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Behavior of osteoblast-like cells on fibronectin or BMP-2 immobilized surface

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ABSTRACT

Property modification of an implant surface is known to influence the behavior of cells surrounding implant material. This study examined the osteogenic cell behavior on protein immobilized surface in vitro. Following plasma surface modification, fibronectin or bone morphogenetic protein-2 (BMP-2) was immobilized. The number of cells adhering to fibronectin-immobilized surface increased after 1 and 2 h of incubation compared with non-immobilized surface. Alkaline phosphatase activity and osteocalcin mRNA expression of the osteogenic cells on the BMP-2 immobilized surface was greater than that on the non-immobilized surface. This study demonstrated that protein can be immobilized to a polystyrene surface after treatment with O_2 plasma and that the osteogenic cells surrounding a biomaterial can be controlled by the immobilization of protein to the biomaterial.

Biomaterials actively influence the area surrounding a healing wound. Generally, the healing of a wound around an implant is influenced by material-related factors, *e.g.*, surface topography, mechanical properties, and physicochemical properties of the implant material. Many reports have dealt with the surface modification and the control of cell behavior on surface topography (11, 13–16, 27). Surface topography can influence cell orientation based on the concept of contact guidance (20) and expressions of cell functions (13–16, 27). Property modification of an implant surface is also known to influence the behavior of the cells surrounding the implant. Plasma surface modification including plasma polymerization and plasma surface treatment is an effective and economical method of surface treatment for many materials. The method is of growing interest in biomedical engineering because it can be used to control the physicochemical properties of biomaterials (3, 17, 18). Plasma surface treatments using various gases such as Ar, O_2, N_2, and SO_2 have been employed to modify blood compatibility, to influence cell adhesion and growth, and to control protein adsorption (2, 8, 21, 22, 26).

Fibronectin is known to play an important role in governing the interactions of biomaterials with their surrounding matrices (1, 19). It was also reported that precoating materials with fibronectin enhanced cell adhesion to the substrate (4, 5). In addition, bone morphogenetic protein-2 (BMP-2) is known to induce ectopic bone formation, which stimulates osteoblastic maturation (24, 25). The purpose of this study is to evaluate the immobilization of protein to an implant surface and the behavior of osteogenic cells on a fibronectin or BMP-2 immobilized surface.

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MATERIALS AND METHODS

Immobilization and demonstration of protein. Polystyrene substrates were treated with O_2 plasma for 10 min. The apparatus was used for plasma-polymerization under the conditions of a flow rate of 50ml/min, pressure discharge of 1.5 Pa, and output of 200 watts. After the O_2-plasma treatment, the protein was immobilized onto the substrate. Rat fibronectin (Biomedical Technologies, MA, USA) or recombinant human BMP-2 (Peprotech EC, London, UK) was dissolved in phosphate-buffered saline (PBS) solution (pH 7.4) at a fibronectin concentration of 10 ppm and a BMP-2 concentration of 1 ppm.

The degree of protein immobilization was measured by the method described by Yoshinari *et al.* (26). Briefly, the QCM-D instrument (QCM-D300; Q-Sense AB, Goteborg, Sweden) was operated with AT-cut single crystal quartz sensors with a resonant frequency of 5 MHz for the adsorption assay. O_2-plasma treated QCM-D sensor crystals were used as sensors. The crystal resonant frequency (Δf) and the dissipation factor (ΔD) of the oscillator were measured simultaneously at a fundamental resonant frequency (5 MHz) and at a number of overtones, including 35 MHz. Monitoring the resonance behavior of piezoelectric oscillation enables measurement of mass adsorption at the surface in real time, usually as a function of the decrease in resonance frequency (f). The frequency shift (Δf) is related to the adsorbed mass (Δm). At 35 MHz, a frequency shift of 1 Hz corresponds to a change in mass of approximately 2.6 ng/cm^2. This relationship is strictly applicable for sufficiently thin and rigid films. A second measurement parameter, dissipation (D), gives qualitative information about the viscoelastic properties of the adsorbed layer.

