Effects of Metallic Concentrations Other Than Ti, Al and V on Cell Viability†

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New titanium alloys containing zirconium, tin, niobium, tantalum and palladium are being developed by our group. Using metallic particles we investigated effects of Zr, Sn, Nb, Ta and Pd concentrations in Eagle’s medium and α medium, added as alloying elements for the new Ti alloy, on the colony formation ratio of fibroblast V79 cells taken from the lungs of Chinese hamsters, and on the relative growth ratios of murine fibroblast L929 and murine osteoblast-like MC3T3-E1 cells. Moreover, we investigated the effects of chromium and molybdenum concentrations on the colony formation ratio of V79 cells and on the relative growth ratios of L929 and MC3T3-E1 cells. The effects of Co, Ni, Fe and Si concentrations on the relative growth ratios of L929 and MC3T3-E1 cells were examined. Various sterilized metallic particles were extracted after incubation for 86.4 ks (1 d) to 432 ks (5 d) under a 95% air-5% CO₂ atmosphere at 310 K (37°C). The maximum concentrations of Zr, Sn, Nb, Ta and Cr released into Eagle’s medium and α medium after 0.2 μm membrane filtration were 2.1, 4.1, 0.5, 0.07 and 0.14 mass ppm, respectively. Therefore, the colony formation of the V79 cells and the relative growth ratios of L929 and MC3T3-E1 cells were nearly unity. For palladium particle extraction, the colony formation of V79 cells and the relative growth ratio of L929 cells gradually decreased from a concentration of approximately 2 mass ppm or higher and reached nearly zero at greater than 20 mass ppm. Influences of Mo, Fe, Ni and Co concentrations on the MC3T3-E1 cell viabilities were more pronounced at lower concentrations than with the L929 and V79 cells. In the case of Mo particle extraction, the relative growth ratio of MC3T3-E1 cells decreased from the concentration of about 10 mass ppm. For iron, nickel and cobalt particle extractions, the MC3T3-E1 cell viabilities decreased from concentrations of about 2, 1 and 1 mass ppm or higher, respectively, and the relative growth ratio of MC3T3-E1 cells decreased to zero at concentrations greater than 20 mass ppm. In the case of Si particle extraction, the relative growth ratio of the L929 cells was nearly unity at the high concentrations of 65 mass ppm and over. However, the relative growth ratio of MC3T3-E1 cells decreased from 1 mass ppm and was nearly zero at concentrations greater than 50 mass ppm.

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I. Introduction

The cytocompatibility of various pure metals has been summarized in groups by Kawahara et al., and metallic titanium, tin, zirconium, palladium and tantalum were reported to be low cytotoxic elements(1)-(3). A correlation between corrosion resistance and biocompatibility of various metals and alloys has also been reported(4). Cobalt, nickel and copper are classified into the sterile abscess (toxic) group. Iron, molybdenum, silver, gold, stainless steel, cast and wrought Co-Cr alloys are classified into the capsule (scar tissue) group. Zirconium, titanium, niobium, tantalum and platinum exhibit excellent biocompatibility and belong to the loose connective vascularized (vital) group in tissue reaction. The toxicology of tin is almost identical to that of tin organic compounds(5)-(7).

