Glow Discharge Plasma Treatment of Titanium Plates Enhances Adhesion of Osteoblast-like Cells to the Plates Through the Integrin-Mediated Mechanism

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Purpose: *Initial adhesion of cells to implant surfaces and subsequent behavior of the cells are important determinants for biocompatibility of the implants. It was previously reported that both adhesion of MC3T3-E1 osteoblast-like cells to titanium (Ti) plates and their differentiation into more mature cells on the plates were stimulated by treatment of the plates with glow discharge plasma (GDP). However, the mechanisms of these processes have not yet been identified. In this study, the adhesion and differentiation mechanism of osteoblast-like cells to Ti with and without GDP were investigated.* ***Materials and Methods:*** *The adhesion and differentiation mechanism of MC3T3-E1 osteoblast-like cells to Ti, with and without GDP, were investigated by cultivation in serum-free medium and use of a competitive inhibition test to examine the influence of extracellular matrix proteins contained in the serum and to identify cell binding proteins. In addition, the amount of fibronectin adsorption on each Ti plate was investigated by enzyme-linked immunosorbent assay and fluorescein isothiocyanate labeling. Furthermore, the stress fiber formation and morphology of cells on each plate were evaluated microscopically.* ***Results:*** *Adherent cells on Ti plates, with and without GDP, were significantly reduced in serum-free conditions and the presence of RGDS (Arg-Gly-Asp-Ser) peptides. Fibronectin adsorption on titanium plates was increased by GDP. Furthermore, stress fiber formation of cells was extremely progressive on the Ti plates treated with GDP and was not observed on the cells inhibited by RGDS peptide.* ***Discussion:*** *These results suggest that RGDS containing serum proteins have a major role in regulating specific adhesion of cells to Ti, and GDP promoted cell adhesion and differentiation on Ti by increasing the adsorption of proteins.* ***Conclusion:*** *According to this study, the adhesion and differentiation mechanism of osteoblast-like cells to Ti, with and without GDP, can be obtained.* (Int J Oral Maxillofac Implants 2002;17:771–777)

Key words: *cell adhesion, cell differentiation, glow discharge plasma treatment, RGDS peptide*

Titanium (Ti) and its alloys have been widely available as implant materials in contact with bone because of the achievement of osseointegration.[1–3] It is obvious that the surface structure of an implant material that interfaces with bone has an important role in osseointegration. Many researchers have reported the effect of surface modifications of Ti on its biocompatibility in vitro and in vivo.[4–9] Nevertheless, the mechanism of its biocompatibility has not been completely explained.

Glow discharge plasma (GDP) treatment is a method for cleaning, surface-activating, and in some cases sterilizing materials by using low-temperature gas plasma.[10,11] It can also be effective in the surface treatment of dental implants.[12,13] GDP has been shown to improve initial cell adhesion and differentiation of osteoblast-like cells to Ti plates using experimentally developed equipment.[13] The surface of the specimen inside a vacuum chamber was cleaned by colliding ions and electrons in low-temperature gas plasma that produced excellent

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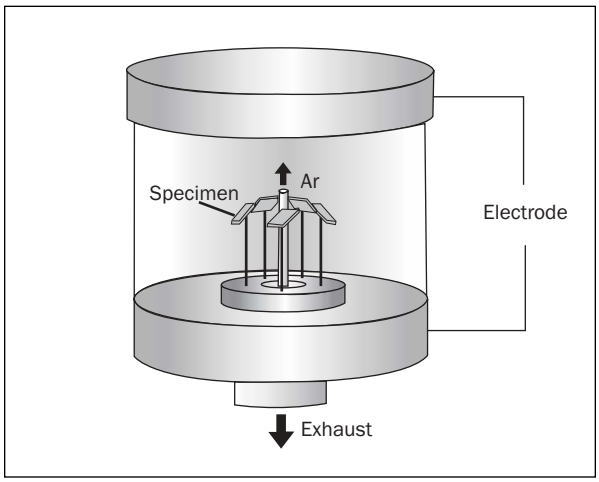


Fig 1 The experimentally developed GDP device.

wettability of the surface without changing the minute structure of the specimen. Swart and coworkers also reported that GDP for between 60 and 120 seconds improved the initial adhesion of osteoblast-like cells to Ti plates because of increasing wettability.[14] However, it remains unclear why GDP promoted not only cell adhesion but also cell growth and differentiation.

