OXIDATION OF TITANIUM, RGD PEPTIDE ATTACHMENT, AND MATRIX MINERALIZATION OF RAT BONE MARROW STROMAL CELLS

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The aim of this study was to compare the efficacy of attachment of arginine-glycine-aspartic acid (RGD) peptide to titanium surfaces oxidized by different methods. Titanium surfaces were treated as follows: (1) treatment A: passivation in nitric acid, (2) treatment B: heated in air at 400°C for 1 hour, (3) treatment C: immersed in 8.8 M H_2O_2/0.1 M HCl at 80°C for 30 minutes, and (4) treatment D: treated as in treatment C and then heated at 400°C for 1 hour. RGD was attached to titanium samples treated as in treatments A through D. The quantity of attached RGD was determined by an enzyme-linked immunoabsorbent assay. Mineralization of a rat bone marrow stromal cell (RMSC) culture on the titanium surfaces after 21 days was determined y atomic absorption spectroscopy. The treatments were ranked according to quantity of RGD attached as C, A, B, and D. Twenty-one days after RMSC culture, the degree of mineralization was significantly higher for treatment C than for treatments A, B, and D and for controls. The efficacy of RGD attachment varies with the oxidation treatment given to titanium. Oxidation in H_2O_2/0.1 M HCl at 80°C provided the best overall surface for RGD attachment as well as calcified matrix formation of RMSCs.

INTRODUCTION

Osseointegration of titanium implants is a prerequisite for their success in restoring the dentition. In current use, bone grows into direct contact with the titanium implant surface to achieve successful integration.[1,2] This process requires a healing period of 3 to 6 months before restoration of the dentition. Improvements in dental implants that would enable their immediate restoration and loading would be beneficial for restoring immediate function for patients. Both clinical- and basic-science approaches to immediate loading of dental implants are actively being pursued.

Bone formation after implant placement can proceed from the implant bed as well as the implant surface. Bone growth from

the implant bed proceeds as a response to injury. The degree to which bone forms from the implant surface toward the implant bed is uncertain but will clearly benefit from having a biologically compatible implant surface.[3] Several surface treatments have been studied for preparing a biomimetic titanium implant surface. A biomimetic implant surface close to undifferentiated osteoblast precursor cells in bone marrow should attract bone precursor cells to the implant surface and promote their attachment and differentiation and bone formation from the implant surface. This process will be expected to accelerate osseointegration. Appropriate biological molecules must be identified and their effects on osteoblast function thoroughly investigated to design effective biomimetic implants.

Earlier techniques of creating a biomimetic surface introduced biological molecules either passively or by physical adsorption[4,5] to the implant surface and placement site to aid osseointegration. Attachment of biological molecules to implant surfaces has been shown to significantly improve cell attachment[6–9] and promote cell differentiation and function.[10–12] The attachment of osteoblast cells to surfaces is mediated by integrin receptors.[13–15] Two integrins, α5β1 and αVβ3, which bind extracellular molecules containing an RGD sequence, are reported to be strongly associated with the attachment of osteoblast-like cells onto implant biomaterials.[15] Therefore, coating implants with RGD peptides is a feasible approach to improve cell attachment.

Although passive attachment of RGD can increase cell proliferation rates and decrease time to reach confluence, lower degrees of mineralization are obtained when compared with surfaces with chemically grafted RGD.[10] Furthermore, physical adsorption techniques require large quantities of bioactive material that may become denatured[11] or leach to cause undesirable effects at locations other than the implant site.[12] Covalent binding of biological molecules provides a chemically stable surface with molecules that can be aligned for optimum effect. Variations in orientation of biomolecules may be designed to promote a more specific or rapid physiological response.[12] The covalent binding of biological molecules onto solid surfaces has been used for creating bioactive surfaces on plastics[16,17] and inorganic materials.[11] Ito and coworkers[18] have reported that increasing the distance between bound protein and its substrate may have a beneficial effect on cell growth in addition to its effect on cell attachment. Healy and coworkers[19] found that surface density of RGD ligands greater than 0.6 pmol/cm^2 bound to an aluminum oxide surface significantly enhanced osteogenic cell attachment and spreading[11,16] compared with a peptide density lower than 0.01 pmol/cm.^{2} A subsequent study by Rezania and Healy[10] showed that ligand densities greater than 0.62 pmol/cm^2 were important for the maturation and mineralization of extracellular matrix in contact with solid surfaces. They believe that an optimum surface concentration of adsorbed fibronectin is needed for cell adhesion and locomotion.

