Effect of microstructure of titanium surface on the behaviour of osteogenic cell line MC3T3-E1

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Three different microstructures were obtained on a titanium surface via immersion in HCl, H_3PO_4, or mixed acid of HNO_3 and HF (HNO_3/HF) solution. The microstructure and R_{max} of the acid-treated surfaces were dependent on the acid type and immersion conditions. The growth rate of the osteogenic cell line MC3T3-E1 on each acid-treated sample, which was measured using MTT-formazan assay, was significantly higher than that of the standard which was ground with #400 SiC grit paper. Moreover, both the H_3PO_4 treated sample and the HNO_3/HF-treated surface showed a tendency to enhance the alkaline phosphatase activity of MC3T3-E1 cells, which were grown on each acid-treated surface. These results suggest that the acid treatment of titanium is effective for the improvement of its osteocompatibility. © *1998 Chapman & Hall*

1. Introduction

Titanium and titanium alloy have recently been used as implant materials instead of SUS 316 or Co–Cr alloy because of their excellent tissue compatibility, but further tissue compatibility is still required. As one of the tissue compatibility factors of implant, the surface property of the implant material is recognized as a major important factor in the initial stage of contacting the surrounding bone tissue. Thus, in order to improve the osteocompatibility of titanium implant material, surface treatments such as bioactive material coating, surface roughning and other methods have been developed and applied [1–7]. The microstructure of the titanium metal surface itself is also considered to be one of the important factors affecting its compatibility with bone tissue [8]. However, only physical or mechanical methods such as plasma coating, anodic oxidation, and wire-type electric discharge machining, have been introduced to modify the surface morphology, and it has been also suggested that these treatments accelerate the attachment of bone tissue and its ingrowth into the modified surface [8]. On the other hand, chemical treatment is aimed specifically to obtain better affinity by modifying the surface chemical conditions, because bone attachment with the implant is considered to be regulated and to result from the chemical reaction with plasma and the implant surface.

It was found that chemical treatment can also modify the surface of titanium by immersion in an acid solution, and varied microstructures are obtained depending on the kind of acid and treating conditions. In this study, three types of surface were prepared by treating with different acids, and the effect of each acid-treated surface on the growth of osteogenic cells was evaluated.

2. Materials and methods

2.1. Samples

Pure titanium metal (KS-40, Kobe Steel Ltd, Japan) plates, 10 mm × 10 mm × 1.5 mm in size, were used. After grinding the titanium plates with #400 SiC grit paper, they were immersed in HCl, H_3PO_4, or a mixed acid of 10% HNO_3 and 5% HF. The surface structure of each specimen was observed with field-emission type scanning electron microscopy (FE-SEM, Hitachi S-4000, Japan) and thin-film X-ray diffraction (XRD) was carried out using a Rint 1500 (Rigaku-Denki, Japan). Titanium plates, ground with #400 SiC grit paper, were used as the standard. All specimens were sterilized at 180 °C for 2 h under dry conditions.

2.2. Cell line and culture

The osteogenic cell line MC3T3-E1 [9–11] was obtained from Riken Gene Bank (Japan), and cultured in alpha modification of Eagle's minimum essential medium (αMEM, Gibco BRL, USA) containing 10% foetal bovine serum, 100 units/ml penicillin, and

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100 μg/ml streptomycin under a humidified 5% CO_2–air atmosphere at 37 °C. Cells were inoculated on to each specimen (plate size 10 mm × 10 mm × 1.5 mm) at 10 000 cells/plate in 24-well tissue culture plates (no. 3047, Falcon, USA), and overlaid with 1.5 ml of culture medium. The medium was exchanged every 3 d. Cultured cells were detached by trypsinization and suspended in new culture medium with foetal calf serum before each experiment.

2.3. Cell growth rate

The cell growth rate was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) formazan assay [12]. It was preliminarily confirmed that the MTT-formazan formation was proportional to the number of living cells in the range from 10^4–10^6 cells; $1OD_{405}$ of MTT-formazan in dimethyl sulphoxide (DMSO) corresponded to 0.75×10^4 cells of MC3T3-E1.

The titanium plates on which the cells grew were transferred into a new 24-well tissue culture plate after 3,7, and 14 d incubation. Each titanium plate was washed twice with 1.5 ml phosphate buffer saline (PBS) and then 1.0 ml αMEM medium and 100 μl 5 mg/ml MTT in PBS were added. After 4 h incubation at 37 °C, the medium was discarded from each well, and 500 μl 100% dimethyl sulphoxide (DMSO) was added to dissolve the MTT-formazan produced. Absorbance at 570 nm of MTT-formazan in DMSO was measured by Beckman DU7400 spectrophotometer.