It is known that the adhesion of epithelial cells and fibroblasts to the substrate strongly depends on fibronectin.[15–18] In addition, many tissue culture cells need the formation of focal adhesion initiated by the specific bindings of extracellular matrix (ECM) proteins and receptors for growing and differentiating.[19–22] Cell-binding domains such as RGDS (Arg-Gly-Asp-Ser) peptide identified in many proteins have a major role in the formation of focal adhesion.[23–26]

It has been hypothesized that GDP might influence cell-specific adhesion and differentiation by inducing the RGDS-containing serum proteins such as fibronectin to adsorb to the surface. In this study, the adhesion mechanism of MC3T3-E1 osteoblast-like cells to Ti plates treated by GDP was investigated by cultivation in a serum-free medium and a competitive inhibition test. Fibronectin adsorption on each Ti plate was investigated by an enzyme-linked immunosorbent assay (ELISA) and fluorescein isothiocyanate (FITC) labeling. The stress fiber formation and morphology of cells were evaluated microscopically.

MATERIALS AND METHODS

Preparation of Specimens

Japanese Industrial Standard (JIS) grade 2 Ti (KS-50; Kobe Steel, Kobe, Japan) was used as the material. The surface of the Ti plates with a dimension of 10×10×1.0 mm was ground gradually with waterproof polishing papers from #500 to #1,200 grit under running water and then polished with alumina particles with an average diameter of 0.3 μm.

Cleaning of Specimens

The prepared specimens were cleaned ultrasonically in acetone, detergent solutions (7 times, ICN, Biomedicals, Aurora, Ohio), and pure distilled water for 15 minutes. Then the specimens were dried and stored for 24 hours in a desiccator that maintained a humidity of 50% at 23°C.

Glow Discharge Plasma Treatment

Figure 1 shows the GDP device used in this study. GDP was performed by the method introduced in a previous study.[12] After the specimens were fixed to the holder connected with an anode in the chamber under argon gas replacement, electric glow discharging was processed under a vacuous degree of 8×10^{-3} Torr for 1 minute. Specimens were removed from the chamber on a clean bench. Then specimens were supplied for the cell culture study.

Cell Culture

An osteoblastic cell line, MC3T3-E1, established from newborn mouse calvaria was obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in α minimal essential medium (αMEM) (Gibco, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Gibco) and 1% antibiotic (penicillin, Gibco) under a 5% CO_2 atmosphere at 37°C. Cells were suspended in serum-free medium at 1×10^5 cells/mL and used for cell adhesion experiments.

Cell Adhesion

Serum-free Culture. Titanium specimens were placed in 24 well culture plates with both 1 mL serum-free αMEM and αMEM containing 20% FBS (Gibco). Then 1 mL floating cells were plated onto each of the specimens with and without serum and incubated at 37°C and 5% CO_2 for 1 hour to study the influence of ECM protein contained in serum.

Cell Inhibition Test. Ti specimens were placed in 24 well culture plates with 1 mL αMEM containing 20% FBS. For the competitive inhibition test, cells were incubated for 2 hours under 3 sets of conditions: *(1)* αMEM containing 50 μg/mL RGDS (Sigma, Tokyo, Japan); *(2)* 50 μg/mL RGES

(Arg-Gly-Gly-Ser) (Sigma), which is known to have no effect on cell adhesion; and *(3)* serum-free αMEM without peptide as a control. One milliliter of floating cells from each set of conditions were then plated onto each of the specimens with and without GDP and incubated at 37°C and 5% CO_2 for 1 hour.

Cell Count. A cell-counting kit (Dojindo, Kumamoto, Japan) was used for the measurement of cell adhesion. After incubation, each specimen was moved to another well and was washed by phosphate-buffered saline (PBS) (-) (without Ca++ and Mg++) (Gibco) 3 times to remove nonadherent cells. Adherent cells were mixed with 1 mL of medium and reagent solution 100 μL. After 1 hour of incubation, absorbance at 450 nm was measured. The number of adherent cells was calculated from the activity of the original cell suspension.

