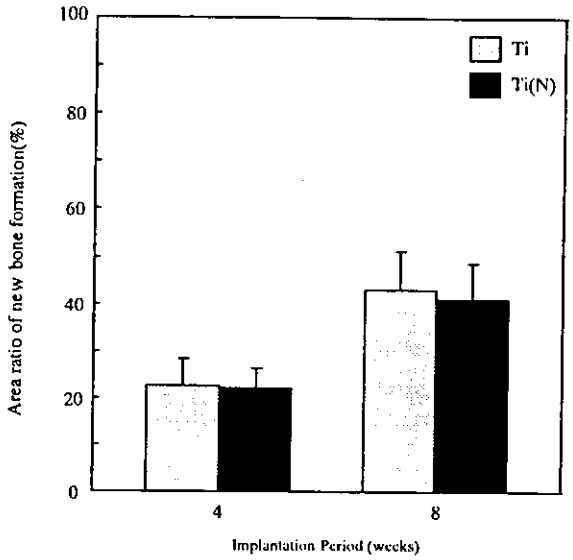

Fig. 13 The area ratio of new bone formation at 4 and 8 weeks.

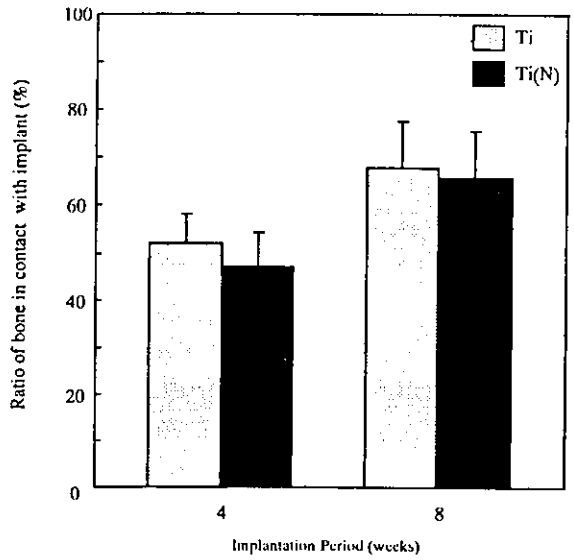

Fig. 14 The ratio of bone in contact with specimens at 4 and 8 weeks.

DISCUSSION

Evaluation of surface quality

Macroscopically, the nitrided layer appeared like surface film with uniform thickness. However, the surface roughness of titanium nitride was greater than that of titanium for both the #2000 and 6μm polished specimens. Ttitanium nitride is formed through the process of nucleation and growth, and is expanded to the full surface from the insular distribution at the beginning. Therefore, as observed in the cross-section of Fig. 5, which consists of polycrystals, the surface layer has some ruggedness and porosity, which differs, depending on the conditions of the nitriding temperatures, N_2 partial pressure and processing time. The surface roughness of nitrided layer is thus coarsened compared with before nitriding treatment.

The composition of the nitrided layer was a mixture of TiN and Ti_2N as revealed from X-ray diffraction. It was confirmed that the surface was sufficiently nitrided, and the thickness of the nitrided layer was about 2μm.

Watari reported the formation of oxide, carbide, and a nitrided layer on the surface of titanium by controlling the gas partial pressure in a vacuum, and showed that the layer thickness grew in proportion to the square root of treatment time and Ti_2N was formed by nitriding[23,24].

Takamura[25] reported that the minute chemical compound layer, mainly consisting of TiN, is formed on the surface, followed in the inside by a hard layer with solid solution hardening of the nitrogen to α-Ti. The thickness of the chemical compound layer and the thickness of the hard layer changed almost proportional to the square root of the nitriding time at a constant temperature. When nitriding was carried out at 850℃ for 16 hours, the thickness of the chemical compound layer was

about 3μm, and that of the hard layer was about 50μm. In the present study, the thickness of the nitrided layer was about 2μm, which is slightly smaller due to the difference in nitriding time. The composition of the nitrided layer was a mixture of TiN and Ti_2N as revealed from X-ray diffraction. In the Ti-N phase diagram[26], TiN and Ti_2N intermingle at a temperature of 850℃. In a previous study on the mechanical properties of nitrided titanium, the present authors reported that Vickers hardness of nitrided titanium was about 1,300, approximately 10 times that of pure titanium and was strongly bonded with 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 and it was estimated that there would be almost no damage on the surface under the clinically used conditions[21].

Corrosion resistance test

Titanium surface has a passive film formed by stable oxide, and this aids biocompatibility under the severe environment at conditions found *in vivo*. Therefore, titanium is used mostly as implant material at present. However, titanium has a few negative points. It was suggested that the dissolution of metal ions can be generated where the oxide film is exfoliated by the abrasion and in implants with a porous structure[27-29]. Surface nitriding may be one of the methods to solve these problems. In the present study, SBF and 1.0% lactic acid were chosen from the general solvent for the dissolution test of biocompatible metals, and the corrosion resistance of titanium and nitrided titanium was compared. The dissolution depends on the material and also surface roughness. The effect of surface roughness would be more influenced in acid etching. Therefore, fine mechanical finishing was performed for lactic acid dipping to compare the dissolution properties of the materials.

The dissolved amount of titanium in SBF was the background level, as low as the detection limit of ICP (Fig. 6). The dissolved amount of titanium in titanium and nitrided titanium in the 1.0% lactic acid showed no significant difference after 10 and 30 days immersion (Fig. 7). The corrosion resistance of titanium nitride was excellent and similar to titanium. The surface of nitrided titanium formed a minute chemical compound layer, therefore, it is suggested that the corrosion resistance was improved in comparison with the titanium[15]. In the present study, no significant difference could be found between titanium and nitrided titanium. This may be due to the roughened Ra after nitriding, and minute cracks induced during the cooling process after heating at 850℃ through which titanium might be dissolved out.

Wettability and S.mutans adhesion

In general the adhesion mechanism of bacteria to a solid surface is affected by nonspecial factors such as the physiochemical binding power of the hydrophobic interaction, the electrostatic interaction and the peculiar interaction by glycoproteins of the bacterial surface layer[30,31]. In the oral cavity environment, the surface material is covered with saliva and serum, and the force to detach bacteria from the surface material by caloric intake and tongue motion always works. Therefore, it is

difficult to examine the adhesive properties of bacteria to surface materials *in vivo*. In the present study, wettability of the surface material was examined by contact angle and adhesion of bacteria *in vitro*.

The contact angle was about 60° and there was no significant difference between titanium and nitrided titanium in wettability (Fig. 8). The quantity of *S.mutans* adhesion, evaluated by absorptiometry, showed no significant difference between titanium and nitrided titanium (Fig. 9). The surface free energy of a solid surface such as a tooth and various restorative materials is low, when the contact angle is large[32]. The quantity of plaque adhesion to a solid surface *in vivo* correlates with the solid surface free energy, and the formation of plaque is small on a surface with small surface free energy[33-35]. These findings suggest that based on the nearly equivalent surface free energy, the wettability of titanium and nitrided titanium were similar.

Biocompatibility

1) Biocompatibility in soft tissue

In the examination of the bioreaction in soft tissue (subcutaneous tissue) of titanium and nitrided titanium, the biocompatibility of both cylindrical specimens and fine particles were investigated. Although titanium specimens of macro size such as the cylindrical implants showed good biocompatibility, fine particles as small as a cell induced cytotoxicity, as reported previously[16,17]. The abrasion resistance of nitrided titanium was reported to be significantly much higher than titanium. The simulated experiments showed that there would be almost no damage on the surface material under general clinical conditions, when nitrided titanium is used as an abutment of dental implants[21]. By the using an ultrasonic scaler or metallic scaler, however, some of the nitrided layer may be detached from the surface and enter the gingival tissue. If the toxicity of abraded powders is high, it may bring about harmful results even if the abrasion resistance is high. Therefore, the biocompatibility of fine particles was also examined in addition to that of cylindrical specimens in this study.

In the case of cylindrical specimens, titanium and nitrided titanium were encapsulated with fibrous connective tissue in which fibroblasts were observed, and no inflammatory response was observed (Fig. 10). In both specimens, the fibrous connective tissue at 8 weeks became thinner than at 4 weeks (Fig. 10). In the tissue response to fine particles, both titanium and nitrided titanium induced phagocytosis by macrophages and inflammation continuously, but no significant differences beyween titanium and nitrided titanium could be recognized (Fig. 11). These results suggest that the tissue reaction to both macroscopic size and fine particles of nitrided titanium were nearly equivalent to those of titanium.

2) Biocompatibility in hard tissue

The abutment of implants has been connected with fixtures of implants, and it is close to the bone which supports the fixture. Therefore, it is necessary to use material without harmful effects on the bone for the abutment. Pure titanium is used for the abutment at present. To use nitrided titanium as the abutment part, it is neces-

sary to confirm its biocompatibility for hard tissue. In this study, biocompatibility in hard tissue was evaluated by comparing the quantity of new bone formation and the ratio of bone in direct contact with the implant surface.

In observations by optical microscopy, no inflammatory reactions could be recognized in either specimens. New bone was in contact in both specimen surfaces, and the thickness of new bone was also almost equivalent. In the quantitative measurement by image analysis, the area ratio of new bone formation and the ratio of bone in contact with the specimens, were increased significantly between 4 and 8 weeks. There were no significant differences between both specimens for each period.

The chemically very stable properties of titanium nitride, especially the excellent corrosion resistance which may be better than titanium, would contribute to its biocompatibility in both soft and hard tissue.

Therefore, the application of nitrided titanium was suggested from the view point of osteocompatibility for the parts which require both biocompatibility and abrasion resistance such as an abutment of dental implants and the sliding part of artificial joints.

CONCLUSIONS

To examine its possible use as an implant material, especially for the abutment, corrosion resistance, wettability of the surface, quality of *S.mutans* adhesion and biocompatibility, tests of nitrided titanium were carried out and the following findings were obtained.

1. The corrosion resistance of titanium nitride was excellent and similar to those of titanium.
2. The wettability of the surface and quality of *S.mutans* adhesion showed no significant difference between titanium and nitrided titanium.
3. The biocompatibility of nitrided titanium in soft and hard tissue suggested that tissue reactions in soft tissue and the formation of new bone in hard tissue were excellent, and equivalent to those of titanium.

