Transforming Growth Factor-β1 Release from a Porous Electrostatic Spray Deposition–Derived Calcium Phosphate Coating

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ABSTRACT

This study evaluated the utilization of a porous coating, derived with electrostatic spray deposition (ESD), as a carrier material for transforming growth factor-β1 (TGF-β1). A porous β-tricalcium phosphate coating was deposited with ESD, and 10 ng of ^{125}I-labeled TGF-β1 was loaded on the substrates. A burst release during the first hour of incubation of >90% was observed, in either culture medium or phosphate-buffered saline (PBS). Ninety-nine percent of the growth factor was released after 10 days of incubation. All samples were able to inhibit epithelial cell growth, indicating that the growth factor had remained bioactive after release. Thereafter, osteoblast-like cells were seeded upon substrates with or without 10 ng of TGF-β1. While proliferation of osteoblast-like cells was increased on TGF-β1-loaded substrates, differentiation was inhibited or delayed. In conclusion, a porous ESD-derived calcium phosphate coating can be used as a carrier material for TGF-β1, when a burst release is desired.

INTRODUCTION

GROWTH FACTORS ARE TISSUE-SPECIFIC POLYPEPTIDES, acting as local regulators of cellular activity.[1] The natural bone matrix contains many growth factors, including a number from the transforming growth factor-β (TGF-β) superfamily. When bone is remodeled or affected by trauma growth factors are released from the bone matrix, which influence bone cell metabolism and help to stimulate natural repair. When treating bone defects it may be desirable to supplement the site of trauma with growth factors to stimulate bone formation. One of the isoforms often studied in this respect is TGF-β1, a 25-kDa polypeptide. *In vivo* studies have shown the stimulating effects of TGF-β1 on osteogenesis in orthotopic sites.[2] During initial healing, TGF-β1 exerts an immunosuppressive effect, stimulates the deposition of extracellular matrix, inhibits the formation of osteoblast-like cells, and is a chemotactic factor for various cell types, among others, osteoblast-like cells.[3,4] On the other hand, when applied in an ectopic site, TGF-β1 does not give rise to new bone formation.[5] Effects of TGF-β1 *in vitro* are dependent on cell type and concentration.[6,7] Nevertheless, it has to be noted that proliferation of mesenchymal cell types, such as osteoblast-like cells, is stimulated on exposure to TGF-β1.[3]

An approach to supply growth factors to the body is by means of a carrier material. This carrier should be site-specific and biocompatible, and has 2 functions. First, the carrier acts as a transport medium, as it is supposed to deliver the growth factor to the desired site. Second, the carrier itself can guide the regeneration process.[8] In view of this, calcium phosphate (CaP) ceramics have been extensively used as carrier materials for growth factors. Recombinant human (rh)TGF-β1 has been adsorbed onto ceramic coatings,[9–11] ceramic discs,[12,13] and mixed into paste-like formulations.[14–16]

Although many studies use CaP ceramics as carrier material for inductive factors, every combination of a specific CaP ceramic and inductive factor has its own specific characteristics. A relatively new technique developed for the

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deposition of CaP coatings is electrostatic spray deposition (ESD), which is based on the generation of an aerosol containing calcium and phosphate ions. The aerosol is directed toward a heated substrate. The solvent will evaporate, leaving a CaP coating on the substrate. The technique has several benefits over the others currently applied, including the control over the morphological and chemical properties of the coating.[17] With the ESD technique, for instance, porous CaP coatings can be deposited. This porosity could enable a better diffusion of nutrients and efflux of metabolites during tissue regeneration. Further, the porosity could be beneficial from a biomaterials aspect for the incorporation of growth factors and osteogenic cells. To date, growth factor release from ESD-derived coatings has not yet been described.

We hypothesize that a porous, ESD-derived CaP coating can serve as a carrier material for TGF-β1. Further, the use of a TGF-β1-loaded CaP coating will have an effect on the proliferation of osteoblast-like cells cultured on this coating. The aims of this study were therefore to determine the release characteristics of TGF-β1 from a porous CaP coating that was deposited with ESD, and to determine the effects on an osteoblast-like cell culture. After deposition, the coating was fully characterized and loaded with TGF-β1. TGF-β1 was labeled with radioactive iodine and incubated in medium or phosphate-buffered saline (PBS) for up to 10 days to determine the release in vitro. In addition, the activity of the released TGF-β1 was determined in a bioassay. Further, osteoblast-like cells were seeded upon these coatings, to examine the effects of TGF-β1. Proliferation, alkaline phosphatase (ALP) activity, and osteocalcin expression were evaluated. Finally, cell morphology was examined via scanning electron microscopy.

