The aim of this study was to investigate the long-term effects of alloys containing silver (mainly Ag-Sn alloy) on oral mucous tissue. We observed biopsy tissue specimens from patients diagnosed as having amalgam tattoo and/or metal pigmentation by light and electron microscopy and electron-probe microanalysis (EPMA). In most cases, Ag-Sn alloy was present in the tissue but it could not be confirmed if the alloy originated from amalgam. Distributions of both Ag-S and Ag-Sn have typical patterns. Most Ag forms Ag₂S and is stably deposited in three patterns along the collagen, basement membrane, and fibrous cells without inducing any host reaction. On the other hand, Sn forms large granules that contain Ag, S, C, N, P, and Ca, and is in soft state in the tissue. Tissue reactions to the alloy become weaker as time passes.

Key words: Amalgam tattoo, Ag₂S salt, Tissue reaction

INTRODUCTION

There are many diseases that involve metals or metal alloys, such as metal allergies, lichen planus, and metal pigmentation. Against this backdrop, metal toxicity must be thoroughly investigated during development of any dental alloy. In order to better understand the effects of dental alloys on oral tissue, it is most useful to study clinical samples such as biopsy specimens. Amalgam tattoo is a good example of a tissue pigmentation disorder caused by dental alloy, and numerous aspects of this condition have been investigated. Many authors have focused on mercury toxicity and concluded that soon after amalgam insertion into the tissue, mercury caused inflammation with numerous giant cells and microphages, but the effects of mercury disappeared over time. Lau reported that leakage of mercury from impacted dental amalgam particles induced metallothionein, while residual Ag₂S and Sn did not induce metallothionein. Doorn stated that mercury in tissue was eliminated over time. Another pigmentation disorder is gingival metal pigmentation beneath the crown. It is well known that in most cases the alloy can remain for long periods of time without any clinical impact, but can raise esthetic issues. In this study, we observed such biopsy specimens and investigated the relationship between dental metals (mainly Ag and Sn) and mucous tissue.

MATERIALS AND METHODS

Excision biopsies of amalgam tattoo were obtained from 16 patients. Biopsies were fixed in neutral formalin and were embedded in paraffin wax according to routine histological procedures. Serial sections were cut (thickness: 3 μm) from each specimen, and were stained with Hematoxylin and Eosin (H-E) for microscopic observation. Unstained sections were placed onto a well-polished carbon stick and were investigated by electron-probe microanalysis (EPMA, JXA-9800R/RL, JEOL, JAPAN) with a wavelength dispersive spectrometer (WDS). Measurement was performed under the following conditions: accelerating voltage, 15 kV; beam current, 0.03 μA; spot size, 1 μm. Compositional (COMP) images, quantitative information, qualitative information, and an element map were obtained. Samples were also investigated using a scanning electron microscope (SEM, JSM-6330F, JEOL, JAPAN).

RESULTS

Light microscopy

The excision sites of the biopsies were as follows: gingiva in 10 cases; oral floor in three cases; buccal mucous membrane in two cases; and palate in one case. Obvious inflammation was observed in only one case on histopathological examination. Rather than clear inflammation, worn-out granulation tissue that was collagen-rich and which contained fewer fibroblasts and more fibrocytes was observed. In a few cases, a large number of giant cells and/or microphages was observed. Two forms of dark aggregation, probably of metal origin, were observed in tissue in the H-E stained and unstained sections (Figs. 1A and 1C). One form had numerous discrete and fine black or brown granules (Fig. 1A, arrow). No scratch marks, except in three cases, on the
tissue section were observed around the large aggregations. Granules formed aggregates of various sizes (maximum 60-μm diameter) among tissue spaces. The other form resulted in pigmentation of the host tissue by fine granules (average size, 0.2 μm). These pigmentations were divided into three types: pigmentation of collagen fibers (five cases, Fig. 1A, arrowhead); "collagen-like structures" (eight cases, Figs. 1C and 1D, arrowhead), which were originally named by us and were observed frequently between collagen fibers; and brown pigmentation. Brown pigmentations were observed in the basement membrane of mucus epithelium (five cases), around strained muscle (two cases), around and/or inside blood vessels (eight cases, Figs. 1C and 1D, arrow), and in fibroblasts and macrophages (eight cases). These findings were easily observed, particularly in unstained sections. "Collagen-like structures" were sometimes atrophic but were ignored by surrounding tissues and/or cells such as macrophages. These pigmentations were observed as artifacts after displacing normal tissue arrangement but were not identified by usual staining methods such as Malloy stain, Silver impregnation stain, PAS stain, and Elastica Van Gieson stain. Through examination using a polarization microscope, these pigmentations were clearly distinguished under positive polarization; but its positive figure remained, even when samples were rotated on the stage (Figs. 1B and 1D).

