Evaluation of the Biocompatibility of a Ti-Ta-Sn Alloy Using Cell Cultures

Masahito Miki^{1,*} and Masafumi Morita²

¹Technical Support Center, Research and Development Bureau, Saitama University, Saitama 338-8570, Japan

²Graduate School of Science and Engineering, Saitama University, Saitama 338-8570, Japan

The purpose of this study was to determine the suitability of a newly developed Ti-Ta-Sn alloy for use as a metallic biomaterial. The *in vitro* cell toxicity was determined by testing the corroding metal solutions using cell culture. Besides the cell adhesion rate, cell proliferation and colony formation were tested on the metal plates. Results of the cytotoxicity tests for higher concentrations of the corroding metal solutions (32 ppm and 64 ppm) revealed that the toxicity for U937 macrophages was lowest for the Ti-Ta-Sn alloy, followed by SUS316L and Co-Cr-Mo, with Ni-Ti being the most toxic. The Ti-Ta-Sn solution showed no cytotoxicity, even at a concentration of 64 ppm. The Co-Cr-Mo and Ni-Ti solutions showed high cytotoxicity for L929 cells. The cytotoxicity that was measured from the lactate dehydrogenase (LDH) release was highest for the Co-Cr-Mo alloy, followed by Ni-Ti, SUS316L, and Ti-Ta-Sn that showed the lowest value. Besides, the success rate of cell adhesion was highest for the Ti-Ta-Sn alloy after culturing for 6, 12, and 24 h. The cell proliferation tests showed that the cell proliferation speed and the relative cell proliferation rate after 3 days were both highest on Ti-Ta-Sn plates. Colony formation was highest on the Ti-Ta-Sn plates, and it was lowest on Co-Cr-Mo and SUS316L plates. These results demonstrated the suitability of the Ti-Ta-Sn alloy for use as a metallic biomaterial at the cellular level. [doi:10.2320/matertrans.M2015062]

(Received February 17, 2015; Accepted April 1, 2015; Published June 25, 2015)

Keywords: titanium alloy, cell adhesion rate, cell proliferation rate, cytotoxicity analysis, metallic biomaterial

1. Introduction

Ti and its alloys possess unique advantages, including high biocompatibility, lightweight, non-magnetic, corrosion resistance, and hypoallergenicity, along with excellent mechanical properties. They are often used at sites that involve high mechanical strength and ductility, or at sites that form an integral part in bone attachment for a long period. Thus far, Ti-6Al-4V alloy has been used as the primary metal alloy for metallic biomaterials. However, the alloy contains V, which is considered to exhibit *in vivo* toxicity. This has increased concerns on the safety of these alloys.¹⁾ Recently, Ni-Ti alloys have attracted considerable attention because of their superior mechanical properties, such as shape memory effect, superelasticity, and low Young's modulus,^{2,3)} which make them suitable for use as stents, guidewires, and orthodontic wires. However, this alloy has a high content of Ni that has been found to trigger allergic responses.^{4,5)} Given this fact, there has been considerable interest on the development and commercialization of Ni-free metallic biomaterials in Europe and the US.⁶⁾

The Ti-23Ta-3Sn (at%) alloy developed in this study (henceforth referred to as Ti-Ta-Sn) is a Ni-free and V-free β shape memory titanium alloy. The transformation temperature is as high as 423 K. The alloy does not show superelasticity at room temperature. However, optimizing heat treatment potentially leads to high strength, low Young's modulus, and high-elastic strain limit. Besides these, the alloy has high-corrosion resistance, and non-magnetism that can be clearly observed from the X-ray patterns.^{7,8)} The Ta used in this alloy was chemically stable and exhibited low-*in vivo* toxicity. Therefore, it has attracted considerable attention as an inert metallic biomaterial, both in pure form and as a constituent of alloys. Ta, in its pure form, has mostly been used in the field of orthopedics as a material for artificial joints or bone markers.⁹⁾

The aim of this study was to investigate the suitability of the newly developed Ti-Ta-Sn alloy as a potential metallic biomaterial using *in vitro* cell culture techniques.