The receptor-mediated attachment of osteoblasts to surfaces and enhancements in receptor activity are dependent on material surface chemistry. On titanium surfaces, the integrin activity is influenced by the nature of the metallic substrate,[20] and hydroxyapatite-coated implants are reported to bind better to RGD–containing proteins than to passivated Ti surfaces.[15] These reports suggest that differences in the quality of oxides on titanium could influence the attachment of RGD peptides and their efficacy. Furthermore, a determination of the integrin binding to a biomimetic surface would provide a method of determining the efficacy of the surface for promoting osteoblast attachment.

The hypothesis of this study was that covalent binding of biological molecules onto titanium might vary depending on the characteristics of its surface oxide. Oxides of different crystal structure, thickness, and porosity will alter the density of RGD peptide binding and, consequently, osteoblast attachment and maturation.

Several methods of varying the oxide on titanium have been proposed as intermediate steps for creating a biomimetic titanium implant surface. Kokubo et al[21] have used sodium hydroxide treatment to form a titania gel that leads to deposition of apatite on storage in simulated body fluid. Wang et al[22] described methods of forming anatase by immersion of titanium in an H_2O_2/HCl mixture followed by heating at 400°C. To form rutile, they heated titanium at 400°C for 1 hour. The effect of different oxidation treatments on the covalent attachment of RGD peptide and its subsequent effect on mineralization of the extracellular matrix deposited by a rat bone marrow stromal cell (RMSC) culture were investigated in this study.

Materials and Methods

Preparation of titanium disks

Cylindrical commercially pure titanium rods (99.7%) 1.27 cm in

diameter were cut into 2-mm thick disks and sequentially ground on SiC paper from 240 to 600 grit. All disks were rinsed in distilled water, sonicated in acetone for 10 minutes, and then air dried. Titanium disks were categorized into 4 groups of 6 disks each. Groups A, B, C, and D were treated as follows:

- A: Passivation in 30% HNO_3 for 30 minutes, rinsed in distilled water, and air dried.
- B: Heated in air at 400°C for 1 hour.
- C: Immersed in a solution containing 8.8 M H_2O_2/0.1 M HCl at 80°C for 30 minutes.
- D: Treated as in treatment C, then heated at 400°C for 1 hour.

Surface roughness

Samples treated as above were scanned in an atomic force microscope (Model D 3000, Digital Instruments, Santa Barbara, Calif) to determine surface roughness by previously reported techniques.[23] Images of at least 3 random sites (100-μm^2 area each) per sample were obtained from 2 samples in each group in a tapping mode under ambient conditions. Roughness determinations were made with silicon tips of approximate radius of curvature of 50 nm in contact with the sample under a nominal spring constant of 20 to 100 N/m. Deflections caused by topographic features during scanning were measured and used to determine average surface roughness.

Immobilization of RGD peptide

Titanium disks treated as in treatment A through D above were given a biomimetic treatment by covalently attaching RGD peptide by using a modification of the techniques described by Nanci et al.[12] Titanium disks were first silanized by immersion for 2 hours in 5% wt/wt 3-glycidoxypropyltrimethoxysilane (Aldrich, St Louis, Mo) in acetone. Samples were air dried for 20 minutes and subjected to vacuum desiccation overnight. Samples were then given a 10-minute sonication in 100% ethanol and air dried. Silanated Ti disks were coated with the RGD peptide (Sigma, St Louis, Mo). To attach RGD peptides to the titanium surface, a peptide solution in phosphate-buffered saline (PBS) was made, and a pyridine (1 mL/100 mL) catalyst was added. Samples were immersed in peptide solution for 2 hours for attachment and then rinsed in PBS.

