OSTEOBLAST PROGENITOR CELL RESPONSES TO CHARACTERIZED TITANIUM SURFACES IN THE PRESENCE OF BONE MORPHOGENETIC PROTEIN–ATELOPEPTIDE TYPE I COLLAGEN *IN VITRO*

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KEY WORDS

Titanium
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The goal of this study was to investigate the effect of bone cell response to titanium (Ti) surfaces in the presence of bone morphogenetic protein (BMP)–atelopeptide type I collagen mixture. The atelopeptide type I collagen was used as a potential carrier for the BMP. Sterilized 600-grit Ti samples were used as substrates for the cell culture study. X-ray photoelectron spectroscopy indicated the presence of TiO_2 on the Ti surface. The *in vitro* cell culture study was performed using an osteoblast progenitor cell line derived from mice (2T9). At confluency, the cells cultured on Ti surfaces were divided into three groups: unstimulated culture, culture stimulated by BMP–atelopeptide type I collagen (40 ng/mL), and culture stimulated by atelopeptide type I collagen (40 ng/mL). The unstimulated and atelopeptide type I collagen cultures were controls in this study. After 4 days of incubation, protein production, alkaline phosphatase (ALP) activity, and hexosaminidase activity were observed to be the highest for cells exposed to the BMP–atelopeptide type I collagen mixture. Statistical differences in cellular protein production and ALP activity were observed between the controls and the surfaces exposed to the BMP–atelopeptide type I collagen mixture. Similarly, a statistical difference in hexosaminidase activity was observed between unstimulated Ti surfaces and surfaces exposed to BMP–atelopeptide type I collagen mixture. However, no statistical differences in protein production, ALP activity, and hexosaminidase activity were observed between cells exposed to atelopeptide type I collagen solution and the unstimulated surfaces.

INTRODUCTION

Titanium (Ti) is currently the material of choice for dental and orthopedic implants. It has excellent corrosion resistance and biocompatibility, permitting establishment of close bony apposition.[1] Studies on surface properties of Ti have indicated the presence of an amorphous titanium dioxide (TiO_2).[2] Depending on the sterilization technique and surface treatments, oxide thicknesses in the range of 30 to 50 Å are often reported.[3–5] Increased oxide thickness and crystallinity have been reported with anodization treatments, and an increase in surface energy has been observed following radio frequency glow discharge.[3,6,7]

Cells have evolved elaborate strategies for interacting with their environment and can sense variations in surface properties, including surface roughness and chemical composition. By altering the surface properties of Ti, researchers have observed different rates of cellular attachment to the surface *in vitro*.[8–20] Currently, plasma-spraying implants with hydroxyapatite (HA) are used as a means of improving osseointegration. However, a major problem associated with plasma-sprayed HA coatings is their poor strength at the coating–implant interface[21]; this problem can lead to implant failure.[22,23]

Recently, bone morphogenetic proteins (BMP) have been used as a means to achieve optimal bone–implant interactions. In experimental fracture sites, increased BMP activity was observed in and around the bone ends.[24] Differentiation of mesenchymal-derived cells into cartilage and bone has been reported to occur when the cells are stimulated with BMP.[25,26] However, because of the rapid diffusion of BMP when implanted *in vivo*, a carrier acting as a slow delivery system is required to deliver the BMP.[27] In this study, we used atelopeptide type I collagen as a potential carrier for the BMP; our goal was to investigate the effect of titanium (Ti) surfaces on osteoblast phenotypic expression in the presence of BMP–atelopeptide type I collagen mixture.

MATERIALS AND METHODS

Substrate material

Ti grade 2 (ASTM F76) disks, 15.24 mm in diameter by 3.18 mm thick, were obtained from Metal Samples (Munford, Ala). The 600-grit Ti surfaces were produced by sequentially grinding the samples with 240-, 400-, and 600-grit silicon carbide. These surfaces were then ultrasonically degreased in benzene, acetone, and ethanol, for 10 minutes in each solvent, rinsing with deionized water between applications of solvents. A passivation procedure was conducted by exposing the Ti samples to a concentrated nitric acid : deionized water solution (40:60, v:v) at room temperature for 30 minutes (ASTM F86-76). After each surface treatment, the Ti samples were rinsed with deionized water and sterilized under ultraviolet light for a minimum of 24 hours prior to cell culture studies.

BMP–atelopeptide type I collagen

Following the procedure previously reported, purified BMP was obtained from bovine bone using liquid chromatography.[28] Atelopeptide type I collagen solution was used as a carrier in this study. As previously characterized and reported, bovine BMPs have a molecular weight of 18 kd.[28] Porcine BMP has also been reported to consist of peptides that contain 163 amino acids. A stock BMP–atelopeptide type I collagen mixture was prepared using 10 μg purified BMP and 6 mg atelopeptide type I collagen solution.