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Mechanical Properties of Surface Nitrided Titanium for Abrasion Resistant Implant Materials

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In order to verify its application for abrasion-resistant implant materials such as abutment in dental implants and artificial joints, mechanical properties of surface nitrided titanium were evaluated by three different tests, the Vickers hardness test, Martens scratch test and ultrasonic scaler abrasion test. The Vickers hardness of a nitrided layer of 2 μm in the thickness was 1300, about ten times higher than that of pure titanium. The Martens scratch test showed high bonding strength for the nitrided layer with matrix titanium. The abrasion test using an ultrasonic scaler showed very small scratch depth and width, demonstrating extremely high abrasion resistance. The results show that a surface-nitrided titanium has sufficient abrasion resistance if it is used under clinical conditions.

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Keywords: *biomaterial, implant, titanium, surface reforming, titanium nitride, abrasion resistance, hardness*

1. Introduction

Titanium is the most commonly used as an implant material at present. Its surface has a passive film formed in stable oxides working for biocompatibility under the severe environment *in vivo*.[1-4] However, titanium also has weak points. One of them is its low Young's modulus, about half that of iron, nickel and chromium. Another is low abrasion resistance.[5] Minute titanium abrasion powder may cause an inflammatory reaction.[6,7] Therefore it is desirable to develop implant materials with both biocompatibility and abrasion resistance.

Titanium nitride is known for its high surface hardness and mechanical strength. It has also been reported that the dissolution of Ti ions from titanium nitride is very low.[8,9] Considering these facts, the surface nitriding method, one of the surface treatments for metallic material,[10,11] seems useful. However, research on titanium nitride to evaluate its physical properties for application to implants has not very often been carried out. Dental implants are composed of various components. The implant abutment division (the mucosa penetration division) is exposed in the oral cavity, and then plaque and dental calculus adhere on it. Removal of them is a necessary requirement to obtain a good prognosis throughout the long-term maintenance of the implant. Therefore, the implant abutment division should have abrasion resistance to scaling treatment.

In this study, surface properties and mechanical properties of surface-nitrided titanium were evaluated by three different tests in either static or dynamic manner, *i.e.*, the Vickers hardness test, Martens scratch test and ultrasonic scaler abrasion test to verify its application for abutments in dental implants.

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

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2. Materials and Experimental Methods

2.1 Sample preparation

JIS type 1 pure titanium plates (10 × 10 × 0.5 mm: KOBE STEEL LTD, Kobe, Japan) were used as specimens. The specimens were prepared with two different types of surface finishing, those polished with #2000 waterproof paper (#2000 specimens) and those polished with 6 μm diamond emulsion (BUEHLER, USA) (6 μm specimens). The nitriding of these specimens was done under a N_2 atmosphere of 1 atm at 850°C for 7 hours.

2.2 Evaluation of surface quality

2.2.1 Observation

The surface structure was observed by the atomic force microscope (AFM) (TMX-2000 Explorer, TopoMetric, Santa Clara, USA). The cross section of the surface-nitrided titanium was observed by SEM (S-4000N, HITACHI, Tokyo, Japan) after the specimen was cut and polished.

2.2.2 Roughness measurement

For the #2000 specimens, a surface roughness measurement machine (Surf Com 200C, TOKYO SEIMITSU, Tokyo, Japan) was used. On the 6 μm specimens, AFM was used with scaning area 50 μm × 50 μm and scanning rate of 5 μm/min. Measurement was done 5 times for each specimen and the average height deviation from the mean plane (Ra) was obtained.

2.3 Hardness test

The Vickers hardness was measured with a micro-Vickers hardness tester (NT-M001, SHIMAZU, Kyoto, Japan) under an applied load of 0.49N–4.9N for 10 sec. Indentation depths for different loads were calculated from the shape of the indenter.

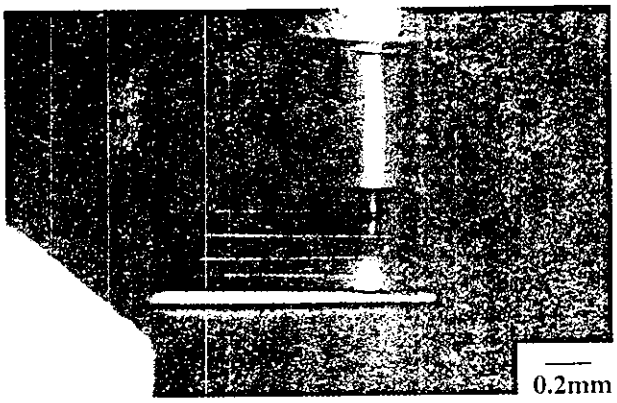

Fig. 1 Martens scratch tester equipment.

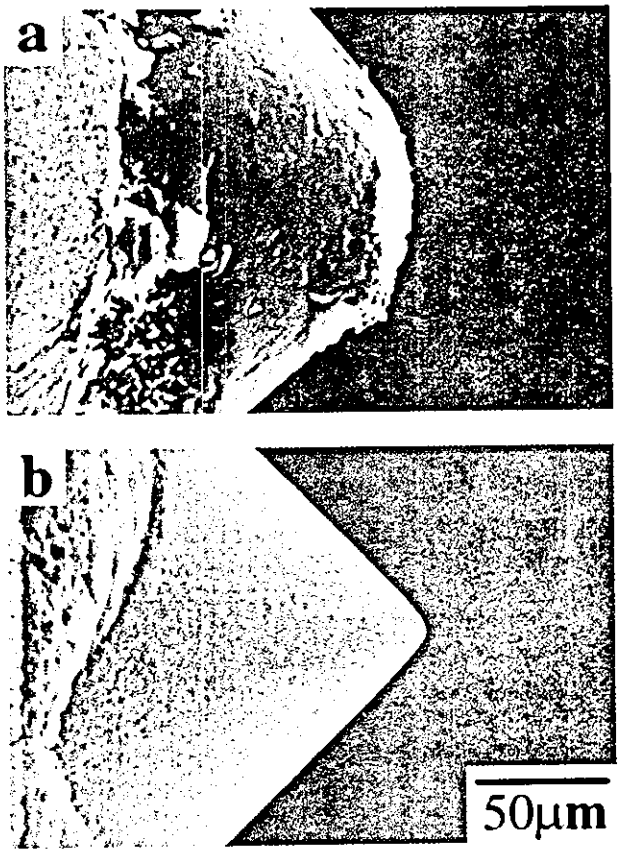

Fig. 2 Indenter of Martens scratch tester observed by SEM: with radius of curvature of 50 μm (a) and 5 μm (b).

2.4 Martens scratch test

The scratch tests were carried out with a Martens scratch tester (IMOTO, Tokyo, Japan), and the specimens were fixed on the specimen mount (Fig. 1). For the #2000 specimens, the tests were done under loads of 0.49, 0.98, 2.45, 4.9 and 9.8N using an indenter with a 50 μm radius of curvature (Fig. 2), and for the 6 μm specimens, under the loads of 0.098, 0.245, 0.49, 0.98 and 2.45N using an indenter with a 5 μm radius of curvature (Fig. 2). The scratch traces were observed with a digital microscope (VH6300, KEYENCE) and the cross-section profiles were obtained with the surface roughness measurement machine. The width and depth of the trace were used for evaluation and comparison.

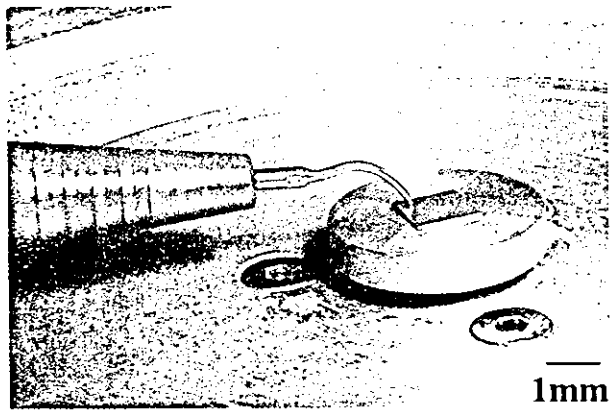

Fig. 3 The abrasion test equipment using a dental ultrasonic scaler.

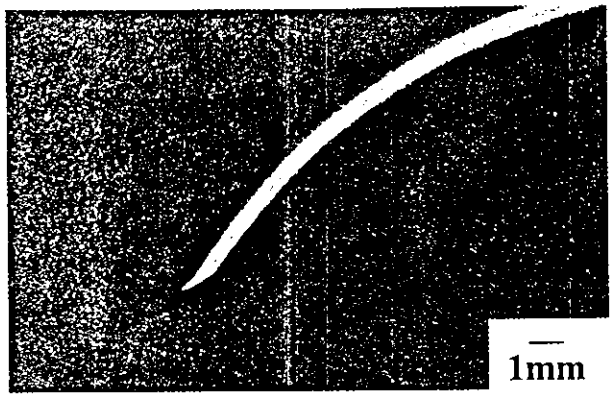

Fig. 4 Dental ultrasonic scaler tip.

2.5 Ultrasonic scaler abrasion test

After the specimen was fixed on a rotating base as shown in Fig. 3, the tip of a dental ultrasonic scaler (OSADA ENAC type 5, OSADA, Tokyo, Japan) shown in Fig. 4 was loaded on the specimen surface at 0.49, 0.98, 2.45 or 4.9N. The specimens were rotated for 30 seconds at the speed of 100 min^{-1}. The abraded trace was observed with a digital microscope. Surface roughness was measured with a surface roughness measurement machine, and the cross sectional area of the abraded trace was deduced. Trace depth and width were measured for top to bottom and top to top of tubercles formed by the abrasion test.

3. Results

3.1 Evaluation of surface quality

Figure 5 shows the typical surface structures of pure titanium (a) and surface-nitrided titanium (b) observed by AFM. On titanium, the roughness formed by polishing was observed nearly parallel in one direction. Surface-nitrided titanium had roughness with an irregular form.

Figure 6 shows a cross section of a nitrided layer observed by SEM. The nitrided layer was about 2 μm thick on the titanium.

