Proliferation and Differentiation of Normal Human Osteoblasts on Dental Au–Ag–Pd Casting Alloy: Comparison with Cytotoxicity to Fibroblast L929 and V79 Cells

Kazuo Isama, Atsuko Matsuoka, Yuji Haishima and Toshie Tsuchiya

Division of Medical Devices, National Institute of Health Sciences, Tokyo 158-8501, Japan

Normal human osteoblast NHOst cells were cultured on the dental Au–Ag–Pd casting alloy using the micromass culture, and the proliferation and differentiation of NHOst cells were determined. 13Au–58Ag–21Pd which met JIS T 6106 and 10Au–62Ag–13Pd which did not meet JIS T 6106 were used in this experiment. 10Au–62Ag–13Pd contained Cu more than 13Au–58Ag–21Pd. The proliferation and differentiation of NHOst cells cultured on 10Au–62Ag–13Pd were significantly decreased more than those on 13Au–58Ag–21Pd, respectively. It was suggested that the content of Cu caused the difference in the proliferation and differentiation between NHOst cells cultured on 13Au–58Ag–21Pd and that on 10Au–62Ag–13Pd. Moreover, the cytotoxicity of the Au–Ag–Pd alloy was evaluated by the MEM elution assay and the colony assay using fibroblast L929 and V79 cells, in order to compare with the cytological effects of the alloy on NHOst cells. 13Au–58Ag–21Pd showed no cytotoxicity to L929 and V79 cells in the MEM elution assay and the colony assay. However, 10Au–62Ag–13Pd showed extremely weak cytotoxicity to L929 cells and weak cytotoxicity to V79 cells in the colony assay, though 10Au–62Ag–13Pd showed no cytotoxicity to L929 cells in the MEM elution assay. NHOst cells expressed the toxicity to the proliferation and differentiation on 10Au–62Ag–13Pd clearly. The in vitro toxicity test based on the proliferation and differentiation of NHOst cells was useful to evaluate the toxicity of medical materials.

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1. Introduction

The Au–Ag–Pd casting alloy is most generally used as a dental prosthesis. The Japanese Industrial Standard (JIS) T 6106: “Dental Casting Au–Ag–Pd Alloy” specifies the chemical composition and mechanical properties of the dental Au–Ag–Pd casting alloy, with the inclusion of test methods. And the approval of the Minister for Health, Labour and Welfare (MHLW) based on Article 14 of the Pharmacists Law is unnecessary for the alloy which meets JIS T 6106 in Japan. Recently, the price of the alloy jumped, and the dental Au–Ag–Pd casting alloy which did not meet JIS T 6106 distributed under the guise of the standardized alloy.

On the other hand, the evaluation of physical, chemical and biological properties of individual dental material is required in “Guidelines for Physical, Chemical and Biological Tests of Dental Materials” notified from MHLW. The cytotoxicity test is a convenient and useful method for evaluating biological safety and required most dental material in the guidelines. The cytotoxicity test methods of medical material are shown in detail in “Guidelines for Basic Biological Tests of Medical Materials and Devices-Part III: Cytotoxicity Test” and USP XXII (87): “Biological Reactivity Tests, In-Vitro.” According to these guidelines, the cytotoxicity test is carried out using fibroblast L929 or V79 cells even in the dental material. However, the cytological effects on the peculiar cells in the dentistry, such as osteoblasts, are unclear only in the cytotoxicity test using fibroblasts.

In this study, the proliferation and differentiation of normal human osteoblast NHOst cells cultured on the dental Au–Ag–Pd casting alloy were examined, and it was compared with the cytotoxicity of the alloy to fibroblast L929 and V79 cells. It was verified that the in vitro toxicity test using NHOst cells was useful to evaluate the toxicity of medical materials.

2. Experimental Methods

2.1 Alloy specimens

Two lots of dental Au–Ag–Pd casting alloy (size: 12.0 mm × 8.0 mm × 1.0 mm) of which the chemical composition differed were commercial products. The chemical composition of the standardized alloy is specified with more than 12 mass% of Au, more than 40 mass% of Ag and more than 20 mass% of Pd in JIS T 6106: “Dental Casting Au–Ag–Pd Alloy.” One lot of the alloy contains 13.0 mass% of Au, 57.9 mass% of Ag and 20.6 mass% of Pd, and meets JIS T 6106 (13Au–58Ag–21Pd). However, another lot of the alloy contains 9.7 mass% of Au, 61.8 mass% of Ag and 12.8 mass% of Pd, and does not meet JIS T 6106 (10Au–62Ag–13Pd).