2.4. Cell attachment

After cells were inoculated and incubated for 4 h at 37 °C, the media were discarded and each titanium plate was washed twice with PBS at 37 °C. The cells which were attached on sample titanium plates were measured by MTT-formazan assay [12].

2.5. Alkaline phosphatase activity

Alkaline phosphatase (ALPase) activity was determined according to the method of Lowry *et al.* [13], with r-nitrophenyl phosphate as a substrate. After cells were incubated on titanium plates for 3, 7, and 14 d, titanium plates were transferred into a new 24-well tissue culture plate and washed twice with PBS at 37 °C. Cells were digested with 500 μl digestion buffer (50 mmol carbonate buffer (pH 10.15), 2 mmol $MgCl_2$ 1% triton X-100) by settling on ice for 30 min. The 100 μl cell extract was mixed with 0.5 ml substrate buffer (100 mmol carbonate buffer (pH 10.15), 2 mmol $MgCl_2$, 10 mmol r-nitrophenyl phosphate, 600 mmol 2-amino-2-methyl-1-propanol) and the mixture was incubated at 37 °C for 30 min or 1 h. The reaction was terminated by addition of 0.4 ml 0.25 N NaOH, and the absorbance at 405 nm was measured. The protein content of the cell extract was simultaneously determined using a Micro BCA Protein Assay Kit (Pierce, USA).