Fibronectin/PBS solution (10 ppm) was introduced into an axial flow chamber comprising a T-loop in order to thermally equilibrate the sample at 37 ± 0.05°C. The sequence of injections into the QCM cell for each experimental run was: 0.5 ml of double-distilled water and 0.5 ml of PBS containing fibronectin, and 0.5 ml of double-distilled water. PBS containing BMP-2 (1 ppm) was done in the same way as that of the PBS containing fibronectin.

The morphological demonstration of immobilized protein morphology was performed by an immunofluorescence technique. First, a cell disk, half of which was covered by parafilm for control, was modified by plasma treatment. Following the peeling off of the parafilm, the fibronectin or BMP-2 was immobilized by immersion in PBS containing protein. After being washed with PBS, the cell disk with fibronectin was stained using mouse anti-human fibronectin (1 : 100; C-20, Santa Cruz Biotechnology, CA, USA) as the first antibody and Alexa Fluor 488 goat anti-mouse IgG (1 : 200; Molecular Probes, Leiden, The Netherlands). The cell disk with BMP-2 was stained using rabbit anti-human BMP-2 (1 : 100; PeprotechEC, London, UK) as the first antibody and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Leiden, the Netherlands). Finally, the specimens were examined with a Biorad MRC 1000 confocal laser scanning microscopic (CLSM) system.

Evaluation for osteogenic cell behavior. For biological evaluation of the test materials, a rat bone marrow (RBM) cell culture technique was used as described by Maniatopoulos (10). Briefly, RBM cells were obtained from the femora of 40- to 43-day-old male Wistar rats (100–120 g). The epiphyses were cut off and both diaphyses were flushed out using alpha-MEM (Gibco Life Technologies B.V., Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS, heat-treated at 56°C for 35 min, Gibco), 50 mg/ml freshly prepared ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA), 10 mM Na beta-glycerophosphate (Sigma), 10^{-8} M dexamethasone (Sigma), and antibiotic (gentamicin). Each femur was processed with 15 ml of this medium. The cells were suspended and cultured in three 80-cm^2 tissue culture flasks (Nunc Products by Gibco). Finally, the cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After 8 days of primary culture, the cells were detached using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA, pH 7.2). Subsequently, the cells were resuspended in the supplemented culture medium described above and used in the experiments.

For the fibronectin immobilized surface, the cell attachment assay was performed as described by Keller *et al.* (9). Briefly, 10×10^4 RBM cells were seeded on cell disks with or without fibronectin immobilization. At the end of 1-, 2-, 3-, and 6-hour incubation periods, the cell suspensions were carefully removed by a pipette and the disk surfaces were rinsed twice with PBS to remove any non-adherent cells on the surface. After being detached with a trypsin solution containing 0.02% EDTA, the adherent cells were counted with a Coulter counter (Coulter, Z-1).

For the BMP-2 immobilized surface, the alkaline phosphatase (ALP) activity was measured. 1×10^4

RBM cells were seeded on the BMP-2 immobilized surface. The cultures were incubated for 3, 7, and 14 days. After the culture medium was removed, the cell layers were rinsed with PBS. Demineralized H_2O (Milli-Q) was then added to each specimen and put on ice. Finally, the cells were harvested with a rubber scraper and the cell suspensions were transferred to 10 ml tubes. The cells were sonicated for 10 min and centrifuged at 2000 rpm for 10 min. Subsequently, a 10 ml aliquot of each cell lysate plate was incubated for 1 h at 37°C in a working reagent consisting of 0.5 M 2S-amino-2-methyl-l-propanol (Sigma), 5 mM p-nitrophenol phosphate (Sigma), and 5 mM magnesium chloride (1 : 1 : 1). The reaction was stopped by the addition of 100 ml 0.3 M sodium hydroxide, and the final absorbance was read at 405 nm using a microplate reader (Biorad 450; Bio-Rad, CA, USA). To determine the specific ALP activity, the protein contents in the same lysates were determined using the Pierce BCA protein assay (Pierce, IL, USA). A 150 ml aliquot of each cell lysate was added to 150 ml BCA working reagent in a 96-well culture plate and incubated for 2 h at 37°C. Absorbances were then measured at 562 nm using the same microplate reader mentioned above. Four independent experiments were performed for all assays, and two specimens of each sample type were used in each experiment.

