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Behavior of in vitro, in vivo and internal motion of micro/nano particles of titanium, titanium oxides and others

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To clarify the effect of micro/nanosizing of materials onto biological organism, the particle size dependence of reaction of cells and tissue as investigated by both biochemical cell functional test and animal implantation test. Especially for nanoparticles the behavior of invasion and internal diffusion inside body was visualized using an XSAM (X-ray Scanning Analytical Microscope). The increase of specific surface area is usually counted as nanosizing effect which causes the enhancement of chemical reactivity and therefore toxicity of materials such as carcinogenicity found in 500 nm Ni particles for the long term implantation in the soft tissue of rat. Even biocompatible materials such as Ti and TiO_2 shows stimulus with the decrease of particle size. They cause phagocytosis to cells and inflammation to tissue when the size of particles is below 3 μm. For the size below 50 nm, they may invade into the internal body through the respiratory or digestive system and diffuse inside body. After compulsory exposure test of 30 nm TiO_2 particles through the respiratory system, the Ti mapping by XSAM showed the internal diffusion inside the whole body. Nanoparticles injected from caudal vein diffused with time course to lung, liver and spleen. The uptake of 30 nm TiO_2 particles through the digestive system and diffusion into these organs was also confirmed. These phenomena observed in biocompatible or bioinert materials are the nonspecific, physical particle and shape effects which occur independent of materials. Nanoparticles might be the objects whose existence has not been assumed by the living body defense system.

Key-words : Nanoparticle, Phagocytosis, Inflammation, Internal diffusion, Biocompatibility, Nanotoxicology, Cytokine, Size effect

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1. Introduction

1.1 Development of nanotechnology

Nanotechnology has been intensively developed and applied in the fields of electronics, chemistry and others. For materials nanosizing brings in quantum effect for less than about 1.5 nm and the formation of activity points such as contained in some catalysts. However the most unambiguous and influential effect is the surface area effect. It is well-known that the specific surface area which is defined as surface area for unit volume is increased with the decrease of particle size and chemical reactivity is pronounced. Therefore high throughput is expected in the functions of material properties and performances of devices. In these developments it is usual that the merit side is emphasized and demerit is neglected.

1.2 Necessity to establish principle for biomedical application

However it is natural that demerit appears as well as merit since merit and demerit are dependent on whether they work as usefulness or not for the purpose of human beings, although both originate from the same mechanism. Especially for the biomedical application of nanotechnology it is necessary to elucidate the phenomena and establish the proper principle in advance to step out to human application. The reaction of biological organism to proteins and saccharides including virus, bacteria, enzyme, and phamacological agents has been investigated in biology and medicine. For materials the reaction to the usual cases, that is, the macroscopic size is well investigated. But the reaction to the micro/nano sizing is not so clear.

1.3 Chemical reactivity enhancement effect

One of the most important factors to affect on the biocompatibility of materials in macroscopic size is ionic dissolution and this is also true for micro and nano size. This is closely related to the specific surface area and becomes apparent in most cases as stimulus or toxicity to nanosizing. Ni is known to cause allergy in macroscopic size. We found that 500 nm Ni particles cause the formation of tumors after one year when implanted in the soft tissue of rats. This is the typical example and nanosizing effect onto biological organism has been usually interpreted from this aspect.

1.4 Stimulus effect in non-soluble materials

On the other hand corrosion-resistent and biocompatible Ti causes inflammation in abraded fine particles[1),2)] which are produced from artificial joint, and asbestos,[3)] a kind of clay minerals, induces mesothelioma after a long-term, large quantity of exposure. These phenomena cannot be explained by the specific surface area effect and understood as the different effect from the material properties of either toxicity or biocompatibility, that is, physical size and shape effect. The abraded fine particles may diffuse inside the body through the cardiovascular system. There is also the possibility that the uptake of nanoparticles occurs through the respiratory and digestive systems.

These strongly suggest the necessity to reveal the micro/nanosizing effect other than the specific surface area effect,[4] such as the biological reactivity of micro/nanoparticles and their internal dynamics.

1.5 Nanotoxicology and DDS originated from internal particle diffusion

Meanwhile Drug Delivery System (DDS) is one of the most typical biomedical applications of nanoparticles. The development of DDS is expected for the administration of anticancer agent and gene transfection. The behavior of nanoparticles in the internal body is necessary to investigate for the assessment of nanotoxicology and this is, in turn, essential to comprehend the diffusion path of DDS to reach the diseased target. Thus internal diffusion is significant from both demerit and merit aspects of nanotechnology.

1.6 Purpose

In the present study both biochemical cell functional test and animal implantation test were done to clarify the particle size dependence of reaction of cells and tissue, and micro/nanosizing effect with the primary attention focussed on non-soluble materials such as Ti and TiO_2.[5),6)] In addition for nanoparticles, the behavior of invasion and internal diffusion inside body was visualized using XSAM (X-ray Scanning Analytical Microscope)[7),8)] for the level of the whole body and organs.

2. Materials and methods

2.1 Specimens

99.9% pure Ti, and TiO_2 particles of the various size were principally used throughout. For in vitro and in vivo implantation tests Fe, Ni, TiO_2 and carbon nanotubes[9),10)] were also used. The particles of nominal size from 500 nm to 150 μm were used for Ti. Usually these contain the size distribution to the considerable amount. To reduce the size distribution as small as possible and equalize the experimental conditions among materials such as metallic Ti, Fe and Ni, the particles of 0.5, 3, 10 μm were extracted by sedimentation method and those less than 300 nm were extracted by ultrafiltration from particle powders of nominal size.

2.2 Dissolution test of Ti particles

After Ti particles were immersed in HBSS (Hanks balanced salt solution) at 37°C for 1 month, the supernatant was filtered through a 0.45 μm membrane to remove Ti particles and then elemental analysis was done by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) using ICPS - 8100, Shimadzu, Tokyo.

2.3 Biochemical analyses of cellular reaction to materials

Human neutrophils, which play a central role in the initial stage of inflammation in a non-specific manner against foreign bodies, were used as probe cell. Particles smaller (0.5, 3 μm) and larger (10, 50, 150 μm) than neutrophils were used to determine the relationship between cell and particle size with respect to cytotoxicity.

Cell survival rate and lactate dehydrogenase (LDH) values, superoxide anion (O^{2-}) production per 10^6 neutrophils were measured. Cytokines of TNF-α and Il-1β were measured using ELISA kits (Endogen, Inc. USA). Morphological change of neutrophils mixed with HBSS containing various particles was observed by optical microscopy (OM: Zeiss, Axioskop, Germany) and scanning electron microscopy (SEM: Hitachi S-4300, Tokyo).

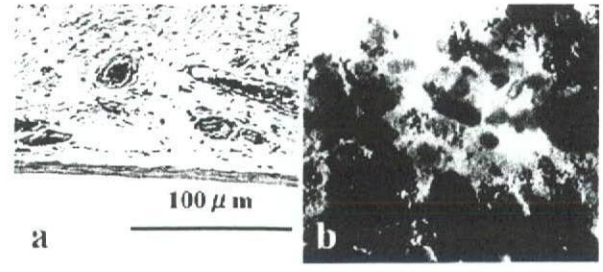

Fig. 1. Comparison of the reaction of rat soft tissue to the macroscopic Ti implant (a) and 3 μm Ti particles (b) by histological observation.

2.4 Animal experiments

Particles were inserted in the subcutaneous connective tissue in the abdominal region of Wistar rats aged between 11 and 12 weeks (weight 350–380 g). Specimens were prepared through the usual process of fixation, embedding, sectioning, staining with hematoxylin-eosin, and then histopathologically observed.

2.5 Visualization of internal distribution of nanoparticles

The compulsory exposure test to the respiratory system was performed to rats using 30 nm TiO_2 particles. The uptake of nanoparticles through the digestive system was also tested for mice by mixing agar gelatin containing 30 nm TiO_2 particles to their foods. To inspect internal diffusion more simply, the experiments were done for mice by injecting nanoparticles directly to the cardiovascular system from caudal vein. The observation of internal distribution of nanoparticles was conducted for the whole body and each organ by elemental mapping in air using X-ray Scanning Analytical Microscope (XSAM: Horiba XGT-2000V, Tokyo) without the pretreatments of fixation, dehydration and staining after sectioning. The distribution inside the organ was inspected by elemental mapping using energy dispersive X-ray spectroscopy (EDS) installed to SEM. The experiments of internal diffusion were also done for the particles Ti, Fe, Ni, Pt, TiC, Fe_2O_3.

3. Results

3.1 Comparison of tissue reaction to macroscopic and nanosize materials

Figure 1 shows the histological observation of the reaction of rat soft tissue to the macroscopic Ti implant (a) and 3 μm Ti particles (b) after 8 weeks, comparatively. For the macroscopic size, Ti implant was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials such as the bulk Ti. For 3 μm Ti numerous inflammatory cells appeared. The macrophages and adjacent collagen show degenerative changes in morphology. Ti particles, observed as small black dots, were phagocytized into the cytoplasm by a macrophage.

3.2 Particle size dependence of cell reaction

Figure 2 shows the SEM images of human neutrophils in HBSS (Hanks balanced salt solution) (a) and exposed to 500 nm Ti particles (b). Figure 2b showed the neutrophil extending its pseudopod to phagocytize Ti particles for the size below 3 μm. For the particles larger than 10 μm, phagocytosis was not observed.

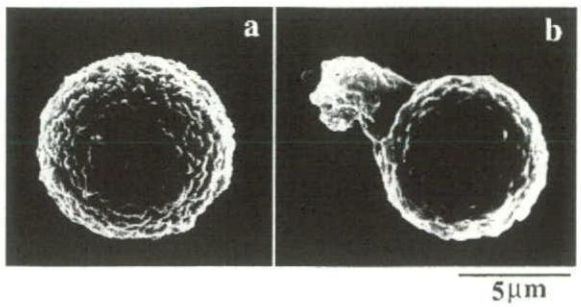

Fig. 2. SEM images of human neutrophils. a: control, b: exposed to particles of 500 nm Ti.

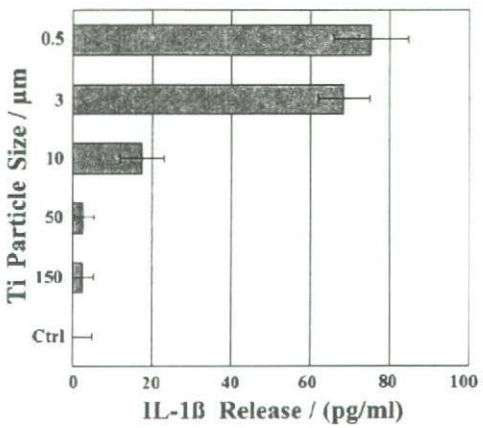

Fig. 3. Dependence of Il-1β release from neutrophils on Ti particle size.