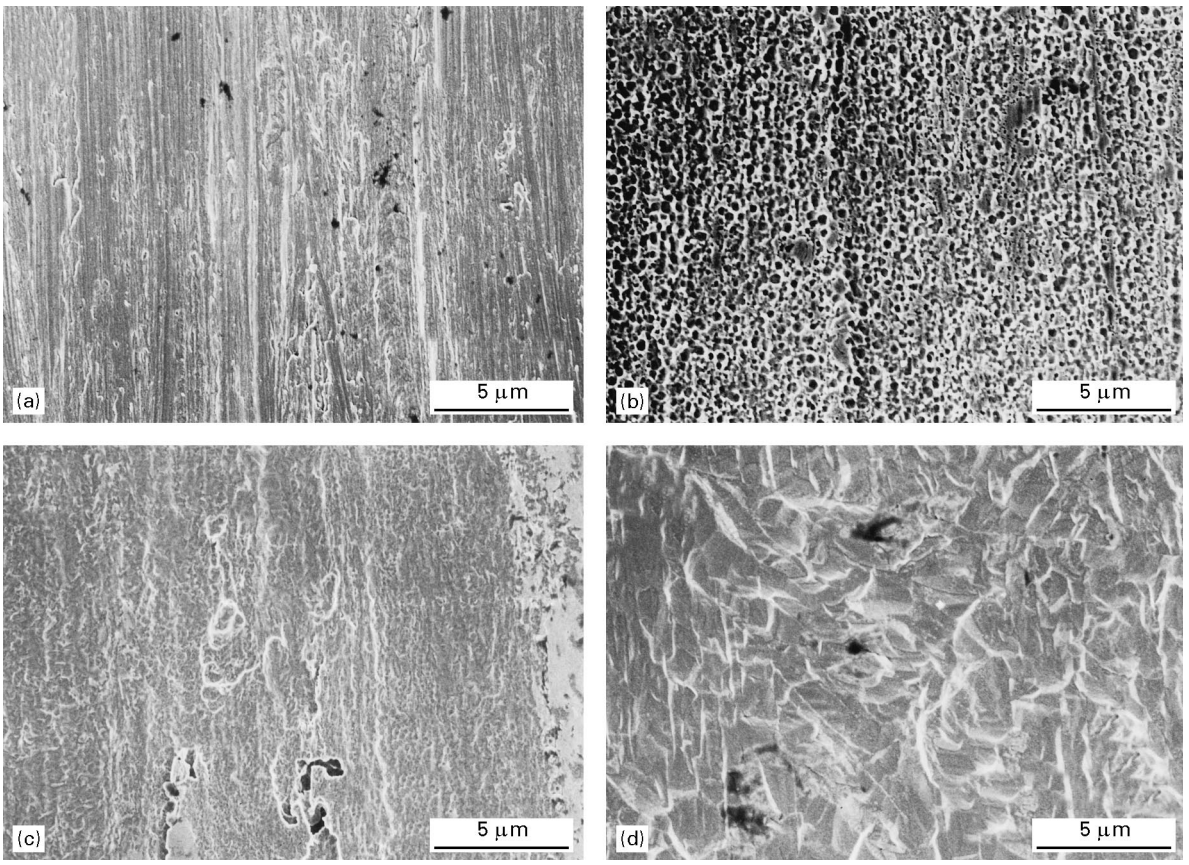

Figure 1 FE-SEM photographs of (a) the standard surface, (b) the HCl-treated surface, (c) the H_3PO_4-treated surface and (d) the HNO_3/HF-treated surface.

3. Results

3.1. Microstructure of the acid-treated titanium surfaces

The microstructure of the titanium surfaces formed via acid treatment was observed with FE-SEM. The microstructure of the acid-treated surface was dependent on the kind of acid and the immersion conditions, and a typical micrograph of each is as shown in Fig. 1. The surface of the HCl-treated sample shows a sponge-like structure with a pore size ranging from 0.1–0.5 μm diameter. The surface of the H_3PO_4-treated sample shows the same surface structure as the #400 SiC ground sample surface, referred to as the standard, according to the FE-SEM observation, but the surface roughness of the H_3PO_4-treated specimen is higher than that of the standard, as shown in Table I. The surface roughness of the HCl-treated sample and the H_3PO_4-treated sample showed the same level, but that of the HNO_3/HF-treated surface was almost five times higher than that of the other acid-treated surfaces. As shown in Fig. 2, the thin-film X-ray diffraction pattern does not show any differences between the standard and each acid-treated specimen.

3.2. Initial attachment and proliferation of MC3T3-E1 cells on acid-treated surfaces

The amount of the initial attached cells on titanium plates was measured as the amount of MTT-formazan formed by the active MC3T3-E1 cells on them after a total of 8 h incubation (4 h incubation in αMEM medium and 4 h incubation in αMEM with MTT medium). The amount of initial attached cells on each acid treated specimen was almost equal to that on the standard. This result indicated that the microstructures formed via acid treatment did not affect the initial attachment of MC3T3-E1 cells to the titanium plates.

The growth rate of MC3T3-E1 cells on each plate is shown in Fig. 3. Even after 3 d incubation, the growth rate of MC3T3-E1 cells on every acid-treated specimen was higher than that on the standard surface. This tendency was also observed at least until 14 d incubation. The highest cell growth at 14 d incubation was obtained on the HNO_3/HF-treated surface, it being almost 1.5 times higher than that on the standard.

TABLE I Surface roughness (R_{max}) of the specimens

Sample	R_{max} (μm)
Standard (as-ground)	2.6
HCl-treated	4.8
H_3PO_4-treated	5.7
HNO_3/NF-treated	19.5

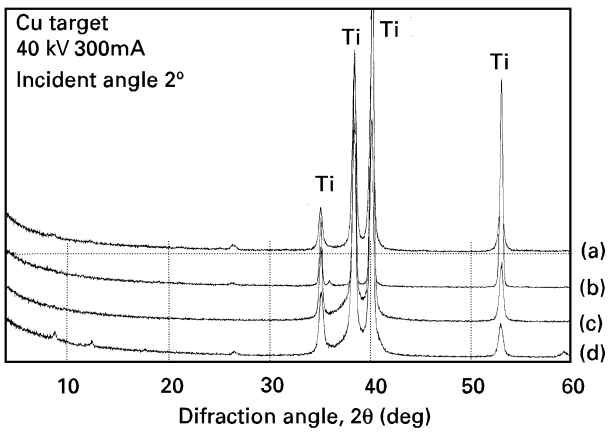

Figure 2 Thin-film X-ray diffraction pattern of the specimens: (a) standard (as-ground); (b) HNO_3/HF-treated; (c) H_3PO_4 treated; (d) HCl-treated.

3.3. ALPase activity of MC3T3-E1 cells grown on acid-treated surface

Fig. 4 shows the ALPase activity of MC3T3-E1 cells grown on the acid-treated surface in reference to the standard surface. After 14 d incubation, the ALPase activity of cells growing on the acid-treated surface was slightly higher than that of cells growing on the standard surface, except for the HCl-treated surface. This suggested that the microstructure introduced via acid treatment may possibly increase the ALPase acitivity of MC3T3-E1 cells, and be influenced by the chemical species of the acid used.