The Ra of the #2000 specimens measured by the surface roughness measurement machine was 0.24 μm (standard deviation: 0.001) for the titanium, and 0.44 μm (0.024) for the titanium nitride. The Ra of the 6 μm specimens measured by AFM was 0.04 μm (0.001) for the titanium, and 0.08 μm

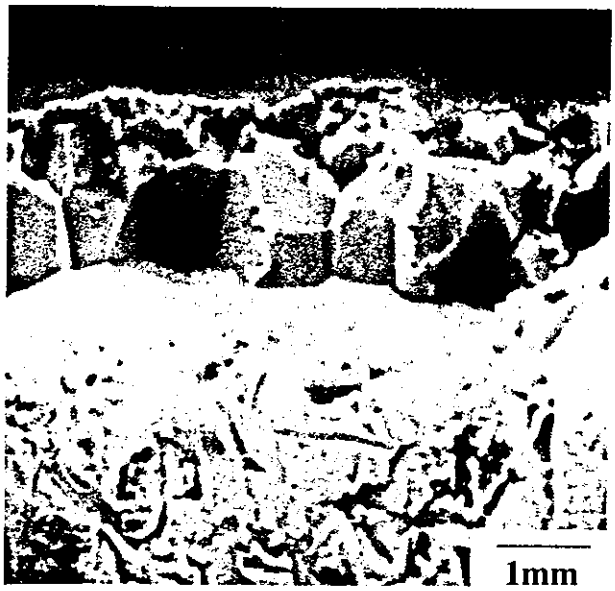

Fig. 6 Cross section of nitrided layer observed by SEM.

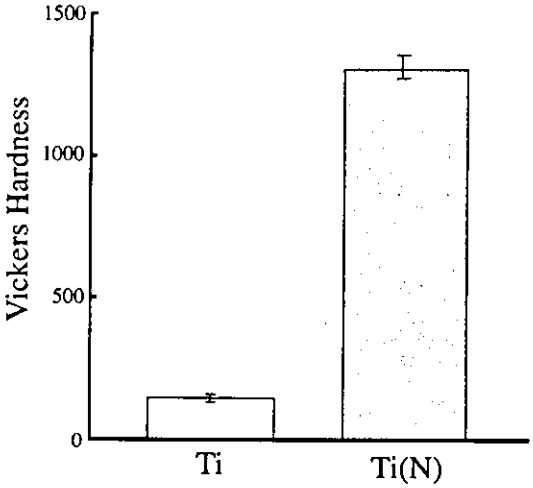

Fig. 7 Vickers hardness of titanium and surface nitrided titanium with a load 0.49N.

(0.002) for the titanium nitride. The surface of titanium nitride was rougher than that of titanium.

3.2 Hardness test

Figure 7 shows the results of Vickers hardness tests under a load of 0.49N. The mean value of the titanium was 146 (SD: 3.5), and for titanium nitride it was 1308 (29.4). Titanium nitride had an about 10 times higher value than titanium.

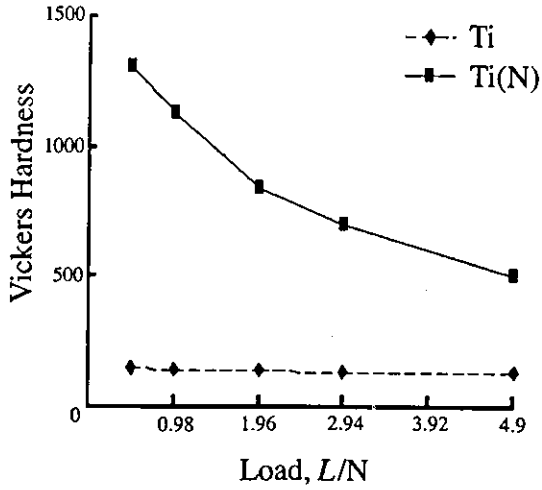

Fig. 8 Load dependence of Vickers hardness.

Figure 8 shows the load dependence of Vickers hardness. For titanium, these values did not change greatly. Vickers hardness of titanium nitride decreases with increasing the load, approaching the value of titanium.

Figure 9 shows the indentation depth formed by the Vickers hardness test as a function of load. The indentation depth increased with an increase in load on each specimen. The depth for titanium nitride was smaller than that for titanium, and it exceeded the nitride layer thickness of about 2 μm at loads higher than 1.96N.

3.3 Martens scratch test

Figure 10 shows a scratched trace after the Martens scratch test (#2000 specimens). The width of the scratched trace widened with the increase of load. The widths for titanium nitride were narrower than those for titanium at identical loads.

Figures 11 and 12 show cross-section profiles after the Martens scratch test. The trace depth and width increased with an increase in load. For the test using a 50 μm indenter (Fig. 11) tubercles on surfaces were recognized on both sides of the trace at loads higher than 2.45N for titanium. For titanium nitride, however, traces were not observed at loads of 0.49 and 0.98N. For the test using the 5 μm indenter no tubercle was observed on either side of the scratched trace at loads of 0.098, 0.245 and 0.49N for titanium nitride.

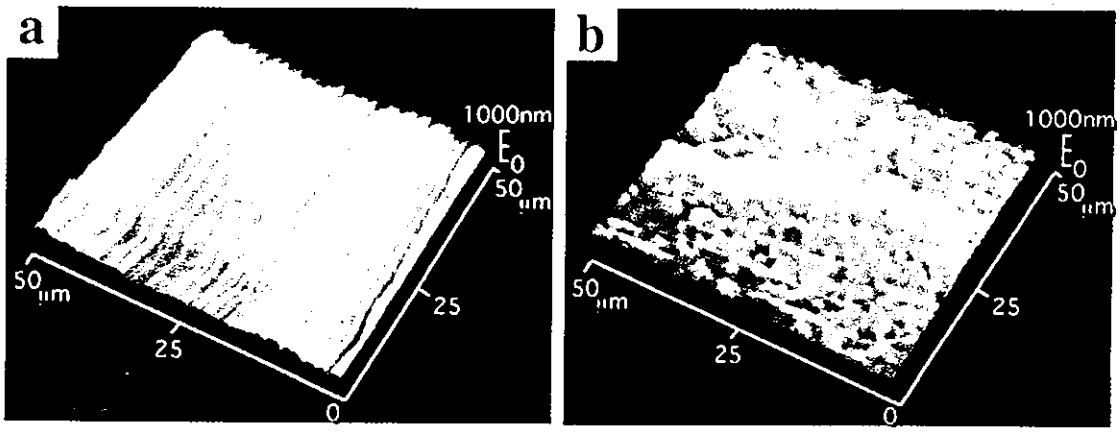

Fig. 5 Surface structure observed by AFM. (a) before nitriding, (b) after nitriding.

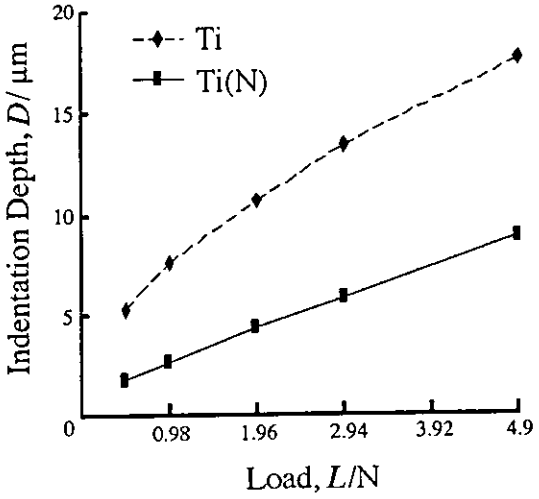

Fig. 9 Indentation depths formed by Vickers hardness tests with different loads.

Figure 13 shows cross sections of scratch traces after the Martens scratch tests observed by SEM. The lower parts with dark contrast are the specimen. The upper parts are the embedding material. The cross-sectional shapes of the scratched trace depth and width in Fig. 13 correspond to those obtained using surface roughness measurement machine shown in Figs. 11 and 12. The depth of the scratched trace of titanium nitride was much smaller than that of titanium.

Figures 14 and 15 show the load dependence of trace depth and width, respectively. Both the trace depth and the width increased with an increase in the load. With the use of a 50 μm indenter, a recognizable trace was observed at loads higher than 2.45N on the #2000 titanium nitride specimens. With the use of a 5 μm indenter, for the 6 μm titanium specimen, the trace depth rapidly increased in a parabolic manner in the low-loaded regions less than 0.49N and was then saturated for the loads higher than 0.98N. The value approached to that obtained with the 50 μm indenter, and was almost equivalent to 2.45N. The difference in the trace width between titanium nitride and titanium in the small load regions lower than 0.98N was remarkable for both the 5 μm and 50 μm indenters. When the load increased, the difference was smaller, and the value of titanium nitride became closer to that of titanium.

3.4 Ultrasonic scaler abrasion test

Figure 16 shows a surface scratched by hand using a dental ultrasonic scaler. Titanium had a clear trace, while a trace was hardly formed in titanium nitride.

Figure 17 shows an appearance of a surface after the abrasion test. For titanium, clear traces were recognized for each load. For titanium nitride, a trace was hardly formed at the load of 0.49N. Black traces were observed at loads higher than 0.98N.

Figure 18 shows the cross sectional profiles after the abrasion tests shown in Fig. 17. For the load of 0.49N, tubercles of scaling were confirmed on titanium. The trace depth was about 9 μm. For titanium nitride, a trace was hardly observed, and no change of the surface roughness could be recognized. For 4.9N, the tubercles were observed on both sides of the trace on titanium. The trace depth was about 25 μm. For titanium nitride, the trace was barely observed and there were no tubercles. The trace depth was about 2.5 μm, about one-tenth that of titanium.

Figure 19 shows the load dependence of the trace cross section area after the dental ultrasonic scaler abrasion test. For titanium, the cross sectional area increased with an increase in the load. For titanium nitride, the increase of the cross section was very small.

4. Discussion

4.1 Formation of nitrided layer

Titanium was nitrided by the gas nitriding method in the present study. For both the #2000 and 6 μm specimens that were surface-finished before nitriding, the surface roughness for titanium nitride was larger than that for titanium. The nitrided layer was a mixture of TiN and Ti_2N as revealed by X-ray diffraction.[12]

Takamura[13] reported that the minute chemical compound layer mainly consists of TiN formed on the surface, followed in the inside by a hard layer of α-Ti hardened by solid-solution hardening of nitrogen. The thicknesses of the chemical compound layer and hard layer are almost proportional to the square root of the nitriding time at a constant temperature. When nitriding was carried out at 1123 K for 16 h, the thickness of the chemical compound layer was about 3 μm, and that of the hard layer was about 50 μm. In the present study, the thickness of the nitrided layer was about 2 μm, which is slightly smaller due to the difference of nitriding time.

4.2 Evaluation of the mechanical properties at the surface

The Vickers hardness test is a rather static indentation hardness test, one of the most fundamental evaluation methods. The ultrasonic scaler abrasion test is a dynamicaly repeated abrasion test. The Martens scratch test has intermediate features of both and is an index to the resistance to peeling off of the coating layer from the base metal. The relations of these three tests are summarized in Fig. 20. As Vickers hardness become higher from titanium to titanium nitride, the Martens scratch depth is decreased and the abrasion resistance is increased, indicating that the bonding strength of the nitrided layer to matrix titanium was sufficiently high.

