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Effects of Ti ions and particles on neutrophil function and morphology

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Abstract

We compared the cytotoxicity of soluble and particulate titanium (Ti), vanadium (V) and nickel (Ni) by biochemical functional analysis and by microscopic morphology with micro-area elemental analysis in vitro using human neutrophils as probes and in vivo in animals. The biochemical analyses of LDH, superoxide anion, TNF-α and scanning electron microscopy (SEM) showed that Ni in solution destroys the cell membrane of neutrophils, whereas Ti and V in solution stimulate neutrophils and increase the quantity of released superoxide anions. Fine Ti particles (1–3 μm), which smaller than neutrophils (about 5 μm), were phagocytized by the cells and the results were similar in vivo. These results showed that the cytotoxic effect of Ti particles is size dependent, and that they must be smaller than that of cells.

The present study demonstrated that the biochemical functional tests are useful for evaluating the biocompatibility of materials.

Keywords: Titanium; Leukocyte; Superoxide; Cytotoxicity

1. Introduction

Metals and alloys are the most generally accepted materials for bone and tooth replacement [1] because of their mechanical and physical properties. However, the potential risk of corrosion and the detrimental consequences of corrosion byproducts over the surrounding tissue [2–4] are issues of clinical importance. The most common metal used in orthopedics and dentistry is titanium (Ti) and its alloys [5–6]. Titanium is highly corrosion resistant at ambient temperature due to a thin and stable protective oxide layer that forms on its surface.

However, Ti ions might slowly diffuse into surrounding tissue where they would be transported into serum and urine [7]. Although bulk Ti is stable and biocompatible in vivo, Dorr et al. measured Ti levels of up to 21 ppm in fibrous membranes encapsulating implants [8]. The influence of released metal ions on mineralization and bone formation has been studied in vitro. Metal ions including Ti can inhibit apatite formation and growth [9], cell proliferation [10] and specific cellular functions such as ALP activity [11], extracellular mineralization [12–14] and bone-released gene expression [15]. Some in vivo studies [16–17] have found Ti pigmentation in soft tissue around Ti implants.

However, little is known about the effect of Ti on cellular function and the relevance between in vivo and in vitro findings. The present study examines the effect of Ti and other metals on the function and morphology the human neutrophils and evaluates its cytotoxicity and biocompatibility in vitro and in vivo. We compared the effects of fine Ti particles and dissolved Ti ions with those of V and Ni ions. Titanium is frequently used in orthopedics and dentistry, while V, which is also used as an alloy for implants (such as Ti–6Al–4V), and Ni are representative metal ions that are cytotoxic.

Shanbhag et al. [18] used macrophages to study the influence of Ti and Ti–6Al–4V particles on NO release from cells. They also reported [19] that interleukin and PGE-2 are released when macrophages coexist with the

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Ti oxide and polystyrene particles. Compared to macrophages, the function of neutrophils is simpler; they play a central role in the initial stage of inflammation in a non-specific manner against foreign bodies. Therefore, we selected human neutrophils to function as probes in this study.

The particles used by Shanbhag et al. were 0.15–1.76 μm in diameter, which was smaller than that of the cells. Here, we used particles that were smaller (1–3 μm) and larger (10 μm) than the neutrophils to determine the relationship between cell and particle size with respect to cytotoxicity.

2. Materials

2.1. Cells

Human peripheral blood was obtained from normal volunteers in our laboratory. Neutrophils were separated from the blood using 6% isotonic sodium chloride containing hydroxyethyl starch and Ficoll-Hypaque.

2.2. Metal samples

Solutions of titanium, vanadium and nickel were prepared from standards for atomic absorption spectrometry (Kanto Chemical, Tokyo, Japan) and the pH of each solution was adjusted using the solution of alkali. The solutions were diluted with Hanks' balanced salt solution (HBSS) to 2 and 10 ppm of metals. Metals were 99.9% pure Ti particles of 1–3 μm (Soekawa Chemicals, Tokyo, Japan) and 10 μm (High Pure Chemicals, Saitama, Japan) and 99.9% pure Ni particles of 0.2 μm in diameter (Soekawa Chemicals, Tokyo, Japan).

3. Methods

3.1. Dissolution test of Ti

Titanium particles (10 and 1–3 μm) were immersed in HBSS for 1 month at 37°C. The supernatant was filtered through a 0.45 μm membrane (ADVATEC, Tokyo, Japan) to remove Ti particles then the Ti concentration was analyzed by inductively coupled plasma (ICP: P-4010 HITACHI, Tokyo, Japan).

3.2. Evaluation of cell disruption

Neutrophils were mixed with the Ti, V and Ni solutions, then incubated at 37°C for 30 min.

3.2.1. Cell survival rate

After samples were stained with Trypan blue, the cell population was counted under an optical microscope (Axioskop; ZEISS, Germany) using Thomas' hemacytometer. The cell survival rate is expressed as mean ± standard deviation ($n = 8$). Data were analyzed by Student's-t test with the level of significance set at 5%.

3.2.2. Measurement of lactate dehydrogenase (LDH)

The LDH values of samples were measured using the Lactate Dehydrogenase CII—Test (Wako Pure Chemical Industries, Osaka, Japan) and by spectrophotometry (HITACHI U-1100, Tokyo, Japan). Values are expressed as mean ± standard deviation ($n = 8$). Data were analyzed by Student's-t test with the level of significance set at 5%.

3.3. Cell function test

3.3.1. Measurement of superoxide anion from neutrophils

Neutrophils were mixed with each metal solution and HBSS containing Ti particles. Superoxide anion production was assayed by measuring the superoxide dismutase-inhibiting reduction of equine ferricytochrome C (550 nm). Samples were incubated at 37°C for 5 min and the reaction was inhibited by adding PMA (139 nM). The values are expressed as mean ± standard deviation ($n = 6$). Data were analyzed by Student's-t test with the level of significance set at 5%.

3.3.2. Measurement of TNF-α

Neutrophils mixed with metal and particle solutions were incubated at 37°C for 30 min. TNF-α in the supernatant was measured using ELISA kits (Endogen, Inc. USA).

3.3.3. SEM observation and EDS elemental analysis

Neutrophils were mixed with 10 ppm Ti, V, Ni solution and HBSS containing 1–3 μm titanium particles, incubated at 37°C for 30 min, then fixed in Karnovskys' solution (pH 7.4), post-fixed in 0.1 mol/l cacodylate buffer and 0.1 M osmium acid for 1 h, dehydrated and coated with Pt–Pd. Morphological changes in the neutrophils were observed by scanning electron microscopy (SEM: HITACHI S-4300, Tokyo, Japan). Elemental analysis and mapping were performed by energy dispersive X-ray spectroscopy (EDS).

3.3.4. Animal experiments

Wistar rats aged between 11 and 12 weeks (weight 350–380 g) were anesthetized with diethyl ether (Wako Pure Chemical Industries, Osaka, Japan), then pentobarbital sodium (30 mg/kg; NEMBUTAL INJECTION, Dainabot, Osaka, Japan) was injected into the abdominal cavity and 1–3 μm Ti particles were inserted into the subcutaneous connective tissue in the abdominal region. The wound was then sutured. The rats were killed 8 weeks after implantation and blocks of connective tissue were resected. After fixation with 10% neutral buffered

formalin, the tissue blocks were conventionally embedded in paraffin, then sectioned and stained with hematoxylin-eosin. The specimens were histopathologically observed using an optical microscope (ZEISS, Axioskop, Germany).

4. Results

4.1. Evaluation of cell disruption

Fig. 1 shows the survival rate of neutrophils in the 2.0 and 10 ppm solutions of Ni, V and Ti. HBSS was the control. Only the Ni solution significantly differed from HBSS; the survival rate was the lowest among the three metal groups. Although the survival rate as a mean value was slightly lower in V than Ti, neither significantly differ from HBSS. The difference between the 2 and 10 ppm solutions was not significant in any group.

Fig. 2 shows the value of LDH in each solution containing neutrophils. Levels of LDH were significantly increased only in the Ni group. The Ti group differed little from the control.

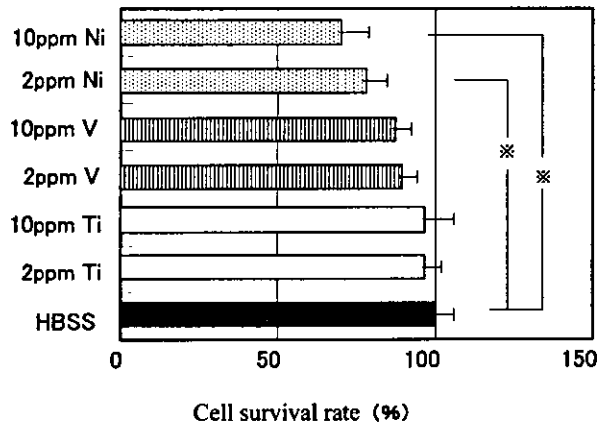

Fig. 1. Survival rate of neutrophils in 2 and 10 ppm solutions of Ni, V and Ti. (※ $p<0.05$) HBSS: Hanks' balanced salt solution.

