The Effects of Particulate Metals on Cell Viability of Osteoblast-like Cells in vitro

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Effects of fifteen particulate objects, fourteen metals and one non-metal on cell viability of osteoblast-like cells were studied in vitro, to determine whether an adverse effect on cells could be induced by the particulate form or soluble ions. The Al, Ti, Zr, Nb, Ta, Cr, Mo, and Fe particulates depressed cell viability at higher particulate concentrations, but their extracts yielded no effect on cells except for Mo. On the other hand, little difference in cell viability between particulates and extracts was observed for Cu, Si, V, W, and Co. However, Mn and Ni yielded more adverse effects on cells in the case of the particulates than the extracts. These findings suggested that the effects of particulates on cells depended upon the direct effects of contact between particulates and cells, the indirect effects of dissolved ions and the kinds of particulate elements.

Key words: Particulate, Cytotoxicity, Metal

INTRODUCTION

Metallic biomaterials such as stainless steel, cobalt-chromium alloy, pure titanium, and titanium alloys have been widely used for the replacement of bones, joints, tooth roots, etc in the medical and the dental fields. Their clinical use is based on their stress-bearing qualities. At the same time, the clinical situation tends to accelerate wear and to produce particulate debris, then leads to promote metal ion dissolution. Particulate wear debris has been recognized as one of the major factors responsible for loosening orthopedic implants. Extensive in vitro studies have shown that particulate wear debris of several metals has adverse effects on macrophages, fibroblasts, and osteoblasts. These studies have revealed that particulate wear debris had different effects on cells than those of bulk metals. We also previously reported that the cell viability of the filtrates through a 0.22 μm filter was higher than that of the extracts under dynamic conditions. The above findings suggest that particulate metals could have a dual action on cells, namely a direct interaction between the particulate surface and cellular membranes and on indirect effect due to dissolution of toxic soluble ions from particulates. Many of these previous studies included mainly information about titanium, Ti-6Al-4V alloy, and Co-Cr alloys. However, the development of new metallic biomaterials has been carried out using many other metallic and non-metallic elements. It is essential to clarify whether the cause of cytotoxicity of particulate metallic or non-metallic elements is due to
particulate forms or metal ion dissolution.

In the present study, we examined the effects of 14 kinds of particulate metals and one non-metal, which are potential constituents of non-precious alloys, to induce cytotoxicity in osteoblast-like cells in vitro. In addition, it was the purpose to identify whether an adverse effect on cells could be induced by the particulate form or soluble ions.

MATERIALS AND METHODS

Cell culture
The MG-63 osteoblast-like cell line was obtained from the American Type Culture Collection (Rockville, MO), and cultured at 37°C and 5% CO₂ in a monolayer in Eagle's minimum essential medium (MEM, ICN Biomedicals, Ohio), supplemented with 10 v/v% heat-inactivated fetal bovine serum (ICN Biomedicals, Ohio), 1 v/v% glutamine (200 mM, ICN Biomedicals, Ohio), and 1 v/v% nonessential amino acids (100×, ICN Biomedicals, Ohio).

Particulate objects
Pure particulate objects used as specimens in this experiment are shown in Table 1. Based on the particulate weight to medium volume ratio determined from preliminary experiments, different concentrations of the specimens were weighed (Table 1), autoclaved at 120°C for 20 min and mixed with the culture medium under sterile conditions. The culture media containing the specimens were ultrasonicated for 10 min. Dispersion of particulates was achieved by continuous vibration throughout the experimental process of the cell culture under sterile conditions.