4.3 Hardness

Ito *et al.*[10] reported that the Vickers hardness of titanium nitride was 1170 and Nukata[14] reported it to be 1370. The measurement conditions for Vickers hardness and nitriding conditions in the reports were slightly different from the present ones. Since the surface hardness of the present specimens was about 1300, nitriding seemed to be sufficiently occur. For the dependence of the indentation load on the Vickers hardness, the value does not change greatly in titanium (Fig. 8). In titanium nitride, the Vickers hardness was the highest at the load 0.49N and decreased with an increase in load. This was mainly because the indentation depth at the load of 0.49N was about 2 μm (Fig. 9) where indentation was completed within the nitride layer, while with an increase in load the indentation depth exceeded the thickness of the nitrided layer as seen in Fig. 6, and reached the softer matrix of titanium. For a larger range of loads, the indentation depth

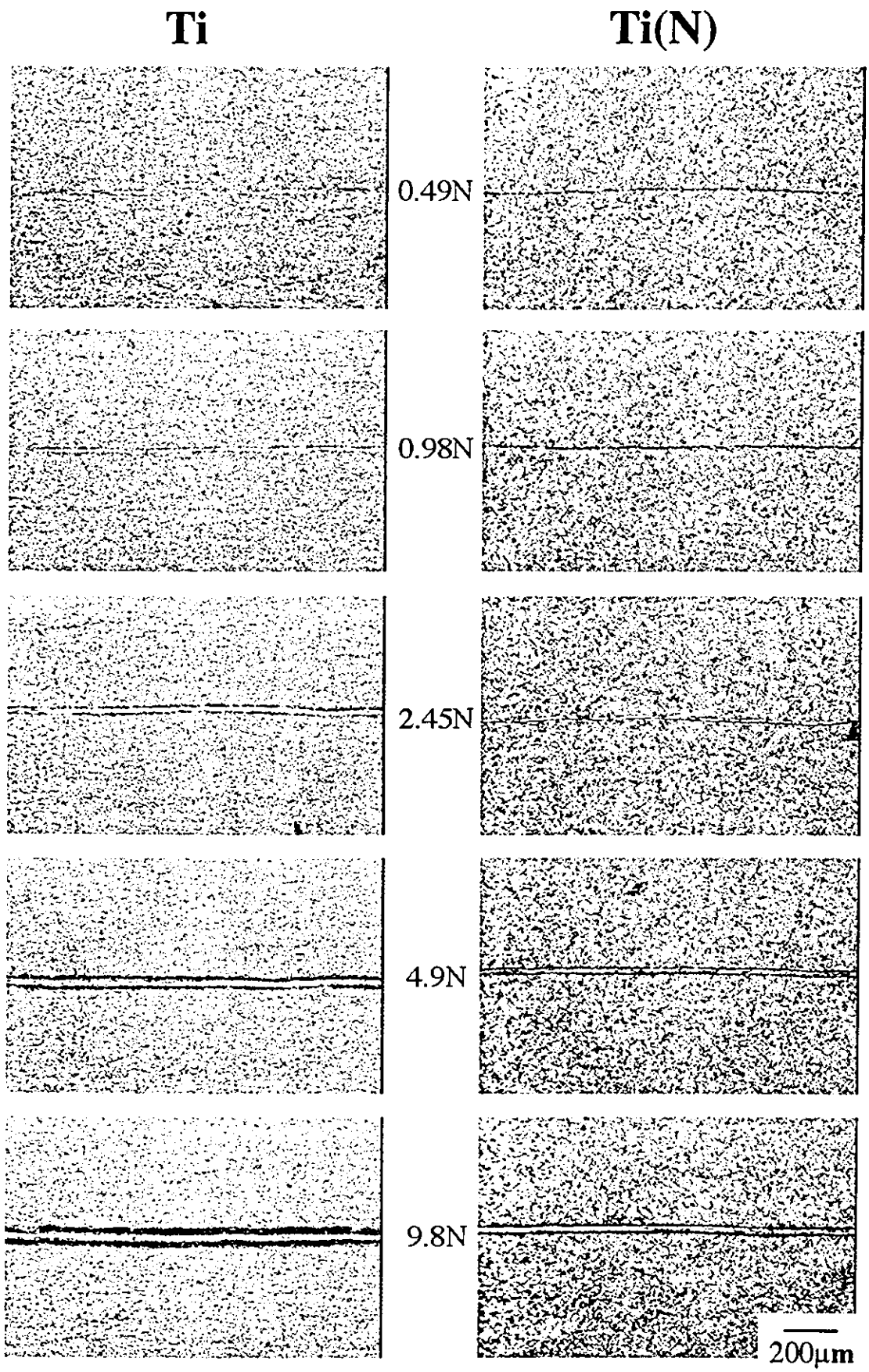

Fig. 10 Scratched traces after Martens scratch tests using a 50 μm indenter on #2000 surface.

of the titanium nitride was, however, still smaller than that of titanium, since the penetration of the indenter was suppressed by the nitrided layer.

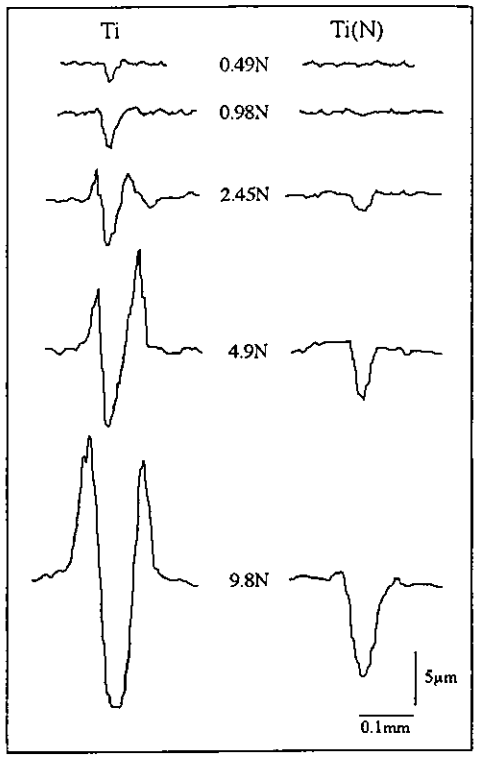

Fig. 11 Cross section profiles of traces after Martens scratch tests Fig. 10.

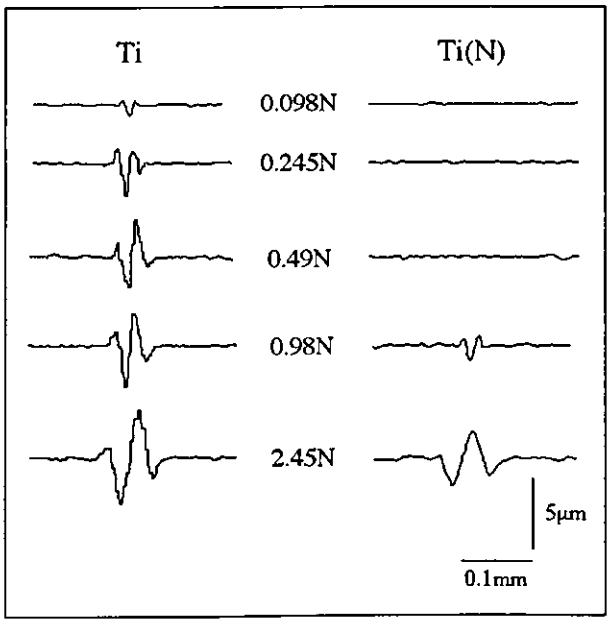

Fig. 12 Cross section profile of traces after Martens scratch tests using a 5 μm indenter on 6 μm specimens.

4.4 Martens scratch test

Martens scratch hardness is usually expressed as the load forming a scratched trace with 0.01 mm width. If this definition is applied in the present case, the test would measure mostly the hardness of matrix titanium, since the surface-nitrided layer was very thin (2 μm). Therefore the depth and width of the scratched trace were used to evaluate Martens hardness.

The trace depth-load curve for titanium using the 5 μm indenter, shown in Fig. 14, has a parabolic form. According to Meyer's law, hardness H is expressed as $H \propto P/d^2$ where P is the load and d is the diameter of the indenter. If hardness H of the tested materials are the same, this equation leads to the relation $d \propto P^{1/2}$. The apex of the tip of the 50 μm indenter is rounded as seen in Fig. 2 and the Meyer' law cannot he applicable when the indentation depth is light. On the other hard the apex of the tip of the 5 μm indenter is so sharpened that the Meyer' law works well. Under this condition the parabolic relation $d \propto P^{1/2}$ clearly appeared as seen in the data for titanium using the 5 μm indenter in Fig. 14.

The depth of the scratched trace of titanium nitride was much smaller than titanium at each load (Fig. 14). This is because the penetration of the indenter was suppressed by the existence of the very hard nitrided layer. A recognizable trace in the nitrided titanium was not observed until 2.45N for the 50 μm indenter and 0.98N for the 5 μm indenter having a more acute angle. As for the trace width (Fig. 15), a large difference between titanium nitride and titanium was observed in the small load region for both the 50 μm and 5 μm indenters. As the load increased, the difference became smaller, and the value of titanium nitride approached to that of titanium. This was because the indentation depth exceeded the thickness of about 2 μm nitrided layer formed on the surface and reached the softer matrix titanium.

These results showed that layer of the titanium nitride was combined with the pure titanium base metal with sufficient bonding strength, since it was hardly exfoliated by the scratch test.

4.5 Abrasion resistance

In dental treatment, when deposits such as dental calculus and plaque adhere on the surface of natural teeth, they are generally removed using a metallic scaler and an ultrasonic scaler in order to maintain healthy conditions for the teeth and periodontium. On dental implants, deposits such as dental calculus have a tendency to adhere to the abutment division. Although pure titanium with specularity polishing treatment is used for the abutment division, it is often necessary to remove deposits to maintain the implant throughout long-term usage. The surface hardnesses of titanium and titanium alloys are, however, much lower than that of the natural tooth. For example, the Hv of the enamel part of the tooth is about 400. When the implant abutment division is scaled using a metallic scaler and ultrasonic scaler, the abutment surface suffers damage. This causes a vicious cycle in which the plaque easily stagnates in the damaged part and dental calculus deposits again. Therefore, the removal of dental calculus is generally carried out using a plastic scaler at present.

