21:00-21:05

Closing Remark

Prof. Kazuyuki Tohji
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Topic 1

Particles and Reaction of Cells and Tissue

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The reaction to fine particles in Ti, Fe, Ni, TiO_2 and carbon nanotubes(CNT) was investigated in vitro using human neutrophils as probe cells and in vivo in animal implantation test. The biochemical functional analyses of cell survival rate, LDH, superoxide anion, cytokines and microscopic observation of cellular morphology, histological observation of tissue reaction revealed that the stimulatory effects on neutrophils and inflammation in soft tissue became prominent as the Ti particle size became smaller for the range less than 100μm. The effect was especially pronounced when the particle size was smaller than 10μm, about the cell size, where the phagocytosis was induced. ICP elemental analysis showed that the dissolution from Ti particles was below detection limit. Fe showed the quantitatively similar results as Ti, although Fe is soluble. All these results indicated that the cytotoxicity arises due to the physical size and shape effect of particles, which is different from the chemical toxicity effect induced by ionic dissolution and usually dominant in bulk materials.

CNT showed the cytotoxicity and tissue inflammation as generally expected from particle reaction as bioinert material. However the specific cytotoxicity was not observed for short term test. Preparation and surface modification of CNT's suitable for biomedical application was developed and investigation for scaffold for cell proliferation and apatite coating was done.

Invited Talk 1

Using Near-Infrared Methods to Explore the Interactions of Carbon Nanotubes with Biological Systems

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Center for Nanoscale Science and Technology, and

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Rice University

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Carbon nanotubes are novel artificial nanomaterials that promise many exciting applications in fields such as microelectronics and materials science. However, very little is yet known about their interactions with biological systems. In part, this is because it is quite difficult to observe all-carbon nanostructures in complex biological environments. The discovery of near-infrared fluorescence from single-walled carbon nanotubes (SWNT) offers a powerful new approach for detecting and imaging nanotubes in cells, tissues, and organisms. Using spectrofluorimetry and a fluorescence microscope modified for imaging beyond 1100 nm, we have selectively and sensitively detected SWNT in biological specimens. Results will be presented on mouse macrophage cells that have been incubated with nanotubes. High contrast near-infrared fluorescence images reveal intracellular nanotubes that have been actively ingested, apparently through phagocytosis. In addition, these cells show no indication of toxicity or growth inhibition from 96 hours of nanotube exposure. Future prospects for using near-IR fluorescence in toxicology and biodistribution studies will be discussed, along with ideas for developing nanotube bioconjugates as targeted agents for medical diagnosis and therapy.

Topic 2

Biological Behaivior of Hat-Stacked-Type Carbon Nanofibers in the Subcutaneous Tissue in Rats

Atsuro Yokoyama*[1], Yoshinori Sato[2], Yoshinobu Nodasaka[1], Satoru Yamamoto[1], Takao Kawasaki[1], Masanobu Shindoh[1], Takao Kohgo[1], Tsukasa Akasaka[1], Motohiro Uo[1], Fumio Watari[1] and Kazuyuki Tohji[2]

Graduate School of Dental Medicine, Hokkaido University 1

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Introduction

Applications for medical device of nano scale substance such as carbon nanotubes (CNTs) and fullerenes have been attracted a great deal of attention. The details on toxicity and biocompatibility of nano scale substances have been not clarified yet. The investigation of tissue response including animal experiment in addition to *in vitro* study is necessary to evaluate toxicity and biocompatibility of nano scale substances and to develop biomaterials with them, however there were a few reports about tissue reaction to nano scale substances. Sato et al. developed a new type of carbon nanofibers (CNFs), whose structures were like as stacked hats, they named it hat-stacked-type CNFs (H-CNFs). H-CNFs were soluble in water and it was possible to control the size of them. The purpose of this study was to investigate tissue response in the subcutaneous tissue and evaluate biocompatibility of H-CNFs.

Materials and Methods

H-CNFs: H-CNFs were produced by thermal CVD using powdered Ni catalyst in the conventional flow reactor system according to the report by N. M. Rodriguez.

Animal experiments: Eight male 6-week-old Wistar strain rats were used in this study. Clusters of H-CNFs were implanted in the subcutaneous tissue in the thoracic region bilaterally in each rat. The rats were sacrificed at 1 and 4 weeks after surgery. Specimens were observed by optical microscopy and transmission electron microscopy.

Results

The structure of H-CNFs resembled the bamboo hats stacked up toward a needle axis. Lengths of CNFs were ranged approximately between 100 nm and 1 um, and the diameters of those were between 30 and 100 nm, respectively. Ni was not detected.

At 1 week after implantation, clusters of H-CNFs were surrounded by granulation tissue with a slight inflammatory change. Some of H-CNFs were englobed by macrophages. At 4 weeks, clusters of H-CNFs were surrounded by fibrous connective tissue. No severe inflammatory response such as necrosis, degeneration and neutrophils infiltration were observed around H-CNFs through the experimental period.

TEM observation showed that some H-CNFs were recognized in phagocytes with many vacuoles such as macrophages at 1 week. There were no changes of the form in H-CNFs after phagocytosis. At 4 weeks,

although the characteristic forms of H-CNFs, hat-stacked shape, were recognized, the degree of aggregation was decreased in comparison with that at 1 week. Furthermore, the lengths of H-CNFs looked shorter than those at 1 week and some of H-CNFs appeared translucent, which was not observed at 1 week.