**Scanning electron microscopy**

We examined the surface images of paraffin sections cut with a microtome. No scratch marks, except in three cases, were seen on tissue sections around the large aggregations (Fig. 2A). The aggregations consisted of many small granules (Fig. 2B). Small granules were scattered around the large aggregations and were found in the external matrix or in
Fig. 2 SEM image of metal pigmentation in paraffin sections.
A) A large foreign object of 20μm to 60μm (arrow) surrounded by connective tissue. No scratch marks are observed in the vicinity of the large foreign object.
B) Magnified area indicated by arrow in Fig. 2A: grain groups consisting of small granules.
C) Vicinity of area shown in Fig. 2A: in fibrous tissue, granules of various sizes are observed (arrow).
D) A large foreign object (arrow) appears to be hard with no soft tissue in specimens that contain Au.
E) Collagen-like structure (arrow) in the vicinity; the area in Fig. 1D is shrunken.
F) COMP image of Fig. 2E: bright area (arrow) indicates the presence of heavy elements in the tissue.
G) Vicinity of area shown in Fig. 1A: fibrous tissue is observed (arrow).
H) COMP image area of Fig. 2G: arrow indicates heavy elements. No clear granules are observed.
Fig. 3 Scatter diagram of all quantitative data obtained via EPMA.
A) Correlation between Ag (W%) and S (W%): Type A
B) Correlation between Ag (W%) and S (W%): Type B
C) Correlation between Ag (W%) and S (W%): Type C
D) Correlation between Sn (W%) and Ag (W%): Type D
E) Correlation between Sn (W%) and Ag (W%): Type E
F) Correlation between Sn (W%) and Ag (W%): Type F
G) Correlation between Sn (W%) and Ca (W%)
H) Correlation between Ca (W%) and P (W%)
I) Number of cases are shown; Total number of type A-F cases; Number of cases containing Hg, Cu, Si, In, and Au; Number of cases with correlation between types A-C and Hg, Cu, Si, In and Au, and between types D-F and Hg, Cu, Si, In and Au.

\[\begin{array}{|c|c|c|c|c|c|c|c|}
\hline
 & type A & type B & type C & total & Hg & Cu & Si & In & Au \\
\hline
\text{type D} & 8 & 1 & 0 & 9 & 3 & 2 & 0 & 1 & 0 \\
\text{type E} & 2 & 0 & 0 & 2 & 0 & 0 & 1 & 0 & 0 \\
\text{type F} & 1 & 1 & 3 & 5 & 0 & 3 & 2 & 3 & 3 \\
\hline
\text{total} & 11 & 2 & 3 & 16 & 3 & 5 & 3 & 4 & 3 \\
\hline
\text{Hg} & 3 & 0 & 0 & 3 & & & & & \\
\text{Cu} & 3 & 0 & 2 & 5 & & & & & \\
\text{Si} & 2 & 0 & 1 & 3 & & & & & \\
\text{In} & 0 & 2 & 2 & 4 & & & & & \\
\text{Au} & 0 & 1 & 2 & 3 & & & & & \\
\hline
\end{array}\]

\(\Delta\) mark in Figs. 3A-3C indicate standard Ag₂S salt.
Fig. 4 Map and line analysis by EPMA.
A) SEM image and distribution map for Ag, Sn, and S in the same tissue area shown in Fig. 1C. Color bars indicate the weight content ratio for each element.
B) Distribution of Ag (W%) by line analysis along the red line shown in Fig. 4A.
C) Distribution of S (W%) by line analysis along the red line shown in Fig. 4A.
D) Distribution of Sn (W%) by line analysis along the red line shown in Fig. 4A.
the cells. These were electron-dense granules and the sizes ranged from 0.1 μm to 0.4 μm (Fig. 2C). Different aggregations having a metallic appearance were observed in three cases (Fig. 2D).

The "collagen-like structures" observed in light microscopy (Fig. 1C) were observed in a shrunken structure (Fig. 2E) that had low and high electron density granules on COMP images (Fig. 2F). Near the area shown in Fig. 1A, the collagen contained no clear granules, but a material of high electron density was observed in the COMP image (Figs. 2G and 2H).

