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RESEARCH ARTICLE

Bone regenerative properties of injectable PGLA–CaP composite with TGF-β1 in a rat augmentation model

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Abstract

The aim of this study was to examine the bone augmentation properties of an injectable composite consisting of PLGA microspheres/CaP cement (20/80), and the additional effect of loading PLGA microspheres with TGF-β1 (200 ng). For this purpose, PLGA/CaP composites (control) and PLGA/CaP composites loaded with TGF-β1 (test group) were injected on top of the skulls of 24 Wistar rats. Each rat received 2 materials from the same experimental group, and in total 48 implants were placed ($n = 8$). After 2, 4, and 8 weeks the results were evaluated histologically and histomorphometrically. The contact length between the implants and newly formed bone increased in time, and was significantly higher for the TGF-β1-loaded composites after 2 weeks. Also, bone formation was significantly higher for the TGF-β1-loaded composites (18.5% $\pm$ 3) compared to controls (7.21% $\pm$ 5) after 8 weeks of implantation. Immunohistochemical staining demonstrated massive inflammatory infiltrates in both groups, particularly at 2 weeks, which decreased substantially at 4 and 8 weeks. In conclusion, injectable PLGA/CaP composites stimulated bone augmentation in a rat model. The addition of TGF-β1 to the composite significantly increased bone contact at 2 weeks and enhanced new bone formation at 8 weeks. Copyright © 2008 John Wiley & Sons, Ltd.

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Keywords TGF-β1; injectable CaP cement; PGLA–CaP composite; bone augmentation; bone; growth factors

1. Introduction

Autogenous bone is still considered to be the ‘gold standard’ for bone augmentation. However, due to the known limitations of autogenous bone grafting, such as donor site morbidity and limited supply, increasing efforts focus on the development of bone substitutes. In order to create a bone graft, which is comparable or superior to bone, allografts are combined with osteoinductive substances, such as growth factors. Transforming growth factor-β1 (TGF-β1) is a member of TGF-β superfamily, which regulates key aspects in bone metabolism (Massagué, 1990). It affects osteoblast differentiation, matrix formation and mineralization. Furthermore, it exerts its functions both during embryogenesis and in adult organisms, orchestrating complex phenomena such as inflammation and tissue repair (Roberts and Sporn, 1993). Studies have shown that TGF-β1 supplied to a variety of biomaterials is able to enhance bone formation *in vivo*, depending on concentration and application method (Okuda *et al*., 1995; Sumner *et al*., 1995; Lind *et al*., 1996). Consequently, TGF-β1 is an appealing factor for use in clinical procedures for bone augmentation.

As mentioned above, various materials have already been used for local delivery of TGF-β1, including calcium phosphate (CaP) ceramics (Okuda *et al*., 1995; Sumner *et al*., 1995; Lind *et al*., 1996). This material has been selected for bone replacement and augmentation,

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due to its excellent bone behaviour. The shortcomings of the prefabricated CaP ceramics, such as lack of shaping and difficult delivery to the defects, have been overcome by the development of injectable CaP cements (Ooms *et al*., 2005). Injectable self-setting cement can fill macroscopic as well as microscopic bone defects. Cements of this kind may be resorbed, allowing gradual replacement by host bone (Jansen *et al*., 1995). To promote degradation and tissue ingrowth injectable CaP cements have been combined with PLGA microspheres (Ruhe *et al*., 2003). Additionally, these degradable PLGA microspheres provide a method for delivery of bone formation promoting proteins, such as TGF-β1. Such an injectable composite has been developed by our group and comprehensively studied *in vitro* and *in vivo* (Ruhe *et al*., 2003, 2005, 2007). However, the direct mode of application in a bone augmentation model has not yet been evaluated.

In view of the above-mentioned, the aim of this study was to examine the bone augmentation properties of an injectable PLGA–CaP composite and to evaluate the additional effect of loading the composite with TGF-β1 in a rat model.