Metallic concentrations in tissue surrounding SUS316 stainless steel and Co-Cr alloy implants have been reported(8)-(10). Regarding the release of metallic ions in vivo from dental implant materials, average concentrations of Co, Cr, Mo and Ni ions in tissue organs (brain, kidneys, liver, lungs and spleen) after mandibular implantation into rabbits for 62 Ms (2 years) have been examined(11). The molybdenum concentrations were about 0.2 mg/kg in the brain and lungs, and approximately 2 mg/kg in the kidneys, liver and spleen for a Co-Cr-Mo alloy implant. Cobalt, chromium and nickel releases into urine and serum for patients with knee or hip prostheses made of porous-coated Co-Cr-Mo alloy have been reported. The mean cobalt concentrations in serum and urine exhibit a slight increase in patients with Co-Cr-Mo alloy knee implants at 3.6 to 72.6 M s (6 to 120 weeks) after surgery. Substantially increased Co levels were observed in the serum and urine of two patients from 4.2 Ms (7 weeks) to 58 Ms (22 months) postarthroplasty associated with the
loosening of the prostheses, and one patient also exhibited elevated Cr levels in the urine and serum\(^9\). The mean nickel concentrations in the serum and urine greatly increased 86.4 to 172.8 ks (1 to 2 d) after implantation, diminishing by 1.2 Ms (2 weeks) in spite of the use of a Co–Cr–Mo alloy containing very little Ni (0.2 mass%). The concentrations of cobalt, chromium, and nickel in the serum for a period of up to 15.8 Ms (6 months) after implantation of total hip replacement have been analyzed by electrothermal atomic absorption spectroscopy. Chromium levels in serum were found to rise to a pronounced postoperative peak and then diminish, although not falling to normal mean levels after 15.8 Ms (6 months). Cobalt levels in serum either remained the same or decreased while nickel levels in serum began to rise 1.2 Ms (2 weeks) to 3.6 Ms (6 weeks) after surgery\(^10\). Moreover, increases in urinary cobalt and nickel excretion were detected in several patients at 15.8 Ms (6 months), and in most patients at 31 Ms (one year) after implantation with a porous-coated total hip replacement\(^11\).

Eliminations of nickel, cobalt and chromium following the repeated injections of high dosage metal salts into Syrian hamsters have been reported\(^9\). Nickel is rapidly eliminated in the urine and cobalt is eliminated much more slowly than Ni. Chromium is released very slowly in the urine. However, metal levels in the organs (liver, lungs, spleen and kidneys) are similar to those of control animals. BF-2 cells, an established fibroblastic cell line derived from the caudal fin of bluegill sunfish (Lepomis macrochirus), have been exposed to 18 metal salts\(^30\). Based on the concentration of metal that reduced the uptake of neutral red by 50% (NR50), the ranking of cytotoxicity for cationic metals is Ag\(^+\) > Hg\(^+\) > Cd\(^+\) > Zn\(^+\) > Cu\(^+\) > Co\(^+\) > Ni\(^+\) > Pb\(^+\) > Sn\(^+\) > Mn\(^+\) > Cr\(^+\), and for the anionic metal complexes it is AsO\(_4\)^{-2} > CrO\(_4\)^{-2} > CrO\(_3\)^{-2} > AsO\(_3\)^{-2} > SeO\(_3\)^{-2} > MnO\(_4\)^{-2} > SeO\(_4\)^{-2}. The cytotoxic effects of cationic and anionic metal salts on BALB/c mice 3T3 fibroblasts were also evaluated with 96-well microtiter plates. Based on the spectrophotometrically determined absorbance of neutral red, the ranking of cytotoxicity for cationic metals is Cd\(^2+\) > Hg\(^2+\) > Ag\(^+\) > Zn\(^2+\) > Mn\(^2+\) > Cu\(^2+\) > Ni\(^2+\) > Cr\(^3+\) at NR50. The anionic metal salts in order of toxicity are CrO\(_4\)^{-2} > CrO\(_3\)^{-2} > AsO\(_3\)^{-2} > AsO\(_4\)^{-2} > SeO\(_3\)^{-2} > SeO\(_4\)^{-2} > MnO\(_4\)^{-2}. Also, the effect of the metal-metal interaction on cytotoxicity shows a marked reduction in Cd toxicity by Zn, and to a lesser degree, by Ni\(^39\). The cytotoxicity of metal salts for murine fibroblast L929 and osteoblastic MC3T3-E1 cells using 21 metal salts have been reported\(^22\). The metal salts were grouped into low (NR50 ≥ 5 × 10^{-4} mol·L\(^{-1}\)), intermediate (10^{-4} ≤ NR50 ≤ 5 × 10^{-4} mol·L\(^{-1}\)) and high (NR50 ≤ 5 × 10^{-5} mol·L\(^{-1}\)) toxic categories. Low toxicity: FeSO\(_4\), FeCl\(_3\), SnCl\(_4\), TiCl\(_4\), ZrCl\(_4\), NbCl\(_4\), MoCl\(_4\), TaCl\(_4\), WCl\(_6\) and Al(NO\(_3\))\(_3\). Intermediate toxicity: CuCl\(_2\), CuSO\(_4\), CuCl\(_3\), SnCl\(_2\), MnCl\(_2\), NiCl\(_2\), PdCl\(_2\), ZnCl\(_2\) and Cr(NO\(_3\))\(_3\). High toxicity: VCl\(_3\) and K\(_2\)Cr\(_2\)O\(_7\).

