Biological evaluation of the effect of magnetron sputtered Ca/P coatings on osteoblast-like cells in vitro

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A rat bone marrow cell culture was used to evaluate the osteogenic potential of amorphous and crystalline thin calcium phosphate (Ca/P) coatings. The coatings were deposited on titanium discs using a radiofrequency magnetron sputter procedure. Amorphous and crystalline plasma spray Ca/P coated and noncoated titanium discs served as reference material. The cellular behavior was analyzed with quantitative (attachment and proliferation rates) and qualitative (scanning electron microscopy) techniques. No significant differences were found in cell attachment and proliferation rates between the various materials. Scanning electron microscopy showed extracellular matrix formation after 18 days of culture on amorphous plasma-sprayed and the two types of magnetron sputtered coatings. Furthermore, no severe degradation of the magnetron sputtered coatings was observed. They even appeared to induce apatite formation. On basis of the results, we conclude that magnetron sputtering appears to be a promising method to manufacture bioactive ceramic coatings. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Since about 10 years, the application of calcium phosphate (Ca/P) coatings has become an accepted technique to improve the biologic behavior of metallic dental and orthopaedic implants.1 Currently, plasma-spraying is the most frequently used method for the deposition of Ca/P coatings on implant materials. However, despite the described beneficial effect of these coatings on the bone response,2–6 there is also some concern regarding their long term performance.7 For example, it is reported that the Ca/P coating resorbs over time and loses its mechanical integrity by lack of adherence.8 Since the clinical consequences of these findings have not been understood completely, more knowledge and experience has to be obtained about the characteristics of Ca/P coatings. To achieve this goal, besides the further improvement of the plasma-spray technique, the efficacy of more appropriate deposition methods has to be investigated. Therefore, in our laboratory experiments have begun on the application of magnetron sputtering for the production of Ca/P coatings on metal and plastic substrates.

Radiofrequent (RF) magnetron sputter-coating is performed using commercially available sputter equipment. In a preliminary study, we showed that this technique produces highly adhesive, uniform coatings.9 Energy-dispersive X-ray analysis, X-ray diffraction, and atomic absorption spectrometry confirmed that the sputtered layers were well-crystallized Ca/P ceramic with a Ca/P ratio varying between 1.9 and 2.5. In vitro and in vivo experiments demonstrated the biocompatibility of the coatings. Based on these results, we concluded that magnetron sputtering is a promising method for forming a Ca/P coating onto an implant material. Nevertheless, several problems, e.g., the endurance and the Ca/P ratio of the coating, have to be solved before clinical use of magnetron sputtered implant systems can be considered. To elucidate these problems and to gain more insight into the physicochemical and biological properties of the sputtered coatings extensive in vitro and in vivo experiments have to be done. In this article we present the first stage of the biological evaluation by using rat bone marrow cell cultures to investigate the

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potential of Ca/P sputtered coatings for osteoblast-like cell growth.

MATERIALS AND METHODS

Materials

Commercially pure titanium (cpTi) discs with a diameter of 12 mm were used. They were polished to 320 grit with abrasive papers, ultrasonically cleaned for 15 min in acetone and placed in 100% boiling ethyl alcohol. After drying, the discs were left untreated or provided with four different Ca/P coatings. Two types of Ca/P coatings were deposited using a plasma-spray technique. The first was used as applied (HA-PS), the second was subjected to a heat treatment for 2 h at 600°C after coating deposition (HA-PS/ht). The thickness of these coatings was about 50 μm.

Two other types of Ca/P coating were produced using the RF magnetron sputter technique. The sputter process was done at a power level of 800 W and a process pressure of 5 x 10^{-3} mbar using argon gas. The titanium discs were mounted on a water-cooled substrate holder. One coating was produced with a rotating substrate holder (Ca/P-r), while the other coating was deposited with the substrate holder in an indexed position (Ca/P-i). The thickness of the magnetron sputtered coatings varied between 2.5-4.0 μm.

All deposited coatings were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier infrared absorption spectrometry (FIR).