The surfaces of these alloys were polished with waterproof abrasive paper up to grit #800 under running deionized water, and then ultrasonically cleaned in ethanol and deionized water each 3 times. The alloy specimens were dry heat sterilized at 180°C for 2 h.

2.2 Micromass culture of NHOst cells

Normal human osteoblast NHOst cells (clone: 5488-6) were purchased from BioWhittaker, Inc. (Walkersville, Maryland, USA). NHOst cells were grown in alpha minimum essential medium (α-MEM) (Gibco Laboratories, Grand Island, New York, USA) supplemented with 10% fetal calf serum (FCS, Intergen, Purchase, New York, USA) in a 37°C humidified atmosphere of 5% CO₂ and 95% air.

The sterilized alloy specimens were placed in the well of type I collagen coated 24-well plate (Asahi Techno Glass Co., Tokyo, Japan). Cell suspensions were prepared in the
culture medium and adjusted to give $2 \times 10^6$ cells/mL. A $20 \mu$L aliquot of the cell suspensions was delivered on the al-
loy specimen or the well of the 24-well plate (for control),
and incubated at 37°C in the humidified atmosphere. Af-
ter the cells were attached on the alloy specimen, 1.0 mL
of the complete medium that contained 5 mmol/L disodium
$\beta$-glycerophosphate ($\beta$-GP) (Sigma Chemical Co., St. Louis,
Missouri, USA) in the culture medium was added. The com-
plete medium was changed three times a week, and the cells
were cultured for 2 weeks.

2.3 Proliferation of NHOst cells
The proliferation of NHOst cells was determined by
using the cell proliferation assay reagent, TetraColor
ONE (Seikagaku Co., Tokyo, Japan). The cell cul-
tures were exchanged with the culture medium containing
0.1 mmol/L 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-
5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt,
$4 \mu$L 1-methoxy-5-methylphenazinium methasulfate and
3 mmol/L NaCl, and were incubated for 2 h. The absorbance
of the medium was read at 450 nm (reference at 600 nm) with
a plate reader. It has been proven that the absorbance and cell
population show a linear relationship.

2.4 Differentiation of NHOst cells
The alkaline phosphatase (ALP) activity and calcium
amount of NHOst cells were determined by the following
methods as an index of the differentiation of NHOst cells.
After the proliferation was determined, the cell cultures
were washed three times with Dulbecco’s phosphate-buffered
saline without calcium and magnesium salts (PBS(-)). One
milliliter of 0.1 mol/L glycine buffer (pH 10.5) contained
$4 \mu$mol/L p-nitrophenylphosphate, 10 mmol/L MgCl2 and
0.1 mmol/L ZnCl2 was added, and the cell cultures were in-
cubated at 37°C for 15 min. The absorbance of p-nitrophenol
liberated was read at 405 nm (reference at 600 nm) with a
plate reader.

The calcium amount of NHOst cells was determined by
using the diagnostic kit, Calcium C (Wako Pure Chemical
Industries, Ltd.). After the ALP activity was determined,
the cell cultures were washed three times with PBS(-) and
fixed with 10% formalin solution. The fixed cell cultures
were washed five times with deionized water and decalci-
fied with 500 µL of 0.1 mol/L HCl for 15 h at room tempera-
ture. Ten microliter of the decalcifying solution and 1.0 mL of
0.88 mol/L monoethanolamine buffer (pH 11.0) were mixed,
and 100 µL of 0.63 mmol/L o-cresolphthalein complexon and
69 mmol/L 8-hydroxyquinoline was added. After 15 min at
room temperature, the absorbance of the reaction solution was
read at 570 nm with a plate reader.