Discussion

In this study, any severe inflammatory response such as necrosis and invasion of neutrophils were not observed. These results suggested that H-CNFs were not acutely toxic in the subcutaneous tissue. It was supposed that these different results of our study from those of other reports on CNTs might be caused on the physicochemical properties of H-CNFs, for example, water solubility and the characteristic structure composed of the stacked graphene hats, apart from the implant site and species of the animals.

Observation by TEM revealed that H-CNFs implanted in the subcutaneous tissue were phagocyted by macrophages. The changes of structure in H-CNFs such as shortening and translucency occured in lysosome and cytoplasm might relate to the characters of H-CNFs.

Conclusion

H-CNFs in the subcutaneous tissue did not induce the acute severe inflammatory reaction. They were englobed by phagocytes such as macrophage and foreign body giant cell.

Invited Talk 2

Peptides that Recognize the Surface of Carbon Nanohorns and Titanium

Kiyotaka Shiba

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Combinatorial polymerization of 20 amino acids can give peptides that have unparalleled specificities and affinities to target molecules. Peptide phage system has been established at 90' to isolate artificial peptides that target to biomacromolecules such as receptors, enzyme and antibodies. [1] The methodology has been recently applied to isolate peptides that specifically recognize the surface of inorganic materials.[2]

I will introduce our studies on the isolation and the characterization of artificial peptides that recognize the surface of inorganic materials including single wall carbon nanohorns (SWNHs)[3] and titanium (Ti). [4]

We used a M13 phage library (diversity ~ 2.7 x 10^9) that displays a linear 12-mer sequence at the N-terminus of coat protein pIII. After cycles of "binding", "washing", "eluting" and "amplification by bacteria", we concentrated phage clones that can bind to the surface of SWNHs or Ti. After several cycles of the bio-panning, we cloned some of the phages and determined the DNA sequences that code for displayed peptides. From these efforts, we identified the SWNHs binding peptide (NHBP-1), DYFSSPYYEQLF, and Ti binding peptide (TBP-1), RKLPDAPGMHTW.

Although numbers of peptide aptamers that bind to various inorganic materials have been isolated using this peptide phage system in the past several years, little is known about the molecular mechanisms and how they interact with the surface of the materials. Therefore, we started to reveal how NHBP-1 and TBP-1 recognize the surface of SWNHs and Ti, respectively.

First we have constructed a series of alanine substitution mutants and investigated their effects on phage binding. From these experiments, we proposed the model for minimal aptamer of TBP-1, minTBP-1 (hexapeptide) recognition, in which electrostatically interactions between the amphoteric surface of titanium and first arginine and fifth asparate have pivotal roles for the recgnition.[4]

To gain further insights into the mechanisms of the interaction between TBP-1 and the surface of Ti, next, we tested its target specificity by using ten different metal targets. Results revealed that TBP-1 binds to the surfaces of confined groups of inorganic materials that share no obvious common features such as isoelectric point, crystal shape, and wettability. The mutant aptamers that lost affinity to titanium did not bind to these materials, indicating that the same mechanism should determine the interactions between TBP and the groups of metals.

I will also talk about the profile of drug adsorption and release of carbon nanohorns.[5]

1) J. K. Scott, G. P. Smith, Science, **249**, 386 (1990).
2) S. R. Whaley, D. S. English, E. L. Hu, P. F. Barbara, A. M. Belcher, Nature, **405**, 665 (2000).
3) D. Kase, I. John L. Kulp, M. Yudasaka, J. S. Evans, S. Iijima, K. Shiba, Langmuir **20**, 8939 (2004).
4) K. Sano, K. Shiba, J Am Chem Soc, **125**, 14234 (2003).
5) T. Murakami K. Ajima, J. Miyawaki, M. Yudasaka, S. Iijima, K. Shiba, Mol Pharmaceutics **1**, 399 (2004)

P01

Water-Soluble Hat-Stacked-Type Carbon Nanofibers for Biomedical Applications

Yoshinori Sato[1], Balachandran Jeyadevan[1], Kazuyuki Tohji[1], Rikizo Hatakeyama[2], KazuchikaTamura[3], Tsukasa Akasaka[3], Motohiro Uo[3], Atsuro Yokoyama[3], Ken-ichiro Shibata[3] and Fumio Watari[3]

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Nanocarbon materials have been widely studied as catalyst supports in heterogeneous catalysis because of their unique morphology and reactivity. Recently, a novel type of carbon nanofiber exhibiting cup-stacked morphology was developed as a unique and functional "tubular" nanosized materials. Generally speaking, the morphology of carbon nanofibers is dependant on the metal catalyst. We have synthesized the carbon nanofibers with the structure in which graphene-hats are stacked toward a needle-axis. Here, we call these "Hat-stacked-type carbon nanofibers (Hat-stacked-type CNFs)". As the edges of the graphene-hat have been exposed, many hydrophilic groups can be added in order to dissolve them into water and to use them biomedical application such as a carrier of DNA transduction. Also, as each hat is van der Waals force in which binding force is weak, hat-stacked-type CNFs can be cut off, that is, it is possible to control their length size. Thus, hat-stacked-type CNFs can be expected as a biomaterial as well as carbon nanotubes [1].

In this presentation, we report simple cutting and water-solubilization of the hat-stacked-type CNFs using sonication in a mixture of concentrated acids [2]. We purified the hat-stacked-type CNFs and characterized the cut hat-stacked-type CNFs by SEM, TEM, XRD, and FT-IR measurements. The hat-stacked-type CNFs were easily cut into 400-nanometer to 1.5-micrometer lengths, and the cut nanofibers formed a stable solution in water.