Fibronectin Measurement

ELISA. The ELISA was used to measure the adhesion quantity of adsorbed fibronectin in each specimen. The specimens treated with and without GDP were soaked in FBS and 10% FBS containing αMEM at 37°C for 30 minutes. After incubation, the specimens were washed with PBS(-) 3 times and fixed with 5% bovine serum albumin (BSA) (Sigma) for 1 hour. After washing with PBS(-) 3 times, they were immersed in 2,000 times dilution monoclonal anti-human fibronectin (TaKaRa, Shiga, Japan) for 2 hours. After washing with PBS(-) 3 times, they were immersed in 4,000 times dilution alkaline phosphatase conjugate anti-mouse–gamma g immunoglobulin (Sigma) for 30 minutes. After washing with PBS(-) 3 times, they were immersed in p-nitrophenyl phosphate (pNPP) (Sigma) for 30 minutes. Finally, after 3 mol/L sodium hydroxide was added, absorbance at 405 nm was measured in the specimens. The amount of fibronectin was calculated from the absorbance of bovine plasma fibronectin (Sigma).

FITC Labeling. FITC labeling was used for the observation of adsorbed fibronectin on each specimen. The specimens treated with and without GDP were soaked in 10% FBS containing αMEM at 37°C for 30 minutes. After incubation, the specimens were washed with PBS(-) 3 times and fixed with 5% BSA (Sigma) for 1 hour. After washing with PBS(-) 3 times, they were immersed in 2,000 times dilution monoclonal anti-human fibronectin (TaKaRa) for 2 hours. After washing with PBS(-) 3 times, they were immersed in 32 times dilution FITC conjugated anti-mouse IgG (Sigma) for 1 hour at room temperature. After washing with PBS(-) 3 times, adsorbed fibronectin on each specimen was observed under a fluorescence microscope (E-600, Nikon, Tokyo, Japan).

Stress Fiber Formation and Cell Morphology

Titanium specimens were placed in 24 well culture plates with 1 mL αMEM containing 20% FBS. Cells were incubated in αMEM containing 50 μg/mL RGDS and serum-free αMEM without peptide for 2 hours. One milliliter floating cells incubated in serum free αMEM were then plated onto each of the specimens with and without GDP. One milliliter floating cells incubated in αMEM containing 50 μg/mL RGDS were plated onto specimens without GDP. Then the specimens were incubated at 37°C and 5% CO_2 for 1 hour.

Adherent cells on each specimen after 1 hour cultivation were dehydrated after washing with PBS(-). The cells were fixed with 3.7% formaldehyde in PBS(-) and permeated by treatment with 0.1% Triton X-100 in PBS(-) for 1 minute. The cells were then incubated for 3 hours with rhodamine-conjugated phalloidin solution. After the cells were washed with water, stress fiber formation and cell morphology were observed under a fluorescence microscope.

RESULTS

Cell Adhesion

Serum-free Culture. The initial cell adhesion of specimens treated with GDP was higher than that of the untreated specimens. In the absence of serum, cell adhesion was reduced significantly, both with and without GDP (Fig 2).

Cell Inhibition Test. After 1 hour of cultivation, the cell adhesion of specimens treated with GDP was higher than that of specimens without GDP treatment. Cell adhesion was not inhibited entirely by RGES peptide, both with and without GDP. On the other hand, cell adhesion was inhibited by RGDS peptide, both with and without GDP but especially without GDP, in which case it was almost completely inhibited (Fig 3).

Fibronectin Adsorption

After 1 hour of incubation, the adsorbed fibronectin in each specimen was evaluated. Specimens with GDP showed higher adsorption of fibronectin than those without GDP, both in αMEM containing 10% FBS and in FBS (Fig 4a) in the ELISA test. In the FITC labeling test, a high amount of adsorbed fibronectin was observed on the specimens with GDP compared with those without GDP (Fig 4b).

