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Bone engineering on the basis of periosteal cells cultured in polymer fleeces

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Large bone defects caused by severe trauma, infection or tumor resection are still a major challenge for orthopaedic surgery. The key concept for successful bone regeneration consists of combining the osteoinductive effect of osteogenic cells with a suitable carrier structure to promote osteoblastic differentiation and optimal matrix production. Therefore, periosteal cells cultured in polyglycolic-polylactid acid (PGLA) fleeces were investigated for their osteogenic differentiation and used to repair critical size bone defects in a rabbit model. Periosteal cells were isolated from New Zealand White rabbits and expanded *in vitro*. Osteogenic differentiation was investigated by analysis of alkaline phosphatase and osteocalcin production *in vitro* depending on culture conditions and passage number. Cells were seeded into PGLA fleeces. After further cultivation, tissue constructs were examined histologically and by immunohistochemistry for cell distribution and osteogenic differentiation. These constructs of defined size were used to repair critical size calvarial defects (group I) in rabbits compared to a defect repair with polymers only (group II) or to untreated defects (group III). Bone healing was evaluated after 4 weeks by radiodensitometry and a special histological scoring system. For early evaluation, radiodensitometry was not sensitive enough to detect differences in calcification. However, on histologic examination the group with cell/fleece constructs revealed intense formation of uncalcified bone. The mean defect closure of the experimental group I was 65%, compared to control groups II and III with 31% and 22%, respectively. The established methods of 3-D-cell culture and *ex-vivo* transplant assessment proved to be a valuable tool for quality assurance. The results demonstrate that the combination of periosteal cells and polymer fleeces is a tissue engineering approach, which may have clinical applications in various fields of reconstructive surgery.

1. Introduction

Critical size bone defects often demand the transplantation of bone tissue or substitutes to restore bone integrity. While autologous bone grafts raise problems of restricted availability and donor site morbidity, transplantation of biomaterials relies only on the osteoconductive and mechanical properties of the substitutes. New tissue engineering approaches for bone repair, therefore, concentrate on combining autologous cells with appropriate resorbable biomaterials.

For bone engineering, sufficient supply of autologous osteogenic cells is of paramount importance for the preparation of large transplants. Well-differentiated cells are difficult to expand in cell culture and they often lose their potential for differentiation. In contrast, mesenchymal stem cells proliferate easily, but directing these cells into osteogenic differentiation is still a major obstacle. The cambium layer of the periosteum contains osteogenic and chondrogenic progenitor cells at different stages of differentiation [1–3]. These cells contribute to normal bone development and repair [2, 3]. Nakahara *et al.* reported that cultured periosteal cells from young chicks, when assayed *in vivo* in athymic mice, differentiate into osteoblasts and finally give rise to bone tissue [4]. Cultured periosteal cells have been used previously for bone repair [5–7].

In this study, cultured periosteal cells were investigated *in vitro* for their potential of osteogenic differentiation and seeded into a three-dimensional polyglycolic-polylactid acid (PGLA) fleece. These nonwoven fleece structures are biocompatible and bioresorbable, provide high internal surface areas for cell attachment and, at the same time, consist of low amounts of biomaterial [8, 9]. The ability of osteogenic cells to maintain their phenotypic properties while seeded into the polymer is very important for later application *in*

vivo. Therefore, *in vitro* studies of cellular viability and differentiation were performed.

For testing the *in vivo* potential to form bone, cell/fleece constructs were transplanted into calvaria defects in a rabbit model. These are critical size defects which usually do not regenerate at the size chosen here [10] and, therefore, serve as a challenging test for bone repair.

2. Materials and methods

2.1. Sampling and cell isolation

The anteromedial portion of the tibial periosteum of 16-week-old New Zealand White rabbits was dissected and cut into small fragments, which were subsequently digested by 0.25% collagenase (Worthington Biochemical Corp., Freehold, NJ, USA) in Ham's F-12 (Biochrom, Berlin, Germany) for 3 h. The resulting cell suspension was passed through a sterile 100 μm polyester mesh filter (Estal mono, Thal, Switzerland), centrifuged, and the pellet was washed three times with Ham's F-12 before the cells were further proceeded to monolayer culture. As control, fibroblasts from rabbit muscle tissue were obtained using the same isolation procedure and cultivation technique as for periosteal cells. After this preparation and at each culture passage, cells were counted and viability by trypsan blue exclusion was found to exceed 90%.

