Influence of Silver Ions in HA Film on Morphology of Macrophages

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ABSTRACT

Ion beam assisted deposition (IBAD) was successfully used to produce a dense and ultra-adherent Hydroxyapatite (HA) film on titanium alloy and alumina. Recently it is also proved that the HA coatings on alumina substrate treated with 20 ppm $AgNO_3$ had the structure of $(Ag,Ca)_{10}(PO_4)_6(OH)_2$, which exhibited excellent antimicrobial effects. The present paper aims to morphologically characterize the adhesion of macrophages on newly developed Ag-HA coated alumina and Ti6Al4V substrates and to evaluate the biocompatibility of the coatings *in vitro*. It can be found that the cell number on alumina substrate is more than titanium substrate under the same conditions. With the increase of the concentration of $AgNO_3$ in the treatment, the cell number on Ag-HA coatings decreased. Up to 20 ppm $AgNO_3$ by Ag-treatment, the morphological development of the cells on Ag-HA coating was similar to that of the cells on HA coating, suggesting the biotolerance of the Ag-HA coatings.

1. INTRODUCTION

Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$, being a widely used implant material, has been demonstrated to be biocompatible and biodegradable, and with the ability to become mechanically interlocked with bone [1]. Coatings of HA on titanium alloy and other substrates have been widely used, especially in orthopedic prostheses [2]. Implant caused infections are considered to be serious and common complications in orthopedic surgery, various methods have been developed for delivering antibiotics at the bone-implant interface [3]. Many types of bioceramics, which contain Ag^+ ions for antibacterial or bacteriostatic applications have been developed in recent years [4-6].

In our previous works, ion beam assisted deposition (IBAD) is successfully used to produce a dense ultra-adherent and pinhole-free HA layer on titanium alloy and alumina [7-8]. Recently it is also proved that HA coatings on alumina substrate treated with 20 ppm $AgNO_3$ had the structure of $(Ag,Ca)_{10}(PO_4)_6(OH)_2$, which exhibited excellent antimicrobial effects [9].

The present paper aims to morphologically characterize the adhesion of macrophages on newly developed Ag-HA coated alumina and Ti6Al4V substrates and to evaluate the biocompatibility of the coatings in vitro. Macrophages are known to play a central role both

in chronic infection and in antibody formation. Macrophage and osteoblast functions on different kinds of biomaterials, such as biodegradable polymers, HA, (-tricalcium phosphate (TCP), have been reported [10-11]. However, nothing is known about the reaction of Ag-HA coating on cells of skeletal system. We wish to know whether the Ag-HA coatings would be harmful to the bone cells like macrophages for the development of antimicrobial implant materials.

2. MATERIALS AND METHODS

2.1 Materials Preparation

The flat 10×10×1 mm alumina and Ti6Al4V samples were coated with HA by sputtering the HA target and bombarding the compactness using IBAD system. The thickness of the film was measured to be 638 nm on alumina and 700nm on Ti6Al4V substrate. The Ag-HA coatings were prepared by incorporating Ag^+ ions in HA crystals through an ion exchange reaction in 5ppm, 20ppm or 100ppm $AgNO_3$ solution at room temperature for 48 hours. The Ag-HA coatings were proved to have antimicrobial effect against *E.coli, Ps. aeruginosa, S. aureus and S. Epidermidis* [9].

Eight kinds of samples were prepared for the SEM study, as shown in Table 1.

Table 1: composition of the samples

Sample	Composition
1	Al_2O_3 + HA
2	Al_2O_3 + HA + Ag (5ppm $AgNO_3$)
3	Al_2O_3 + HA+ Ag (20ppm $AgNO_3$)
4	Al_2O_3 + HA+ Ag (100ppm $AgNO_3$)
5	Ti6Al4V + HA
6	Ti6Al4V + Ag (5ppm $AgNO_3$)
7	Ti6Al4V + Ag (20ppm $AgNO_3$)
8	Ti6Al4V + Ag (100ppm $AgNO_3$)

2.2 Macrophage culture

Murine macrophages cell line, RAW 264.7, was cultured on 10 cm diameter Petridish in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % fetal bovine serume (FBS) using a 37℃, 5 % CO_2 incubator.