However, it is very difficult for the operator to place the scaler accurately and remove dental calculus, since the form of the abutment is cylindric and the surface is glossy. The application of the instrument to the lingual surface is especially difficult because the form of the superstructure is completely different from the natural tooth. To resolve this problem, abrasion-resistant materials with biocompatibility are necessary for the abutment division. A test with a dental ultrasonic scaler used in a general clinic was thus carried out to evaluate the abrasion resistance of titanium nitride quantitatively. After the cross-sectional profile was measured, the area of the cross-sectioned trace was used for evaluation rather than the

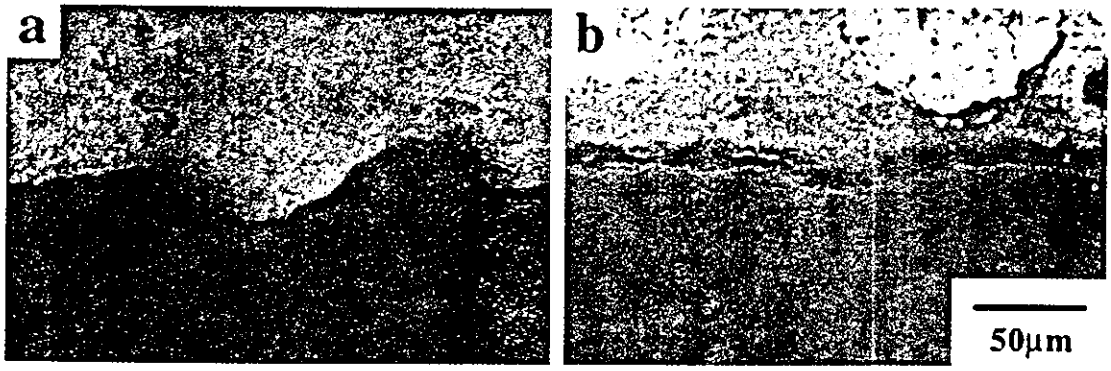

Fig. 13 Cross section of scratch trace observed by SEM after Martens scratch test using a 50 μm indenter with a 9.8N load: Lower side with dark contrast is the specimen. (a) titanium, (b) nitrided titanium.

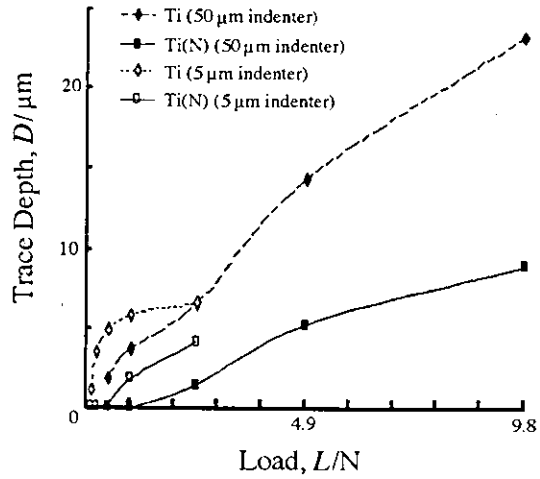

Fig. 14 Load dependence of the trace depth by Martens scratch test.

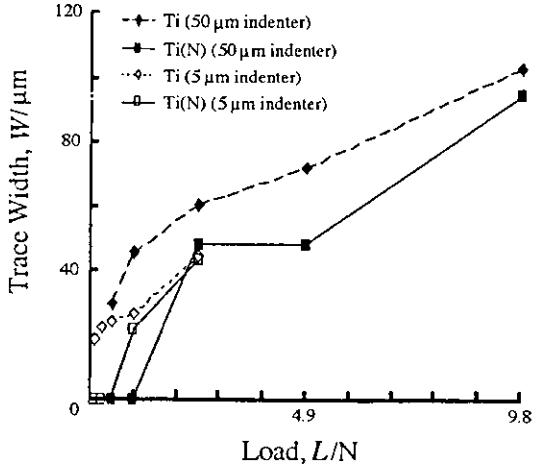

Fig. 15 Load dependence of the trace width by Martens scratch test.

depth of abrasion, since the abrasion trace moved in the radial direction during the abrasion test and the integration of these traces reflected the total abrasion more accurately.

In the cross-section profile after the abrasion test, the trace and the tubercles on both sides of the trace were observed at each load for titanium (Fig. 18). With titanium nitride, the trace was hardly observed at the loads of 0.49 and 0.98N, since a hard nitrided layer existed on the titanium nitride surface and the penetration of the probe tip was obstructed. Traces were observed at loads of 2.45 and 4.9N, but there

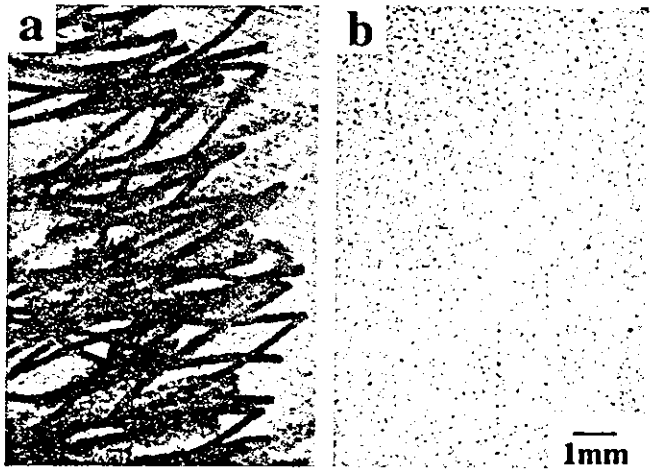

Fig. 16 Specimen surfaces scratched by hand using a dental ultrasonic scaler. (a) titanium, (b) surface-nitrided titanium.

were no tubercles such as observed on titanium. The trace depth was about 1/10 that of the titanium at 4.9N. A black trace was observed at loads greater than 0.98N. This was considered to be the abraded powders of stainless steel of the probe tip, which was softer than TiN, because the profile of the cross section had no deepened trace on the specimen.

In dental clinics, the operation to remove the dental calculus by ultrasonic scaler is carried out with light pressure of about 0.49N. In the results shown in Fig. 19, a clear trace was observed at the 0.49N load in titanium, but no trace was observed in titanium nitride. Therefore, for clinical use, it is anticipated that there would be very little damage on the abutment surface made of nitrided titanium.

4.6 Clinical application

In order to examine the biocompatibility of surface-nitrided titanium, hypodermic implantation of cylindrical implants and fine particles into the rat abdomen was carried out, and biocompatibility nearly equivalent to that of pure titanium was confirmed.[12]

The mechanical properties and fracture toughness of the implant material should be close to those of bone. The surface-nitrided titanium tested in this study was very hard, and the inside base metal, pure titanium, had mechanical properties closer to bone. Therefore, surface-nitrided titanium is more suitable for implants than whole titanium nitride. The

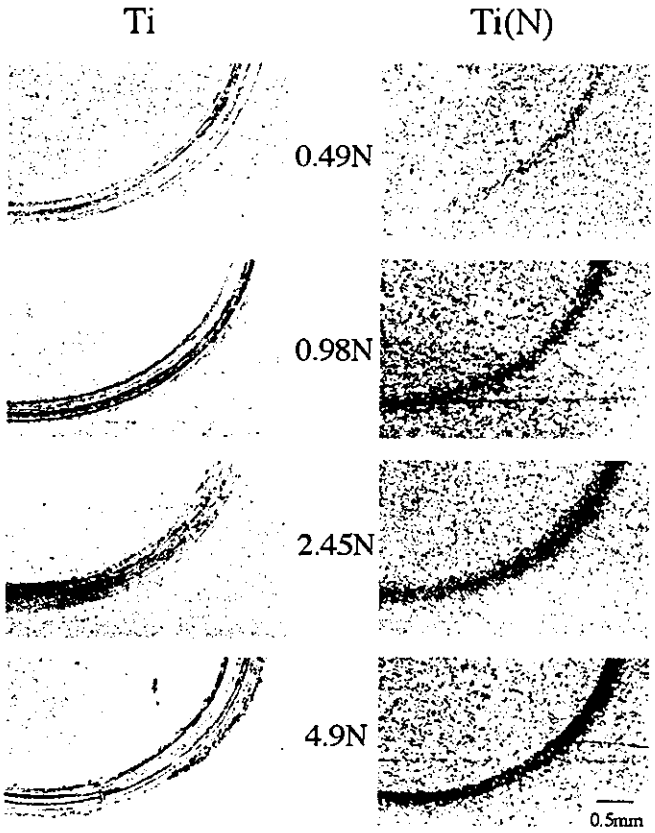

Fig. 17 Specimen surfaces after abrasion tests using a dental ultrasonic scaler.

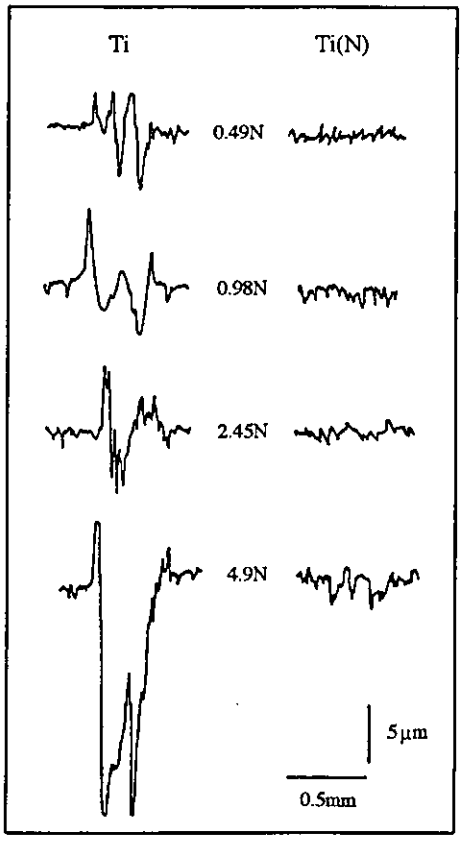

Fig. 18 Cross sectional profiles after the ultrasonic scaler abrasion tests.