2.2. Monolayer culture

Isolated cells were plated at 2×10^4 cells cm^{-2} in culture flasks with Ham's F-12 supplemented by 10% fetal bovine serum, 100 IE ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Biochrom, Berlin, Germany) (NM). The medium was replaced every second day and cells were subcultured for five passages for transplant preparation. To harvest the cells, 0.02% trypsin/ethylene diamine tetra-acetic acid (EDTA) (Biochrom, Berlin, Germany) was applied in phosphate-buffered saline (PBS) for 5 min. Differentiation analysis was performed for each passage. Cells were seeded onto six-well plates at 2×10^4 cells cm^{-2}. Cells were treated with osteogenic medium (OM) (NM supplemented with 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, 0.3 mM ascorbic acide, Sigma, Deisenhofen, Germany). After 4, 8, 12 and 16 days, cells were analyzed for alkaline phosphatase and osteocalcin expression. Four days prior to osteocalcin assay, medium of corresponding samples was supplemented by 10^{-9} M 1,25-OH-vitamin D_3.

2.3. Immunohistochemistry for alkaline phosphatase and osteocalcin

Cells were plated on chamber slides at 2×10^4 cells cm^{-2} and cultured for 1 day. For immunohistochemistry cells were washed with PBS, fixed for 5 min in acetone and dried at 37 °C for 30 min. After rinsing in 1% H_2O_2 (Merck, Darmstadt, Germany) for 5 min and incubation with 10% rabbit serum (Biochrom) in PBS for 30 min, slides were incubated for 60 min with primary antibodies (monoclonal mouse anti-human alkaline phosphatase antibody (Dako, Hamburg, Germany), dilution 1 : 40; monoclonal mouse anti-human/bovine osteocalcin antibody (Dako), dilution 1 : 20). Control stainings were performed with isotype-matched irrelevant antibodies. Slides were then incubated with biotinylated rabbit anti-mouse antibody (Dako) (dilution 1 : 600) for 30 min. After subsequent washes, slides were covered with peroxidase conjugated streptavidin (Dako) (dilution 1 : 1000). The color reaction was developed by 3,3′-diaminobenzidine or new fuchsin substrate kit (Dako), followed by counterstaining with haematoxylin (Merck).

2.4. Preparation and culture of three-dimensional carriers

Fifth passage cells were suspended in OM and mixed with fibrinogen (Tissucol®, Immuno GmbH, Heidelberg, Germany) at a ratio of 3 : 1. After seeding into Ethisorb®-fleeces (Ethicon, Norderstedt, Germany, 15 mm diameter, 2 mm height), thrombin diluted 1 : 10 with PBS was added to achieve fibrin polymerization (group I). Identically manufactured fleeces without cells were used for control (group II). Carriers were cultured in OM for 1, 2, 3 and 4 weeks at standard culture conditions.

2.5. Histology of cell/fleece constructs

Cryosections were mounted on aminoalkylsilane-coated slides (Sigma), fixed for 5 min in acetone, and stored at − 20 °C. Sections were stained with haematoxylin and eosin (HE) (Sigma). Immunohistochemistry for alkaline phosphatase and osteocalcin was performed as described above.

2.6. MTT-test

For testing cell viability in fleece structures, the MTT-test [11] was performed using equal size cell/fleece constructs which had been cultivated over 1, 2, 3 and 4 weeks. These carriers were incubated for 4 h in 550 μl MTT-solution (0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) in medium) at 37 °C and subsequently exposed to 400 μl lysis buffer (96.4% v/v DMSO (Sigma), 0.6% v/v acidic acide (Merck), 3% v/v SDS (Merck)) at 4 °C overnight. Cell viability was assessed photometrically by measuring the amount of formazan in the lysis buffer solution at 560 nm.

2.7. Surgical procedure

A total of 10 critical size calvarial defects on five rabbits, weighing 3–4 kg, were created. The rabbits were operated on under general anaesthesia with 0.4 ml kg^{-1} Hypnorm® (fluanisone 10 mg ml^{-1} and fentanyl 0.315 mg ml^{-1}, Janssen-Animal Health, UK) given intramuscularly 25 min preoperatively. Under aseptic conditions the calvaria was exposed and a 15 mm full thickness defect was created bilaterally in the parietal bone using a trephination drill. The periosteum was removed completely. All defects were rinsed with normal saline to remove any residual bone powder. The defects were filled with periosteal cell/fleece constructs (group I; four defects), with polymers alone (group II; three

defects) or left untreated (group III; three defects). The wound was closed in layers with Vicryl®-sutures (Ethicon, Norderstedt, Germany). All animals were handled according to the standard procedures for laboratory animals and were sacrificed after 28 days.