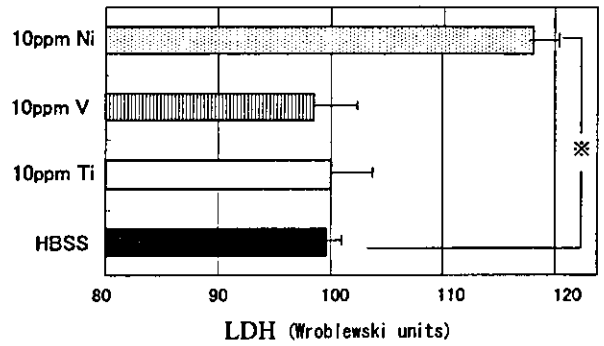

Fig. 2. LDH released from human neutrophils on the exposure to various metal solutions. (※ $p<0.05$).

4.2. Cell function test

Fig. 3 shows the quantity of superoxide anion produced from neutrophils in solutions of three metals. The Ti and V solutions significantly differed from HBSS while the Ni solution did not statistically differ from HBSS.

Fig. 4 shows the amount of TNF-α released from neutrophils in metal solutions and in HBSS containing metal particles. The TNF-α levels increased only in HBSS containing 1–3 μm Ti particles. No significant changes were associated with any of the other metal solutions, or with HBSS containing 10 μm Ti and with 0.1 μm Ni particles.

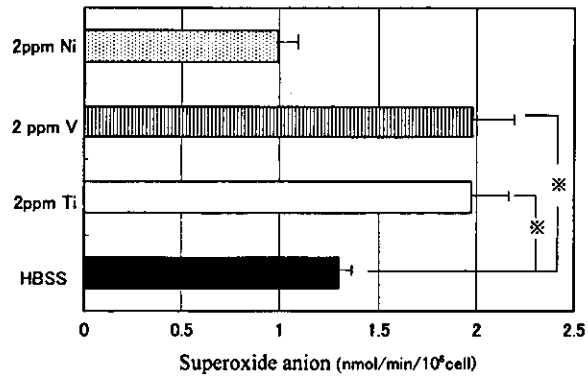

Fig. 3. Superoxide anion production of human neutrophils in 2 ppm solutions of Ni, V and Ti. (※ $p<0.05$).

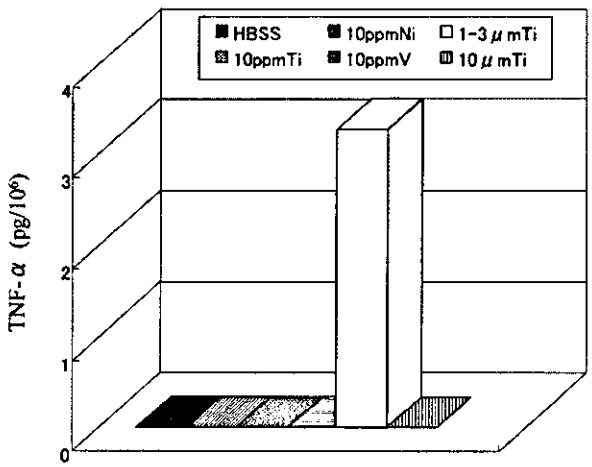

Fig. 4. Amount of TNF-α released from human neutrophils exposed to metals in solution and as particles.

4.3. Dissolution test of Ti

The Ti concentration measured in HBSS containing 1–3 and 10 μm Ti particles was below the detection limit of ICP, which is in the order of 0.001 ppm.

4.4. SEM and EDS

Fig. 5 shows the morphology of human neutrophils exposed to 10 ppm solutions of Ni, V and Ti determined by SEM. The cell membrane of the V and Ti groups was expanded compared with the normal condition, and the fine surface morphology was more complex than in the control group (HBSS). The cell membrane was obviously deformed and disrupted by Ni.

Fig. 6 compares the SEM image of human neutrophils exposed to 1–3 μm Ti particles in solution (a) with an elemental Ti map image of the same area (b). Phagocytosis of the particle by human neutrophils of about 5 μm is indicated in the SEM photograph. EDS mapping confirmed that the engulfed particle was Ti. Such phagocytosis occurred only with the 1–3 μm Ti particles in solution.

Fig. 7 shows that the EDS spectrum obtained from the square area of Fig. 6 detected the elements Na, Al, Si, Pt, Pd, K and Ti. Sodium, Al, Si, K originated from

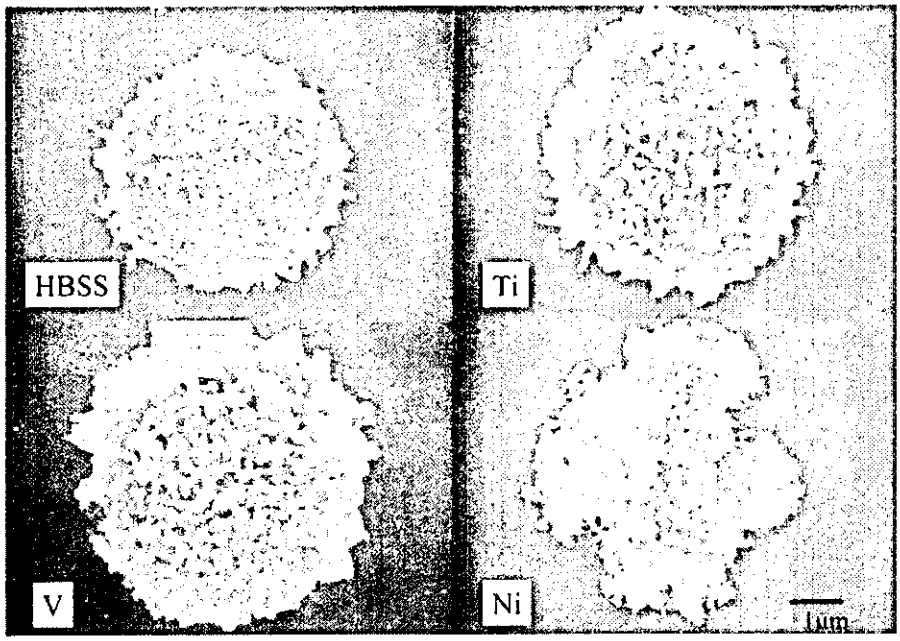

Fig. 5. Morphological changes in human neutrophils exposed to 10 ppm metals in solution determined by SEM observation. HBSS, control; Ti, titanium; V, vanadium; Ni, nickel.

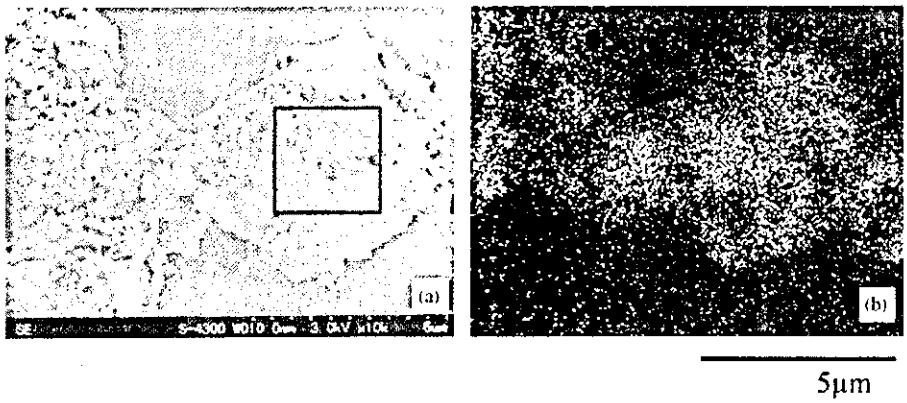

Fig. 6. SEM image of human neutrophils exposed to 1–3 μm pure Ti particles in solution (a) and corresponding Ti elemental maps (b).

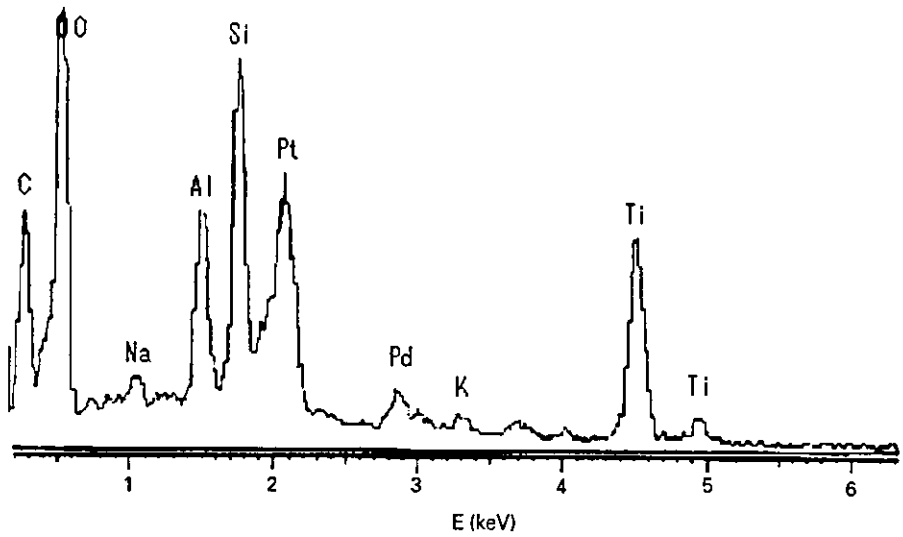

Fig. 7. Energy dispersive spectrum obtained from square part of Fig. 6. Titanium, Na, Al, Si, K originated from glass substrate, and Pt, Pd arose from coating film for SEM observation.

