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Collagen Formation and Adhesion of Human Gingival Fibroblasts on the IBAD Ca-P Coating on Ti

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Abstract

Coatings of hydroxyapatite (HA) and tricalcium phosphate/HA (TCP/HA) on titanium were fabricated by ion beam assisted deposition (IBAD). Significant effect of the Ca-P coatings on human Gingival Fibroblasts (HGFs) attachment and formation of type I collagen were found by using immunofluorescence microscope. TCP/HA and HA coatings exerted more HGFs attachment and collagen I formation. Comparing with HA coating, TCP/HA coating exhibited better responses during the late period of the tests. This investigation indicated that this surface modification method may enhance the biological seal at the cervical level of the titanium dental implants.

Keywords : Ca-P coating, IBAD, cell adhesion, collagen

1. Introduction

Dental implants meet two kinds of tissue in oral environment: soft tissue and bone. Although the success rate of dental implant is very high today, there are still failure cases which have often been reported [1]. One of the diseases leading to implant failure is marginal infection resulting from the absence of attachment of the soft tissue to dental implant surfaces. Thus, obtaining an effective perimucosal seal of soft tissue to the implant surface should be one of the prerequisites for successful treatment with endosseous dental implants. So there have been some attempts to enhance the interface attachment and binding strength between soft tissue and implant by modifying the surfaces of dental implants. It has been demonstrated that the reactions of soft tissue to implants be influenced by altering the topography of implant surface. Among the attempts, hydroxyapatite (HA) coating is the common choice. Various coating methods were employed to deposit hydroxyapatite on CP titanium, one of the most frequently method used is plasma spraying technique [2-4]. However, according to the clinic failure cases, much concerns about the stability of plasma-sprayed HA coatings have been raised, which typically are relative thick, poorly bonded to the metal substrates, and difficult in controlling over the Ca/P ratio, which has been proved to be one of the key factors affecting the bioactivity of implant coatings. In order to overcome these shortcoming, we prepared two thin and defect-free calcium phosphate coatings, HA and TCP/HA, by ion beam-assisted deposition (IBAD) method, and analyzed the morphology and component of the coatings. Also we examined the number of attached HGFs and ECM collagen I formation to evaluate the bioactivity of the coating samples.

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2. Experimental

2.1 Sample preparation

The samples of CP titanium were cut into 10 mm in diameter and 2 mm in thickness. The sample surfaces were ground and polished through 1micron diamond paste. Calcium phosphate films of HA and TCP/HA were deposited to a thickness of 1 μm by IBAD method. Granules of pure beta-TCP, prepared with high purity calcium carbonate and reagent-grade phosphoric acid, were used as an evaporants. For the calcium phosphate coating, an electron beam evaporator (15kW rated power supply, Telemark, USA) and an end-hall type ion gun (Commonwealth Scientific, USA) were employed. Details of deposition were described elsewhere [5]. Before used in cell experiments, all samples were cleaned with 70% ethanol and then sterilized by exposing to ultraviolet light for 24 h.

2.2 Morphology and composition analysis

The morphologies of all the samples were examined by SEM (JSM-5600LV) with the accelerated voltage of 15 keV. The surface roughness of samples was measured by a profilometer (Handy Surf E-30A, Japan). The compositions of Ca and P of HA and TCP/HA coatings were determined with an SEM XEDS (SEM x-ray energy dispersive spectrometers) method.

2.3 Cell experiments

Human gingival fibroblasts (HGFs, passages 4-9) were obtained from healthy donors with the same procedure as described earlier [6]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 15 mM Hepes, and the antibiotics penicillin (100,000U/L) and streptomycin (100mg/L) in a humidified atmosphere of 95% air-5% CO_2 at 37℃, and were inoculated directly on to the top of each type of disks in 24-well cell culture plates at a density of 25,000 cells/ml. Cell counting was carried out using acridine orange staining method. After appropriate time interval of incubation (2, 6, 12, 24, 72 or 120 h), samples were fixed in 95% ethanol and stained in 4×10^{-4} mg/ml acridine orange for 1 min. After rinsed in PBS, the cells were viewed by a fluorescent microscope (Olympus BX60, Japan). The number of attached cells was counted in a randomly selected 2 mm^2 area.