MATERIALS AND METHODS

Preparation of the substrates

To obtain a completely smooth substrate surface, silicon wafers (WaferNet GmbH, Eching, Germany) with a thickness of 0.5 mm were cut into square substrates (0.8×0.8 cm). These silicon substrates were coated with 50 nm thick titanium (Ti) layer using the RF magnetron sputter technique (Edwards ESM 100), to mimic the surface of a Ti implant.[18] The duration of sputtering was 5 min while the substrates were rotated (the sputter power was 200 W, the process pressure was 5×10^{-3} mBar, and the sputter rate 10 nm/min). Subsequently, substrates were cleaned ultrasonically in isopropanol for 5 min.

Thereafter, the ESD technique was used to deposit a 1.0–1.5 μm thick, porous CaP coating on the substrates.[17] The ESD coating procedure was performed using a vertical ESD setup (Advanced Surface Technology, Bleiswijk, the Netherlands) with a 2-component nozzle. The precursor solutions used for the coating were 5.66 mM Ca(NO_3)_2·4H_2O in butylcarbitol (C_8H_18O_3) and 3.33 mM H_3PO_4 in butylcarbitol. The applied voltage was 6.3–6.5 kV, the flow rate was 1 mL/

h, and the deposition time was 45 min. During the deposition, substrates were heated to 350°C. The distance between the nozzle and the substrate was 20 mm.

After deposition, substrates were subjected to an additional heat treatment for 2 h at 1000°C to induce crystallinity. Subsequently, substrates were sterilized via autoclaving for 15 min at 121°C.

Physicochemical analysis

A physicochemical analysis was performed on the substrates. The physicochemical analysis consisted of the following techniques:

- X-ray diffraction (XRD; to assess phase composition and crystallinity). For XRD, a thin film Philips X-ray diffractometer was used, using CuKα-radiation (PW3710, 40 kV, 40 mA).
- Fourier transform infrared spectroscopy (FTIR; to assess molecular composition). The infrared spectra of the films on the substrates were obtained by reflection FTIR (PerkinElmer).
- Energy dispersive spectrometry (EDS; to assess Ca/P ratio). To determine the calcium/phosphate ratio of the coatings, discs were examined using a Jeol 6310 SEM which was equipped with an energy disperse X-ray detector.
- Scanning electron microscopy (SEM; to assess surface morphology). Discs were sputter-coated with gold and examined and photographed using a Jeol 6310 SEM.

Recombinant human TGF-β1 release in vitro

Recombinant human (rh)TGF-β1 (R&D Systems, Minneapolis, MN) was radioiodinated with ^{125}I according to the iodogen method.[19] Briefly stated, 2 μg rhTGF-β1 was solubilized in 10 μL of 4 mM HCl, centrifuged (20 s, 2500 rpm), and the content was transferred to an 0.5-mL Eppendorf vial coated with 50 μg of 1,3,4,6-tetrachloro-3α,6α-diphenylglucouril (Iodogen®) (Pierce, Rockford, IL). The vial containing latent rhTGF-β1 was rinsed twice with 40 μL of 50 mM phosphate buffer, which was added to the iodogen vial. To the iodogen vial (=5 μL) was added 0.7 mCi Na^{125}I (Amersham Intl., Amersham, UK) and the vial incubated at room temperature for 5 min. One hundred microliters of saturated tyrosine solution was added to the reaction mixture. The reaction mixture was separated on a disposable Sephadex G25M column (PD10, Amersham-Pharmacia, Uppsala, Sweden) and was eluted with PBS–0.1% BSA. The void fractions containing ^{125}I-TGF-β1 were pooled fraction. The specific activity of the ^{125}I-TGF-β1 was 200 μCi/μg. The radiochemical purity was determined by instant thin-layer chromatography (ITLC) and exceeded 95%.

