Effects of Aluminum Oxide Film on Fibroblast L929 and V79 Cell Viabilities[†]

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The effects of Ti and Al oxide films on the relative growth ratio of murine fibroblast L929 and the colony formation ratio of fibroblast V79 cells derived from the lung of a Chinese hamster were investigated. Titanium and aluminum particles were oxidized inside an oven at 453 K (180°C) for 7.2 ks. These particles were suspended in Eagle's medium and kept for 173 ks (48 h) inside an incubator under a 95%air-5%CO₂ atmosphere. Eagle's medium without metallic particles was also kept inside the incubator under similar conditions for the control. The mediums were filtered with a 0.2 µm membrane filter to remove metallic particles (Eagle's medium extract). 3.0×10^4 L929 cells/dish and 10^2 V79 cells/dish were seeded in both the control and in the Eagle's medium extracts. In the case of oxidized Al particle extraction, the relative growth ratio of L929 cells was 0.8 ± 0.07 after 346 ks (4 d) of incubation. A relatively strong oxide film formed on the surfaces of the Al particles, hence the relative growth ratio of the L929 cells markedly increased.

Al and Ti plates were polished to a mirror-like finish using diamond paste and alcohol; well proliferated colonies of V79 cells were observed on the mirror-like finished Ti plate ($Ra = 4.8 \pm 0.2$ nm), whereas no colonies existed on the mirror-like finished Al plate ($Ra = 38.5 \pm 8.4$ nm). However, the colonies appeared when the Al plates were acid-treated with a solution of NaHCO₃ and C₂H₄O₂ ($Ra = 43.4 \pm 10.1$ nm) after mirror-like finishing, and also when roughening with #240 water-proof emery paper ($Ra = 1548 \pm 169$ nm). The colony formation ratio of the V79 cells was found to depend on the surface roughness (Ra) and strength of the Al oxide film.

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I. Introduction

The colony formation of V79 cells using two pure metals: Ti and Ni; two ceramics: alumina ceramic and tricalcium phosphate (TCP); and two polymers: high-density polyethylene (HDP) and polyvinylchloride (PVC) have been reported⁽¹⁾. Colonies on the Ti, alumina ceramic and HDP proliferated to the same extent as the colonies of the control. However, no colonies were detected on either the disks or semidisks of nickel and TCP, or on the PVC film disk because of the release of cytotoxic substances. In our previous paper, we examined the influences of Al and V concentrations used as alloying elements for Ti-6%Al-4%V ELI (Extra Low Interstitial) alloy, an attractive alloy for medical implants, by estimating the relative growth ratio of murine fibroblast L929 cells and the colony formation ratio of V79 cells derived from the lungs of a Chinese hamster⁽²⁾. The ultrapure water extract containing Al particles resulted in the formation of aluminum hydroxide gel, while the relative growth ratio became almost unity when the Eagle's medium

was prepared with ultrapure water after filtration with a 0.2 µm membrane filter. Aluminum concentration in this Eagle's medium was 0.6 mass ppm. The relative growth ratio of L929 cells was high because the effect of the aluminum hydroxide gel in the medium after filtering with a 0.2 µm membrane filter was low. On the other hand, the relative growth ratios of the L929 cells and murine osteoblastic MC3T3-E1 cells sharply decreased as the Al concentration in the Eagle's medium extract with Al particles increased to approximately 0.2 to 0.5 mass ppm. Titanium and aluminum particles sterilized at 453 K (180°C) for 7.2 ks inside an oven were not toxic for the L929 cells⁽³⁾. Since the result was different for the oxide film formed on the Al particles, we investigated the effects of oxidation and surface finishing on the relative growth ratio of the L929 cells and on the colony formation of V79 cells using Al and Ti plates.

II. Experimental Methods

1. Cell culture using oxidized Ti and Al particles

Five grams of high-purity Ti (99.3%) and Al (99.95%) particles were oxidized inside an oven at 453 K (180°C) for 7.2 ks (2 h). The oxidized and unoxidized Ti and Al

