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Specific response of osteoblast-like cells on hydroxyapatite layer containing serum protein

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Abstract Accelerations of bone-like apatite deposition and cell growth on an electrically polarized ceramic hydroxyapatite have been reported. A relationship between these phenomena was investigated in a previous report, and then it was suggested that osteoblast-like cell's (MC3T3-E1) growth had relevance to the mineral growth. The effect of the formed apatite layer especially appeared to be on the cell adhesion. The acceleration of cell proliferation on the polarized HAp has been shown using fibroblastic cell (L929) and nerve cell (SK-N-SH) lines, therefore the effect of the layer on L929 and SK-N-SH was investigated to support the mechanism of acceleration of cell proliferation by polarization of HAp. In this study, the effect of the bone-like apatite layer was not confirmed on L929 cell's growth. On the other hand, the acceleration of nerve cell's proliferation was confirmed on the formed apatite layer. However, the remarkable improvement of the cell adhesion of SK-N-SH was not confirmed on the apatite layer. Consequently, it was considered that the bone-like apatite containing serum protein obtained by the coprecipitation of bone-like apatite and serum protein has a pronounced role only in the activity of osteoblast-like cells.

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1. Introduction

Cell attachment, adhesion, and spreading are the first phase of the cell/biomaterial interaction, and influence the capacity of cells to proliferate and differentiate on contact with the implant [1–4]. Accordingly, surface character must be considered during the production of biomaterials to control the interaction between the biomaterial and the tissue. To modify the biocompatibility of the surface, hydroxyapatite (HAp) has been used in coatings in the dental and orthopedic fields [5, 6]. Biomimetic and plasma-spraying techniques constitute the prevailing coating methods used with HAp [7–9]. The biomimetic technique relies upon the formation of an apatite layer on substrates within simulated body fluid (SBF) with ionic concentrations nearly identical to those of human blood plasma [10, 11].

An effective treatment of the HAp for improvement of bioactivity was discovered. The HAp can be electrically polarized [12], and then the proliferation of osteoblast-like cells (MC3T3-E1) [13] is accelerated on the negatively charged surface of the polarized HAp [14]. The effect of the polarization was confirmed not only the proliferation of MC3T3-E1 cells, but also other cell's proliferation, mineral growth [15, 16], bacteria growth [17], and osteoconductivity [18]. The relationship between these phenomena was investigated, and it was suggested that the cell's proliferation had relevance to the mineral growth. The bone-like apatite growth was controlled on the charged surface of the polarized HAp in SBF

and cell culture medium. The growth behavior was related to the cell proliferation with acceleration on a negatively charged surface and deceleration on the positively charged surface. The proliferation of MC3T3-E1 cells was significantly accelerated on the bone-like apatite layer formed in the culture medium supplemented with 10% FBS [19–21]. Furthermore, all of the adherent MC3T3-E1 cells were closely contacted to the apatite layer formed in culture medium with FBS for 7 days. Accordingly, it was decided that the bone-like apatite deposition was one of the factors responsible for the acceleration of the MC3T3-E1 cell's proliferation. The acceleration of cell proliferation on polarized HAp had been confirmed using fibroblastic cells (L929) [22] and nerve cells (SK-N-SH) [23], therefore the effect of the layer on L929 and SK-N-SH was investigated to support the mechanism of acceleration of cell proliferation by polarization of HAp. In this study, the normal HAp ceramic was used as a substrate to estimate the effect of the bone-like apatite except the effect of the polarization.

The amount of the bone-like apatite with adsorption of serum protein was lower than that of the bone-like apatite without serum protein adsorption. Inhibition of the nucleation of bone-like apatite by some proteins has been reported [24–28]. However, a preparation of a large amount of the bone-like apatite layer is desirable for activation of MC3T3-E1 cells, because the effect of the bone-like apatite layer clearly appeared to be increased with an increasing of the amount of the layer. Therefore, in this study, the condition of the bone-like apatite layer formation was modified to obtain the larger amount of the layer that should have the greater cell activation in a short period, and then the effect of the obtained layers on the growth of MC3T3-E1 cells.

2. Materials and methods

2.1. Formation of bone-like apatite containing serum protein

Hydroxyapatite slurry obtained by a precipitation reaction was filtered and freeze-dried, and then calcined at 800°C in air for 2 h. The single phase of the obtained HAp was verified by X-ray diffraction (XRD; Phillips PW-1700) and infrared spectroscopy (IR; Hitachi I-2000). After pelletization by a uni-axial press, the ceramic HAp was prepared by sintering of the HAp pellet at 1250°C for 2 h under flowing H_2O vapor. The obtained ceramic HAp with a diameter of 11 mm had 96% relative density.