[1] A. Bianco and M. Prato, *Adv. Mater.* **15**, 1765 (2003).
[2] J. Liu et al., *Science* **280**, 1253 (1998).

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P02

Carbohydrate Coating of Carbon Nanotubes for Biological Recognition

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2) Graduate school of Environmental Studies, Tohoku University

Carbon nanotubes (CNTs) have been attracting considerable attention because of their unique physical properties and potential for a variety of applications. Modifications of CNTs by covalent and non-covalent methods have been examined in recent studies. However, no facile method for incorporating carbohydrate chains as recognition signaling molecules into a CNT has been reported. Here we describe a simple method for surface modification of a CNT with carbohydrate chains as recognition signaling molecules. In this study, we used commercially available lactose-carrying polystyrene (PVLA), which has both pendant β-galactose moieties for use as recognition signaling molecules and a polystyrene backbone that can be adsorbed onto the surface of a CNT via hydrophobic interactions.

The procedure used for coating a MWNT (n-MWNTs: from NanoLab, m-MWNTs: from MTR Co. Ltd.) with PVLA was as follows. FITC-labeled PVLA and MWNT material were put into water and ultrasonicated for 15 min. After 1 h of incubation at room temperature, the

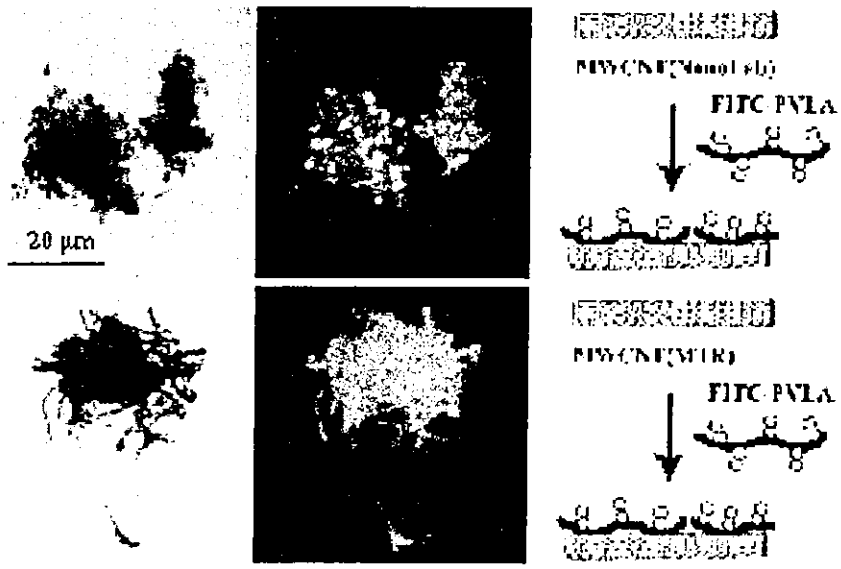

Fig. 1 CLSM Images of FITC-PVLA/MWNTs

mixture was centrifuged. The aggregated MWNT was carefully washed with PBS and deionized water to remove the unreacted polymer. Fluorescence observation by confocal laser scanning microscopy showed that the carbohydrate-carrying polymer was uniformly and densely localized along needle shape of the m-MWNT (Fig. 1).

To evaluate biological recognition affinity, interactions of the MWNT with lectins were examined by binding tests. The resultant MWNT was found to acquire a selective binding affinity to the corresponding lectin without a nonspecific interaction. On the other hand, a bare MWNT nonspecifically interacted with lectins. These results showed that a MWNT coated with a carbohydrate-carrying polymer has biological recognition signals.

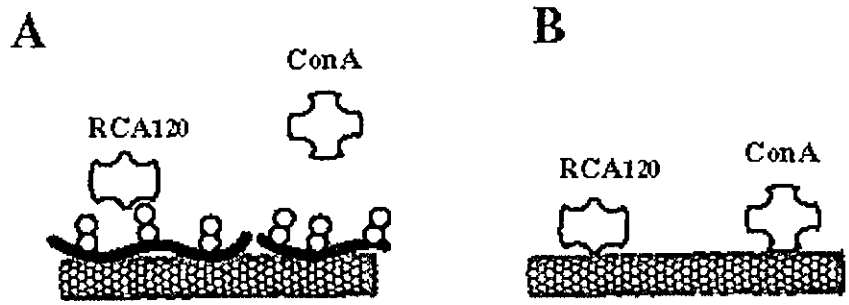

Fig.2 Interactions of (A) PVLA/MWNTs with lectins. (B) bare MWNTs with lectins

In summary, we have demonstrated that multi-walled carbon nanotubes (MWNTs) coated with a carbohydrate-carrying polymer for use as biological recognition signals can be easily prepared by a non-covalent method via hydrophobic interactions. A MWNT coated with a carbohydrate-carrying polymer was found to acquire a selective binding affinity to the corresponding lectin (Fig. 2). Modification of a CNT with various carbohydrate chains will be a useful protocol for molecular designs of biomaterials, nanoarchitecture, and biosensors.

