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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

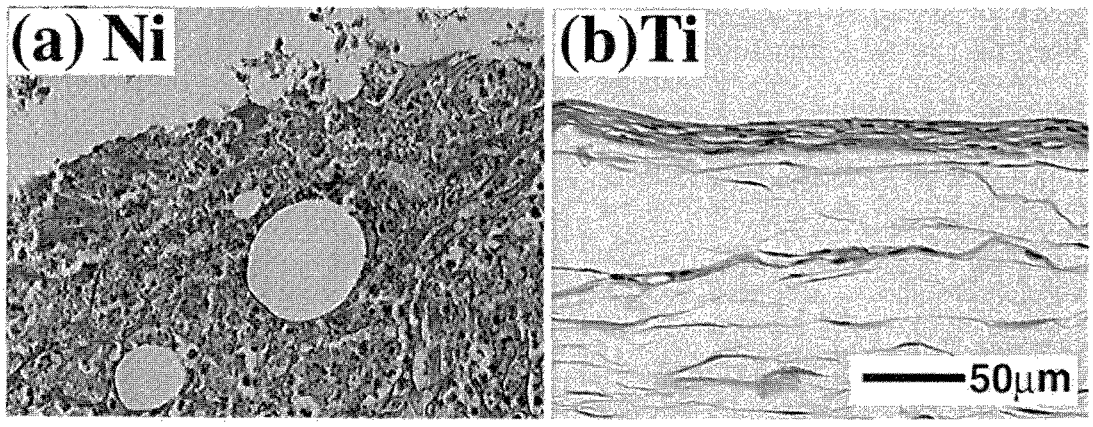
Fig.1 shows the comparison of tissue reaction to the macroscopic size (1 mm ϕ x 10 mm) of Ni (a) and Ti (b) after 1 week implantation in the dorsal thoracic region of rat. Implant had been situated in the upper space of each photograph. In Ni the expansion of capillary vessels was observed. Tissue in 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



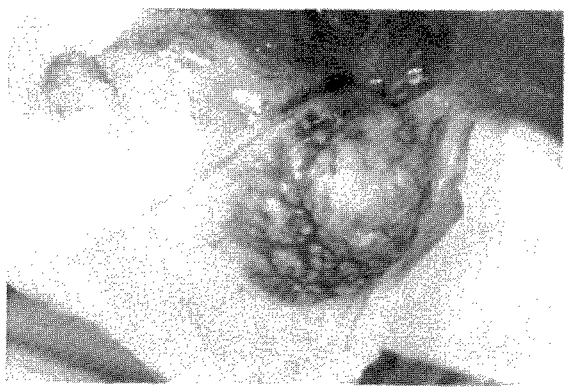


Fig.2 Tumor induced after 1 year implantation of 500nm Ni particles [4].

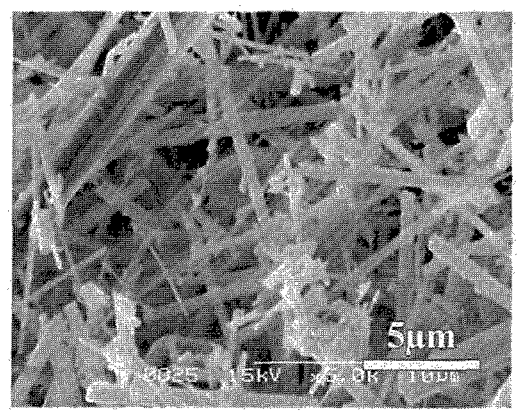


Fig.3 Morphology of asbestos (crocidolite: blue asbestos) observed by SEM.