Figure 3 shows the amount of Il-1β released from neutrophils in HBSS containing Ti particles. Il-1β is one of the most representative cytokines of inflammation. Il-1β showed the increase against the decrease of particle size. The increase was pronounced for 0.5 and 3 μm. The release of LDH, superoxide and cytokine TNF-α showed the similar behavior as Il-1β, while cell survival rate showed the inverse decreasing tendency. ICP elemental analysis showed that the dissolution from Ti particles was negligible below detection limit. The pronounced phenomena of biochemical cell reactivity observed for the particle size below 3 μm in Fig. 3 are closely related to the phagocytosis shown in Fig. 2.

3.3 Particle size dependence of tissue reaction

The histological image of tissue reaction of rat to the different size of Ti particles for the long term implantation test showed the similar size dependence to those in vitro shown in Figs. 2 and 3. **Figure 4** is the tissue reaction to 10 μm (a) and 150 μm Ti (b) particles after 30 week implantation. For 150 μm Ti, each particle was surrounded by fibrous connective tissue layer, which is similar to the case of macroscopic Ti implant shown in Fig. 1a. Tissue reaction to 10 μm Ti was inflammatory where there was inflammatory cell infiltration as well as fibrous connective tissue formation.

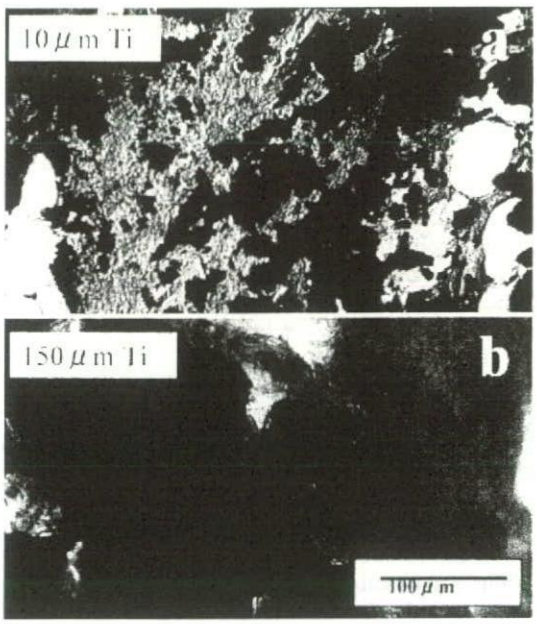

Fig. 4. Histopathological image of tissue reaction to 10 μm (a) and 150 μm (b) Ti particles after 30 week implantation.

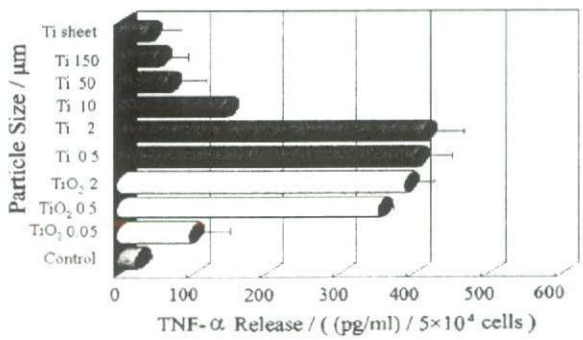

Fig. 5. Dependence of TNF-α release from neutrophils on particle size down to nm size.

3.4 Stimulus in nm size

Figure 5 shows the dependence of TNF-α release from neutrophils on particle size down to nm size. Stimulus, represented as amount of TNF-α release, which is pronounced below 3 μm, exhibited the maximum from around μm down to 500 nm, similar to the case of Il-1β shown in Fig. 3, and then for further smaller size decreased below 200 nm. This means that the biophylactic system does not work well any more against the invasion of nanoparticles into the inside of body.

3.5 Internal diffusion of nanoparticles

Figure 6 is the Ti mapping of the internal whole body of rats by XSAM after compulsory exposure test to respiratory system, and reveals the distribution of 30 nm TiO_2 particles. The condensation occurred from the respiratory system to urinary bladder by diffusion in the body through the cardiovascular system after the direct uptake into blood

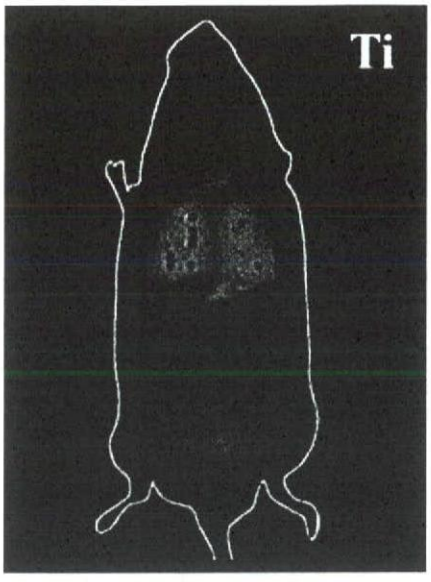

Fig. 6. XSAM Ti mapping of internal distribution of 30 nm TiO_2 particles after compulsory exposure test to respiratory system.

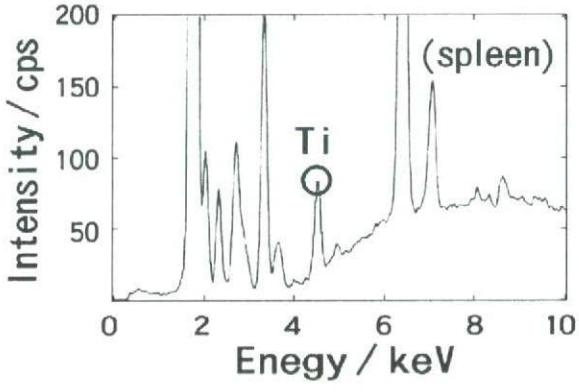

Fig. 7. Elemental analysis of spleen of mouse by XSAM after 10 days of oral administration of 30 nm TiO_2 particles.

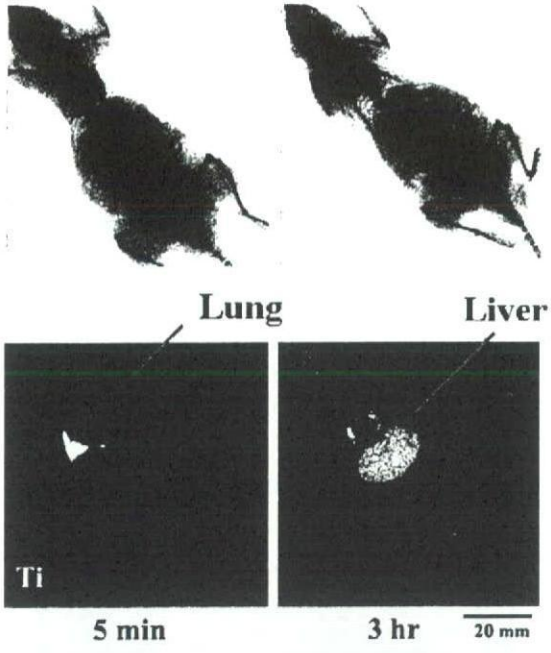

Fig. 8. Time course of internal diffusion of 30 nm TiO_2 particles after injection to caudal vein.

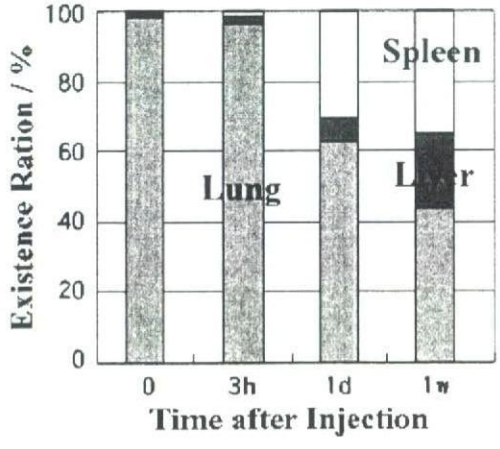

Fig. 9. Change of existence ratio of TiO_2 particles in each organ with time.

vessels from lung cells.

Figure 7 is the XSAM elemental analysis from spleen for the case after 10 d of oral administration of 30 nm TiO_2 particles. Although peak height is small in this case, Ti-Kα peak undoubtedly exists other than Fe-Kα peaks around 6.5 keV and peaks of incident X-ray from Rh target below 4 keV. This confirms the phenomenon that nanoparticles were taken into the internal body through digestion system.

Figure 8 shows the X-ray transmission image and the corresponding Ti elemental mapping by XSAM for 5 min and 3 hr after injection of 30 nm TiO_2 particles to caudal vein. TiO_2 nanoparticles diffused to lung just after injected from caudal vein, then liver and spleen with time course.

Figure 9 shows the change of existence ratio of TiO_2 particles in each organ with time. Particles reach lung shortly after injection, then the content in lung decreases and the content in liver and spleen increases with time.

To observe the more detailed distribution of nanoparticles in each organ, EDS elemental mapping was applied. **Figure 10** is the SEM image (a) and corresponding Ti elemental mapping by EDS (b) for spleen of mouse at 3 hr after injection of 30 nm TiO_2 particles to caudal vein. The distribution is not uniform and in dotted manner.

4. Discussion

4.1 Particle size dependence of reaction of cells and tissue

Comparison of the reaction of tissue to the macroscopic Ti and 3 μm Ti particles in Fig. 1 showed clearly the micro/nanosizing effect on biological organism. Both biochemical cell functional test and animal implantation test showed the toxicity due to fine particles and its size dependence. Both

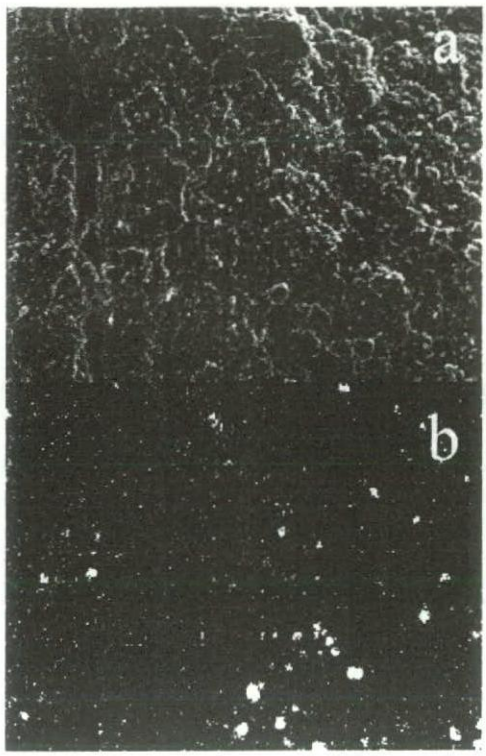

Fig. 10. SEM image (a) and corresponding Ti elemental mapping by EDS (b) for spleen of mouse at 3 hr after injection of 30 nm TiO_2 particles to caudal vein.

results of in vitro and in vivo are in accordance each other in their size dependence. Ti particles larger than approximately 100 μm was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials such as the bulk size of Ti implant.[11),12)] As the particle size was smaller, stimulus was induced due to the physical size effect in the range less than about 100 μm as shown in Fig. 4. The inflammation was especially pronounced when the particle size was typically below 3 μm which is smaller than 10 μm, about the cell size, where phagocytosis was induced.