The cytotoxic effects of pure metals like Cu, Ti, AI, V, Cr, Mo, Fe, Co and Ni on the L929 mouse fibroblasts have been spectrophotometrically examined by absorbance at 540 nm with 24-well microtiter plates using extracts obtained by dynamic extraction, and the order of cytotoxicity expressed in mass ppm is Cr > V > Co > Fe > Mn > Cu > Ni > Mo\(^9\). On the other hand, the order in millimol is Cr > Co > V > Fe > Mn > Cu > Ni > Mo. Titanium and aluminum exhibited no cytotoxicity. In our two previous papers, the effects of Ti, AI and V concentrations in Eagle’s medium and α medium, added as alloying elements for the popular Ti-6%Al-4%V ELI implant alloy, on the colony formation ratio of V79 cells, and on the relative growth ratios of L929 and MC3T3-E1 cells were reported using metallic particles\(^30\). For the AI particle extraction, the colony formation ratio of the V79 cells and the relative growth ratios of the L929 and MC3T3-E1 cells sharply decreased as the AI concentration in the medium was increased to approximately 0.2 to 0.5 mass ppm. However, the colony formation ratio of the V79 cells and the relative growth ratio of the L929 cells sharply decreased when the V concentration in the medium was increased to 0.2 mass ppm and became zero at approximately 0.5 mass ppm. The relative growth ratio of the MC3T3-E1 cells decreased from the 0.002 mass ppm V concentration and approached zero at about 0.2 mass ppm. Moreover, the effects of oxide film on the colony formation ratio of the V79 cells and on the relative growth ratio of the L929 cells were also reported\(^30\). In the case of oxidized AI particles extracted using an oven, the change in the relative growth ratio of the L929 cells was very small, from 43.2 ks (0.5 d) to 345.6 ks (4 d), and it was almost 0.8 after 345.6 ks (4 d) of incubation. The colony formation ratio of the V79 cells also depends on the strength of the aluminum oxide film.

In the United States, Ti-13%Zr-13%Nb (ASTM1713-96) alloy without AI and V was developed for medical implants\(^29\). New Ti alloys such as Ti-15%Zr-4%Nb-4% Ta-0.2%Pd and Ti-15%Sn-4%Nb-2%Ta-0.2%Pd containing Zr, Sn, Nb, Ta, Pd are being developed by our group\(^29\). The corrosion resistance, corrosion fatigue strength in physiological saline solution and mechanical properties at room temperature of these new Ti alloys were better than those of the Ti-6%Al-4%V ELI implant alloy.

In this paper, the effects of Zr, Sn, Nb, Ta and Pd concentrations in Eagle’s medium and α medium, added as alloying elements for the new Ti alloy, on the colony formation ratio of V79 cells, and on the relative growth ratios of L929 and MC3T3-E1 cells were investigated using metallic particles. The effects of chromium and molybdenum concentrations on the colony formation ratio of the V79 cells and on the relative growth ratios of the L929 and MC3T3-E1 cells were also investigated. Moreover, to compare the influence of the metallic particles and metal salts on cell viability by optical density measurements\(^22\), the effects of Co, Ni, Si and Fe concentrations on the relative growth ratios of L929 and MC3T3-E1 cells were examined.
II. Experimental Methods

1. Metallic particles and extraction method

High-purity metallic particles (Nilaco Corporation, Sn: 99.999%, Nb: 99.9%, Ta: 99.9%, Pd: 99.9%, Mo: 99.95%, Si: 99.9%, Co: 99%, Ni: 99.9%, Cr: 99%, Fe: 99%) except for Zr particles (Nilaco Corporation, 94 +4%Hf containing ultrapure water) were sterilized under an ultraviolet lamp for more than 14.4 ks (4 h). Particle size distributions were measured using a laser diffraction particle size analyzer (Shimadzu Corporation; SALD-2000A) within a particle diameter range of 0.03 to 1000 μm\(^2\). Average and standard deviation values of particle size obtained from two measurements before the particle extraction to avoid adsorption of the medium on the surface of metallic particles are shown in Fig. 1. Particle sizes of Zr, Si, Pd, Ni, Co, Mo, Sn, Ta, Nb and Fe were greater than 0.4, 0.5, 0.5, 0.7, 0.7, 1, 14, 14, 17 and 17 μm, respectively.