2.3 Attachment studies

The speciments were inserted in 6 well dishes, 2ml of DMEM were added in each well. 1 ml of full-grown macrophages with 2 ml DMEM in Petridish was treated with 0.5 ml trypsin-ethylene diamine tetraacetic acid (trypsin-EDTA). And then 9.5 ml DMEM was put in the petridish with cells. The cells in Petridish were separated occasionally by giving a little shock. 1ml of the solution with about 10^5 cells/ml of activated macrophages in Petridish was

dropped onto the samples in 6 well dishes. Samples with the 6 well dishes were incubated in a 37℃ humidified atmosphere of 5% CO_2 for 48 hours. After that the samples were treated with 3ml of 3.7% formalin solution for 10 minutes, and washed with phosphate buffer saline (PBS) solution for 3 times.

2.4 Scanning electron microscopy (SEM)

Macrophages on the Samples were fixed *in situ* with 3ml of 2.5% glutaraldehyde in 0.1 Fvl sodium cacodylate buffer for 2 hours in room temperature. The samples were gently washed with 3ml of PBS (3 × 5 min) to remove any unattached cells, followed by dehydrated through a graded alcohol series (50-100%), each for 1 hour. As a final stage of the preparation, the samples with the cells on them were coated with gold. The samples were observed using a JSM model 5200 SEM at an accelerating voltage of 15-25 kV.

3. RESULTS

The effect of silvers ions in HA coatings on morphology of macrophages on both alumina and titanium substrates were studied. The HA coated alumina and titanium without Ag-treatment were served as control samples.

Fig.1-2 demonstrates the cells in contact with the coating. There are multiple layers of macrophages on the surface of HA coating on alumina (sample 1) after 2 days culture (Fig.1(a)). Fig. 1(b) is the enlargement of the cells shown in Fig. 1(a). The shape of the cells is either spherical or oval, with multiple ruffling and round knobs on the surface of the cells. On the surface of the coating, the cells are well attached, spread and proliferated on the surface, with their lamellipodia (arrow) covering on the HA coating. No morphological sign of cell damage was found. Fig. 2(a) shows the cells on Ag-HA coating (sample 3) on alumina after 2 days culture and Fig. 2(b) is the enlargement of the cells. Comparing the cells on Ag-HA coating with the one on HA coating, there is no obvious change in morphology. The spreading of the arm-like pseudopodia (arrow) suggests that the cells are in an active state. Most often the cells were observed to spread their filopodia to make themselves covering the material both in HA and Ag-HA coated alumina samples. The only difference between the cells shown in Fig. 1(a) and Fig. 2(a) is the number of cells. The cell number of the upper layer on the HA coating is about 11240/mm^2 , whereas the one on Ag-HA (5ppm $AgNO_3$) is about 7300/mm^2, on Ag-HA (20ppm $AgNO_3$) is about 6400/mm^2, as shown in Fig. 3(a).

On the Ag-HA film of alumina samples treated with 100 ppm $AgNO_3$ solution (sample 4), the macrophages show significant different form (Fig. 4). There is no lamellipodia or filopodia of the cells to see, suggesting that the cells are not in the active state. Besides, the number of the cells is much fewer than the one showed in Fig. 1(a) and 2(a).