These phenomena occur commonly in any bioactive and bioinert materials other than Ti, such as Fe and TiO_2 where particles induce nonspecifically phagocytosis to cells and inflammation to tissue for the size below 3 μm. It is different from the usually observed toxicity due to the ionic dissolution effect in the macroscopic size.[4)]

4.2 Stimulus in nm size

Nanosizing effect is usually interpreted by the increase of specific surface area, which pronounces chemical reactivity with the decrease of particle size. Effects related to the ionic dissolution correspond to this category, such as the acceleration of toxicity observed in Ni where tumor was generated in the long-term implantation for 500 nm particles, compared with necrosis occurred in short term for macroscopic size.

Specific surface area effect is based solely on the material properties, and indifferent from biological body, while physical particle size effect has the origin in the relative size relationship between particles and cell/tissue. Stimulus arises by biological process which induces the occurrence of functionality of body defense system.

4.3 Internal diffusion of nanoparticles

Figure 5 shows that particles become less stimulative when the particle size becomes in the level of 50 nm or less and the recognition by body defense system becomes lower. The invasion of nanoparticles into the body occurs for this range of particle size. The present results showed both cases of uptake of nanoparticles through the respiratory (Fig. 6) or digestive system (Fig. 7). Figures 8 and 9 show that nanoparticles diffuse with time course to lung, liver and spleen after injection from caudal vein. SEM image and EDS elemental mapping in Fig. 10 show the distribution of TiO_2 nanoparticles inside the organ of spleen.

5. Conclusions

Particles cause nonspecifically phagocytosis to cells and inflammation to tissue for the size below 3 μm. For the size below 50 nm particles may invade directly into the internal body through the respiratory or digestive system and diffuse inside body. Nanoparticles might be the objects whose existence has not been assumed by living body defense system. Thus the visualization of the internal dynamics of nanoparticles is essential for the proper treatments based on risk assessment and biomedical applications such as DDS. The present study could successfully visualize the internal diffusion of nanoparticles inside the whole body using XSAM.

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Reaction of cells and tissue to material nanosizing

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Micro/nanosizing effect of materials onto biological organism was investigated by both in vitro biochemical cell functional test and in vivo animal implantation test. Dependence of reaction of cells and tissue, and that of bone formation of apatite on particle size were studied. The increase of specific surface area causes the enhancement of chemical reactivity and therefore toxicity in many cases. This is the most usually and easily recognizable and strongest effect in most cases. However there is the other effect which becomes prominent especially for biocompatible materials such as Ti and TiO_2. Stimulus was increased with the decrease of particle size and pronounced below 3 μm by inducing phagocytosis to cells and inflammation to tissue. For the size below 50nm, particles invade into the internal body through the respiratory or digestive system and diffuse inside body. For bone, synthetic hydroxyapatite exhibits excellent osteoconductivity but it is not substituted with natural bone and remains permanently in the body. When the composite with collagen and nanoapatite synthesized in the biomimetic aspects is implanted, resorption of nanocomposite through phagocytosis by osteoclasts and new bone formation by osteoblasts occurred simultaneously after inflammation. Nanocomposite leads to the bone substitutional properties, which resembles the remodeling process in natural bone. Thus nanosizing induces the intrinsic functions of biological organism and results in the conversion of functions such as from biocompatibility to stimulus and from osteoconductivity but non-bone substitutional to bone substitutional properties through biological process.

Key words: nanosizing, biomaterial, tissue regeneration, inflammation, nanotoxicology, titanium, apatite

1. INTRODUCTION

One of the important fields of nanotechnology is biomedical application. DDS (Drug Delivery System) is one of the most typical bioapplications of nanoparticles. It is well-known that the specific surface area is increased with the decrease of particle size and chemical reactivity is pronounced. Nanosizing effect related to the ionic dissolution which affects on biocompatibility is usually interpreted from this aspect. On the other hand corrosion-resistent and biocompatible Ti causes inflammation in abraded fine particles [1, 2] which are produced from artificial joint, and asbestos [3], a kind of clay minerals, induces mesothelioma after a long-term, large quantity of exposure. These phenomena can be understood as the physical size and shape effect, apart from the material properties of either toxicity or biocompatibility.

On the other hand, hydroxyapatite (HAP), the main component of bone, has the difference in behavior between synthetic apatite and bone. Synthetic hydroxyapatite, in the usual case, of a macroscopic size, exhibits excellent osteoconductivity. However it is not substituted to natural bone and remains permanently in the body. Natural bone is composed of collagen and nanocrystallites of apatite with the size of approximately 50nm. Bone is continuously remodeled by resorption and new bone formation. Thus there exist apatites with the different behavior, non-resorbable and resorbable apatite.

These strongly suggest the necessity to reveal the micro/nanosizing effect of materials onto living organism [4]. In the present study both biochemical cell functional test and animal implantation test were done to clarify the micro/nanosizing effect and particle size dependence of reaction of cells and tissue [5, 6]. The behavior of invasion of nanoparticles and internal diffusion inside body was visualized using XSAM (X-ray Scanning Analytical Microscope) [7, 8] for the level of the whole body and organs. Then the nanosizing effect in apatite was investigated using biomimetic bone-resembling nanoapatite/collagen composite and the mechanism of the different behavior from macroscopic apatite was discussed.

2. EXPERIMENTAL PROCEDURE

Both biochemical cell functional tests and animal implantation tests were done using the fine particles of 99.9% pure Ti, Fe, Ni and TiO_2 for the various sizes from 300 nm to 150 μm [5,6]. Human neutrophils were used as probe cells for various cell toxicity tests, after mixed with particles in HBSS (Hank's balanced salt solution) at 37°C. Histological investigations were done after implanted in the subcutaneous connective tissue of rats.

The compulsory exposure test to the respiratory system was performed using 30nm TiO_2 particles. The

experiments of internal diffusion was done for the particles Ti, Fe, Ni, Pt, TiO_2, TiC, Fe_2O_3 by injection to caudal vein. XSAM observation for the whole body and each organ was conducted in air without the pretreatments of fixation, dehydration and staining after sectioning.

Hydroxyapatite-collagen composites were synthesized biomimetically on mineralized collagen type I. They have the three-dimensional scaffold structures with the interconnecting pores. They were implanted into the subcutaneous tissue and bone defects made in the femur of rats for 1-12 weeks and observed histopathologically [9].

3. RESULTS

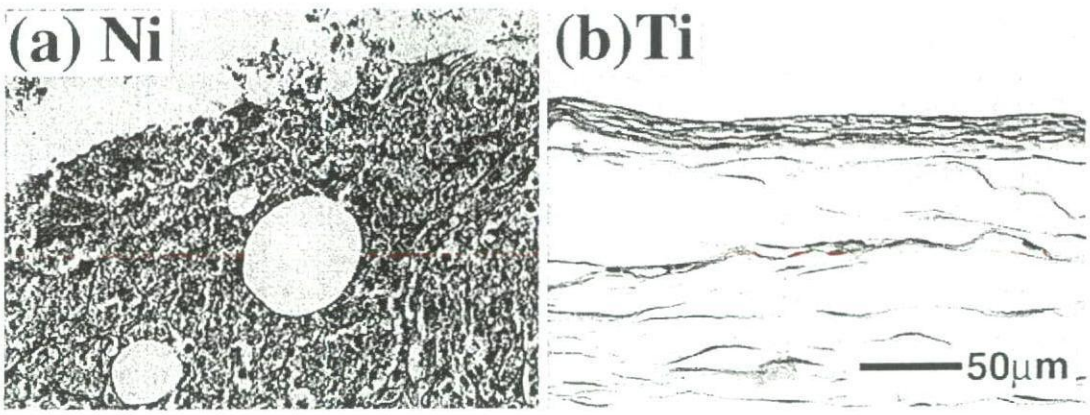
Fig.1 shows the comparison of tissue reaction to the macroscopic size (1 mm ϕ x 10 mm) of Ni (a) and Ti (b) after 1 week implantation in the dorsal thoracic region of rat. Implant had been situated in the upper space of each photograph. In Ni the expansion of capillary vessels was observed. Tissue in the photograph was in necrosis and in degeneration in the distant region. For Ti fibrous connective tissue was already formed surrounding implant from the earlier stage, which is the feature of biocompatible materials [10].

Fig.2 showed the tumor occurrence in the subcutaneous tissue of rat after 1 year implantation of 500nm Ni particles. Ni is already toxic in a macroscopic size as seen in Fig.1. When it becomes fine particles, toxicity is enhanced remarkably. This is the typical example of specific surface effect which increases reciprocally to particle size and leads to the enhancement of chemical dissolution and therefore toxicity.

Fig.3 shows the morphology of one of the asbestos (crocidolite: so-called blue asbestos) observed by SEM. The diameter of asbestos is a few nm to a few μm and the length is ranged from submicron to tens of μm. Crocidolite has a distinctly straight needle shape.
There are also very fine particles which may easily disperse as dust.

Asbestos is a kind of clay minerals and silicate in composition. The dissolution level is very low. It is known that asbestos induces mesothelioma after a long-term, large quantity of exposure to respiratory system. This is the result by the different mechanism from that occurred in Ni particles of Fig.2 and related more to the particle size and shape.

Fig.4 shows the histological observation of the reaction of rat soft tissue to the macroscopic Ti implant (a) and 3μm Ti particles (b) after 8 weeks, comparatively. The macroscopic size of Ti implant was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials. For 3μm Ti numerous inflammatory cells appear. The



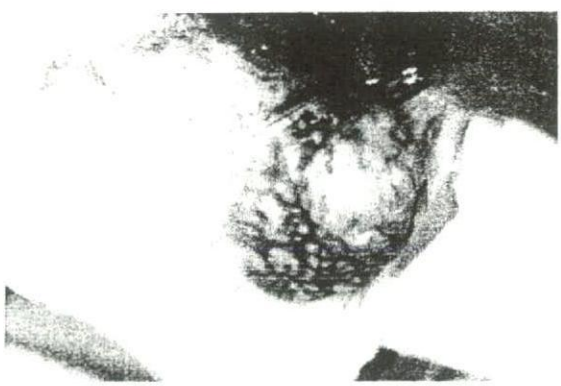


Fig.2 Tumor induced after 1 year implantation of 500nm Ni particles [4].

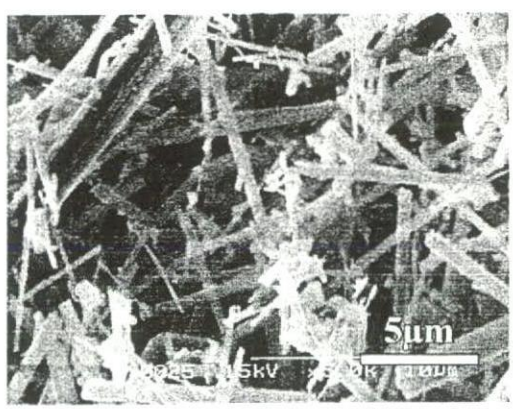


Fig.3 Morphology of asbestos (crocidolite: blue asbestos) observed by SEM.

macrophages and adjacent collagen show degenerative changes in morphology. Ti particles, observed as small black dots, were phagocytized into the cytoplasm by a macrophage.