Stress Fiber Formation and Cell Morphology

After 1 hour of incubation, adherent cells on specimens on which GDP was deposited already had begun to form stress fibers and widely extended cytoplasm (Fig

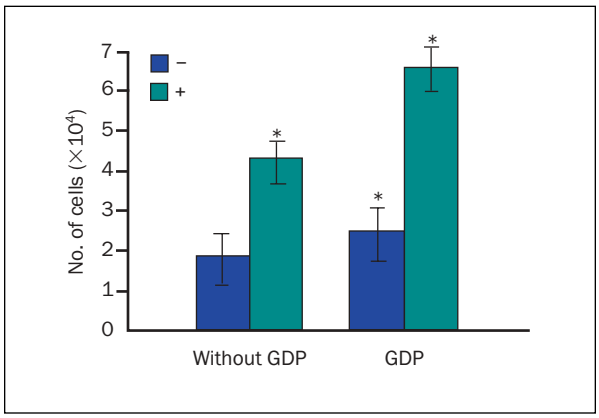


Fig 2 Number of adherent cells on Ti plates after 1 hour of cultivation in the serum-free culture. − indicates absence of serum; + indicates presence of serum. Data represent means of 10 samples. *$P < .05$ (*t* test).

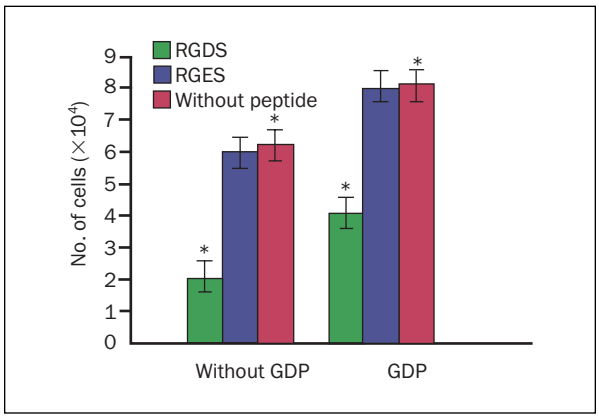


Fig 3 Cell inhibition test. Number of adherent cells on Ti plates after 1 hour of cultivation in the cell inhibition test. RGES was used as a negative control. Data represent means of 10 samples. *$P < .05$ (*t* test). RGES was not compared.

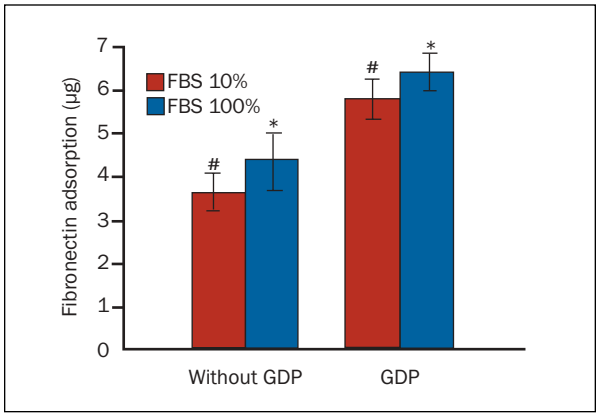
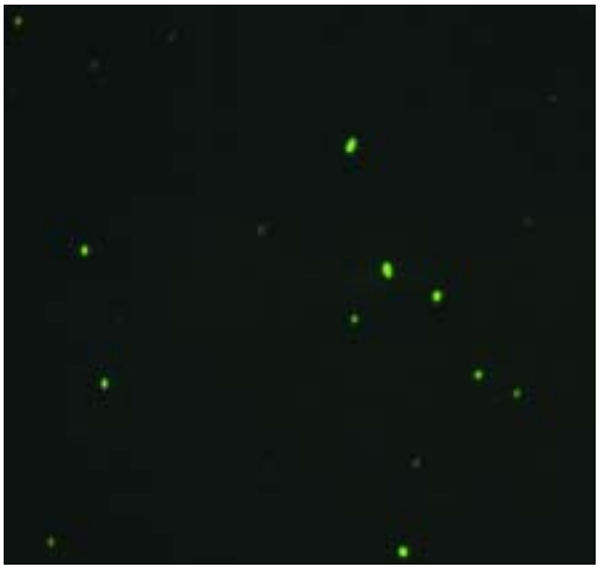
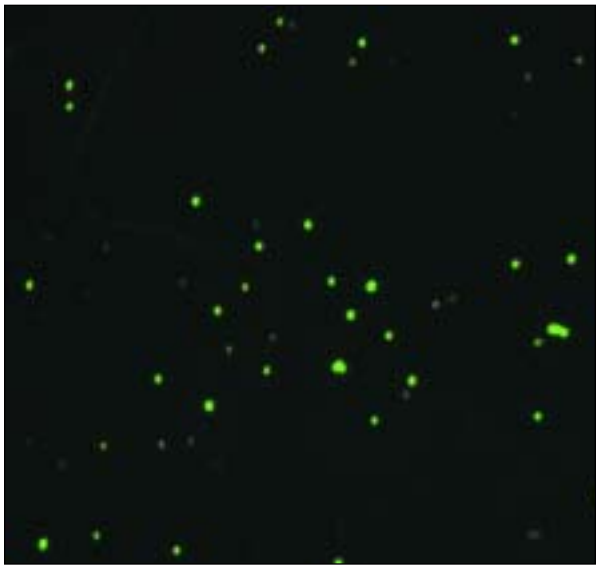