P03

Effects of Ti, Ni and Fe Particles on Cell Function and Particle size Dependent Cytotoxicity

Kazuchika Tamura[1,*1], Noriyuki Takashi[2], Fumio Watari[1], and Yasunori Totsuka[2]

1 Biomaterials and Engineering, Graduate School of Dental Medicine

2 Oral and Maxillofacial Surgery, Graduate School of Dental Medicine, Hokkaido University, Sapporo, 060-8586, Japan

The cytotoxicity by metal ion is one of the most important problems in the use as biomaterial. Ti is regarded as the most stable metallic material, which is difficult to ionize in the living body. It is noted, however, that Ti has the harmful effect on tissue when it is the abrasion powder.

Our previous study showed that Ti fine particles caused inflammation, especially when smaller than cell size, about 5 μm. The phagocytes uptake the Ti particles, when their size decreases down to the cell size. The neutrophils in phagocytosis released superoxide anions and cytokines. They discharge superoxides and produce many chemical transmitters. Part of phagocytes die, or injury cells physically, after fine particle is up-taken in and then the intracellular enzyme escapes. The reaction of cell and tissue depended on the particle size in Ti. Especially the xenobiotic reaction of tissue was obviously enhanced by the particles of 3 μm or less. By these processes, fine particles lead to the inflammatory reaction.

In this study we examined and compared the size dependency of cell reaction against metallic particles with different characteristics: Ni with the solubility and tendency for toxicity, iron with high solubility and rather low cytotoxicity, in addition to Ti with very little solubility and high biocompatibility.

The bio-reaction of cells to fine particles was investigated by biochemical analysis and by microscopic observation *in vitro* using human neutrophils as probes. Attentions were focused on the dependence of cell functions on metallic particles size. The three materials with different characteristics in corrosion, dissolution and biocompatibility were compared: highly corrosion-resistant and biocompatible titanium (Ti), very little corrosive but cytotoxic nickel (Ni) and easily corrosive but little toxic iron (Fe). The chemical dissolution from these particles in HBSS was measured by ICP for up to 4 weeks.

Fig.1

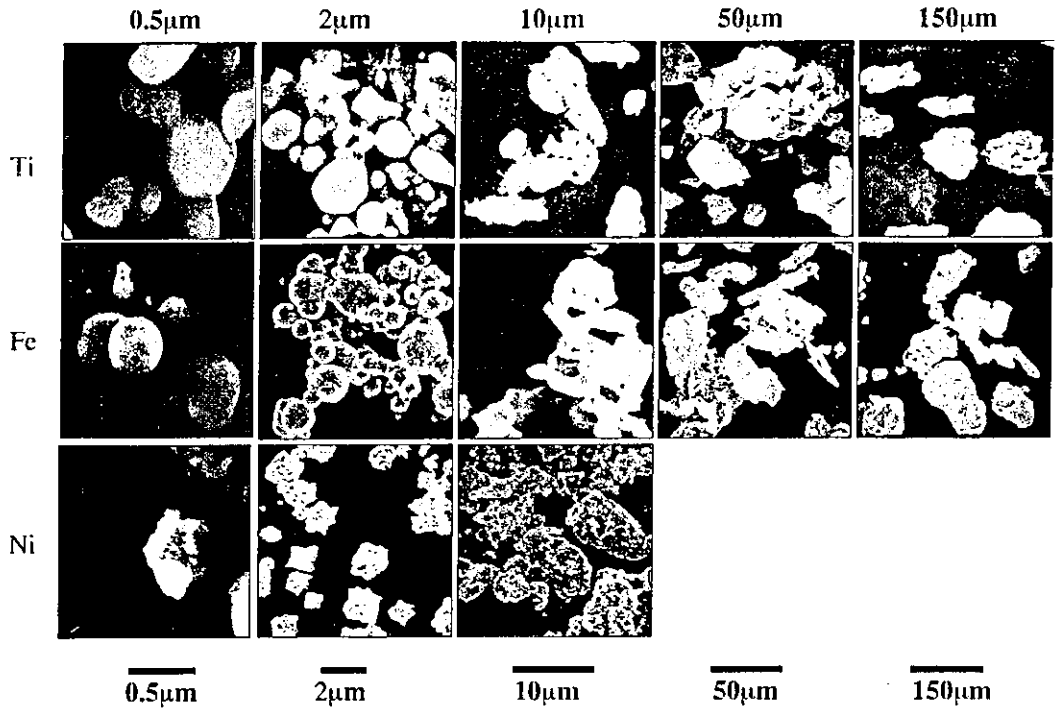

Fig. 1 shows the SEM images of various sizes of Ti, Fe and Ni particles used for the experiment. The nominal size was 0.5 μm (a), 3 μm (b), 10 μm (c), 50 μm (d) and 150 μm (e) for Ti particles, 0.5 μm (f), 3 μm (g) and 10 μm (h) for Ni particles, and 0.5 μm (i), 3 μm (k), 10 μm (l), 50 μm (m) and 150 μm (n) for Fe particles respectively. The average diameter closely matched the sizes given by the suppliers.

