, Germany, 2002.

Lieb, E., Milz, S., Hacker, M., Dauner, M., Göpferich, A., Schulz, M.B.; 3-D cell culture on poly(L-lactic-co-glycolic acid) fiber meshes for the evaluation of TGF-β1 effects in bone tissue engineering. 5th Annual Meeting of the GZG / ETCS, Regensburg, Germany, 2002.

Neubauer, M., Fischbach, C., Lieb, E., Hacker, M., Teßmar, J, Schulz, M.B., Göpferich, A., Blunk, T.; Adipogenesis of Rat Marrow Stromal Cells in Two- and Three-Dimensional Cell Culture. 5th Annual Meeting of the GZG / ETCS, Regensburg, Germany, 2002.

Neubauer, M., Fischbach, C., Lieb, E., Hacker, M., Teßmar, J, Schulz, M.B., Göpferich, A., Blunk, T.; Exposure to Basic Fibroblast Growth Factor Enhances Adipogenesis of Rat Marrow Stromal Cells in vitro. Keystone Symposia 2002 "Molecular Control of Adipogenesis and Obesity", Keystone, CO, USA, 2002.

Lieb, E., Milz, S., Dauner, M., Göpferich, A., Schulz, M.B.; In vitro effects of TGF-β1 on rat marrow stromal cells for bone tissue engineering. 1st Biennial Meeting of the European Tissue Engineering Society (ETES), Freiburg, Germany, 2001.

Neubauer, M., Fischbach, C., Lieb, E., Hacker, M., Teßmar, J, Schulz, M.B., Göpferich, A., Blunk, T.; Adipogenesis of Rat Marrow Stromal Cells in Two- and Three-Dimensional Cell Culture. 1st Biennial Meeting of the European Tissue Engineering Society (ETES), Freiburg, Germany, 2001.

Hacker, M., Teßmar, J., Neubauer, M., Lieb, E., Blunk, T., Göpferich, A., Schulz, M.B.; Anhydrous scaffold fabrication from sensitive biodegradable polymers. MRS Fall Meeting USA, 2001.

Hacker, M., Lieb, E., Neubauer, M., Blunk, T., Göpferich, A., Schulz, M.B.; Entwicklung einer Methode zur Herstellung dreidimensionaler Zellträger für das Tissue Engineering. DPhG, Halle, Germany, 2001.

Lieb, E., Teßmar, J., Fischbach, C., Blunk, T., Schulz, M.B., Göpferich, A.; Me.PEG-PLA Diblock Copolymers Modulate Adhesion and Shape of Rat Marrow Stromal Cells for Bone Tissue Engineering. 3rd Biennial Tissue Engineering Society Meeting, Orlando, USA, 2000.

Lieb, E., Teßmar, J., Blunk, T., Göpferich, A., Schulz, M.B.; Me.PEG-PLA diblock copolymers influence differentiation of rat marrow stromal cells. 3rd Biennial Tissue Engineering Society Meeting, Orlando, USA, 2000.

Lieb, E., Teßmar, J., Fischbach, C., Blunk, T., Schulz, M.B., Göpferich, A.; PEG-PLA Diblock Copolymers Modulate Cell Behaviour for Tissue Engineering of Bone. DPhG, Münster, Germany, 2000.

Lieb, E., Teßmar, J., Fischbach, C., Blunk T., Schulz, M.B., Göpferich, A.; PEG-PLA Diblock Copolymers Modulate Adhesion of Rat Marrow Stromal Cells for Tissue Engineering of Bone. Item 2000, Innsbruck, Austria, 2000.

Award

Item 2000 Award for the poster presentation, International Tissue Engineering Meeting, Item 2000, Innsbruck, Austria.

Danksagung

Die vorliegende Arbeit wurde von Februar 1999 bis Juni 2003 am Lehrstuhl für Pharmazeutische Technologie der Universität Regensburg angefertigt.

Mein besonderer Dank gilt:

- Herrn Prof. Dr. Achim Göpferich für die wissenschaftlichen Anregungen und Diskussionen, das große Interesse am Fortgang der Arbeit und für die Förderung zur Teilnahme an Kongressen im In- und Ausland.
- Frau Dr. Michaela Schulz für die Betreuung der Arbeit, das stete Interesse am Fortgang der Arbeit und für ihre Unterstützung, Kontakte zu anderen Arbeitskreisen zu knüpfen, um neue Methoden am Lehrstuhl zu etablieren.
- Herrn Dr. Torsten Blunk für die wissenschaftlichen Diskussionen.
- der Universität Regensburg für die zeitweise finanzielle Unterstützung in Form des Stipendiums zur Förderung des künstlerischen und wissenschaftlichen Nachwuchses.
- Herrn. Dr. Stefan Milz, Anatomische Anstalt, LMU München für die Hilfe beim Erlernen der Techniken, Gefrierschnitte und immunhistologischen Färbungen anzufertigen.
- Herrn Dr. Peter Sandner, Lehrstuhl für Physiologie für die Unterstützung beim Erlernen der RT-PCR.
- Frau Dr. Leoni Kunz-Schughart, Pathologie, Klinikum, Universität Regensburg für die Hilfe bei den Immunfluoreszenzfärbungen.
- Herrn Michael Hacker und Herrn Dr. Dieter Rose für die Anfertigung der elektronenmikroskopischen Aufnahmen.
- Herrn Dr. Johann Vancea, Lehrstuhl für Physik für die Durchführung der ESCA Untersuchungen.

- Herrn Florian Truksa, Lehrstuhl für Anorganische Chemie für die Durchführung der EDX-Messungen.
- Herrn Dr. Jörg Teßmar für die Synthese der Me.PEG-PLAs und aktiven Polymere.
- dem Arbeitskreis von Herrn Prof. Dr. Horst Kessler, Institut für Organische Chemie und Biochemie, Technische Universität München für die gute Zusammenarbeit im RGD-Projekt.
- Herrn Dr. Martin Dauner, ITV Denkendorf für die Versorgung mit Matrices aus PLLGA.
- Frau Allison Dennis für die gewissenhafte Korrektur des Manuskripts.
- dem Sekretariat für die schnelle Hilfe bei allen Verwaltungsangelegenheiten.
- meinen Mitdoktoranden am Lehrstuhl für die Diskussionsfreudigkeit, sowie für die außerfachlichen Gespräche und Aktivitäten.
- und allen übrigen Mitarbeitern des Lehrstuhls für ihre kollegiale Hilfsbereitschaft.