Fig 4a *(Left)* Fibronectin adsorption on Ti plate. Data represent means of 10 specimens. *$P < .05$; #$P < .05$ (*t* test).

Fig 4b *(Below)* Fibronectin adsorption on Ti plate observed with FITC labeling (*left,* without GDP; *right,* with GDP) (magnification ×200).





5). On the other hand, stress fiber formation of cells on the specimens without GDP was poor, and cells adhered loosely to the surface (Fig 6). Cell adhesion and extension, especially by stress fiber formation, were inhibited completely by the RGDS peptide (Fig 7).

DISCUSSION

The present study indicated that cells were not able to adhere sufficiently to Ti, both with and without GDP, in the absence of serum. Gronowicz and McCarthy

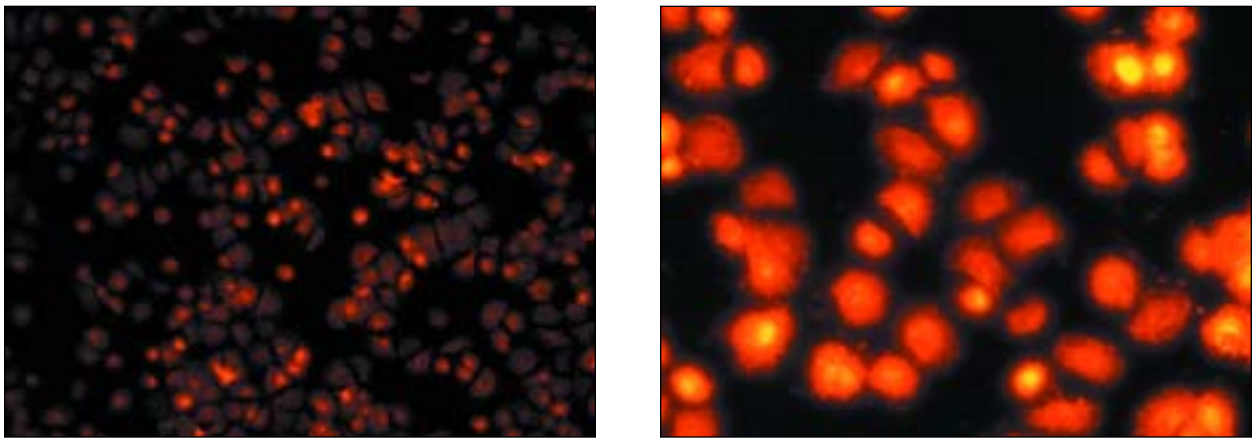


Fig 5 Stress fiber formation and morphology of cells on Ti plate with GDP (low magnification ×200; high magnification ×800).