Fig.5 shows the dependence of superoxide production from human neutrophils on Ti particle size , Superoxide was increased with the decrease of particle size. The increase was pronounced for 3μm and 500nm. The release of LDH and cytokines TNF-α and Il-1ß showed the similar behavior as superoxide, while cell survival rate showed the inverse decreasing tendency. Under these conditions ICP elemental analysis indicated that the dissolution from Ti particles was negligible below detection limit [5].

Fig.6 shows the SEM image of a human neutrophil exposed to 500nm Ti particles in HBSS. The neutrophil is extending its pseudopod and going to phagocytize a 500nm Ti particle. For the particles larger than about 10μm, phagocytosis was not observed. The pronounced phenomena of biochemical cell reaction for below 10μm in Fig.5 are closely related to the phagocytosis shown in Fig.6.

The histological image of in vivo tissue reaction of rat to the different size of Ti particles showed the similar size dependence to those in vitro shown in Figs.5 and 6.

Fig.7 shows the dependence of TNF-α release from neutrophils on particle size down to nm size. TNF-α is one of the most representative cytokines related to inflammation. Stimulus, represented as amount of TNF-α release, which is pronounced below 3μm, exhibited the maximum from around μm down to 500nm and then for further smaller size decreased below 200nm. This means that the biophylactic system does not work well any more against the invasion of nanoparticles into the inside of body.

Fig.8 is the Ti mapping of the internal whole body of rats by XSAM after compulsory exposure test to respiratory system, and shows the distribution of 30nm TiO_2 particles. The condensation occurred from the respiratory system to urinary bladder by diffusion in the body through the cardiovascular system after the direct uptake into blood vessels from lung cells.

Fig.9 shows the X-ray transmission image and the corresponding Ti elemental mapping by XSAM for 5min and 3hr after injection of 30nm TiO_2 particles to caudal vein. TiO_2 nanoparticles diffused to lung just after injected, then liver and spleen with time course.

Fig.10 is the dental implant composed of hydroxyapatite-coated titanium. Apatite has excellent biocompatibility and induces new bone formation to its surface after implanted in the bone circumstances.

Synthetic hydroxyapatite in the usual case, that is, in

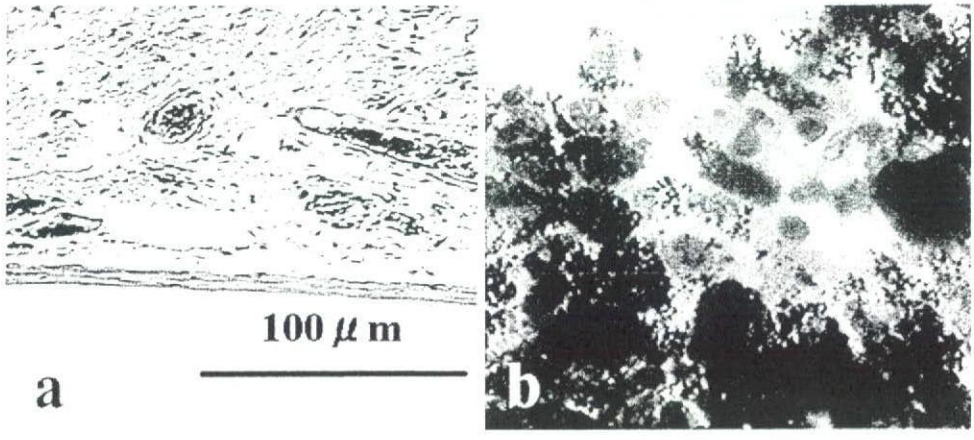


Fig.4 Comparison of reaction of rat soft tissue to the macroscopic Ti implant (a) and 3μmTi particles (b) in histological observation.

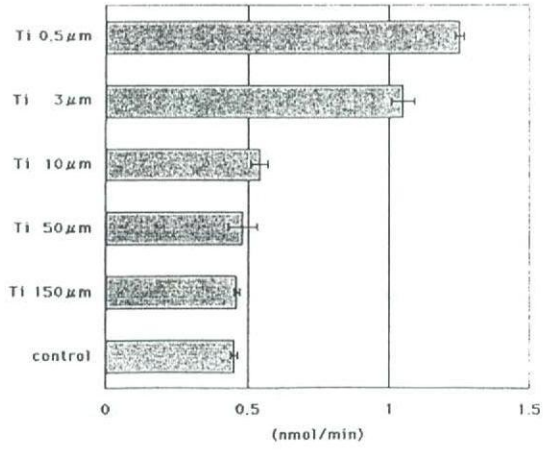


Fig.5 Dependence of superoxide production from neutrophils on Ti particle size [6]

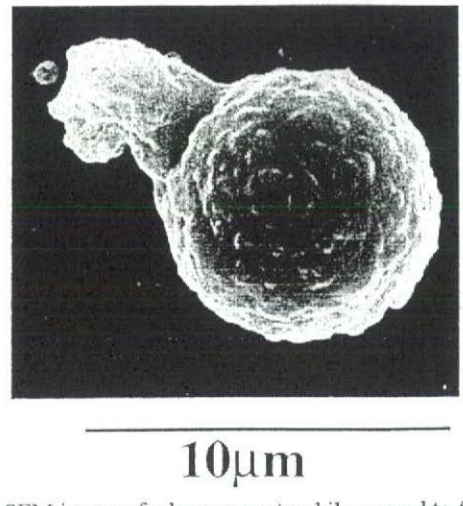


Fig.6 SEM image of a human neutrophil exposed to 500nm Ti particles [6].

a macroscopic size, exhibits excellent osteoconductivity. However it is not substituted to natural bone and remains permanently in the body, therefore it is suitable for the use as implant.

On the other hand it is well-known that natural bone is composed of collagen and nanocrystallites of apatite with the size of approximately 50nm. Fig.11 is the SEM photographs, comparing the difference of morphology of hydroxyapatite for sintered synthetic apatite (a) and natural hard tissue, in this case, enamel of molar of rat (b). In synthetic apatite the size of particles is a few microns and they agglomerate at random, while in enamel enamel prizm of about 5 μm is composed of a bunch of apatite crystallites of about 50nm. It is known that apatite crystallites are grown in their c-axis along collagen fibrils. Thus natural hard tissue is regarded as a kind of composite with the preferably oriented structure of nanocrystallites.

Fig.12 shows the comparison of morphology of hydroxyapatite synthesized without (a) and with (b) collagen by SEM observation. The particle size of apatite is mostly a few microns for without-collagen, while under the coexistence of collagen the product becomes the agglomerate of apatite crystallites of less than 100nm with the lower crystallinity, as revealed from X-ray diffraction analysis.

When the biomimetic nanocomposites of apatite and collagen fibrils were implanted in the subcutaneous tissue, they were covered with fibrous connective tissue and then resorbed mostly at 8 weeks by phagocytosis.

Fig.13 shows the histopathological image when nanocomposites were implanted in the bone marrow of rat for 8 weeks. The area of nanocomposites (asterisks) was decreased and covered with new bone (white asterisks) of lamellar structures. Resorption of the nanocomposites and replacement by new bone proceeded. This tendency was progressed with time by 12 weeks. Phagocytosis of nanoapatite by osteoclasts and osteogenesis by osteoblasts occurred adjacently each other. Resorption and remodeling were similar to the case of autologous bone graft. As a result nanoapatite composites work as bone substitute materials for

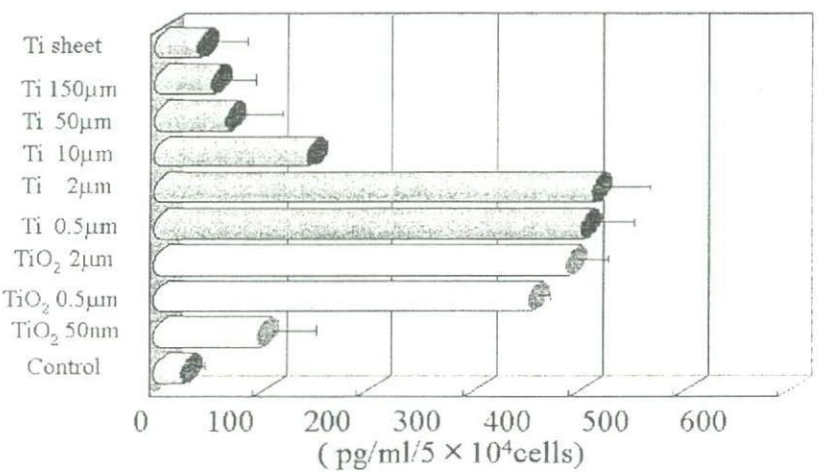


Fig.7 Dependence of TNF-α release from neutrophils on particle size down to nm size [4]

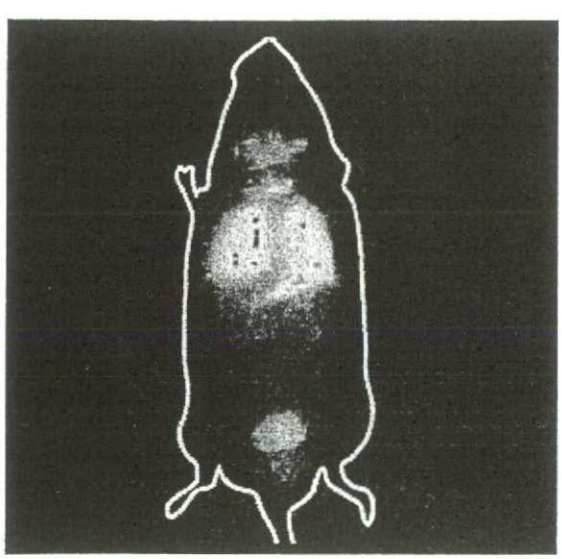


Fig.8 XSAM Ti mapping of internal distribution of 30 nm TiO_2 particles after compulsory exposure test [4].

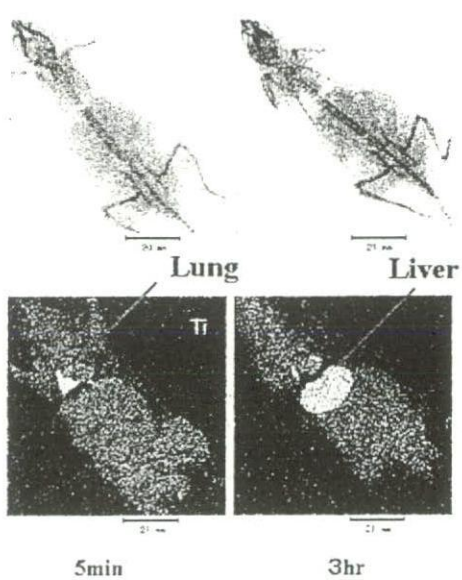


Fig.9 Time course of internal diffusion of 30nm TiO_2 particles after injection to caudal vein

hard-tissue reconstruction.