2. Experimental Procedure

2.1 Metal ion elution in simulated body fluid (SBF)

The elution of metal ions in simulated body fluids was tested using the powdered constituent metals of the alloys, such as SUS316L, Co-Cr-Mo, and Ti alloys. The metal powders used in this study were Fe (53 μ m pass), Ni (63 μ m pass), Cr (63–90 μ m), Mo (63 μ m pass), Co (75 μ m pass), Ta (45 μ m pass), Sn (38 μ m pass), Ti (63–90 μ m), and Al (53–106 μ m) (Kojundo Chemical Laboratory Co., Ltd., Japan). In the typical process, a 50-mL polypropylene centrifuge tube contained 25 mL of RPMI-1640 (simulated body fluid). Subsequently, the metal powder sterilized under dry heat (180°C, 1 h) was added to the SBF at a concentration of 0.1 g/mL. The resulting mixture was shaken for 10 days at 37°C in a thermostatic shaker to dissolve the metal ions. Subsequently, 1 mL of the sample solution was collected from each centrifuge tube and subjected to inductively coupled plasma (ICP) analysis after adding 1 mL of concentrated nitric acid and heating at 160°C to denature all proteins.

2.2 Cytotoxicity analysis of the oxidized metal solutions

The cytotoxicity was analyzed using L929 cell culture (Riken Brc, Japan) that is used to evaluate orthopedic and dental implant materials, and U-937 (Riken Brc, Japan) immune cells that ingest foreign bodies by phagocytosis and cause inflammation. The metal solutions were obtained from the metals corroded in simulated body fluid, such as SUS316L (The Nilaco Corporation, Japan), Co-Cr-Mo alloy (provided by NEC Tokin, Japan), Ni-49Ti alloy (provided by NEC Tokin, Japan), and Ti-Ta-Sn alloy samples, using a potentiostat. The alloy except Ti-Ta-Sn has been already used as metallic biomaterials. A synthetic cell culture medium was

*Corresponding author, E-mail: miki@mech.saitama-u.ac.jp

used as the simulated body fluid. RPMI-1640 and Eagle MEM media were used for U-937 cells and L929 cells, respectively. In the typical process, 400 mL of the SBF was added to an electrochemical analysis cell. Subsequently, solutions from the corroded metals were prepared by applying electric potential through each sample metal as a working electrode. A potentiostat was used to apply the voltages SUS316L, Co-Cr-Mo, and Ni-Ti +5 V (vs. SCE) and Ti-Ta-Sn +10 V (vs. SCE). Following that, 50 mL of the corroded metal solution was taken from each sample and diluted to 64, 32, 16, 8, 4, 2, 1, and 0 ppm in the synthetic cell culture medium. This solution was performed sterilization (121°C, 20 minutes) of in an autoclave. FBS and L-glutamine, sodium hydrogen carbonate were added in this solution afterwards.

The cytotoxicity of the metal solution was analyzed using the following procedure: In the typical process, U-937 human thymus-derived macrophage progenitor cells were adjusted to a concentration of 1×10^5 cells/mL and seeded into 96-well plates. Phorbol 12-myristate 13-acetate (PMA) was subsequently added, and the mixture was cultured at 37°C under 5% CO₂ for 24 h in an incubator to allow the cells to differentiate into macrophages. Similarly, the L929 cells suspended at a cell concentration of 1×10^5 cells/mL were seeded in 96-well plates and cultured at 37°C under 5% CO₂ for 24 h in an incubator. Following this, the medium was replaced with the corroded metal solutions (64 ppm~1 ppm) that were prepared as described earlier, and the culturing was continued for another 72 h. Subsequently, a new set of 96-well plates was prepared with the sample of each test solution. The lactate dehydrogenase concentration released in each well was measured with an absorption photometer using the LDH cytotoxicity detection kit (Takara Bio Inc., Japan). Triton X-100 was used for the total cell destruction.

