Journal of Hard Tissue Biology 15[2] (2006) p65-68

CODEN-JHTBFF, ISSN 1341-7649

Original

Improvement of Adhesion and Spreading of Human Gingival Fibroblasts to RGD-grafted Titanium Surface

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(Accepted for publication, April 10, 2006)

Abstract: Objective: Arg-Gly-Asp(RGD)-containing peptide was coupled on titanium surface of dental implants. It is confirmed that this peptide promotes human gingival fibroblasts (HGFs) adhesion during the early stage after implantation was tested. Methods: RGD peptides were attached covalently to the titanium surface by 1,1'-carbonyldiimidazole (CDI). Primary HGFs were inoculated on commercially pure titanium and RGD-grafted titanium respectively. Cell attachment and proliferation was assessed by acridine orange staining technology. Cell spreading were checked by immunofluorescence staining and confocal laser scanning microscope. Results: The number of attached and proliferated HGFs and their average spreading area were higher on RGD modified titanium than on unmodified titanium. The actin cytoskeleton reorganization existed earlier on RGD coated samples than on uncoated ones. Conclusions: This research may develop a new potential method to enhance the integration between human gingival soft tissue and titanium implant surfaces.

Key Words: dental implant, RGD, Human gingival fibroblasts, Cell adhesion, Actin cytoskeleton

Introduction

Dental implant has become one of the main therapy methods in clinic. Although the success rate of dental implant is high today, postoperative implant failure has often been reported. One of the diseases leading to implant failure is marginal infection resulting from implant penetration of the oral mucosa. A previous study showed that ligature-induced infection may lead to clinical and histopathological alterations of the tissue integration of dental implants. 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.

When being inserted into the body, the implant is immediately conditioned by proteins and other components from exposure to blood at the implant site, and the dynamic process of their adsorption and desorption determined the final protein composition on the implant surfaces[1]. The original surface characteristics of the materials will influence the adsorption and conformation of proteins, which will influence host cells interacting with the conditioned metal surface [2,3]. One potential approach is the immobilization of extracellular matrix adhesion proteins (collagen, fibronectin, laminin, vitronectin) and growth factors (BMP, GF-b) onto an implant surface in order to induce a specific cellular response and promote long-term device integration [4-7]. Although these methods have significant function in promoting the integration between implant and tissue, however, the use of full-length proteins and intact growth factors for coating applications may be limited by their stability, availability, and expense.

The RGD sequence is a ubiquitous adhesive motif found in proteins throughout the body including many extracelluler proteins such as vitronectin, fibrinogen, von Willebrand factor, collagen, laminin, osteopontin, tenascin and bone sialoprotein as well as in membrane proteins, in viral and bacterial proteins, and in snake venoms [8]. They play not only a major role as anchoring molecules but are also important in processes like embryogenesis, cell differentiation, immune response, wound healing and hemostasis [9-11]. RGD peptides have been proved to promote cell adhesion in many previous researches. The RGD sequence is by far the most effective and most often employed peptide sequence for stimulated cell adhesion on synthetic surfaces. Biological effect of RGD peptide on various cell types such as osteoblast, fibroblast,

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endothelial cell have been already demonstrated [12-14]. But few studies have been published focusing on the influence of RGD peptides on primary human gingival fibroblasts. In this study, the RGD peptides were covalently attached to the titanium surface to check the attachment, proliferation and spreading of human gingival fibroblasts to the titanium dental implants by immunofluorescence staining and confocal laser scanning microscope.

Materials and methods

Activation of the surface of titanium and Peptide coupling

The samples of CP titanium were cut into 10 μm in diameter and 2 μm in thickness. The cylinders were then immersed in the hydrogen peroxide solution (30%) and kept at 60°C for 24 h. The Ti samples were activated with 1,1'-carbonyldiimidazole (CDI) (Sigma- Aldrich, St. Louis, MO) using the protocol described in the previous report [15]. The outline of the procedure is shown in Fig.1. The titanium samples were washed in acetone for several times then in dry acetone for three times. The Ti samples were immersed in CDI solution in dry acetone (20 mg/mL). After 30 min reaction with gentle stirring, the Ti samples were washed with dry acetone five times to remove the uncoupled CDI. The peptide Arg-Gly-Asp (RGD) from laminin chain purchased from Sigma (St. Louis, MO) was dissolved in disinfected 100 mM $NaHCO_3$ (pH=8.5) at the concentration of 1 mg/mL.

Peptide coupling to CDI activated Ti surface was performed for 36 hours with gently shaking at room temperature. Then the Ti samples were washed thoroughly by PBS five times.

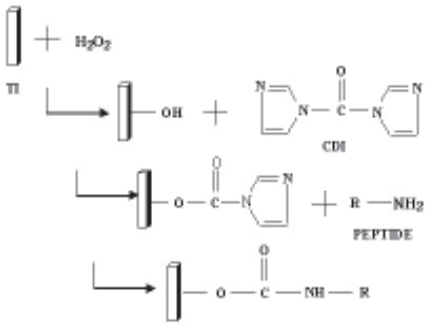

Fig.1 Outline of the peptide-immobilization process.