X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) was performed using a Perkin-Elmer 5400 system (Physical Electronics, Eden Prairie, Minn) at a base pressure of less than 10^{-7} Pa. Duplicate samples before and after immersion were analyzed using a take-off angle of 45°. All surface spectra over a range of 0 to 1100 eV were obtained using Mg K_α radiation (1253.6 eV) at 15 kV and 20 mA. The pass energy used for obtaining the surface spectra was 90 eV. Relative atomic concentrations for all identified elements were quantified from the multiplex spectra (pass energy of 18 eV) using peak areas and elemental sensitivity factors obtained from the Physical Electronics (Perkin-Elmer) 5400 spectrometer. High-resolution Ti 2p and O 1s spectra were collected with a pass energy of 9 eV. From the high-resolution spectra, peak separation between Ti $2p_{1/2}$ and Ti $2p_{3/2}$ and full width at half maximum (FWHM) for Ti $2p_{3/2}$ were measured. Photoelectron peak positions were corrected for charging by referencing to the adventitious C 1s peak at 284.6 ± 0.4 eV.

Cell culture

A mouse osteoblast progenitor cell line, 2T9, was used to evaluate the cellular response on Ti surfaces. The 2T9 is a mesenchymal cell line from the calvaria of a transgenic mouse expressing SV40 large T antigen under transcriptional control of mouse BMP promoter. The Ti disks fit tightly into the wells of a 24-well culture plate, completely covering the bottom of the well. The cells were seeded at a concentration of 10,000 cells/mL in alpha-minimal essential medium (α-MEM) containing 7% fetal bovine serum, 1% antibiotic–antimycotic solution, and 50 μg/mL ascorbic acid. One milliliter of the cell suspension was plated per well of a 24-well plate containing a Ti disk. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

At confluency, the medium was removed and replaced with mineralizing medium containing α-MEM, 2% fetal bovine serum, 1% antibiotic–antimycotic solution, 50 μg/mL ascorbic acid, and 4 mM β-glycerophosphate. At confluency, the Ti samples were divided into three groups. We added 40 ng/mL of BMP–atelopeptide type I collagen

mixture to one group, and we added 40 ng/mL of atelopeptide type I collagen (first control) to another group. The last group (second control) was untreated. The mineralizing medium was changed every 2 days. No additional atelopeptide type I or BMP–atelopeptide type I collagen mixture was added when the mineralizing medium was changed. Four days after stimulation, we measured protein assay, hexosaminidase activity, and ALP specific activity.

Protein assay

Protein was determined using the Pierce BCA protein assay (Pierce, Rockford, Ill). Four samples from each treatment were used to measure the protein production. On the day of assay, the α-MEM from the cell culture was removed and discarded. Prior to measuring protein contents from the cell layers, a series of bovine serum albumin (BSA) concentrations was prepared to generate a standard curve. The cell layers were lysed with 1 mL of Triton X-100 (0.2%). A 30-μL aliquot of the cell lysate (or BSA) was added to 200 μL of BCA working reagent in a 96-well culture plate and incubated for 30 minutes at 37°C. Absorbance was measured at 600 nm using a microplate reader. The amount of proteins produced by the cells was read off of the standard BSA curve. At an α value of 0.05, the protein levels were statistically evaluated using analysis of variance, with the differences compared using the Student-Newman-Keuls test.

ALP activity

Four samples from each treatment were used to measure the ALP activity. Before measuring protein contents from the cell layers, a series of *p*-nitrophenol concentrations was prepared to generate a standard ALP curve. On the day of assay, a 50-μL aliquot of the same cell lysate (or *p*-nitrophenol) was added to 50 μL of working reagent in a 96-well culture plate and incubated for 1 hour at 37°C. The working reagent consisted of 1.5 M 2-amino-2 methyl-1-propanol (Sigma, St Louis, Mo), 20 mM *p*-nitrophenol phosphate (Sigma), and 1 mM magnesium chloride (1:1:1). The reaction was stopped using 100 μL of 1 N sodium hydroxide; at this point, the absorbance was read as 410 nm using a microplate reader. The ALP produced by the cells was read off of the standard ALP curve. ALP specific activity was calculated by normalizing to protein contents. At an α value of 0.05, the ALP specific activities were statistically evaluated using the analysis of variance, with the differences compared using the Student-Newman-Keuls test.