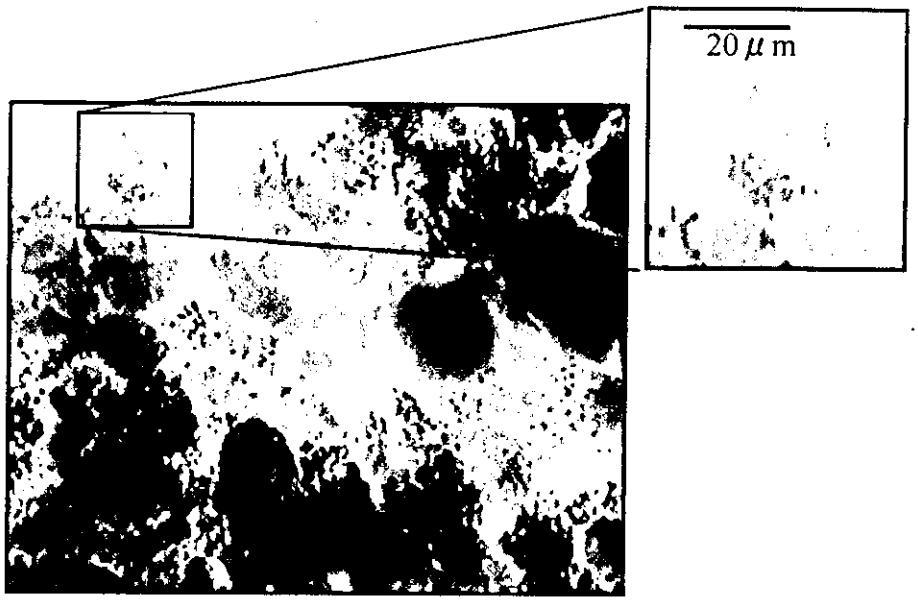

Fig. 8. Tissue response to 1–3 μm Ti particles inserted in subcutaneous tissue for 8 weeks. (HE stain; original magnification × 400).

the glass substrate and Pt, Pd from the coating film for SEM observation.

Detection of high titanium peaks shows that the deformed neutrophils in Fig. 6 contain Ti particles.

4.5. Animal studies in vivo

Fig. 8 shows a histological image of rat soft tissue inserted with pure titanium particles of 1–3 μm after 8 weeks. Inflammatory cells are numerous and the small black dots are Ti particles. The macrophages and adjacent collagen show degenerative changes in morphology. The enlarged view shows that Ti particles were phagocytized into the cytoplasm by a macrophage.

5. Discussion

5.1. Probe cells

The most representative leukocytes with the function of phagocytosis are neutrophils and macrophages. In

response to foreign objects, neutrophils react first then macrophages follow. Neutrophils account for the largest proportion (about 54–65%) of human leucocytes. Their function is to initially react non-specifically to foreign objects, while macrophages have a more complex function. They have a specific relationship with individual cytokines and respond to the various cytokines emitted after neutrophils react with foreign objects. Shanbhag et al. [18] investigated the influence of Ti and Ti–6Al–4V particles on NO release from murine macrophages. They also examined the effect of Ti oxide and polystyrene particles that were smaller than macrophages on cytokine and PGE-2 release and showed that the release of these cytokines is dependent on particle size [19]. We found that Ti particles that are smaller than cells cause inflammation in vivo and in vitro. Our results agree with those of Shanbhag et al. who showed that that the functional reaction of cells in response to particulate debris appears to be dependent on the size of the particles. We used human neutrophils rather than macrophages to monitor the effect of Ti ions and particles for the above reasons.

5.2. *Cytotoxicity of Ti, V and Ni in solution*

We examined the cytotoxicity of Ti in solution and as particles as well as V and Ni in solution by biochemical functional analysis as well as by microscopic morphology observation and elemental analysis.

The effect of Ti can be compared with that of other metal ions. We therefore selected V and Ni ions, as they are also cytotoxic. Ti is chemically stable and insoluble in vivo. Nickel dissolves into the surrounding tissues where it is highly toxic. Vanadium is cytotoxic and soluble in artificial solutions [3–4].

We examined the cytotoxic effects of ions at concentrations of 2 or 10 ppm. According to Liao et al. [21], concentrations of Ti above 10 ppm inhibit cell proliferation, whereas 5 ppm or less had no effect on stimulated proliferation. A preliminary experiment showed that LDH (Fig. 2) was not significantly changed in the presence of 2 ppm of Ti, therefore we performed studies at 10 ppm, which induced significant changes. To examine the effects on superoxide anions (Fig. 3), the tendency for the two solutions was similar. We therefore used 2 ppm since sensitivity to the lower ion concentration has more significance when judging cell toxicity.

Fig. 2 shows that LDH values were significantly higher only in Ni solution. LDH is an intracellular enzyme involved in the glycolytic pathway. The LDH value only increased when the cell membrane was destroyed. Fig. 1 showed that cell survival rate was significantly lower only in Ni solution. These biochemical analyses indicate that neutrophils are destroyed by the exposure to Ni solution. The cell morphology shown in Fig. 5 confirmed that neutrophils are disrupted in Ni solution.

Cell survival rates and LDH activities did not significantly differ among Ti, V or HBSS solution (Figs. 1 and 2). However, the quantity of superoxide produced by neutrophils (Fig. 3) significantly increased in Ti and V solutions. Superoxide anions are released from intracellular organs and the cell membrane when the membrane of neutrophils is stimulated. The results of the biochemical analyses suggest that V and Ti solutions do not destroy, but rather stimulate neutrophils. The SEM observations showed that neutrophils were inflated and that a minute membrane structure was extensively developed. This morphological change would be related to the increase in superoxide anion release (Fig. 5).

On the other hand, Ni produced less superoxide (Fig. 3), which appears to contradict its effects in solution. This could be due to the low survival rate of neutrophils (Fig. 1) caused by destruction in Ni solution that surpasses the stimulatory effect of Ni.

The V solution was relatively more cytotoxic than the Ti solution, according to the cell survival rate and the results of the superoxide test (Figs. 1 and 3). An increased superoxide content in vivo can affect the cell circumference and damage the DNA.

To summarize the above results, the decreased cell survival rate (Fig. 1), the increased LDH activity (Fig. 2) and the disrupted morphology in the SEM observation (Fig. 5) suggest that Ni is cytotoxic to neutrophils.

On the other hand, the release of superoxide anion (Fig. 3), the deformed morphology (Fig. 5) and no significant change in the cell survival rate (Fig. 1) and LDH value (Fig. 2) suggest that Ti and V in solution do not destroy the cell membrane, but rather stimulate human neutrophils. Our results suggest that Ti in solution can excite neutrophils, although there was no difference between 2 and 10 ppm of Ti in solution.

5.3. *Cytotoxicity of Ti particles*

We evaluated the influence of fine Ti particles of 1–3 and 10 μm in diameter. Our experiments indicated that the TNF-α value increased only in the solution of 1–3 μm Ti particles. Neutrophils release TNF-α from the moment foreign matter is taken up by the cells. Our SEM observation (Fig. 6) and EDS elemental analysis (Figs. 6 and 7) showed that only 1–3 μm Ti particles were phagocytized by neutrophils of about 5 μm in diameter.

Our initial studies of dissolution showed that Ti was detected by ICP in HBSS mixed with fine Ti particles. We determined whether the Ti was generated from ions or particles, by passing the mixture through a 0.45 μm membrane. Only ions or particles <0.45 μm can pass through the membrane. The results showed that Ti was

below the detection limit of ICP after filtration. We therefore concluded that the increase in TNF-α was not caused by Ti ions but mostly by Ti particles larger than 0.45 μm.

The 1–3 μm Ti particles excited neutrophil activity and increased the production of superoxide anions, (Figs. 4 and 6) while 10 μm Ti particles did not. The increased quantity of TNF-α caused neutrophil activation, resulting in inflammation.