2. Materials and Methods

2.1. Materials

The calcium phosphate cement Calcibon® (Biomet Merck, Darmstadt, Germany) was used in this study. The cement powder consisted of 62.5% α-tri-calcium phosphate (α-TCP), 26.8% $CaHPO_4$, 8.9% $CaCO_3$ and 1.8% precipitated hydroxyapatite (PHA). An aqueous solution of 1% Na_2HPO_4 was used as a liquid component with a liquid : powder ratio of 0.35 ml/g. Low-molecular weight poly(DL-lactic-co-glycolic acid) (PLGA) Purasorb® was provided by Purac (Gorinchem, The Netherlands). Human rh-TGF-β1 was supplied by R&D Systems (Abingdon, UK).

2.2. Preparation of PLGA microspheres

PLGA microspheres were prepared using a water-in-oil-in-water (w/o/w)-double emulsion solvent evaporation technique, as described previously by Ruhe *et al.* (2003). Briefly, 1.4 g low molecular weight (LMW) PLGA was dissolved in 2 ml dichloromethane (DCM) inside a 50 ml polypropylene (PP) tube; 500 µl demineralized water (ddH_2O) was added while vortexing vigorously for 1 min, subsequently adding 6 ml 0.3% polyvinylalcohol (PVA) solution. Vortexing was continued for another 1 min. The content of the 50 ml tube was transferred to a stirred 1000 ml beaker and another 394 ml 0.3% PVA was added slowly. This was directly followed by adding 400 ml 2% isopropylic alcohol (IPA) solution. The suspension was stirred for 1 h. The spheres were allowed to settle for 15 min and the solution was decanted. The suspension left was centrifuged and the clear solution at the top was decanted. ddH_2O (5.0 ml) was added, the spheres were washed, centrifuged and the solution was aspirated. Finally, the spheres were frozen, freeze-dried for 24 h and stored under argon at −20 °C.

2.3. Adsorption of TGF-β1 on PLGA microspheres

Recombinant human TGF-β1 (rh-TGF-β1; 1 µg) was dissolved in 800 µl 0.1% acetic acid and added to 1.0 g microspheres, after which the TGF-β1 was freeze-dried onto the microspheres. The entrapment efficiency of the protein was determined by normalizing the amount actually entrapped with the amount added to the fabrication process.

2.4. Preparation of PLGA–CaP composites

Composites were made by adding PLGA microspheres to the CaP cement in a PLGA : CaP cement weight ratio of 20 : 80. The cement powder was sterilized by γ-irradiation of 25 kGy (Isotron B.V., Ede, The Netherlands) and the cement liquid was filter-sterilized (0.2 µm filter). First, 256 mg CaP cement powder provided with 64 mg PLGA microspheres was added to a 2 ml plastic syringe (Sherwood Medical Monojet, Schwallbach, Germany). The mixture was shaken vigorously for 15 s, using a Silamat® mixing apparatus (Vivadent, Schaan, Liechtenstein). Subsequently, 125 mg 1% Na_2HPO_4 was applied to the mixture, shaken again for 15 s and then delivered by means of the syringe to the surgical site. PLGA–CaP composites provided with TGF-β1 were made by adding a fraction of TGF-β1-adsorbed PLGA microspheres to a fraction of as-prepared PLGA microspheres, followed by the same procedure as described for the unloaded composites. The average amount of delivered material was 60 mg and the growth factor-loaded composites contained 200 ng TGF-β1.

2.5. Surgical procedure

For the animal experiment, 24 healthy, skeletally mature male Wistar rats, with an average weight of 250 g, were used. National guidelines for care and use of laboratory animals were observed, and the Animal Ethical Committee of the Radboud University Nijmegen Medical Centre approved the study protocol. In total, 48 composite implants were placed; each animal received two implants from the same group on its parietal cranial bone for evaluation periods of 2, 4 and 8 weeks ($n = 8$ for each material and evaluation time).

Anaesthesia was induced with 4% Isoflurane by a non-rebreather mask and maintained with 2% Isoflurane, 0.4% N_2O and 0.4% O_2 during surgery. The animals were premedicated by an intramuscular injection of fentanyl (2.7 ml/kg) to reduce the operative pain, and a subcutaneous injection of buprenorphine (150 µg/kg) was applied to reduce the postoperative pain.