2.5 MEM elution assay
The MEM elution assay using L929 cells nearly followed
USP XXII (87): “Biological Reactivity Tests, In-Vitro.”
Murine fibroblast L929 cells were obtained from Japanese
Cancer Research Resources Bank (Tokyo, Japan). L929 cells
were grown in Eagle’s minimum essential medium (MEM,
Gibco Laboratories) supplemented with 10% horse serum and
nonessential amino acids in a 37°C humidified atmosphere of
5% CO2 and 95% air.

Several pieces of the sterilized alloy specimens were put
in the well of the multi-well plate, and Eagle’s MEM supple-
mented with 5% FCS (5% FCS-MEM) was added so that the
sample/medium ratio might be 6 cm²/mL. After incubation at
37°C in the humidified atmosphere for 24 or 48 h, the extract
designated to be 100% extract was separated by decantation.
The 100% extract was serially diluted with 5% FCS-MEM to
give 50% and 25% extracts.

L929 cells of 6 or $8 \times 10^4$ cells in 0.5 mL 5% FCS-MEM
were seeded in each well of the 24-well plate, and incubated
at 37°C in the humidified atmosphere. After 24 h incubation,
the medium was exchanged with 0.5 mL of the serially di-
luted medium extract or the medium without the extract (for
control). The cell cultures were observed at 24 or 48 h expo-
sure, microscopically. After 48 h incubation, the medium was
exchanged with 0.5 mL of medium containing 50 µg/mL of
neutral red (NR). The cell cultures were incubated for another
3 h, and fixed with formalin solution. Then 0.5 mL of 50% ethanol
and 1% acetic acid solution was added, and NR was extracted
from the cell cultures for 2 h. The absorbance of the extract
solution was read at 540 nm with a plate reader. The cell vi-
ability of L929 cells was calculated as a ratio of the absorbance
in the sample to that in the control.

2.6 Colony assay
The colony assays using L929 and V79 cells followed
“Guidelines for Basic Biological Tests of Medical Materials
and Devices-Part III: Cytotoxicity test.”
Chinese hamster fibroblast V79 cells were obtained from
Japanese Cancer Research Resources Bank (Tokyo, Japan).
V79 cells were grown in Eagle’s MEM supplemented with
10% FCS in a 37°C humidified atmosphere of 5% CO2 and
95% air.

Several pieces of the sterilized alloy specimens were put
in the well of the multi-well plate, and 10% FCS-MEM for L929
cells or Eagle’s MEM supplemented with 5% FCS, nonessen-
tial amino acids and 1 mmol/L sodium pyruvate (5% FCS-
GMNP) for V79 cells was added so that the sample/medium
ratio might be 3 or 6 cm²/mL. After incubation at 37°C in
the humidified atmosphere for 24 or 72 h, the extract desig-
nated to be 100% extract was separated by decantation. The
100% extract was serially diluted with 10% FCS-MEM (L929
cells) or 5% FCS-GMNP (V79 cells) to give 50% and 25%
extracts.

The cell suspension containing 100 cells/mL of 0.5 mL was
placed in each well of the 24-well plate, and incubated at
37°C in the humidified atmosphere. After 24 h incubation,
the medium was exchanged with 0.5 mL of the serially di-
luted medium extract or the medium without the extract (for
control), and the cells were cultured for 7 days (L929 cells) or
6 days (V79 cells). The cell cultures were fixed with 10% for-
malin solution, and the colonies formed were stained with 5%
Giemsa staining solution. The number of colonies on each
well was counted, and relative plating efficiency was calcu-
lated as a ratio of the number of colonies in the sample to
that in the control. The cytotoxic potential of the medium
extract was expressed as a concentration of the extract which
inhibited plating efficiency to 50% (IC50). The IC50 value was
calculated by the probit method.
Table 1  The proliferation and differentiation of NHOst cells cultured on the dental Au–Ag–Pd casting alloy.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Proliferation (%)</th>
<th>ALP activity (%)</th>
<th>Calcium amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 3.29</td>
<td>100.0 ± 4.52</td>
<td>100.0 ± 1.90</td>
</tr>
<tr>
<td>13Au–58Ag–21Pd</td>
<td>36.8 ± 2.99**</td>
<td>28.6 ± 4.90**</td>
<td>27.6 ± 2.45**</td>
</tr>
<tr>
<td>10Au–62Ag–13Pd</td>
<td>22.4 ± 6.32****</td>
<td>13.1 ± 4.83****</td>
<td>22.1 ± 2.12****</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD for 4 dishes. Significant difference compared with control at **P < 0.01 and compared with 13Au–58Ag–21Pd at #P < 0.05 and ##P < 0.01 by Tukey-Kramer test in one-way ANOVA.