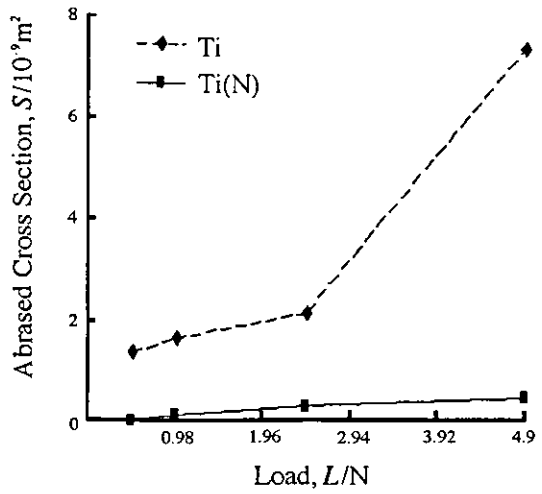

Fig. 19 Load dependence of the trace cross section area after the ultrasonic scaler abrasion test.

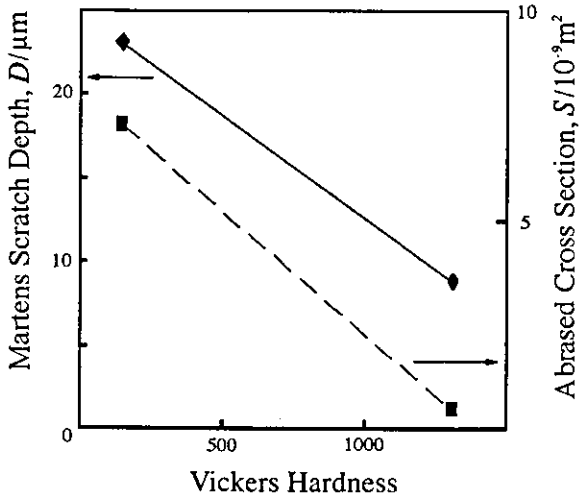

Fig. 20 Relation of Martens scratch depth and abraded cross section by ultrasonic scaler with Vickers hardness.

thickness of the nitrided layer can be varied by changing the nitriding conditions. In the abrasion resistant test, there was almost no damage of the surface under the conditions assuming the clinical application and the thickness 2 μm would be sufficient against abrasion in the practical use. It was also confirmed that the bonding strength of the coating layer to matrix was sufficiently high based on the Martens scratch test. When the thickness of the nitrided layer exceeds 2 μm, the coating layer may become easily exfoliated from the base metal due to the lack of coherency at the interface. Therefore, it is considered that the 2 μm thickness of the nitrided layer is adequate for clinical usage.

These facts suggest that surface-nitrided titanium, which has very high abrasion resistance under clinical conditions, is promising for dental implants especially for abutments. Application in orthopaedics was also suggested for the sliding parts of artificial joints in which abrasion powders cause cytotoxicity.

5. Conclusions

(1) To quantitatively evaluate the characteristics of surface-nitrided titanium under clinically used conditions, the abrasion-resistance test with a dental ultrasonic scaler was developed. The Vickers hardness test and Martens scratch test, which are more basic methods for evaluation of mechanical properties were also carried out, and the relations among the tests were examined.

(2) A nitrided layer on titanium with a thickness of 2 μm showed Vickers hardness of about 1300, about 10 times higher than that of pure titanium and was strongly bonded with the pure titanium base metal.

(3) In both the Martens scratch test and abrasion-resistance test with a dental ultrasonic scaler, the trace depth and trace width of titanium nitride were smaller than those of pure titanium. Titanium nitride showed very high abrasion resistance and it was estimated that there would be almost no damage on the surface under clinically used conditions.

(4) Surface-nitrided titanium is a promising material for dental implants, especially for abutments and sliding parts of artificial joints.

Acknowledgments

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Effects of Particle Size on Cell Function and Morphology in Titanium and Nickel

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The dependence of cytotoxicity on particles size in titanium (Ti) and nickel (Ni) was investigated by biochemical functional analysis and by microscopic observation of cellular morphology, *in vitro* using human neutrophils as probes and *in vivo* in animal implantation test. The biochemical analyses of cell survival rate, LDH, superoxide anion, cytokines of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and observation by scanning electron microscopy (SEM) showed that Ti fine particles (2 μm) stimulate neutrophils and increases the quantity of released superoxide anions, whereas Ni particles deform or disrupt the cell membrane of neutrophils. The 2 μm Ti particles, smaller than neutrophils of about 5–10 μm, were phagocytized by cells *in vivo* and the results were similar *in vitro*, which lead to the remarkable release of TNF-α. These results showed that there is the size-dependent cytotoxic effect in Ti fine particles and the effect is the most pronounced when they are smaller than cells. On the other hand, Ni particles caused the disruption of neutrophils *in vitro* and necrosis of tissue *in vivo* mainly through ions produced by their dissolution.

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Keywords: *titanium, nickel, fine particle, neutrophil, superoxide, cytokine, cytotoxicity*

1. Introduction

The metal titanium (Ti) and its alloys are most commonly used in orthopedics and dentistry.[1,2] Titanium is highly corrosion-resistant at ambient temperature due to its thin and stable protective oxide layer formed on its surface and is one of the most biocompatible metals. In this sense Ti is the nearly ideal metallic material for implant.[3–7] However there are not only good properties but also short points. Although the bulk Ti is stable and biocompatible *in vivo*, some *in vivo* studies[8,9] found Ti pigmentation in soft tissue around Ti implants and plates. Our previous study with X-ray scanning analytical microscope (XSAM) revealed that Ti particles were included in the pigmented soft tissue. These Ti particles may be produced during the implanting and plating operation.[10]

Low abrasion resistance is one of the few short points of Ti. The abraded fine particles produced in its sliding parts of artificial joints often cause inflammation in the surrounding tissue.[10] However, little is known about the effect of metallic particles on cellular function and the relevance between *in vivo* and *in vitro* findings.[11] On the other hand, nickel (Ni) is used as an alloying element such as Ni–Ti for orthopedics and orthodontics, although nickel is known with its allergy and harmful effect to tissue.[12–14]

The present study examined the effects of fine Ti and Ni particles and their size dependence on the function and morphology of the human neutrophils to evaluate their cytotoxicity and biocompatibility *in vitro* and *in vivo*.

Human neutrophils, which play a central role in the initial stage of inflammation in a non-specific manner against foreign bodies, were used as probes. Particles smaller (0.4 μm, 2 μm, 5 μm) and larger (10 μm, 45 μm, 150 μm) than the neutrophils were used to determine the relationship between cell and particle size with respect to cytotoxicity. Then the effects of Ti particles and Ni particles were compared.

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2. Materials and Methods

2.1 Specimens

2.1.1 Metallic particles

99.9% pure Ti particles (JIS class 1type) of 2 μm (Soekawa Chemicals, Tokyo, Japan), 10 μm (High Pure Chemicals, Saitama, Japan), 45 μm (Sumitomo Sitix, Tokyo, Japan) and 150 μm (Sumitomo Sitix, Tokyo, Japan), and 99.9% pure Ni particles of 0.4 μm (Soekawa Chemicals, Tokyo, Japan) and 5 μm (Nilaco, Tokyo, Japan) were used and mixed with HBSS (Hanks' balanced salt solution) in concentration 2000 mg/ml, respectively.

2.1.2 Cells

Human peripheral blood was obtained from healthy volunteers in our group. Neutrophils were separated from blood using 6% isotonic sodium chloride containing the hydroxyethyl starch and lymphocyte isolation solution (Ficoll-Hypaque™, Amersham Pharmacia Biotech AB, Sweden). After metallic particle solution was kept at 37°C for two weeks, then neutrophils were added, and incubated at 37°C for 30 minutes, then they were used for various cell toxicity tests.[15]

2.2 Evaluation of cells and their functions

2.2.1 Cell survival rate

After stained with trypan blue, the cell population was counted under an optical microscope (Axioskop; ZEISS, Germany) using Thomas' hemacytometer.

2.2.2 Lactate dehydrogenase (LDH)

The LDH values of samples which included 10^6 neutrophils were measured using the lactate dehydrogenase C II-test (Wako Pure Chemical Industries, Osaka, Japan) and by spectrophotometry (HITACHI U-1100, Tokyo, Japan).

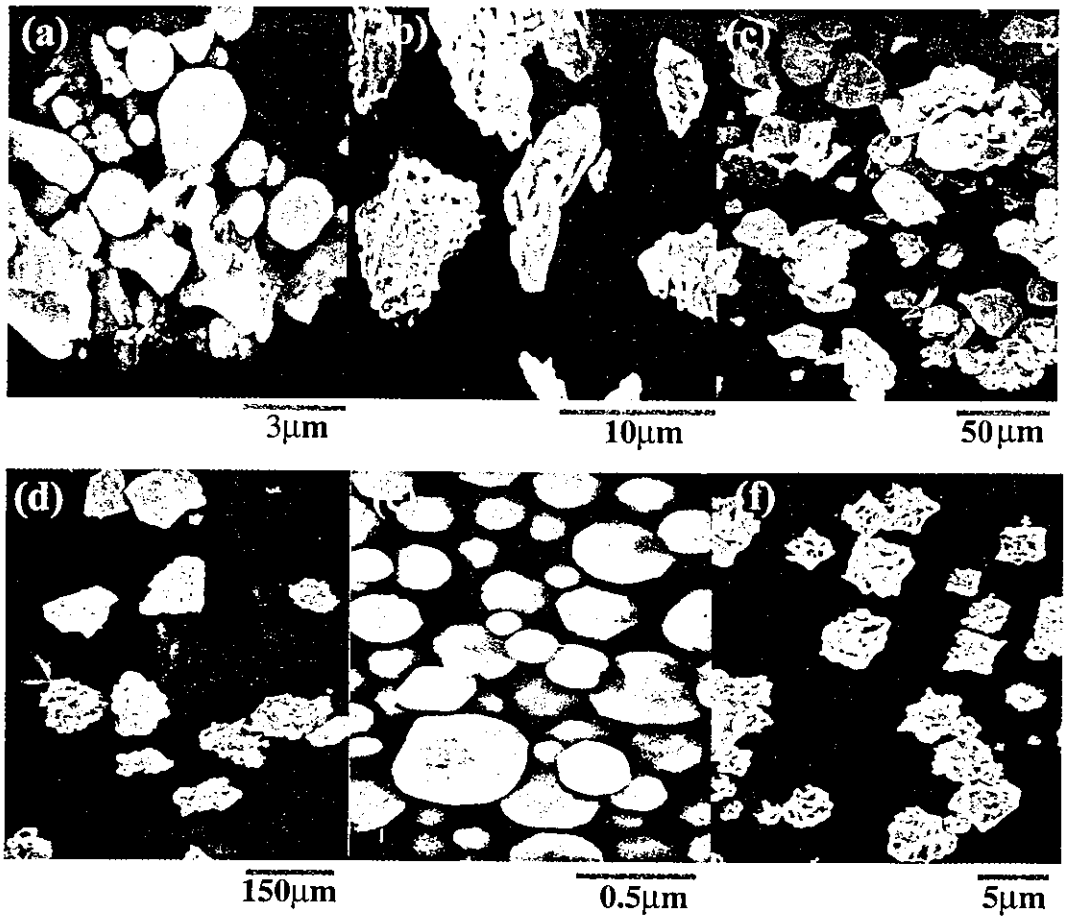

Fig. 1 SEM images of Ti 2 μm (a), 10 μm (b), 45 μm (c) and 150 μm (d) particles and Ni 0.4 μm (e) and 5 μm (f) particles.