The ceramic HAp was sterilized by an autoclave. The ceramic HAp was placed in a 24-well microplate with 2 ml per well of α-MEM (GIBCO^{TM}) supplemented with 10% FBS (GIBCO^{TM}) for 3 days in an incubator containing 5% CO_2 in air at 37°C. It has already been reported that the bone-like apatite containing serum protein on the ceramic HAp was formed in α-MEM supplemented with 10% FBS for 3 days, and the formed layer showed greater cell adhesion and proliferation of MC3T3-E1 [19–21]. After soaking, the specimens were washed with 0.1 M phosphate buffer solution (pH = 7.4). The surfaces of the formed layers on the soaked ceramic HAp were observed by scanning electron microscopy (SEM; Hitachi S-4500).

2.2. Culture of L929 and SK-N-SH cells

The responses of L929 cells and SK-N-SH cells on the bone-like apatite containing serum protein were estimated. L929 cells and SK-N-SH cells were cultured in the culture media supplemented with 10% FBS in an incubator containing 5% CO_2 in air at 37°C respectively. Medium for the formation of the bone-like apatite layer were drawn from each well, and then 2 ml of suspensions of cells (L929: 2.30×10^4 cells $\cdot$ ml^{-1}, SK-N-SH: 7.38×10^4 cells $\cdot$ ml^{-1}) were divided into the well of the 24-well microplate containing the samples.

After cultivation for 3 days, cell growth was estimated by counting the adherent cells and by morphological observation. For cell counting, the adherent cells were separated from the surface of each specimen using 1 ml of 2.5% trypsin solution (GIBCO^{TM}). The same volume of culture medium was added into the suspension to stop the trypsin treatment. Then, the number of adherent cells was measured using a hematocytometer (Erma, Tokyo). The cell cultivation was carried out 2 times, and the standard deviation and statistical evaluation of the cell count was calculated by using 8 data ($n = 8$). Morphological observation of adherent cells was carried out using SEM. Before SEM observation, adherent cells were fixed by 2.5% glutaraldehyde solution (TAAB) and 1% osmium tetroxide solution (Nissin EM Co. Ltd.) for 1 h respectively. The cells were dehydrated by ethanol (50–100%) and dried with a critical point dryer after fixation. The specimens were coated by Pt-Pd ion sputtering (Eiko IB-5). The effect of the bone-like apatite containing serum protein on cell growth was estimated by the comparison with the cell's responses on the ceramic HAp without the layer.

2.3. Formation of three types of bone-like apatites

The bone-like apatite layers were classified into three types by the period of contact with the serum protein. Type-I layer was formed in α-MEM supplemented with 10% FBS for 3 days, and it had been in contact with serum protein since the beginning of the apatite deposition. Type-II layer was formed in α-MEM without FBS for 3 days, and it did not adsorb serum protein until the cell cultivation stage. Type-III layer was formed by soaking for 2 days in α-MEM, and then by soaking in FBS for 1 day. The base of this layer is

bone-like apatite similar to the type-II layer, and its surface is covered with the adsorbed serum protein.

2.4. Culture of MC3T3-E1 osteoblast-like cells

MC3T3-E1 cells were cultured in α-MEM supplemented with 10% FBS in an incubator containing 5% CO_2 in air at 37°C. The MC3T3-E1 cell suspension (1.17×10^4 cells $\cdot$ ml^{-1}) was divided into the 24-well microplate (2 ml/well) containing with the samples. After cultivation for 3 days, cell growth was estimated by counting the adherent cells and by morphological observation. The cell cultivation was carried out 2 times, and the standard deviation and statistical evaluation of the cell count was calculated by using 8 data (n = 8).

