Chemical modification of pure titanium surfaces to enhance the cytocompatibility and differentiation of human mesenchymal stem cells

Takashi KADO¹, Hideki AITA², Yuki ICHIoka¹, Kazuhiko ENDO³ and Yasushi FURUICHI¹

¹Division of Periodontology and Endodontology, Department of Oral Rehabilitation, School of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa Ishikari-Tobetsu, Hokkaido 061-0293, Japan
²Division of Geriatric Dentistry, Department of Human Biology and Pathophysiology, School of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa Ishikari-Tobetsu, Hokkaido 061-0293, Japan
³Division of Biomaterials and Bioengineering, Department of Oral Rehabilitation, School of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa Ishikari-Tobetsu, Hokkaido 061-0293, Japan

Corresponding author, Takashi KADO; E-mail: kado@hoku-ryo-u.ac.jp

The aim of this study was to improve the cytocompatibility and differentiation of human bone marrow-derived mesenchymal stem cells on the surface of titanium implants by immobilizing biofunctional molecules on their surface. Gly-Arg-Gly-Asp-Ser (GRGDS) peptides, human plasma fibronectin (pFN), or type I collagen from calf skin (Col) was covalently immobilized on the titanium surfaces. Twice as many cells attached to the Col- and pFN-immobilized titanium surfaces than attached to the as-polished surface control. The ALP activity of the cells, as well as the mineralized nodule formation, was significantly higher on the Col- and pFN-immobilized titanium surfaces than on the as-polished surfaces. These results indicate that the immobilization of biofunctional molecules such as Col and pFN on titanium surfaces enhances the attachment, spreading, proliferation, and differentiation of human bone marrow-derived mesenchymal stem cells, which may lead to a more rapid bone-titanium integration.

Keywords: Titanium, Implant, Chemical modification, Biofunctional molecules, Human bone marrow-derived mesenchymal stem cells

INTRODUCTION

Dental implants have been widely used as replacements for missing teeth. A dental implant fixture made of pure titanium or titanium alloys integrates with the surrounding bone, thus supporting the dental prosthesis. The formation of a direct interface between the implant and bone without any intervening soft tissue is defined as osseointegration¹. The immediate loading of dental implants has recently gained popularity because it is reported to have various benefits, including a reduction in the treatment time and trauma, as well as aesthetic and psychological benefits for the patient. Poor bone quality and quantity and osteoporosis are risk factors for implant failure. Both experimental animal and clinical data indicate that challenging bone conditions negatively affect bone wound healing²–⁵. Therefore, methods to improve or enhance bone formation around dental implants installed in challenging bone conditions are needed. Chemical modifications of titanium implant surfaces may be one effective method for achieving rapid bone formation around the implant⁶.

Immediately following implant placement, a series of events occurs between the host and the implant surface, which includes the initial interaction of blood with the implant surface, wherein proteins and ligands are dynamically adsorbed onto and released from the implant surface⁷. Because biological tissues are known to interact mainly with the outermost atomic layers of dental implants, the surface oxide properties of the implant play an important role in protein adsorption and immobilization⁸. Specifically, the surface chemistry, topography, roughness, and wettability of the surface can affect the type, quantity, and conformation of the adsorbed protein layer⁹–¹⁰. Several approaches involving the alteration of the surface physicochemical, morphological, and/or biochemical properties are actively being investigated to improve the bone-implant interface¹¹. The biochemical surface modification methods, wherein biofunctional molecules are immobilized or adsorbed onto biomaterials, are of particular interest for the purpose of inducing specific cell and tissue responses, i.e., controlling the tissue-implant interface using the direct delivery of biofunctional molecules.

One disadvantage of adsorption is that it provides little control over the delivery, release/retention, or orientation of the molecules. The proteins are initially retained on the surface by weak physical adsorption forces. Later, depending on the implant microenvironment, the proteins may desorb from the surface in an uncontrolled manner to initiate their desired responses⁶. In contrast, covalent immobilization is often used when a rigid fixation of the molecule is needed to help prevent subsequent conformational changes or inactivation of the biofunctional molecules¹². The immobilization of biofunctional molecules onto titanium surfaces through the formation of covalent bonds to enhance cytocompatibility has been reported using several techniques. For example, Endo showed that fibronectin can be immobilized on titanium surfaces through Schif's base formation while retaining its native function¹³. Peng et al. reported that collagen can be immobilized on titanium surfaces through poly(ethylene-co-vinyl alcohol)¹⁴. Hayakawa et al. reported that fibronectin can be immobilized on titanium surfaces using a tresyl chloride activation technique¹⁵.

Received Aug 22, 2018: Accepted Feb 3, 2019
doi:10.4012/dmj.2018-257 JOI JST JSTAGE/dmj/2018-257
Oya et al., Khatayevich et al., and Park et al. have all reported the immobilization of Arg-Gly-Asp peptide on titanium surfaces through poly(ethylene glycol). In a previous study, we reported that we successfully immobilized Gly-Arg-Gly-Asp-Ser, fibronectin, and type I collagen on titanium through surface carboxyl groups induced by bonded p-vinylbenzoic acid (pVBA) through peptide bonds, which improved the cytocompatibility of the surface to human periodontal ligament cells. However, little information is available on how effective these various biofunctional molecules are at enhancing the compatibility of human bone marrow mesenchymal stem cells (hBM-MSCs) with titanium and whether they can accelerate the differentiation of hBM-MSCs seeded on titanium surfaces.