Ten nanograms of the ^{125}I-labeled rhTGF-β1 was loaded on the coatings (n = 3 for all samples). For this, a 10-μL droplet of PBS–BSA 0.1% containing 10 ng of rhTGF-β1 was placed on the coatings, and distributed over the entire

surface area. After the droplets solution was placed on the coatings, substrates were frozen at –20°C and lyophilized overnight to dehydrate the substrates. Subsequently, the substrates were placed in glass vials containing either 5 mL of osteoblast culture medium (α-minimal essential medium (α-MEM), supplemented with 10% fetal calf serum (FCS; Gibco BRL, Life Technologies B.V. Breda, the Netherlands), 50 μg/mL of ascorbic acid (Sigma-Aldrich, St. Louis, MO), 50 μg/mL of gentamicin, 10 mM Na-β-glycerophosphate (Sigma-Aldrich), and 10^{-8} M dexamethasone (Sigma-Aldrich), or 5 mL of PBS, and incubated at 37°C. After 1, 4, 24, 72, and 240 h of incubation, substrates were transferred to a fresh glass vial containing 5 mL of culture medium or 5 mL of PBS. The radioactivity of the old vials (with remaining culture medium–PBS) as well as the fresh vials (culture medium/PBS with substrate) was counted with a gamma counter (1480 Wizard 3″, PerkinElmer Life Sciences, Boston, MA). Both release and retention were calculated independently: the retention was measured as percentage of the originally loaded rhTGF-β1. The release was measured as the accumulated percentage of the originally loaded rhTGF-β1.

TGF-β1 activity after release

Substrates were loaded with 10 ng of TGF-β1 as described in the preceding text, albeit unlabeled ($n = 2$). To test whether released TGF-β1 was still bioactive, a mink lung epithelial (MLE) cell test was used, as MLE cells show drastic inhibition of proliferation after exposure to bioactive TFG-β1. First, substrates were placed in a vial containing 2 mL of α-MEM supplemented with 10% FCS and 50 μg/mL of gentamicin. Then substrates were incubated at 37°C, 5% carbon dioxide(CO_2), and 95% humidity. After 72 h, culture medium was removed and added to a population of MLE cells (cell line CCL-64, Mv-1-Lu, ATCC, Manassas, VA) seeded in a 24-well plate at a density of 56,000 cells/cm². The cells were incubated at 37°C, 5% CO_2, and 95% humidity.

After 3 days of culture, the DNA content of every well was examined. Culture medium was removed from the cell layer. Cells were washed twice with PBS and subjected to 2 freezing–defrosting cycles in 1 mL of deionized water. Subsequently, the total amount of DNA was measured using a PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR), according to the instructions of the manufacturer. Briefly stated, dsDNA stock was used to generate a standard curve ranging from 0 to 2000 ng/mL. A 100-μL sample and 100 μL of PicoGreen working solution were added to the wells of a 96-well plate. The plate was incubated at room temperature in the dark for 2 to 5 min. Thereafter, the plate was read at 480–520 nm. With the aid of the standard curve, DNA content could be determined. Samples and standards were assayed in duplicate.

Osteoblast-like cell isolation and culture

Rat bone marrow cells were obtained from the femora of 1 male, 40–43-day old Wistar WU rat per culture run. Both femora were removed and washed 3 times with α-MEM supplemented with 0.5 mg/mL of gentamicin and 3 μg/mL of fungizone (Gibco). Subsequently, the epiphyses were cut off and diaphyses were flushed out with osteoblast culture medium to remove the bone marrow. The bone marrow was divided over 3 culture flasks (75 cm²) and cultured in osteoblast culture medium. The cells were incubated at 37°C, 5% CO_2, and 95% humidity. The following day, culture medium was refreshed to remove all nonadherent cells. After this, culture medium was refreshed every 2–3 days.

After 7 days of primary culture, cells were detached using trypsin–EDTA (0.25% w/v trypsin–0.02% EDTA). The cells were concentrated, resuspended in 10 mL of culture medium, and counted using a Coulter® Counter. For seeding, 11,500 cells/cm² were added to either the substrates with 10 ng of TGF-β1, or to nonloaded control substrates. Substrates with adherent cells were incubated at 37°C, 5% CO_2, and 95% humidity. Medium was refreshed every 2–3 days.

Proliferation

After 1, 4, 8, and 12 days of cell culture, proliferation of the cells was examined by determining the DNA content of every substrate ($n = 4$). Culture medium was removed from the cell layer. Subsequently, total DNA was collected and measured using a PicoGreen dsDNA Quantitation Kit, as described earlier. Samples and standards were assayed in duplicate.