<table>
<thead>
<tr>
<th>particulates*</th>
<th>purity(%)*</th>
<th>particulate size (μm)*</th>
<th>original concentration (mg/ml)</th>
<th>Item No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>99.8</td>
<td>100</td>
<td>1.25</td>
<td>CU-114125</td>
</tr>
<tr>
<td>Al</td>
<td>99.95</td>
<td>74-147</td>
<td>2.50</td>
<td>AL-014100</td>
</tr>
<tr>
<td>Si</td>
<td>99.9</td>
<td>147</td>
<td>1.25</td>
<td>SI-500101</td>
</tr>
<tr>
<td>Ti</td>
<td>99.98</td>
<td>&lt;44</td>
<td>2.50</td>
<td>TI-454100</td>
</tr>
<tr>
<td>Zr</td>
<td>-</td>
<td>-</td>
<td>1.25</td>
<td>ZR-494110</td>
</tr>
<tr>
<td>V</td>
<td>99.5</td>
<td>&lt;75</td>
<td>0.625</td>
<td>V-474100</td>
</tr>
<tr>
<td>Nb</td>
<td>99.9</td>
<td>&lt;45</td>
<td>2.50</td>
<td>NB-324100</td>
</tr>
<tr>
<td>Ta</td>
<td>99.9</td>
<td>&lt;44</td>
<td>2.50</td>
<td>TA-414051</td>
</tr>
<tr>
<td>Cr</td>
<td>99</td>
<td>&lt;50</td>
<td>2.50</td>
<td>CR-094500</td>
</tr>
<tr>
<td>Mo</td>
<td>99.9</td>
<td>3-5</td>
<td>2.50</td>
<td>MO-294100</td>
</tr>
<tr>
<td>W</td>
<td>99.95</td>
<td>1</td>
<td>1.25</td>
<td>W-464101</td>
</tr>
<tr>
<td>Mn</td>
<td>99.98</td>
<td>1.5</td>
<td>0.625</td>
<td>MN-284101</td>
</tr>
<tr>
<td>Fe</td>
<td>99.998</td>
<td>&lt;74</td>
<td>2.50</td>
<td>FE-224111</td>
</tr>
<tr>
<td>Co</td>
<td>99.9</td>
<td>&lt;50</td>
<td>0.625</td>
<td>CO-104100</td>
</tr>
<tr>
<td>Ni</td>
<td>99.99</td>
<td>10</td>
<td>0.625</td>
<td>NI-314010</td>
</tr>
</tbody>
</table>

*The Nilaco Corp., Tokyo
Cell viability assay

Exposure of the cells to particulate metals and non-metal was conducted in two ways. The experimental conditions in the present study were determined as follows. Preliminary experiments revealed that the cytotoxicity of particulates depended on the kinds of elements. Thus, the highest concentration was determined from concentrations where the particulates did not completely surround the whole surface of the wells, and the lowest concentration was from the limitations of measuring the weight of particulates. For the exposure periods, 6 days of exposure was the longest period in the present study, because cells were confluent and stable within this period.

1) Experiment 1

In experiment 1, the direct effects of 15 elements on cell viability were examined. One hundred $\mu l$ of suspension of $5 \times 10^3$ cells/ml was poured into each well of 96-well plates and incubated in CO$_2$ for 24 hr at 37°C. Particulate concentrations are given as serial dilutions of the original exposed concentrations (Table 1). One hundred $\mu l$ of the particulate suspension at various concentrations was added into each well with 100 $\mu l$ of culture medium containing cells and the combined volume was 200 $\mu l$. After the addition of the particulates, the cells were cultured in CO$_2$ for an additional 3 and 6 days at 37°C. Cell viability was examined using neutral red assays.

2) Experiment 2

In experiment 2, a 200 $\mu l$ particulate suspension at various concentrations, given as serial dilutions of the original exposed concentrations, was added into each well and extracted in CO$_2$ for an additional 3 days at 37°C under cell-free conditions. After three days, 100 $\mu l$ of extracts was added into each well with 100 $\mu l$ culture medium containing $5 \times 10^3$ cells/ml. The cells were cultured in CO$_2$ for an additional 3 days at 37°C and the cell viability was examined using neutral red assays. Three replicates for each particulate and concentration were used in each assay and repeated four times.

3) Neutral red assay

Neutral red assay was used to determine the cell viability. At the end of the culture periods, the medium was removed and replaced with the culture medium containing 50 mg/ml neutral red. The 96-well plate was further incubated in CO$_2$ for 2 hr at 37°C, washed, fixed with 1% formaldehyde in PBS (-), and finally extracted with 200 $\mu l$ of a mixture of 1% acetic acid and 50% ethanol. The optical density was measured at 540 nm using a microplate reader (3550, BioRad, CA).

4) Statistic analysis

The survival rates of the untreated controls were set to represent 100% viability. Experimental results were expressed as a percentage of the control. Values were expressed as means and standard deviations, and the differences observed between groups of particulate- and extracts-exposed cells and control cells were evaluated by Student’s $t$ test and considered significant for $p$ values lower than 0.05.
RESULTS

Experiment 1
Cell viability after exposure to the particulates is shown in Figs.1-6. Nb and Zr particulates did not reduce cell viability at the concentration of 2.50 mg/ml for Nb.