The aims of this study were to determine the detailed surface structure of titanium surfaces modified with proteins and a peptide using pVBA as a cross-linking agent using Fourier transform infrared spectroscopy (FT-IR) and X-ray photoelectron spectroscopy (XPS) and to compare the cytocompatibility and differentiation of hBM-MSCs seeded on the titanium surfaces modified with the different biofunctional molecules.

MATERIALS AND METHODS

Titanium substrate
Commercially pure grade 2 titanium disks (JIS, Japan Industrial Specification H 4600, 99.9 mass% Ti, GC, Tokyo, Japan) with a diameter of 6, 15, or 33 mm were purchased. The disks were mechanically as-polished to a mirror finish using colloidal silica. The 6-mm-diameter titanium disks were used for XPS analysis, the 33-mm-diameter titanium disks were used for the gene expression analysis, and the 15-mm-diameter titanium disks were used for the other cell behavior analyses. The as-polished disks were ultrasonically cleaned in distilled and deionized water for 5 min and then dried with oil-free flowing air. The titanium disks using for cell analyses were placed into the wells of a 24-well plate, and then the titanium disks were sterilized by 70% ethanol. Titanium powder (ϕ<45 μm, Wako Pure Chemicals, Osaka, Japan) was used for FT-IR.

Covalent immobilization of biofunctional molecules
Covalent immobilization of biofunctional molecules has been previously described. Briefly, pVBA (Wako Pure Chemicals) was applied to the titanium disk to introduce carboxyl groups onto the surface. This treatment was performed by immersing the polished pure titanium disk in a 1% (v/v) aqueous solution of pVBA (pH 10.8) for 2 h at room temperature. The disk was then removed from the solution and washed with water. Then, cell-adhesive molecules were covalently immobilized on the titanium surface forming amide-type bonds between introduced carboxylic acid groups of pVBA and amino groups of cell-adhesive molecules in the presence of condensing agent [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC); Wako]. The titanium disk with surface carboxyl groups was immersed into a 1.47% EDC solution (pH 7.0) containing 0.1 mg/mL of either Gly-Arg-Gly-Asp-Ser (GRGDS; MW: 490 Da; Peptide institute, Osaka, Japan), human plasma fibronectin (pFN; MW: 440 kDa; Sigma-Aldrich, St. Louis, MO, USA), or type I collagen from calf skin (Col; MW: 100 kDa; Sigma-Aldrich) and stored at 4°C. After 72 h, the disks were removed from the solution and rinsed with phosphate buffered saline (PBS; pH 7.4) to remove protein molecules non-covalently immobilized on the surface (Fig. 1).

Characterization of the modified surfaces
The titanium surfaces with bound pVBA were analyzed with an FT-IR (Frontier, Perkin Elmer, Boston, MA, USA) equipped with the diffuse reflectance accessory (Perkin Elmer). The spectra were recorded at 4.0 cm⁻¹ resolution, and 64 scans were made over the range of 450–4,000 cm⁻¹. The FT-IR spectra of the pVBA powders and pVBA molecules bound on the titanium were measured using KBr and titanium powder as references. The absorption spectra were obtained from the reflectance spectra using the Kubelka-Munk function.

The modified titanium surfaces were characterized spectroscopically to confirm that the GRGDS, pFN, and Col molecules were covalently immobilized on the titanium surfaces. The XPS spectra were obtained using an X-ray photoelectron spectrometer (ESCA-850, Shimadzu, Kyoto, Japan) with an Al Kα source, 7 kV accelerating voltage, and 30 mA currents under a vacuum of 1.0×10⁻⁶ Pa. The binding energy scale was calibrated using the Au 4f₇/₂ and Cu 2P₃/₂ peaks at 83.8 and 932.8 eV. The N 1s spectra were obtained from the as-polished and GRGDS-, pFN-, and Col-immobilized titanium surfaces.
Human bone marrow mesenchymal stem cell (hBM-MSC) culture

hBM-MSCs (Poietics™, Lonza, Visp, Switzerland) were cultured on 10-cm tissue culture polystyrene dishes (Falcon BD, Franklin Lakes, NJ, USA) in Lonza mesenchymal stem cell basal medium with the mesenchymal stem cell growth supplement (MSCGM; Lonza), containing fetal bovine serum (FBS), L-glutamine, and GA-1000 (Gentamicin/Amphotericin-B). The cells were cultured at 37°C in a humidified incubator with 5% CO2-in-air. After seven passages, the cells were cultured in MSC osteogenic induction medium (SingleQuots, Lonza), which was MSC basal medium combined with an osteogenic induction supplement that contained FBS, L-glutamine, penicillin/streptomycin, dexamethasone, ascorbate, and β-glycerophosphate. After reaching 80% confluency during the last passage, the cells with and without osteogenic induction were detached using 0.05% trypsin-0.53 mM EDTA-4 Na (Invitrogen, Paisley, UK) and seeded onto the as-polished and modified titanium disks. The culture medium was replaced every 3 days. The cells cultured in the osteogenic inductive medium were assessed for alkaline phosphatase activity, gene expression, and bone mineral staining, and the non-induced cells were used for the initial attachment and morphology assays.