4. Discussion

Surface morphology is considered to be one of the important factors in the behaviour of cells *in vitro*. Miyazaki *et al.* indicated that a titanium surface with 15–20 μm roughness, which had been prepared using a wire-type electric discharge machine (wire-EDM), resulted in an increased growth rate of fibroblasts and epicelial cells [14], and ALPase activity of cultured osteoblastic cells on a titanium plate [15].

In this study, we prepared three different surface morphologies using acid treatment. The microstructure

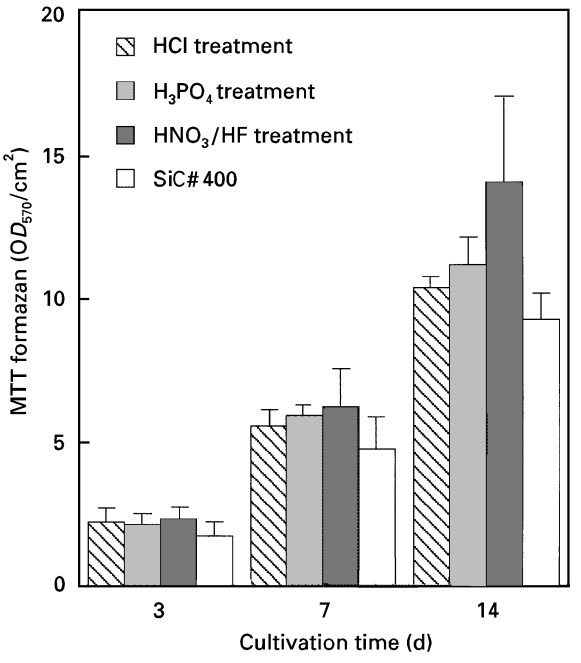

Figure 3 MC3T3-E1 cell growth on the acid-treated titanium plates as a function of cultivation time.

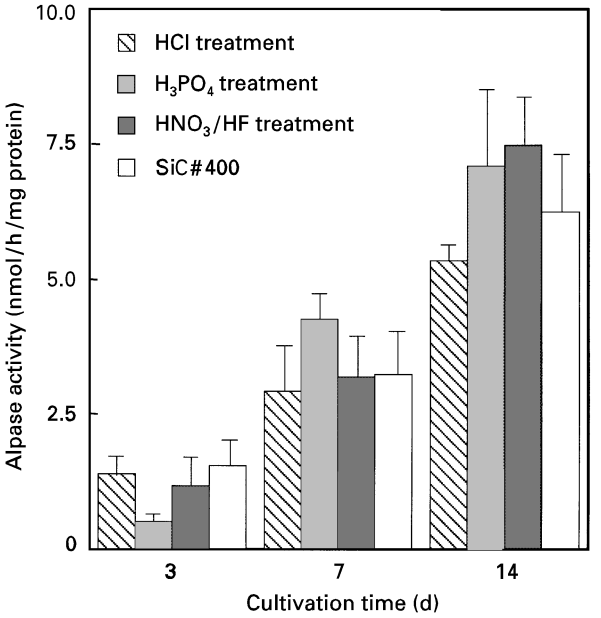

Figure 4 Alkaline phosphatase activity of MC3T3-E1 cells grown on acid-treated titanium plates as a function of cultivation time.

and surface roughness (R_{max}) of each acid-treated titanium plate differed from each other. The HCl-treated sample showed a porous structure with pore size ranging from 0.1–0.5 μm, and the HNO_3/NF-treated titanium plate showed a rough and sharp-edged surface. R_{max} of the HCl, H_3PO_4 and HNO_3/NF treated titanium surfaces were 4.8, 5.7, and 19.5 μm, respectively, whereas the R_{max} of the standard titanium plate, which was ground with #400 SiC grit paper, was 2.6 μm.

The order of effectiveness for the increase in cell growth rate increased from the HNO_3/NF-treated surface, to the H_3PO_4 treated surface, and finally the HCl treated surface. This order corresponded to the R_{max} of the titanium surface, indicating that the roughness of the titanium surface might be one of the key factors of the growth stimulation of MC3T3-E1 cells and showed the same tendency as that reported by Miyazaki *et al.* [15]. However, the effect of the sponge-like structure is not recognized, because the pore size is too small for the cell to grow into and form the osteon [16].