Fig. 1 Viability of cells grown for 3 days in the presence of Al, Ti, Nb, Ta, Cr, Mo, and Fe particulates. Errors bars represent the standard deviation. *Significant differences (p<0.05) from control cultures

Fig. 2 Viability of cells grown for 6 days in the presence of Al, Ti, Nb, Ta, Cr, Mo, and Fe particulates. Errors bars represent the standard deviation. *Significant differences (p<0.05) from control cultures
Fig. 3 Viability of cells grown for 3 days in the presence of Cu, Si, Zr, and W particulates. Errors bars represent the standard deviation. *Significant differences (p<0.05) from control cultures

Fig. 4 Viability of cells grown for 6 days in the presence of Cu, Si, Zr, and W particulates. Errors bars represent the standard deviation. *Significant differences (p<0.05) from control cultures
Fig. 5 Viability of cells grown for 3 days in the presence of V, Mn, Co, and Ni particulates.
Errors bars represent the standard deviation.
*Significant differences (p<0.05) from control cultures

Fig. 6 Viability of cells grown for 6 days in the presence of V, Mn, Co, and Ni particulates.
Errors bars represent the standard deviation.
*Significant differences (p<0.05) from control cultures
or 1.25 mg/ml for Zr after 3 and 6 days of exposure, respectively (Figs. 1-4). Ti, Ta, Al, Cr, Mo and Fe particulates brought cell viability lower than that of controls at the concentration of 2.50 mg/ml or double dilution after 3 days of exposure (Fig. 1). For Ta and Cr particulates, the cell viability after 6 days of exposure was similar to that of the controls. Al and Ti particulates showed slightly higher cell viability after 6 days of exposure than that after 3 days of exposure. Mo and Fe particulates showed slightly reduced cell viability with exposure time.

The viability of cells grown for 3 days in the presence of Cu, Si, and W particulates were similar to that of controls after 8- to 32-fold dilution of the concentration of 1.25 mg/ml (Fig. 3). For V, Mn, Co, and Ni particulates, a 32- to 256-folds dilution of the concentration of 0.625 mg/ml was required to make the cell viability equal that of the control (Fig. 5). The cell viability of Cu, Si, W, V, Mn, Co, and Ni particulates was slightly lower or equal after 6 days of exposure compared with that after 3 days of exposure (Figs. 3-6).

The order of cytotoxicity for particulates was Mn>V>Co≡Ni>W>Cu>Si>Mo, Fe>Al, Ti, Nb, Cr≡Zr, Ta.

Experiment II
Cell viability after exposure to extracts is shown in Figs. 7-9. Al, Ti, Nb, Ta, Cr, Fe, or Zr extracts revealed the same level of cell viability as that of controls even at the highest concentration of 2.50 mg/ml or 1.25 mg/ml (Fig. 7). Mo extracts depressed cell viability down to the concentration of double dilution. When cells were exposed to Cu, Si, W, V, and Co extracts, the cell viability was similar to those of cells ex-
Fig. 8 Cell viability of extracts obtained from Cu, Si, Zr, and W particulates. Errors bars represent the standard deviation. *Significant differences (p<0.05) from control cultures exposed to particulates (Figs. 8, 9). However, Mn or Ni extracts showed higher cell
viability compared with particulates (Fig. 9).

The order of cytotoxicity for extracts was V > Co > Ni > W ≡ Cu ≡ Si > Mn > Mo > Al, Ti, Zr, Nb, Ta, Cr, Fe.

DISCUSSION

The present findings demonstrated that the effects of particulates to osteoblast-like cells depended upon the direct effects due to the contact between particulates and cells, the indirect effects of dissolved metal ions and the kinds of particulate elements. The present results suggested that cytotoxicity due to particulates and their extracts could be categorized into three types. The first type was that extracts yielded detrimental effects to cells. The second type was that both particulates and extracts affected cell viability. The third type was that particulates depressed cell viability, but extracts did not.