5.4. Titanium cytotoxicity depends on particle size

The 1–3 μm Ti particles were phagocytized by neutrophils, which then produced superoxide anions and TNF-α. These effects were absent in solutions of 10 μm Ti particles. This suggests that Ti cytotoxicity is related to cell size. Shanbhag et al. [19] reported that the release of cytokine and PGE-2 from macrophages depends on the size of Ti oxide and polystyrene particles. They used particles of 0.15–1.76 μm in diameter, which were smaller than the cells and found some size dependence. We examined particles that were smaller (1–3 μm) and larger (10 μm) than the neutrophils to determine what affect the relationship between cell and particle size has on cytotoxicity. The amounts of TNF-α released by 1–3 and 10 μm particles were much more remarkable. Neutrophils phagocytized Ti particles only when the particles were smaller than the cells (Ti, <3 μm; cells, about 5 μm). Therefore, fine Ti particles may cause cytotoxicity, although the macroscopic size of the Ti implant was quite biocompatible.

5.5. Relationship to in vivo findings

We mainly examined the effect of Ti particles in rats since dissolution from Ti particles was undetectable in vitro. Therefore, the main effect appears to be caused by particles rather than by ions. The in vitro cytotoxicity findings discussed above were closely related to the in vivo results, in which a long-term inflammatory reaction was elicited by 1–3 μm Ti particles. We have observed tissue reactions to various sizes of Ti particles (1–3, 10, 40, 50 μm) inserted into the soft tissues of rats (data not shown). We found that regions inserted with 40–150 μm Ti particles, which were bigger than inflammatory cells, were more quickly engulfed by fibrous connective tissue. Numerous inflammatory cells appeared when Ti particles smaller than cells were inserted (Fig. 8). These results can be explained by the increased amount of superoxide, TNF-α release and subsequent cytotoxic stimulation that partly resulted from phagocytosis into the soft tissue around the inserted titanium particles.

5.6. Clinical cytotoxicity of particles

Black pigmentation is sometimes recognized in soft tissue around titanium plates used to fix jawbones. We have found by optical microscopic observation and by X-ray scanning analysis microscopy (XSAM) (XGT-2000V, HORIBA, Tokyo, Japan), that this type of pigmentation contains minute particles of Ti. These particles were derived from the Ti plate, probably during fixation by screwing and other procedures. Other investigators have also found Ti in soft tissue around Ti plates in humans [11–12]. The results of the present study suggest that minute abraded particles can induce cytotoxicity rather than the dissolved Ti ions, since the dissolution of Ti from the Ti particles may be negligible. These results suggest that as much metal debris as possible should be removed from tissue during surgery and that the resistance of materials against abrasion in artificial joints and other sliding parts should be increased.

Titanium is usually biocompatible because a native passivating oxide layer normally covers its surface. Our experiments also indicated that the dissolution of Ti particles in HBSS is negligible. Others, however, have described that the protective oxide layer is not entirely stable and that titanium ions are released [20,22] when the TiO_2 overlayer is removed. Liao et al. described that Ti in solution at 5 ppm and above interferes with calcium mineralization [21]. Cellular alterations against ions in solutions in vitro depend on the concentration of ions exposed to the cells [23–24]. Our present results suggest that Ti in solution can excite neutrophils although the findings of 2 and 10 ppm Ti in solution were essentially identical. Most of studies on the biological effect of Ti dissolution, however, did not distinguish between Ti ions and Ti particles. This is especially important when analyzing the tissue surrounding implanted materials in vivo. This is because ionic dissolution and mechanical separation can occur with debris in such circumstances.

The cytotoxicity described in some studies may have been due to Ti particles. One mechanism of Ti dissolution in vivo and the subsequent cytotoxicity, may be the cellular production of superoxide anions, which causes oxidation to progress on a Ti surface. This conjecture requires further study.

6. Conclusion

The present study differentiated the cytotoxicity of Ti ions and particles in human neutrophils. Large pieces of Ti, as in implants, are normally biocompatible because it is covered with a natural passivating oxide layer. However, solutions and fine particles of Ti may influence the circumferential organization. Although

we have shown in animals that Ti is biocompatible down to 40 μm, the present study demonstrated that fine Ti particles below 10 μm in diameter induce cytotoxicity both in vivo and in vitro. When the Ti particle is smaller than the cell (~10 μm), cytotoxicity is induced, resulting principally from engulfment by inflammatory cells.

Release of superoxide anion and TNF-α due to stimulation and phagocytosis are factors that can enhance the further process of phagocytosis and inflammation. Therefore, the effects of fine particles of Ti that are produced during various clinical processes must be carefully considered.

Finally, this study demonstrated that this experimental technique can evaluate and bio-functionally analyze material even when biocompatibility is relatively high.

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Original paper

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Surface Properties and Biocompatibility of Nitrided Titanium for Abrasion Resistant Implant Materials

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Corrosion, other related properties and biocompatibility of surface nitrided titanium were investigated to examine its possible use as an abrasion resistant implant material. The nitrided layer about 2μm thick composed of TiN and Ti_2N was formed on titanium by a gas nitriding method. The dissolved amount of titanium ion in SBF was as low as the detection limit of ICP, and that in the 1% lactic acid showed no significant difference from titanium. The tissue reaction of the cylindrical implant in soft tissue of rats showed no inflammation, and fine particles of 1μm induced phagocytosis, which was similar to titanium. The implantation in the femor showed the new bone formed in direct contact with implants. All the results suggested that the wettability, corrosion resistance, *S.mutans* adhesion and biocompatibility were nearly equivalent to those of titanium. The surface of nitrided titanium was promising, with biocompatibility comparable with titanium, as an implant material such as for an abutment part of a dental implant, which requires high abrasion resistance.

Key words : Titanium nitride, Implant, Biomaterial

INTRODUCTION

In the increasingly aged society, implants as a method for recovering the function of lost teeth will become more and more important. Titanium is used most as the implant material at present with several variations including apatite coated titanium implants and functionally graded implants[1-9]. Corrosion resistance is one of the most important factors to affect the biocompatibility of implant materials[10-14]. The titanium surface has a passive film formed by a thin and stable oxide, which aids biocompatibility under the severe environments found *in vivo*. Titanium has, however, also negative points. One of them is low abrasion resistance[15]. Minute titanium abrasion powders may cause an inflammatory reaction[16,17]. Therefore, it is desirable to develop implant materials with both biocompatibility and abrasion resistance. The surface nitriding method would be one of the surface treatments of metallic material to solve this problem.

Titanium nitride is known for its high surface hardness and mechanical

strength[18,19]. It was also reported that the dissolution of Ti ion is very low[19,20]. From these findings, the surface nitriding method appears to be useful. However, research to evaluate the surface properties and biocompatibility of nitrided titanium for application as an implant has rarely been carried out.

As for dental implants it is comprised various components. The implant abutment part (the mucosa penetration part) is exposed in the oral cavity and so plaque and dental calculus easily adhere on it. Removal of the plaque and dental calculus is necessary to obtain a good prognosis throughout the long term maintenance of the implant. Therefore, it is desirable for the abutment part to have abrasion resistance against the scaling treatment.

In a previous study on the mechanical properties of nitrided titanium, the present authors reported that a nitrided layer on titanium with a thickness of 2μm showed a Vickers hardness of about 1,300, approximately 10 times that of pure titanium, and was strongly bonded with the pure titanium base metal. In both the Martens scratch test and abrasion resistance test with a dental ultrasonic scaler, nitrided titanium showed very high abrasion resistance[21].

The purpose of the present study was to evaluate corrosion resistance, wettability of the surface, quality of *S.mutans* adhesion and biocompatibility of the surface nitrided titanium and to examine its possible use, as an abrasion resistant implant material, especially for the abutment part of dental implants.

Pure titanium and surface nitrided titanium are abbrevicted as Ti and Ti(N), respectively in the following figures.

MATERIALS AND METHODS

Specimen preparation

A 1 mmϕ $\times$7 mm length 99.9% titanium wire (NILACO Ltd., Tokyo, Japan) and JIS type 1 pure titanium plate (10$\times$10$\times$0.5 mm: KOBE STEEL Ltd., Kobe, Japan) were used as the cylindrical and plate specimens, respectively. Both specimens were polished with #2000 waterproof paper (the #2000 polishing specimen). Part of the plate specimens were also polished with 6μm diamond emulsion (BUEHLER, Illinois, USA)(the 6μm polishing specimen). These specimens were treated with 0.1% hydrofluoric acid solution for 10 sec to clean the surface. They were then ultrasonically cleaned with distilled water, 100% ethanol and 99.5% acetone for 15 min each. Nitriding was performed under the conditions of a N_2 atmosphere of 1 atm at 850 ℃ for 7 hours. (N_2 flow rate: 50 L/min, Furnace volume: 0.574 m^3)

Evaluation of surface quality

1) Surface observation

Surface structure was observed by atomic force microscopy (AFM) (TMX-2000 Explorer, TopoMetric, Santa Clara, USA).

2) Confirmation of the nitrided layer

The cross-section of the surface nitrided titanium was observed by SEM (S-4000N,

HITACHI, Tokyo, Japan) after the specimen was cut and polished. X-ray diffraction of the specimen surface was performed using X-ray diffraction equipment (JDX-3500, JEOL, Tokyo, Japan).