Determination of attached peptide by enzyme-linked immunosorbent assay

The amount of RGD attached to titanium surfaces was determined by enzyme-linked immunosorbent assay. The Ti surface was blocked with 2% bovine serum albumin in PBS and exposed to human integrin αVβ3 (Chemicon International, Temecula, Calif) for 1 hour at room temperature.[24] The αVβ3 integrin was detected with a biotin-labeled antibody clone (Chemicon International). Alkaline phosphatase (AP) conjugated strept avidin was then attached to biotin by immersion for 1 hour at 37°C. A color reaction was developed by reacting AP with p-nitrophenyl phosphate to release p-nitrophenol for 1 minute. The resulting increase in absorbance at 410 nm was used to determine the AP activity as a comparative indicator of RGD attached to the titanium surface. For each experiment, the optical density values obtained for each surface was normalized to the optical density obtained for the control HNO_3-passivated surface. The mean normalized values obtained for 3 experiments were compared by Kruskal-Wallis analysis of variance (ANOVA) on ranks.

Expression of the osteoblastic phenotype

RMSCs were obtained from the femurs of 2 female 100-g Wistar Furth rats for each experiment. Cells were maintained in alpha minimum essential medium (α-MEM) according to previously reported protocols.[25,26] Upon reaching confluence, cells were seeded at a density of 10 000 cells/cm^2 onto titanium surfaces and maintained in growth medium. After 24 hours the medium was replaced with fresh medium containing 10^{-8} M dexamethasone and 50 μg/mL ascorbate phosphate. Culture medium was replaced every other day. Calcium assays were performed after 21 days of culture. Each experiment was performed at least 3 times.

Determination of calcium

Ti disks were washed twice in Hanks buffered salt solution to remove medium. Calcium was extracted by incubating Ti disks with 1 mL of 0.2 N HCl for 1 hour at room temperature with gentle rocking. Each disk was extracted twice. Each extract was diluted to a final volume of 2 mL, and the amount of extracted calcium was quantified by atomic absorption spectroscopy (422.7 nM) with a calcium standard curve. Each experiment was performed a minimum of 5 times.

Statistical analysis

Statistical analysis was performed with Sigma Stat software (Jandel Scientific Software, San

TABLE 1

Surface roughness of oxidized titanium*

Treatment	Average Roughness (nm)
HNO_3 passivation	208.1 ± 18.2[a]
H_2O_2 + HCl	197.2 ± 12.6[a]
Heat at 400°C for 1 hour	178.5 ± 10.2[b]
H_2O_2 + HCl + heat at 400°C	173.9 ± 13.3[b]

*Values with the same superscript letters are not significantly different ($P < .05$).

Rafael, Calif). To derive meaningful information from several sets of experiments, data obtained from each calcium-determination experiment were ranked from highest to lowest. The ranked data were compared by Kruskal-Wallis 1-way ANOVA. Where significant differences were observed, Student-Newman-Keuls method was used for pairwise comparisons.

RESULTS

Surface roughness

The average roughness of the oxidized titanium can be ranked as follows (Table 1) from highest to lowest roughness: treatment A (30% HNO_3), treatment B (H_2O_2 + HCl), treatment C (heat at 400°C), and treatment D (H_2O_2 + HCl + heat). Roughness of HNO_3-passivated (treatment A) and H_2O_2 + HCl–treated (treatment C) surfaces were not significantly different but were significantly higher than treatments that involved heating the samples (treatments C and D).

RGD attachment

RGD attachment to titanium as measured by αVβ3 integrin binding was highest for H_2O_2/0.1 M HCl–oxidized surfaces followed by HNO_3-passivated surfaces (Table 2). The experimental surfaces rank as follows from highest quantity of RGD attached to lowest: treatment C (H_2O_2/HCl), treatment A (HNO_3), treatment D (H_2O_2/HCl + heat at 400°C), and treatment B (heat at 400°C). Samples that were heated after chemical oxidation (treatments B and D) and the silanated control showed low RGD binding.