ALP activity assay

After DNA analysis, the same samples were used to determine ALP activity. 4-Nitrophenol (4-NP) stock was diluted in buffer solution (5 mM $MgCl_2$, 0.5 M 2-amino-2-methyl-1-propanol), to generate a standard curve ranging from 0 to 25 nM. A 80-μL sample and 20 μL of buffer solution were added to the wells of a 96-well plate. Subsequently, 100 μL of substrate solution (5 mM para-nitrophenylphosphate) was added to the wells, and the plate was incubated at 37°C for 1 h. The reaction was stopped by adding 100 μL of 0.3 M NaOH. Finally, the plate was read via an enzyme-linked immunosorbent assay (ELISA) reader at 405 nm. With the aid of the standard curve, ALP activity was determined in nM 4-NP produced per hour per nanogram of DNA. Samples and standards were assayed in duplicate.

Osteocalcin content

The osteocalcin content was measured with an enzyme immunoassay (EIA; Biomedical Technologies, Stoughton, MA). After 8, 16, and 24 days of culture, cell layers were washed twice with PBS, scraped off in 1 mL of EIA sample buffer, and stored at –20°C until use ($n = 3$).

For analysis, osteocalcin stock was diluted in sample buffer to generate standards ranging from 0 to 40 ng/mL. A

100-μL sample was added to the wells of a 96-well plate, and the plate was incubated at 4°C for 24 h. Hereafter, the wells were washed 3 times with wash buffer. One hundred microliters of osteocalcin antiserum was added, and the plate was incubated at 37°C for 1 h. Subsequently, the wells were washed 3 times with wash buffer. One hundred microliters of donkey anti-goat IgG peroxidase was added, and the plate was incubated at room temperature for 1 h. The wells were washed 3 times with wash buffer. One hundred microliters of substrate mix was added, and the plate was incubated at room temperature in the dark for 30 min. One hundred microliters of stop solution was added, and absorbance was read with an ELISA reader at 450 nm. With the aid of the standard curve, total osteocalcin content could be determined. Samples and standards were assayed in duplicate.

SEM

After 1, 4, 8, and 16 days of culture, substrates ($n = 2$) were washed twice with PBS. Cells were fixed for 5 min in 2% glutaraldehyde. Subsequently, substrates were washed for 5 min with 0.1 M sodium-cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried with tetramethylsilane. The substrates were sputter-coated with gold and examined via a Jeol 6310 SEM.

Statistical analysis

The complete experiment was performed in duplicate to ensure reproducibility. Further, every sample was measured in duplicate. Statistical analysis was performed using an unpaired t-test. Calculations were performed in GraphPad InStat, Version 3 (GraphPad Software, San Diego, CA).

RESULTS

Physicochemical analysis

XRD and FTIR spectrometry showed that after the heat treatment, the CaP coating was a crystalline β-tricalcium phosphate (β-TCP) coating. ESD analysis showed a calcium over phosphate ratio of 1.4–1.5. SEM revealed that the CaP coating had a porous surface structure (Fig. 1).

rhTGF-β1 release in vitro

Figure 2 shows the release characteristics of ^{125}I-labeled rhTGF-β1 in culture medium and PBS. After 1 h of incubation, the 10 ng-loaded coatings had released 95.3% of their rhTGF-β1 content into the culture medium. This increased in time up to 99.4% after 10 days. Similar results were found for samples placed in PBS. After 1 h, a release of 95.5% was observed, which increased to 99.6% after 10 days of incubation. As mentioned, the retention was also measured. The retention added to the release was always 100%.
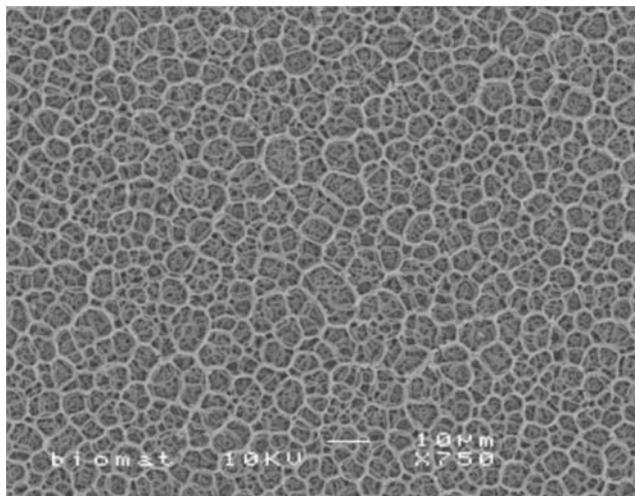

FIG. 1. Scanning electron micrograph of the porous CaP coating before cell culture.