Initial cell attachment

The initial attachment of the cells onto the titanium disks and culture dishes were evaluated by counting the number of attached hBM-MSCs. The disks were placed into the wells of a 24-well plate, and cells were seeded at a density of 6.0×10⁴ cells/cm². As controls, cells were also seeded onto the wells of commercially available 24-well plates coated with Col (Celltight C-1, Sumitomo Bakelite, Tokyo, Japan) and pFN (BioCoat fibronectin, Cultureware, BD, Franklin Lakes, NJ, USA) at a density of 6.0×10⁴ cells/cm². After 4 h of incubation at 37°C, the disk surfaces were rinsed with PBS to remove any unattached cells. The adherent cells were then detached from the disks using 0.05% trypsin-0.53 mM EDTA-4 Na (Invitrogen). The cells were pelleted to remove the trypsin and then resuspended in fresh growth medium. The number of cells in the solution was counted with a hemocytometer.

Analysis of attached cell density by scanning electron microscopy (SEM)

SEM was used to assess the density of the cells attached onto the titanium surfaces. Each disk was placed in a single well of a 24-well plate. The hBM-MSCs were seeded at a density of 5,700 cells/cm² and incubated for 4 h. After incubation, the samples were washed twice with PBS and fixed in 2.5% glutaraldehyde buffered with PBS. The samples were washed with PBS six times for 5 min each, dehydrated in ethanol/water mixtures of 50, 70, and 80% for 5 min each, 90% for 10 min, and finally 100% for 20 min. The samples were then critical-point dried with CO₂, sputter-coated with gold, and examined using a scanning electron microscope (SSX-550, Shimadzu) at an accelerating voltage of 15 kV.

Immunofluorescent staining of actin cytoskeleton

Confocal laser scanning microscopy was used to examine cell morphology and the cytoskeletal arrangement in the hBM-MSCs seeded onto the titanium surfaces. The disks were placed into individual wells of a 24-well plate, and the cells were seeded at a density of 5,700 cells/cm². After 4 h of culture, the cells were stained with the fluorescent dye rhodamine phalloidin (Molecular Probes, Eugene, OR, USA). The area, perimeter, Feret’s diameter, and circularity of the cells were quantified using an image analyzer (Image J, NIH, Bethesda, MD, USA).

Vinculin expression analysis

The expression and localization of vinculin were analyzed using microscopy and densitometry. The disks were placed into individual wells of a 24-well plate, and the cells were seeded at a density of 5,700 cells/cm². After 4 h of culture, the cultures were stained with a mouse anti-vinculin monoclonal antibody (Abcam, Cambridge, MA, USA) and then by a FITC-conjugated anti-mouse secondary antibody (Abcam). The vinculin expression was quantified as a pixel-based density using an image analyzer (Image J). The vinculin expression density was calculated in two different ways: based on cell number (total pixels/cell number) and based on cell area (total pixels/total cell area).

Alkaline phosphatase activity

The ALP activity of the hBM-MSCs cultured in the osteogenic induction medium was examined by staining and colorimetry. The disks were placed into individual wells of a 24-well plate, and cells were seeded at a density of 3.0×10⁴ cells/cm². After 7 days of culture, the cells were washed twice with PBS and incubated with 120 mM Tris buffer at pH 8.4 containing 0.9 mM naphthol AS-MX phosphate and 1.8 mM fast red TR for 30 min at 37°C. For colorimetry, the cells were lysed with 0.4% IGEPAL CA-630, 10 mM Tris-Cl at pH 7.5, and centrifuged at 10,000 rpm at 4°C for 10 min. The total cellular ALP activity in the lysate was measured with p-nitrophenyl-phosphate (LabAssay ALP, Wako Pure Chemicals) as the substrate. The amount of DNA in the lysate was measured with a DNA quantitative kit (Hokudo, Hokkaido, Japan) according to the manufacturer’s protocol.

Gene expression analysis

The gene expression of the cells was semi-quantitatively analyzed using reverse transcription polymerase chain reaction (RT-PCR). The disks were placed into individual wells of a 24-well plate, and cells were seeded at a density of 3.0×10⁴ cells/cm². After 14 days of culture, the total RNA in the cultures was extracted using Isogen (Nippongene, Toyama, Japan) according to the manufacturer’s protocol. The complementary DNA (cDNA) was synthesized with the Omniscript reverse transcriptase (Qiagen, Valencia, CA, USA) using (dT)₅ primers (1 mM). The PCR reaction was performed
using Taq DNA polymerase (Qiagen). Amplification for the detection of the cDNA from the hBM-MSCs was performed using the requisite number of cycles under the following conditions: 94°C for 30 s, an annealing temperature optimized for each primer pair for 45 s, and then 72°C for 60 s. Forward and reverse primers were used for the target genes, which were runt-related gene 2 (Runx2; GeneBank accession number NM 001024630), Osterix (GeneBank accession number AF 477981), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GeneBank accession number NM 002046). The amplified products were separated by agarose gel electrophoresis and stained with ethidium bromide. The band intensity was quantified under ultraviolet light and normalized to the GAPDH mRNA reference. The PCR experiments were performed using samples from at least three different cell preparations, and the results were obtained from triplicate PCR experiments on the same cell samples.