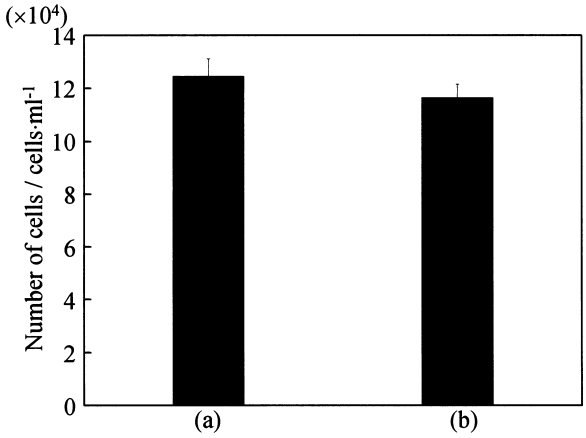

Fig. 2 Number of L929 cells adhering after cultivation for 3 days, on each surface. The ceramic HAp had 1.25×10^5 cells $\cdot$ ml^{-1} adhering (a). The bone-like apatite containing serum protein had 1.16×10^5 cells $\cdot$ ml^{-1} adhering (b).

3. Results

3.1. Responses of L929 cells and SK-N-SH cells to the bone-like apatite containing serum protein

The surface of the ceramic HAp as the substrate of the bone-like apatite deposition is shown in Fig. 1 (a). Nano-size particles of the bone-like apatite containing serum protein were deposited on the substrate by soaking in α-MEM with 10% FBS for 3 days (Fig. 1 (b)). The morphology was similar to that of previous report [19].

The number of adherent L929 cells on the ceramic HAp was counted as 1.25×10^5 cells $\cdot$ ml^{-1} (Fig. 2 (a)). The number of adherent L929 cells on the bone-like apatite containing serum protein was 1.16×10^5 cells $\cdot$ ml^{-1}, and it was estimated to be 7% lower than that on the ceramic HAp (Fig. 2 (b)). However, the difference between these values was not statistically significant difference in t-test ($\rho > 0.1$). The morphology of the adherent L929 cells was polygonal-shaped on the ceramic HAp (Fig. 3 (a)), while that on the bone-like apatite containing serum protein was similar to that on the ceramic HAp (Fig. 3 (b)). Both adherent cells formed cell/cell junctions and these had not reached to confluent state.

The number of SK-N-SH cells adhering to the ceramic HAp was counted as 1.06×10^5 cells $\cdot$ ml^{-1} (Fig. 4 (a)). The number of adherent SK-N-SH cells on the bone-like apatite containing serum protein was 1.19×10^5 cells $\cdot$ ml^{-1}, and it was estimated to be 12% greater than that on the ceramic HAp (Fig. 4 (b)). A statistically significant difference was confirmed between these values by t-test ($\rho < 0.1$). On the ceramic HAp, SK-N-SH adhered and formed many colonies (Fig. 5 (a)). On the other hand, the morphology of colonized SK-N-SH cells on the bone-like apatite containing serum protein had not been confirmed because of the adherent SK-N-

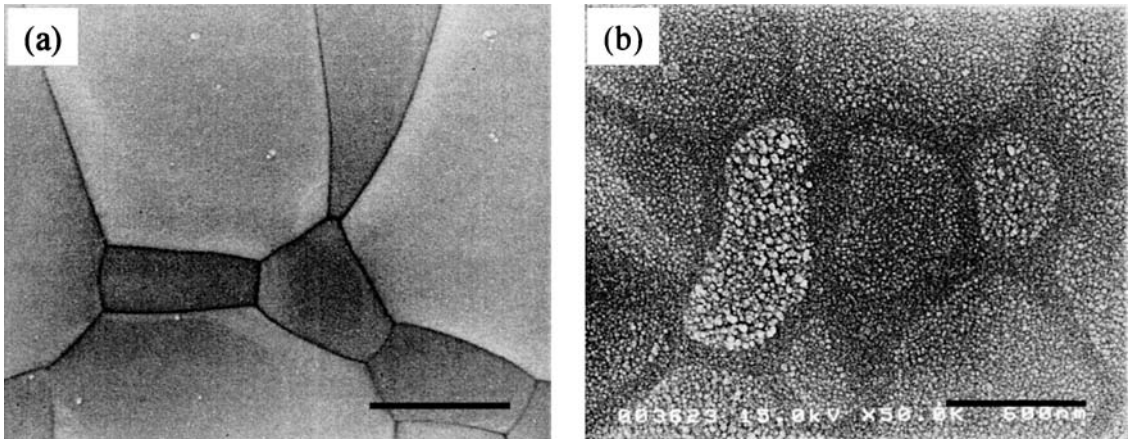

Fig. 1 SEM photographs of ceramic HAp and formed layers. The HAp grain is clearly visible on the surface of the ceramic HAp (a). The bone-like apatite containing serum protein was formed in α-MEM with 10% FBS for 3 days by a concerted reaction with the deposition of bone-like apatite and the adsorption of serum protein; this appears as an aggregation of particles with diameters of 10–20 nm (b). (bar: 600 nm.)

