Effects of Six Particulate Metals on Osteoblast-like MG-63 and HOS Cells *in vitro*

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The effects of six particulate metals (Al, Ti, Zr, Nb, Ta and Cr) on cell viability and alkaline phosphatase (ALP) activity were studied *in vitro* using two types of osteoblast-like cells, MG-63 and HOS cells. The cell viability in the presence of Al, Ti and Zr was depressed at lower concentrations than in the presence of Nb, Ta and Cr. The average sizes of the Al, Ti, Zr, Nb, Ta and Cr particulates were 6.48 μm , 16.99 μm , 5.07 μm , 14.18 μm , 8.32 μm and 23.27 μm respectively. The interaction of HOS cells with the particulates was more sensitive than that of MG-63 cells. ALP activity increased at higher concentrations only with the Al particulates; other experimental conditions did not exert an influence on ALP activity. These findings suggest that the cell viability of osteoblast-like cells might be influenced by particulate size and metal type, but ALP activity was not influenced by these factors.

Key words : particulate metals, osteoblast-like cells, cytotoxicity

INTRODUCTION

Metallic biomaterials have been widely used as stress-bearing implants in the medical and dental fields due to their good mechanical properties. As a result, their stress-bearing functions promote metal ion dissolution and wear debris. Extensive *in vitro* studies have shown that particulate wear debris and dissolved metal ions have adverse effects on macrophages^{1–4)}, monocytes^{5,6)}, fibroblasts^{7–9)}, and osteoblasts^{10–12)}. These studies have demonstrated that particulate wear debris had different effects on cells from those of bulk metals. For example, the response of cells to wear debris has been recognized as one of the major factors responsible for the loosening of orthopedic implants¹³⁾. We previously reported that 12 kinds of pure particulate metals can be roughly divided into three groups: those with direct effects due to the contact between particulates and cells, those with indirect effects due to leaching of toxic soluble ions from the particulates, and those with both direct and indirect effects¹⁴⁾. Evans⁸⁾ suggested that the adverse effects depended more on the particulate nature of the material than its chemical biocompatibility. In addition, Yao *et al.*¹⁵⁾ stated that particulates of 21 μm in diameter showed no effect on MG-63 or HOS osteoblast-like cells.

The objective of the present study was, therefore, to evaluate the dose-effect of six kinds of particulate metals which did not have indirect effects due to dissolution in the previous study¹⁴⁾ and passed through a 20 μm -sieve, on the two human osteoblast-like cells, by analyzing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) reduction and alkaline phosphatase (ALP) activity. These particulate metals were selected because they were important and/or additional elements for dental as well as medical uses. Moreover, there was little information about the particulate metals, except for Ti.

MATERIALS AND METHODS

Particulate metals

Six commercial pure particulate metals were used as shown in Table 1. Before use, each kind of commercial particulate metal was passed through a sieve with a 20- μm aperture (IIDA Seisakusho, Osaka). The particulate size distribution of each particulate metal was measured using a laser diffraction particle size analyzer (SALD-2100, Shimadzu, Kyoto).

Cells and cell cultures

The MG-63 and HOS human osteoblast-like cell lines (American Type Culture Collection, VA) were cultured in monolayers in Dulbecco's modified Eagle medium (DMEM) (ICN Biomedicals, OH), supplemented with 2 mM glutamine (ICN Biomedicals, OH) and 10 v/v% fetal bovine serum (FBS) (ICN Biomedicals, OH) in a humidified atmosphere at 5% carbon dioxide in air at 37°C.