For the measurement of ECM formation, cells seeded on the samples were incubated for 3, 12, 24, and 48h. Then, the cells were rinsed in PBS, fixed with 4% formaldehyde for 10 min and permeabilized in 0.1% (V/V) Triton X-100 for 5 min. After blocked in 1 % bovine serum (BSA, Sigma), the cells were incubated for 1 h at 37℃ with rabbit polyclonal antiserum against human collage I (dilution 1:30, Sigma, Poole, UK). Then the specimens were incubated with TRITC-conjugated goat anti-rabbit IgG (dilution 1:30, Sigma). Samples were examined with a fluorescence microscope. Thirty cells were selected and viewed randomly for each sample. All the results were treated with Image-Pro software, One-way ANOVA with Fisher LSD test was performed, with values of $p<0.05$ considered statistically significant.

3. Results and discussion

Our previous studies showed that the Ca/P ratio could be controlled and the bone fixation of calcium phosphate coated implants could be improved by IBAD method [7,8]. The surface morphology of titanium, HA and TCP/HA coating examined by SEM showed no difference (As shown in Fig. 1.). The Ra values of surface roughness of all samples are similar to about 1.0 μm. It indicated that calcium phosphate coating does not change the surface morphology and roughness of titanium. The component of calcium and phosphate were measured by SEM XEDS (As shown in Fig. 2). Statistical analysis showed that there was no significant difference in the content of phosphate between HA and TCP/HA samples. While the content of calcium in

Fig. 1.
SEM analysis.

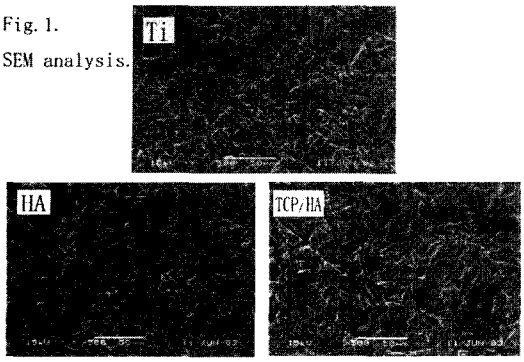

Fig. 1. SEM morphology of three samples, 500×.

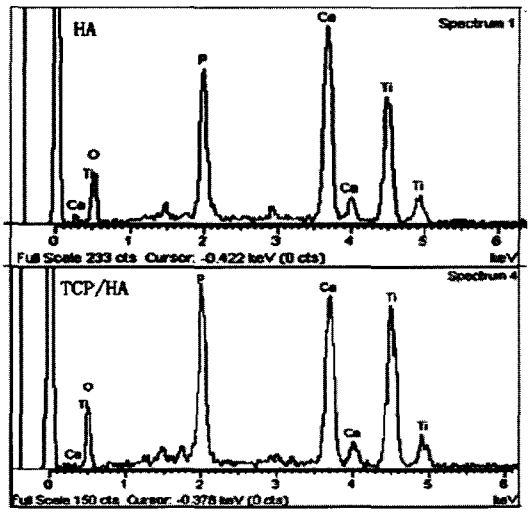

Fig. 2. SEM XEDS(SEM x-ray energy dispersive spectrometers) analysis. There was no significant difference in the content of phosphate between HA and TCP/HA samples. While the content of calcium in HA (20.66±0.46, W%) is more than that in TCP/HA (16.63±0.76, W%) ($p<0.01$).

HA (20.66±0.46, W%) is more than that in TCP/HA (16.63±0.76, W%), and the difference showed statistical significance ($p<0.01$), thus the ratio of TCP/HA is lower than that of HA.