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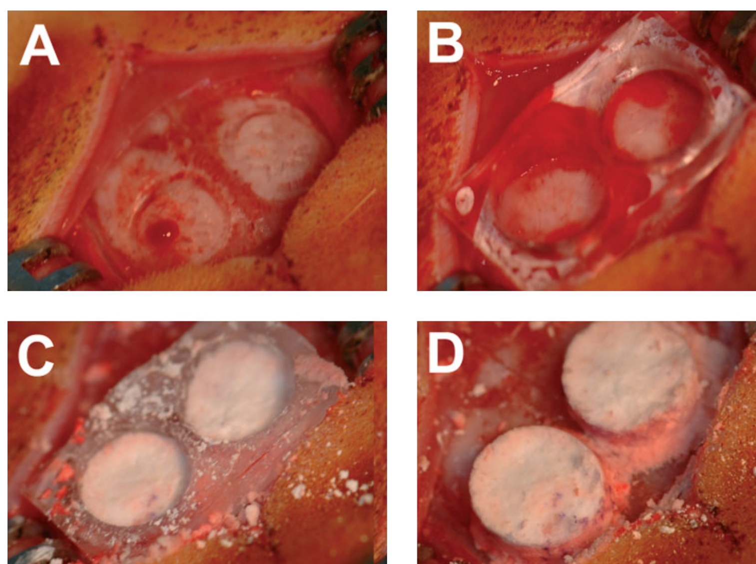

Figure 1. Rat model for bone augmentation. (A) Defect; (B) silicon mould; (C) PLGA–CaP composite in mould; (D) PLGA–CaP composite after setting and removal of the mould

Before surgery, the rat cranium was shaved and swabbed with iodine. A medial sagittal incision extending from the nasofrontal area to the occipital protuberance was made and soft tissues were sharp dissected to visualize the cranial periosteum. Subsequently, the periosteum was undermined and reflected, exposing the parietal bones. A hollow trephine bur (ACE Dental Implant System, Brockton, MA, USA) with an outer diameter of 5 mm in a dental handpiece was used to mark the position of two augmentation sites on the parietal bone, aside from the sagittal sinus. The trephine burr did not penetrate into the whole thickness of the skull, but was stopped just in the upper part of the cortical plate, next to the periosteum. Within the marked areas, the cranial surface was scratched in order to remove part of the cortical bone and to achieve exposure of the osteogenic cells. Afterwards, a silicon mould with a diameter of 5 mm, specifically designed for this purpose, was placed on the cranium and the PLGA–CaP composite was injected (Figure 1). In this way, implants in the form of discs were created, with a diameter of 5 mm and a height of 2 mm. After setting of the composite, the mould was removed and the periosteum and overlying skin were closed in separate layers with 5-0 and 4-0 Vicryl resorbable sutures, respectively.

2.6. Histological preparation

The animals were sacrificed 2, 4, and 8 weeks after surgery, using an overdose of carbon dioxide. The skin was dissected and the defect sites were removed, along with the surrounding bone and soft tissues, and fixed in 10% neutral formalin for 1 week. Half of the specimens ($n = 4$ per material and evaluation time) were dehydrated in gradual series of alcohol and embedded in polymethylmethacrylate (PMMA). Before the embedding procedure, samples were cut exactly through the middle of the implants to ensure that the microtome sections were made in the area of interest and the same region was analysed for each sample. After polymerization, sections were prepared using a modified diamond-blade sawing microtome technique (Leica Microsystems GmbH, Wetzlar, Germany). The sections were approximately 10 µm thick and were stained with methylene blue and basic fuchsin. The remaining specimens ($n = 4$ per material and implantation time) were decalcified for 2 weeks in Formical-2000 (Immunodiagnostika & Biotech GmbH, Berlin, Germany) and then embedded in Paraplast paraffin (Klinipath B.V., Duiven, the Netherlands). Sagittal microtome sections were made, 5 µm in thickness, which were stained with haematoxylin and eosin (H&E) and immunohistochemically using an antibody against CD68 (Macrosialin; Janeway *et al.*, 1999) to detect inflammatory cells, including monocytes, macrophages and neutrophils. Again, before the embedding procedure, samples were cut exactly through the middle of the implants.