The LDH cytotoxicity detection kit is highly sensitive to LDH released by the cells that is an indicator of cytotoxicity. In principle, LDH is a cytoplasmic enzyme that normally does not cross the cell membrane. However, if the membrane is damaged, it is released into the extracellular medium. The kit used in this study creates a red formazan dye that absorbs light at 490 nm.

Cytotoxicity calculation

$$\text{Cytotoxicity (\%)} = \frac{[(\text{experimental value} - \text{low control}) / (\text{high control} - \text{low control})] \times 100}{1} \quad (1)$$

Low control: Natural release of LDH from the cells.

High control: Maximum release of LDH from cells to which Triton X-100 has been added.

2.3 Determination of cell adhesion and proliferation

The cell adhesion and proliferation tests were conducted in accordance with the JIS T0301 using alloys of Ti-Ta-Sn, Ti-6Al-4V, Co-Cr-Mo (donated by NEC TOKIN), and SUS316L. All the alloys analyzed in this study had ϕ 12.8 mm and a thickness of 1 mm. Each metal plate was polished with waterproof sandpaper under running water, starting at #120 and gradually increasing the number to final polish at #2000. After polishing, the plates were washed ultrasonically in ethanol and ultra-pure water. Subsequently, the samples were autoclaved at 121°C for 20 min. Three

samples of each were placed in 24-well plates. Following this, 1 mL of L929 cell suspension adjusted to 1×10^5 cells/mL was added to each well. For the control, cells were added without the metal plate. Subsequently, the samples were cultured in an incubator at 37°C under 5% CO₂. For the cell adhesion test, the cells were cultured for periods of 6, 12, and 24 h. On the other hand, the cells were cultured for 1, 2, and 3 d for the proliferation tests. The cells were washed with PBS(–) and detached with 0.5 mL Trypsin-EDTA solution. These were then suspended by adding 0.5 mL of the medium. Subsequently, the cells in the suspension were counted using a hemocytometer. Cells were counted 3 times for each sample, and the average was taken as the final value. The tests were repeated 5 times in the same manner. The cell adhesion and relative proliferation rates were calculated according to the JIS T0301 equation given as follows. The cell proliferation speed was calculated according to the eq. (4).

$$\text{Cell adhesion rate} = (N_n/N_0)/(N_n^{\text{control}}/N_0) \quad (2)$$

$$\text{Relative cell proliferation rate} = N_n/N_n^{\text{control}} \quad (3)$$

$$\text{Cell proliferation speed} = N_n/N_1 \quad (4)$$

N_n : Number of cells in the sample after culturing for n hours
 N_n^{control} : Average number of cells in the control after culturing for n hours

N_0 : Number of cells seeded initially

N_1 : Number of cells in each sample after culturing for 1 day

2.4 Colony formation test (direct contact method)

The colony formation test was performed using V79 cells (Riken Brc, Japan) that tend to proliferate rapidly and exhibit high colony formation rate. In the typical process, metal plates were placed in 24 well-plates, as described in section 2.3. Subsequently, 1 mL suspension of V79 cells adjusted to 50 cells/mL was added to each well. For the control, a cell suspension without the metal plate was used. The samples were cultured in an incubator at 37°C under 5% CO₂ for 7 d. Following this, the medium was removed from the wells, washed with PBS(–), fixed in 10% formalin, stained with Giemsa stain, and the number of colonies was counted. The colony formation test was repeated 5 times. The colony formation rate was calculated according to the JIS T0301 equation given below.

$$\text{Colony formation rate} = N/N^{\text{control}} \quad (5)$$

N : Colony count in the sample after culturing for 7 days

N^{control} : Average colony count in the control after culturing for 7 days

3. Results and Discussions

3.1 Release of metal ions in the simulated body fluid

The release of ions from the pure constituents of the alloys is shown in Fig. 1. High levels of ions were released from Ni, Mo, and Co that are constituents of the SIS316L, Co-Cr-Mo, and Ni-Ti alloys. In contrast, the level of metal ion release from Ti, Ta, and Sn that are constituents of the Ti-Ta-Sn alloy was too low to be detected. According to the standard electrode potential of these metals, dissolution and oxidation are expected to occur more readily in the order of Al, Ti, Cr,

Fig. 1 Metal ions eluted from the metal particles.