Before use in the cell culture experiments, all discs were autoclaved for 30 min at 120°C.

Cell isolation and culture

Osteoblast-like cells were prepared using the rat bone marrow (RBM) culture method as described by Maniatopoulos,10 Davies,11 and De Bruijn.12 Briefly, both femora of young adult male Wistar rats (weight 100-120 g, age 40-43 days) were removed and washed four times with α-Minimal Essential Medium (α-MEM, Gibco, Life Technologies B.V., Breda, The Netherlands), containing 0.5 mg/mL gentamycin (Gibco) and 3.0 μg/mL fungizone (Gibco). Afterwards, the epiphyses were cut off and the diaphyses flushed out, using α MEM supplemented with 15% fetal calf serum (FCS, heat induced at 56°C for 35 min, Gibco), 50 μg/mL of freshly prepared ascorbic acid (Sigma, Chemical Co., St. Louis, MO., USA), 10 mM Na β-glycerophosphate (Sigma), 10^{-8}M dexamethasone (Sigma) and antibiotics at one-tenth of the concentration described above. Finally, cultures were incubated in a humidified atmosphere of 95% air, 5% CO₂ at 37°C. The phase contrast photomicrograph of Figure 1 shows the general appearance of a primary culture. After 5 to 7 days in culture, cells were harvested by trypsin treatment (0.25% w/v trypsin) and used for the experiments.

The osteogenic phenotype and function of the RBM cells were confirmed by several parameters, including the presence of alkaline phosphatase, deposition of calciumphosphate material by Von Kossa’s method, and immunostaining. For the immunocytochemical identification we used a specific monoclonal antibody (E11)13 against a cell membrane associated antigen of rat osteoblasts. A goat-antimouse/FITC conjugate was used to locate the monoclonal antibody. We noted that 12-day-old cultures on microscopic glass slides showed a positive anti-osteoblast reaction. No attempts were made to detect the possible influence of different substrates on the osteoblast expression of the cell cultures.

Cell attachment assay

The test substrates were positioned on the bottom of sterile 24-well plates (Greiner, Greiner B.V., Alphen a/d Rijn, The Netherlands) and a total of 1.0 mL of culture medium containing 5 x 10^4 cells was added to each substrate. The cultures were incubated for 8 h at 37°C in 5% CO₂/air. After incubation, the wells were washed twice using phosphate buffered saline (PBS) to remove nonattached cells. Then the substrates were taken out of the wells and placed into counting tubes. To detach the attached cells, 1 mL of trypsin was added and the tube was placed for 8 min at 37°C. Isotone solution was added and the cells

Figure 1. Phase contrast micrograph of a primary rat bone marrow cell culture.
were counted using a Coulter Counter. After the counting procedure the presence of nondetached cells was checked by scanning electron microscopy of the various substrate surfaces.

Two runs of experiments were carried out. In each run all materials were present in quintuple.

**Cell proliferation assay**

RBM cell suspensions, containing $5 \times 10^4$ cells, were seeded on the experimental substrates as described before and incubated at 37°C in 5% CO$_2$/air. After 8 h of incubation, the nonattached cells were removed by PBS rinses. To each well 1 mL of fully supplemented medium was added and the specimens were incubated for 5 days. The medium was changed every other day. At the end of the incubation period, the medium was discarded and the substrates were rinsed twice with PBS. Then, the substrates were taken out of their wells and placed into counting tubes. After detachment by trypsinization, isotope solution was added to count the number of adherent cells using a Coulter Counter. Similar to the attachment assay, noncoated and coated surfaces were checked on the presence of nondetached cells.

The presented results are based on the average of two separate experiments. In each experiment all materials were present in quintuple.