The light microscopical evaluation of all sections was done using an optical microscope (Leica BW, Rijswijk, The Netherlands), and consisted of a complete morphological description of the tissue response to different implants as well as of histomorphometrical analysis.

2.7. Histomorphometry

For the histomorphometrical analysis, a Leica Qwin Proimage analysis system (Leica BV, Rijswijk, The Netherlands) was used. Sections were digitized at low magnification (×2.5) and measurements were done on three sections per cranial specimen. All PMMA sections were used for evaluation of bone response and all paraffin sections for assessment of inflammatory reaction.

To quantify the amount of bone augmentation, newly formed bone, as stained and discerned by its woven

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structure and location, was marked manually and its area was measured. Afterwards, this ROI was subtracted from the whole area of the implant. From these two data, the amount of bone formation (%) was calculated.

In order to measure the contact length between the implants and the bone, lines were drawn manually at the direct interface between the composites and newly formed bone. The sum of these intersections was calculated and interpreted as contact bone–composite length.

Further, the intensity of the inflammatory response around the implants was quantified. For this purpose, pictures with higher magnification (i.e. $\times 10$) were used. The presence of macrophages was detected with anti-CD68 staining and subsequently digitized by a computer program in the region of interest (implant + surrounding tissue; ROI). The areas with positive anti-CD68 staining were given as a percentage (%) of the total ROI.

2.8. Statistical analysis

For the statistical analyses, the GraphPad InStat program (GraphPad Software, San Diego, CA, USA) was used. One-way analysis of variance (ANOVA) was performed on the data obtained from the histomorphometric analyses. ANOVA assumes that the data are sampled from populations with identical standard deviations. This assumption was tested using the method of Bartlett. Further, ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the Kolmogorov–Smirnov method. In addition, a Tukey–Kramer multiple comparisons test was done. Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. General observations

The injection of the composite into the silicon mould placed on the scratched cranial surface went without complications. The setting time of both loaded and unloaded composites was about 10 min. Setting and handling properties of the composites were influenced by the presence of blood. When the composite was injected on dry, good isolated cranial surfaces the setting time was shorter, the composite was easily shaped, and after removal of the mould the implant was secured more stable. Mixing with blood affected the handling properties of the composites and made them more fragile. In addition, the composites loaded with TGF-β1 appeared to be more fragile after setting than the unloaded composites, as indicated by easy cracking.

During the implantation periods, all animals remained in good health. At sacrifice, macroscopic signs of inflammation and adverse reaction were apparent around loaded as well as unloaded implants, predominantly after 2 and 4 weeks. After 8 weeks, the signs of inflammation disappeared. Upon retrieval, all implants in both groups were *in situ* and surrounded by a fibrous capsule. The capsule thickness differed between different time points, with a tendency to reduction for the later periods. No differences between the loaded and unloaded composites were observed.

3.2. Descriptive histology

Histological examination of PMMA-embedded samples revealed new bone formation in both groups at all time points. Newly formed bone was more abundant at 4 and 8 weeks than at 2 weeks (Figure 2). Bone formation was observed at the defect margins and along the lower part of the cement at the side of the cranium. No bone formation was seen from the periosteum side on the top of the implants, or throughout the implant. At higher magnification, newly formed bone revealed thin trabeculae and large marrow spaces filled with fat tissue and haematopoietic cells. In addition, a direct contact between the composites and newly formed bone, without any fibrous tissue interface, was apparent in both experimental and control groups (Figure 3). Furthermore,