TGF-β1 activity after release

Substrates that were loaded with 10 ng of TGF-β1 were placed in culture medium for 3 days. This medium was removed and added to a culture of MLE cells. The control group received normal culture medium. Figure 3 shows the DNA content of MLE cells after 3 days of culture in medium containing released TGF-β1 or normal culture medium. Compared to the control group, growth of the MLE cells was significantly inhibited ($p < 0.001$) when cultured in medium with released TGF-β1, which proved that the released TGF-β1 was still bioactive.

Proliferation of osteoblast-like cells

The results of the proliferation assay from the 2 performed runs with osteoblast-like cells were similar. Results from the first run are depicted in Fig. 4. DNA content was measured after 1, 4, 8, and 12 days of incubation. An increase in DNA content is seen in time for both groups.
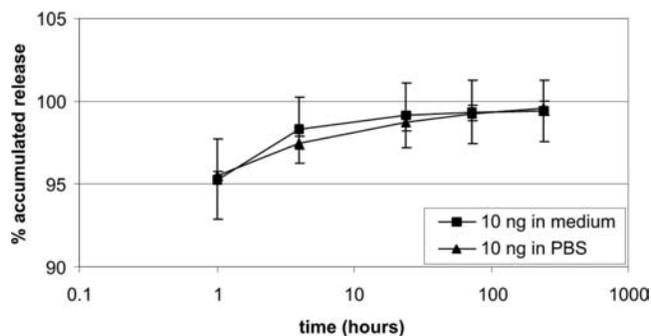
FIG. 2. Release profiles of TGF-β1 in culture medium and PBS *in vitro*. Ten nanograms pf [125] I-labeled TGF-β1 was loaded on the substrates. A burst release is seen, followed by a slow release up to the 240 h time point.
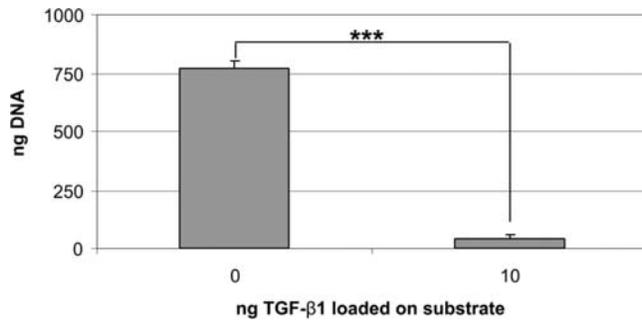
FIG. 3. The DNA content of MLE a cell culture after 3 days, in normal culture medium, or medium containing released TGF-β1. Note the inhibition of growth induced by TGF-β1. Values represent the means ± SD. ***$p < 0.001$.

After 1 and 4 days of incubation, no differences were seen between cells cultured on substrates with or without TGF-β1. However, proliferation was markedly stimulated after 8 and 12 days of incubation on the substrates loaded with TGF-β1, compared to the substrates without TGF-β1.
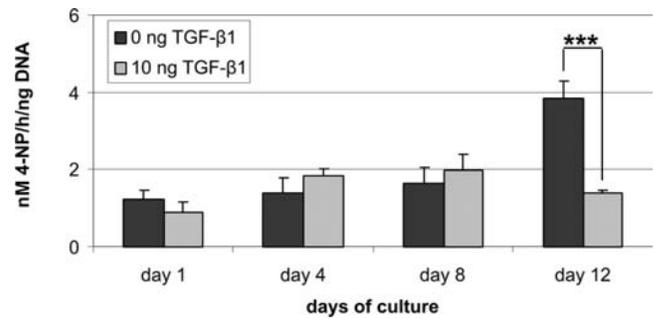
ALP activity assay

Again, the results of the 2 performed runs were similar, and the results of the first run are shown in Fig. 5. After 1, 4, and 8 days of culture, no differences were found between cells cultured on loaded and unloaded substrates. After 12 days of culture, ALP activity is increased for cells cultured on unloaded substrates, compared to cells cultured on substrates loaded with 10 ng of TGF-β1.