Hexosaminidase activity

The hexosaminidase activity assay was used to determine the cell numbers in the lysate suspension. Prior to measuring hexosaminidase activity in the lysate suspension, a standard cell number curve was generated by measuring the hexosaminidase activity of different known cell numbers. Four samples from each treatment were used to measure the hexosaminidase activity. On the day of the assay, 50 μL of each cell suspension dilution was added to wells in a flat bottom plate. A solution of 40 μL substrate buffer was used. The substrate consisted of 0.0684 g of *p*-nitrophenyl*N*-acetyl-β-D glucosamide, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D glucopranoside, and 10 mL of a 0.1 M citrate buffer. The solutions were incubated at 37°C for 1 hour. After 1 hour, we added 80 μL of a stop buffer that consisted of 0.05 M glycine, 5 mm ethylenediaminetetraacetic acid (pH 10.5), and water. The plates were read using a microplate reader at 405 nm, and the cell numbers from each lysate suspension were determined from the standard cell number curve. Differences among the cell numbers were statistically compared using analysis of variance.

Results and Discussion

By means of XPS, carbon, oxygen, titanium, and trace amounts of nitrogen were observed on the surfaces. Figure 1 shows a representative surface spectrum of the 600-grit Ti surface. Carbon (24–30% of atoms) and nitrogen (less than 2% of atoms) were observed for both surfaces. This high concentration of carbon on Ti surfaces was not surprising. Titanium surfaces are very active, and thus the adsorption of the atmospheric carbon and nitrogen is unavoidable. Nitrogen was also adsorbed onto the Ti surfaces during the passivation treatment.

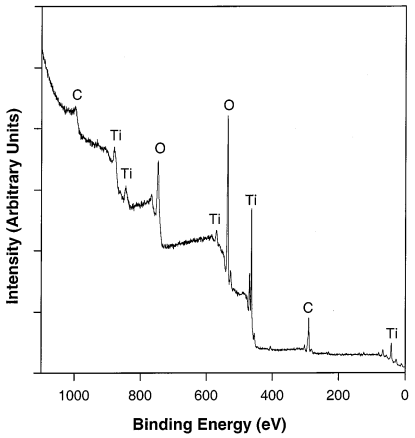

FIGURE 1. Representative XPS survey scan of Ti surfaces.

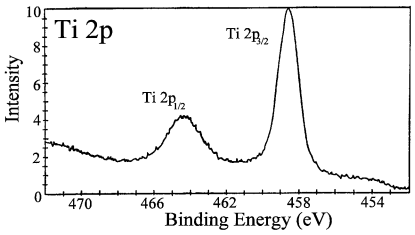

FIGURE 2. Representative high-resolution spectrum of Ti 2p on Ti surfaces.

High-resolution Ti 2p spectra show the binding energies of Ti $2p_{1/2}$ and Ti $2p_{3/2}$ to be 464.0 and 458.2 eV, respectively (Fig 2). The binding energies of Ti 2p and TiO_2 single crystal standard are shown in Table 1. The peak separation between the Ti 2p doublet was observed to be 5.8 eV for all of the Ti samples, including the TiO_2 single crystal standard. In concurrence with other investigators, this peak separation and peak position indicated the presence of TiO_2 on the nonpassivated, the passivated, and the anodized Ti

TABLE 1
Binding energies (eV), peak separation (eV), and FWHM (eV) of Ti 2p

Sample	Binding Energies		Peak Separation*	FWHM†
	Ti $2p_{3/2}$	Ti $2p_{1/2}$		
Grade 2 Ti	458.2	464.0	5.8	1.3
TiO_2 single crystal	458.3	464.1	5.8	1.1

*Peak separation (eV) between Ti $2p_{3/2}$ and Ti $2p_{1/2}$.
†FWHM (eV) of Ti $2p_{3/2}$.

surfaces.[29–31] This separation value indicated an oxidation state of 4+ for the Ti surface.

The FWHM at Ti $2p_{3/2}$ for all the Ti samples was observed to be 1.3 eV, whereas the FWHM at Ti $2p_{3/2}$ for the TiO_2 single crystal standard was observed to be sharper (FWHM = 1.1 eV). This suggests a difference in the crystallinity of TiO_2 on the Ti samples as compared to the TiO_2 single crystal. FWHM values of 1.2 to 1.4 for Ti $2p_{3/2}$ have been reported in other studies for TiO_2 powder-pressed pellets, and for TiO_2 produced by reactive e^--beam evaporation, by reactive plating, and by dip coating.[29,32] Other studies, using Raman spectroscopy, have indicated the presence of an amorphous oxide on Ti surfaces.[6] In addition, conversion of the Ti metal to TiO_2 is indicated by the chemical shift of the dominant 2p peaks and by measuring the binding energy (BE) between O 1s (529.7 eV) and Ti $2p_{3/2}$ ($BE_{O\ 1s} - BE_{Ti\ 2p_{3/2}}$) indicated a 71.5 ± 0.2 eV difference for all of the surfaces analyzed.