4. DISCUSSION

4.1 Specific surface area effect and physical particle size effect by nanosizing

Nanosizing effect is usually interpreted in the aspects of the increase of specific surface area. Since chemical reactivity is pronounced with the decrease of particle size, effects related to the ionic dissolution, dominant on biocompatibility of macroscopic materials, accelerate toxicity such as in Ni which generated tumor in the long term implantation for 500nm particles as shown in Fig.2. This effect has the most serious influence, toxicity in many cases, and most commonly taken into account for nanosizing effect. There are, however, other kind of effects. Biocompatible Ti causes inflammation in abraded fine particles [1,2,11], and asbestos [3], a kind of clay minerals, induces

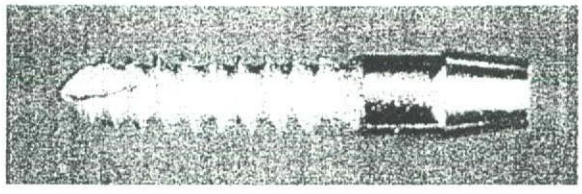


Fig.10 Dental implant composed of apatite-coated titanium

Fig.11 Difference of morphology of hydroxyapatite. a) sintered synthetic apatite, b) enamel of molar of rat.

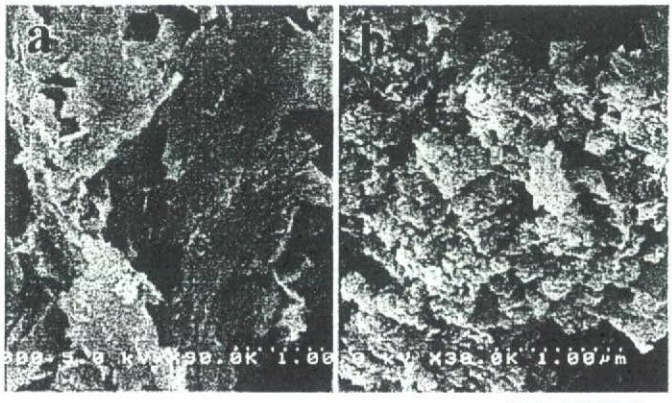


Fig.12 Hydroxyapatite synthesized without (a) and with (b) collagen.

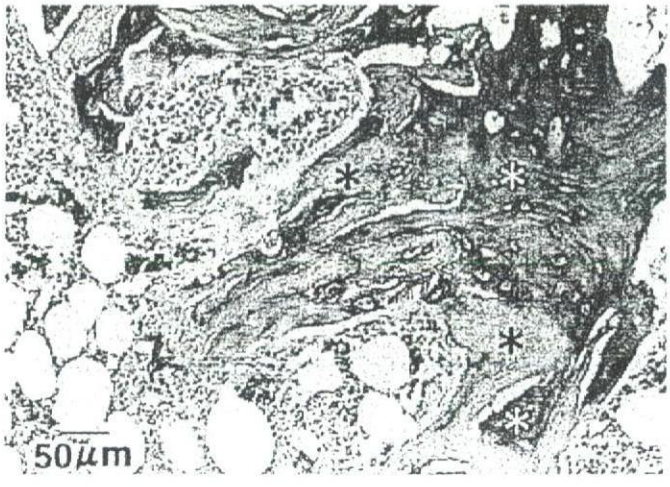


Fig.13 Histological image at 8 weeks after implantation in the bone marrow of rat. Materials (asterisks) were decreased and covered with new bone (white asterisks) with lamellar structures [9].

mesothelioma after a long-term, large quantity of exposure. These phenomena can be understood as the physical particle and shape effect, apart from the material properties of either toxicity or biocompatibility. Particles below 10 μm cause phagocytosis to cells and inflammation to tissue even for biocompatible materials such as Ti and TiO_2.

4.2 Invasion of nanoparticles into internal body

By compulsory exposure test, the 30nm TiO_2 particles diffuse directly from the respiratory system into the internal body. Nanoparticles injected from caudal vein diffused with time course to lung, liver and spleen. The uptake of the 30nm TiO_2 particles through the digestive system. was also confirmed. Particles below 50nm might be the objects whose existence has not been assumed by the living body defense system and can invade into the internal body through the respiratory or digestive system.

4.3 From non-resorbable to resorbable apatite by nanosizing

Synthetic hydroxyapatite exhibits excellent osteoconductivity in a macroscopic size, but it is not substituted to bone and remains permanently in the body, therefore it is suitable for the use as implant [12]. It is well-known that natural bone is composed of collagen and nanoapatite crystallites of approximately 50nm [13]. When the biomimetically synthesized nanoapatite composite with collagen [9,14] is implanted, phagocytosis and inflammation is induced. Osteoclasts and osteoblasts are then differentiated. Phagocytosis by osteoclasts and new bone formation by osteoblasts is simultaneously activated and proceeded as shown in Fig.13. As a result, nanoapatite composite leads to the bone substitutional properties.

4.4 Induction of bioactive properties and conversion of functions by nanosizing

The conversion of functions is attained for apatite by nanosizing - from osteoconductivity in macroscopic size to bone substitutional properties in nano/micro scale. Nanoparticles cause the reaction of cells/tissue and stimulate to the occurrence of inflammation, which works as toxicity in most cases and, for some cases depending on the situation, pronounces the conversion of functions leading to the bioactive properties. Nano structure is essential for these stages to be processed.

5. CONCLUSIONS

Nanosizing of materials induces the reaction of cells and tissue and the intrinsic functions of biological organism, which leads to the conversion of functions such as from biocompatibility to stimulus and from osteoconductivity but non-bone substitutional to bone substitutional properties through biological process. This is different from specific surface area effect originated solely from material properties. There are controversial arguments as to whether carbon nanotubes may have the serious toxicity due to their acicular or fibrous particle shape, associated with lung carcinogenicity of asbestos, whilst we have rather found the favorite properties as biomaterials [15-19]. The physical particle size and shape effect in micro/nanosizing is the essential basis for the proper understanding of such phenomena and for the development of biomedical applications of nanotechnology.

6. ACKNOWLEDGEMENTS

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特集 ナノ粒子材料とその安全性

ナノ粒子の生体反応性と為害性発現

材料のサイズがマクロからミクロ，ナノに微小化したときの効果として代表的なものは比表面積増大効果による化学反応の活性化であるが，これとは別に生体との相互作用に基づく微粒子特有の効果も現れる．これらは人間の意図する目的と合致すれば高機能性というメリットとして作用するが，一方また意図せずして為害性というデメリットとして発現する可能性もある．マクロとは異なり，ナノ粒子は本質的に高機能性と刺激性の二面性を併せ持つことを認識する必要がある．

亘理　文夫

生体に対する材料と微粒子の効果とは？

栄養摂取にせよ毒性発現にせよ，材料と生体との相互作用の多くは水溶性としてイオン化して体内に吸収されることから開始する．可溶性のNaは容易に摂取され摂取過剰になることもあるのに対し，難溶性のCaは吸収されにくい．試薬のカタログを見るとBa化合物のほとんどが劇物・毒物といったラベルがしるされているにもかかわらず，硫酸バリウムのみなにも記されず胃カメラで用いられるのは溶解度がきわめて低いからであろう．結婚式の食事で出て来るスープに金粉がちりばめられていることがあるが，健康に問題ないのだろうか．純金であればイオン化傾向が最も小さく，イオン溶出が無いと見なせるから，消化器を単に通過するだけであれば，何事も起きないであろう．このようにマクロでの材料の生体適合性には溶出性（だけではない）が，大きな影響を及ぼす．この傾向はミクロ/ナノになっても変わらない．コーヒーへの角砂糖と粉末状の砂糖の溶けやすさからも明らかのように，微粒子になりサイズが小さくなると，比表面積は増大するから，溶解性や化学反応性は著しく増大する．この効果は目に見えて大きく，多くの場合，微粒子化またはミクロ/ナノサイズ化（以下ナノサイジングとも記載）の効果はこの比表面積効果で説明されている．この効果は生体と無関係に生ずる材料の効果である（図1）[1]．

しかしナノサイジング効果はこれだけに留まらない．生体親和性にすぐれるチタンが人工関節骨頭摺動部で使用され，摩耗粉となると周囲組織に炎症を起こし骨融解を導いて使用寿命が10年程度になる場合があることや，粘土鉱物の1種アスベストを長期大量に被曝すると中皮腫を発症する現象には，単に材質が毒性か生体親和性かという特性とは別に，微粒子という物理的サイズに起因する効果が寄与していると考えられる[2]．

ここではまず，マクロでもアレルギー性等の為害性を呈するNiを溶出性金属の代表として，生体親和性[3]に富みインプラント[4]に多く使用されているTiを非溶出性の代表的金属として取り上げ，マクロからミクロ/ナノへ微小化したときの生体

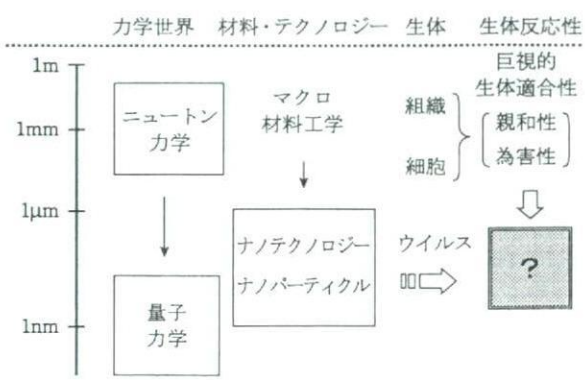

図1　ナノテクノロジーの力学世界，生体との関係，および材料のマイクロ/ナノサイジングと生体反応性の関連

反応性を比較して見てみよう.

ミクロ/ナノ粒子化と生体反応

図2はマクロサイズの材料に対する生体軟組織の反応 (in vivo 組織埋入試験), 図3は微粒子化 (0.5μm) したときの個々の細胞に対する反応 (in vitro 細胞毒性試験), 図4はこの微粒子を軟組織に長期埋入した結果 (in vivo) である[5].

図1では直径1mmの棒状インプラントをラット皮下軟組織に1週埋入後の周囲組織の一部で, 図の上部に挿入されていたインプラントは取り除かれている. Niでは組織が壊死し, 強い為害作用を示すが, Tiではインプラントを被包化する線維性結合組織が形成されており, 生体親和性に富む材料の典型的な反応を示している.

材料がμm程度の微粒子になると細胞は図3のように, Niに対しては強い為害性のために細胞は破壊され, Tiでは貪食が誘発されている. ここで用いた細胞のヒト好中球は白血球の約50%を占める5～10μmと比較的小さな顆粒球で, 生体内の異物に対して非特異的に反応する貪食細胞の一種である[6) 7)].

この条件下で各微粒子からのイオン溶出量をICP (誘導結合プラズマ) 元素分析すると, Ti微粒子では検出限界値以下であり, イオン溶出は無視できると考えられる. 即ち, Ti微粒子に対する反応は物理的サイズ・形状に由来する効果である[6) 7)].

これらの微粒子を大量に半年～1年間軟組織に埋入すると (図4), Niでは腫瘍が発生し, Tiでは貪食を繰返し微粒子群が次第に凝集する中, 慢性的な炎症が継続する.

物理的サイズ効果の特徴

溶出性材料における比表面積効果は図3(a), 図4(a)のように作用が顕著で認識しやすい. 一方, そうした化学的溶出効果が無視し得る非溶出性材料やbioactive, bioinert材料でもμm～nmになると, 材質によらず微粒子の物理的サイズ効果による刺激性が顕現化する (図3(b), 図4(b)).