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particles were put into a 100 mL bottle (Iwaki Glass Co., Ltd.) with 50 mL of Eagle's medium containing fetal bovine serum (10 vol%), 7.5% NaHCO_3 (2.3 vol%) and 3% L-Glutamin (1 vol%), and then kept for 172.8 ks (2 d) inside the incubator under a 95%air–5% CO_2 atmosphere at 310 K (37°C). The Eagle's medium was stirred every 43.2 ks (12 h) so that the surfaces of the particles could mix adequately with the Eagle's medium. After extraction, the Eagle's medium was centrifuged at 50 s^{-1} (3000 rpm) for 0.9 ks (15 min). The supernatant was filtered with a $0.2 \mu\text{m}$ membrane filter (Advantec Toyo Co., Ltd.) inside the clean bench. The 2.8 mL Eagle's medium extracted with the Ti and Al particles was poured into culture dishes (Iwaki Glass Co., Ltd.). 0.2 mL L929 cells (Dainippon Pharmaceuticals Co., Ltd., frozen and stored) suspension adjusted to 1.5×10^5 cells/mL were seeded in the culture dishes. They were then incubated for a maximum of 345.6 ks (4 d) inside the incubator under a 95%air–5% CO_2 atmosphere at 310 K (37°C). After incubation, the L929 cells were counted using a Coulter counter. Eagle's medium similarly kept inside the incubator without metallic particles was used as the control. The effects of oxidation on cell viability of the L929 cells were estimated by the relative growth ratio calculated as the (average number of cells per dish after 86.4 ks (1 d) to 345.6 (4 d) incubation with particles extracted medium)/(average number of cells in the control). Twelve dishes or more were used to obtain average and standard deviation values by repeating the cell culture test 3 to 5 times.

2. Cell culture using mirror-like finished Ti and Al plates

To determine the effects of surface roughness and surface protection by the Al oxide film on the colony formation ratio of V79 cells, three types of specimens were prepared in this study. First, high-purity Al and Ti plates (Nilaco Corporation; 99.9%), 49 mm in diameter and 1 mm in thickness were polished to a mirror-like finish using diamond paste and alcohol to obtain a smooth sur-

face after lapping (mirror-like finishing). The surface roughness values (R_a) of these Ti and Al plates obtained by mirror-like finishing were $4.8 \pm 0.2 \text{ nm}$ and $38.5 \pm 8.4 \text{ nm}$, respectively as shown **Table 1**. Secondly, some of these mirror-like finished plates were treated by wiping them with cotton saturated with a solution of concentrated acetic acid and nitric acid (1:1) for 10 s to strengthen the Al oxide film and to protect from Al ion release (hereafter defined as acid-treated Al plate, $R_a = 43.4 \pm 10.1$). Thirdly, aluminum plates were polished with #240 waterproof emery paper to change the surface roughness (#240 roughening, $R_a = 1548 \pm 169 \text{ nm}$). These three differently treated Ti and Al plates were sterilized with alcohol, and put into dishes 55 mm in diameter. Thereafter, 6 mL of Eagle's medium was poured into each dish. 2 mL of the V79 cells (obtained from The Institute of Physical and Chemical Research) suspension-adjusted to 50 cells/mL were seeded in the dishes. The dishes were then rotated slowly so that the cells were uniformly spread. The dishes were incubated for 604.8 ks (7 d) in a 95%air–5% CO_2 atmosphere at 310 K (37°C) and the colonies were counted. The effects of oxide film and surface finishing on the viability of the V79 cells was estimated by the colony formation ratio, calculated by (number of colonies per dish after 604.8 (7 d) incubation with surface treated plates)/(average number of colonies in the control without a metallic plate). Three dishes or more were used to obtain average and standard deviation values.

3. Surface analysis

(1) Surface roughness

The surface roughness values (R_a) of various Ti and Al plate surfaces were measured using a Mitsutoyo SURFTEST-701. The surface roughness was measured for a length of 1.25 mm ($0.5 \text{ mm} \times 5 \text{ span}$). These surface roughness measurements were repeated 3 times for every specimen. The average and standard deviation values of surface roughness were calculated by repeating for 2 or 3 specimens as per JIS B 0601-1994.

Table 1 Comparison of the mean and standard deviation values of surface roughness (R_a) for mirror-like finished Ti and Al plates.

Culture dish	Mirror-like finished Ti plates		Mirror-like finished Al plates	
	Before cell culture	After cell culture	Before cell culture	After cell culture
R_a/nm	R_a/nm	R_a/nm	R_a/nm	R_a/nm
13.2 ± 1.6	4.8 ± 0.2	4.1 ± 0.6	38.5 ± 8.4	55.5 ± 5.9
Surface-treated Al plates after mirror-like finishing				
	Before cell culture	After cell culture		
	Etching with $\text{NaHCO}_3 + \text{C}_2\text{H}_4\text{O}_2$	Etching with $\text{NaHCO}_3 + \text{C}_2\text{H}_4\text{O}_2$		#240 Roughening
	R_a/nm	C.F.R: 0.87	C.F.R: 0.58	R_a/nm
43.4 ± 10.1		R_a/nm	R_a/nm	1548 ± 169
		50.1 ± 2.9	89.9 ± 5.4	

C.F.R.: Colony Formation Ratio of V79 cells.