The results of 14 d incubation on each specimen showed that the growth rates were significantly higher than that of the standard. However, it seems that ALPase activity exhibits a tendency to correspond to the cell growth rate, but this is not at a statistically significant level except for the HCl-treated specimen. According to Kodama *et al.* [17], ALPase activity of the MC3T3-E1 cell shows a degree of differentiation into the osteoblast; therefore, it is considered that the osteoblastic differentiation of MC3T3-E1 does not exhibit a remarkably clear difference between them. However, this does explain the reason why the clear difference in AlPase activity between the HCl-treated specimen and H_3PO_4 and HNO_3/HF-treated specimen is not only because of the physical properties of the surface of treated specimen, such as pore size and surface roughness, but also because of the surface chemical influence.

The acid treatment is simpler and easier than the other physical methods to modify the surface morphology of titanium metal, such as wire-EDM, plasma coating, and ion-beam etching, in addition, it is confirmed that the acid treatment is effective for the improvement of the osteogenic cell growth on a titanium plate. It suggests that this method is expected to improve the osteocompatibility of implants made of titanium and titanium alloy.

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References

1. M. SHIRKHANZADEH, *J. Mater. Sci. Lett.* **10** (1991) 1415.
2. M. H. ZABLOTSKY, *Implant Dentistry* **1** (1992) 253.
3. H. ISHIZAWA, M. FUJINO and M. OGINO, *Bioceramics* **5** (1992) 43.
4. A. MORONI, V. CAJA, E. EGGER, V. PEZZUTO and E. Y. CHAO, *Biomaterials* **5** (1992) 299.
5. A. MORONI, V. L. GAJA, E. L. EGGER, L. TRINCHESE and E. Y. S. CHAO, *ibid.* **15** (1994) 926.
6. K. J. J. PAJAMÄKI, T. S. LINDHOLM, Ö. H. ANDERSSON, K. H. KARLSSON, E. VEDEL, A. YLI-URPO and R.- P. HAPPONEN, *J. Mater. Sci. Mater. Med.* **6** (1995) 14.
7. T. KAWAI and N. NISHIMURA, in "Proceeding of International Symposium on Microsystems, Intelligent Materials and Robots" edited by J. Tani and M. Esashi (The Committee of the Seventh Sendai International Symposium Committee, Sendai, 1995) p. 305.
8. D. M. BRUNETTE, *Int. J. Oral Maxillofac. Implants* **3** (1988) 231.
9. H. KODAMA, Y. AMAGAI, S. KASAI and S. YAMAMOTO, *Jpn. J. Oral Biol.* **23** (1981) 899.
10. H. SUDO, H. KODAMA, Y. AMAGAI, Y. YAMAMOTO and S. KASAI, *J. Cell Biol.* **96** (1983) 191.
11. M. KUMEGAWA, M. HIRAMATSU, K. HATAKEYAMA, T. YAJIMA, H. KODAMA, T. OSAKI and K. KURISU, *Calcif. Tissue Int.* **35** (1983) 542.
12. D. A. SCUDIERO, R. H. SHOEMAKER, K. D. PAULL, A. MONKS, S. TIERNEY, T. H. NOFZIGER, M. J. CURRENS, D. SENIFF and M. R. BOYD, *Cancer Res.* **48** (1988) 4827.
13. O. H. LOWRY, N. R. ROBERTS, M. L. WU, W. S. HIXON and E. J. CRAWFORD, *J. Biol. Chem.* **207** (1954) 19.
14. T. MIYAZAKI, S. FUJIMORI, H. ITAHSHI, S. KAKUTA and M. NAGUMO, *J. Dent. Res.* **72** (1993) 390.
15. S. FUJIMORI, H. ITAHSHI and T. MIYAZAKI, in "Proceeding of Transactions of 2nd International Congress on Dental Materials" edited by Y. Tani (The Committee of the 2nd International Congress on Dental Materials, Hawaii, 1993) p. 238.
16. J. J. KLAWITTER, J. G. BAGWELL, A. M. WEINSTEIN, B. W. SAUER and J. R. PRUITT, *J. Biomed. Mater. Res.* **10** (1976) 311.
17. H. KODAMA, Y. AMAGAI, H. SUDO, T. OHNO and K. IIJIMA, in "Cell mediated calcification and matrix vesicles" edited by S. Yousuf (Elsevier Science, 1986) p. 297.

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