Fig. 1 The cell viabilities of L929 cells cultured with the medium extract of dental Au–Ag–Pd casting alloy. Medium extraction of 13Au–58Ag–21Pd (circles) and 10Au–62Ag–13Pd (triangles) with 5% FCS-MEM at 6 cm²/mL (sample/medium ratio) for 24 h (a) and 48 h (b). Values are expressed as means ± SD for 3 dishes.

Fig. 2 The plating efficiencies of L929 cells cultured with the medium extract of dental Au–Ag–Pd casting alloy. Medium extraction of 13Au–58Ag–21Pd (circles) and 10Au–62Ag–13Pd (triangles) with 10% FCS-MEM at 3 cm²/mL ((a), (b)) and 6 cm²/mL ((c), (d)) for 24 h ((a), (c)) and 72 h ((b), (d)). Values are expressed as means ± SD for 3 dishes. Significant difference compared with control at **P < 0.01 and compared with 13Au–58Ag–21Pd at ##P < 0.01 by Tukey-Kramer test in two-way ANOVA.

2.7 Statistical analysis
All measured values were collected in 3 or 4 sets and expressed as means ± standard deviation (SD). Differences among the groups were evaluated with analysis of variance (ANOVA). When significant differences among the groups were found, Tukey-Kramer test was applied for multiple comparisons. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1 Proliferation and differentiation of NHOst cells
The proliferation, ALP activity and calcium amount of normal human osteoblast NHOst cells cultured on the dental Au–Ag–Pd casting alloy for 2 weeks were determined using the micromass culture. The proliferation of NHOst cells cultured on 10Au–62Ag–13Pd was significantly decreased more than that on 13Au–58Ag–21Pd (Table 1). The ALP is the representative enzyme of osteoblastic differentiation, and the calcification is the last phenotype of osteoblasts. The ALP activity and calcium amount of NHOst cells cultured on 10Au–62Ag–13Pd were also significantly decreased more than those on 13Au–58Ag–21Pd, respectively (Table 1).

3.2 MEM elution assay using L929 cells
In the MEM elution assay, there was no morphologic difference between fibroblast L929 cells cultured with the medium extract of 13Au–58Ag–21Pd and that of 10Au–62Ag–13Pd on the observation at 24 and 48 h exposure, including the controlled L929 cells.

The cell viability of L929 cells was determined after 48 h culture with the medium extract of the Au–Ag–Pd alloy (Fig. 1). There was no significant difference between the cell viability of L929 cells cultured with the medium extract of 13Au–58Ag–21Pd and that of 10Au–62Ag–13Pd.

3.3 Colony assay
3.3.1 L929 cells
The relative plating efficiency of L929 cells cultured with the medium extract of the Au–Ag–Pd alloy was determined in the colony assay. There was no significant effect of the medium extract of 13Au–58Ag–21Pd on the plating efficiency of L929 cells in any extraction conditions: the sample/medium ratio of 3 cm²/mL for 24 and 72 h, and that of 6 cm²/mL for 24 and 72 h (Fig. 2). However, the plating efficiency of L929 cells cultured with the medium extract of 10Au–62Ag–13Pd was significantly decreased in the most severe extraction condition: the sample/medium ratio of 6 cm²/mL for 72 h (Fig. 2(d)).

The IC₅₀ values of the medium extract of the Au–Ag–Pd alloy to L929 cells are shown in Table 2. The IC₅₀ value of the...
medium extract of 10Au–62Ag–13Pd with 10% FCS-MEM at 6 cm²/mL (sample/medium ratio) for 72 h was 77.8%.