Experiment

Before use, the weighed particulate metals were sterilized by ultraviolet irradiation for 48 hr. The original exposed concentration was prepared by suspending each particulate metal at 0.8 mg/ml in DMEM containing 2 mM glutamine, 10 v/v% FBS, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. The culture media containing the particulates were ultrasonicated for 5 min. Dispersion of particulates was achieved by continuous vibration. Four serial dilutions were then prepared by the addition of the culture medium. This resulted in five suspensions with particulate concentrations of 0.40 mg/cm², 0.20 mg/cm², 0.10 mg/cm², 0.05 mg/cm², and 0.025 mg/cm² for each type of particulate metal. The highest concentration was determined from the concentration where the particulates did not completely surround the whole surface of

Table 1 Particulate metals and sizes used in this experiment

Metals*	Average (μm)	SD (μm)	Distribution (μm)		
			10% D	50% D	90% D
Al	6.48	0.58	0.86	8.96	30.44
Ti	16.99	0.14	11.08	17.21	25.59
Zr	5.07	0.19	2.76	5.30	8.57
Nb	14.18	0.20	7.47	14.70	25.11
Ta	8.32	0.31	3.14	9.17	19.31
Cr	23.27	0.13	15.85	23.32	34.17

*The Nilaco Corp., Tokyo

wells.

Cells were plated in 24-well plates at 5,000 cells/cm² in DMEM containing 2 mM glutamine and 10 v/v% FBS. After a 24-hr incubation, the medium was removed and replaced with 1 ml of an experimental medium containing the particulate metals. After the addition of the particulate metals, the cells were cultured in a humidified atmosphere of 5% carbon dioxide in air for an additional 4, 7 days and 14 days at 37°C.

Cell viability assay

Cell viability was examined using the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo- phenyl)-2H-tetrazolium, inner salt (MTS) (CellTiter 96 AQueous, Promega, WI). The experimental medium was removed and replaced with a medium containing 100 μ l of MTS reagent in 500 μ l of culture medium. Then the plate was incubated for 2 hr at 37°C in a humidified atmosphere of 5% carbon dioxide in air. After 2 hr, 150 μ l of the medium was transferred into each well of the 96-well assay plate. The optical density was measured at 490 nm with a reference wavelength of 650 nm in an ELISA plate reader. The untreated controls were set to represent 100% viability. Experimental viability results were expressed as a percentage of the control.

Alkaline phosphatase assays

At the end of the culture time, the culture medium was removed and the cell layers were rinsed twice with phosphate-buffered saline (PBS(-)). Distilled water with 0.01% Triton X-100 was added to each well. The plates were put on ice and ultrasonicated for 5 min, and then subjected to three cycles of freezing and thawing to lyse the cells.

The protein content of the cell lysates was assayed using a modified Lowry protein assay (Pierce, IL). Forty microliters of the substrate was incubated with 200 μ l of the modified Lowry protein assay reagent for 10 min at room temperature. Twenty microliters of diluted 1N Folin-Ciocalteu reagent was added and then incubated for 30 min at room temperature. Serial dilutions of bovine serum albumin (5-1000 μ g/ml) were used for a standard curve. The plate was read at 750 nm on an ELISA plate reader.

ALP activity was measured in the cell lysates by spectrophotometry. The substrate, 100 μ l of p-nitrophenyl phosphate in the appropriate buffer (SIGMA, MO), was added to 100 μ l of the cell lysate. The mixture was allowed to react at 37°C for 60 min and then stopped by the addition of 50 μ l of 3N NaOH. Optical density was determined at 405 nm on an ELISA plate reader and compared with the values of a series of p-nitrophenol standards. ALP activity was expressed as nmol of p-nitrophenol per hour per μ g of cellular protein.

Statistic analysis

Each experiment was performed five times. Results for cell viability after 4 days

were first analyzed for statistical significance using three-way ANOVA. Significant differences were observed with respect to cells, particulate metals and concentrations. Values were expressed as means and standard deviations, and the differences observed between the groups of particulate-exposed cells and the control cells were evaluated by Student's *t* test and considered significant for *p* values lower than 0.05.