The experimental sequence of using metallic particles have been carried out\(^2\). Ten grams of each sterilized particle were mixed with 100 mL of the Eagle’s minimum essential medium (Eagle’s medium) and the α-modified Eagle’s medium (α medium) containing fetal bovine serum (10 vol%) and 7.5% NaHCO\(_3\) in a 100 mL bottle. These media were then kept in the incubator for 86.4 to 432 ks (1 to 5 d) under a 95% air–5% CO\(_2\) atmosphere at 310 K (37°C). The medium containing metallic particles was stirred at intervals of 43.2 ks (12 h) so that the surfaces of the particles could mix adequately with the medium. Thereafter, these media were centrifuged at 50 s\(^{-1}\) for 0.9 ks and the supernatant was filtered with a 0.2 μm membrane filter in the clean bench. In all the experiments, Eagle’s medium and α medium were kept in the incubator under similar conditions without metallic particles for the control.

Also, in the case of Zr, Nb and Ta particle extractions, to examine the influences of denaturing in the α medium due to adsorption of proteins, aminocids for the surfaces of the metallic particles on the relative growth ratio of MC3T3-E1 cells, α MEM solution (pH: 5.3) without fetal bovine serum and 7.5% NaHCO\(_3\) and different quantities of the Zr, Nb and Ta particles were prepared. One to five grams of Zr and Ta particles (1, 1.5, 2, 2.5, 3 and 5 g) were mixed with the 50 mL α MEM solution and the α medium. They were then kept for 172.8 ks (2 d) in the incubator and were then filtered using a 0.2 μm membrane filter. Later, the α medium was prepared with α MEM filtrates. Moreover, to examine the effects of pH in solvent on the metallic ion release, α MEM solutions were used. Hereafter, we refer to the α MEM solution not containing fetal bovine serum or 7.5% NaHCO\(_3\) and the α medium containing fetal bovine serum and 7.5% NaHCO\(_3\). α medium prepared with extracts using the various metallic particles is defined as the α medium extract in this paper.

Moreover, the effects of mixed particles of Ti+

Zr + Nb + Ta and Ti + Sn + Nb + Ta on the relative growth ratio of the L929 cells were investigated, as these are the main alloying elements in the new Ti alloys. Three grams each of Ti, Zr, Nb and Ta as one group and Ti, Sn, Nb, Ta particles as the other group were mixed. These particles were suspended in the Eagle’s medium after sterilization under an ultraviolet lamp for more than 14.4 ks.

The Eagle’s medium and α medium prepared with the metallic particle extracts were diluted by adding freshly
prepared Eagle’s medium or α medium. The Eagle’s medium and α medium extracts with Fe, Ni, Co and Pd particles were diluted up to 40 times, while Eagle’s medium and α medium extracts with Mo particles were diluted up to 400 times. To dilute the α MEM extract, α medium extracts prepared from the α MEM extract were diluted using fresh α medium.