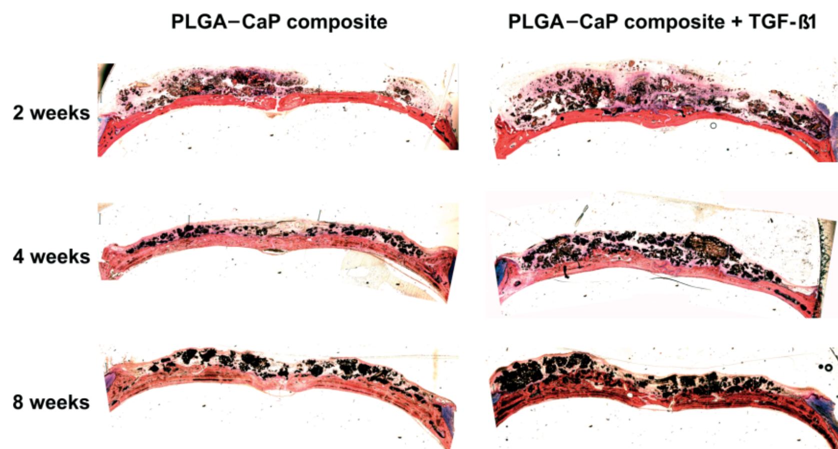

Figure 2. Light micrographs of PMMA-embedded specimens showing bone augmentation at different time points (×2.5 magnification)

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implants managed to stay in place during all investigation periods. However, the integrity of the composite structure appeared to be affected. Fractures, as observed inside the implants, were filled with fibrous tissue and inflammatory cells. Signs of inflammation were best visible in paraffin sections stained for CD68. The inflammatory infiltrate, consisting of macrophages and lymphocytes, surrounded the implants and penetrated into the composite structure. In the course of time, a substantial decrease in the inflammatory response was observed in both groups (Figure 4). Together with the decrease in inflammation, a consolidation of the implant fragments and their incorporation in the bone tissue was noticed. Paraffin sections stained with H&E did not provide any information additional to that provided by the PMMA sections.

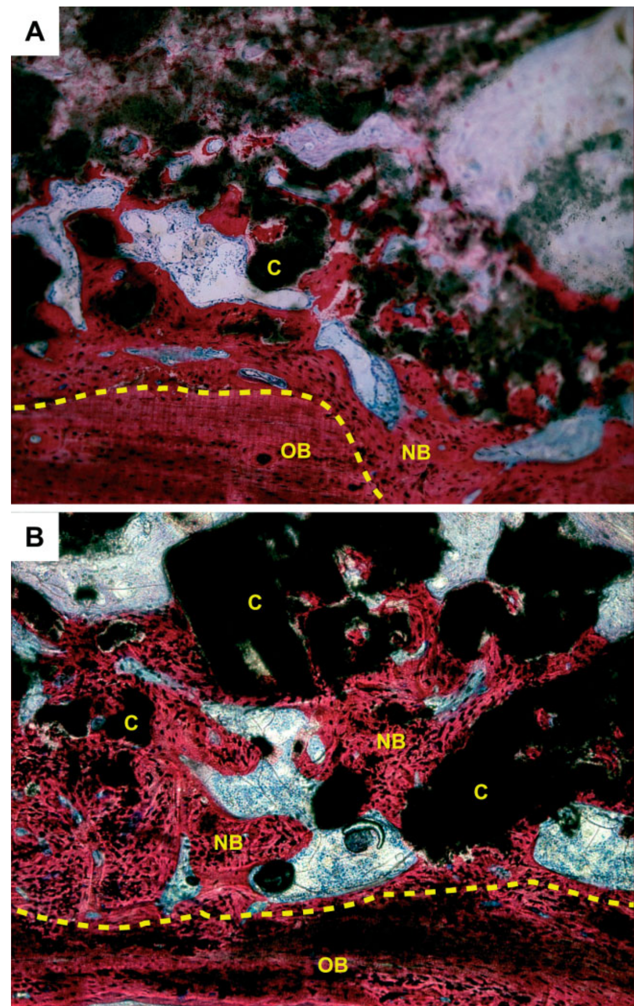

Figure 3. Histological section of PMMA embedded specimen (×10 magnification). (A) unloaded PLGA–CaP composite; (B) TGF-β1 loaded PLGA–CaP composite. Guided bone formation is visible with direct contact composite–newly formed bone without any fibrous tissue interface. Dashed line in yellow indicates the transition between the newly formed bone and the old bone. OB, old bone; NB, newly formed bone; C, composite

3.3. Histomorphometry

The amount of newly formed bone was almost the same for both experimental and control groups after 2 and 4 weeks. At 8 weeks, there was more bone formation in the group of TGF-β1 loaded implants ($18.5 \pm 3\%$) than in the group of the unloaded ones ($7.2 \pm 5\%$), which was statistically significant ($p < 0.05$; Figure 5).