Test of peptide immobilized to titanium surface

To detect the peptide immobilized to the titanium surface, the peptides RGD was labeled by fluorescein isothiocyanate (FITC) as the fluorescent probe. The labeling reaction was initiated by the addition of three aliquots of 0.2 mg/ml peptides to one aliquot of FITC. The mixtures were then kept in darkness at room temperature overnight. The labeled peptide was further purified by HPLC (High Performance Liquid Chromatography) and was immobilized to the Ti samples as the procedure mentioned above. Then the titanium was visualized by fluorescence microscopy.

Cell attachment and proliferation assay

Human gingival fibroblasts (HGFs) were obtained from healthy donors as described earlier [16]. 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 37C, and were inoculated directly on to the top of each type of disks in 24-well cell culture plates at a density of 20,000 cells/ml. Cell counting was carried out using acridine orange staining method. After appropriate time interval of incubation (3, 24, 72h), samples were fixed in 95% ethanol and stained in 4×10^{-4}mg/ml acridine orange for 1 min. After rinsing in PBS, the cells were viewed by a fluorescent microscope (Olympus BX60, Japan). The number of attached cells was counted in a randomly selected $2mm^2$.

Immunofluorescence staining

For the measurement of reorganization of cell actin cytoskeleton, the cells seeded on the samples were incubated for 2 and 24h. Then, the cells were rinsed in PBS, fixed with 4% formaldehyde for 10 min and permeabilized in 0.1% (V/V) Triton X-100 at 4 °C for 5 min. Then cells were incubated with Tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (20ug/ml, Sigma) for 30 min at 37°C, washed in PBS. The formation of cells actin cytoskeleton was viewed by confocal laser scanning microscope (CLSM, Leica TCS-NT, Germany). Statistic analysis was done with PSS 10.0 software for Window. One-way analysis of variance (ANOVA) was used for multiple comparisons. Values of $p<0.05$ was considered to be significant.

Results

The test of peptide immobilized to Ti samples

To demonstrate the presence of peptide, the Ti samples immobilized with FITC labeled peptide were analyzed by fluorescence microscopy. The representative fluorescence micrograph (Fig.2) clearly showed that the green fluorescence

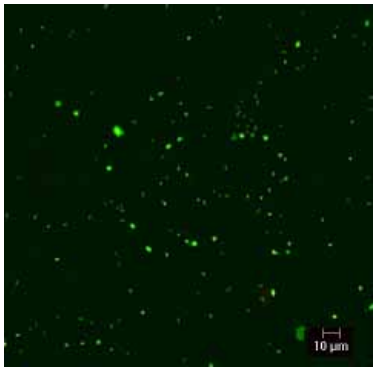

Fig. 2. The fluorescence micrograph of Ti sample immobilized with FITC labeled peptide.

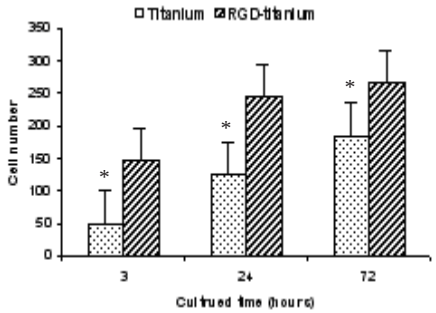

Fig.3. The number of attached and proliferated cell on Titanium and RGD-grafted titanium. From 3h to 72 h after cell seeding, the cell number on the RGD-grafted Titanium is more than that on Titanium (*: $p<0.05$).

spots evenly distributed on the surface of Ti samples which illustrated that the bioactive peptide had been successfully grafted to the surface. However, no fluorescence probe could be detected in the unmodified Ti samples.

Cell attachment and spreading

The initial attachment is crucial to the subsequent behaviors such as spreading, proliferation, and differentiation of the cells on substrates. As shown in Fig.3, there was a time-dependent increase of the number of attached and proliferated cells on both samples

From 3 hours after cell seeding, the cell number on RGD-Ti is significantly larger than that on CP titanium ($p<0.05$). This trend became more and more obvious as the culture time went by. So modifying Ti with the RGD peptide caused a significant increase in the number of adherent fibroblasts. This may be due to the receptor recognition sequence

Arg–Gly–Asp (RGD) contained in many cell adhesion proteins, including fibronectin, vitronectin, and fibrinogen [17,18]. Therefore, these RGD coupled surfaces produce adhesive substrates. The extent of cell spreading is one of the important parameters of the biocompatibility of substrates. Cell attachment is normally followed by cell spreading, in which the cells reorganize the F-actin cytoskeleton, resulting in the flattening and spreading of the cell, and subsequently form the focal adhesion plaques. Fig.4 shows the flurorescein conjugated phalloidin binding to the F-actin cytoskeleton of HGFs on the surface of CP titanium and RGD-grafted titanium after seeding 2hrs. It can be seen in Fig. 4.b that the polymerized actin forms numerous stress fibers throughout the cytosol which terminate at the cell periphery, which indicates that HGFs cultured on the RGD-Ti have spread quite well after seeding 2 hours. However, fewer actin fibers formed in HGFs cultured on the uncoated substrates (Fig.4.a) compared with HGFs on RGD-Ti, which showed that HGFs spread with lower extent. These results are consistent with the spreading area as shown in Fig.5. The cells on all substrates showed increased time-dependent cell spreading area. From 2 to 24 h, the cells on RGD-