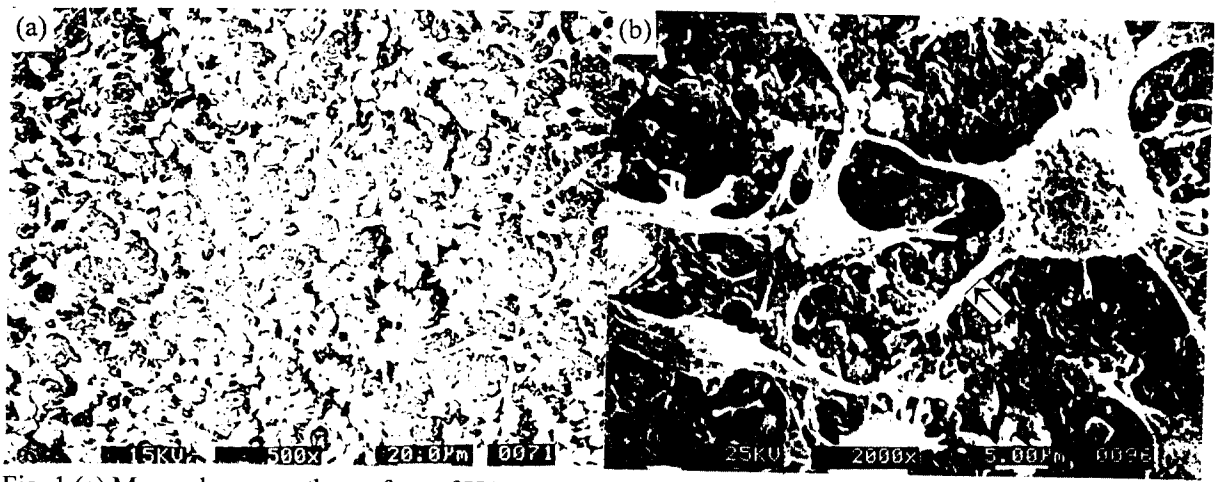

Fig. 1 (a) Macrophages on the surface of HA coated alumina (sample 1) after 2 days culture. (b) The enlargement of the macrophages, arrows indicate the lamellipodia covering the HA coating.

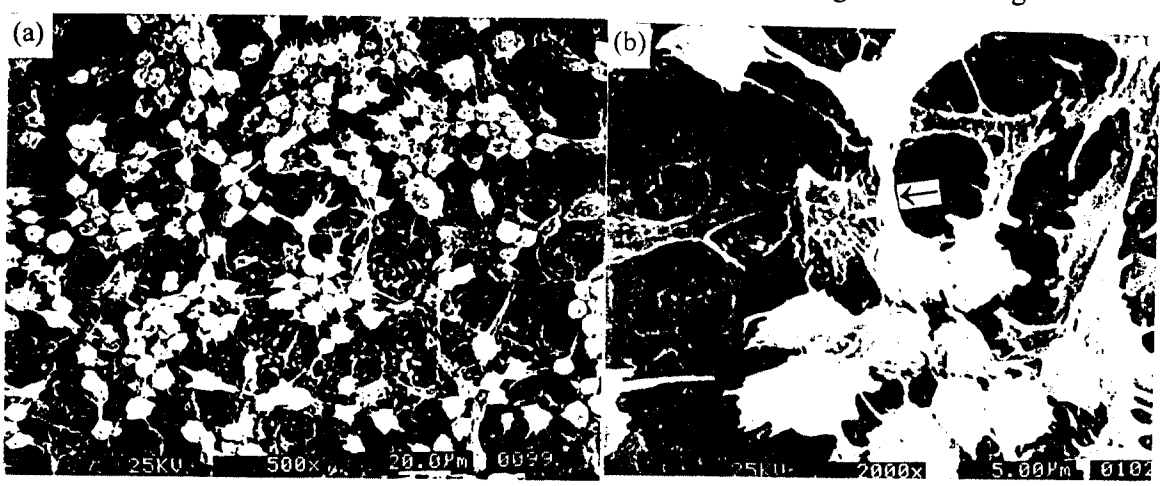

Fig. 2 (a) The cells on Ag-HA coating (sample 3) after 2 days culture. (b) The enlargement of the cells, arrows indicate the lamellipodia covering the Ag-HA coating.

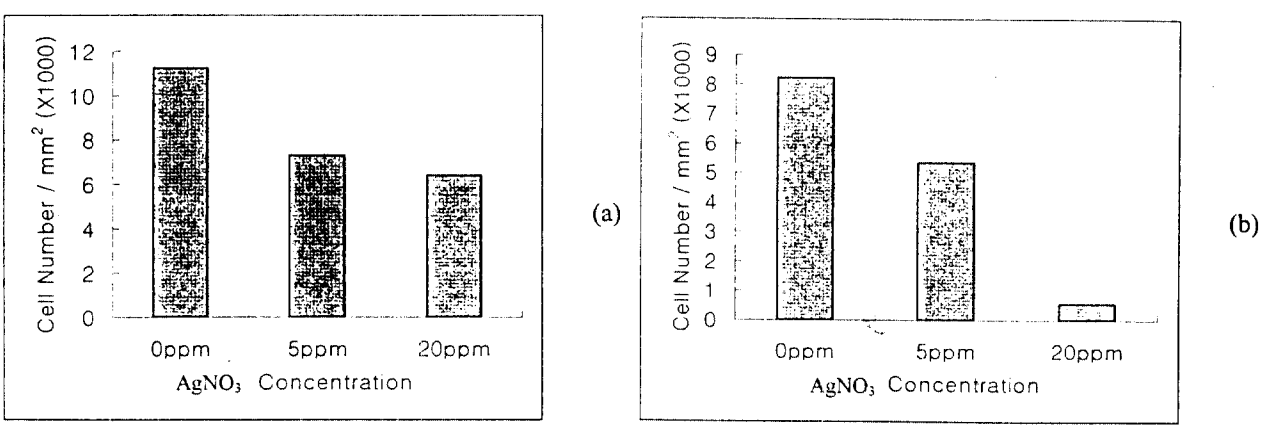

Fig. 3 Number of macrophages cultured on alumina (a) and titanium (b) substrate for 2 days.