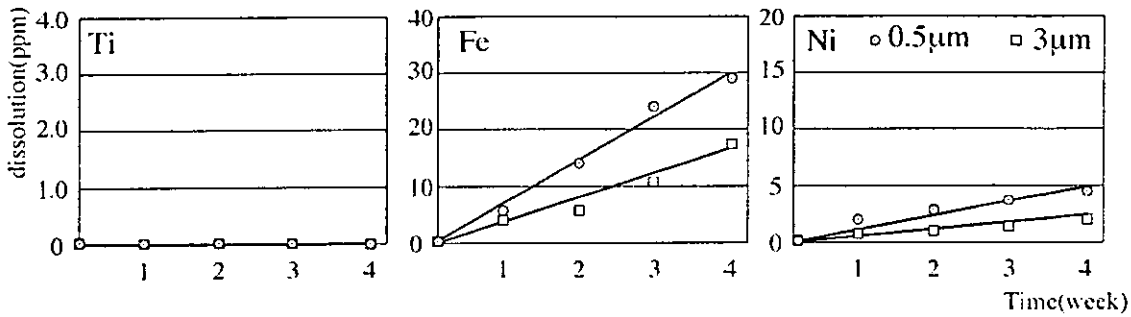

Figure 2 shows the dissolved amount of Ti, Fe and Ni ions from the 0.5 μm and 3 μm particles of 1mg Ti, Fe and Ni after 1, 2, 3 and 4 weeks in the 10ml physiological saline detected by ICP. The dissolved amount of Ti was below detection limit, and the amount of Fe and Ni increased proportionally with time.

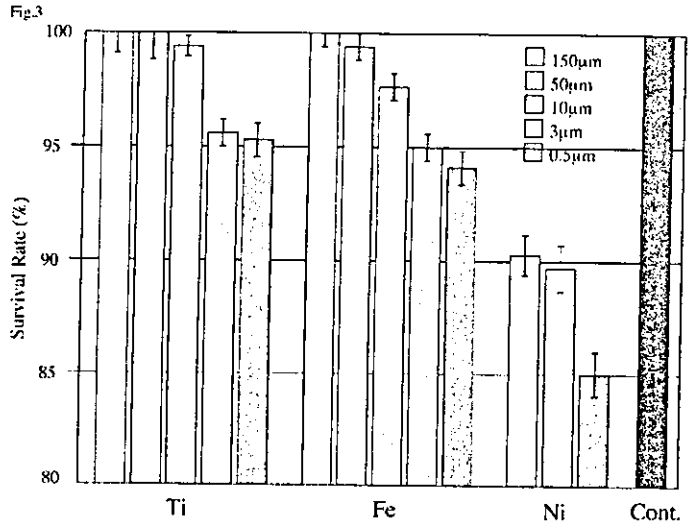

Figure 3 shows the survival rate of neutrophils in the HBSS solution containing Ti, Fe and Ni particles. The mean value for Ti was the smallest in the size 0.5 μm and 3 μm and followed by 10 μm. The other Ti particles had no significant difference from control. Ni particles showed the much lower survival rate, significantly differed from the HBSS. The survival rate was clearly lower in the 0.5 μm Ni particles than 10 μm. The survival rate for Fe particles resembled Ti and showed the size dependence.

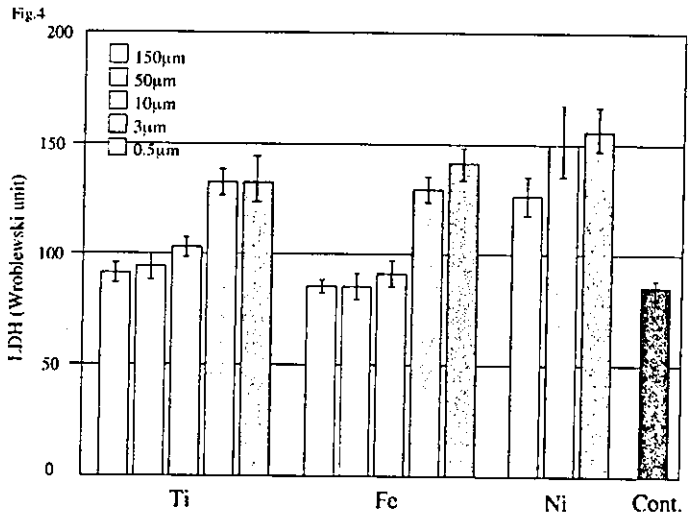

Figure 4 shows the value of LDH from neutrophils in each particle solution. The difference between each Ti particle and the control was significant except 150 μm. Levels of LDH were significantly higher in the 3 μm or less than the other larger sizes. LDH showed the tendency to increase as the Ti particle size became smaller. The Ni group showed higher values than Ti for the same particle size. They had also the tendency to increase as the particle size became smaller. LDH leakage amount for Fe particles resembled Ti and showed the similar size dependence.

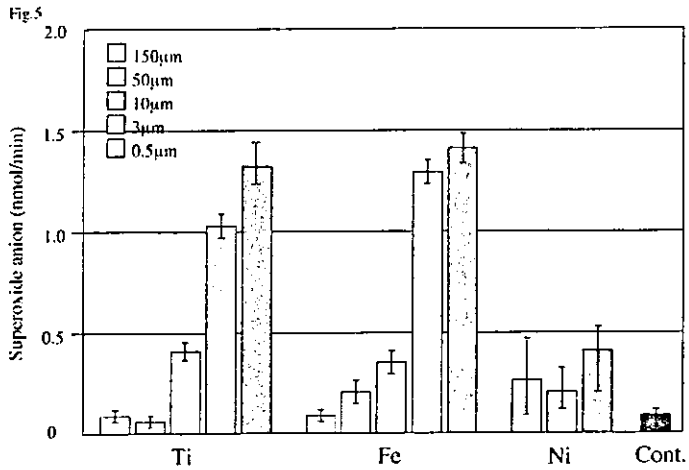

Figure 5 shows the quantity of superoxide anion produced from neutrophils. Ti and Fe group showed for the size less than 10 μm the typical dependence on particle size. The produced amount increased as the particle size decreased and the 0.5 μm particles showed the largest value. The value for the larger size is nearly the same level or slightly higher than control. On the other hand, Ni had significantly much smaller values compared with Ti and Fe.