2.8. Analysis by radiodensitometry

After the animals were sacrificed, X-rays of the excised calvarias were taken. Defect area and the surrounding bone were analyzed densitometrically, using NIH Image software (National Institute of Health, Bethesda, Maryland, USA). The difference between bone and defect area density was used as a measure for bone formation. Analysis was performed blind and repeated three times. The mean value was used as a measure for quantitation.

2.9. Histological analysis

Excised calvarias were partially decalcified and embedded in paraffin. Vertical slices were stained with hematoxylin and eosin (HE) and Masson's trichrome.

3. Results and discussion

3.1. Investigation of periosteal cells in monolayer culture

In general, cell proliferation and cell differentiation are almost contradicting situations in cell and tissue development [12] and are modulated by different culture conditions. Cell proliferation causes dedifferentiation, whereas stimulation of differentiation reduces proliferation. In osteoprogenitor cells, the media supplements ascorbic acid, β-glycerophosphate and vitamin D are known to induce cell differentiation [13].

Therefore, periosteal cells from rabbit tibia were expanded in monolayer culture using regular medium. Prior to differentiation analysis, osteogenic supplements were added. Alkaline phosphatase is an early, but unspecific marker of osteogenic differentiation, which is progressively expressed upon differentiation [13]. Osteocalcin, in contrast, is very specific for osteoblasts; however, it is expressed later on during differentiation [12]. Furthermore, it is essential for the formation of mineralized matrix [14]. Immunohistochemical analysis for both markers revealed that periosteal cells produce significant amounts of alkaline phosphatase and osteocalcin even after five passages of cell expansion, when finally stimulated with osteogenic supplements to differentiate (Fig. 1). Testing proliferating periosteal cells in regular medium prior to medium supplementation, these cells still expressed AP and osteocalcin up to five passages, but at a much lower level (data not shown). As a control, fibroblasts from muscle tissue showed production of only minor amounts of AP and no production of osteocalcin. These results demonstrate that periosteal cells were successfully isolated and expanded *in vitro* without losing the expression of osteogenic markers. These findings are in accordance with investigations by others [15, 16]. Of importance for subsequent experiments, the number of passages performed under these conditions allowed sufficient amplification of osteogenic precursor cells for tissue engineering of large autologous transplants.