Bone mineral staining
The mineralization of cultured hBM-MSCs was assessed using alizarin red staining and colorimetry. The disks were placed into individual wells of a 24-well plate, and cells were seeded at a density of 3.0×10^4 cells/cm². After 28 days of culture, the cells were fixed with 10% formalin neutral buffer solution at room temperature for 30 min. The monolayers were washed with distilled water three times and then treated with 40 mM alizarin red solution for 60 min. The cells were washed again with distilled water at least three times. The alizarin red was extracted from the monolayer by incubation in 10% cetylpyridium chloride buffer for 1 h. The dye was removed, 150-μL aliquots were transferred to wells of a 96-well plate, and the absorbance was read at 577 nm with a plate reader (Bio-Rad Laboratories, Boston, MA, USA).

Statistical analysis
Differences between groups were assessed using analysis of variance with Tukey’s post-hoc tests. p Values less than 0.05 were considered statistically significant.

RESULTS

Surface characterization
The FT-IR spectra of the pVBA powders and titanium powder after immersion in 1% pVBA solution are shown in Fig. 2(a) and (b). The FT-IR spectrum obtained from the pVBA powders was characterized by bands at 3,017 and 2,974 cm⁻¹ attributed to asymmetric and symmetric vibrations of the C-H bonds, and at 1,604, 1,561, 1,508, and 1,411 cm⁻¹ attributed to the stretch vibrations of the C=C bonds of benzene in pVBA. The band representing the stretch vibration of the C=O bond of the carboxyl group was also observed. An almost identical spectrum was obtained from the monolayer by incubation in 10% cetylpyridium chloride buffer for 1 h. The dye was removed, 150-μL aliquots were transferred to wells of a 96-well plate, and the absorbance was read at 577 nm with a plate reader (Bio-Rad Laboratories, Boston, MA, USA).

Bone mineral staining
The mineralization of cultured hBM-MSCs was assessed using alizarin red staining and colorimetry. The disks were placed into individual wells of a 24-well plate, and cells were seeded at a density of 3.0×10^4 cells/cm². After 28 days of culture, the cells were fixed with 10% formalin neutral buffer solution at room temperature for 30 min. The monolayers were washed with distilled water three times and then treated with 40 mM alizarin red solution for 60 min. The cells were washed again with distilled water at least three times. The alizarin red was extracted from the monolayer by incubation in 10% cetylpyridium chloride buffer for 1 h. The dye was removed, 150-μL aliquots were transferred to wells of a 96-well plate, and the absorbance was read at 577 nm with a plate reader (Bio-Rad Laboratories, Boston, MA, USA).

Statistical analysis
Differences between groups were assessed using analysis of variance with Tukey’s post-hoc tests. p Values less than 0.05 were considered statistically significant.

RESULTS

Surface characterization
The FT-IR spectra of the pVBA powders and titanium powder after immersion in 1% pVBA solution are shown in Fig. 2(a) and (b). The FT-IR spectrum obtained from the pVBA powders was characterized by bands at 3,017 and 2,974 cm⁻¹ attributed to asymmetric and symmetric vibrations of the C-H bonds, and at 1,604, 1,561, 1,508, and 1,411 cm⁻¹ attributed to the stretch vibrations of the C=C bonds of benzene in pVBA. The band representing the stretch vibration of the C=O bond of the carboxyl group was also observed. An almost identical spectrum was obtained from the monolayer by incubation in 10% cetylpyridium chloride buffer for 1 h. The dye was removed, 150-μL aliquots were transferred to wells of a 96-well plate, and the absorbance was read at 577 nm with a plate reader (Bio-Rad Laboratories, Boston, MA, USA).

Statistical analysis
Differences between groups were assessed using analysis of variance with Tukey’s post-hoc tests. p Values less than 0.05 were considered statistically significant.
culture dish or between the pFN-immobilized titanium surfaces and the pFN-coated cell culture dish.

**Spreading of hBM-MSCs on the biofunctional molecules immobilized titanium surfaces**

Fluorescent images of the hBM-MSCs on the as-polished titanium surfaces and the titanium surfaces modified with the biofunctional molecules are shown in Fig. 5A. The confocal microscopy images of the hBM-MSCs after staining with rhodamine phalloidin showed that the cells were clearly larger after 4 h of incubation on the Col- and pFN-immobilized titanium surfaces than on the as-polished and GRGDS-immobilized titanium surfaces. The cells on the Col- and pFN-immobilized titanium surfaces were enlarged by distinct regions of lamellipodia-like actin projections showing stress fibers within their cytoplasm. Morphometric analysis of the area, perimeter, and Feret’s diameter of the cells showed larger values of these parameters on the Col- and pFN-immobilized titanium surfaces than on the as-polished and GRGDS-immobilized titanium surfaces (Fig. 5B).

**Expression of vinculin by hBM-MSCs on the biofunctional molecules immobilized titanium surfaces**

Confocal microscopy images of the hBM-MSCs after anti-vinculin and anti-actin staining are shown in Fig. 6A. After 4 h of incubation, the vinculin was expressed by the cells on all of the titanium surfaces. Vinculin was found to be localized at the tip of the stretching cytoplasmic projections in the hBM-MSCs on all of the titanium surfaces (Fig. 5A). However, the hBM-MSCs on the Col- and pFN-immobilized titanium surfaces showed a more extensive expression of vinculin at the tips of the cell projections, and the expression was more contiguous along the cell outline than in the cells cultured on the as-polished and GRGDS-immobilized titanium surfaces. The densitometry data shown in Fig. 6B demonstrated that the vinculin expression per cell and per area were larger in the hBM-MSCs seeded on the Col- and pFN-immobilized titanium surfaces than on the as-polished and GRGDS-immobilized titanium surfaces.