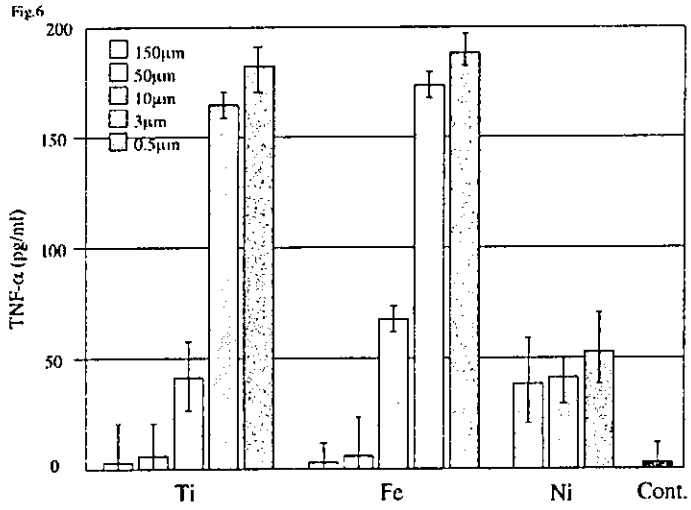

Figure 6 shows the amount of TNF-α released from neutrophils in HBSS containing metallic particles. The TNF-α levels increased significantly for the 10 μm or less Ti and Fe particles. For the particles smaller than 3 μm the released amount was pronounced. The amount was lower for Ni particles.

Fig.7

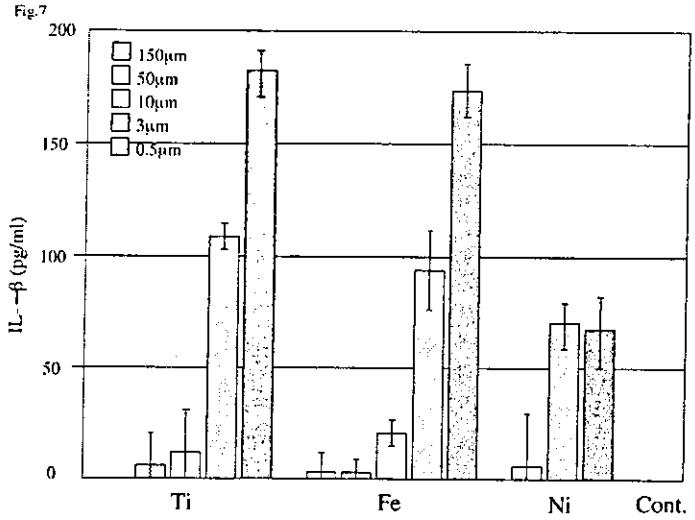

Figure 7 shows the released amount of IL-1β. Similarly to TNF-αthe IL-1β levels were increased with the decrease of particle size and significantly pronounced for 3 μm or less particles. The particles larger than 50 μm released slightly and they had not much difference from the control.

Fig.8

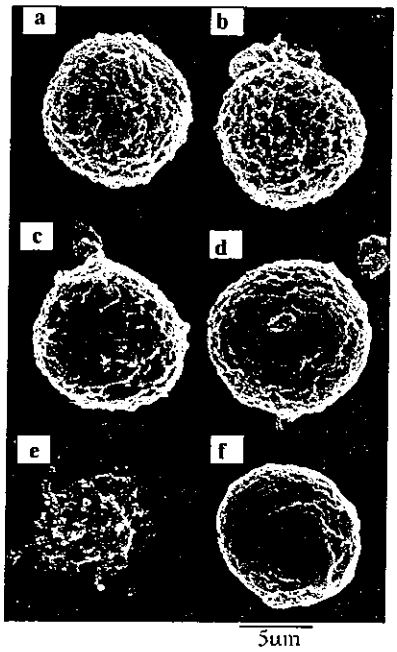

Figure 8 shows the SEM images of human neutrophils exposed to the 0.5 μm particles: a normal neutrophil in HBSS (a), neutrophil stimulated by Ti (b), Fe (c) and Ni (d), broken down with Ni particles (e), and an atrophied neutrophil transformed with Ti particles (f).

1.Size control of metallic particles with different nature

Previously, our studies[1] demonstrated the importance of size of titanium particles in mediating

inflammation and injury. The association between titanium particles and tissue response was investigated both in *in vivo* and *in vitro*. The ability of well-known pathogenic particles to cause tissue injury appears to be related to the their shape, intrinsic reactivity and their accumulating dose. We showed that the particles with varying sizes have the different potencies to induce the inflammatory reactions to neutrophils. However, the particle size was not the same for different materials. Therefore there was an ambiguity in the results when the behavior for different materials was compared.

In this study, particles were selected from the different metals of Ti, Fe and Ni.

Results of ICP analysis showed that Ni and Fe particles were ionized in the HBSS in proportion to immersion time (Fig.2). Fe showed the several times larger ionization tendency than Ni. Dissolution of Ti was below detection limit. As shown in the dissolvability by ICP analysis and the stimuli observed as deformation of morphology in neutrophils by SEM (Fig.1), the three representative metals have the typically different nature: non-dissolvable and biocompatible Ti, very much soluble and rather bio-inert Fe, and dissolvable to some extent and highly toxic Ni. To make clear, the size were controlled to be commonly set equal for these different metallic particles, Ti, Ni, Fe, through the process of filtration and condensation. Weight of particles was also controlled equal for different size of particles. The present particles size covers the range below (0.5 and 3 μm), approximate the same (10 μm) and over (50 and 150 μm) the cell size.