Nonpassive particulates such as Cu, W, V, Mn, Co, and Ni and the amorphous Si particulate depressed cell viability at low concentrations. These particulates dissolve easily due to nonpassivation. An adverse effect on cells could be induced by soluble ions. There was, in fact, little difference in cell viability between the particulates and the extracts in the present study. The difference in cell viability among the elements was suggested to depend on the intensity of the soluble ions’ cytotoxicity and the amount of soluble ions. According to the results obtained in Yamamoto’s study, the order of cytotoxicity for metal ions was V$^{3+}$ > Co$^{2+}$ > Cu$^{2+}$ > Mn$^{2+}$ > Ni$^{2+}$ > W$^{6+}$. On the other hand, the present results suggested that the order of cytotoxicity was Mn > V > Co ≡ Ni > W > Cu for particulates and V > Co > Ni > W ≡ Cu > Mn for the extracts. The discrepancies between the two study findings appear to be due to the difference in the amount of dissolved ions. Clark et al. suggested that the rate of corrosion depended on the kinds of metals and that the presence of protein solutions markedly accelerated the corrosion of some metals. Moreover, particulate size was presumed to play an important part in the rate of ion dissolution. In the present study, the particulate size was 100 μm for Cu and 1 μm for W. The rate of dissolution from Cu particulates was possibly slow, because the surface area per weight for Cu was smaller than that of W. This appears to be one of the reasons why the cytotoxicity of the extracts was similar to Cu and W. A difference in cell viability between the particulates and the extracts was observed for Ni and Mn. The cytotoxicity of the particulates was stronger than that of the extracts. This result shows that the effects of particulate exposure depended not only on the soluble ions, but also on the interaction between particulates and cells owing to their slow dissolution. However, the cytotoxicity of the extracts could be influenced by the concentration of free soluble ions, valence number, complex, or the precipitate, in the medium. Therefore, it is necessary to analyze their chemical species or states and the concentrations in the medium. The cell viability of Si particulates, a non-metal, was higher than that of the extracts. Unfortunately, knowledge about the cytotoxicity of Si and Si$^{4+}$ is scarce. Therefore, the results in this study could not
It is suggested that the nonpassivated metals such as Mo and Fe dissolve metal ions easily. Despite the nonpassivation, the cell viability after 6 days of exposure for Mo and Fe particulates was similar to the controls after 8- and 4-fold dilution, respectively. Both particulates yielded weak cytotoxicity compared with Cu, Si, W, V, Mn, Co, and Ni. Moreover, only Mo extracts depressed cell viability slightly. The cytotoxicity of extracts depended on the amounts of dissolved ions and their intensity of cytotoxicity. Yamamoto et al.\(^{17}\) investigated the cytotoxicities of 43 metal salts and found that the IC\(_{50}\) of the Mo and Fe salts were appreciably higher than that of other metal salts such as Cu, W, V, Mn, Co, and Ni. Although metal ions were dissolved from Mo and Fe, the amounts of dissolved ions might not have exceeded the concentration level that strongly depressed cell viability. It is conceivable that the weak suppression of the cell viability of Mo and Fe may be due to the high local concentration of metal ions dissolved in contact areas between particulates and cells. Therefore, Mo and Fe particulates increased ion concentrations of the contact area and depressed cell viability with exposure time.

Exposure of the cells to Al, Ti, Ta, or Cr extracts resulted in a small effect on the cell viability. This result may be explained as follows. Highly passivated metals such as Ti, Ta, Nb, Zr, Cr, and Al are known to remain uncorrosive\(^{17,19}\). The present results suggested the particulates yielded a slight effect on cells, but not the extracts. The dissolution of soluble ions was apparently negligible. Therefore, the effects of particulates on cells is considered to be due to the direct contact of particulates with the cell membrane\(^ {15}\) and the phagocytosis of osteoblast-like cells. In particular, care was taken to ensure that the particulates were small enough to be phagocytosed. Particulates less than 15\(\mu\)m are known to be phagocytosable\(^ {10,20-22}\). Since the present study did not measure the distribution of particulate size, the number of particulates less than 15\(\mu\)m in size remained unknown. However, the depression of cell viability might be attributed to particulates less than 15\(\mu\)m. Cell viability after 6 days of exposure was higher than that after 3 days of exposure. These results suggest that the cells can proliferate after first cell damage. Pioletti et al.\(^ {11}\) reported that an exposure level below the threshold particulate concentration value allowed recovery of viability. The present results may be explained thus; although the particulates were taken into the osteoblasts within the first 24 hr, adequate numbers of cells could remain to proliferate, and finally remained similar to controls\(^ {11}\).