2. Cell cultures

(1) L929 and MC3T3-E1 cells

The culture medium for the L929 cells (Dainippon Pharmaceuticals Co., Ltd., frozen and stored) was prepared by adding 7.5% NaHCO₃ solution (2.3 vol%), fetal bovine serum (10 vol%, Gibco BRL) and 3% L-Glutamine solution (1 vol%) to Eagle’s MEM solution. For the MC3T3-E1 cells (donated by Meikai University), the culture medium was prepared by adding fetal bovine serum (10 vol%) and 7.5% NaHCO₃ solution (2.4 vol%) to the α MEM solution. 0.2 mL of the cell suspension was adjusted to 1.5×10⁶ or 2.5×10⁶ cells/mL and was poured into the culture dish containing 2.8 mL of the Eagle’s medium extract for the L929 cells and α medium extract for the MC3T3-E1 cells. These dishes were then incubated up to 345.6 ks (4 d) under a 95%air–5%CO₂ atmosphere at 310 K (37°C). After incubation, the cells were completely separated with pipet trypsin. The cells were then counted using a Coulter counter. The number of cells in 0.5 mL of the medium containing trypsin and electrolyte for each dish were counted four times and the average number of cells in each dish was estimated. The culture medium placed in the incubator without metallic particles was used as the control. The relative growth ratios of the L929 and MC3T3-E1 cells were estimated using the following formula: (average number of cells per dish after 345.6 ks (4 d) incubation using Eagle’s medium or α medium prepared with various extracts)/(the average number of cells in the control). Four to six dishes were used for each cell culture and repeated 3 to 5 times to evaluate the average and standard deviation values of the relative growth ratio of the cells for more than 15 dishes.

(2) V79 cell

For the V79 cells (obtained from The Institute of Physical and Chemical Research) seeding, Eagle’s medium was prepared by adding 7.5% NaHCO₃ solution (1.5 vol%) and fetal bovine serum (10 vol%) to Eagle’s MEM solution. Two mL of cell suspension adjusted to 50 cells/mL was poured into the cell dish containing 6 mL of the Eagle’s medium, which was prepared using extracts with various particles. These dishes were kept for 604.8 ks (7 d) in the incubator in 95%air–5%CO₂ atmosphere at 310 K (37°C). After incubation, the colonies were fixed, and the number of colonies was then counted. Culture dishes containing only Eagle’s medium were used as the control. The colony formation ratio was estimated using the formula: (average number of colonies per dish after 604.8 ks (7 d) incubation with the Eagle’s medium extracts)/(the average number of colonies in the control).

The average and the standard deviation values of the colony formation ratio were calculated using three to five dishes.

3. Chemical analysis

The exact chemical states of various metals in the medium could not be clarified in this experiment. Hence, the effects of metallic concentrations in the medium on the relative growth ratios of the L929, MC3T3-E1 cells, and on the colony formation ratio of the V79 cells were estimated. Metallic concentrations in Eagle’s medium and α medium were measured by inductively coupled plasma mass spectrometry (ICP-MS), electrothermal atomic absorption spectrometry (EAAS), inductively coupled plasma emission spectrometry (ICPES) and atomic absorption spectrometry (AAS). The zirconium, tin, niobium, tantalum, palladium, silicon, cobalt, nickel and chromium concentrations in the Eagle’s medium and α medium were analyzed by ICP-MS, the Fe concentration by EAAS and ICPES, and the Mo concentration by ICP-MS and AAS. The analytical methods have been described elsewhere. Silicon concentration was measured by EAAS and ICP-MS after the measurement medium was diluted 10 times with 1 vol% concentrated nitric acid solution. The average value obtained from three repetitive runs was defined as the analytical value. All the culture mediums were stored in Corning disposable plastic centrifugal tubes or in Falcon centrifugal containers with 0.2 vol% concentrated HCl solution (pH<2) added to avoid any concentration change before analysis. The lower limit in all the analysis techniques was 0.001 mass ppm.

4. Anodic polarization test

To compare the stability of passive films formed on block specimens and metallic particle surfaces in Eagle’s medium, anodic polarization tests carried out on various high purity metals (Soekawa Chemicals Co., Ltd., Zr: 98%, Sn: 99.9%, Nb: 99.9%, Ta: 99.9%, Pd: 99.9%, Mo: 99.9%, Co: 99.9%, Cr: 99.9%, Ni: 99.9%, Fe: 99.9%, Si: 99.99%). Test specimens of 8 mm diameter and 5 mm height were coated with epoxy resin except for 0.5 cm², polished with #600 water-proof emery paper, and then ultrasonically cleaned in ethanol for 0.9 ks (15 min). Anodic polarization tests were conducted with an automatic potentiostat in Eagle’s medium. The testing solution was deaerated with high-purity nitrogen gas at a rate of 3.3×10⁻⁶ m³/s (200 cm³/min) for 0.9 ks. The specimen was held initially at −1 V for 300 s, followed by natural electrode potential for 300 s in medium. Anodic polarization tests were carried out from −1 V to 5 V at a sweep rate of 6.67×10⁻⁴ V/s (40 mV/min). A platinum electrode and a saturated calomel electrode (SCE) were used as counter and reference electrodes, respectively.