Ta, Fe, Co, Ni, Mo, and Sn. Metals, such as Ti, Cr, and Ta, which are important constituent metals of metallic biomaterials, are extremely prone to oxidation. Thus, a thin film of oxidized material covers the surface, thereby inhibiting oxidation. Therefore, these materials often exhibit noble metal-like properties. Hence, for practical consideration of oxidation, it is more appropriate to use the corrosion potential of the metal instead of the standard electrode potential. Accordingly, the order from most stable to least stable becomes Ta, Ti, Sn, Al, Cr, Fe, Ni, Co, and Mo.¹⁰⁾ This corresponds to the release of ions by these metals in the RPMI-1640 simulated body fluids observed in this study.

3.2 Cytotoxicity tests on the oxidized metal solutions

According to the anodic polarization test, the initial potential for transpassive dissolution implies that the Ti-Ta-Sn alloy is more stable than the Co-Cr-Mo or Ni-Ti alloys. The alloy is immediately re-passivated upon the disruption of the passivation film, thereby providing extremely high-corrosion resistance.⁷⁾ For this reason, the release of ions in the body is thought to be low. In this experiment, the alloy was forcibly corroded using a potentiostat, and the corroded metal concentration was the same for each metal.

Figure 2 shows the LDH activity of the U937 human thymus-derived macrophage cells when the corrosion solutions from each alloy was administered to them. Macrophages are phagocytes of the innate immune system. Their function is to ingest any foreign matter entering the body via phagocytosis, degrade it, absorb it, and subsequently expel it from the body. If a metal is absorbed during this break down and absorption process, it may lead to cell damage; this depends on the type and the amount of metal absorbed. High toxicity will lead to the death of macrophages, and LDH will pass through the membrane and diffuse. The cytotoxicity can be estimated by measuring the concentration of LDH released.

At low concentrations of 1 ppm and 2 ppm, there was no major difference among the various alloys tested in this study. However, at concentration of approximately 4 ppm, the cytotoxicity of Co-Cr-Mo alloy increased. With further increase in concentration to 16 ppm, the cytotoxicity of Ni-Ti also increased. At high concentrations of 32 and 64 ppm, the Ni-Ti alloy was the most toxic, followed by Co-Cr-Mo, SUS316L, and Ti-Ta-Sn, in this order. The Ti-Ta-Sn alloy did not show any toxicity even at 64 ppm.

Fig. 2 Cytotoxicity of eluted metal ions administered to U937 macrophage cells.

Fig. 3 Cytotoxicity of eluted metal ions administered to L929 cells.

Figure 3 shows the LDH activity when the eluted ions from each alloy were administered to the L929 cells. The L929 cells are basic fibroblasts that make up the biological tissue. Unlike macrophages, they have no phagocytosis function. However, when they are surrounded by toxic material, the membranes may get damaged and LDH is released, thereby acting as an indicator for cytotoxicity.

At low concentrations of 1 and 2 ppm, there was no significant difference among the various alloys considered in this study. For concentration of 8 ppm, the cytotoxicity of the Co-Cr-Mo alloy increased. With further increase in concentration to 32 and 64 ppm, the alloys Co-Cr-Mo and Ni-Ti exhibited high toxicity. The order of LDH value (highest to lowest) was Co-Cr-Mo, Ni-Ti, SUS316L, and Ti-Ta-Sn.

3.3 Cell adhesion and cell proliferation tests

3.3.1 Cell adhesion rate

Cell adhesion is the first step for cells to proliferate on the surface of metals. Figure 4 shows the cell adhesion to the Ti-Ta-Sn, Ti-6Al-4V, Co-Cr-Mo alloys, and SUS316L. After 6 h, the cell adhesion rate was high for the Ti-Ta-Sn and Ti-6Al-4V alloys. For all the times tested, the cell adhesion rate for the Ti-Ta-Sn alloy was the highest. However, after 24 h, the difference was not as large as that seen after 6 h.