Osteocalcin content

The osteocalcin content for both runs were similar and the results of run 1 are shown in Fig. 6. No osteocalcin content could be identified after 8 days for cells cultured on substrates with or without TGF-β1. After 16 and 24 days of culture, still no osteocalcin content was observed for cells cultured on substrates loaded with TGF-β1. In contrast, osteocalcin content was detected for cells cultured on substrates without TGF-β1.
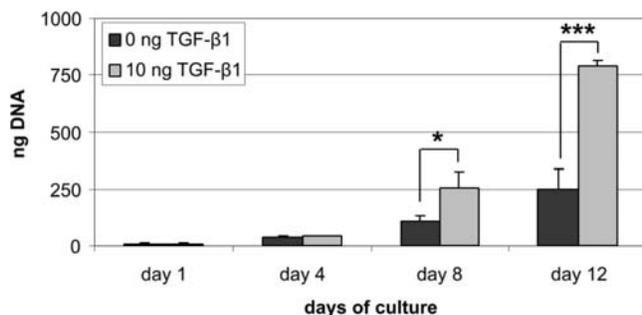
FIG. 4. DNA content of osteoblast-like cells cultured on substrates with and without 10 ng of TGF-β1. Values represent the means ± SD. *$p < 0.05$; ***$p < 0.001$.
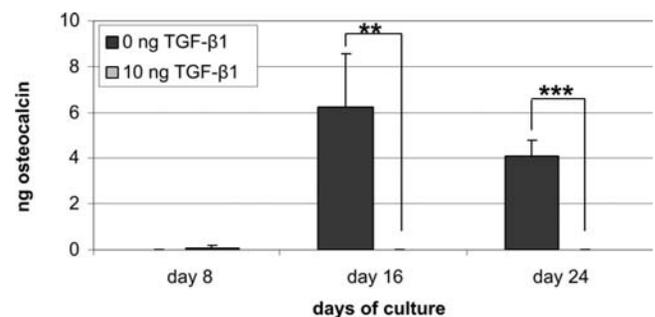

FIG. 5. ALP activity of osteoblast-like cells cultured on substrates with and without 10 ng of TGF-β1. Values represent the means ± SD. ***$p < 0.001$.

SEM

SEM observations revealed no differences between loaded and unloaded substrates after 1 and 4 days of culture. After 8 days of culture, a confluent cell layer covered the entire surface in both groups. However, cells cultured on substrates without TGF-β1 showed the beginning of the formation of a mineralized extracellular matrix. Cells cultured on substrates loaded with TGF-β1 did not show signs of mineralization. After 16 days of culture, the difference in the extent of mineralization was more pronounced. Cells cultured on substrates without TGF-β1 showed many mineralized nodules (Fig. 7A). Cells cultured on substrates with 10 ng TGF-β1 did not show any signs of mineralization (Fig. 7B). Cross-sections of the samples presented in Fig. 7A and B are shown in Fig. 7C and D, respectively. Visible are the silicon substrate, the coating, and the cell layer on top of the coating. In Fig. 7C, the mineralized matrix is visible. In Fig. 7D, a confluent cell layer is present on top of the coating.

DISCUSSION

A CaP coating applied on a titanium implant provides the metal with an osteoconductive layer. In addition to increasing osteoconductive properties, osteoinductive agents,