Mineralization of the RMSC matrix

Calcium deposits were highest for treatment C surface (H_2O_2/0.1 M HCl/RGD) followed by the treatment A surface (HNO_3/RGD) (Figure). Calcium obtained from Ti surfaces that received treatment C was significantly ($P < .05$) higher than all other experimental surfaces. Oxidation treatments B and D that had a heat treatment or a combined chemical and heat oxidation before RGD attachment had lower amounts of calcium deposition. Calcium deposition on these surfaces was not significantly different ($P > .05$) from tissue-culture plastic and silane-attached controls.

TABLE 2

Relative quantities of arginine-glycine-aspartic acid (RGD) on titanium surfaces*

HNO_3 + RGD	1.9 (1.7)[a]
HNO_3 + silanes	0.4 (0.1)
Heated at 400°C + RGD	0.4 (0.1)
H_2O_2/HCl + RGD	3.5 (2.8)[a]
H_2O_2/HCl + heating at 400°C + RGD	0.9 (0.3)[b]

*Values with the same superscript letters are not significantly different ($P < .05$).

DISCUSSION

The chemical attachment of RGD peptide onto titanium surfaces oxidized by different methods and its effect on the expression of the osteoblastic phenotype were investigated. A comparison of the quantity of chemically attached RGD to the experimental titanium surfaces (Table 2) shows that differences in oxidation of the titanium surface result in differences in RGD attachment. Of the experimental surfaces investigated, oxidation with a mixture of H_2O_2/HCl provided the surface with the highest RGD attachment, followed by HNO_3 passivation. The 2 surface treatments that yielded the highest RGD attachment also showed significantly higher surface roughness (Table 1). A correlation coefficient test for the effect of roughness on RGD attachment yielded a value of 0.45 for the experimental surfaces. Surface roughness may play a role in the quantity of peptide attached by providing an increased surface area for attachment. However, treatment A, which had the highest roughness, did not show the highest level of mineralization. Because silane is the first chemical agent that interacts with the Ti surface during RGD attachment, the interaction between silane and the oxide on the titanium surface may also be important in determining the quality and quantity of RGD attachment.

The use of integrins to determine the quantity of RGD

attached to the implant surface provides a useful indication of RGD present in conformations that would permit osteoblast attachment and differentiation.[15] Although material surface with a high degree of RGD attachment is desirable to provide more sites for integrin-mediated osteoblast attachment, the quantity of RGD attached alone may not be a sufficient indicator of osteoblast activity. RGD attachment in conformations that do not favor the binding of integrins will not be useful in promoting osteoblast cell attachment. As such, physical methods of determination of the total quantity of RGD on titanium may have a limited predictive value for the efficacy of RGD attachment and osteoblast function. Our experimental results show differences in RGD attached in conformations beneficial to matrix mineralization depending on the oxidation treatment given to the titanium surface. In addition to surface roughness, the differences in RGD attachment may be related to various qualities of the oxides such as thickness, density and porosity, and chemical composition. A highly porous surface would be expected to provide an increased surface area for peptide attachment. However, treatments that would be expected to lead to high oxide thickness, such as combined H_2O_2/HCl + heat or heat alone, were not conducive to RGD attachment in favorable conformations to osteoblast attachment. This finding may be related to the decrease in surface roughness that was observed to accompany heat treatment of titanium. Heating at 400°C, as was done in this study, will be expected to lead to high oxide thickness.