III. Results and Discussion

Figure 2 shows the change in relative growth ratio of
the L929 cells as a function of incubation time from 86.4 ks (1 d) to 345.6 ks (4 d) incubation for the Eagle’s medium extract with Zr, Sn, Pd and Mo particles. The change in relative growth ratio of the L929 cells with V particle extraction is also shown for comparison. In the case of Eagle’s medium extracts with Zr and Sn particles (Zr: 2.1 mass ppm, Sn: 0.02 mass ppm, respectively), the L929 cells proliferated in the same ratio as those of the control and hence the relative growth ratio of L929 cells was nearly unity at all the incubation times. For the Eagle’s medium extract with Pd particles (3.9 mass ppm), the relative growth ratio of the L929 cells decreased at 172.8 ks (2 d) incubation and thereafter remained constant at approximately 0.8. On the contrary, the Eagle’s medium extract with V particles (192 mass ppm) strongly inhibited cell growth, causing a significant decrease in the relative growth ratio of the L929 cells as early as 43.2 ks (0.5 d). It is interesting that the relative growth ratio for the Eagle’s medium extract with Mo particles (481 mass ppm) gradually decreased and became to 0.1 at 345.6 ks (4 d) incubation in spite of the high concentration.

The effect of extraction time on the release of metallic ions from various metallic particles into Eagle’s medium is shown in Fig. 3. The palladium and iron concentrations increased with longer extraction time. However, changes in zirconium, tin, niobium, tantalum, chromium and silicon concentrations in the Eagle’s medium were small at all extraction times.

The effects of extraction time on the relative growth ratio of the L929 cells are also shown in Fig. 4. The relative growth ratios for Eagle’s medium extracts with Zr, Sn, Nb, Ta and Si particles were approximately unity at all extraction times. The relative growth ratio for the Pd particles extraction markedly decreased as extraction time increased because the Pd concentration in Eagle’s medium increased with an increase in the extraction time. A slight decrease in the relative growth ratio of the L929 cells for Cr particle extraction was noted. Based on these results, we believe that degree of denature in the Eagle’s medium is small even after 432 ks (5 d) of extraction. On the other hand, in the case of Eagle’s medium extracts with Mo, Ni, Co and Fe particles, the metallic concentration in the medium was high, and hence the relative growth ratios of L929 cells were almost zero at all extraction times.

The various metallic concentrations in α medium after metallic particle extraction with α MEM solution and α medium are compared in Fig. 5. The zirconium, cobalt and nickel concentrations for α MEM extraction were slightly higher than those in the α medium extract. Otherwise, niobium and silicon concentrations in α MEM extraction were lower than those in the α medium extracts. Tantalum, tin and palladium concentrations were the same in both α MEM and α medium extracts.

Figure 6 compares the anodic polarization curves in the Eagle’s medium. The passive films that formed on the Nb, Ta and Si block specimens were fairly stable and stronger than those of the Fe, Co, Ni and Pd block specimens because of the much lower current density exhibited within the potential region up to 5 V. Small passivation peaks were also seen for the Mo, Cr, Zr and Sn block specimens.

Figure 7 compares the metallic concentrations in the Eagle’s medium and α medium extracts before incubation (after 0.2 μm filtration) and after 345.6 ks (4 d) of incubation. Metallic concentrations after 0.2 μm filtration and incubation were in good agreement. Figure 8 shows the effects of dilution on the metallic concentration in Eagle’s medium and α medium. Metallic concentrations in Eagle’s medium and α medium decreased according to
Fig. 5 Comparison of metallic concentrations in \( \alpha \) medium with \( \alpha \) MEM solution and \( \alpha \) medium extracts.

Fig. 7 Comparison of metallic concentrations in Eagle's medium and \( \alpha \) medium after 346 ks (4 d) of incubation and 0.2 \( \mu \)m filtering.

Fig. 6 Comparison of anodic polarization curves for different metals in Eagle's medium at 310 K (37°C).

Fig. 8 Effects of dilution ratio on metallic concentrations in Eagle's medium (a) and \( \alpha \) medium (b).