### 3.3.2 V79 cells

The relative plating efficiency of fibroblast V79 cells cultured with the medium extract of the Au–Ag–Pd alloy was determined in the colony assay. There was no significant effect of the medium extract of 13Au–58Ag–21Pd on the plating efficiency of V79 cells as well as L929 cells in any extraction conditions (Fig. 3). However, the plating efficiency of V79 cells cultured with the medium extract of 10Au–62Ag–13Pd was significantly decreased with concentration dependence in the following extraction conditions: the sample/medium ratio of 3 cm²/mL for 72 h (Fig. 3(b)) and that of 6 cm²/mL for 24 h (Fig. 3(c)) and 72 h (Fig. 3(d)).

The IC₅₀ values of the medium extract of the Au–Ag–Pd alloy to V79 cells are shown in Table 2. The IC₅₀ value of the medium extract of 10Au–62Ag–13Pd with 5% FCS-GMNP at 3 cm²/mL (sample/medium ratio) for 72 h was 90.7%, and those at 6 cm²/mL (sample/medium ratio) for 24 and 72 h were respectively 67.5% and 65.7%.

### 4. Discussion

In the present study, normal human osteoblast NHObst cells were cultured on the dental Au–Ag–Pd casting alloy using the micromass culture, and the proliferation and differentiation of NHObst cells were determined.

Quarles et al. reported that before attaining confluence mouse osteoblastic MC3T3-E1 cells actively proliferated, but failed to express an ALP activity and did not accumulate a mineralized extracellular collagenous matrix at this stage. After the cultures underwent growth arrest with the attainment of confluence, the ALP activity and mineralized extracellular collagenous matrix were expressed. The cell density of the micromass culture is high, and the situation of the micromass culture is similar to state of confluence at initial stage of culture and formed 3-dimensional spheroids similar to the in vivo tissues. Therefore, the micromass culture was carried out for the present experiment, and the differentiation of NHObst cells cultured on the alloy succeeded in the short period of 2 weeks.

Thompson and Puleo reported that the ALP activity, osteocalcin content and calcium amount of bone marrow stromal cells greatly rose in the later stage of culture. They indicated that the osteoprogenitor cells first differentiated into immature osteoblasts characterized by the expression of ALP and then into mature osteoblasts characterized by the expression of osteocalcin and calcification. Schwartz et al. also observed that the ALP activity which was low at initial stage of culture rose at middle stage of culture, and then lowered again at later stage of culture of NHObst cells. Thus, the ALP activity and calcium amount were both determined as the characteristics of the differentiation of NHObst cells.

The proliferation, ALP activity and calcium amount of NHObst cells cultured on the Au–Ag–Pd alloys were significantly decreased in comparison with those of control, respectively (Table 1), because the culture area of the alloy was narrower than that of the control dish. On the other hand, the proliferation of NHObst cells cultured on 10Au–62Ag–13Pd was decreased more than that on 13Au–58Ag–21Pd. And the ALP activity and calcium amount of NHObst cells cultured on 10Au–62Ag–13Pd were also decreased more than those on 13Au–58Ag–21Pd, respectively. Table 1 demonstrated the proliferation and differentiation of NHObst cells were more strongly inhibited by 10Au–62Ag–13Pd than 13Au–58Ag–21Pd.

By X-ray fluorescence spectroscopy, 13Au–58Ag–21Pd and 10Au–62Ag–13Pd contained respectively 6.7 and 13.2 mass% of Cu. 10Au–62Ag–13Pd contained Cu more
than 13Au–58Ag–21Pd. In our recent study, Cu$^{2+}$ had high toxicity in which IC$_{50}$ in the proliferation and differentiation of NHOst cells were respectively smaller than $10^{-5}$ mol/L.\textsuperscript{7)} And Miura and Takeda detected 22.9 ppm of Cu in the filtrate of the Eagle’s MEM after the Au–Ag–Pd alloy was dynamic extracted, though the amounts of other metals were less than 1 ppm. They described that Cu was selectively dissolved in the Eagle’s MEM from the Au–Ag–Pd alloy by the dynamic extraction.\textsuperscript{8)} Akagi et al. also reported that Cu was most abundantly found in the extracts of Au–Ag–Pd alloy by the dynamic extraction. Then the only element found in the filtrates in the same amount as in the extracts.\textsuperscript{9)} The content of Cu should cause the difference in the proliferation and differentiation between NHOst cells cultured on 13Au–58Ag–21Pd and that on 10Au–62Ag–13Pd.