3.3.2 Cell proliferation test

The cell proliferation speed is an important parameter in biocompatibility evaluation that indicates the mechanism of

Fig. 4 Cell adhesion rate on each metal plate.

Fig. 5 Changes in cell proliferation on metal plates during 3-day culturing.

cell proliferation after adhesion to a metal surface. Figure 5 shows changes in the cell proliferation speed during 3-day culturing. This figure shows the comparative ratio of the cell number increased every 24 h in reference to the number of cells after 1 day. After 1–2 days, the cell proliferation speed was approximately equal to that of the control Ti-Ta-Sn and Ti-6Al-4V alloys, while that of SUS316L was lower. On the 2nd day and thereafter, the cell proliferation speed was higher on the surface of the control and SUS316L. After 3 days, the highest value among the alloys was seen on the surface of Ti-Ta-Sn, while the lowest value was on the surface of Co-Cr-Mo alloy. The Ti-Ta-Sn alloy consistently showed high values of the relative cell proliferation rate. While the lowest value of proliferation was observed for SUS316L on the 2nd day, that observed for Co-Cr-Mo dropped sharply on the 3rd day (Fig. 6).

3.4 Colony formation test

Cells attached to the bottom of the well or to the metal surface proliferate and form colonies. The colony formation is considered to be indicative of the manner in which they grow on the surface of implants. Therefore, in the present study, we investigated the rate of colony formation on the surface of different alloys. As shown in Fig. 7, the colony formation was different for each type of metal plate. In particular, the colony formation rate was the highest for the Ti-Ta-Sn alloy. There was no statistically significant difference between the Ti-Ta-Sn and the control or between the Ti-Ta-Sn and Ti-6Al-4V alloys. However, there was a significant difference ($p < 0.01$) between the Ti-Ta-Sn and Co-Cr-Mo

Fig. 6 Relative cell proliferation rates on metal plates during 3-day culturing.

Fig. 7 Colony formation rate of V79 cells on each metal plate.

alloys and between Ti-Ta-Sn and SUS316L alloys. This confirmed the high colony formation rate on the Ti alloys, Ti-Ta-Sn, and Ti-6Al-4V.

4. Conclusion

The following points summarize the key findings of this study:

With regards to corrosion, high concentrations of Mo, Ni, and Co ions were eluted in the RPMI-1640 simulated body fluid, whereas the elution of Ti, Ta, and Sn ions as a constituent of the Ti-Ta-Sn alloy was low.

Results of cytotoxicity tests from the corroded metal solutions at concentrations of 32 and 64 ppm indicated cytotoxicity (highest to lowest) in the order of Ni-Ti, Co-Cr-Mo, SUS316L, and Ti-Ta-Sn in U-937 macrophages. No toxicity was detected from the Ti-Ta-Sn solution, even at a high concentration of 64 ppm. At concentrations of 32 and 64 ppm, the cytotoxicity of Co-Cr-Mo and Ni-Ti alloys with regard to the L929 cells was high, with the order of LDH release values (highest to lowest) being Co-Cr-Mo, Ni-Ti, SUS316L, and Ti-Ta-Sn.

The cell adhesion rate was highest for the Ti-Ta-Sn alloy for all the culture times tested (6, 12, and 24 h) in this study.

Besides, cell proliferation tests indicated that both the speed of proliferation and the relative proliferation rates after 3 days were highest in the case of the Ti-Ta-Sn alloy.

Colony formation was high in the Ti-Ta-Sn alloy, and it was low in the Co-Cr-Mo and SUS316L alloys.

These results suggest that the Ti-Ta-Sn alloy is highly biocompatible at the cellular level.

Acknowledgments

Part of this study was conducted under the auspices of the Japan Science and Technology Agency Revitalization Promotion Program (A-STEP), seeds validation type (241FS0064).

We sincerely thank Messrs. Shunsuke Takeguchi, Yoshiki Ishikawa, Takasumi Kubo, and Taiki Takahashi, Nippon Piston Ring Co., Ltd. for their generosity in providing the Ti-Ta-Sn alloy.

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