**Electron-probe microanalysis**

We obtained COMP images from paraffin sections of 16 samples by EPMA. The lighter parts on the COMP images were metal-rich parts, and we randomly selected several of these points to obtain qualitative information. We then took several measurements for each case and obtained a total of 278 sets of qualitative information for all sections. C (all cases), O (all cases), N (all cases), Ag (all cases), Sn (12 cases), Fe (all cases), S (all cases), Ca (11 cases), Si (three cases), Cu (six cases), Au (three cases), and In (four cases) were detected. We found a strong relationship between Ag and S (correlation coefficient, r = 0.92) (Fig. 3A). No relationships were observed between Ag and Sn (Fig. 3D), while strong relationships were observed between Sn and Ca and between Ca and P (Figs. 3G and 3H). There were three types of Ag-S distribution (Figs. 3A-3C) and three types of Ag-Sn distribution (Figs. 3D-3F). The relationship between the three types of Ag-S distribution and the three types of Ag-S distribution is shown by the number of cases (Fig. 3I). The relationship between the three types of Ag-S distribution and contents of Hg, Cu, Si, In, and Au, as well as the relationship between the three types of Ag-Sn distribution and contents of Hg, Cu, Si, In, and Au are also shown by the number of cases (Fig. 3I). Type A and type D were most prevalent, while the type of distribution changed when Au and In were included. In order to compare the relationship between Ag and S in tissue, we measured powdered Ag2S salt (Wako Ltd.) by EPMA and confirmed its relationship with Ag2S salt (Fig. 3, △mark). Cu has been a component of amalgam and was found in five cases, but was not related to any other data. The relationship between inflammation and distribution of Ag and S was unclear.

By analyzing the sample using EPMA near the area shown in Fig. 1C, we obtained a map that illustrates the two-dimensional distribution of Ag, Sn, and S, as well as an SEM image (Fig. 4A). Distributions of Ag and S in the map were almost the same, but distributions of Ag and Sn in the map differed substantially. This relationship is shown in the line analysis graphs indicating the intensities of Ag (Fig. 4B), S (Fig. 4C), and Sn (Fig. 4D) by weight percent (W%) in analysis of the same area of Fig. 4A. This analysis confirmed that the small granules were composed of Ag and S, but not Sn.

**DISCUSSION**

In this study of 16 biopsy specimens, which were doubted to be malignant melanoma, Ag was detected in all cases and Sn was detected in 12 cases. Based on the results shown in Fig. 4, we doubt that the clinical cause of pigmentation is Ag2S.

One of the best ways to determine the type of dental alloy causing the pigmentation is the sampling method used by atomic absorption spectroscopy (AAS)\(^\text{15}\). However, even with such information, one cannot be certain that the alloy is actually present in the tissue. We must therefore precisely study the biopsy specimens.

We found more than 10% tin in most samples containing silver (Fig. 4), and thus we believe that the \(\gamma\)-type silver-tin alloy\(^{17}\) was present—hence suggesting the likely presence of amalgam. There have been numerous studies on amalgam that helped the present study\(^{18-20}\). These included studies into the corrosion of amalgams by mechanical fracture test\(^{19}\), and into the ions released from various types of amalgam in oral environments or artificial saliva\(^{19}\). Ag, Sn, Hg, and Cu have also been measured\(^{20}\) in the released liquid. On the other hand, with regard to tissue reactions, there also have been numerous studies on amalgam. Most of these focused on the hazards of mercury\(^{9,10,12,13,17,21}\). Harrison\(^{9}\) detected mercury in only four macrophages in 41 cases of amalgam tattoo. Schedle et al.\(^{16}\) used fresh amalgam and aged amalgam that was left in culture medium for six weeks. They found that fresh amalgam had a greater inflammation factor than the aged amalgam, and this was largely due to the presence of Hg. Eley\(^{21}\) showed in an animal experimental model that mercury in the tissue disappeared within 25 weeks. Eley\(^{17}\) concluded that the cause of fibrous capsules was corrosion of the \(\gamma\) phase of amalgam. These researchers\(^{9,10,17,21}\) believe that there are inflammatory reactions due to mercury soon after amalgam is inserted into the tissue, but over time, inflammation disappears and mercury is not detected. One of the reasons that Hg disappears from tissue is that macrophages move the mercury from the amalgam implantation site to remote sites, such as the lymph nodes\(^{17}\). On the other hand, Forsell\(^{22}\) detected mercury in various tissues using autometallography, which is more sensitive in detecting mercury than our system. Lau\(^{31}\) found mercury with metallothionein in the histocyte-like cells near large granules. We therefore believe that the difference stems from the Hg amount, but most mercury leaves the site over time. In our study, we confirmed...
three cases with mercury counts within the measurement limits of EPMA on a WDS system, but it is impossible to say whether smaller amounts of amalgam were present in other biopsy samples. It is thus possible that most cases of our study were amalgam tattoo, but it is also possible that most cases were due to the use of silver-tin alloys, such as dental abutment.