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

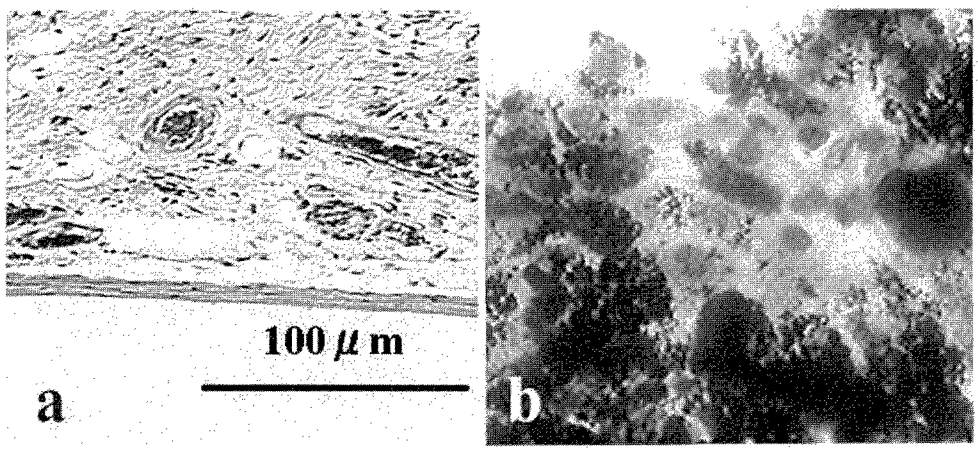


Fig.4 Comparison of reaction of rat soft tissue to the macroscopic Ti implant (a) and 3μmTi particles (b) in histological observation.

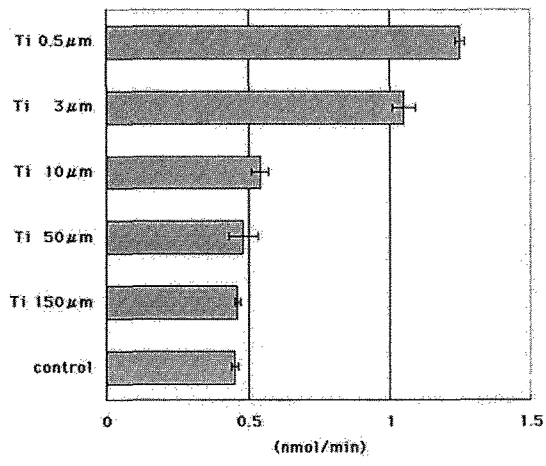


Fig.5 Dependence of superoxide production from neutrophils on Ti particle size [6]

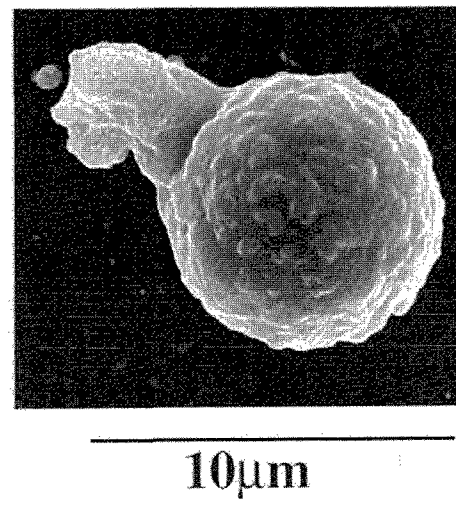


Fig.6 SEM image of a human neutrophil exposed to 500nm Ti particles [6].

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

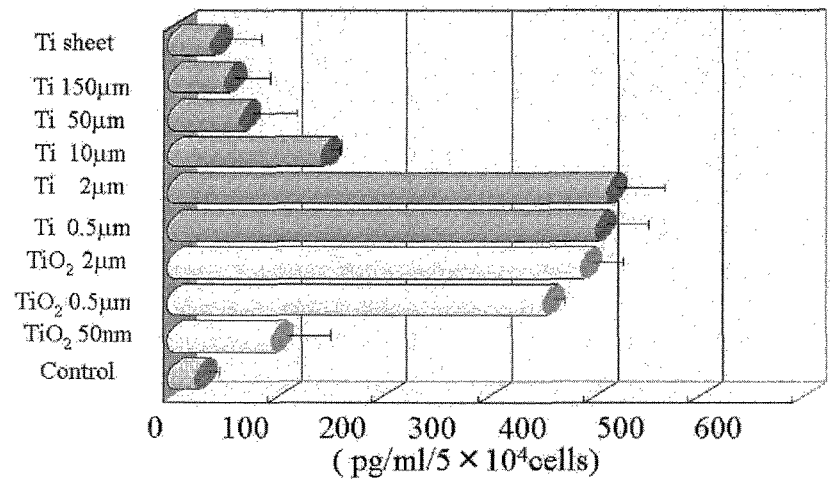


Fig.7 Dependence of TNF-α release from neutrophils on particle size down to nm size [4]