The contact length between the composites and newly formed bone increased with time. At 2 weeks TGF-β1 loaded samples showed significantly better bone contact than unloaded controls (19 ± 7 mm vs. 2.7 ± 4 mm; $p < 0.05$). After 4 and 8 weeks, no difference in the contact length was observed between the groups (Figure 6).

The amount of inflammation after 2 weeks was substantial for both groups. However, after 4 weeks it decreased to $9.0 \pm 2\%$ for the TGF-β1-loaded implants and to $13.3 \pm 2\%$ for the controls. This difference between the groups appeared to be statistically significant ($p < 0.01$). At 8 weeks, minor inflammation was observed in both groups (Figure 7).

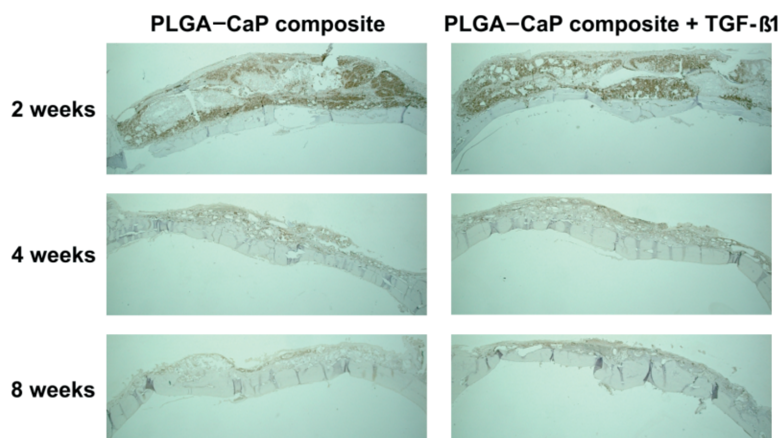

Figure 4. Light micrographs of paraffin sections stained for CD68, showing the inflammatory response at different time points (×2.5 magnification)

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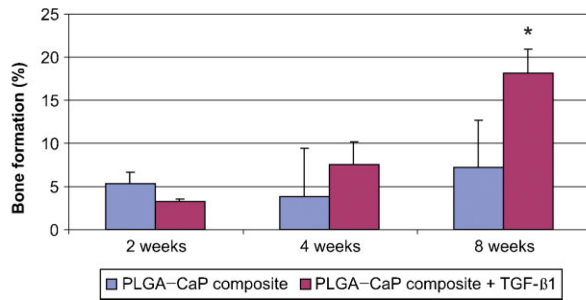

Figure 5. Histomorphometric measurements of bone formation (%) for all evaluation periods. *$p < 0.05$ (statistically significant)

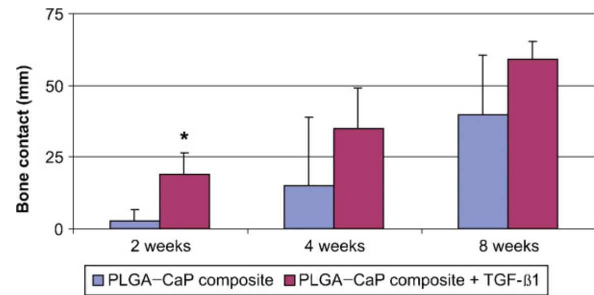

Figure 6. Histomorphometric measurements of composite–bone contact length (mm) for all evaluation periods. *$p < 0.05$ (statistically significant)