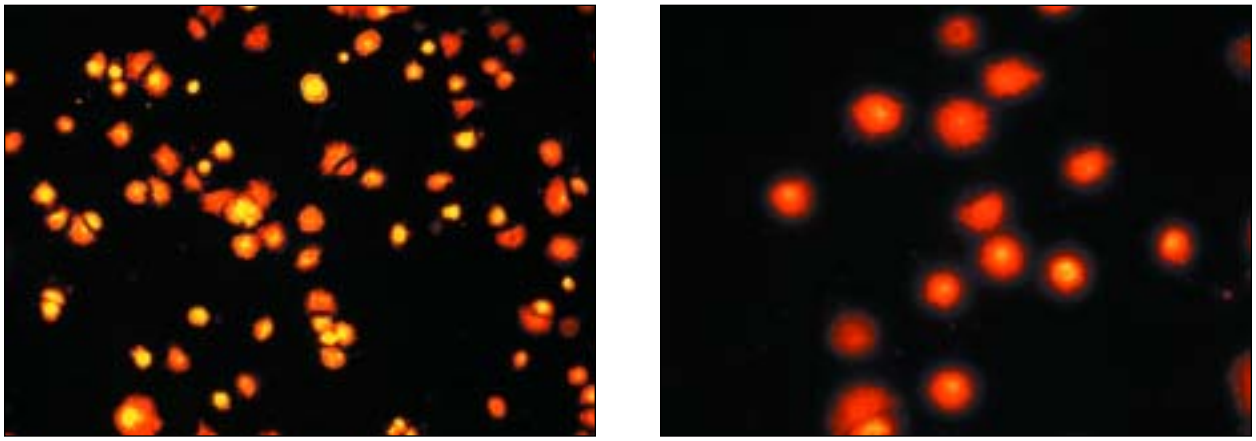


Fig 6 Stress fiber formation and morphology of cells on Ti plate without GDP (low magnification ×200; high magnification ×800).

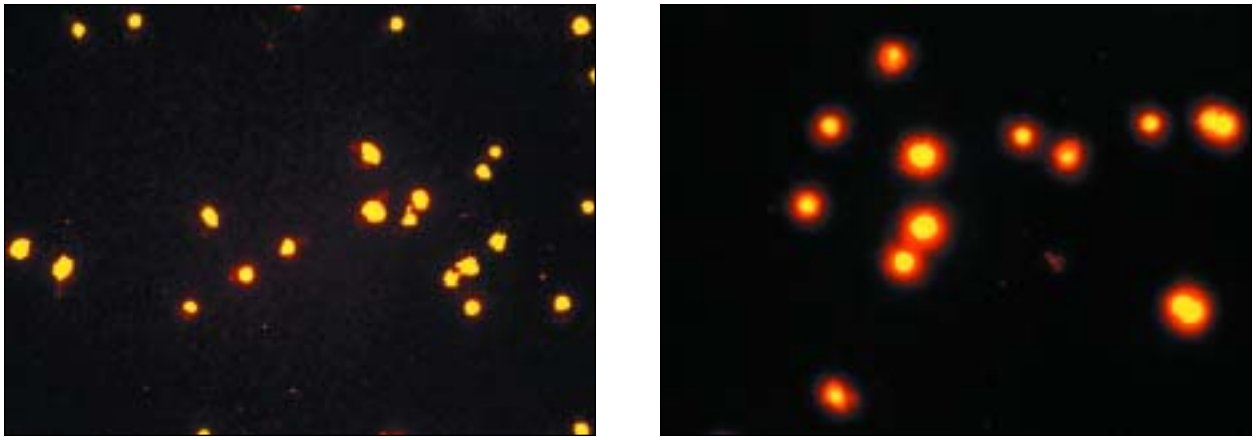


Fig 7 Stress fiber formation and morphology of cells on Ti plate. Cells were pre-incubated in the serum-free medium containing RGDS peptide (low magnification ×200; high magnification ×800).

suggested that serum has no effect on cell adhesion and osteoblast-like cells are able to bind directly to a Ti surface.[2] Since a small number of cells were found to adhere to Ti in the absence of serum, the possibility of direct adhesion of cells to the Ti surface cannot be ruled out. However, since cell adhesion is significantly reduced in the absence of serum, it is obvious that serum proteins play a major role in cell adhesion.