FIG. 6. Osteocalcin content of osteoblast-like cells cultured on substrates with and without 10 ng of TGF-β1. Values represent means ± SD. **$p < 0.01$, ***$p < 0.001$. Color images available online at www.liebertpub.com/ten.
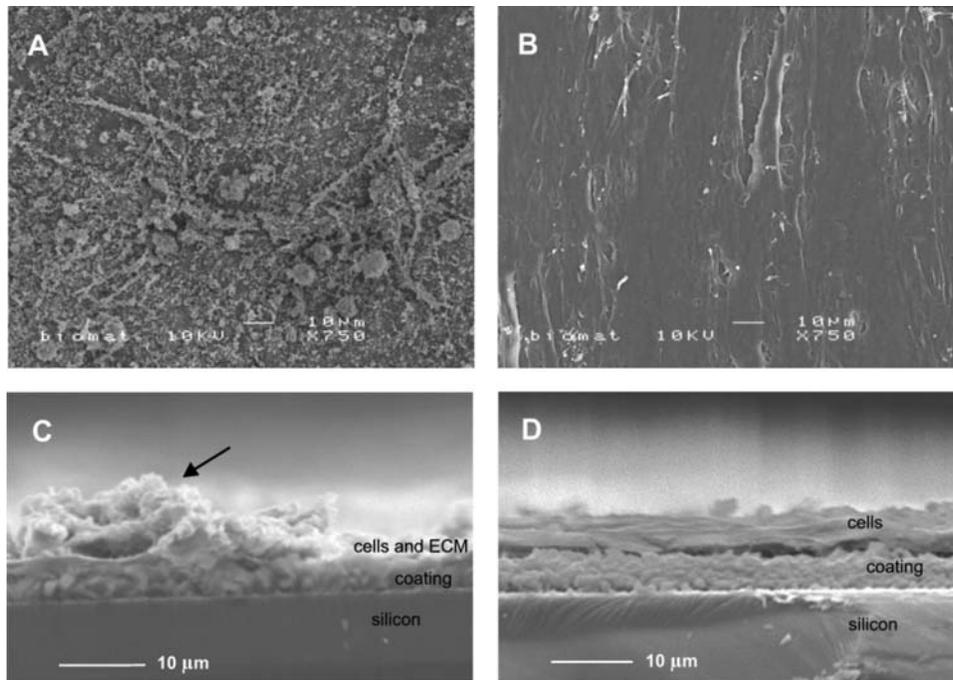
FIG. 7. Scanning electron micrographs of osteoblast-like cells cultured for 16 days. (**A**) Substrate without TGF-β1. Note the formation of a mineralized extracellular matrix. (**B**) Substrate with 10 ng of TGF-β1. Note that mineralization has not occurred. (**C, D**) Cross-sections of the samples shown in **A** and **B**, respectively. Visible are the silicon substrate, coating, and cell layer. In **C**, mineralized matrix is visible (arrow). In **D**, a thick confluent cell layer is present.

such as TGF-β1, can also be applied to a coating to enhance bone formation. This study examined the utilization of a porous ESD-derived CaP coating as carrier material for TGF-β1. Results show a burst-release of bioactive TGF-β1 from the coatings already during the first hour of incubation. When osteoblast-like cells were seeded on substrates loaded with TGF-β1, their proliferation was stimulated and differentiation was inhibited, or delayed. Therefore, results show that ESD coatings can be carriers for the growth factor, if a burst release is desired.

Appropriate methods to analyze the parameters of interest had to be selected before commencing this study. To measure the release of TGF-β1, literature describes the use of ELISAs,[2,20] as well as radioactively labeled growth factors.[9,16,21] In a preliminary study, the release of TGF-β1 was determined via both methods. However, it was found that data obtained from the ELISAs were largely variable, whereas data from the radiolabeling study were consistent. Another major benefit of the use of radioiodinated TGF-β1 over ELISAs was that, in addition to the release, the retention of TGF-β1 in the coating could also be determined. Therefore, we have selected radioactive labeling as the preferred technique in this study.

Another technical decision involved the conditions of the release, as the release can be determined under static or dynamic conditions. In the body, release of a growth factor such as TGF-β1 from a carrier will take place under the dynamic conditions of tissue and fluid movements. Further, macro-

phages and osteoclasts can remodel and degrade the coating, thereby influencing the release of TGF-β1. As currently no tissue culture method is available that accurately mimics such effects, it was decided to determine the release under static conditions, which seemed also the most logical choice in relation to the subsequent cell culture experiment. This discrepancy between *in vitro* and physiological *in vivo* conditions can also be the reason that *in vivo* results do not always match the *in vitro* data and that less activity of growth factors is seen *in vivo* compared with *in vitro* studies.

For the *in vitro* release characteristics, CaP coatings were loaded with 10 ng of ^{125}I-labeled TGF-β1, and release was determined in culture medium and PBS. These release media were chosen as many researchers use a buffered solution to measure the release. However, to identify the effects of proteins and ions, release was also determined in culture medium. Preliminary studies already confirmed that an increase of the dose results in the same kind of burst release, which is a valid method to increase the final concentration close to the implant. Regarding literature, it is difficult to compare the results of the present study, as many experimental parameters are involved, which can all influence the outcome of the release studies, like the growth factor used, the method employed to load the carrier material (e.g., incorporation or adsorption), type of carrier material (e.g., ceramic coating or cement), release media (e.g. serum, culture medium, or PBS), and techniques to measure the release (e.g., ELISA or radioactive labeling). A study by