**Cell morphology assay**

RBM cell suspension (200 μL per well, containing $1 \times 10^4$ cells) was added to the test substrates as previously described for the cell attachment and proliferation experiments. The cultures were incubated for 6 and 18 days at 37°C in 5% CO$_2$/air. The culture medium was changed every other day. After the various incubation periods, the nonattached cells were removed by PBS rinses. The attached cells were fixed in situ with 2% v/v glutaraldehyde in 0.1 M sodium cacodylate buffered solution for 30 min at 4°C, rinsed twice in cacodylate buffered solution, followed by dehydration through a graded series of ethanol. Subsequently, the specimens were dried by tetramethylsilane. Finally, after sputter-coating with gold, they were examined using a Philips SEM-500 scanning electron microscope at an accelerating voltage of 12 kV.

The possible influence of culture medium on the noncoated and Ca/P coated titanium discs was also examined. Therefore, some discs were incubated for 6 and 18 days with fully supplemented culture medium, but without cells. After incubation, the discs were processed for scanning electron microscopy.

**RESULTS**

**Characterization of the Ca/P coatings**

A complete description of the characteristics of the coatings has already been given elsewhere.$^{14,15}$

In summary, SEM examination revealed that the specimens were covered with an uniform coating. Further, it was observed that the Ca/P-r coatings had a smoother surface microstructure, while the Ca/P-i surfaces showed a polycrystalline appearance.

XRD patterns demonstrated that the HA-PS and HA-PS/ht coatings showed an amorphous/crystalline structure. The crystallinity of the HA-PS coatings appeared to be 60%. After heat-treatment (HA-PS-ht specimen) the crystallinity of the coatings increased slightly to 65%.

In Figure 2 XRD patterns of the Ca/P-i and Ca/P-r are shown. The analysis of the diffractograms showed that the indexed procedure resulted in a crystalline Ca/P coating with a preferred (001) crystallographic orientation with the C-axis perpendicular on the substrate surface (reflections 002, 102, 112, 202, respectively; 25.9, 28.1, 32.4, and 34.0° 2-Theta). Coatings prepared with the rotating substrate holder showed an amorphous structure, without any specific reflection lines.

Infrared measurements of the plasma-sprayed samples showed absorption bands characteristic of P—O and O—H bonds. In the spectra of both sputtered coatings no OH bonds were detectable. These coatings showed a wide peak over the region from 2800 to 4000 cm$^{-1}$ indicative for water absorption at the surface.$^{16}$

**Cell attachment**

Table I shows the results of the osteoblast attachment experiments. Statistical testing of the findings, using an one-way analysis of variance (ANOVA) and a multiple comparison procedure (Newman–Keuls) revealed that no significant differences existed between the attachment percentages of RBM cells to the various materials. Data are unavailable for Ca/P-i coatings due to detachment of the coating during cell trypsinization, which obstructed the orifice of the counting tube. SEM inspection demonstrated that no cells were left on the various substrates after trypsinization.
Cell proliferation

The results of the proliferation assay of RBM cells on the various substrate materials are given in Table I. SEM examination of the trypsinized substrates, after the 5-day culturing period of the proliferation assay, demonstrated incomplete removal of the cells from HA-PS-ht surfaces (Fig. 3). Therefore, these data were excluded from the statistical analysis. One way analysis of variance (ANOVA) and multiple comparison procedure (Newman–Keuls) revealed no significant difference between titanium and the other materials.

Cell morphology

Scanning electron microscopy showed a comparable cell morphology on all test surfaces. Cells cultured on cpTi surfaces formed multilayers. No systematic signs of bonelike tissue formation were observed (Fig. 4).

The morphology of RBM cells cultured on the different plasma-sprayed coatings was similar as de-

<table>
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<tr>
<th>Substrate</th>
<th>Attachment Mean</th>
<th>Attachment SEM</th>
<th>Proliferation Mean</th>
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<td>2736</td>
<td>255854</td>
<td>53650</td>
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<tr>
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*SEM represents standard error of the mean.
Figure 3. Scanning micrograph showing cells left on a plasma-spray coated substrate left after trypsinization; bar = 12.8 μm.

scribed by De Bruijn. In short: after culture periods of 6 days the cells were spread and exhibited filopodia spanning the macropores produced by the plasma-spraying. Extracellular matrix formation was observed after 18 days of culture. No difference in coverage with extracellular matrix existed between the heat treated and nonheated plasma-sprayed coatings.