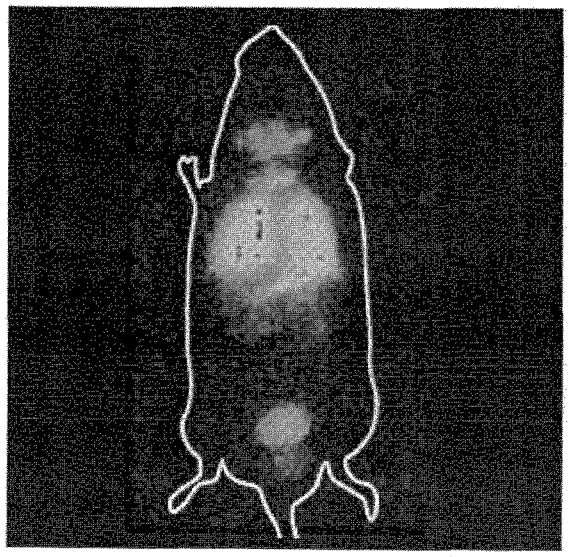


Fig.8 XSAM Ti mapping of internal distribution of 30 nm TiO_2 particles after compulsory exposure test [4].

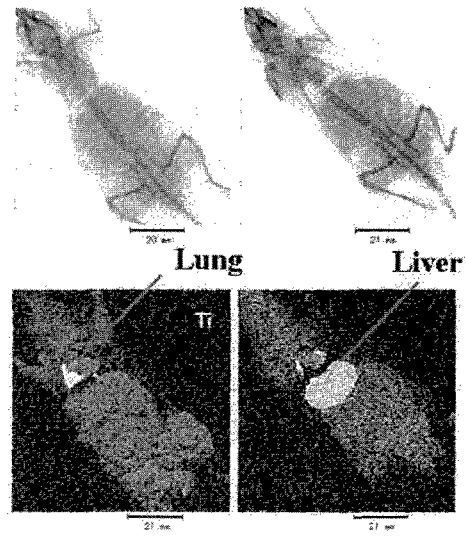


Fig.9 Time course of internal diffusion of 30nm TiO_2 particles after injection to caudal vein

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

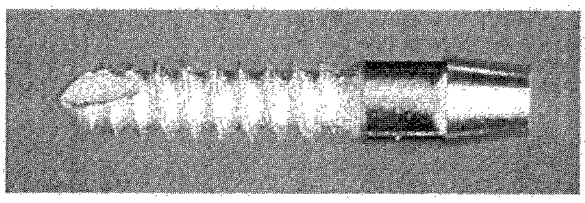


Fig.10 Dental implant composed of apatite-coated titanium

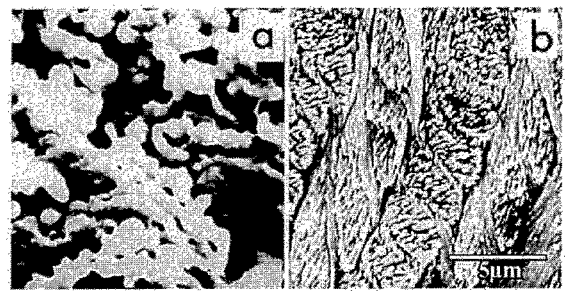


Fig.11 Difference of morphology of hydroxyapatite. a) sintered synthetic apatite, b) enamel of molar of rat.

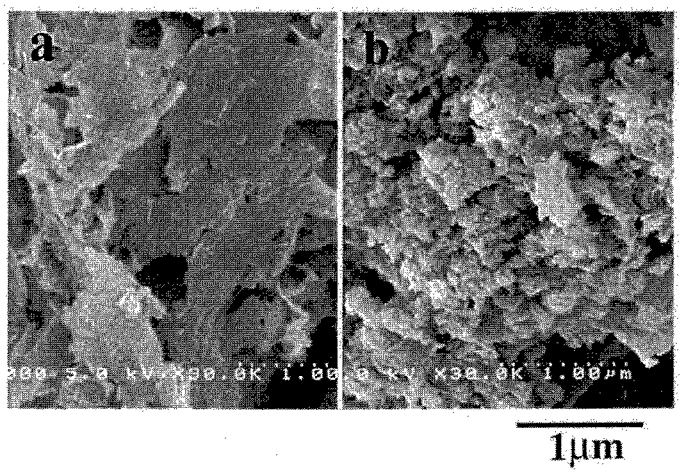


Fig.12 Hydroxyapatite synthesized without (a) and with (b) collagen.