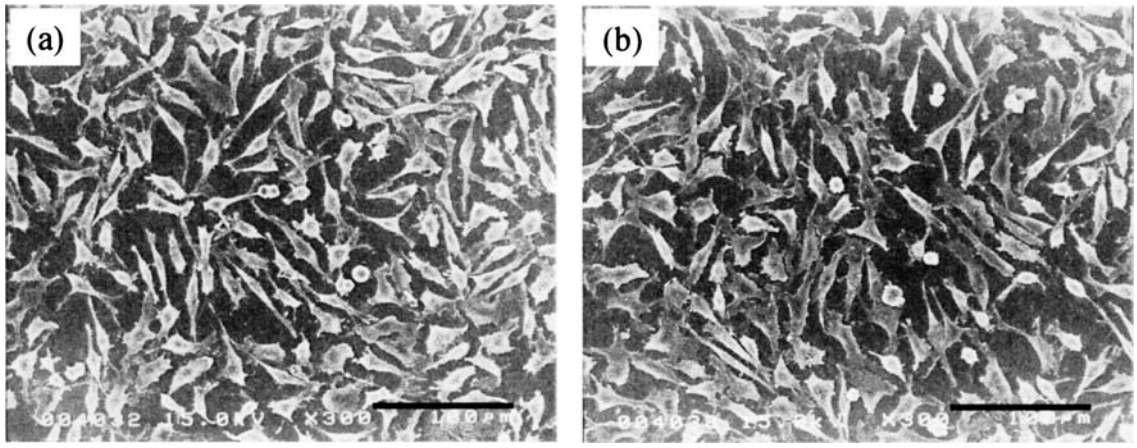

Fig. 3 SEM photographs of L929 cells adhering to each surface. Evenly distributed cells and junctions were observed on the ceramic HAp (a). Cells adhering to the bone-like apatite containing serum protein are shown in (b). There was no remarkable difference in the cells adhering to each surface. (bar: 100 μm.)

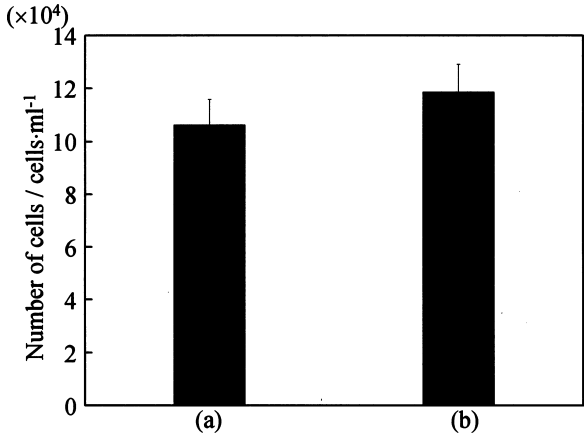

Fig. 4 Number of SK-N-SH cells adhering after cultivation for 3 days, on each surface. The ceramic HAp had 1.06×10^5 cells $\cdot$ ml^{-1} adhering (a). The bone-like apatite containing serum protein had 1.19×10^5 cells $\cdot$ ml^{-1} adhering (b).

SH cells easily exfoliated from the bone-like apatite containing serum protein. Although cell exfoliation occurred on the bone-like apatite containing serum protein, lamellipodia and junctions were observed at a few survival cells (Fig. 5 (b)).

3.2. Effects of three types of bone-like apatites on MC3T3-E1 cell's growth

The bone-like apatites were deposited on the ceramic HAp after soaking in culture media for 3 days. Surfaces of ceramic HAp and bone-like apatite containing serum protein (type-I layer) were shown in Fig. 1. On the sample soaked in α-MEM, the bone-like apatite without serum protein (type-II layer) took a network structure (Fig. 6 (a)). Although the base of the bone-like apatite covered with serum protein (type-III layer) was type-II layer, the surface of the bone-like apatite was modified by soaking in FBS. Its morphology was like the type-II layer but with a filling of the holes (Fig. 6 (b)).