The finding that In and/or Au was present in four cases suggests that the origin of alloy could be the crown or metal bond. These cases also showed different distributions of Ag-Sn and Ag-S (Fig. 3) and had metallic appearance on SEM images (Fig. 2). We believe that more than two types of dental alloy — such as silver alloy, amalgam, dental abutment, crown cast, and metal bond — were present among the cases in this study. The aim of this study was not to identify the origins of diseases but rather to investigate the effects of silver-related alloys. It is thus interesting that the histopathological findings in most cases were similar, despite the likelihood of different disease origins.

Fig. 4 shows that silver, with its higher tendency to ionize, is easily released into the tissue and readily bonds with S when compared with tin. Many authors have shown that in patients with amalgam, silver is independently present in the tissue. This same finding is seen in Argyria, which is a silver pigmentation disease.

We categorized three pigmentation patterns in the tissue (Figs. 1-3). These patterns should have strong relationships with the size of Ag$_2$S particles, which would depend on the mechanism of release from the alloy. The peculiar finding of “collagen-like structures” — which were found near collagen — likely contained sulfated mucosubstance (a component of the extracellular matrix) and were connected with Ag$_2$S. Harrison reported similar findings by TEM, and Mohr reported this as pigmentation of elastic fibers. We believe that this structure is not recognized as foreign body by host because no host reactions were induced. However, we were unable to fully identify this structure, and we therefore need further histological identification.

The finding of direct pigmentation of collagen (Fig. 1B), which had the same appearance as tissue stained with silver-stain and was observed by TEM (Figs. 2G and 2H), showed the ionization properties of silver. All three types of pigmentation also exhibited false polarization. This could be due to the diffusion of light by small granules of Ag$_2$S in the tissue, and is thus a useful method for confirming metal pigmentation.

Most of the big particles, with sizes ranging from several microns to dozens of microns, appeared to be composed of alloy corroded by organic substances on SEM images (Figs. 2A and 2B) and were in soft state in the tissue. Giant cells were rarely found around these particles, and old granulation tissue was also found. We believe that a granulation factor exists in connection with these particles, possibly due to the presence of alloy and/or mercury. As time passes, the granulation factor becomes weaker and stimulation factors that gather giant cells lose their potency. Sakai reported that metal particulate granule size (3-100 μm) was a more important factor than the metal type for cell activity using MG-63 osteoblast-like cell culture. Tamura also demonstrated the toxicity of small granules of titanium in subcutaneous tissue of rats. We also believe that the relationship between tissue reaction and size of granules is important in the corrosion process of silver-containing alloys in tissue. But it is difficult to draw a firm conclusion on this based on the observations in this study. Therefore further studies are required.

The correlations between Ca and Sn and between Ca and P were strong. External classification should have occurred, and inflammation controlled as a result. Indeed the effects of Ca have been harnessed for bioactive treatment of metal implant surfaces. However, the Ca volume was insufficient and the P to Ca ratio was not suitable for calcification. Using WDS with EPMA is a reasonable approach because the peaks of Ca-Kα = 3.69 kV and Sn-Lβ = 3.67 kV were near, which means that detection with the EDS system that most researchers use would have been impossible.

From a toxicological perspective, Feng showed — by using Escherichia coli and Staphylococcus aureus — that DNA lost its ability to replicate and proteins became inactive in solutions containing silver ions. Wataha reported that silver ions caused rapid death in macrophages. These examples indicate that silver acts in its ionized state in most cases, and is thus safe when stable, as in Ag$_2$S salt (solubility product, $1.8 \times 10^{-17}$ mol/l (10°C)). Pigmentation by Ag is probably not harmful from the viewpoint of inflammation and foreign body reactions. However, Ag remains in the mucus membranes for long periods of time without being eliminated from the body. In this study, we did not investigate the tissue with regard to function. Therefore, although the total amount of Ag was relatively small, it still remains unknown whether Ag in mucosa is harmful to the host.

It remains unclear why the immune system cannot attack pigmented tissue and/or recognize it as a foreign body. To answer this question will surely open up new ideas and new frontiers on immunity. Thus the study of metal pigmentation remains important, and further research on silver will certainly help to elucidate dental alloy pigmentation in oral mucosa.
REFERENCES