図5は微粒子と共在させたヒト好中球から産生したサイトカインTNF-αの微粒子サイズ依存性を示したものである (in vitro細胞機能性試験)[2]. サ

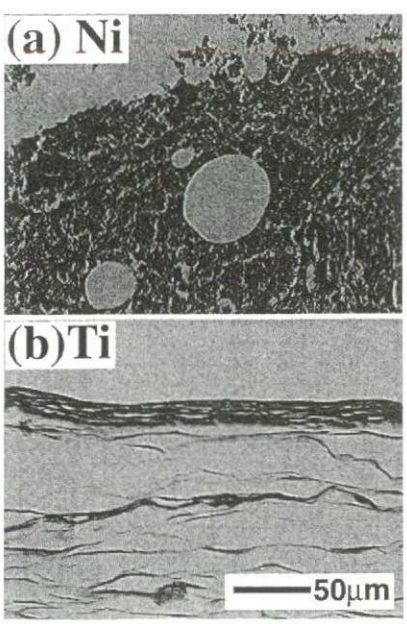

図2　マクロサイズの各種金属に対する軟組織の反応 (ラット皮下埋入1週後). (a) Ni, (b) Ti

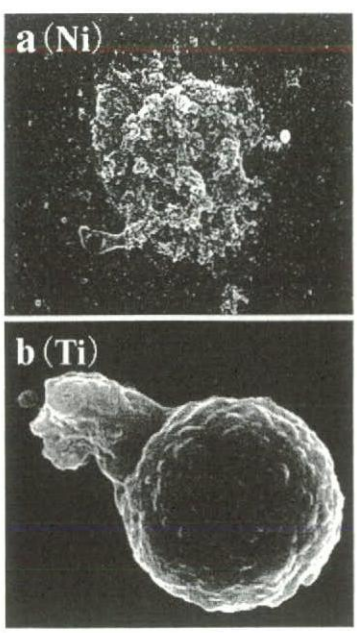

図3　各種金属微粒子に対するヒト好中球の反応 (SEM像). (a) Ni (0.5μm), (b) Ti (0.5μm)

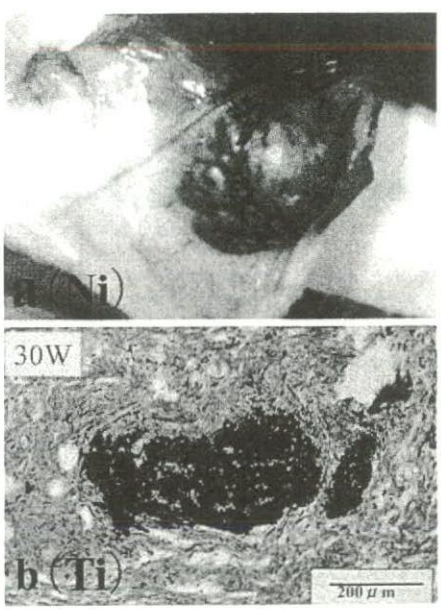

図4　ラット軟組織に長期 (6ヵ月～1年) 埋入後の各種金属微粒子に対する反応. (a) Ni (0.5μm), (b) Ti (0.5μm)

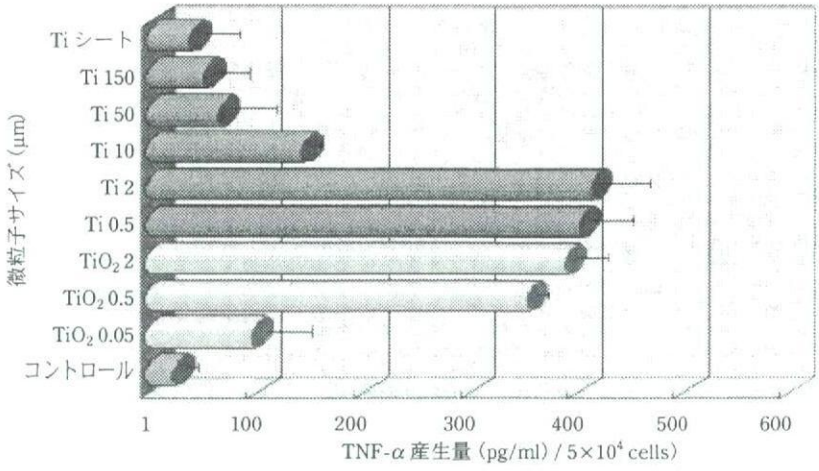

図5　炎症性サイトカインTNF-α産生の微粒子サイズ依存性

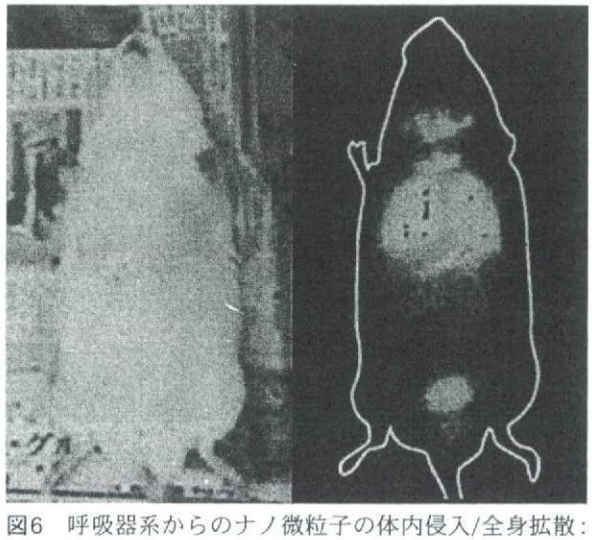

図6　呼吸器系からのナノ微粒子の体内侵入/全身拡散：30nmTiO_2粒子の強制露曝試験後のXSAMマッピングによるラット体内の全身Ti元素分布像[5].

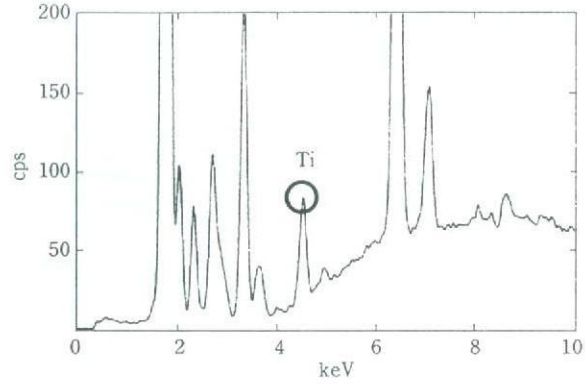

図7　消化器系からのナノ微粒子の体内取込/全身拡散：30nmTiO_2粒子を10日間投与後の脾臓からのX線元素分析．Tiが検出される[11].

イトカインは細胞から放出される比較的低分子量の蛋白質で細胞間のシグナル伝達，新たな細胞の分化・誘導等の機能を果たしている．サイトカインは多数存在するが，TNF-αは代表的な炎症性サイトカインの一種で，刺激性や炎症の程度を示す指標と考えることができる．Ti微粒子サイズが150μmから0.5μmへ順次小さくなるにつれ，放出量は増加する傾向にあるが，とりわけ10μm以下になると急激に増加している．

in vitro, in vivo試験の結果を総合すると，100μm以上では巨視的サイズと同様の親和性を示すが，50μm以下では刺激性が亢進し，特に10μm以下になると細胞に貪食（図3(b)）を誘発して，刺激性が増大し（図5），組織に炎症（図4(b)）を引き起こす現象が金属，セラミックス，ポリマー共通に起きる．これは微粒子と細胞・組織との相対的なサイズの大小関係に由来し，生物学的プロセスによって非特異的に刺激性を示現する効果である（図1）．

ナノ粒子の体内侵入・全身拡散

この材料に非特異的な物理的粒子サイズに由来する刺激性は，μm近傍で最大値を示した後，さらに小さくなりnm領域になるとむしろ低下する（図5）[2]．ナノ物質の応用の観点からは一見都合がよさそうに見えるが，別の見方をすれば異物に対する生体の認識能力あるいは防御能力が低下するということにも通ずる．

微粒子の大きさが約10μmを切ると気管支を通過するが，図6は化粧品に使われている30nmの

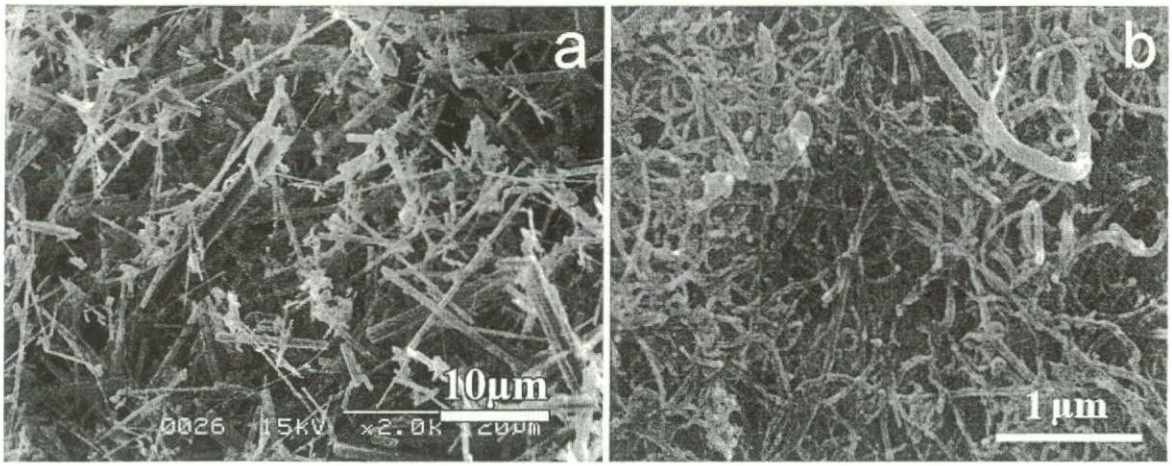

図8　(a) アスベスト (クロシドライト：青石綿) と (b) カーボンナノチューブの粒子形態[5]

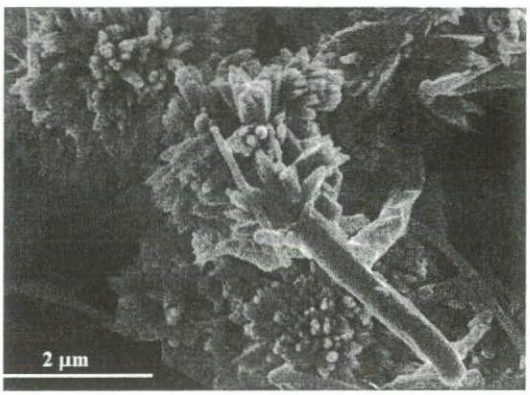

図9　人工体液浸漬によるカーボンナノチューブへのアパタイト析出

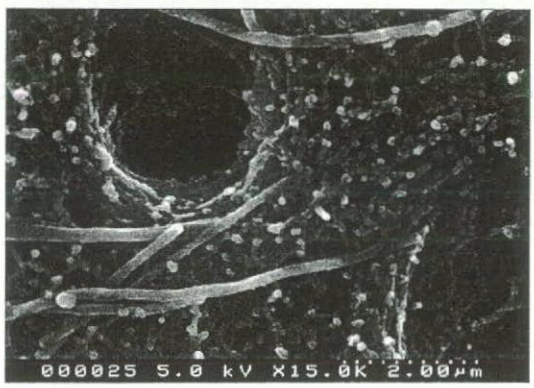

図10　脱灰象牙質表面に露出したコラーゲン線維へのカーボンナノチューブの吸着

TiO_2粉末をラットに強制曝露試験したときのX線走査型分析顕微鏡 (XSAM)[8)~10)] によるTi元素分布像である[5]. 呼気にのって肺胞に到達したナノ粒子が肺から直接血中に取り込まれ, 全身に拡散することがわかる.