RESULTS

Particulate size

The average sizes of each particulate metal are given in Table 1 and the size distributions are shown in Fig. 1. The average diameters of the Al, Zr, and Ta particulates were smaller than $10\text{ }\mu\text{m}$. For the Zr particulates, 90% of the particulates had a diameter smaller than $8.56\text{ }\mu\text{m}$. The Al and Ta particulates were widely distributed, as shown in Fig. 1. The Ti and Nb particulates had a somewhat similar diameter, but the distribution curve of the Nb particulates shows a smaller spread than that of the Ti particulates (Fig. 1). The Cr particulates had the largest diameter of all particulates used in this study, and only 10% of the Cr particulates had a diameter smaller than $15.85\text{ }\mu\text{m}$.

Cell viability

The cell viabilities of MG-63 cells exposed to each particulate metal for 4 days and 7 days are shown in Figs. 2 and 3, respectively. For all particulates after 4 days, cell viability decreased at the particulate concentration of 0.40 mg/cm^2 . Cell viability with the Al particulates was significantly lower than that with the Nb, Ta, and Cr particulates. The cell viability increased as the particulate concentration decreased and was similar to that of the control at the concentration of 0.025 mg/cm^2 . After

Fig. 1 Particulate distribution.

Fig. 2 Cell viability of MG-63 cells grown for 4 days in the presence of Al, Ti, Zr, Nb, Ta and Cr particulates.

*Significant differences ($p < 0.05$) from control cultures

Fig. 3 Cell viability of MG-63 cells grown for 7 days in the presence of Al, Ti, Zr, Nb, Ta and Cr particulates.

7 days, the cell viability was greater than 90% for all experimental conditions (Fig. 3) with no significant differences being observed between the experimental groups and controls. In other words, cell viability increased with culture time.

The cell viability of HOS cells exposed to each particulate for 4 and 7 days are shown in Figs. 4 and 5, respectively. After 4 and 7 days of cultivation, the cell viability of HOS cells decreased at the lower concentration compared with that of

Fig. 4 Cell viability of HOS cells grown for 4 days in the presence of Al, Ti, Zr, Nb, Ta and Cr particulates.
*Significant differences ($p<0.05$) from control cultures

Fig. 5 Cell viability of HOS cells grown for 7 days in the presence of Al, Ti, Zr, Nb, Ta and Cr particulates.
*Significant differences ($p<0.05$) from control cultures

MG-63 cells ($p<0.05$). At the particulate concentration of 0.40 mg/cm², cell viability was 34.63% for Al particulates, 32.89% for Ti particulates, 32.13% for Zr particulates, 49.91% for Nb particulates, 46.94% for Ta particulates, and 51.23% for Cr particulates. As the particulate concentration decreased, cell viability increased and was greater than 80% at 0.025 mg/cm² for all cells except those with the Al

Fig. 6 Effects of particulates on ALP activity of MG-63 cells after 4 days cultivation.

Fig. 7 Effects of particulates on ALP activity of MG-63 cells after 7 days cultivation.

particulates, which had a viability of 53.75%. After 7 days, cell viability increased and was greater than 90% at 0.025 mg/cm² for all cells except those with the Al particulates.

Alkaline phosphate activity

The results from the ALP assays of MG-63 cells are shown in Figs. 6, 7, and 8, for 4, 7 and 14 days of cultivation, respectively. The ALP activity after 4 days of cultivation did not show a statistically significant difference between the controls and each experimental group in irrespective of concentrations and particulate metals.

Fig.8 Effects of particulates on ALP activity of MG-63 cells after 14 days cultivation.

Fig.9 Effects of particulates on ALP activity of HOS cells after 4 days cultivation.

*Significant differences ($p < 0.05$) from control cultures

ALP activity tended to decrease with culture time. The ALP activity after 7 days of cultivation also did not show a significant difference between the controls and each experimental group. The addition of particulates, except for Cr, tended to decrease ALP activity after 14 days of cultivation, but the effect was not significant.