The cellular response to the sputter-coated ceramic substrates was identical to the plasma-sprayed substrates, although the occurring processes were easier to follow due to the smooth surface texture of the sputtered coatings. Following incubation, the RBM cells adhered and spread over the sputtered Ca/P surface, resulting in a confluent cell layer at the sixth day. After 18 days formation of a granular mineralized layer was seen. No differences in appearance and coverage of this ECM layer was observed between both types of sputtered Ca/P ceramic (Figs. 5 and 6). Examination of cracks in this layer, which were due to the drying process, made apparent that this layer was rather thick (about 1 μm). In areas where parts of the ECM were elevated, afibrillar mineralization foci were observed. In addition, exposure of the underlying ceramic surface proved that 18 days after incubation a substantial thickness of the coating was still present. Further inspection revealed the precipitation of tiny spherulites (about 0.35 μm) covering this original coating surface.

This formation of spherulites on the surface of the sputtered coatings was also seen after 6 days of incubation in fully supplemented culture medium without cells. Figure 7(a) and (b) shows the SEM photograph of these specimens. The small cracks present in the coatings are a result of the SEM preparation process. This kind of spherulites was also formed on the control HA-PS specimens, while on the heat treated...
DISCUSSION AND CONCLUSIONS

The observations presented here again confirm the suitability of *in vitro* experimentation for studying cell-substrate interactions, enabling not only the evaluation of cellular behavior, but also its visualization on the electron microscopical level. Moreover, cell culture techniques have the advantage of offering a completely defined environment.

The rat bone marrow cells attached and grew on all substrate surfaces. The question was raised whether this was correlated to the induction and formation of extracellular matrix. For example, no systematic signs of bone like tissue formation could be found on titanium substrates. Although, a definite reason for this effect of titanium is difficult to express, a possible explanation is given by Massas. He cultured rat parietal bone cells on titanium and hydroxyapatite substrates. After an incubation period of 14 days the cells were equally proliferating on both materials. On the other hand, the alkaline phosphatase expression and parathyroid hormone response, characteristic of osteoblastic phenotype expression, were higher in cultures grown on hydroxyapatite. Massas suggested that, since bone cell populations are heterogeneous, this increase is caused by the higher capacity of hydroxyapatite to support the proliferation and differentiation of osteogenic cells. A similar phenomenon can have occurred in our RBM cell cultures, resulting in more osteoblast-like cells together with ECM formation on the Ca/P coated specimens. This supposed influence of the substrate material upon osteoblast phenotype is further supported by Lian. She demonstrated that osteoblast proliferation is related to the synthesis of ECM. The significance of these findings for the *in vivo* behavior of titanium and Ca/P coated implants will be clear and has already been confirmed in various ultrastructural studies.

In contrast to these findings Davies reported ECM formation on titanium surfaces. A reason for this difference may be that in these studies the cells were enzymatically released from the tissue culture flasks with 0.01% trypsin solution, while we used 0.25% solution. Since various proteolytic enzymes and concentrations can have a different damaging effect on morphology, growth rate, and cellular activity, this might result in a loss of osteoblastic phenotype and ECM secretion.

Considering the attachment and proliferation as-

Figure 6. Scanning micrographs of RBM cells cultured for 18 days on a Ca/P-i coated specimen. A portion of the cell multilayer and ECM are raised, resulting in a good view on the afibrillar mineralization foci in the ECM. Further, parts of the coating became detached from the titanium disc during the preparation and procedure and remained adhered to the ECM. It can be seen that the coating has followed perfectly the original titanium surface. (a) bar = 13 μm; (b) bar = 3 μm.
IN VITRO EVALUATION OF MAGNETRON SPUTTERED Ca/P COATINGS