3) Roughness measurement

On the #2000 polishing specimen, AFM was used for a scaning area 50×50μm and with a scanning rate 5μm/min. Measurements were performed 5 times for each specimen and the average height deviation from the mean plane (Ra) was obtained.

Corrosion resistance test

The dissolution test of titanium was carried out using Simulated body Fluid (SBF) and 1% lactic acid in order to evaluate corrosion resistance of nitrided titanium. A plate specimen with a 220 mm^2 surface area (the #2000 polished specimen in the SBF and the 6μm polished specimen in the 1% lactic acid) was used as a test piece. They were ultrasonically cleaned and then sterilized with ethylene oxide gas. These specimens were soaked in SBF and in 1% lactic acid 5 ml for 10 and 30 days. The test was performed 5 times for each specimen. The vessel of the blank condition where the specimen was not soaked was prepared as a control population. The composition of SBF followed the methods of Kokubo *et al.*[22]. The SBF was prepared by dissolving reagent grade chemicals of NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$ and $CaCl_2$ into deionization distilled water so that ion concentrations of the fluid were almost equal to those in human blood plasma. The ion concentrations are shown in Table 1. The lactic acid used was high-pure lactic acid (reagent prime class, Wako PURE CHEMICAL INDUSTRIES, Ltd., Osaka, Japan) with 90% purity and was diluted with deionization distilled water at 1.0%. The sealing polypropylene vessel was used for the dipping test. The vessel was set at 37℃ under sealed conditions for the whole period of the dipping. After dipping, the titanium concentration (ppm=mg/L) was analyzed using the ICP emission analysis equipment (P-4010 type, HITACHI, Tokyo, Japan), and the amount (ng/mm^2) of Ti dissolution per unit area was deduced.

Wettability of the specimen surface

To evaluate the wettability of the specimen surface, measurement of contact angle was carried out by the dropping test. First, 2μl of distilled water was dropped on the specimen surface, and the droplet was photographed from the horizontal direction. The contact angle was defined as the double of angle ∠CAB (Fig. 1). Measurements were performed 5 times on each specimen, and the mean value was

Table 1 Ion concentrations (mM) in the simulated body fluid (SBF) and human plasma

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}
SBF	142.0	5.0	1.5	2.5	148.8	4.2	1.0
Human plasma	142.0	5.0	1.5	2.5	103.0	13.5	1.0

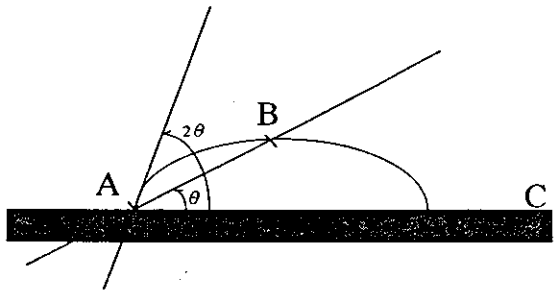

Fig. 1 Measuring method of the contact angle.
A: Intersection point of the droplet surface and specimen surface
B: Top of the droplet. C: specimen surface

obtained.

S.mutans adhesion

To compare the adhesion of *S.mutans* on titanium and nitrided titanium, the *S.mutans* attachment test was carried out. The 6μm polished specimen was used as a test piece. They were ultrasonically cleaned and then sterilized with ethylene oxide gas.

1) Bacteria and culture

Streptococcus mutans JC 2 which was divided from the Department of Oral Pathobiological Science Hokkaido University was used. *S.mutans* was inoculated in Brain Heart Infusion Broth (EIKEN CHEMICAL Co., Ltd, Tokyo, Japan) with 5% sucrose added (5% sucrose BHI culture medium). Precultivation was carried out at 37℃ for 24 hours. This fungus liquid of 60μl was added to 5% sucrose BHI culture medium of 3 ml. These specimens were suspended with 5-0 nylon thread for oral surgery, and cultivation was carried out at 37℃ for 24, 48, and 72 hours. Measurements were performed 5 times on each specimen, and the mean value was obtained.

2) Measurement of adhered bacteria

After the culture, specimens were washed with 10 ml distilled water. These specimens were invested in 2 ml 1N sodium hydroxide solution, elimination and solubilization of the fungus were by ultrasonic cleaning for 60 seconds. The absorbence was measured at 550 nm using the spectrophotometer (U-1100, HITACHI, Tokyo, Japan), and the mean value was regarded as adhesion of *S.mutans*.

Biocompatibility

To compare the biocompatibility of titanium and nitrided titanium, these materials were implantation.

1) Implantation in subcutaneous tissue

(1) Animal experiment

Wistar strain rats aged 14 weeks (weight, 350-380 g) were used. After anesthesia

with diethyl ether (Wako Pure Chemical Industries, Osaka, Japan), pentobarbital sodium (30 mg/kg; NEMBUTAL INJECTION, Dainabot, Osaka, Japan) was injected into the abdominal cavity of the rats, and the cylindrical implants (1 mm ϕ × 7 mm length) were inserted in the subcutaneous connective tissue in the abdominal region. The wound was then sutured. Four specimens were implanted for each mateial. These rats were sacrificed at 4 and 8 weeks after surgery, and tissue block involved specimens were harvested.

(2) Histological evaluation

Implants inserted in the subcutaneous tissue were carefully removed from the tissue blocks after fixation in 10% neutral buffered formalin, and the tissue blocks were then embedded in paraffin by a conventional method. The tissue blocks were sectioned and stained with Hematoxylin-Eosin. These specimens were histopathologically observed with an optical microscope (Axioskop, ZEISS, Germany).

2) Implantation test of fine particles

(1) Specimens

Titanium fine particles (1~3μm) (SOEKAWA CHEMICALS Co., Ltd, Tokyo, Japan), nitrided titanium fine particles (1~1.5μm) (KISHIDA CHEMICAL Co., Ltd, Osaka, Japan) were used as experimental specimens. Each specimen was sterilized by ethylene oxide gas after it was washed in 100% ethanol, and then it was used for the experiment.

(2) Animal experiment

Wistar strain rats aged 14 weeks (weight, 350-380 g) were anesthetized by the methods similar to those for the subcutaneous implant, and fine particles were inserted in the subcutaneous connective tissue in the abdominal region. The wound was then sutured. Four specimens were implanted for each material. These rats were sacrificed at 1, 4, and 8 weeks after surgery, and tissue block specimens were harvested.

(3) Histological evaluation

After fixation in 10% neutral buffered formalin, and the tissue blocks were then embedded in paraffin by a conventional method. The tissue blocks were sectioned and stained with Hematoxylin-Eosin. These specimens were histopathologically observed with an optical microscope.

3) Implantation in the hard tissue

(1) Animal experiments

Wistar strain rats aged 14 weeks (weight, 350-380 g) were anesthetized by the methods similar to the subcutaneous implant. A hole was carefully made in the lateral surface of the diaphysis of the femur using a dental round bur (ϕ 1 mm), with a physiological saline external coolant, and the cylindrical implants (1 mm ϕ × 7 mm length) were inserted in the bone marrow. The wound was then sutured. Four specimens were implanted for each material. These rats were sacrificed at 4 and 8 weeks after surgery, and tissue block specimens were harvested.

(2) Histological evaluation

The tissue blocks from the rat femurs were fixed in 10% neutral buffered formalin, washed, stained with Villanueva bone stain, and then embedded in PMMA. The

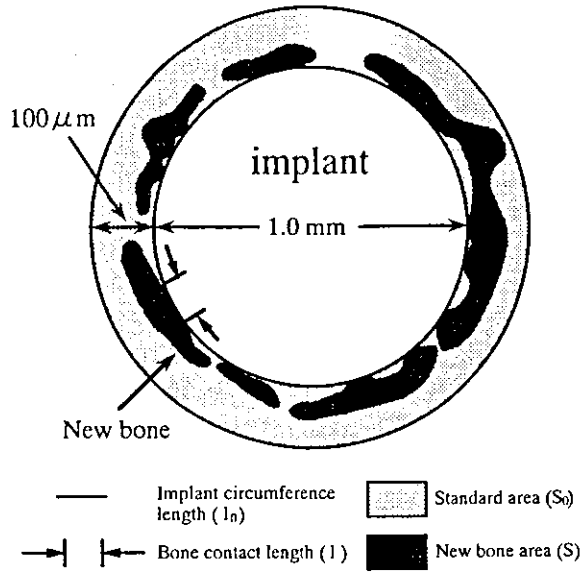

Fig. 2 Measurement methods of the area ratio of new bone formation and the ratio in contact with a specimen.

tissue blocks were then sectioned at 400 μm with a precision sawing machine (ISOMET 2000, BUEHLER, Illinois, USA) and thinner sections of about 100 μm thickness were prepared by mechanical polishing. These specimens were histopathologically observed with an optical microscope. In addition, histomorphometric investigation by image processing was carried out according to the method of Matsuno *et al.*[4]. As shown in Fig. 2, new bone (S) that formed in the area of the region within 100 μm from the specimen surface (standard area: S_0) was measured, and the area ratio of new bone formation (A) was obtained as $A=S/S_0$. The proportion of bone contact length (l) for each specimen circumference length (l_0) (The ratio of bone in contact with the specimen: C) was obtained as $C=l/l_0$. Eight polishing specimens were used per implantation period for histomorphometric analysis.