図7は同じ粒子を10日間経口投与後, 脾臓から取得したX線元素分析スペクトルである. Tiが検出されており, 消化器系からも体内侵入・全身拡散を起こすことが示される[11].

カーボンナノチューブとアスベスト

カーボンナノチューブは主として電子エミッター, 燃料電池など電子, 化学分野で注目され, 応用開発がなされている. 欧米からは肺ガン誘発性のあるアスベストとの形状の類似性 (図8)[5] の連想から重大な為害性があるという論調が出されていた[12]. しかしそれに相当する実際のデータは無いままに, 議論されてきたところがあるが, 最近, 発癌しやすいラットを使用して中皮に直接曝露する腹腔内埋入試験により, 中皮腫が発生したという報告が出された[13]. 長期大量に被爆した場合, アスベストと同様な発癌性を呈するかについては, カーボンナノチューブ[14]には様々なものがあり, また特性や挙動がアスベストとは異なる面があり[15], 今後なお検討を要するが, その為害性の有無如何に関わらず, 生産現場等, 長時間大量に扱う環境では, 被曝を避ける予防的措置を取ることは当然必要である.

カーボンナノチューブのバイオ応用に注目した研究は今日に至るまできわめて少ないが, 筆者らの行った細胞機能性試験[16)17)], 動物埋入試験[18]か

らは，bioinert材料一般に起きる程度の微粒子刺激性は有するものの，短中期的には特異的な生体為害性は認められず[19]，むしろ生体材料として有利な細胞・組織に対する特徴的な種々の親和性が多数見出され，バイオ用カーボンナノチューブの開発[20]，糖鎖・アパタイトによる表面修飾，組織再生用スカフォールド等[21]，バイオ応用へ向けた開発を行っている．図9はカーボンナノチューブを人工体液に浸漬した際に生ずるアパタイトの析出，図10は酸で脱灰した歯の象牙質部の表面に露出したコラーゲン線維に，蛋白質や糖鎖への親和性に富むカーボンナノチューブが吸着する例である．

おわりに

健康と環境問題は，2008年7月に開催されたG8北海道洞爺湖サミットでも取り上げられたように，21世紀に直面する主要課題である．ナノテクノロジーの進展とともに，ナノ物質の開発が進行し，新しい機能性や高効率化が実現されつつあるが，一方，ナノサイズ化により化学反応は著しく促進されることから，メリット（高機能性）とともに，意図せずしてデメリット（為害性）もまた昂進される可能性がある．これらはしかし人間にとって有効か逆効かという価値判断に依存した評価であり，同一種の現象の表裏の関係にあると言える．

例えば抗癌剤を投与するとしばしば癌患部に到達する前に健常組織に吸収され体力を損なう副作用に働いてしまうが，DDS（ドラッグデリバリーシステム）はその欠点を回避するために，癌患部にのみ特異的に結合する修飾基を付けて血流中を移送し薬剤を投与しようとするナノ粒子の代表的なバイオ応用例である．こうした薬物体内動態は意図的に血液に投与した作用であるのに対し，図6に示したナノ粒子の体内侵入・全身拡散[22]は意図せずして血中を回流する効果であって，人間の目的にかなうか否かの違いだけで現象的には全く同一である．

2000年以来，筆者らはほぼ世界に先駆け，材料のミクロ/ナノサイジングに対する生体反応性を調べ，ナノテクノロジーの人体へのバイオ応用にはあらかじめ起こり得る生体反応と条件を把握する必要があるということをデータに基づき繰返し提起してきた．またその中でカーボンナノチューブのバイオ応用性も見出してきた．しかしこうした話題はナノテクのイメージに対してマイナスであるとして必ずしも好まれないところがある．

ナノ微粒子は生体防御機構が想定していない対象である可能性があり，高機能性と刺激性の両面を併せ持ち，その制御が重要であるということを基本的な認識として持つ必要がある．アスベストの二の轍を踏むことのないよう，産業労働衛生[23]や地球環境保全の観点からナノテクノロジーのリスクアセスメントもまた国民の安心できるナノテク開発の確立のために必須である[24]．

通常，ナノテク関連の学会は応用性を追求するがリスク評価のテーマは好まれず，トキシコロジー（毒性）分野では為害性のみ強調する傾向があり，両極に分かれがちである．筆者らはナノテクノロジーのバイオ医用応用と安全性評価の両者を同一の場で検討することを意図し，研究会を開催してきたが[25]，この種の会議は世界的にも類例がなく，今後，機会を得て学会設立へと発展させたいと考えている．

繰返しになるが，ナノ物質の有害性発現はナノ物質の生体反応性から言えばその一局面に過ぎず，材料の微細化に伴う生体反応自体はきわめて興味深く本質的な研究分野である．

例えば骨の主成分はリン酸カルシウムの一種ハイドロキシアパタイトであるが，我々の骨の構造はナノアパタイトから成る複合材料と見なすことができ，骨吸収と形成のプロセスを繰返すリモデリングを常に行っている．実際マクロな人工アパタイトはすぐれた骨伝導性を示すが，骨に置き換わらないのに対し，生体を模倣したナノアパタイト－コラーゲン/コンポジットを骨欠損部に埋入すると，骨置換性に機能性転換する[26]．こうした現象はナノ微粒子であるから生物学的プロセスにおいて可能なのであり，ナノ構造は生体に必須とも言うことができる[27]．

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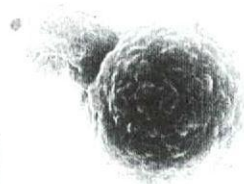
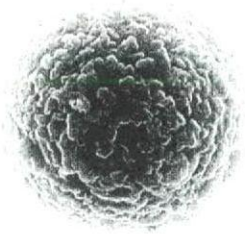


ナノトキシコロジー入門

亘　理　文　夫



ナノテクノロジーの進展に伴って，ナノサイズの微粒子が環境や生体へ及ぼす影響への関心が高まってきた．リスクが未知である現状では，断片的なデータを誇張した議論を避け，まず，基礎データを蓄積することが重要である．環境中や生体内でのナノ粒子の挙動・反応性に関する正しい理解は，ナノテクノロジーの健全な発展にも役立つはずだ．

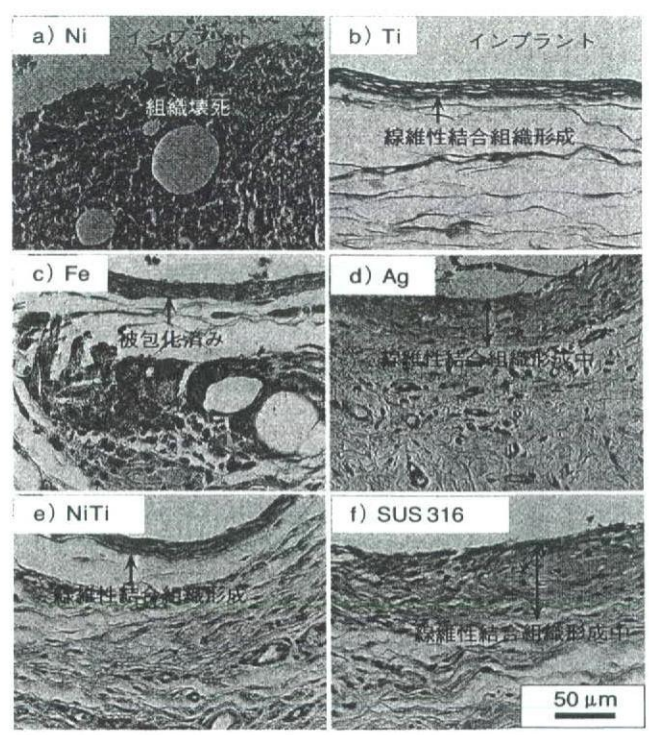
材料の微細化と生体刺激性

チタンは金属中，最も生体親和性に優れ，インプラント（体内埋植）に最もよく使われているが，人工関節の骨頭摺動部に使用された場合には摩耗粉を発生し，周囲組織に炎症をひき起こす．最近問題が顕在化したアスベストは，材質的には粘土鉱物の一種であり，本来毒性のあるものではないが，長期間大量に吸引曝露すると，20～30年を経て中皮腫を発症するに至る．こうした現象には，単に材質が毒性か生体親和性かという特性とは別に，微粒子特有のサイズ，形状に起因する効果が寄与しているものと考えられる．

材料のナノサイジングは新たな機能性を産み出すことから，ナノテクノロジーの展開が図られている．抗がん剤を健常組織に吸収させず，がん患部にのみ選択的に移送するドラッグデリバリーシステム（DDS）は，バイオ応用をめざした典型的なナノ粒子の応用例である．一方でナノ粒子は，人体が生体防御機構の対象として想定してこなかった新たな異物であり，組織傷害性もまた亢進する可能性がある．今後のナノテクノロジーの本格的なバイオ応用開発には，ナノ/マイクロ粒子の生体および環境への影響と，その注意点を把握しておくことが必要である．

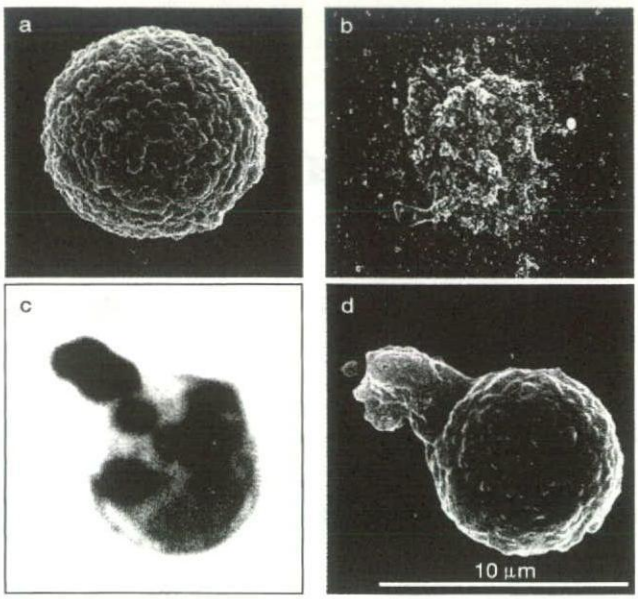
筆者らは2000年以来，材料のマイクロ/ナノサイジングによる生体反応性（文献1～3）と，それに基づくバイオ応用，とりわけカーボンナノチューブを中心とした研究開発を行い，このことを提起してきた．この間，2003年に“ナノトキシコロジー”という用語が欧米から現れ，たとえば，つぎのような主張がなされている．