**Expression of osteogenic differentiation markers by the hBM-MSCs on the biofunctional molecules immobilized titanium surfaces**

The m-RNA expression of Runx2 and Osterix as evaluated by RT-PCR are shown in Fig. 7A. After 14 days, the hBM-MSCs seeded on the Col-immobilized titanium surfaces showed increased expression of Runx2 and Osterix compared with that by cells on the as-polished titanium surfaces.
Fig. 5 (A) Initial spreading and cytoskeletal arrangement of hBM-MSCs 4 h after seeding onto the titanium surfaces. Representative confocal microscopy images of the cells dual stained with rhodamine phalloidin for actin filaments (red) and DAPI for nuclei (blue). (B) Morphometric evaluations were performed using the images. Data are shown as the mean±SD (n=13), and values with the same letter indicate that the groups are not statistically different.

Fig. 6 (A) Representative confocal microscopy images of the cells stained with anti-vinculin (green), rhodamine phalloidin for actin filaments (red), and DAPI for nuclei (blue). The cells were cultured for 4 h on the titanium surfaces. (B) Vinculin expression per cell was evaluated by densitometry using the confocal microscopic images. Data are shown as the mean±SD (n=13), and values with the same letter indicate that the groups are not statistically different (p<0.05 for significance).
Fig. 7 Osteogenic differentiation of the hBM-MSCs.

(A) The mRNA expression levels of the osteoblastic markers Runx2 and Osterix were evaluated by RT-PCR. Data are shown as the mean±SD (n=3), and values with the same letter indicate that the groups are not statistically different (p<0.05 for significance). (B) The ALP activity of the hBM-MSCs was assessed by staining (top panels) and colorimetry (lower histogram). (C) The mineralization of cultured hBM-MSCs was assessed by alizarin red staining (top panels) and colorimetry (lower histogram). Data are shown as the mean±SD (n=4) for panels B and C, and values with the same letter indicate that the groups are not statistically different (p<0.05 for significance).

Alkaline phosphatase activity of the hBM-MSCs cultured on the biofunctional molecules immobilized titanium surfaces

The ALP activity of the hBM-MSCs was assessed by staining and colorimetry, as shown in Fig. 7B. At day 7, the ALP-positive areas of the hBM-MSCs cultured with osteogenic induction medium on the Col- and pFN-immobilized titanium surfaces were larger than those found on the as-polished and GRGDS-immobilized titanium surfaces. In addition, the ALP activity, which was optically quantified and normalized to the DNA content, was significantly higher on the Col- and pFN-immobilized titanium surfaces than on the as-polished and GRGDS-immobilized surfaces.

Mineralization of the hBM-MSCs cultured on biofunctional molecules immobilized titanium surfaces

The mineralization of cultured hBM-MSCs, as examined by alizarin red staining and colorimetry, is shown in Fig. 7C. At day 28, mineralized nodule formation in the hBM-MSCs cultured with osteogenic induction medium on the Col- and pFN-immobilized titanium surfaces was substantially larger than that by the cells cultured on the as-polished and GRGDS-immobilized titanium surfaces. In addition, the quantified amount of mineralized nodule formation was significantly larger on the Col- and pFN-immobilized titanium surfaces than on the as-polished and GRGDS-immobilized surfaces.
DISCUSSION

Structure of the modified titanium surfaces with pVBA and biofunctional molecules
In addition to the bands attributed to the pVBA molecules, the band assigned to the CH₂ deformation vibration appeared in the spectra after the titanium powders were immersed in 1% pVBA solution (Fig. 2(b)). This finding suggested that the pVBA molecules were covalently bound to the titanium surface. The results of the present study demonstrated that the GRGDS, pFN, and Col molecules immobilized on the titanium surfaces in the presence of EDC remained on the titanium surfaces after ultrasonically washing in deionized water. The GRGDS peptides, pFN, and Col were successfully immobilized onto the titanium surface by forming amide-type bonds between the carboxyl groups of the pVBA molecules and the amino groups on the biofunctional molecules, as suggested by Kado et al.¹⁹ and Timkovich²².