For quantitative osteocalcin mRNA expression, total RNA was extracted using the Isogen reagent (Nippon Gene, Japan) according to the manufacturer's instructions. Briefly, the cultured osteoblast-like cells were homogenized and solubilized in an Isogen/chloroform solution at 4°C. Supernatants were obtained by centrifugation at 12,000 × g for 20 min at 4°C. The precipitates were obtained by decantation and washed with 75% ethanol. Finally, the RNA pellets were dissolved in RNAase free water and preserved at −20°C until used. Using the extracted RNA as a template, reverse transcription reactions were conducted with an RT-PCR kit (RNA-PCR kit Ver. 2.1; Takara Biomedicals, Japan) to synthesize cDNA. A quantitative PCR was then conducted using osteocalcin and GAPDH primers with a LightCyclerR using the double-stranded DNA dye SYBR Green I (Roche Diagnostics, Germany). The PCR conditions and primer sequences used in the LightCycler® are listed in Table 1. Quantification was performed by comparing the levels obtained with standardized samples. In the present study, the concentrations of cDNA in the unstimulated samples were 0.2, 0.5, 1.0, and 2.0 μl. The PCR conditions used in the LightCycler™ were 40 cycles. A melting curve analysis was also performed after the PCR amplification to confirm the absence of the primer dimer in the PCR products. The ratios of osteocalcin mRNA expressions were supplemented by the value of a housekeeping gene such as GAPDH. The results were analyzed by t-test at $p = 0.05$.

RESULTS

The degree of protein immobilization was measured by the QCM-D instrument, which was operated with AT-cut single crystal quartz sensors with a resonant frequency of 5 MHz for the adsorption assay. The frequency curve shows a decrease over time during the early stages of adsorption until a certain frequency was reached after about 120 min. The estimated amount of fibronectin or BMP-2 on the QCM sensor is shown in Fig. 1. The difference in frequency was approximately 100 Hz after the action of PBS containing fibronectin, indicating that 260 ng/cm^2 of fibronectin was immobilized on the surface. In contrast, the frequency changed from 0 to −17 Hz after the action of PBS containing BMP-2, indicating adsorption of approximately 44.2 ng/cm^2 of BMP-2 on the surface.

The morphological demonstration of immobilized protein morphology was performed by an immuno-

Table 1 *Primers used in quantitative PCR (LightCycler™)*

Target cDNA Primer sequence (5' to 3')	Polymerase chain reaction condition
Osteocalcin	
Forward: GGT GCA AAG CCC AGC GAC TCT	95°C 10 s 60°C 5 s 72°C 12 s
Reverse: GGA AGC CAA TGT GGT CCG CTA	
GAPDH	
Forward: GAC ATT GTT GCC ATC AAC GAC	95°C 10 s 56°C 5 s 72°C 12 s
Reverse: CCA GTA GAC TCC ACG ACA TAC	