(2) XPS analysis

The oxide film formed on the mirror-like finished Ti and acid-treated Al plates during 604.8 ks (7 d) incubation was analyzed by XPS (X-ray photoelectron spectroscopy). The measurement conditions for the XPS were an Al $K\alpha$ X-ray source with 200 W output, and the spot size was set at 300 μm . The argon ion sputtering conditions were, incident angle: 30°; acceleration voltage: 3 kV; acceleration current: 3 mA; and an ellipse shaped sputtering spot: 4 mm $\times$ 2 mm. The sample was sputtered with Ar^+ ions for 5 s to remove atmospheric carbon before measuring the binding energy by MRS (Multi-Reason Scan)⁽⁴⁾. Moreover, a TiO_2 film 100 nm thick was formed on a silicon substrate using plasma spray. The sputtering rate of the TiO_2 film was measured using Ar^+ ions.

4. Anodic polarization test

The anodic polarization properties of pure grade 2 Ti and high-purity Al (Nilaco Corporation; 99.9%) blocks were tested to compare the corrosion resistance in the Eagle's medium. The 10 mm $\times$ 10 mm $\times$ 5 mm block specimens were coated with epoxy resin except for 1 cm^2 . The specimen surfaces were polished using up to #600 water-proof emery paper and ultrasonically cleaned in ethanol for 0.9 ks (15 min). The anodic polarization test was conducted with an automatic potentiostat (Hokuto Denko Ltd.; Model HZ-1A). The test solution was deaerated with high-purity nitrogen gas for 0.9 ks (15 min). The specimen was initially held at -1 V for 300 s followed by natural electrode potential for 300 s in Eagle's medium. The anodic polarization test was carried out from -1 V to 2 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⁽⁴⁾⁽⁵⁾.

III. Results and Discussion

1. Effects of oxidation with Ti and Al particles on the relative growth ratio of L929 cells

Figure 1 shows the effects of using oxidized and unoxidized Ti and Al particles on the relative growth ratios of the L929 cells. The relative growth ratios were unity for both oxidized and unoxidized Ti particles from 43.2 ks (0.5 d) to 345.6 ks (4 d) of incubation. The titanium concentrations in the Eagle's medium extracts for oxidized and unoxidized particles were 0.09 and 0.16 mass ppm, respectively. In the unoxidized Al particle extraction, the relative growth ratio of the L929 cells gradually decreased from 86.4 ks (1 d) and became nearly zero at 345.6 ks (4 d). On the other hand, for the oxidized Al particle extraction, the relative growth ratio of the L929 cells slightly increased from 43.2 ks (0.5 d), reaching 0.8 ± 0.07 at 345.6 ks (4 d) of incubation. This suggests that the oxide film formed on the Al particle surfaces was strong. The aluminum concentration was 0.37 mass ppm, hence a small effect on the relative growth ratio of

Fig. 1 Effects of oxidation with Al and Ti particles on the relative growth ratio of L929 cells as a function of incubation time.

the L929 cells was observed. On the contrary, the Al concentration in the Eagle's medium extract with the unoxidized Al particles was 0.53 mass ppm.

2. Effects of surface finishing with Ti and Al metallic plates on the colony formation ratio of V79 cells

(1) Mirror-like finished Ti and Al plates

The V79 cell was chosen because of its relatively long cell culturing period to examine the effects of various surface-finished Al plates on the colony formation ratio. Figure 2 shows the effects of surface finishing on the colony formation ratio of V79 cells for Ti and Al plates. The number of colonies formed for both the control without a plate and the mirror-like finished Ti plate were almost similar as shown in Fig. 2(a). This result is similar to the findings of Kotoura *et al.*⁽¹⁾ In the case of the mirror-like finished Al plate, no colony formation was observed as shown in Fig. 2(b). However, colonies were seen on the Al plate acid-treated with a mixed solution of acetic acid and nitric acid (etching with HNO_3 and $\text{C}_2\text{H}_4\text{O}_2$), and also on an Al plate polished with #240 water-proof emery paper (#240 roughening) as shown in Figs. 2(c) and (d), respectively. The effects of surface finishing, *i.e.* surface roughing and surface protection by Al oxide films, on the colony formation ratio of the V79 cells is shown in Fig. 3. The colony formation ratio of the V79 cells on the mirror-like finished Ti plate was nearly unity, whereas the colony formation ratio on the mirror-like finished Al plate was zero. However, the colony formation ratio significantly increased when the mirror-like finished Al was acid-treated or roughened with #240 water-proof emery paper. The standard deviation of the colony formation ratio of the V79 cells on acid-treated Al plate was high as seen in Fig. 3.