In each test, a significance test was carried out on the statistical analysis using the Mann-Whitney U test (Stat View 5.0, HULINKS Ltd., Tokyo, Japan).

RESULTS

Evaluation of surface quality

1) Surface observation

The specimen surface after nitriding showed a gold color. Fig. 3 shows the typical surface structure of pure titanium (a) and surface nitrided titanium (b) observed by AFM. In titanium, the ruggedness formed by mechanical polishing was observed nearly parallel in a unidirection. The surface nitrided titanium showed a ruggedness with an irregular form. In the roughness measurement, the Ra of the #2000 polishing specimen was 0.13 μm (Standard deviation: 0.015) in the titanium, and 0.32 μm

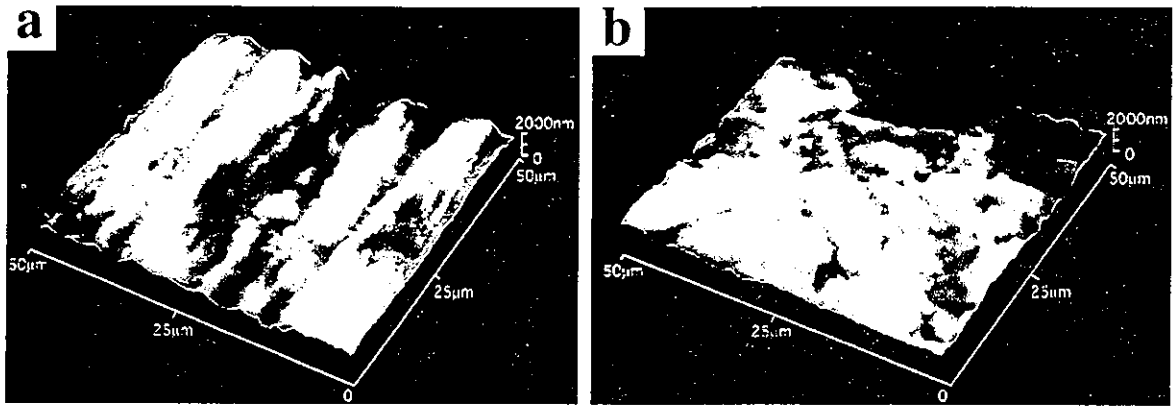

Fig. 3 Surface structure of titanium observed by AFM (#2000 polished specimen). a: before nitriding, b: after nitriding

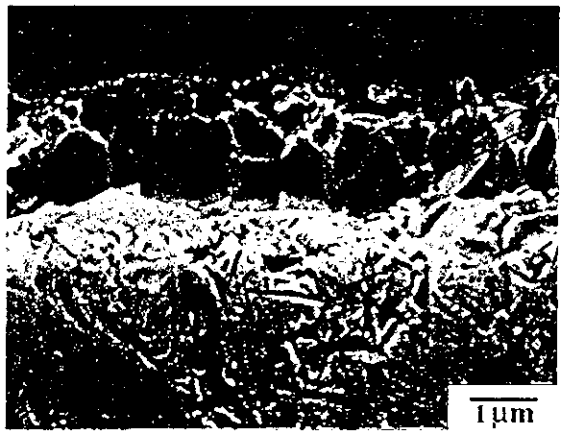

Fig. 4 Cross-section of nitrided layer observed by SEM.

(0.020) in the nitrided titanium. The Ra of the 6μm polished specimen was 0.04μm (0.001) in the titanium, and 0.08μm (0.002) in the nitrided titanium. The surface of nitrided titanium was rougher than that of titanium.

2) Confirmation of the nitrided layer

Fig. 4 shows the cross-section of the nitrided layer observed by SEM. The nitrided layer was approximately 2μm thick on surface of the pure titanium. Fig. 5 shows the X-ray diffraction of nitrided titanium. The composition of the nitrided layer was a mixture of TiN and Ti_2N. The Ti peaks were very few. It was confirmed that the surface was almost completely nitrided.

Corrosion resistance test

1) Simulated body fluid immersion test

Fig. 6 shows the dissolved amounts of titanium in the SBF at 10 and 30 days. In both specimens, dissolution was almost equal to the blank condition. This

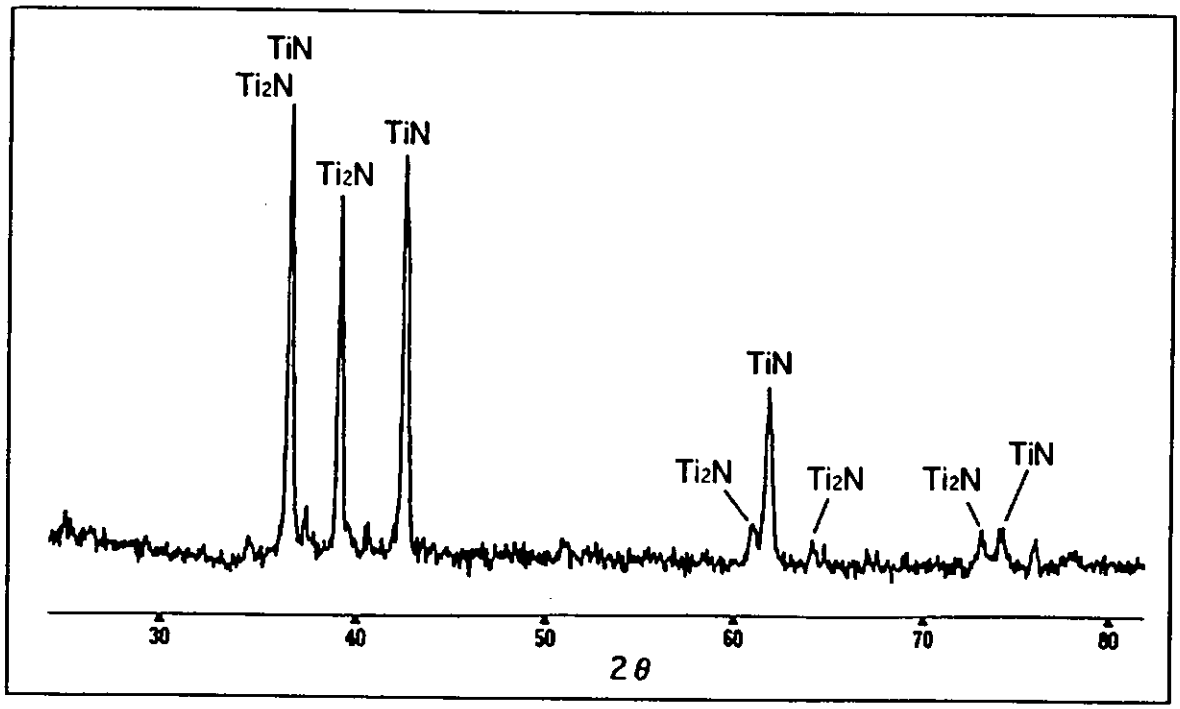

Fig. 5 X-ray diffraction of the surface nitrided titanium.

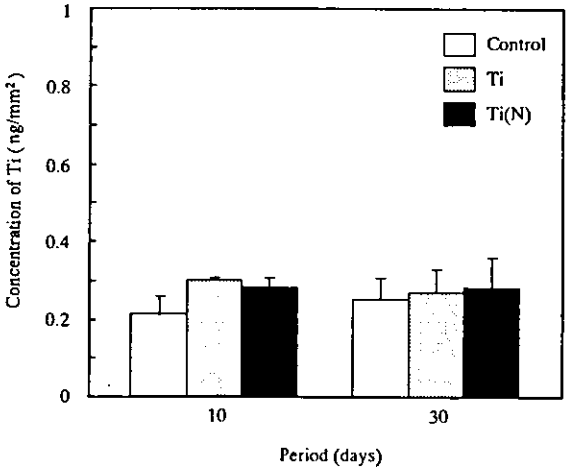

Fig. 6 Titanium dissolution in simulated body fluid from titanium and nitrided titanium.

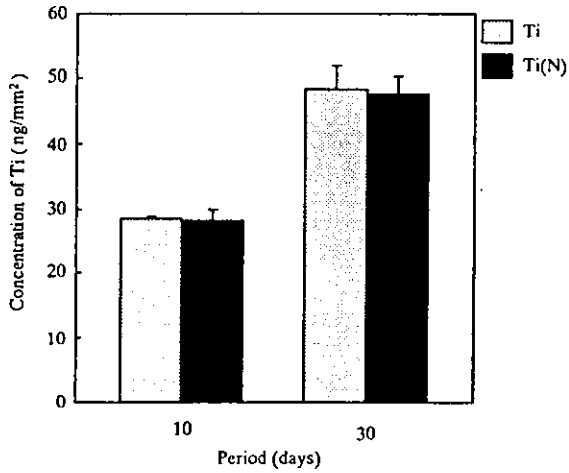

Fig. 7 Titanium dissolution in 1% lactic acid from titanium and nitrided titanium.

background level was the detection limit of ICP.