The dilution ratio. Therefore, we think that the effect of metallic particles of less than 0.2 \( \mu \)m after filtering on the metallic concentration in the medium might be negligible.

Figures 9 and 10 show the change in the relative growth ratios of the L929 and MC3T3-E1 cells as a function of the metallic concentrations in Eagle's medium and \( \alpha \) medium, respectively. Maximum concentrations of Zr, Sn, Nb, Ta, Cr and Ti\(^{2+}\) released during metallic particle extraction were 2.1, 4.1, 0.5, 0.07, 0.14 and 0.3 mass ppm, respectively, and hence did not exhibit any effect on the relative growth ratios of the L929 and
MC3T3-E1 cells. Also, the particle mixtures of Ti + Zr + Nb + Ta and Ti + Sn + Nb + Ta did not exhibit any clear effect on the relative growth ratio of the L929 cells as shown in Table 1. The concentrations of Ti, Zr, Sn, Nb, and Ta were too low (Ti, Zr, Sn, Ta < 0.05, Nb < 0.7 mass ppm) in the medium to cause any significant effect on the relative growth ratio of the L929 cells. Palladium particle extraction had an effect on the colony formation ratio of the V79 cells and the relative growth ratio of the L929 cells at metallic concentrations of 3 mass ppm or above. The influences of Mo, Fe, Ni and Co concentrations on cell viability were seen at lower concentrations for both L929 and V79 cells. For iron, nickel and cobalt particle extraction, the relative growth ratio gradually decreased from approximately 2, 1 and 1 mass ppm, respectively, and approached zero at approximately 20 mass ppm or higher. For molybdenum particle extraction, the relative growth ratio of the MC3T3-E1 cells decreased from concentrations of about 10 mass ppm and above. Otherwise, in the case of silicon particle extraction, the influence of concentration on cell growth was different for the L929 and MC3T3-E1 cells. This may be due to the difference in fibroblastic and osteoblastic types of cells.

Figure 11 shows the effects of Zr, Sn, Nb, Ta, Pd, Ti, Cr and Mo concentrations on the colony formation ratio.

Table 1 Effects of mixed particles on the relative growth ratio of L929 cells and metallic concentrations in Eagle's medium released from mixed particles during 172.8 ks extraction.

<table>
<thead>
<tr>
<th>Mixed particles</th>
<th>Relative growth ratio</th>
<th>S.D.</th>
<th>Metallic concentration (mass ppm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ti</td>
</tr>
<tr>
<td>Ti + Zr + Nb + Ta</td>
<td>0.996</td>
<td>±0.011</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ti + Sn + Nb + Ta</td>
<td>0.988</td>
<td>±0.020</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
of V79 cells. The colony formation ratio of the V79 cells showed a similar tendency in the relative growth ratio as the L929 cells, as shown in Fig. 9. The concentrations at 20% (IC50, relative growth ratio: 0.8) and 50% (IC50, relative growth ratio: 0.5) cell inhibitions for the L929, MC3T3-E1 and V79 cells are compared in Fig. 12. In this figure, IC50 and IC50 values for the V79 cells are results reported by Ito. Similar tendencies for fibroblastic L929, osteoblastic MC3T3-E1 and fibroblastic V79 cells were seen at both IC50 and IC50, except for V. The effects on cell growth for Fe, Ni, Co and Mo, except for Cr, approximately agreed with the results measured by the optical density method. The effects of weights for zirconium, tantalum and niobium particles on the metallic ion release and on the relative growth ratio of the MC3T3-E1 cells are shown in Fig. 13. As shown in Fig. 13(a), the relative growth ratios were about unity for both α MEM and α medium extracts with Nb particles. The relative growth ratio of the MC3T3-E1 cells was nearly up to 5 g of Zr and Ta particle extractions for the α MEM solution. On the other hand, the relative growth ratio for the α medium extract decreased as the weight of Zr and Ta particles was increased from 3 to 5 g. It was suggested that the decrease in the relative growth ratio of the MC3T3-E1 cells might not be influenced by the released metallic concentrations as shown in Fig. 13(b), but was due to gel formation caused by the reaction for the α medium and the metallic particles.