Matrix proteins in bone have been shown to play a crucial role in the calcification and architectural construction of these hard tissues.[33] No statistical difference in the amount of cell layer and matrix-associated protein was observed between the unstimulated Ti surfaces and the Ti surfaces stimulated with atelopeptide type I collagen (Fig 3). However, protein production was observed to be statistically higher when the Ti surfaces were stimulated with BMP–atelopeptide type I collagen mixture.

The ALP activity and hexosaminidase activity are other markers used in this study. The ALP and cell numbers

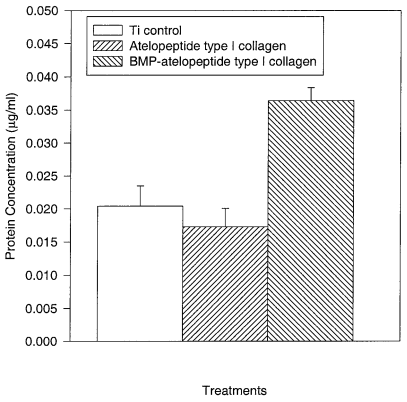

FIGURE 3. Cell- and matrix-associated protein content produced by cells on untreated Ti surfaces, on Ti surfaces in the presence of atelopeptide type I collagen, and on Ti surfaces in the presence of BMP–atelopeptide type I collagen. The error bars represent 1 standard deviation.

are widely recognized as biochemical markers for osteoblast differentiation and proliferation, and they may be an important factor in bone mineralization.[34] The hexosaminidase activity of cells has been reported to be directly proportional to cell numbers.[35] In this *in vitro* cell culture study, ALP activity and hexosaminidase activity were observed to be the highest in cells exposed to BMP–atelopeptide type I collagen mixture (Figs 4, 5). Statistical differences in the ALP activity were observed between the controls and the surfaces exposed to BMP–atelopeptide type I collagen mixture. Similarly, a statistical difference in hexosaminidase activity was observed between unstimulated Ti surfaces and surfaces exposed to BMP–atelopeptide type I collagen mixture. However, no statistical differences in the ALP activity and hexosaminidase activity were observed between cells exposed to atelopeptide

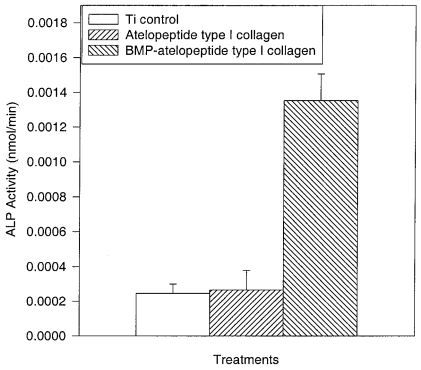

FIGURE 4. Mean ALP specific activity of cells on untreated Ti surfaces, on Ti surfaces in the presence of atelopeptide type I collagen, and on Ti surfaces in the presence of BMP–atelopeptide type I collagen. The error bars represent 1 standard deviation.

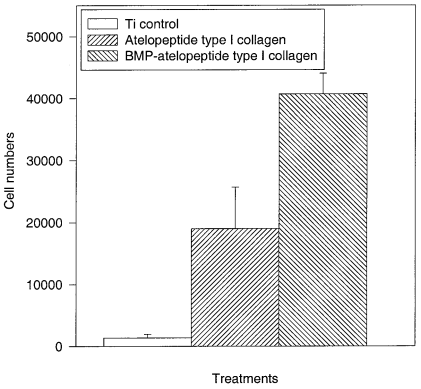

FIGURE 5. Average cell numbers on untreated Ti surfaces, on Ti surfaces in the presence of atelopeptide type I collagen, and on Ti surfaces in the presence of BMP–atelopeptide type I collagen. The error bars represent 1 standard deviation.

type I collagen solution and unstimulated surfaces.

Overall, this study indicates the *in vitro* enhancement of osteoblast activity in the presence of BMP–atelopeptide type I collagen mixture. This study also suggests that the atelopeptide type I collagen can be an effective carrier for BMP and that in the presence of other carriers, such as hydroxyapatite or biodegradable polymers, different rates of osteoblast differentiation and mineralization may be induced. Further *in vitro, in vivo,* and clinical studies are needed to fully investigate the combined effect of bioactive proteins and biomaterial substratum on bone–biomaterials interfaces.

Conclusion

This study suggests that the atelopeptide type I collagen can be an effective carrier for BMP and that the presence of BMP–atelopeptide type I collagen mixture enhanced bone cell activities on Ti surfaces when compared with the untreated cells on Ti surfaces or Ti surfaces in the presence of atelopeptide type I collagen alone.

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