4. Discussion

The present study demonstrates that the injectable PLGA–CaP (20 : 80) composite used stimulated bone augmentation in a rat model. Moreover, the addition of TGF-β1 to the composite significantly enhanced new bone formation at 8 weeks when compared to the control (i.e. unloaded PLGA–CaP composite). The osteopromotive effect of TGF-β1 has been demonstrated in many studies (Arnaud *et al*., 1994; Zhou *et al*., 1995; Sumner *et al*., 1995; Lind *et al*., 1996; Beck *et al*., 1991, 1993; Moxham *et al*., 1996). However, there are studies that describe the absence or even an adverse effect of TGF-β1 on bone formation (Lind *et al*., 1994; Aspenberg *et al*., 1996). Since it is known that follow-up period, as well as animal species, may play role in this aspect (Janssens *et al*., 2005), it was decided to compare the outcomes of the current study with similar experimental studies. Our findings corroborate the results of Vuola *et al*. (2002). In their rat cranial model, TGF-β1-loaded coral-based implants enhanced bone regeneration, and the amount of newly formed bone was also not significantly higher compared to the controls until the 8th week. The authors suggested that the influence of TGF-β1 on bone formation lasted several weeks and diminished with age. In the study of Vuola *et al*., 6–8 month-old rats, weighing 550–560 g, were used. In our study the experimental animals were younger, yet skeletally mature, and with a weight of approximately 250 g. Further, the concentration of TGF-β1 incorporated in our composites (200 ng) was much lower than that used in the study of Vuola *et al*. (1, 2 and 25 µg).

The effect of TGF-β1 on bone formation is known to be dose-dependent; however, it has to do more with an optimal than with a maximal dose (Beck *et al*., 1993). For example, Blom *et al*. (2001) reported enhanced bone formation for the same implantation period with very low concentrations of TGF-β1 (10 and 20 ng) incorporated in a similar type of CaP cement. In the current experiment, enhanced bone formation was also observed with a lower TGF-β1 dose than applied in similar studies (Okuda *et al*., 1995; Bosch *et al*., 1996). Therefore, it can be suggested that the amount of TGF-β1 needed to exert an effect may depend on the type of carrier used.

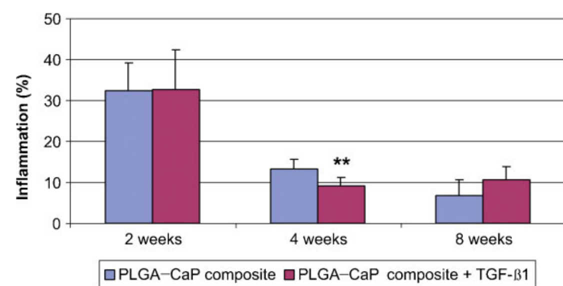

Figure 7. Histomorphometric measurements of the amount of inflammation (%) for all evaluation periods. **$p < 0.01$ (statistically significant)

Delivery systems are of great importance when growth factors (GFs) are utilized to enhance the bone-healing process (Puolakkainen *et al*., 1995). In previous studies (Ruhe *et al*., 2003, 2005) it was demonstrated that: (a) PLGA–CaP composite is a suitable carrier for GFs; and (b) the method of loading GFs on the scaffold (freeze-drying on top of the PLGA microspheres) does not affect their osteoinductive properties. However, the high protein-binding affinity of CaP cement must be taken into account. Therefore, it can be hypothesized that after release from the PLGA microspheres TGF-β1 may have bound to the CaP cement, resulting in a delayed release from the composite. This might explain why no increase in bone formation was seen after 4 weeks for TGF-β1-loaded materials compared to the controls. At the moment, it is still unclear whether the TGF-β1, as bound to the CaP cement, has maintained its osteopromotive properties. We take note that this has to be an issue of investigation.

It has also to be emphasized that the effect of TGF-β1 on bone formation differs among species. In general, studies using rodents and primates as experimental models do not report such favourable outcomes concerning the osteopromotive ability of TGF-β1 compared to studies using dogs (Janssens *et al*., 2005).