The results from the ALP assays of HOS cells are shown in Figs. 9, 10, and 11, for 4, 7 and 14 days of cultivation, respectively. For all three cultivation periods, the level of ALP activity in HOS cells was higher than that in MG-63 cells. The ALP activity of the Al particulates at 0.40, 0.20, 0.10, and 0.05 mg/cm^2 was significantly

Fig. 10 Effects of particulates on ALP activity of HOS cells after 7 days cultivation.

Fig. 11 Effects of particulates on ALP activity of HOS cells after 14 days cultivation.

higher after 4 days of cultivation than that of the controls. However, the other experimental conditions were not significantly different from the controls. After 7 and 14 days of cultivation, none of the experimental conditions were significantly different compared with the controls. That is to say, ALP activity had nothing to do with concentrations and particulate metals.

DISCUSSION

In the present study, we investigated the reaction of osteoblast-like cells to six kinds

of particulate metals. Cell viability decreased at the highest concentration of each metal. However, the Al, Zr, and Ti particulates were cytotoxic compared with the Nb, Ta, and Cr particulates. The interaction of HOS osteoblast-like cells with the particulates was more sensitive than that of MG-63 osteoblast-like cells. On the other hand, ALP activity was not influenced by the particulates, except for the Al particulates at higher concentrations.

All particulates depressed the cell viability of both cell lines. Cell viability with the Al, Ti, and Zr particulates decreased compared to cell viability with the Nb, Ta, and Cr particulates. It is conceivable that cell viability is influenced by particulate size, concentration, composition, and dissolved ions. Okazaki *et al.*¹⁶⁾ reported that Ti, Zr, Nb, and Ta particulates released only a small amount of ions and hence their extracts did not exhibit any effect on the relative growth ratio of cells. The effect on cells due to the solubility of those particulate metals was said to be negligible. On the other hand, Al and Cr particulates were reported to dissolve and decrease cell viability^{16,17)}. However, those studies were conducted at higher concentrations than the present study and/or under dynamic extraction conditions. Therefore, the effects of the particulates in the present study may be considered to be mainly due to the direct contact of particulates with the cell membrane and the phagocytosis of osteoblast-like cells.

Particulates less than $15\mu\text{m}$ in diameter are known to be phagocytosable^{10,11,15,18-21)}. Pioletti *et al.*¹¹⁾ demonstrated that the phagocytosis of titanium particulates by osteoblasts is an important factor affecting their viability. Particulates less than $5\text{--}10\mu\text{m}$ in diameter might be a more important cause of cell damage than composition, as seen by their direct effect on osteoblast viability⁸⁾. Evans *et al.*²²⁾ reported that toxicity due to the direct contact of hydroxyapatite particulates with cells only occurred with particulates smaller than about $5\mu\text{m}$ in diameter. Of particulate sizes in the present study, 90% of the Zr particulates and 50% of the Al and Ta particulates were smaller than $10\mu\text{m}$. For the Nb particulates, 10% were smaller than $10\mu\text{m}$. On the other hand, less than 10% of the Ti and Cr particulates were smaller than $10\mu\text{m}$. However, the cell viability with the Al, Ti, and Zr particulates was lower than that with the Nb, Ta, and Cr particulates. Our study suggested that the cell viabilities with the Al and Zr particulates were depressed by the number of particulates smaller than $10\mu\text{m}$. In particular, the cell viability with the Al particulates was most strongly depressed. This result suggests that the Al particulates may be dissolved and contain particulates smaller than $1.0\mu\text{m}$. On the other hand, the Cr particulates hardly influenced cell viability because they are larger particulates. However, cell viability was not always in agreement with the results of particulate size. For example, the Ti particulates contained larger-sized particulates than the Nb and Ta particulates. Cell viability with the Ti particulates was nevertheless depressed compared to that with the Nb and Ta particulates. The particulate number in each plate well might be related to cell viability. Since the particulate sizes and densities of the metals are different, the number of particulates exposed was different for each type at equal concentrations. When the volume was calculated from the average size by