Function of the molecules immobilized on the titanium surfaces
In this study, no significant difference in the number of attached cells was found between the Col-immobilized titanium surfaces and the Col-coated culture dish or between the pFN-immobilized titanium surfaces and the pFN-coated culture dish (Fig. 4A). Because the pFN- and Col-coated culture dishes were optimized for cell culture, these findings suggest that the pFN- and Col-immobilized titanium surfaces maintain the native function of the biofunctional molecules and that the hBM-MSCs were able to attach to the modified titanium surfaces as easily as they do to coated cell culture dishes. Three factors may explain how the native function of these biofunctional molecules was maintained throughout the immobilization process. First, there was a pVBA molecule between the titanium surface and the biomacromolecule that acted as a cross-linking agent. The relative permittivity of the surface oxide film determines the change in the conformation of any proteins adsorbed onto it²⁰. The conformation of proteins always changes when they are adsorbed onto a solid surface, and their conformation plays an important role in the function of the proteins. For example, the structure of pFN typically changes when it is adsorbed onto a titanium surface, resulting in a modification of the pFN behavior, including how it modulates cell adhesion, spreading, and migration²⁴,²⁵. When the biofunctional molecules are separated from the titanium surface, the amount of cultured osteoblastic cell calcification is larger than that found in cells cultured on such surfaces with biofunctional molecules immobilized directly²⁶. In the present study, the pVBA molecule between the titanium surface and biofunctional molecules acted as a spacer that may have helped maintain the native function of the immobilized biofunctional molecules. Second, the aromatic ring of the pVBA is resistant to the hydrolytic attack of water molecules. The mechanical properties of resin composite restorations change because of hydrolysis of the coupling layer at the interface between the matrix resin and the inorganic filler particles²⁶,²⁷. Söderholm et al. demonstrated that when filler particles were pretreated with hydrophobic silanes, the resulting composites were more durable because the coupling layer was more resistant to the hydrolytic attack of absorbed water molecules²⁹. Similarly, the hydrophobic aromatic ring of pVBA may protect the pVBA molecules that are bound onto the titanium surface from hydrolytic leaching. The stable pVBA binding probably also helps to maintain the native function of the immobilized molecules for a longer period of time. Third, the chemical modification of the titanium surfaces used in the present study included only dipping and washing steps. Because these processes are so simple and all the treatments were performed at 4°C, the protocol used here was successful in immobilizing several types of biofunctional molecules onto the titanium surfaces without disrupting their native functions.

Enhanced attachment, spreading, and differentiation of hBM-MSCs by immobilized Col and pFN
The attachment of adherent cells to extracellular matrix components, including pFN and Col, plays critical roles in osteoblast survival, proliferation, differentiation, and matrix mineralization, as well as in bone formation²⁸,²⁹. The adhesion of cells to extracellular matrix ligands is primarily mediated by integrins, a widely expressed family of transmembrane adhesion receptors³². Extracellular matrix-binding integrins rapidly associate with the actin cytoskeleton and cluster together to form focal adhesions, discrete complexes that contain structural and signaling molecules such as talin, vinculin, actin filaments, focal adhesion kinase, and paxillin³³,³⁴. Focal adhesions actively modify the cytoskeleton and signaling pathways, such as the mitogen-activated protein kinases that regulate transcription factor activity and direct cell growth and differentiation³⁵. The critical role of specific integrin-extracellular matrix interactions, particularly a₅β₁-pFN and a₅β₁-Col, increase osteoblastic gene expression, protein secretion, and matrix mineralization³⁶-³⁹. The increased expression of vinculin in the cells cultured on the Col- and pFN-immobilized titanium surfaces observed here likely contributed to the enhanced growth and differentiation of the hBM-MSCs by influencing their mitogen-activated protein kinases through the formation of focal adhesions.

There are two possible explanations for why the differentiation of the hBM-MSCs was enhanced by the immobilized Col and pFN. First, focal adhesions activate signaling pathways to regulate transcription factor activity and enhance direct cell growth and differentiation, as mentioned above. Second, increased cell attachment and spreading may have promoted differentiation through increased cell-to-cell interactions⁴⁰. In the present study, the hBM-MSCs were seeded at a confluent density on each of the titanium surfaces before assessing the ALP activity, gene expression, and alizarin red staining. Therefore, the cell-to-cell interactions of the hBM-MSCs should
have been equivalent among the as-polished and modified titanium surfaces. These facts suggested that the acceleration of hBM-MSC differentiation by the Col- and pFN-immobilized titanium surfaces was mediated by integrin interaction.

In this study, the hBM-MSCs seeded on pFN-immobilized titanium surfaces did not show significant increase in the m-RNA expression of Runx2 and Osterix compared with that by cells on the as polished titanium surface. Runx2 and Osterix triggers the expression of major bone matrix genes during the early stages of osteoblast differentiation, but Runx2 is not essential for the maintenance of these gene expressions in mature osteoblasts. Because the hBM-MSCs possibly have already begun to differentiate before being seeded on titanium, cells on pFN-immobilized titanium surfaces did not show significant increase in the m-RNA expression of Runx2 and Osterix compared with that by cells on the as polished titanium surface. However, the ALP activity and the mineralization of hBM-MSCs were enhanced on the Col- and pFN immobilized titanium surfaces. Therefore, our results clearly demonstrate that the differentiation of the hBM-MSCs was enhanced by the immobilized Col and pFN.

In contrast to the Col- and pFN-immobilized surfaces, the GRGDS-immobilized titanium surface was less effective at promoting attachment, spreading, and differentiation of the hBM-MSCs. The short polypeptide sequence RGD, which is located in the 10th type III repeating unit of FN, is a key attachment site for a variety of integrin cell surface receptors. The αβ integrin plays an important role in osteogenic differentiation because it is expressed by osteoblasts and osteoprogenitors and promotes cell survival and matrix mineralization. The binding of integrin αβ requires both the Pro-His-Ser-Arg-Asn (PHSRN) sequence in the 9th type III repeating unit of FN and the RGD sequence. Each domain alone shows little effect on binding, but in combination, they synergistically bind to αβ to provide stable adhesion. Therefore, it is assumed that the PHSRN sequence must be present for the RGD sequence to promote the attachment, spreading, and differentiation of hBM-MSCs.