Figure 7. Scanning micrographs of the magnetron sputtered coatings soaked in fully supplemented culture medium for 6 days. All coatings show cracks, which are caused by the SEM preparation process. Spherulitic accretions are visible at the surface of both coatings (arrows). (a) Ca/P-r, bar = 3.2 \mu m; (b) Ca/P-i, bar = 3.2 \mu m.

say, there are two other findings that need further explanation. First, the number of attached cells seeded on Ca/P-i coatings could not be measured by Coulter Counter. Examination revealed that detached coating particles obstructed the orifice of the counting tube. SEM inspection of the Ca/P-i coatings before their use in the cell studies, showed that these surfaces had a polycrystalline appearance. This grain structure is induced by internal stresses in the coating due to the combination of heating of the titanium substrates during the magnetron sputter process and differences in thermal expansion coefficient to substrate and deposited ceramic coating. Consequently, after short incubation periods, when still no additional surface layers are deposited, this stressed Ca/P-i coating can easily be removed by rinsing procedures as used in the counting process.

A second issue is the incomplete removal of dissociated cells from the heat-treated plasma-spray coated substrates in the growth experiments. This observation justifies the conclusion that cellular proliferation has to be considered similar on all investigated surfaces. Besides, it emphasizes the importance of visual surface inspection in these kinds of experiments.

Finally, the study to the influence of culture medium on the Ca/P coated discs revealed that small spherulites were deposited on Ca/P-i, Ca/P-r, and HA-PS substrates, but not on the HA-PS/ht specimens. This surface behavior corresponds well with one of our physicochemical studies, in which the dissolution and precipitation properties of amorphous, amorphous/crystalline, and crystalline sputtered and plasma-sprayed coatings were determined after incubation in simulated body fluid (SBF). The Ca, P, and Mg concentration of SBF is almost equal to human blood plasma. The coated specimens were soaked for 7 days in SBF and the fluid was changed daily. Spectrometric analysis showed, that for amorphous and amorphous/crystalline substrates the Ca and P concentration of SBF, after an initial increase, decreased until a constant concentration was obtained. HA-PS/ht did not change the ion concentration of SBF. SEM examination of the samples demonstrated the formation and growth of apatite nuclei on all surfaces, except for heat treated substrates. On basis of the spectrometric data and similar as described by Li for silica gel, the start of this precipitation process can be attributed to a local supersaturation of both Ca and P caused by dissolution of these ions from the ceramic coating. The subsequent growth of the nuclei is controlled by the Ca and P ions already present in SBF. Tissue culture media contain also physiological concentrations of Ca, P, and Mg ions. In addition, they are buffered at the same pH as SBF. Therefore, it can be assumed that, comparable to SBF, apatite induction processes will take place after immersion of Ca/P coated specimens in fully supplemented culture medium without cells. It will be evident that this apatite formation will influence the bonding properties and the final bone-substrate interface. Nevertheless, the full implication and clinical consequences of this phenomenon for sputter deposited Ca/P coatings have to be investigated further in transmission electron microscopical studies. Also, in these experiments, the required coating thickness to induce the most optimal apatite deposition has to be determined.

In summary, the experiments demonstrated that RBM cells cultured on magnetron sputtered Ca/P coatings stimulated the formation of ECM. Although the sputtered coatings detached in the short term attachment studies, no severe degradation of the coatings was observed in the prolonged culturing assays. In contrast even, sputtered coatings appeared to induce apatite formation. Based on these results, it can be concluded that magnetron sputtering is a promis-
FIGURE 8. Scanning electron micrographs of all experimental cultures observed in fully supplemented culture medium for 18 days. Compared with Figure 7, spherical formation increased on the Ca/P-2, Ca/P-1, and HA-P-5 coatings. At the HA-Ps/HL bar = 3.2 μm. (d) HA-Ps/HL bar = 3.2 μm. (e) HA-Ps/HL bar = 3.2 μm. (f) Ca/P-1 bar = 3.2 μm. (g) Ca/P-2 bar = 3.2 μm.
A new method to manufacture bioactive ceramic coatings. If this can be confirmed in vivo, this coating process may be of advantage over the currently used techniques.

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