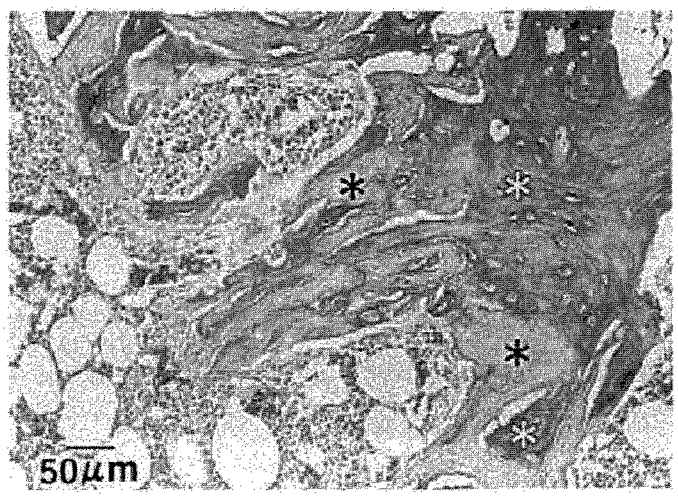


Fig.13 Histological image at 8 weeks after implantation in the bone marrow of rat. Materials (asterisks) were decreased and covered with new bone (white asterisks) with lamellar structures [9].

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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REFERENCES

[1] Y.Tamura, A.Yokoyama, F.Watari and T.Kawasaki, Dental Materials J., 21, 355-372 (2002)
[2] Y.Tamura, A.Yokoyama, F.Watari, M.Uo and T.Kawasaki, Mat.Trans., 43, 3043-3051 (2002)
[3] F.Watari, M.Inoue, T.Akasaka, N.Sakaguchi, H.Ichinose and M.Uo, Proc.6th Asian BioCeramics Symp.2006, 142-145 (2006)
[4] F.Watari, K.Tamura, A.Yokoyama, K.Shibata, T.Akasaka, B.Fugetsu, K.Asakura, M.Uo, Y.Totsuka, Y.Sato and K.Tohji, "Handbook of Biomineralization, Vol.3", Ed. by E.Bauerlein, Wiley-VCH, Weinheim (2007) pp127-144
[5] R.Kumazawa, F.Watari, N.Takashi, Y.Tanimura, M.Uo and Y.Totsuka, Biomaterials, 23, 3757-3764 (2002)
[6] K.Tamura, N.Takashi, R.Kumazawa, F.Watari and Y.Totsuka, Mat.Trans., 43 , 3052-3057 (2002)
[7] M.Uo, F.Watari, A.Yokoyama, H.Matsuno and T.Kawasaki, Biomaterials, 20, 747-755 (1999)
[8] M.Uo, M.Tanaka and F.Watari, J.Biomed.Mater.Res., Part B:Appl.Biomater., 70B, 146-151 (2004)
[9] A.Yokoyama, M.Gelinsky, T.Kawasaki, T. Kohgo, U.König, W.Pompe and F.Watari, J. Biomed Mater Res Part B:Appl Biomater, 75B, 464-472 (2005)
[10] H.Matsuno, A.Yokoyama, F.Watari, M.Uo and T.Kawasaki, Biomaterials, 22, 1253-1262 (2001)
[11] Y.Zhu and F.Watari, Dent.Mat.J., 26, 245-253 (2007)
[12] F.Watari, A.Yokoyama, M.Omori, T.Hirai, H.Kondo, M.Uo and T.Kawasaki, Composites Science and Technology, 64, 893-908 (2004)
[13] F.Watari, J.Electron Microscopy, 54, 299-308, (2005)
[14] S.Liao, W.Wang, M.Uo, S.Ohkawa, T.Akasaka, K.Tamura, F.Cui and F.Watari, Biomaterials, 26, 7564-7571 (2005)
[15] N.Aoki, T.Akasaka, F. Watari and A.Yokoyama, Dent.Mat.J., 26, 178-185 (2007)
[16] N.Aoki, A.Yokoyama, Y.Nodasaka, T.Akasaka, M.Uo, Y.Sato, K.Tohji and F.Watari, J.Biomed. Nanotechnology, 1, 402-405 (2005)
[17] A.Yokoyama, Y.Sato, Y.Nodasaka, S.Yamamoto, T.Kawasaki, M.Shindoh, T.Kohgo, T.Akasaka, M.Uo, F.Watari and K.Tohji, Nano Letters, 5, 157-161 (2005)
[18] M.Ushiro, K.Uno, T.Fujikawa, Y.Sato, K.Tohji, F.Watari, W.Chun, Y.Koike and K.Asakura, Phys. Rev., B 73, 144103/1-11 (2006)
[19] S.Liao, G.Xu, W.Wang, F.Watari, F.Cui, S.Ramakrishna and C.K.Chan, Acta Biomaterialia, 3, 669–675 (2007)

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