The surface roughness values (R_a) of the culture dish and various surface-treated Ti and Al plates before and after cell culture are compared in Table 1. The surface roughness values for the mirror-like finished Ti plates the

Fig. 2 Effects of surface treatment on the colony formation of V79 cells. (a) Mirror-like finished Ti plate, (b) to (d) Surface-treated mirror-like finished Al plates.

Fig. 3 Comparison of the colony formation ratio of V79 cells using surface-treated mirror-like finished Ti and Al plates.

before and after cell culture were 4.8 ± 0.2 nm and 4.1 ± 0.6 nm, respectively. In the case of the mirror-like finished Al plate before cell culture, the surface roughness was 39 ± 8 nm. On the other hand, the surface roughness increased to 56 ± 6 nm after the cell culture. Hence, it is thought that Al ions from the Al plate were released into the Eagle's medium. The before and after cell cul-

ture surface roughness values of the acid-treated Al plates were 43 ± 10 nm and 50 ± 3 nm, respectively. Also, the colony formation ratio of the V79 cells on the acid-treated Al plate tends to decrease as the surface roughness values increased, as shown in Table 1. The aluminum concentrations in the unfiltered Eagle's medium with the mirror-like finished and acid-treated Al plates after 604.8 ks (7 d) of incubation were 1.7 and 1.0 mass ppm (S.D. < 10%), respectively. By filtering with a 0.05 μ m filter (Millipore Co., Ltd.), the Al concentration for the mirror-like finished Al decreased less than 0.1 mass ppm. Therefore, the high Al concentration may be due to the presence of aluminum hydroxide. But the influence on the colony formation ratio of the V79 cells is small because the aluminum hydroxide particles may be suspended in the medium. This result is similar to the case of Eagle's medium prepared using ultrapure water extract with Al particles⁽²⁾. Even though the surface roughness of the acid-treated Al plate (43 ± 10 nm) was slightly higher than that of the mirror-like finished Al plate (39 ± 8 nm), the Al concentration (1.0 mass ppm) in the case of the acid-treated Al plate became slightly lower than that of the mirror-like finished Al plate (1.7 mass ppm). The formation of V79 cell colonies on polycrystal aluminum ceramic is reported to be similar to that of the control⁽¹⁾. It is considered that the colony formation of the V79 cells on the acid-treated Al plate was high due to the formation of a more stable aluminum oxide film on the acid-treated Al plate than on the non-acid-treated Al plate. The surface roughness of the Al plate roughened with #240

water-proof emery paper was quite high (1548 ± 169). It is interesting to note that the colony formation ratio of the V79 cells was high even though the surface roughness was high.

(2) Mirror-like finished Al half plates

Figure 4 shows the effects on the colony formation of V79 cells of acid treatment on mirror-like finished half-plates. Approximately 100 colonies in one dish were seen for dishes without the Al plate (control) (Fig. 4(a)), while on the mirror-like finished Al half-plate (Fig. 4(b)) colony formation was not observed. Smaller colonies formed on the half-culture dish containing mirror-like finished Al half-plate. On the other hand, on acid-treated Al half-plates, colonies of the V79 cells were seen as shown in Fig. 4(c). The colonies of the V79 cells in the half-culture dish, as shown in Fig. 4(c), proliferated greatly compared to the non-acid-treated Al half-plate, as shown in Fig. 4(b). This result might suggest that Al ions other than aluminum hydroxide had a slight effect on the colony formation ratio of the V79 cells. The effects of surface treatment the half-plate on the colony formation ratio of the V79 cells are summarized in Fig. 5. Figure 5 compares the colony formation ratios of the half-plates and half-culture dishes. The standard deviation of the colony formation ratios for the mirror-like finished Al half-plates increased. Otherwise, on the acid-treated Al half-plates, the colony formation ratio in the half-culture dishes increased approximately 0.5.

Figure 6 compares the anodic polarization curves measured in the Eagle's medium. The passive film formed on the pure grade 2 Ti was stable in the Eagle's medium. Passive film did not form on the Al surface in the Eagle's medium, thus the corrosion resistance was low. The current density markedly increased compared to that of V in

Fig. 5 Comparison of the colony formation ratio of V79 cells using surface-treated mirror-like finished Al half-plates.