The initial attachment of cells to materials is crucial in the subsequent spreading, proliferation, and differentiation of the cells to substrates. As shown in Fig. 3, there was a time-dependent increase of the number of attached and proliferated cells on all samples. At 2 and 6 h after cell seeding, there was no significant difference

Fig. 3. Cell attachment and proliferation

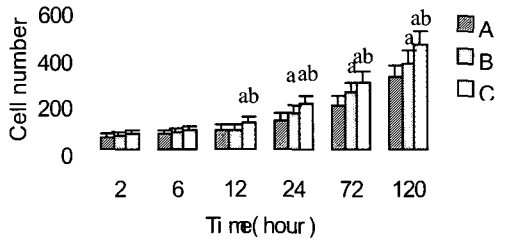

Fig. 3. The number of attached and proliferated cell on Titanium (A), HA (B), and TCP/HA(C). From 12h to 120 h after cell seeding, the cell number TCP/HA is more than that of Titanium and HA($p<0.05$). From 24 h to 120 h, the cell number of HA is more than that of Ti ($p<0.05$). (a=compared with titanium, $p<0.05$; b=compared with HA,$P<0.05$)

among three substrates. But after 12 h and 24 h, the cell number on TCP/HA and HA increased rapidly, began to exceed that on titanium ($p<0.05$). This trend became more and more obvious as the culture time went by. From these results we can concluded that the calcium phosphate coatings does promote the HGFs attachment and proliferation compared with titanium. This result is consistent with that of Grill [9]. Studies [10,11] showed that the mechanism of osteoblasts spreading on HA and titanium was diverse. Serum protein including vitronectin and fibronectin were found on the surface of HA but not on titanium. Cell attachment process is dependent on the properties and conformation of adhesion proteins presented at the biomaterial surface. This distinct difference between coated and uncoated titanium in the present study may due to different adhesive protein adsorbed to the materials and the specific interaction of different integrin and ECM, but the influence of the cell types cannot be overlooked either.

Collagen type I is the most abundant protein in mammals and is the major structural component of most connective tissues. It provides the proper environment for cellular migration, attachment, and differentiation.

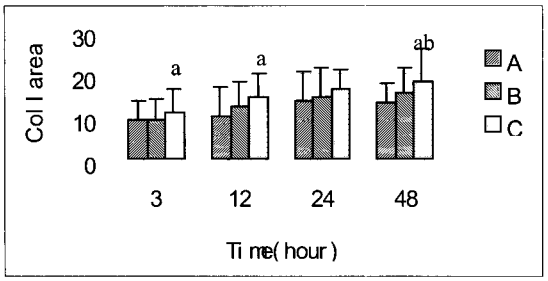

Fig. 4. The coverage area of type I collagen formation on Titanium (A), HA (B), and TCP/HA(C). At 3, 12,and 48h after cell seeding, the formation of type I collagen on TCP/HA is more than that on Titanium ($p<0.05$). At 48h, the formation of type I collagen on TCP/HA is more than that on HA ($p<0.05$). (a=compared with titanium, $p<0.05$; b=compared to HA, $P<0.05$)

Also collagen contributes to control of cell shape, differentiation, and many other cellular activities. Formation and secretion of ECM protein, such as type I collagen, are required for cell spreading and anchorage, so the production of this protein would provide a useful insight into the biological potential. The results of collagen I fluorescent staining is showed in Fig 4. At 3, 12, and 48 h after cell seeding, collagen I area on TCP/HA was larger than that on titanium ($p<0.05$). These results indicated that the different response between TCP/HA and titanium may be influenced by the type I collagen secreted on materials.

Compared with HA, the cell number on TCP/HA was significantly higher than that on HA from 24 h after cell plating ($p<0.05$), and collagen I formation on TCP/HA was significantly higher than that on HA at 48 h ($p<0.05$). This difference may due to the lower Ca/P ratio of TCP/HA. It has been demonstrated that the reactivity of calcium phosphate is generally increased with decreases in the Ca/P ratio [8]. The lower the Ca/P ratio, the higher the dissolution rate, thus the porous TCP/HA released more calcium ion into the medium and participated in cell metabolism. Further research should be carried out to testify this assumption.

4. Conclusion

TCP/HA and HA coatings were prepared by IBAD method. The surface morphology and roughness are similar among coated and uncoated samples, but the Ca/P ratio of TCP/HA is lower than that of HA. TCP/HA and HA coatings exerted the significant influence on cell attachment and collagen I formation. Comparing with HA coating, TCP/HA coating exhibited better responses during the late period of the tests, possibly due to the lower Ca/P ratio. It implied that the adherence of soft tissue to implant surface could be enhanced by TCP/HA coating, thus the biological seal at the cervical level of the implants could be reinforced.

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