Analogous to Fig. 1(a), Fig. 5(a) shows the morphology of multiple layered macrophages on the surface of HA coating on Ti6Al4V substrate (sample 5). From the enlargement of the cells Fig. 5(b), it is clearly shown that the cells have many knobs on their surface. And also

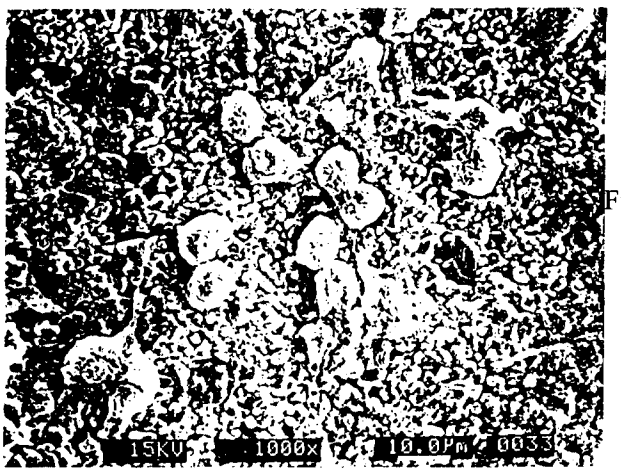

Fig. 4 Macrophages on the Ag-HA film (sample 4) after 2 days culture, no pseudopodia of the cells can be seen.

the cells spread their lamellipodia and filopodia on the surface to cover the material (arrow). The cells in Fig. 6(a) (sample 7) have the similar morphology as the one in Fig. 5(a), but the number of the cells is much fewer. It was calculated that the number of cells in Fig. 5(a) and Fig. 6(a) is about 8230/mm^2 and 550/mm^2 respectively, as shown in Fig. 3(b). The few cells on the Ag-HA coating are still well spread with their filopodia on the coating, as shown in Fig. 6(b) (arrow).

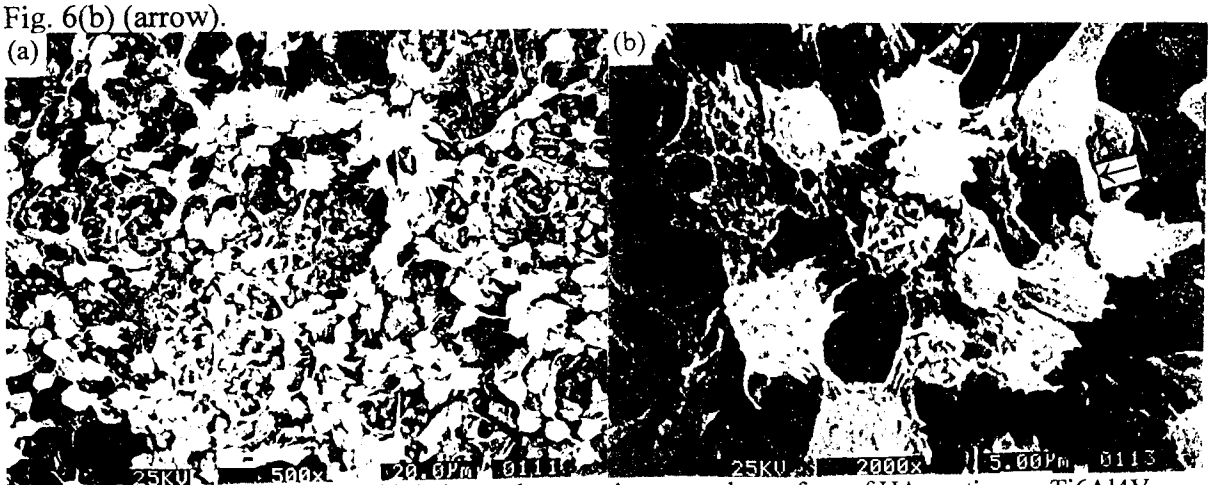

Fig. 5 (a) The morphology of multiple layered macrophages on the surface of HA coating on Ti6Al4V (sample 5). (b) The enlargement of the cells, arrows indicate the lamellipodia covering the HA coating.