2) 1% lactic acid immersion test

Fig. 7 shows the dissolved amount of titanium in the 1% lactic acid at 10 and 30 days. In the dipping for 10 days, the dissolved titanium was 28.3 ng/mm^2 in the titanium, 28.1 ng/mm^2 in the nitrided titanium, and after 30 days, 48.8 ng/mm^2 in the titanium, 47.2 ng/mm^2 in the nitrided titanium. No significant difference was found

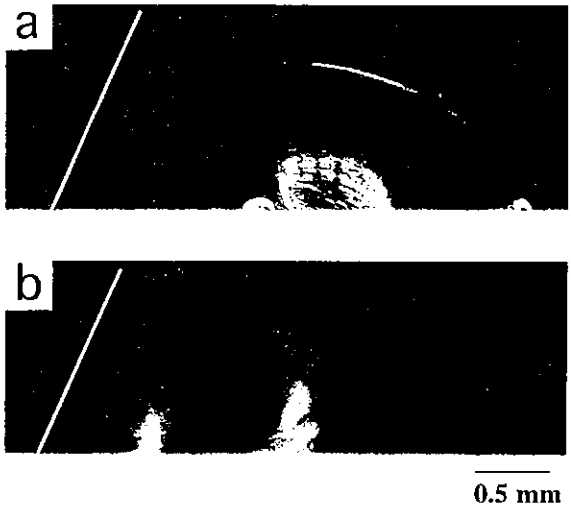

Fig. 8 Image of water droplet and contact angle on a specimen's surface.
a: titanium, b: nitrided titanium

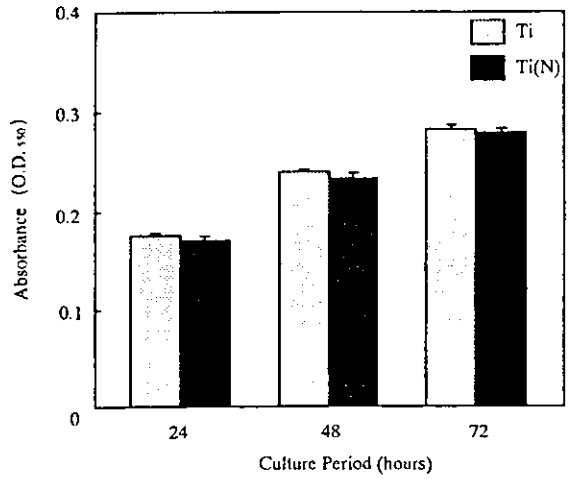

Fig. 9 Quantity evaluation of *S.mutans* adhesion by absorptiometry on titanium and nitrided titanuim.

between titanium and nitrided titanium for either 10 or 30 days.

Wettability evaluation of the specimen surface

Fig. 8 shows an image of a water droplet and the contact angle on the specimen surface. The mean value of the contact angle was 59.0° in the titanium, 60.6° in the nitrided titanium. There was no significant difference between titanium and nitrided titanium.

S.mutans adhesion

Fig. 9 shows the quantity of *S.mutans* adhered on titanium and nitrided titanium by absorptiometry. The average absorbance after the 24, 48, and 72 hours was 0.170, 0.226, 0.278 in the titanium, and 0.175, 0.238, 0.282 in the nitrided titanium, respectively. There was no significant difference between titanium and nitrided titanium.

Biocompatibility

1) Implantation in subcutaneous tissue

Fig. 10 shows tissue responses to titanium and nitrided titanium implanted in subcutaneous tissue of rats at 4 and 8 weeks after surgery. The blank, upper part of each photograph is the position where the specimen was implanted. In both titanium and nitrided titanium at 4 weeks, the specimens were encapsulated with fibrous connective tissue in which fibroblasts were observed, and no inflammatory response was observed. At 8 weeks, the fibrous connective tissue became much thinner than at 4 weeks. No inflammatory change was observed around the specimens. There was significant difference between titanium and nitrided titanium in the tissue response.

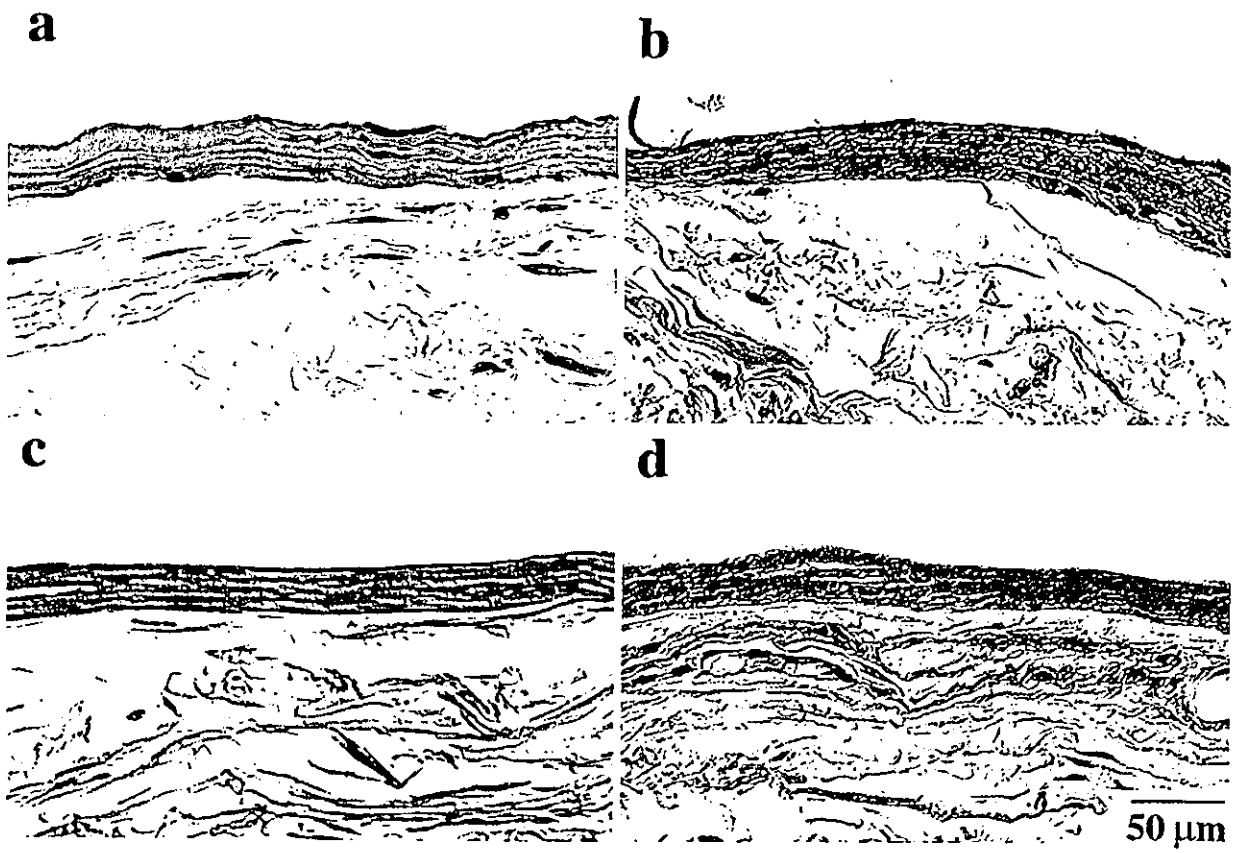

Fig. 10 Tissue response to titanium and nitrided titanium in subcutaneous tissue.
a: Ti 4w, b: Ti(N) 4w, c: Ti 8w, d: Ti(N) 8w

2) Implantation test of fine particles

Fig. 11 shows the tissue response to fine particles of titanium and nitrided titanium in subcutaneous tissue at 1, 4 and 8 weeks. In both titanium and nitrided titanium, numerous inflammatory cells were observed at 1 week. The distribution of the fine particles was widely extended. The high magnification shows that fine particles were phagocytized into the cytoplasm by macrophages. At 4 week, numerous inflammatory cells were still observed, and fine particles were phagocytized by macrophages as at 1 week. The distribution region of fine particles was reduced a little in comparison with at 1 week. At 8 week, the inflammatory response became slightly. Fine particles were arranged with a high density in the distributed region in comparison with at 4 weeks. The difference between titanium nitride and titanium could not be recognized during the experimental period in the degree of inflammatory response and the phagocytosis by the macrophages.