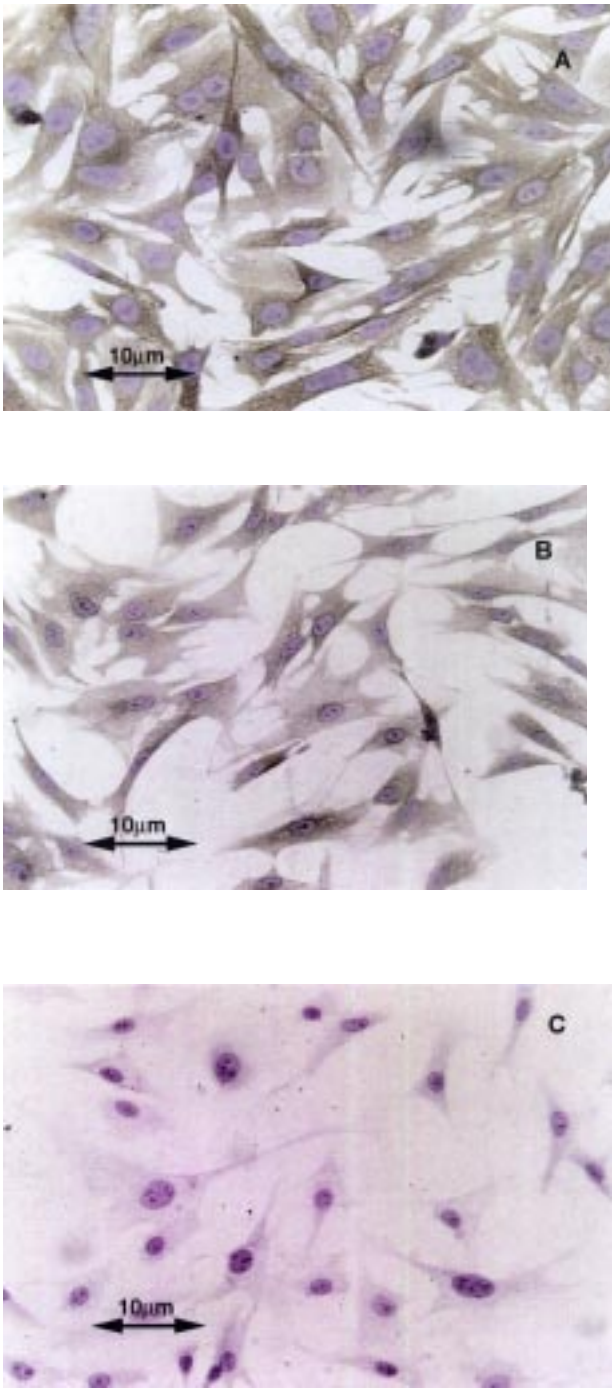

Figure 1 Immunohistochemistry of periosteal cells in monolayer cell culture after five passages and 4 days exposure to osteogenic medium. (A) Alkaline phosphatase, (B) osteocalcin, (C) negative control.

3.2. Investigation of periosteal cell/fleece constructs *in vitro*

Fifth passage periosteal cells were seeded into resorbable biocompatible PGLA fleeces to study the development of three-dimensional bony tissues. Cell/fleece constructs were analyzed regarding overall metabolic activity, AP and osteocalcin expression and cell distribution at 1, 2, 3 and 4 weeks of cultivation. The MTT-test revealed a constant total mitochondrial dehydrogenase activity over this 4-week period. The test result is dependent on cell

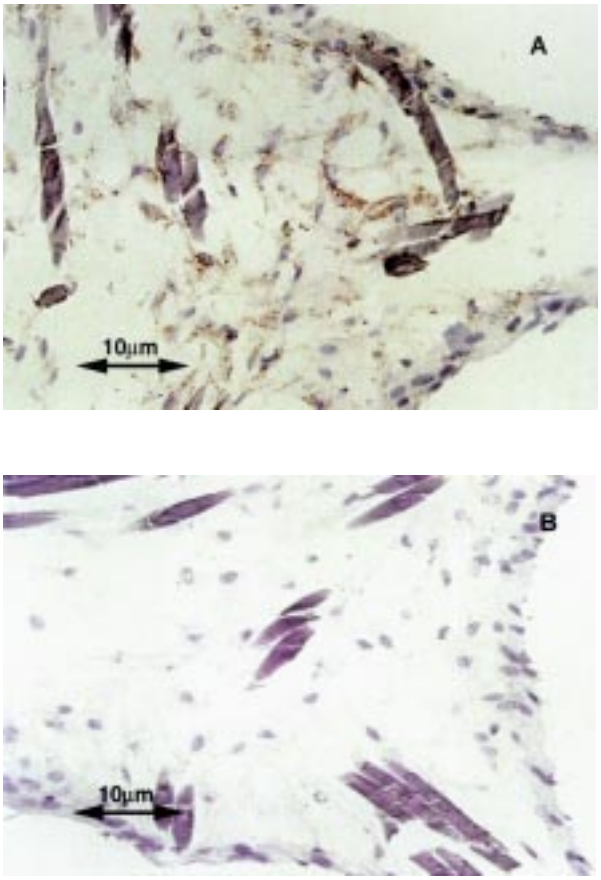

Figure 2 Immunohistochemistry of periosteal cell/fleece construct after 4 weeks of culture. (A) Osteocalcin, (B) negative control.