2.2.3 Superoxide anion

Superoxide anion (O_2^-) production per 10^6 neutrophils was assayed by measuring the superoxide dismutase-inhibiting reduction of equine ferricytochrome C (550 nm). Specimens were incubated at 37°C for 5 min and the reaction was inhibited by adding PMA (139 mM).[16–18]

2.2.4 Cytokines (TNF-α and IL-1β)

Tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) production per 10^6 neutrophils in the supernatant was measured using ELISA kits (Endogen, Inc. USA).

For the measurement of 2.2.1 $\sim$ 4, the values are expressed as means $\pm$ standard deviation ($n = 6$). Data were analyzed by Student's t-test[19] with the level of significance set at 5%.

2.3 SEM observation

Neutrophils mixed with HBSS containing metallic particles were 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 were observed by scanning electron microscopy (SEM: HITACHI S-4300, Tokyo, Japan).

2.4 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. Ti or Ni particles were inserted into the subcutaneous connective tissue in the abdominal region. The wounds were then sutured. After one week of implantation the rats were sacrificed and blocks of connective tissue were extracted. 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).

3. Results

Figure 1 shows the SEM images of various sizes of Ti and Ni particles used for the experiment. The nominal size was 2 μm (a), 10 μm (b), 45 μm (c), 150 μm (d) for Ti particles and 0.4 μm (e), 5 μm (f) for Ni particles, respectively.

Figure 2 shows a histological image of rat soft tissue inserted with pure titanium particles of 2 μm (a) and nickel particles of 0.4 μm (b) for one week. The agglomeration of small black dots in the center area of Fig. 2(a) are Ti particles. Numerous inflammatory cells were observed in the surroundings. The macrophages or neutrophils show the degenerative changes in morphology. The enlarged view in Fig. 2(c) shows that Ti particles were phagocytized into the cytoplasm by an inflammatory cell. Figure 2(b) shows that Ni particles in the lower left area caused strong inflammation and necrosis. Figure 2(d) shows necrosis surrounding the region inserted Ni particles and (e) shows the strong inflammatory response

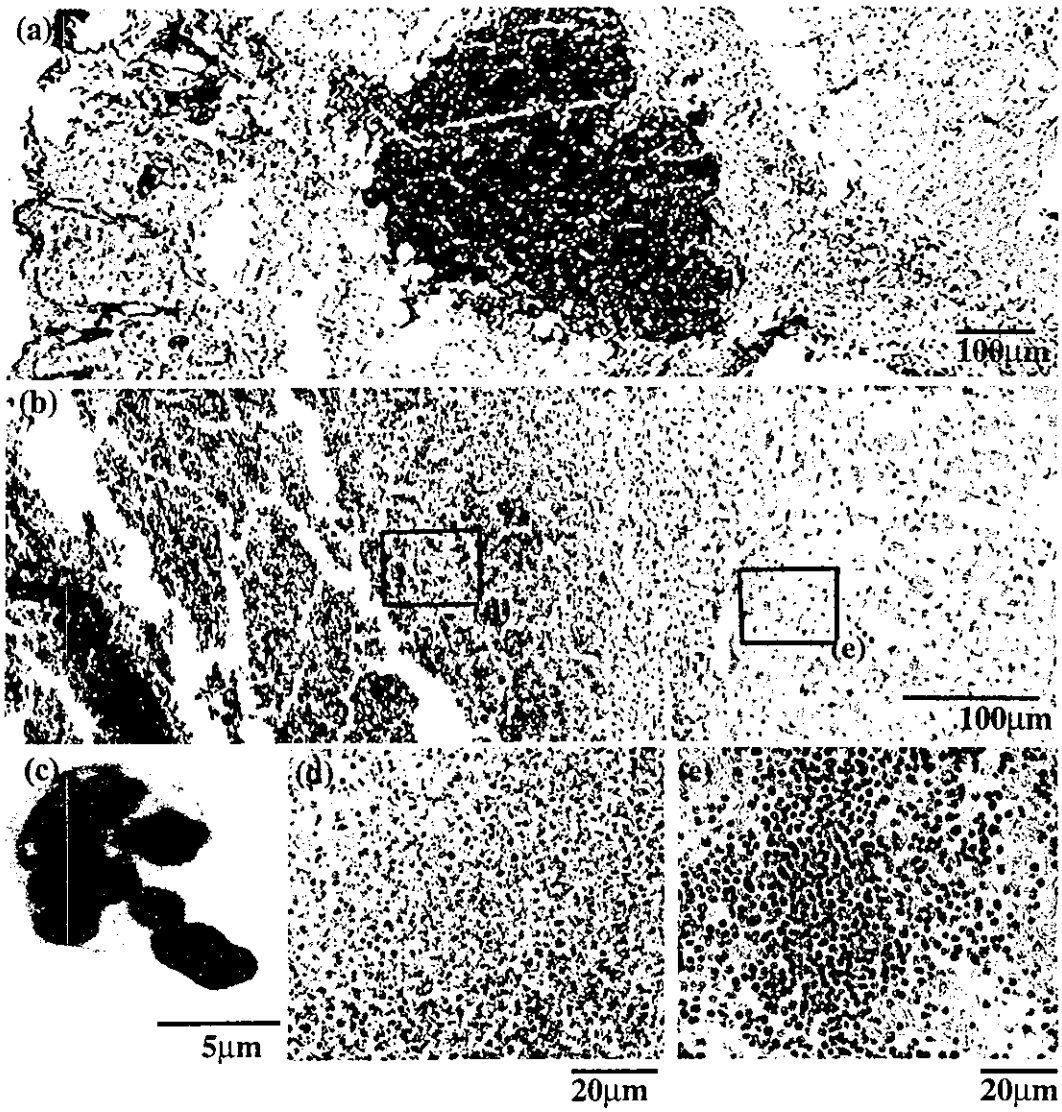

Fig. 2 Tissue response to particles inserted in subcutaneous tissue of rats for one week (HE stain). (a) 2 μm Ti, (b) 0.4 μm Ni, (c) and (d), (e) enlargement of (a) and (b). The agglomeration of 2 μm Ti (a) particles is seen in the center and 0.4 μm Ni particles (b) in the lower left. A neutrophil might phagocytize the 2 μm Ti (c). Ni particles caused necrosis (d) and strong inflammation (e).

by infiltration of lymphocytes and histiocytes, approximately 0.6–0.7 mm apart from Ni particles.

Figure 3 shows the SEM image of human neutrophils exposed to Ti and Ni particles in HBSS solution. Figure 3(a) shows the control neutrophil in HBSS. When a neutrophil was stimulated by 2 μm Ti particles, the surface changed to smoother (b) by the transformation of the cell membrane. Figure 3(c) show that a neutrophil may extends its pseudopod to phagocytize a Ti particle. The morphology of neutrophils exposed to Ni particles were often transformed (d) or destructed (e). Figure 3(f) is the typical SEM image for the case of 10 μm Ti particles or larger. 45 μm Ti particles are observed in the top of Fig. 3(f) and the form of neutrophils in the lower left changed very little.

Figure 4 shows the survival rate of neutrophils in the Ti and Ni particle solution. HBSS solution was the control. Although significant difference from control was not observed in all Ti particle solutions, the smallest value was 2 μm and followed by 10 μm. The other Ti particles had no difference from control. Ni particles showed the lower survival rate significantly differed from the HBSS. The survival rate was clearly lower in the 0.4 μm Ni particles than 5 μm.

Figure 5 shows the value of LDH in each particle solution containing neutrophils. The difference between each Ti particle and the control was significant except 150 μm. Levels of LDH were significantly higher in the 2 μm than the other sizes. LDH showed tendency to increase as the Ti particle size became smaller. The Ni group showed higher values than Ti. They also had the tendency to increase as the particle size became smaller.

Figure 6 shows the quantity of superoxide anion produced from neutrophils in the solutions of Ti and Ni particles. Ti group showed the significant increase from HBSS. The 2 μm particle showed the largest value. The other size is slightly higher than control. On the other hand, Ni had significantly much smaller values from HBSS and the smallest in the 0.4 μm size.

Figure 7 shows the amount of TNF-α released from neutrophils in HBSS containing metallic particles. The TNF-α levels increased only in HBSS containing 2 μm Ti particles.

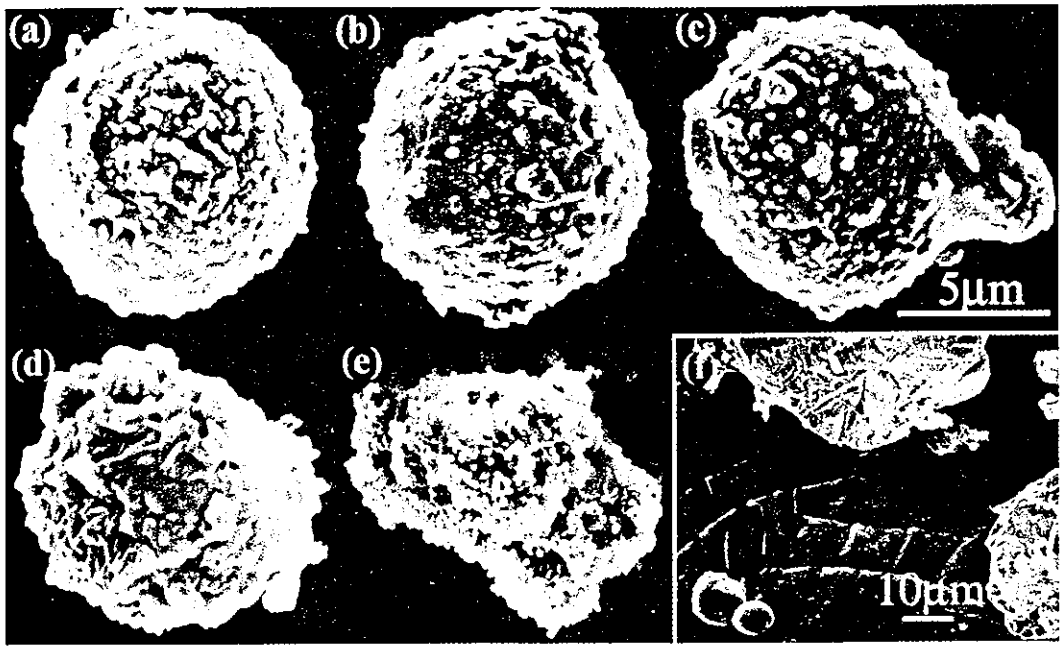

Fig. 3 SEM image of human neutrophils exposed to particles: a normal neutrophil in HBSS (a), neutrophil stimulated by Ti (b), a neutrophil phagocytizing 2 μm Ti particle (c), a neutrophil transformed (d) or broken down (e) with Ni particles, and neutrophils associated with 45 μm Ti particles (f).