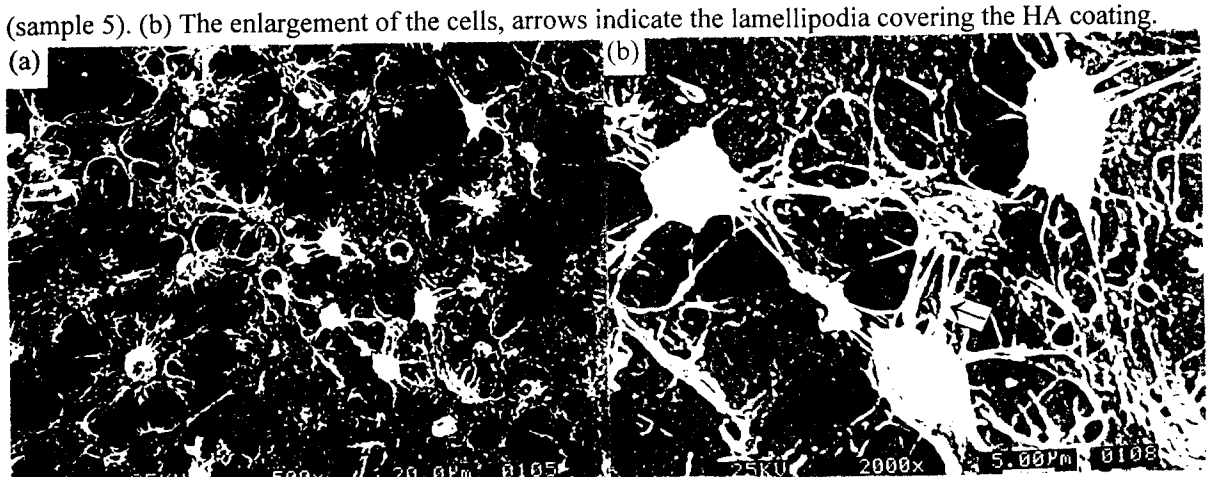

Fig. 6 (a) The cells on Ag-HA coating (sample 7) after 2 days culture. (b) The enlargement of the cells, arrows indicate the filopodia covering the Ag-HA coating.

On the specimens treated with 100 ppm $AgNO_3$ (sample 8), the cells are seriously damaged, they do not only have any pseudopodia, but loose their round form, as shown in Fig. 7.

Fig. 7 The macrophages on the Ag-HA film (sample 8) after 2 days culture, the cells are seriously damaged.

4. DISCUSSION

The cell number on alumina substrate is more than titanium substrate under the same conditions. It means that the cell adhesion and/or growth was effected by the morphology of the coatings. It was found by SEM that the surface morphology of HA on alumina is quite different from that on titanium alloy. There are many Ag-substituted HA crystals on the surface of alumina (Fig. 4), but the HA surface on titanium is flat (Fig. 7). The HA crystals on titanium may much finer than that on alumina. The silver ions on titanium may distribute more homogeneously that that on alumina. It is perhaps more easy for silver ions to release from the surface of titanium than from that of alumina. The more amount of Ag^+ released from titanium surface may affect the adhesion and/or proliferation of macrophages in hydrous environment.

The observations demonstrate that the silver ions in HA have influence on the activity of the macrophages *in vitro*. Up to the concentration of 20 ppm $AgNO_3$, there is no significant morphological changes of the cells, suggesting that the function of macrophages is not interfered. In one of our previous works, the HA coated alumina samples immersed in 20 ppm $AgNO_3$ (12.7 ppm Ag) solution showed excellent antimicrobial effect [9]. So it is suggested that the Ag-treatment of implant materials to prevent infections should be limited to the amount of 12.7 ppm Ag.

The phagocytosis function of macrophages cannot be determined from the present experiment. The studies on dissolution of the crystals of $(Ag,Ca)_{10}(PO_4)_6(OH)_2$ by macrophages and the reaction of osteoblasts to Ag-HA coatings are going on.

5. CONCLUSION

The attachment and spreading of macrophages on alumina substrate is more easy than on titanium substrate under the same conditions *in vitro*. With the increase of the Ag ions in Ag-HA coatings, the cell number decreased. Up to 20 ppm $AgNO_3$ by Ag-treatment, the

morphological development of the cells on Ag-HA coatings was similar to that of cells on HA coatings, suggesting that the Ag-HA coatings with limited silver ions on alumina and titanium alloy can provide a suitable substrate for culture of macrophages.

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