「ナノテクが新たな有害物質を生む？――ナノテクノロジーによって生み出された新しい微粒子が人体にもたらす健康リスクについて，懸念が寄せられている．粒子は小さくなるにつれて化学反応性が高まるが，毒性もまた高くなると危惧される…」（メールマガジンhttp://www.hotwired.co.jp/news/news/20040115302.html）



直径1 mmの各金属インプラントは，各写真の生体組織の上部に接するように挿入されていた（埋入試験後，組織摘出時に取除かれている）．

図1　各種金属に対するラット皮下軟組織の反応（1週間埋入後）（文献11）



a) コントロール，b) Ni（0.5 μm），c) TiO_2（0.3 μm），d) Ti（0.5 μm）

図2　各種微粒子に対するヒト好中球の反応：SEM像（a, b, d）および光学顕微鏡像（c）（文献2）

日本では2004年，ナノテクノロジー開発の社会的影響・責任などの検討会が各省庁で設置されている．しかし，国内外とも，こうした関連の研究会ではデータがないままの議論が多く，断片的なデータが誇張して取上げられることも多い．カーボンナノチューブは特に係争中のテーマである．ここでは以下，筆者らのデータを示しながら説明を進めたい．

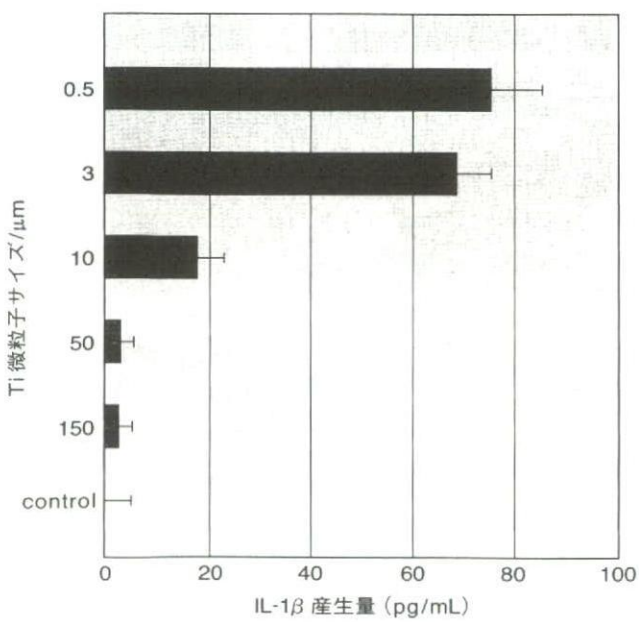


図3　ヒト好中球からのサイトカインIL-1β産生量のTi微粒子サイズ依存性（文献2）

材料と生体反応：マクロ / 生体内（*in vivo*）試験

微粒子の生体反応を見る前に，まず通常の場合，すなわちマクロサイズの材料に対する組織反応性を見ておこう．

図1は各種金属（a：Ni，b：Ti，c：Fe，d：Ag，e：NiTi，f：SUS316）をラット皮下に1週間埋入したときの周囲軟組織を示したものである．Niでは組織が壊死し，強い為害作用（生体親和性に対して，アレルギー性，組織傷害性，毒性など強い生体刺激性全般を指す）を示すが，NiTi，Tiでは，インプラントを被包化する線維性結合組織が形成されており，生体親和性に富む材料の典型的な反応が見られる．NiTiでは，表面でのTiによる強固な不動態酸化皮膜形成によるNi溶出の低減の結果として生体親和性を示したと考えられる．TiとNiTiへの反応は類似しているが，より詳細に見ると細胞核の密度，染色濃度はNiTiのほうがTiよりも高く，相対的に生体がより強く反応していることを示している．純Tiのほうが，長期的には生体親和性がより良好と予想される．Feは，溶出は大きいが為害性は小さく，すでに被包化している．AgおよびNiを含有するステンレス鋼（SUS316）では，まだ線維性結合組織は形成途中である．各材料の生体適合性の程度を敏感に反映して組織反応性が変化していることがわかる．

以上のように，通常（すなわちマクロサイズでは），材料の為害性の程度に差異はあるものの，生体適合性は，まず材質，すなわちイオン化傾向に代表されるような材料の化学的イオン溶出性に第一義的な影響を受ける．

微粒子の生体反応性：生体外（*in vitro*）試験

つぎに材料がミクロサイズ化したとき，すなわち微粒子の生体反応性を*in vitro*細胞機能性試験の結果で示す．

図2は各種微粒子を混和した細胞培養用Hanks溶液（HBSS）中のヒト好中球の顕微鏡像である．図2aは通常（コントロール）の好中球の走査型電子顕微鏡（SEM）像である．

好中球は白血球の約50％を占める5～10 μmと比較的小さな顆粒球で，生体内の異物に対して非特異的に反応する貪食細胞の一種である．

図3はこのときのヒト好中球からのサイトカインIL-1βの放出をTi微粒子サイズに対して示したものである．サイトカインは細胞から放出される比較的低分子量のタンパク質で，細胞間のシグナル伝達，新たな細胞の分化・誘導などの機能を果たしている．サイトカインは多数存在するが，IL-1βは炎症性反応の程度を示す最も代表的な指標の一つである．

Ti微粒子サイズが150 μmから0.5 μmへ順次小さくなるにつれ，IL-1βの放出量は増加する傾向にあるが，とりわけ10 μm付近から急激に増加している．同様にヒト好中球からの活性酸素産生量を測定すると，粒径が小さくなるとともに産生量が増加し，細胞生存率については順次低下した（文献1, 2）．これらの指標はいずれも微粒子サイズの減少とともに，細胞刺激性が増加し，細胞がTi微粒子を異物とみなして反応し，特に10 μmを切ると著しく刺激性が増大することで一致している．

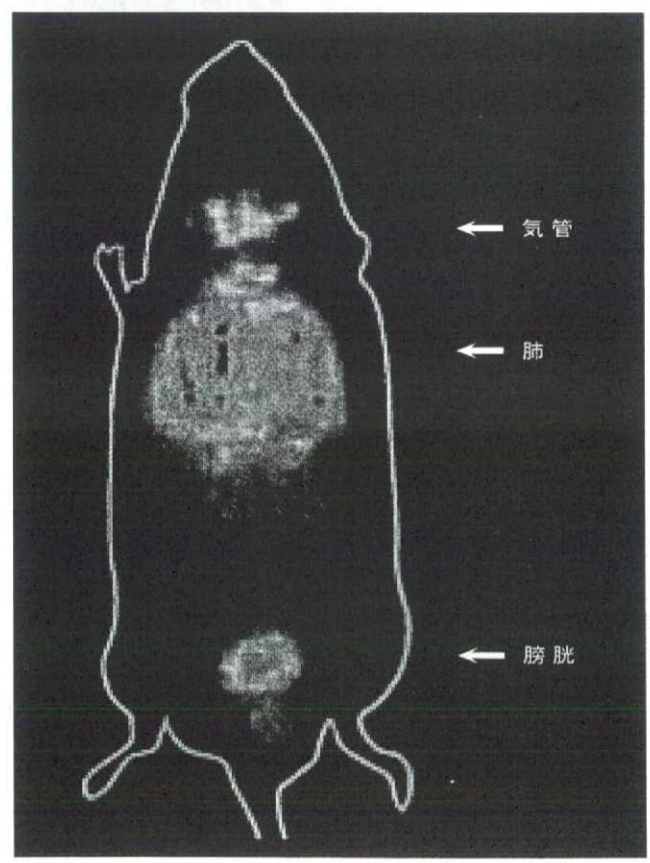
図3の実験条件における液中での好中球の形態を観察すると，3 μm以下の粒子でのみ好中球による貪食像が観察された（図2）．図2c，dは，0.3 μm TiO_2，0.5 μm Ti粒子混和液中で観察された好中球の光学顕微鏡像，SEM像である．それぞれ，偽足を伸ばし貪食しようとしている様子をとらえている．この好中球をエネルギー分散型X線分光（EDS）で元素分析すると，Tiが検出され，好中球によるTi粒子の貪食が確認される．図2bはNi微粒子（0.5 μm）について同様な実験を行ったときの好中球のSEM像で，Niの強い為害性のために細胞は破壊されている．好中球の大きさは約5～10 μmであり，3 μm粒子は貪食可能であるが，10 μm以上の粒子では不可能と考えられる．図3に示された刺激性は，こうした異物に対する細胞の防御作用と密接に関連しており，微粒子サイズが細胞以下になり，貪食を誘発するようになると刺激性は一段と亢進する．

以上の条件下でTi微粒子からの溶出量の高周波誘導結合プラズマ（ICP）元素分析を行うと，検出限界値以下であり，Tiイオン溶出は無視できると考えられる．すなわち，図3でTi微粒子が示した刺激性・為害性は，化学的イオン溶出が無くても発生するのであり，同様なサイズ依存性はFeなどの金属，TiO_2，カーボンナノチューブなどのセラミックス，ポリ乳酸などのポリマーでも，バイオアクティブ*1，バイオイナート*2材料全般に見いだされる．微粒子特有の物理的サイズ・形状に由来するものであることがわかる．もともと為害性のあるNiでは，サイズ依存性を示すが，産生量の絶対値などは，やや異なる挙動を示す．

ナノトキシコロジー効果

上述のように微粒子になり，材料のサイズが細胞と同程度（約10 μm）になると刺激性が亢進し，Tiのような生体親和性材料でも炎症を誘発する．一方，アレルギー性を示す代表的な金属のNiでは，組織埋入試験を行うと，マクロサイズでは周囲組織の壊死，炎症をひき起こすが，マイクロ/ナノ微粒子になると1年埋入後には腫瘍を誘発する（文献12）．もともと為害性を示す材料では，マイクロ/ナノサイジングに伴う比表面積増大効果（同一体積に対する微粒子の全体の総表面積は，粒径が小さいほど増大する）により，為害性も著しく亢進する典型的なナノトキシコロジー効果である．図3で示された微粒子の細胞刺激性は，約0.5 μmを頂点に，さらに小さくなるとむしろ低下する．応用の観点からは，これは一見都合がよさそうに見えるが，また異物に対する生体の認識能力あるいは防御能力が低下するという意味ももつ．

微粒子は大きさが約10 μmを切ると気管支を通過する．図4は化粧品に使われている30 nmのTiO_2粉末をラットに強制曝露試験をしたときのX線走査型分析顕微鏡（XSAM）によ



ナノ粒子は呼吸器系を通じて体内に取込まれ，全身に拡散する．

図4　ナノトキシコロジー：30 nm TiO_2粒子の強制曝露試験後の全身のTi元素分布像

*1　バイオアクティブ（bioactive，生体活性）；骨誘導性を示すアパタイトなどのように積極的に生体の活性を引出す物質．

*2　バイオイナート（bioinert，生体不活性）；Ni，Cd，Beのような為害性は示さないものの，アパタイトのように積極的に生体の作用を誘導することもない物質．アルミナ，カーボンなど．