Fig. 6 Comparison of the anodic polarization curves for pure grade 2Ti, V, and Al in Eagle's medium at 310 K (37°C).

Fig. 4 Effects of surface treatment on the colony formation of V79 cells using mirror-like finished Al half-plates.

the Eagle's medium. A small passivation peak appeared for the Al specimens after the acid treatment (concentrated nitric acid: acetic acid = 1:1).

3. XPS analysis of passive films

The surfaces of the Ti and Al plates after incubation were thoroughly washed with ultrapure water, and the surface of the oxide film without colonies was analyzed using XPS. XPS wide-scanning spectra for the surfaces of the oxide film are shown in Fig. 7, where Fig. 7(a) shows the mirror-like finished Ti plate and Fig. 7(b) shows the acid-treated Al plate. Peaks of Ca and P were observed on the Ti oxide film surfaces, besides those of C, O and Ti. Silicon peaks also appeared before incubation, which indicated that these peaks were caused by the mirror-like finishing. In the case of the Al plate, calcium peaks were weaker than that of Ti plate, and showed no P peaks. Figure 8 shows XPS narrow-scanning spectra of Ti, O, Ca, P, N and C. For Ti, the peaks are attributed

to TiO_2 and Ti_2O_3 , as can be seen from the observed binding energies. The peaks of Ca and P were close to the peak of hydroxyapatite ($\text{Ca}2p_{3/2}$: 348, $\text{Ca}2p_{1/2}$: 351, $\text{P}2p$: 133, $\text{O}1s$: 531 eV). This result is similar to others reported in the literature⁽⁶⁾⁻⁽¹¹⁾. The $\text{O}1s$ peak was found to be that of the Ti-O and Ti-OH bonds, and the $\text{C}1s$

peak to be minor peaks of the C-N and C-S bonds. **Figure 9** shows XPS narrow-scanning spectra of the oxide film formed on the acid-treated Al plate. The Al peak appeared at a binding energy higher than that of Al_2O_3 ($\text{Al}2p$ in sapphire: 74 eV)⁽¹²⁾. The Ca2p peak was from the Eagle's medium, and the binding energy profile of the $\text{O}1s$ orbital was found to be complicated. **Figure 10** compares the sputtering time to remove the oxide film using Ar^+ ions. No substantial change was observed during Ar^+ ion sputtering for Ti plates before and after cell

Fig. 7 XPS wide-scanning spectra for the surfaces of oxide film on pure Ti (a) and Al (b) plates after 0.6 Ms (7 d) of incubation with V79 cells.

Fig. 9 XPS narrow-scanning spectra for the surfaces of oxide film formed on acid-treated mirror-like finished Al plate after 0.6 Ms (7 d) of incubation with V79 cells.

Fig. 8 XPS narrow-scanning spectra for the surfaces of oxide film formed on mirror-like finished pure Ti plate after 0.6 Ms (7 d) of incubation with V79 cells.

Fig. 10 Comparison of Ar^+ ion sputtering time for the removal of oxide film from the mirror-like finished pure Ti and Al plates after 0.6 Ms (7 d) of incubation with V79 cells.

culture. Since the sputtering rate of TiO_2 under the conditions of this measurement was approximately 0.05 nm/s, the thickness of the oxide film was estimated to be 9 nm. This thickness is greater than the thickness of the TiO_2 film (2 to 5 nm) formed in air⁽¹³⁾. In the case of the mirror-like finished Al plate, the Ar^+ ion sputtering time was longer after 604.8 ks (7 d) of incubation than before cell culture. On the other hand, the Ar^+ ion sputtering time for the acid-treated Al plate after the cell culture tended to decrease compared with that of the acid-treated Al plate before the cell culture.

IV. Conclusions

The effects of Ti and Al oxide films and surface finishing on the relative growth ratio of L929 cells and the colony formation ratio of V79 cells were investigated. The following conclusions were derived.

(1) The relative growth ratio of L929 cells decreased gradually from 86.4 ks (1 d) and became close to zero at 345.6 ks (4 d) of incubation for the unoxidized Al particle extract. On the contrary, 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) for the oxidized Al particle extract and was 0.8 ± 0.07 after 345.6 ks (4 d) of incubation.

(2) While the colony formation ratio of the V79 cells on the mirror-like finished Ti plate was almost unity, no substantial colony formation was observed on the

mirror-like finished Al plate. However, V79 cell colonies proliferated on both the acid-treated and #240 roughened Al plates. The colony formation ratio of the V79 cells was found to depend on the surface roughness and strength of the Al oxide film.

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