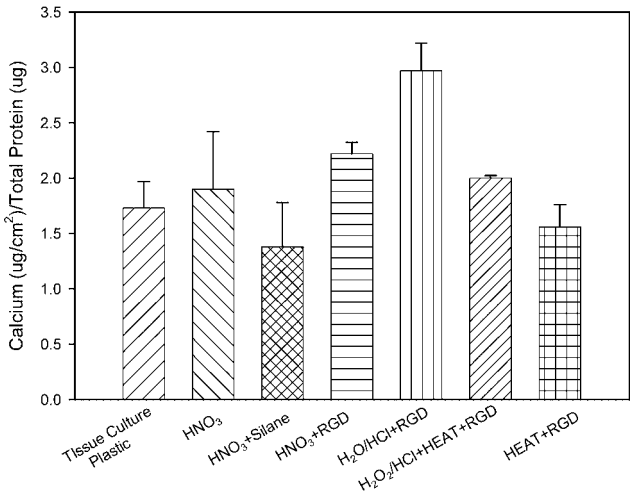

FIGURE. Calcium content of mineral deposits on titanium surfaces.

Marrow stromal cells were induced to transform into bone-forming cells for this experiment. The characteristics of bone-forming cells are a sequential expression of specific genes for AP, production of bone-specific matrix proteins such as osteopontin, and the capacity for matrix mineralization.[25] In this study, matrix mineralization was used as a surrogate measure of osteoblast function in our cultures on Ti surfaces. Increased mineralization is a desirable end product of increased osteoblast function and is appropriate for the purpose of screening surface treatments for implant materials. Bone marrow stromal cells are appropriate for this study because we are interested in conditions that will promote bone formation from the implant surface. Initiation of bone formation from the implant surface requires that osteoprogenitor cells be attracted to the implant surface where they attach, proliferate, and differentiate into committed osteoblasts and form bone. Indeed, modification of implant surfaces to optimize the osteogenic ability of marrow stromal cells could lead to greater clinical implant success.[27]

Oxide thickness [28] and surface roughness[29] are known to influence the response of osteoblasts and osteoblast-like cells to titanium surfaces. The attachment of marrow stromal cells is improved by passivation in HNO_3.[27] This study shows that passivation in nitric acid preserves the roughness of the surface and its well-known benefits to osteoblast activity.[29] Treatments involving heating of titanium would be expected to result in thicker oxides. Our results show that treatments that lead to increased oxide thickness also lead to decreases in surface roughness. Treatments C and D, which would be expected to lead to high oxide thickness, do not attach to high quantities of RGD or yield high quantities of a mineralized matrix.

The results of this study show that RGD attachment on the oxidized titanium surfaces varies depending on the method of oxidation and results in significant differences in the activity of osteoblasts as measured by mineralization. Surface roughness of the experimental surfaces contributes to the increased RGD attachment as well as osteoblast activity. The increase in degree of mineralization corresponds with the findings of Rezania

and Healy,[10] who showed that covalent bonding of cell-adhesion proteins fibronectin and vitronectin, which contain the RGD cell surface recognition sequence[30] to inorganic surfaces, led to increased mineralization.

Titanium spontaneously forms a surface oxide when exposed to the atmosphere. The spontaneously formed Ti oxide is irregular and contaminated with elements in its surroundings.[12] The procedure recommended by the American Society for Testing and Materials (ASTM) for titanium implant treatment involves a passivation in nitric acid for 30 minutes before implantation. This procedure has been reported to lead to unpredictable oxide coverage of titanium and its alloys with a likelihood of adverse effects.[12,31] A secondary oxide treatment has been recommended for formation of an oxide with a reproducible increase in surface thickness.[32–34] On the basis of the results of this study, an increase in oxide thickness may lead to a decrease in surface roughness. Because surface roughness is known to have a favorable effect on bone formation,[33–35] there is a need to balance oxide thickness and roughness for optimum osteoblast activity. The ASTM-recommended nitric acid passivation yielded a surface with a high degree of roughness, high RGD binding, and a high level of mineralization. Treatment of Ti with H_2O_2/HCl forms a titania gel.[22] This gel surface provided the highest surface roughness and the best overall surface for RGD attachment as well as mineralized matrix formation.

CONCLUSIONS

The efficacy of RGD attachment varies with the oxidation treatment given to titanium. Of the treatments investigated, H_2O_2/0.1 M HCl provided the best overall surface for RGD attachment as well as calcified matrix formation by RMSCs. Oxidized titanium surfaces with high surface roughness provided good RGD attachment and mineralized matrix.

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