2. Particle size dependence of cell reaction

2-1 Cell survival rate and LDH

The effect of the particles on cell survival rate and LDH outflow quantity showed the size dependency. As the size of particles decreased, the cell survival rate was lowered and the leakage of LDH was increased. There was very little or no significant difference from control for the larger than 50 μm. 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 viewpoint, the results of the cell survival rates and LDH production showed the very good accordance. Their changing manner is inversely well corresponding to each other (Fig. 3 and 4). As for Ni particles the cell survival rate was remarkably lowered, although the survival rate still showed the size dependency. The survival rate of neutrophils became 90% or less, when they were mixed with 2 μm Ni particles and lower than 70% in the 0.5 μm particles. This is due to the addition of toxicological effect of Ni through its ionic dissolution.

2-2 Superoxide anion

The quantity of superoxide produced by neutrophils significantly increased in the 10 μm or less Ti and Fe particles, especially for particles smaller than 3 μm, and very little differed from control in the other larger sizes. Neutrophils produce several kinds of active oxygen radicals such as hydroxyradical,

hydrogen peroxide, and superoxide anion. Superoxide anions are released from intracellular organs and the cell membrane when the membrane of neutrophils is stimulated, which may affect the cell circumference and damage the DNA in vivo. The results showed that the Ti and Fe particles arise the stimulatory effect to cells (Fig.3). Ni produced much less superoxide. This can be explained as the influence of decrease in cell survival rate.

2-3 Cytokine

Cytokines produced by a variety of cell types in response to pro-inflammatory stimuli and play a role in a normal and pathological tissue reaction. Neutrophils exposed to the different metallic particles were activated to produce the stress responsive pro-inflammatory cytokines IL-1β and TNF-α (Fig. 6 and 7). Ti and Fe particles induced the varying levels of cytokine release depending on their size. However, Ni induced only a small increase compared to Ti and Fe. This is mainly because Ni ion induced cell death. Both Ti and Fe particles of 3 μm and 0.5 μm were far more potent about cell stimulus than the larger particles. Neutrophils may phagocytize fine particles when the particles are smaller than the cell size of 5-10 μm, while IL-1β is released when neutrophils are stimulated by foreign matter and it causes the inflammation reaction cascade. The distinct release of TNF-α observed only in 3 μm or less particles is closely related to the phagocytosis by neutrophils. The increased quantity of TNF-α by phagocytosis caused neutrophil to the further activation: reproduction of superoxide anions and TNF-α. The phagocytosis is difficult for the 10, 50 and 150 μm Ti and Fe particles and the above effects were absent in the present results.

3. Different cell stimulation effect by Ti, Fe, and Ni particles

3-1 Ti

Ti is an insoluble, chemically stable metal and one of the most biocompatible metals. [11-20] The macroscopic size of Ti is most frequently used as implant and plate in vivo. Ti particles, however, had the stimulatory effect to cell as shown in the present study. The 3 μm or smaller particles showed clearly the difference from the control. Since Ti particle elutes very little ion, the cell stimulation effect dependent on particle size is based on physical matter. Phagocytosis of particles leads to activation of cells to produce inflammatory mediators since both phagocytosis and cytokine production occur when smaller particles were incubated with neutorphills.

3-2 Fe

Cell survival rate and LDH outflow quantity by Fe particles showed the size dependency. The effect was little for the cell survival rate for the iron particles over 10 μm, and the LDH activity did not show the significant difference from the control. Superoxide anion production increased, as Fe particle was smaller. There was a significant difference between 3 μm and 10 μm particles. Especially, the 0.5 μm and 3 μm particles stimulated the remarkable increase of cell products. Fe was nearly the same as Ti to show the

size dependency in the respect of superoxide anion and cytokines production. The release of IL-1β and TNF-α resembled for Ti and Fe even in a quantitative aspect when the same size was compared. Fe resembled Ti in the biofunctional reaction to cells and may not affect much on cell functions even if Fe ions were dissolved.

3-3 Ni

Although the survival rate by Ni particles showed the size dependency, the value was much lower than Ti and Fe. It became less than 70% in the 0.5 μm particles. The ionization of nickel in the simulated body fluid was evident in proportion to the inversion time, which is in contrast with Ti where no dissolution was recognized (Fig.2). The morphology of neutrophils exposed to Ni particles was often destructed (Fig.9 (d)). Ni ion is one of the most toxic and known as carcinogenic. The lower survival rate, higher LDH, less cell products than Ti, and cell death showed that chemical effect is predominant and size effect works additionally for cytotoxicity of Ni.

4. Particle size dependent cytotoxicity

Fe particles showed the resembled size effect as Ti in cell toxicity. The chemical cytotoxicity of Fe ion is suggested to be very weak. In both biocompatible and bio-inert materials, Ti and Fe, the cytotoxicity due to fine particles is mainly size effect. Although both Fe and Ni dissolved in HBSS, bio-functional results are very different. Ni particles induced cell death by chemical effect. The lower superoxide anion and cytokine production in Ni was explained due to the low survival rate of neutrophils. In Ni, which has the stronger toxicity as ionized[16-17], particle size is supplementary effect. Thus the present study using human neutrophils in vitro under the controlled conditions of particle size clearly showed the importance of cytotoxicity due to physical size effect in the micro to nano range.