In the current study, the release kinetics of the TGF-β1 from the PLGA–CaP composite was not examined. On the other hand, similar designed PLGA–CaP composites were recently used in another study for the delivery of enamel matrix derivate (EMD) (Plachokova *et al*., in press). An *in vitro* assay revealed that after 4 weeks only 60% of EMD was released from these scaffolds, and this

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sustained release continued as observed during extended follow-up periods. In view of this, it can be speculated that such a release pattern is too slow for TGF-β1 to support early bone development. Therefore, the TGF-β1-promoted bone formation after 4 weeks was enhanced but not statistically significant compared to the control. Nevertheless, it must be taken into account that EMD and TGF-β1 are completely different proteins. Additionally, the EMD release profile was determined *in vitro*, which is known to differ from the *in vivo* situation. In this regard, *in vitro* and *in vivo* TGF-β1 release profiles from PLGA–CaP composites are necessary to prove the above-mentioned hypothesis.

Another reason for lack of significant difference in the amount of newly formed bone between the groups at 2 and 4 weeks could be the material used as a control. The PLGA–CaP composite material has already demonstrated excellent bone regenerative capacity in previous rat studies (Plachokova *et al*., in press). Therefore, it appears unlikely that an osteopromotive agent, such as TGF-β1, can substantially increase its initial effect on bone formation, especially when applied in such a low concentration (i.e. 200 ng).

The PLGA–CaP composite material is a fast-setting type of cement, which still provides sufficient time for moulding and contouring in order to achieve precise adjustment to the defect or augmentation site (Ginebra *et al*., 1994; Kairoun *et al*., 1997). The initial material–bone (material–implant) contact is of utmost importance for integration of the filler material and early onset of remodelling. In cases of discontinuity in the material–bone (material–implant) interface, fibrous tissue ingrowth between the filler and the bone–implant interface will delay bone formation and might result in failure. To estimate the material–bone interface, bone contact measurements were performed. In the current study, the bone contact length for the PLGA–CaP composite increased from 2.76 to 39.63 mm during 8 weeks of implantation. Moreover, TGF-β1 loading resulted in an additional increase of the initial bone contact, since this material showed significantly higher values of contact length at 2 weeks in comparison with the unloaded material. The reason for this significantly increased bone contact in favor of TGF-β1 loaded composites appears to be unknown and will be subject of a future investigation.

Concerning the material application method in this study, direct injection of the PLGA–CaP composite without fixation was chosen due to the close resemblance of this mode of application to the final clinical approach. Nevertheless, it should be taken into account that lack of rigid fixation of the implants to the skull may have influenced bone formation. Rigid fixation is known to decrease movement and shear forces on the graft, thereby improving revascularization and resulting in earlier bone apposition and improved osteoconduction (La Trenta *et al*., 1989; Lin *et al*., 1990; Phillips and Rahn, 1990).

Finally, at 2 weeks of implantation, a serious inflammatory response to the PLGA–CaP material was observed. In view of this, it has to be noted that the mechanical properties of the PLGA–CaP composite are limited. As a consequence, tight closure of the periosteum and subcutaneous tissue over the implants may have caused high pressure, resulting in the occurrence of fractures in the cement material. These fractures can be associated with the appearance of small CaP particles. Orthopaedic implant studies have already highlighted that CaP particles can induce an inflammatory response (Pioletti *et al*., 2000; Shanbhag *et al*., 1998; Glant and Jacobs, 1994) or can alter osteoblast functions if their size is <10 μm (Puleo *et al*., 1991). As a result, bone formation will be delayed until the particles are completely resorbed. A study performed on calvarial rat osteoblasts confirmed the adverse effect of small CaP particles (<10 μm) on bone formation (Pioletti *et al*., 2000). In addition to the presence of small CaP particles, the cement setting and transformation may have contributed to the observed inflammatory response. These processes involve phase transformation of the ceramic with an associated substantial decrease in pH (in this study approximately 5.5) (Habraken *et al*., 2006). The increased acidity around the implant may have been responsible for the appearance of inflammatory cells. All these observations are in agreement with the study of Huse *et al*. (2004), who also found an extensive inflammatory infiltrate into the pores of similar injectable CaP cement loaded with TGF-β1 in an onlay rat model.

5. Conclusion

In summary, the injectable PLGA–CaP composite stimulated bone augmentation in a rat model. The addition of TGF-β1 to the composite significantly increased bone contact at 2 weeks and enhanced new bone formation at 8 weeks. For clinical application, mechanical properties of the injectable composite after setting have to be improved.

Acknowledgements

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