Figure 14 compares concentrations at 50% cell inhibition (IC50) for the metallic particles extractions obtained in this study, as reported by Takeda et al., and the addition of metal salts into Eagle’s medium reported by Yamamoto et al. and Fujimoto. The influence of V, Mo, Co, Ni, Cu, Mn and Pd concentrations on the relative growth ratio of the L929 cells showed a similar tendency, except for Cr and Fe. The toxicity of various metals has been discussed using Mandaleef’s periodic table. The effects of various metals on cell viability obtained in our experiments are is shown in Mandaleef’s periodic table together with elements prone
Fig. 13 Effects of different weights of metallic particles on the relative growth ratio of MC3T3-E1 cells (a) and on the metallic concentration (b).

to inducing allergic reactions\(^{(30)(41)}\) and cancer-causing elements\(^{(28)}\). The results are shown in Fig. 15. Good cell growth refers to the relative growth ratio of cells that is almost unity at the extraction time of 345.6 ks (4 d) and to a concentration at 20% cell inhibition (IC\(_{20}\)) that was greater than 20 ppm. Fair cell growth refers to those metals for which the relative growth ratios of the L929 and MC3T3-E1 cells decreased after longer extraction times as seen for Pd (5 < IC\(_{20} < 20\)). Poor cell growth refers to a growth ratio for which the concentration at 20% cell inhibition was less than 5 ppm. In Fig. 15, the results for Cu, Zn, Cd, Hg and Pd are those reported by Kawahara\(^{(1)(2)}\). It is interesting to note that Ti, Zr, Nb, Ta, Mo and Cr, except for V, are non cytotoxic elements. It is also noted that the group IIb in Mandeleef’s periodic table are toxic elements.

IV. Conclusions

The effects of Zr, Sn, Nb, Ta and Pd concentrations in Eagle’s medium and \(\alpha\) medium, added as alloying elements for new Ti alloys, on the colony formation ratio of V79 cells and on the relative growth ratio of murine fibroblast L929 and murine osteoblast-like MC3T3-E1 cells were investigated using metallic particles. The effects of chromium and molybdenum concentrations on the colony formation ratio of fibroblast V79 cells, and on the relative growth ratios of the L929 and MC3T3-E1 cells, were also investigated. To compare the influences using metal salts and metallic particles on cell viability, the effects of Co, Ni, Fe and Si concentrations on the relative
growth ratio of the L929 and MC3T3-E1 cells were examined. The following are our conclusions.

1. The maximum concentrations of zirconium, tin, niobium, tantalum and chromium released into the Eagle’s medium and α medium after 0.2 μm membrane filtering were 2.1, 4.1, 0.5, 0.07, 0.14 mass ppm, respectively. Therefore, the colony formation ratio of the V79 cells and the relative growth ratios of the L929 and MC3T3-E1 cells were nearly unity. For palladium particle extraction, the colony formation ratio of the V79 cells and the relative growth ratio gradually decreased from the concentrations of approximately 3 mass ppm or higher, and reached nearly zero above 20 mass ppm. In the case of Mo particle extraction, the colony formation of the V79 cells and the relative growth ratio of the L929 cells decreased from the concentrations of about 20 mass ppm. Also, the relative growth ratio of the MC3T3-E1 cells decreased from the concentrations of about 10 mass ppm.

2. The influences of Fe, Ni, Co and Si concentrations on cell viability were seen at lower concentrations as compared to those of the L929 and V79 cells. For iron, nickel and cobalt particle extractions, the MC3T3-E1 cell viabilities increased from concentrations of approximately 2, 1 and 1 mass ppm or above, respectively, and the relative growth ratio of the MC3T3-E1 cells decreased to nearly zero at concentrations over 20 mass ppm. In the case of Si particle extraction, the relative growth ratio of the L929 cells was nearly unity at high concentrations of 65 mass ppm and higher. However, the relative growth ratio of the MC3T3-E1 cells decreased from 1 mass ppm and was nearly zero at the concentrations over 50 mass ppm.

REFERENCES

(36) Y. Ito: The Special Steel, 42 (1993), No. 11, 23.
(41) M. Inoue: Kinzoku, 62 (1992), No. 12, 18.