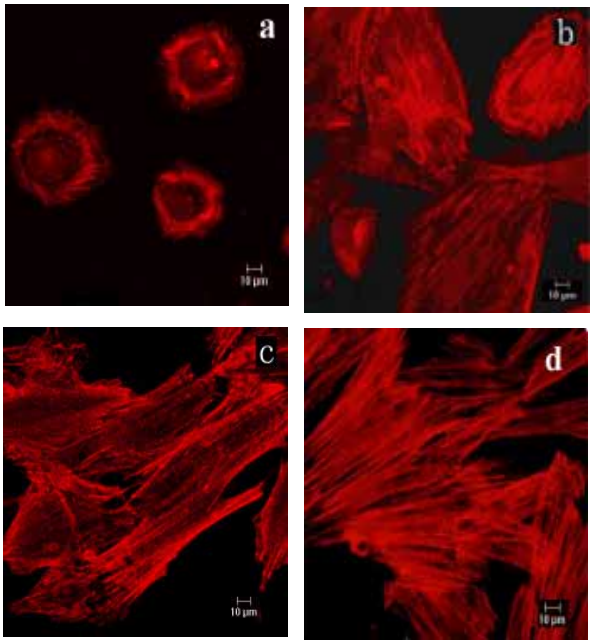

Fig.4 Actin fibers of the cytoskeleton localized using fluorescently conjugated phalloidin (a) Ti-2h-actin, (b)RGD-2h-actin, (c) Ti-24h-actin, (d) RGD-24h-actin.

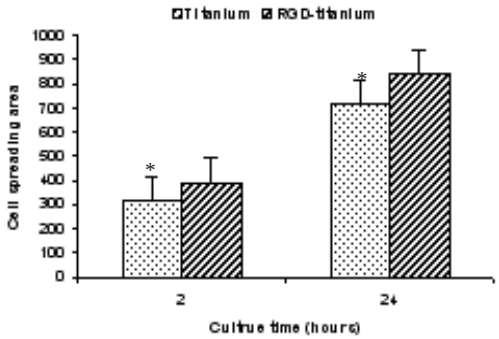

Fig 5. The area of cell spreading on Titanium and RGD-titanium (*: $p<0.05$).

Ti spread wider than that on titanium ($p<0.05$).

Discussion

Cell adhesive RGD sites were identified in many ECM proteins, including vitronectin, fibrinogen, von Willebrand factor, collagen, laminin, osteopontin, tenascin and bone sialoprotein as well as in membrane proteins, in viral and bacterial proteins, and in snake venoms (neurotoxins andisintegrins). About half of the 24 integrins have been shown to bind to ECM molecules in a RGD dependent manner: $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha IIb\beta 3$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, and to some extent $\alpha 2\beta 1$ and $\alpha 4\beta 1$ [19].

In many cases RGD peptides were proven to serve as a useful tool to stimulate cell adhesion on various materials. A great wealth of knowledge has been gained about the biology of integrin mediated cell adhesion on two-dimensional surfaces [20,21]. Techniques to immobilize and detect RGD peptides on biomaterials surfaces were established, as well as *in vitro*

evaluation of adhered cells. Methods to introduce modifiable groups on the implant surface are chemical and physical treatment, e.g. alkaline hydrolysis, reduction, oxidation, tracketching, or plasma deposition. In this study, the RGD peptide was attached to the surface of Ti samples by CDI activation method in which hydroxy was introduced into the surface of Ti samples by oxidation of hydrogen peroxide.

The adherence of fibroblasts to biomaterial surfaces is a complex process involving cell attachment, spreading, focal adhesion plaque formation, and ECM production and reorganization. In comparison with titanium, the number of attached cells, as well as the cell spreading area on the surfaces of RGD-grafted titanium was more than that on the surface of titanium. This result is consistent with that of Cadenaro [22]. These findings indicated that the RGD peptide does promote cell attachment, spreading and actin cytoskeleton reorganization

Conclusion

In summary, the bioactive peptide RGD was grafted on CP titanium by CDI activation method. The RGD grafted titanium exerted the greatest influence on cells behavior involving cell attachment, spreading and actin cytoskeleton reorganization. It implied that the adherence of soft tissue to implant surface could be enhanced by RGD-grafting, thus the biological seal at the cervical level of the implants could be reinforced.

Acknowledgements

This work was supported by a grant (No. 2004F008) of educational department of Liaoning province, China. This work was also partially supported by grants in aid for scientific researches frpm the Ministry of Edition, Culture, Sports, Science and Technology (#15209060,17406027, 17591910, 17591911). This work was also supported by grant-in aid from Chugoku Industrial Innovation Center (#1039908059).

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