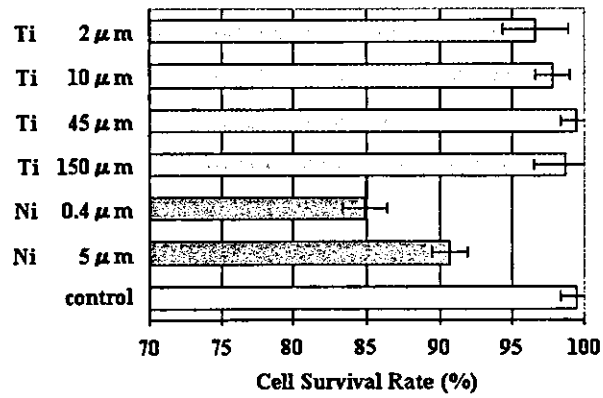

Fig. 4 Survival rate of neutrophils in the solutions of Ti and Ni particles.

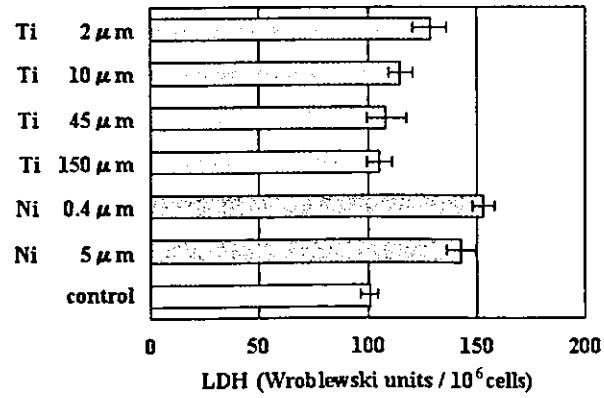

Fig. 5 LDH released from human neutrophils on the exposure to Ti and Ni particles.

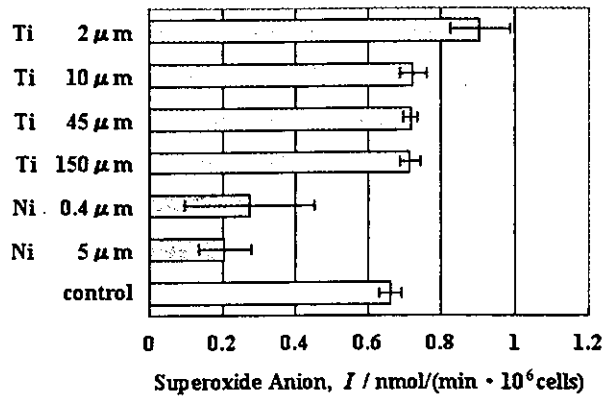

Fig. 6 Superoxide anion production of human neutrophils exposed to Ti and Ni particles.

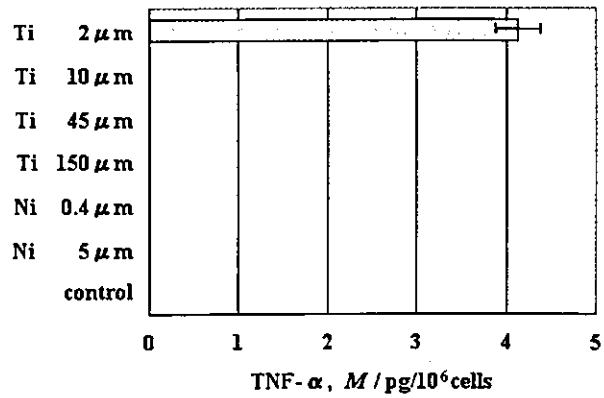

Fig. 7 Amount of TNF-α released from human neutrophils exposed to Ti and Ni particles.

No significant changes were associated with any other Ti and Ni particles.

Figure 8 shows the amount of IL-1β released from neutrophils in HBSS containing metal particles. The IL-1β levels significantly increased only in HBSS containing the 2 μm Ti particles. The other Ti and Ni particles released slightly but they had not much difference from the control.

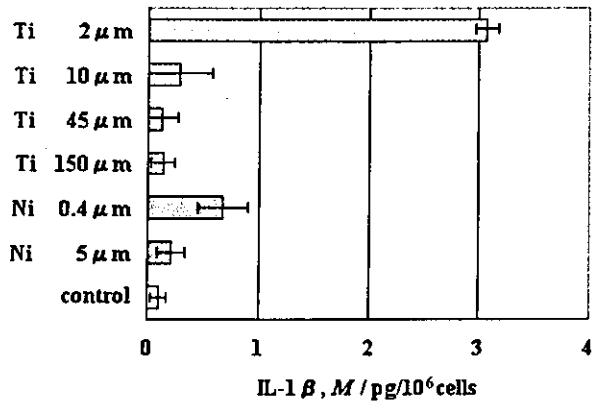

Fig. 8 Amount of IL-1β released from human neutrophils exposed to Ti and Ni particles.

4. Discussion

4.1 Probe cells

The most representative leukocytes with the function of phagocytosis are neutrophils and macrophages. In response to foreign objects, neutrophils react first, followed then by macrophages. Neutrophils account the largest proportion (about 54–65%) of human leucocytes. Their function is initially a non-specific reaction to foreign objects, whereas macrophages have a more complex function. Macrophages have a specific relationship with individual cytokines and respond to certain cytokines emitted from neutrophils after they have reacted with foreign objects. Our results showed that Ti fine particles, especially when they are smaller than cell size, cause inflammatory reaction *in vivo* and *in vitro*. To investigate the initial and simple reaction of cells to fine particles we used human neutrophils[20] rather than macrophages for monitoring the effect of Ti and Ni particles in this study.

4.2 Cell disruption

Cytotoxicity of Ti and Ni particles was examined by biochemical functional analysis as well as by microscopic morphology observation. LDH is an intracellular enzyme involved in the glycolytic pathway. The LDH value increases when the cell membrane is destroyed. Therefore LDH is the representative indication of cell disruption. From this point of view, the results of the cell survival rates (Fig. 4) and LDH production (Fig. 5) showed the very good accordance. LDH values were increased as the survival rate was lowered. Both results had qualitatively the very good correspondence each other in orders to particle size and particle group of Ti and Ni.

4.3 Cytofunctional reaction on exposition to particles

Neutrophils produce several kinds of active oxygen such as hydroxyradical, hydrogen peroxide, and superoxide anion. The superoxide dismutase-inhibiting reduction of equine fericytochrome C method, which was used in the present study, detect superoxide anion (O_2^-). Superoxide anions are released from intracellular organs and from the cell membrane of neutrophils when the latter is stimulated. The amount of superoxide produced by neutrophils (Fig. 6) significantly increased, especially in 2 μm Ti and slightly in the other sizes of Ti particles. These results suggest that Ti particles stimulate neutrophils and its effect depends on the size of the particles.

On the other hand, Ni produced much less superoxide (Fig. 6), which seems to contradict its cytotoxical effects. This could be explained partly due to the low survival rate of neutrophils (Fig. 4) caused by disruption in Ni particle solution, which might surpass the stimulatory effect of Ni.

The biochemical analysis indicated that the TNF-α and IL-1β release was significantly increased only in the solution of 2 μm Ti particles throughout the solutions in the different size of particles of Ti and Ni. One of the causes of TNF-α release from neutrophils occurs when the foreign matter is taken up by the cells. Our SEM observation (Fig. 3(c)) showed that only 2 μm particles in Ti group were phagocytized by neutrophils of about 5–10 μm in diameter.

IL-1β is released when neutrophils are stimulated by foreign matter and it causes the inflammation reaction cascade. We may assume that the 2 μm Ti particles stimulate neutrophils and make them release superoxide and IL-1β. The 2 μm Ti particles are also phagocytized by neutrophils, which then produces superoxide anions again and TNF-α. The phagocytosis is difficult for the 10 μm, 45 μm and 150 μm Ti particles and the above effects were absent in the present results.

The 0.4 μm and 5 μm Ni particles showed the different results from Ti, but they also had some tendency that small particles had the stronger influence.

4.4 Size dependence of cell toxicity

We examined particles that were smaller (2 μm) and larger (10 μm, 45 μm, and 150 μm) than the neutrophils, to determine the relationship between cell and particle size on cytotoxicity. 2 μm Ti particles stimulated the neutrophil activity and increased the production of superoxide anions (Fig. 6), while Ti particles larger than 10 μm did very little. Only 2 μm Ti particles clearly released TNF-α (Fig. 7) and IL-1β (Fig. 8).

All the results of cell survival rate (Fig. 4), LDH production (Fig. 5), superoxide production (Fig. 6) and TNF-α (Fig. 7), IL-1β (Fig. 8) release had the good accordance that they have the size dependence and the size effect becomes more remarkable as the size becomes smaller.[11] This effect is especially pronounced when particles are smaller than the cell size, although there are some differences in the effects between Ti and Ni.

Neutrophils may phagocytize Ti particles when the particles are smaller than the cell size of 5–10 μm. The increased quantity of TNF-α (Fig. 7) by phagocytosis caused neutrophil to further activation, resulting in inflammation. An increased superoxide content *in vivo* may affect the cell circumference and damage the DNA. These fine Ti particles may cause cytotoxicity, although the macroscopic size of Ti implant was quite biocompatible.

4.5 Cytotoxicity of Ti and Ni

Titanium is an insoluble, chemically stable metal and the most frequently used as implant and plate *in vivo*. On the other hand, nickel is dissolved into the surrounding tissues where it works highly toxic, although Ni–Ti alloy is often used as implant and orthodontics wire.

The SEM observations showed that the morphology of