Small effects on cell viability were observed on Nb and Zr particulates. The high density of Nb decreased the particulate number per volume compared with Ti and Al. On the other hand, Zr particulates showed low exposure concentrations because of their fine particulate size. It was suggested that particulate concentration and the number of particulates less than 5-10\(\mu\)m in diameter might be more important factors than the composition for a direct effect on osteoblast viability\(^ {10,11}\). Nb and Zr particulates may yield no effect on the cell viability owing to low amounts of particulates of less than 5-10\(\mu\)m diameter, although the distribution of particulate size was not examined in this study. Further studies are required to clarify the cell
response of Nb and Zr particulates.

Ti and Ta are important elements for dental as well as medical use. Others, such as Cr, Mo, Co, Mn, Ni, and Si have been used as compositional elements accordingly. However, it is expected that new metallic biomaterials with superior mechanical properties and biocompatibility will be developed. The properties of non-precious metals will be influenced by the addition of trace elements. The findings obtained in the present study may provide information on the assessment of the potential cytotoxicity of additional elements in such cases.

Metallic biomaterials have been used for the replacement of bone, joints, tooth roots, etc. Thus, the interaction between biomaterials and osteoblast cells are of importance. The human osteogenic cell line MG-63 is an appropriate cell line for studying osteoblast function, because these cells have been well characterized and are known to express the phenotypic features of osteoblasts\(^{23,24}\). Furthermore, osteoblast-like cells appear to be capable of phagocytosis\(^{25}\). However, caution should be taken when extrapolating the present results to human osteoblasts. Interaction with particulates could provide different cytotoxic results among different cell lines\(^{26}\).

The effect of particulates on cells could be classified by the use of measuring the cell viability of particulates and extracts. The different cell viabilities are attributable to the particulate solubility and the intensity of the soluble ion's cytotoxicity. Particulates of high solubility and the intensity of the ion's cytotoxicity will lower cell viability by the soluble ions. Consequently, differences in cell viability between particulates and extracts were not observed. However, particulates with low solubility or a low intensity of the ion's cytotoxicity will lead to differences in cell viability between particulates and extracts. Cell viability of passivated particulates will be affected by direct contact with cells. As a direct result of this, cell viability will be depressed by particulates, but not extracts.

The wear debris and the promotion of dissolution are important themes to study in order to develop biomaterials used in tissue that is susceptible to wear. The present results demonstrated that cell viability was depressed at extremely low concentrations of nonpassivated particulates. It was suggested that particular elements could be selectively dissolved from alloys\(^{26}\). These results suggest that care must be taken to avoid selecting corrosive and cytotoxic elements for alloy composition. Moreover, even the passivated alloys should be treated with care, since their particulates dissolved compositional ions into the culture medium. Previous results suggested that even though the concentration of dissolved ions caused no effect on the cell viability of osteoblasts, the osteoblast phenotype might be affected\(^{27,28}\). The effects of the extracts on the phenotype and cytokines release of osteoblast-like cells should be investigated in greater detail. Corrosion of stainless steel and cobalt-chromium alloys might lead to the dissolution of biologically active hexavalent chromium into the body\(^{29}\). The severe cytotoxicity of hexavalent chromium was reported by in vitro studies\(^{17,30}\). The present findings did not support the dissolution of hexavalent chromium from pure chromium. It is conceivable that chromium may
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easily dissolve once the protective surface layer is broken due to wear. Therefore, it is important to evaluate wear debris produced under dynamic conditions, which mimics tissue conditions. The combined effects of wear debris and metal ions on cells needs to be clarified, because stress-bearing metallic biomaterials might tend to produce wear debris and dissolved ions while in use.

The observations made in the present study showed that exposing osteoblast-like cells to passivated metals affected cell viability at high exposure levels. However, Wang et al.31) reported that osteoblast-like cells exposed to very low concentrations of Ti and Co-Cr particulates were not found to show changes in cell growth or viability after 72 hr of incubation, but responded by releasing cytokines. In addition, Evans et al.32) suggested that particulates showed a reduced toxicity when ground in serum. These findings are important factors in clinical situations. The effects of particulate size, particulate number, particulate geometry, and protein adsorption on the osteoblast phenotype and the release of cytokines should be a matter of further investigation to clarify the effects of particulate metals with biological systems.

ACKNOWLEDGEMENTS

This study was partially supported by a Grant-in-Aid for General Scientific Research (C) (2) (No.13672067) from the Ministry of Education, Science, Sports and Culture of Japan.

The authors thank the members of the Department of Biomaterials, Osaka Dental University for kind advice and help. Part of this study was carried out using the Institute of Dental Research, Osaka Dental University.

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