Feature of the chemical modification method of titanium with biofunctional molecules using pVBA for providing desirable biological properties
Several methods for the immobilization of biofunctional organic molecules onto titanium surfaces through the formation of covalent bonds have been reported. The immobilization of biofunctional molecules using pVBA has several advantages over the other methods proposed previously. Firstly, the immobilization of the biofunctional molecules can be achieved through a very simple process which includes just dipping and washing steps at 4°C, unlike electrodeposition techniques. This is quite favorable for the immobilization of biological macromolecules without disrupting their native functions. Secondly, pVBA has a more suitable molecular structure than other cross-linking agents used previously for immobilizing biofunctional molecules. The pVBA has longer molecular length than treosyl chloride, which is desirable for a spacer between titanium surface and biomolecules to avoid the conformational change of the immobilized biomolecules. The pVBA has also hydrophobic domain in the molecular chain, which probably contributes to resist to the hydrolytic attack of water molecules. In addition, pVBA molecules are chemically stable in an aqueous solution and don not undergo the self-condensation reaction unlike silane coupling agents.

CONCLUSIONS
Biofunctional molecules, including Col and pFN, were successfully immobilized onto titanium surfaces by a chemical modification method employed in the present study, which maintained the native function of the molecules. The titanium surfaces immobilized with pFN and Col accelerated the attachment, spreading, and differentiation of hBM-MSCs, suggesting that these two biofunctional molecules immobilized on titanium implant surfaces may lead to a more rapid bone-titanium integration.

ACKNOWLEDGMENTS
This work was supported by GC. This study was supported in part by a Grant-in-Aid for Scientific Research (C) (22592191) from the Japan Society for the Promotion of Science.

REFERENCES
1) Albrektsson T, Branemark PI, Hansson HA, Lindstrom J. Osseointegrated titanium implants. Requirements for ensuring a long-lasting, direct bone-to-implant anchorage in man. Acta Orthop Scand 1981; 52: 155-170.
2) Chen H, Liu N, Xu X, Qu X, Lu E. Smoking, radiotherapy, diabetes and osteoporosis as risk factors for dental implant failure: a meta-analysis. PLoS One 2013; 8: e71955.
3) Herrmann I, Lekholm U, Holm S, Kultje C. Evaluation of patient and implant characteristics as potential prognostic factors for oral implant failures. Int J Oral Maxillofac Implants 2005; 20: 220-230.
4) Liddeelow G, Klineberg I. Patient-related risk factors for implant therapy. A critique of pertinent literature. Aust Dent J 2011; 56: 417-426; quiz 41.
5) Sanfilippo F, Bianchi AE. Osteoporosis: the effect on maxillary bone resorption and therapeutic possibilities by means of implant prostheses-a literature review and clinical considerations. Int J Periodontics Restorative Dent 2003; 23: 447-458.
6) Puleo DA, Nanci A. Understanding and controlling the bone-implant interface. Biomaterials 1999; 20: 2311-2321.
7) Lemons JE. Biomaterials, biomechanics, tissue healing, and immediate-function dental implants. J Oral Implantol 2004; 30: 318-324.
8) Kasemo B, Lausmaa J. Surface science aspects on inorganic biomaterials. CRC Crit Rev Clin Neurobiol 1986; 4: 333-380.
9) Deligianni DD, Katsala ND, Koutsoukos PG, Missirlis YF. Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength. Biomaterials 2001; 22: 87-96.
10) Healy KE, Thomas CH, Rezania A, Kim JE, McKeown PJ, Lom B, et al. Kinetics of bone cell organization and mineralization on materials with patterned surface chemistry. Biomaterials 1996; 17: 195-208.

11) Junker R, Dimakis A, Thoneick M, Jansen JA. Effects of implant surface coatings and composition on bone integration: a systematic review. Clin Oral Implants Res 2009; 20 Suppl 4: 185-206.

12) Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb Tech 2007; 40: 1451-1463.

13) Endo K. Chemical modification of metallic implant surfaces with biofunctional proteins (Part 1). Molecular structure and biological activity of a modified NiTi alloy surface. Dent Mater J 1995; 14: 185-198.

14) Peng C, Tsutsumi S, Matsumura K, Nakajima N, Hyon SH. Morphologic study and syntheses of type I collagen and fibronectin of human periodontal ligament cells cultured on poly(ethylene-co-vinyl alcohol) (EVA) with collagen immobilization. J Biomed Mater Res A 2001; 54: 241-246.

15) Hayakawa T, Yoshinari M, Nemoto K. Direct attachment of fibronectin to tressyl chloride-activated titanium. J Biomed Mater Res A 2003; 67: 684-688.

16) Oya K, Tanaka Y, Saito H, Kurashima K, Nogi K, Tsutsumi H, et al. Calcification by MC3T3-E1 cells on RGD peptide immobilized on titanium through electrodeposition PEG. Biomaterials 2009; 30: 1281-1286.

17) Khatarevich D, Gungormus M, Yazici H, So C, Cotinel S, Ma H, et al. Biofunctionalization of materials for implants using engineered peptides. Acta Biomater 2010; 6: 4634-4641.