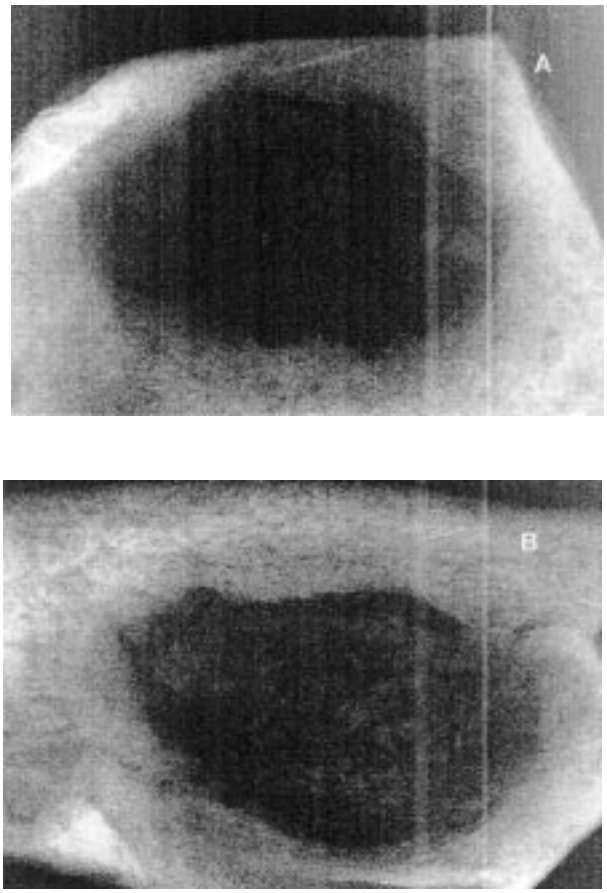

Figure 3 X-ray of calvaria defects after explantation: (A) defect repair with periosteal cell/fleece construct, (B) untreated defect.

number and cell activity. Assuming a constant metabolic activity of the periosteal cells at all times after initiation of differentiation in the three-dimensional culture, it could be concluded that cells do not further proliferate inside the fleeces. Immunohistochemical staining of periosteal cells in PGLA fleeces demonstrated expression of AP and osteocalcin and, therefore, confirmed differentiation into osteoblasts (Fig. 2). Thus, both analyses were in accordance with a switch from cell expansion towards differentiation and bone tissue formation. HE-sections of cell/fleece constructs showed a homogeneous distribution of cells in the fleece structure at 1 and 2 weeks of culture. This confirms the reliability of the fibrin embedding technique introduced earlier by us [17]. During week 3 and 4, a change in distribution occurred with an increase of cell density towards the surface of the artificial tissue construct. This may result from a high nutritional demand for matrix synthesis counteracted by a decreasing supply by diffusion inside the tissue with accumulation of extracellular matrix. As *in vitro* conditions are less favorable compared to *in vivo* ''nursing'' shown earlier for cartilage transplants [18], we decided to implant fleeces after one week of *in vitro* cultivation.

3.3. Repair of rabbit calvarial defects

To challenge the capacity of these engineered osteogenic tissues for bone repair, the established model of critical size calvarial defects in rabbits was used [10]. Radiodensitometry did not reveal significant calcification in any of the treated or untreated groups (Fig. 3). Therefore, no significant radiological differences between cell/polymer constructs and control groups could be established at this early stage of 4 weeks. Although there is a significant delay of healing compared to equivalent treatment in ulnar defects (unpublished data), this lack of early calcification in calvarial defect repair was also observed by others [7]. The most evident explanation for this phenomenon in these two models is the difference of mechanical stress, which is known to be an important promoter of bone healing if applied in limited quantity [19].

To establish a reliable method for the analysis of the repair capacity early in these critical size defects, histological studies were performed. Vertical sections spanning the full diameter of the circular defects were investigated. HE and Masson's trichrome staining were sufficient to distinguish between osteogenic and fibrotic tissue formation (Fig. 4b). Samples derived from group I showed a clearly improved bone regeneration compared to controls. In general, bone formation was not preceded by cartilage formation. This corresponds to the pattern expected for formation of membranous bone like calvaria.

Furthermore, in group I, bone regeneration was initiated not only by ingrowth from the margin. There were also found growing islands of bone in the central portion of the defect. In the control groups osseous repair was confined to marginal bone formation. This finding was considered most important. It was used as a main