Conclusion

In the present study the effect of micro to nano size metallic particles on biocompatibility in vitro was investigated by biofunctional analysis of cell functions; cell survival rate, LDH, superoxide anion, and cytokines, using human neutrophils. The three most typical metals with different characteristics in corrosion, dissolution and biocompatibility were selected (Ti, Fe, Ni) and their influence was compared.

1. The controlled particle size adjusted commonly equal to the different material clearly showed the cytotoxicity due to fine particles and its size dependence.
2. The results of cell survival rate, LDH production, superoxide anion production, TNF-α and IL-1β release, and SEM observation are in accordance each other in that they have the particle size dependence and its effect is enhanced as the size becomes smaller.
3. Phagocytosis occurs for less than 3 μm, irrespective of materials. The distinct release of TNF-α and superoxide anion in this range is closely related to the phagocytosis.

4. ICP analysis showed no ionic dissolution in Ti particles after 4 week immersion in HBSS. The cytotoxicity by Ti particles is induced solely by physical size effect.
5. Fe was dissolved most of all the metals. However, the cytotoxicity by chemical effect of Fe ion is very weak and cytotoxicity is mainly induced by size dependent effect.
6. . Non-soluble Ti and very soluble Fe showed quantitatively the same level of superoxide anion and cytokine production. This indicates that the cytotoxicity due to fine particles is material independent and size dependent.
7. The relatively less soluble Ni showed more severe toxicity than very soluble Fe. In Ni the toxicity due to chemical ionic dissolution effect is dominant and the particle size effect works additionally.
8. Generally in macroscopic scale the chemical ion dissolution effect is dominant. For less than 10 μm, particle size effect becomes pronounced. The present study clearly showed the importance of cytotoxicity due to the physical size effect in the range of micro to nano meter, which occurs non-specifically for any materials.

PO4

The Cytotoxicological Study of Metal Encapsulating Carbon Nanocapsules

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[Introduction]

In the biomedical and biochemical fields, new drug delivery systems, transgenic systems and biomaterials using nanomaterials are of great interest. On the other hand, the toxicity of these materials is also of concern. The authors are interested in the metal-encapsulating carbon nanocapsules (MECNCs) which are several tens of nanometers in diameter and consist of a graphene sheet structure encapsulating a metallic carbide. MECNCs have a surface covered by a graphene sheet, so they have quite high chemical stability. In the capsule, lanthanide is usually encapsulated as a carbide. The metallofullerenes which contain one metal atom in the fullerene cage (e.g. $Gd@C_{60}$) have been studied as the contrast agent in X-ray or magnetic resonance imaging (MRI). To apply MECNCs for these purposes, the toxicity of MECNC was estimated in this study.

[Experimental procedures]

MECNCs were synthesized by a direct current arc-discharge between a pure graphite cathode and a metal-loaded graphite anode in a helium atmosphere. A pure graphite rod and a graphite

rod loaded with CeO_2 powder were used as the cathode and anode, respectively. The arc discharge was carried out in helium gas. A deposit containing MECNCs formed on the end of the cathode and was carefully removed. The purified MECNCs were dissolved in saline to the concentration of 100 ppm.

Rat fibroblasts were used for the evaluation of the cell survival rate and lactate dehydrogenase (LDH) release with MECNC addition. Rat alveolar cavity humor was obtained from the Wistar rat. The macrophages were separated from this humor using RPMI medium. The MECNCs dispersed in saline were added to cells at concentrations from 0.01 to 10ppm. After 1-day incubation at 37°C, the survival rate of fibroblasts was estimated with a MTT assay. The LDH release, the release of cytokines (tumor necrosis factor-alpha (TNF-a), interleukin-1 beta (IL-1b) and macrophage colony stimulation factor [M-CSF]) were measured using the assay kits.

[Results and Discussions] Figure 1 shows a high magnification TEM images of MECNCs. In the multiwalled carbon layers, (002) fringes of graphitic layers with 0.34 nm spacing are clearly observed. The interior metal composite is the cerium dicarbide CeC_2 single crystal one, as confirmed by electron diffraction. The average particle size is approximately 20 to 30 nm in diameter. Figure 2 shows the survival rates and the amounts of LDH release of the rat fibroblasts for various concentrations of MECNCs. The survival rate was decreased and the LDH release was increased with concentrations of MECNCs higher than 1ppm.

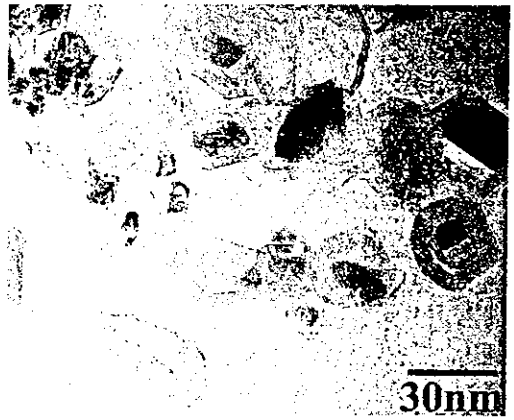

Fig.1

Figure 3 shows the amounts of the cytokines released from rat alveolar macrophages for various concentrations of MECNCs. The TNF-α release was increased with the addition of the MECNCs at concentrations of more than 5ppm. For less than 1ppm, TNF-α release showed no