18) Park JW, Kurashima K, Tsutsumi Y, An CH, Suh JY, Doi H, et al. Bone healing of commercial oral implants with RGD immobilization through electrodeposited poly(ethylene glycol) in rabbit cancellous bone. Acta Biomater 2011; 7: 3222-3229.

19) Kado T, Hidaka T, Aita H, Endo K, Furuchi Y. Enhanced compatibility of chemically modified titanium surface with periodontal ligament cells. Appl Surf Sci 2012; 262: 240-247.

20) Nakanishi K. INFRARED ABSORPTION SPECTROSCOPY: Nankodo Company; 1962.

21) Lindberg B, Maripuu R, Siegbahn K, Larsson R, Gölander CG, Eriksson JC. ESCA Studies of heparinized and related surfaces : 1. Model surfaces on steel substrates. J Colloid Interface Sci 1983; 95: 308-321.

22) Timkovich R. Detection of the stable addition of carbodiimide to proteins. Biochem Biochem 1977; 79: 135-143.

23) Sundgren JE, Bodo P, Ivarsson B, Lundstrom IG. Adsorption of fibrinogen on titanium and gold surfaces studied by ESCA and ellipsometry. J Colloid Interface Sci 1986; 113: 530-543.

24) Garcia AD, Vega MD, Boettiger D. Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation. Mol Biol Cell 1999; 10: 785-798.

25) Grinnell F, Feld MK. Adsorption characteristics of plasma fibronectin in relationship to biological activity. J Biomed Mater Res 1981; 13: 363-381.

26) Schrader ME, Block A. Tracer study of kinetics and mechanism of hydrodynamically induced interfacial failure. J Polym Sci C 1971; 34: 281-291.

27) Söderholm KJ. Degradation of glass filler in experimental composites. J Dental Res 1981; 60: 1867-1875.

28) Söderholm KJ, Zigan M, Ragan M, Fischlaschweiger W, Bergman M. Hydrolytic degradation of dental composites. J Dent Res 1984; 63: 1248-1254.

29) Globus RK, Doty SB, Lull JC, Holmuhamedov E, Humphries MJ, Damsky CH. Fibronectin is a survival factor for differentiated osteoblasts. J Cell Sci 1998; 111: 1385-1393.

30) Linsley C, Wu B, Tawil B. The effect of fibrinogen, collagen type I, and fibronectin on mesenchymal stem cell growth and differentiation into osteoblasts. Tissue Eng Part A 2013; 19: 1416-1423.

31) Zimmerman D, Jin F, Leboy P, Hardy S, Damsky C. Impaired bone formation in transgenic mice resulting from altered integrin function in osteoblasts. Dev Biol 2000; 220: 2-15.

32) Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002; 110: 673-687.

33) Geiger B, Bershadsky A, Pankov R, Yamada KM. Transmembrane crosstalk between the extracellular matrix-cytoskeleton crosstalk. Nat Rev Mol Cell Biol 2001; 2: 793-805.

34) Sastry SK, Burridge K. Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. Exp Cell Res 2000; 261: 25-36.

35) Giancotti FG, Ruoslahti E. Integrin signaling. Science 1999; 285: 1028-1032.

36) Hamidouche Z, Fromigue O, Ringe J, Haupl T, Vaudin P, Pages JC, et al. Priming integrin alpha5 promotes human mesenchymal stromal cell osteoblast differentiation and osteogenesis. Proc Natl Acad Sci U S A 2009; 106: 18587-18591.

37) Jikko A, Harris SE, Chen D, Mendrick DL, Damsky CH. Collagen integrin receptors regulate early osteoblast differentiation induced by BMP-2. J Bone Miner Res 1999; 14: 1075-1083.

38) Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha2beta1 integrin interaction. J Cell Physiol 2000; 184: 207-213.

39) Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem 1998; 273: 32988-32994.

40) Aita H, Horii N, Takeuchi M, Suzuki T, Yamada M, Anpo M, et al. The effect of ultraviolet functionalization of titanium on integration with bone. Biomaterials 2009; 30: 1015-1025.

41) Keselowsky BG, Wang L, Schwartz Z, Garcia AJ, Boyan BD. Integrin alpha5 controls osteoblastic proliferation and differentiation responses to titanium substrates presenting different roughness characteristics in a roughness independent manner. J Biomed Mater Res A 2007; 80: 700-710.

42) Aota S, Nomizu M, Yamada KM. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. J Biol Chem 1994; 269: 24756-24761.

43) Adachi M, Linsley P, Wu B, Tawil B. The effect of fibrinogen, collagen type I, and fibronectin on mesenchymal stem cell growth and differentiation into osteoblasts. Tissue Eng Part A 2003; 67: 684-688.

44) Redick SD, Settles DL, Briscoe G, Erickson HP. Defining the role of osteoblast-specific gene expression and activation of the Osteopontin transcription factor. J Biol Chem 2004; 279: 24756-24761.

45) Garcia AJ, Schwarzsteiner J, Boettiger D, Distinct activation states of alpha5beta1 integrin show differential binding to RGD and synergy domains of fibronectin. Biochemistry 2002; 41: 9063-9069.

46) Redick JD, Settles DL, Biscoe G, Erickson HP. Defining fibronectin’s cell adhesion synergy site by site-directed mutagenesis. J Cell Biol 2000; 149: 521-527.