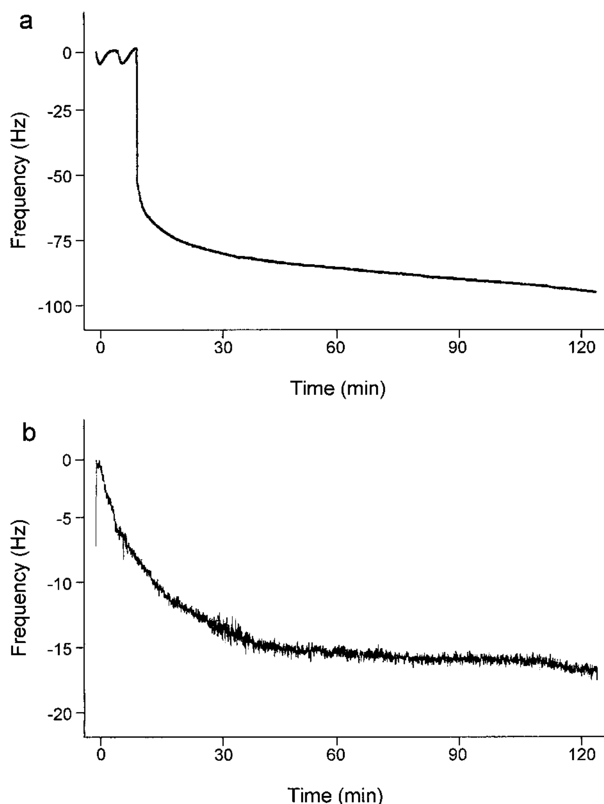

Fig. 1 Frequency shift over time for exposure of an O_2 plasma specimen to fibronectin (a) and BMP-2 (b) obtained via QCM-D measurement.

fluorescence technique. Fig. 2 shows the CLSM images after the immobilization of fibronectin or BMP-2. The control side of either fibronectin or BMP-2 exhibited no immunoreaction, but the side with protein immobilization did exhibit immunoreaction. The fluorescence of the fibronectin immobilized surface was stronger than that of the BMP-2 immobilized surface (Fig. 2).

The results of cell attachment on fibronectin immobilized materials are shown in Fig. 3. The number of cells adhering to each disk tended to increase for up to 6 h. The number of cells attached to the fibronectin immobilized surface was greater than that on the control surface after 1 and 2 h of incubation. However, no significant differences with and without fibronectin immobilization were found after 3 and 6 h of incubation.

ALP activity of the osteoblast-like cells on the surfaces both with and without BMP-2 immobilization increased from 3 to 7 days of incubation, but decreased from 7 to 14 days. After 7 days of incubation, ALP activity of the osteoblast-like cells on the BMP-2 immobilized surface was greater than that on the control surface, though there was no significant difference between them (Fig. 4). In contrast, osteocalcin mRNA expression of the osteoblast-like cells on the surfaces both with and without BMP-2 immobilization was the greatest after 3 days of incubation, and then decreased to 14 days of incubation. In the 14 days of incubation, the expression of osteocalcin mRNA on the BMP-2 immobilized surface was significantly greater than that on the control surface (Fig. 5).

DISCUSSION

Immobilization of protein to biomaterial enables control of the behavior of the cells surrounding the materials. Fibronectin is known to play an important role in governing the interactions of biomaterials with their surrounding matrices, and BMP-2 is known as an ectopic bone formation derivative. This

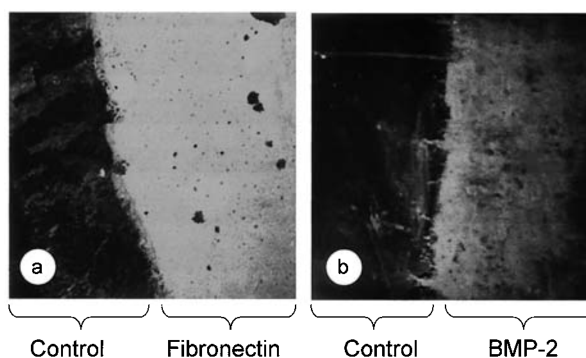

Fig. 2 Immunofluorescence confocal laser scanning microscopic images. a: fibronectin immobilized surface, b: BMP-2 immobilized surface

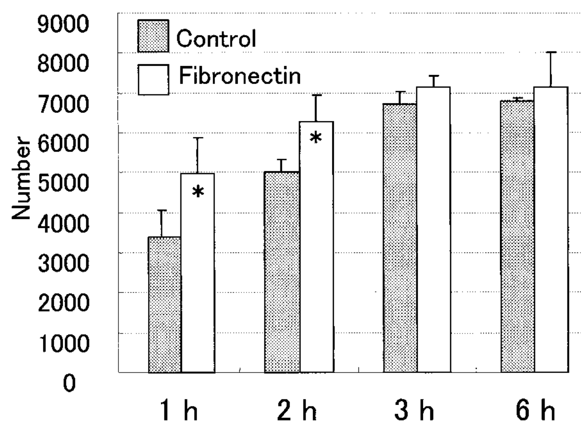

Fig. 3 Number of osteoblast-like cells initially attached on fibronectin immobilized surface. *significantly difference compared with control