The cytotoxicities of the Au–Ag–Pd alloy to fibroblast L929 and V79 cells were evaluated using the MEM elution assay and the colony assay, in order to compare with the cytological effects of the alloy on NHOst cells. Both 13Au–58Ag–21Pd and 10Au–62Ag–13Pd showed no cytotoxicity in L929 cells in the MEM elution assay (Fig. 1). However, though 13Au–58Ag–21Pd showed no cytotoxicity, 10Au–62Ag–13Pd showed extremely weak cytotoxicity to L929 cells in the colony assay (Fig. 2). Because the sensitivity of the MEM elution assay was lower than that of the colony assay and/or the extraction period of the MEM elution assay was short, 10Au–62Ag–13Pd should show no cytotoxicity to L929 cells in the MEM elution assay.

In the colony assay using V79 cells, 13Au–58Ag–21Pd showed no cytotoxicity, but 10Au–62Ag–13Pd showed weak cytotoxicity (Fig. 3). The IC$_{50}$ value of the medium extract of 10Au–62Ag–13Pd was always lower to V79 cells than to L929 cells, when it was compared on the same extraction conditions (Table 2). In the comparison of the IC$_{50}$ value of CuCl$_2$ in the colony assay, the cytotoxicity of Cu was two times stronger to V79 cells than to L929 cells (unpublished data). Also the content of Cu should cause the difference in the cytotoxicity of 10Au–62Ag–13Pd between L929 cells and V79 cells. Anyhow these results indicate that cytotoxicity potentials differ among cell lines. Yamamoto et al. reported that the cytotoxicity potentials of 12 metal salts to 6 cell lines. There was a difference from 1 to 3 orders of magnitude in the IC$_{50}$ value among the cell lines.\textsuperscript{10,11)} The selection of a cell line would be important in the cytotoxicity test of medical materials.

Moreover, in the colony assay using V79 cells, the IC$_{50}$ value of the medium extract of 10Au–62Ag–13Pd changed to 65.7% from > 100% depending on the extraction conditions. This result indicates that the different cytotoxicity potentials are obtained under the different extraction conditions. The extraction condition would be also an important factor in the cytotoxicity test of medical materials. Several extraction conditions are recommended even in ISO 10993-5: “Biological Evaluation of Medical Devices-Part 5: Tests for Cytotoxicity: In Vitro Methods.” Recently, the dynamic extraction has been proposed to the cytotoxicity test especially of dental prosthetic materials.\textsuperscript{5,9,12,13)} As indicated above, NHOst cells expressed the toxicity to the proliferation and differentiation on 10Au–62Ag–13Pd, and the cytotoxicity of this alloy was detected in the colony assay but not detected in the MEM elution assay. The proliferation and differentiation of NHOst cells were also used to examine toxicity of metal salts\textsuperscript{7)} and effectiveness of the gamma-ray irradiation for poly(l-lactide).\textsuperscript{14)} The \textit{in vitro} toxicity test based on the proliferation and differentiation of NHOst cells would be useful to evaluate the toxicity of medical materials, especially metal implants such as artificial bones.

5. Conclusions

The proliferation and differentiation of normal human osteoblast NHOst cells cultured on the dental Au–Ag–Pd casting alloy were determined using the micromass culture. The proliferation, ALP activity and calcium amount of NHOst cells cultured on 10Au–62Ag–13Pd were significantly decreased more than those on 13Au–58Ag–21Pd, respectively. The difference in the content of Cu between these alloys was suspected as a cause of these results. 13Au–58Ag–21Pd showed no cytotoxicity to L929 and V79 cells in the MEM elution assay and the colony assay. However, 10Au–62Ag–13Pd showed extremely weak cytotoxicity to L929 cells and weak cytotoxicity to V79 cells in the colony assay, though 10Au–62Ag–13Pd showed no cytotoxicity to L929 cells in the MEM elution assay. NHOst cells expressed the toxicity to the proliferation and differentiation on 10Au–62Ag–13Pd clearly, and the \textit{in vitro} toxicity test using NHOst cells would be useful to evaluate the toxicity of medical materials.

**REFERENCES**