3) Implantation in the hard tissue

(1) Observation by optical microscopy

Fig. 12 shows the new bone formation around titanium and nitrided titanium in femoral bone marrow at 4 and 8 weeks. At 4 weeks, a 30μm thick layer of newly

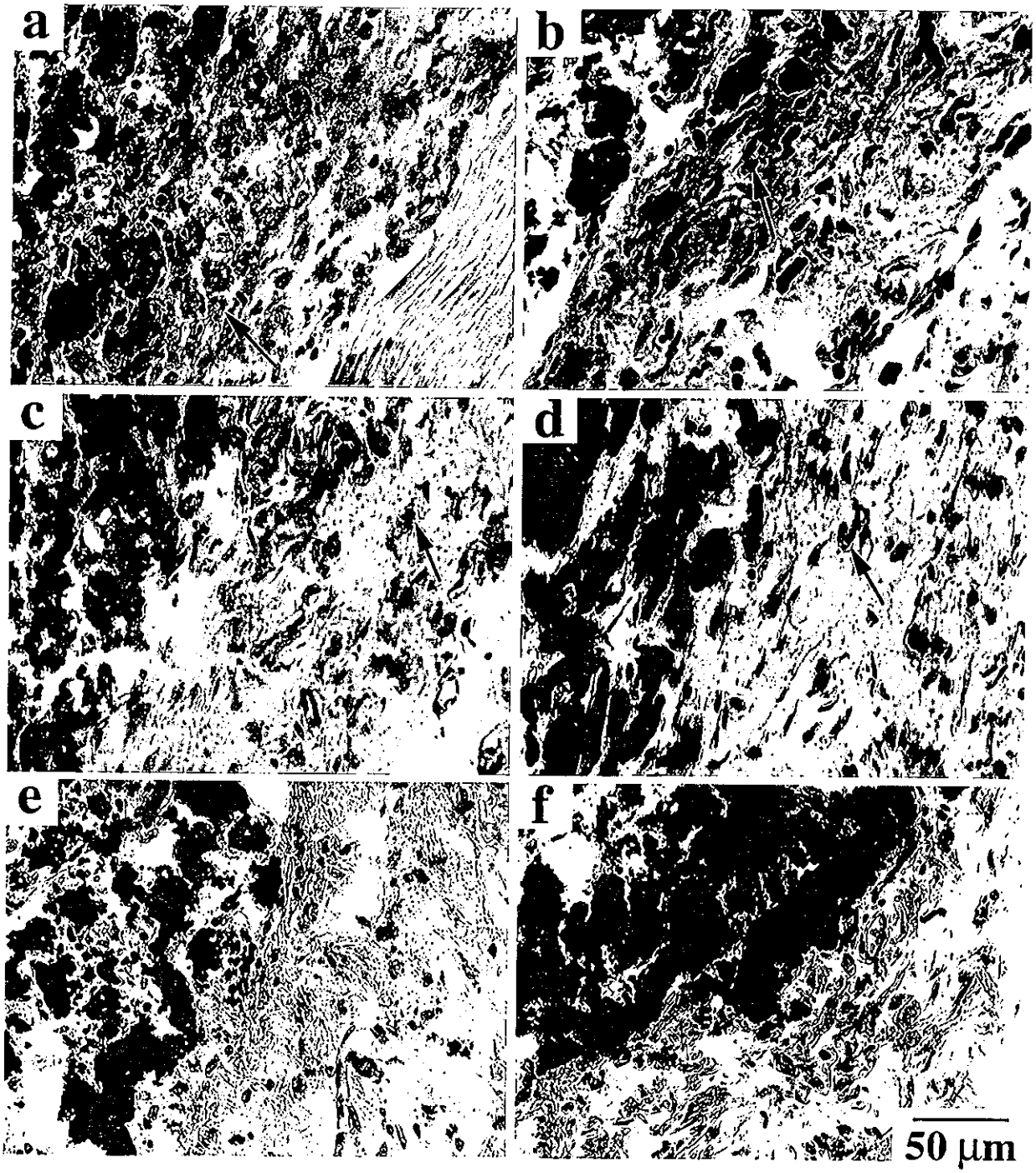

Fig. 11 Tissue response to fine particles of titanium and nitrided titanium in subcutaneous tissue. (Arrow: Phagocytosis by a macrophage)
a: Ti 1w, b: Ti(N) 1w, c: Ti 4w, d: Ti(N) 4w, e: Tl 8w, f: Ti(N) 8w

formed bone was in direct contact with some parts of the surface of the metal implants. The area of direct contact at the bone-metal interface appeared to have increased between 4 and 8 weeks in both specimens.

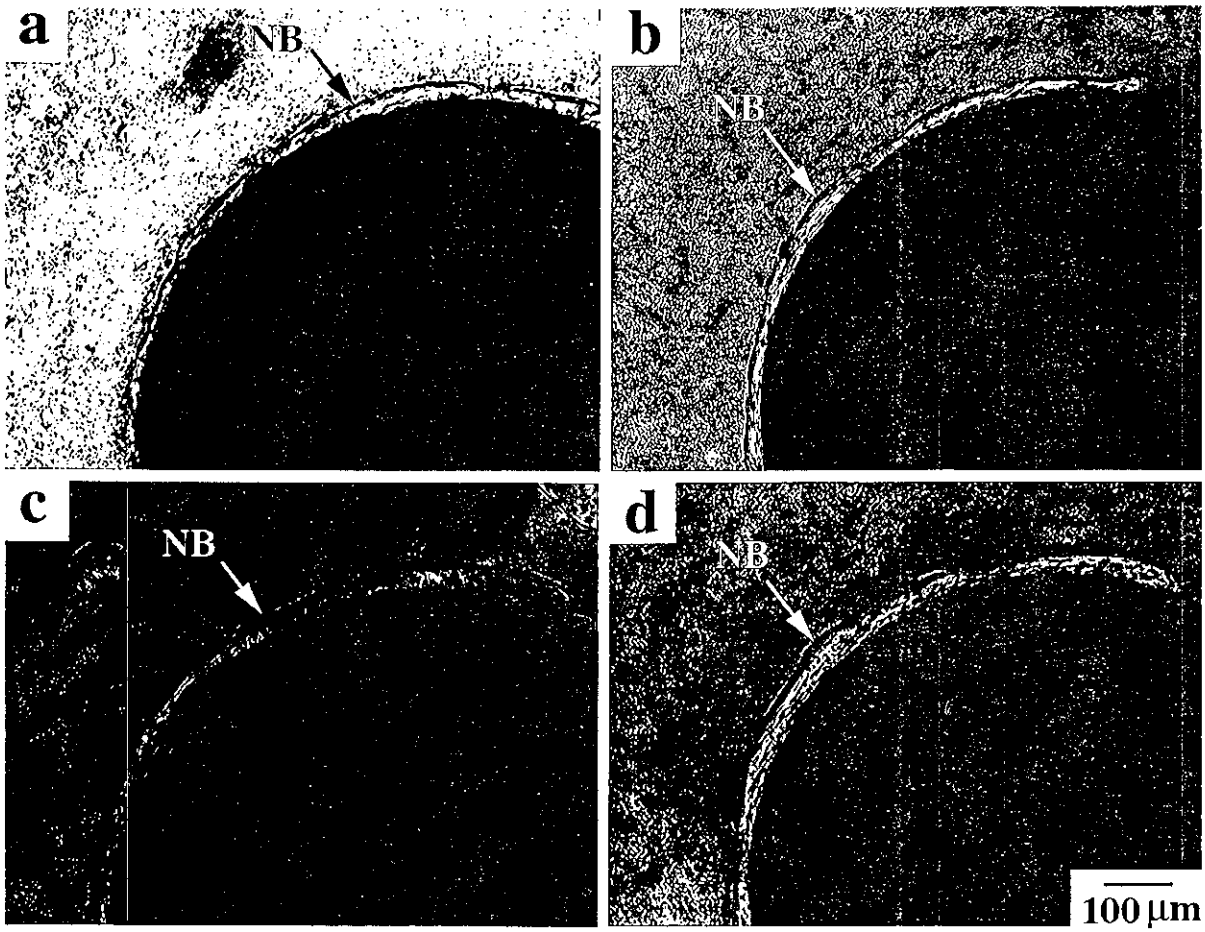

Fig. 12 New bone formation around titanium and nitrided titanium in femoral bone marrow.
NB: New bone
a: Ti 4w, b: Ti(N) 4w, c: Ti 8w, d: Ti(N) 8w

(2) Quantitative analysis of new bone formation

Fig. 13 shows the area ratio of new bone formation in femoral bone marrow at 4 and 8 weeks. At 4 weeks, the area ratio of new bone formation was 22.4% in the titanium, 22.0% in the nitrided titanium. At 8 weeks, the area ratio of new bone formation was 42.6% in the titanium, and 40.8% in the nitrided titanium. There was a significant increase between 4 and 8 weeks. There were no significant differences between titanium and nitrided titanium for each period. Fig. 14 shows the ratio of new bone in direct contact with the implant surface at 4 and 8 weeks. At 4 and 8 weeks, the ratio was 51.5% and 67.8% in the titanium, and 46.6% and 65.8% in the nitrided titanium. There was a significant increase between 4 and 8 weeks. There were no significant differences between titanium and nitrided titanium for each period.