Ruhé *et al.* confirmed already that release characteristics are dependent upon the carrier composition and nanostructure as well as the pH of the release medium.[22] On the other hand, studies with lyophilized growth factors consistently show burst-release profiles, although the intensity of the burst release is influenced by the carrier composition. For instance, a burst release of 70% was observed when TGF-β1 was lyophilized on Ti-fiber mesh implants,[2] while a 50% burst release occurred when lyophilizing TGF-β1 on a porous poly(propylene fumarate) scaffold.[23] Further, Lind *et al.* found an 80% release of TGF-β1 when adsorbed to a plasma sprayed TCP coating.[9] Incorporation of a growth factor into a bulk material generally leads to no, or a very low release. Blom *et al.* found releases of 20%[15] and 1%[14,20] of TGF-β1 incorporated in CaP cement. Release in such a situation can be accomplished only after degradation of the carrier material. We have to emphasize that literature is not conclusive on the preferred release profile for growth factors to promote bone healing. Both systems for sustained as well as burst release have resulted in success. For instance, bone formation was stimulated with a sustained release of bone morphogenetic protein-2 (BMP-2) from a porous CaP cement,[24] and from poly-L-lactic glycolic acid microspheres in sodium carboxymethylcellulose.[25] In contrast, bone formation was also stimulated following a burst release of TGF-β1 from a titanium fiber mesh.[24] One recent study described a comparison between burst and sustained release of the osteoinductive protein TP508 in a rabbit radial defect model. There it was shown that a large burst release of TP508 induced more bone formation.[26] Overall, it can be said that the release profile of every combination of growth factor and carrier material has to be tailored to comply with the desired release profile for the effects of a specific growth factor and the site of implantation. Future studies have to elucidate whether growth factor components can also be incorporated in ESD-derived coatings to create a slow release carrier or an optimized combination of both release profiles.

The osteoblast-like cell assay revealed an increase in proliferation as well as a decrease in ALP activity and osteocalcin levels, on all TGF-β1-loaded substrates. This corroborates with several other studies, which also demonstrated a similar increase in cell proliferation.[27,28] However, literature data on the effect of TGF-β1 on ALP activity are somewhat diverse. Centrella *et al.* reported a decrease in ALP activity,[29] and Harris *et al.* found a decrease in gene expression coding for ALP.[30] On the other hand, Blom *et al.* observed no influence on ALP activity at all,[15] whereas an increase in ALP activity was described by others.[27,28] Similar discrepancies are observed for osteocalcin levels. Still, in accordance with the present results, most available literature reports a reduced osteocalcin content after addition of TGF-β1 during cell culture,[12,28] and a decreased gene expression for osteocalcin.[30] A contradictory observation was, for example, done by Lu *et al.,* who found an enhanced osteocalcin production after 21 days.[27] Differences in cellular behavior can possibly be explained by the cell type used. It is known

that cells with the osteoblastic phenotype can have different responses to TGF-β1. In the ROS 17/2.8, UMR-106, and MG-63 cell lines TGF-β1 inhibits cell replication and stimulates ALP activity,[7] while in the MC3T3, and SaOS-II cell line, and freshly isolated calvarial cells TGF-β1 increases cell replication and decreases ALP activity.[7] Further, a concentration-related effect of TGF-β1 can elicit different responses from the cultured cells. For example, in one of our previous pilot studies, loading of 5 ng of TGF-β1 upon the porous ESD-derived CaP coating induced no effect at all on osteoblast-like cells cultured on the substrates (data not shown). In addition, Centrella *et al.* observed that in sparse cultures of osteoblast-enriched cells, 0.15 ng/mL of TGF-β1 stimulated proliferation, while higher doses gave an inhibitory effect. In subconfluent cultures, the peak response was at 15 ng/mL of TGF-β1.[29] Therefore, we can conclude that the number of cells proportionally to the concentration TGF-β1 is determining the final cellular response.

CONCLUSIONS

In conclusion, the presented data verify that a porous ESD-derived CaP coating can be used as a carrier material for TGF-β1 when a burst release is desired. As confirmed by the mink lung epithelial cell assay, the released TGF-β1 is still bioactive. In addition, the released TGF-β1 stimulated the proliferation of osteoblast-like cells, while differentiation was inhibited or delayed. Future *in vivo* studies have to prove the final clinical efficacy of porous ESD-derived coatings as a carrier material for bone regeneration supporting growth factors.

ACKNOWLEDGMENTS

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