In addition, Brighton and Albelda demonstrated that bone cells that were adhered to a variety of

ECM proteins containing RGD sequences were capable of recognition by integrins of cells, including collagen types I and IV, fibronectin, vitronectin, fibrinogen, and laminin.[27] In the present competitive inhibition test, cell adhesion was not inhibited by RGES peptide, which is known to have no effect on cell adhesion. Since RGES exhibited no toxicity at the concentration used in this study, it was possible to confirm that the peptide concentration in this study was appropriate. On the other hand, cell adhesion was inhibited on Ti, both with and without GDP, by RGDS peptide, because the cells connected to RGDS selectively before plating with their integrin during preparation and could not combine with the protein adsorbed on the Ti after plating.

Fibronectin adsorption on Ti was significantly increased by GDP. This was because GDP cleaned the surface and increased its wettability. Cell adhesion to Ti with GDP apparently depended on the adsorption of ECM proteins containing RGDS peptide, such as fibronectin, on the Ti surface.

In this study, high stress fiber formation of cells was observed on Ti treated with GDP. Cells seemed to adhere strongly to the large amount of protein induced by GDP. On the other hand, cell adhesion and extension on Ti, especially stress fiber formation, were completely inhibited when cells were treated with RGDS peptide.

The initial adhesion of cells induces stress fiber formation, phosphorylation of focal adhesion kinase, and activation of other intracellular signal transduction molecules that affect cell proliferation, differentiation, and apoptosis.[28–32] These results suggest that cell-RGDS sequence binding is important for the differentiation of adherent cells on Ti, both with and without GDP treatment.

CONCLUSION

Cell adhesion and differentiation on Ti depend strongly on the protein adhesion containing RGDS sequence, such as fibronectin, and are promoted by GDP. Further analysis to identify the detailed interaction between the protein and the surface of Ti treated by GDP is needed.

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REFERENCES

1. Albrektsson T, Brånemark P-I, Hansson HA, Lindström 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. Gronowicz G, McCarthy MB. Response of human osteoblasts to implant materials: Integrin-mediated adhesion. J Orthop Res 1996;14:878–887.
3. Howlett CR, Evans MDM, Walsh WR, Johnson G, Steele JG. Mechanisms of initial attachment of cell derived from human bone to commonly used prosthetic materials during cell culture. Biomaterials 1994;15:213–225.
4. Puleo DA, Nanci A. Understanding and controlling the bone-implant interface. Biomaterials 1999;20:2311–2321.
5. Pilliar RM. Overview of surface variability of metallic endosseous dental implants: Textured and porous surface-structured designs. Implant Dent 1998;7:305–314.
6. Norton MR. Marginal bone levels at single tooth implants with a conical fixture design. The influence of surface macro- and microstructure. Clin Oral Implants Res 1998;9: 91–99.
7. Nanci A, Wuest JD, Peru L, et al. Chemical modification of titanium surfaces for covalent attachment of biological molecules. J Biomed Mater Res 1998;40:324–335.
8. Aronsson BO, Lausmaa J, Kasemo B. Glow discharge plasma treatment for surface cleaning and modification of metallic biomaterials. J Biomed Mater Res 1997;35:49–73.
9. Filiaggi MJ, Pilliar RM, Abdulla D. Evaluating sol-gel ceramic thin films for metal implant applications. II. Adhesion and fatigue properties of zirconia films on Ti-6AI-4V. J Biomed Mater Res 1996;33:239–256.
10. Baier RE, DePalma VA. Electrodeless Glow Discharge Cleaning and Activation of High-Energy Substrates to Insure their Freedom from Organic Contamination and Their Receptivity for Adhesives and Coatings. Buffalo, NY: Cornell Aeronautical Laboratory, 1970:176.
11. Aronsson BO, Lausmaa J, Kasemo B. Glow discharge plasma treatment for surface cleaning and modification of metallic biomaterials. J Biomed Mater Res 1997;35:49–73.
12. Shibata Y. Glow discharge treatment on titanium plate [in Japanese]. J Jpn Soc Dent Mater Device 2000;19:84–91.
13. Shibata Y, Fujimori S. Glow discharge treatment is effective for cell proliferation and differentiation of osteoblast-like cells on titanium surface [in Japanese]. J Showa Univ Dent Soc 2000;20:24–29.
14. Swart KM, Keller JC, Wightman JP, Draughn RA, Stanford CM, Michaels CM. Short-term plasma-cleaning treatments enhance in vitro osteoblast attachment to titanium. J Oral Implantol 1992;18:130–137.
15. Sanders MA, Basson MD. Collagen IV-dependent ERK activation in human Caco-2 intestinal epithelial cells requires focal adhesion kinase. J Biol Chem 2000;275:38040–38047.
16. McMillan R, Meeks B, Bensebaa F, Deslandes Y, Sheardown H. Cell adhesion peptide modification of gold-coated polyurethanes for vascular endothelial cell adhesion. J Biomed Mater Res 2001;54:272–283.
17. Webb K, Caldwell K, Tresco PA. Fibronectin immobilized by a novel surface treatment regulates fibroblast attachment and spreading. Crit Rev Biomed Eng 2000;28:203–208.
18. McClary KB, Ugarova T, Grainger DW. Modulating fibroblast adhesion, spreading, and proliferation using self-assembled monolayer films of alkylthiolates on gold. J Biomed Mater Res 2000;50:428–439.