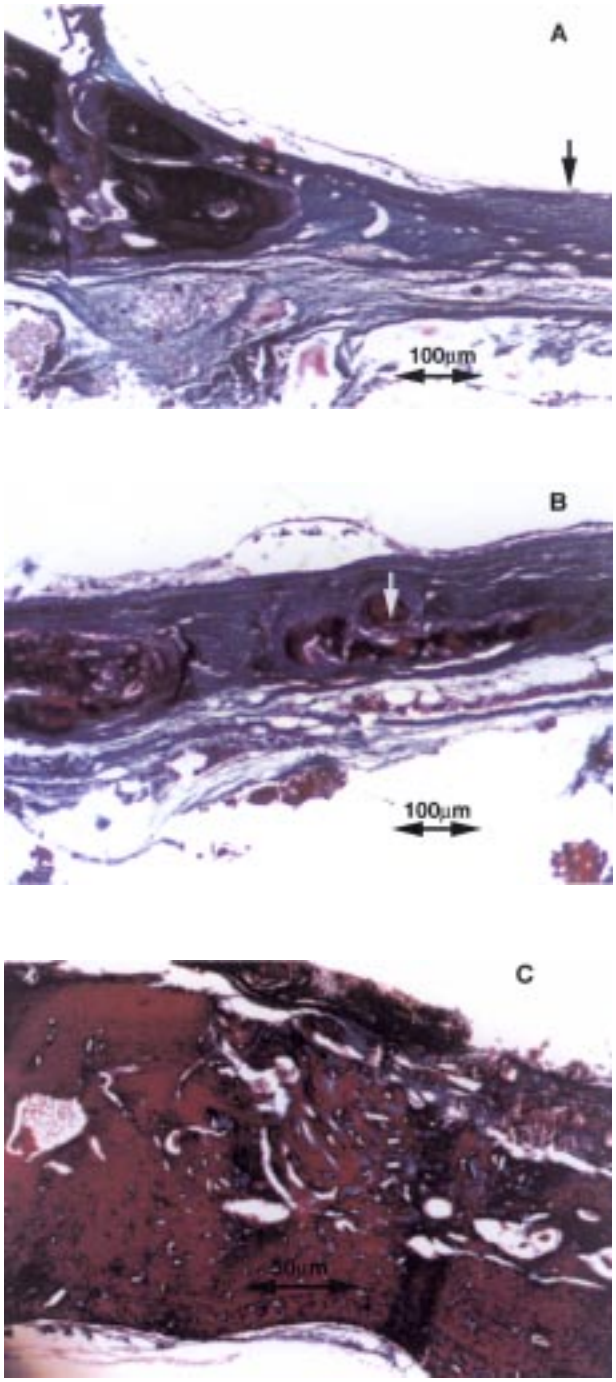

Figure 4 Section of defects after 4 weeks, Masson's trichrome staining. (A) Untreated defect. There is only minor ingrowth of bone from the margin. In the middle of the defect, only fibrous tissue and no islands of bone can be detected. (B) Defect treated with periosteal cell/fleece construct. Formation of bony islands in the middle of the defect can be seen. (C) Defect treated with periosteal cell/fleece construct. Complete bony bridging is demonstrated.

criterion for a scoring system, which was designed for a qualitative and reproducible grading in the early stage of bone repair. Grade 1 was given for ingrowth of bone from the borders of the defect only (Fig. 4a), grade 2 was assigned, if, in addition to marginal growth, islands of bone formation in the middle of the defect were detected (Fig. 4b), grade 3 was assigned for complete osseous bridging of the defect (Fig. 4c). This revealed a mean score of 2.5 for the cell/fleece group, while defect repair with polymers alone and untreated defects received 1.0 and 1.3, respectively. Compared to controls, these data suggest a new quality of bone formation in the experimental group I with foci of osteogenesis originating from the implanted periosteal cells themselves. Similar repair mechanisms were observed in ulnar defects filled with engineered periosteal tissues (unpublished data). In addition, for quantification of bone repair, the percentage of the defect area covered by osteogenic tissue was determined by measuring the portion of bony bridging in relation to the diameter of the whole defect. Mean defect closure for group I was found to be 65%, whereas in groups II and III, defects were closed only by 31 and 22%, respectively.

4. Conclusion

The data presented demonstrate that periosteal cells as progenitor cells for physiologial bone repair are a suitable source for bone engineering regarding their potential for cell expansion and osteogenic differentiation. This osteogenic differentiation *in vitro* is supported by cultivation in three-dimensional PGLA fleeces and fibrin gels under appropriate medium conditions. Using a scoring system for quantification of early bone repair, *in vivo* results in the calvarial defect model show that the combination of periosteal cells with PGLA fleeces is a promising approach to engineered bone repair.

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