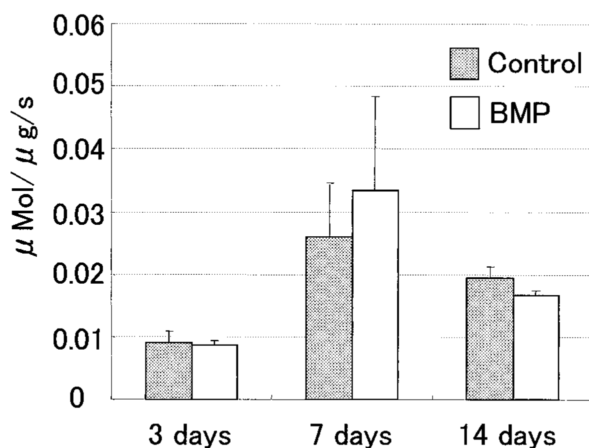

Fig. 4 ALP activity of osteoblast-like cells on BMP-2 immobilized surface.

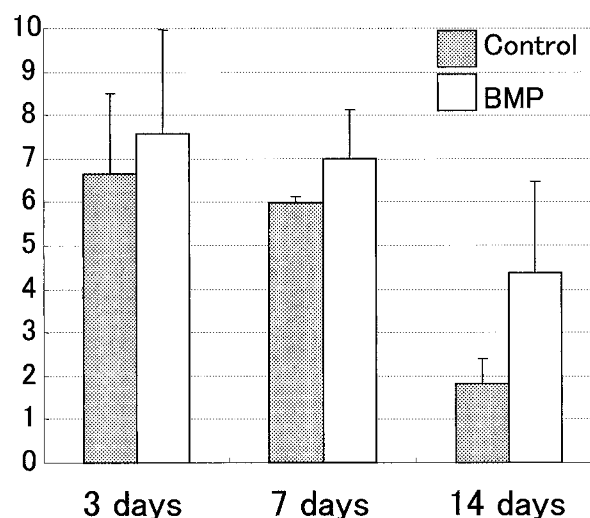

Fig. 5 Osteocalcin mRNA expression of osteoblast-like cells on BMP-2 immobilized surface. Data is shown as the folds of the corresponding each data.

study introduced methods of protein immobilization and examined the adhesion and differentiation of RBM cells on a protein immobilized surface. Modification of the properties of an implant surface is also known to influence the behavior of the cells surrounding the implant. Plasma surface treatment is an effective and economical technique for use with biomaterials. The technique, which can control physicochemical properties, is of particular interest in biomedical engineering (3, 17, 18). Suzuki *et al.* demonstrated an increase in carbonyl carbon bonds, C = O, after the plasma treatment (6, 23). In addition, there is a report that the plasma technique is suitable for generating functional, synthetic polymeric surfaces that can initiate enzyme coupling reactions (23). In this study, the adherence of fibronectin and BMP-2 to polystyrene surface was demonstrated by immunofluorescence using antibodies. The degree of fluorescence was proportional to the amount of protein as measured by Q-Sense.

Fibronectin is a cell-binding protein, which is 270 kDa, and is believed to play an important role in governing the interactions of biomaterials with their surrounding matrices. It has been reported that precoating titanium with fibronectin enhanced the adhesion of cells to the titanium (1, 19). The biological function of fibronectin was evaluated by counting initially attached cells (7, 9). In contrast, BMP-2 is a bone inductive protein and contributes to bone formation around dental implants. The biological evaluation for BMP-2 immobilized surface was carried out by the mesurement of ALP activity and expression of osteocalcin mRNA in this study. These methods are commonly used at this moment (7, 12, 14). So this study indicated that a fibronectin immobilized surface increased the number of adhering cells in the early stages, and that a BMP-2 immobilized surface stimulates an osteogenetic function. Thus, immobilization of the appropriate protein to a biomaterial is beneficial for controlling cell behavior around the material and is beneficial for treatments involving medical and dental implants.

In conclusion, this study demonstrated that protein can be immobilized to a polystyrene surface after treatment with O_2 plasma and that the osteogenic cells surrounding a biomaterial can be controlled by the immobilization of protein to the biomaterial.

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