19. Cowles EA, Brailey LL, Gronowicz GA. Integrin-mediated signaling regulates AP-1 transcription factors and proliferation in osteoblasts. J Biomed Mater Res 2000;52:725–737.
20. Rhee S, Lee KH, Kim D, Kwon YK, Kang MS, Kwon H. Sustained formation of focal adhesions with paxillin in morphological differentiation of PC12 cells. Mol Cells 2000;10: 169–179.
21. Levy L, Broad S, Diekmann D, Evans RD, Watt FM. Beta-1 integrins regulate keratinocyte adhesion and differentiation by distinct mechanisms. Mol Biol Cell 2000;11:453–466.
22. Cao Z, Huang K, Horwitz AF. Identification of a domain on the integrin alpha-5 subunit implicated in cell spreading and signaling. J Biol Chem 1998;273:31670–31679.
23. Singer II, Kawka DW, Scott S, Mumford RA, Lark MW. The fibronectin cell attachment sequence Arg-Gly-Asp-Ser promotes focal contact formation during early fibroblast attachment and spreading. J Cell Biol 1987;104:573–584.
24. Straus AH, Carter WG, Wayner EA, Hakomori S. Mechanism of fibronectin-mediated cell migration: Dependence or independence of cell migration susceptibility on RGDS-directed receptor (integrin). Exp Cell Res 1989;183: 126–139.
25. Maeda T, Oyama R, Ichihara-Tanaka K, et al. A novel cell adhesive protein engineered by insertion of the Arg-Gly-Asp-Ser tetrapeptide. J Biol Chem 1989;264:15165–15168.
26. Rezania A, Healy KE. The effects of peptide surface density on mineralization of a matrix deposited by osteogenic cells. J Biomed Mater Res 2000;15:595–600.
27. Brighton CT, Albelda SM. Identification of integrin cell-substratum adhesion receptor on cultured rat bone cells. J Orthop Res 1992;10:766–773.
28. Moro L, Vnturio M, Bozzo C, et al. Integrins induce activation of EGF receptor: Role in MAP kinase induction and adhesion dependent cell survival. EMBO J 1998;17:6622–6632.
29. Bellis SL, Perrotta JA, Cutis MS, Turner CE. Adhesion of fibroblasts to fibronectin stimulates bone serine and tyrosine phosphorylation of paxillin. Biochem J 1997;325:375–381.
30. Moursi AM, Globus RK, Damsky CH. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. J Cell Sci 1997;110: 2187–2196.
31. Green J, Schotland S, Stauber DJ, Kleeman CR, Clemens SL. Cell-matrix interaction in bone: Type I collagen modulates signal transduction in osteoblast-like cells. Am J Physiol 1995;268:1090–1103.
32. Lynch MP, Capparelli C, Stein JL, Stein GS, Lian JB. Apoptosis during bone-like tissue development in vitro. J Cell Biochem 1998;68:31–49.