assuming the particulates to be spheres, the particulate number per cm^2 was roughly $2,382 \times 10^3$ for Al particulates, 107×10^3 for Ti particulates, $2,827 \times 10^3$ for Zr particulates, 98×10^3 for Nb particulates, 250×10^3 for Ta particulates, and 26×10^3 for Cr particulates. Although the Ti particulates had nearly the same size and number as the Nb particulates, the cell viability with the Ti particulates decreased compared to that with the Nb particulates. The difference in the cell viabilities with the Ti and Nb particulates was not clarified by this study. Maloney *et al.*²³⁾ reported that particulates of different shapes might potentially influence cellular reactivity. In addition, cell viability might be influenced by composition.

Cell viability was higher after 7 days of cultivation than after 4 days for both types of cells and all six types of the particulate metals. After a cell is damaged due to the particulates, the remaining cells can proliferate, reduce the number of particulates per cell, and then allow the viability to recover.

Lohmann *et al.*²⁴⁾ reported the possibility that particulate size plays a role in differential cell response. The present results did not show significant differences in ALP activity among the particulates. However, Al particulates at higher concentrations tended to stimulate ALP activity. Treatment of osteoblasts with TGF- β was reported to stimulate ALP activity and inhibit osteocalcin production²⁵⁾. In addition, Thompson *et al.*²⁶⁾ reported that Al^{+3} at low concentrations appeared to stimulate ALP activity. A small amount of Al^{+3} might be dissolved from Al particulates and subsequently release the cytokines. We shall investigate this in our future studies, in which we will examine the effects of particulates on the release of cytokines and metal ion dissolution.

The six kinds of particulate metals demonstrated differences in cell viability according to the particulate size, number, and composition. These results suggest that morphological information, such as sizes, shapes and so on, might be important in evaluating the biocompatibility of wear debris produced *in vivo*. In addition, the findings in this present study may provide information for the assessment of the potential biocompatibility of selecting the elements in metallic biomaterials.

MG-63 and HOS cells are derived from human osteosarcoma cells and could be used as valuable tools for investigating specific aspects of bone cell function²⁷⁾. However, each cell line has a different morphology: MG-63 cells are fibroblasts and HOS cells are a mixture of fibroblasts and epithelial-like cells^{28,29)}. HOS cells have been frequently used to study transformations caused by metals and chemical carcinogens because of their sensitivity to chemical transformation³⁰⁾. The difference in sensitivity might therefore be one reason for the difference between the two cell lines. In addition, these results show that the extrapolation of cytotoxic results from one kind of cell to human osteoblasts should be performed with caution. Allen *et al.*¹⁰⁾ suggested that cell interaction with particulates could produce different cytotoxic results among different cell lines.

The findings of the present study demonstrate that particulate metals can affect the cell viability of osteoblasts. The effects will depend not only on particulate concentration and size but also on composition. It is unclear which factor contributes

the most. Further studies are required to clarify this point. However, the decrease of cell viability at higher concentrations of all particulates suggests that the number of osteoblasts may decrease because of local accumulation of particulate wear debris due to long periods of use, and that new bone formation may be affected. Moreover, these results demonstrate that the selection of metallic biomaterials used in stress-bearing locations would make it necessary to take into account not only dissolution but also wear. On the other hand, our results show that ALP activity was hardly influenced by the different kinds of particulate metals. These results suggest that other differentiation markers and pro-inflammatory mediators should be investigated in future work to obtain information about the direct interaction between particulate wear debris and osteoblasts. In addition, Evans *et al.*³¹⁾ suggested that particulates show a reduced toxicity when ground in serum. Therefore, the interaction between cells and wear debris produced *in vivo* should be a matter of further investigation to clarify the effects of particulate wear debris with biological systems.

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