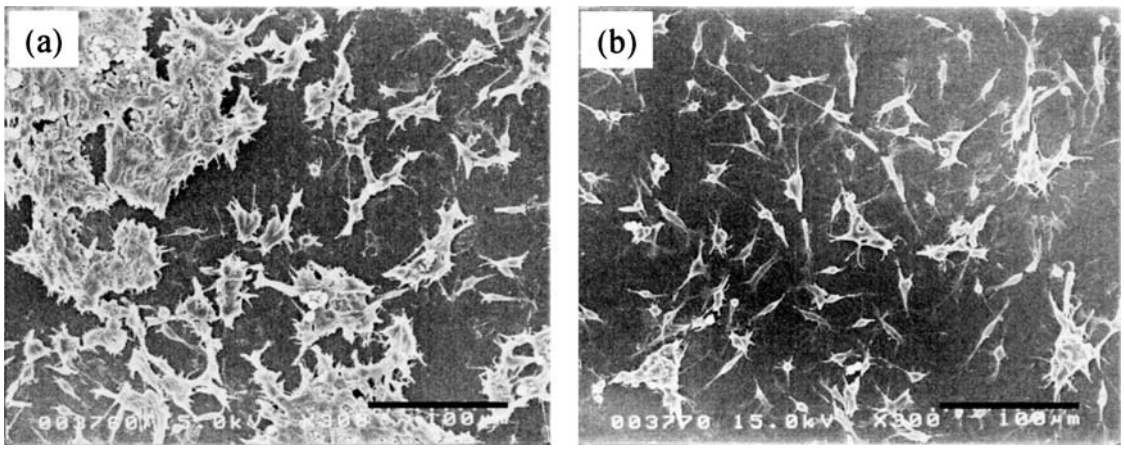

Fig. 5 SEM photographs of SK-N-SH cells adhering to each surface. Colonies of adherent cells were observed on the ceramic HAp (a). Many exfoliations from the bone-like apatite containing serum protein was observed during preparation for SEM. Accordingly, only a few colonies of adherent cells were observed (b). (bar: 100 μm)

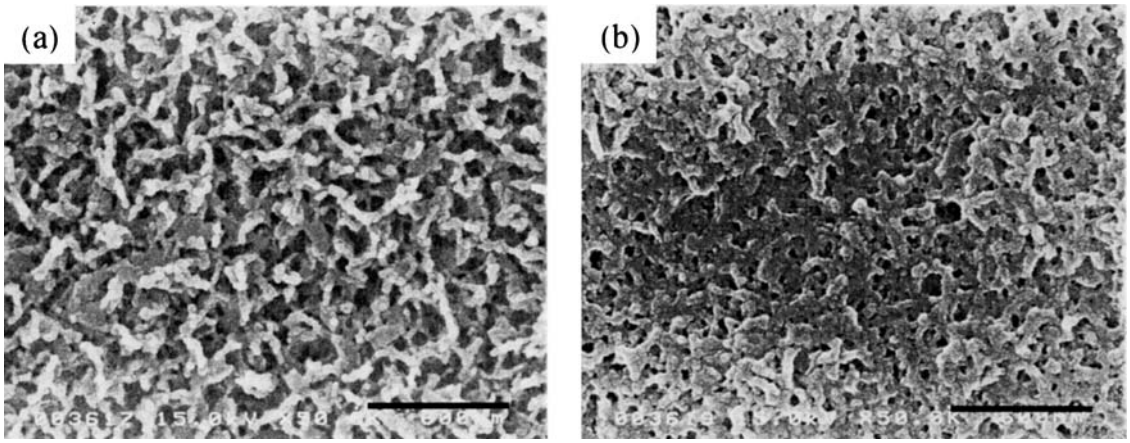

Fig. 6 SEM photographs of type-II layer (bone-like apatite without serum protein (a)) and type-III layer (bone-like apatite covered with serum protein (b)). The bone-like apatite was formed in α-MEM for 3 days. The base of the type-III layer was a type-II layer, and then the surface of the bone-like apatite was covered with serum protein and modified by soaking in FBS for 1 day. (Bar: 600 nm.)

The thickness of the deposited type-II layer was sufficient to measure, and it was more than 2 μm (Fig. 7). However, the thickness of type-I layer was insufficient to measure in cross sectional SEM observation. Lines of EDS spectra clearly showed the interface between the substrate and the formed layer, and then the thickness of the type-I layer was measured as 500 nm by EDS line analysis (Fig. 8).

The number of adherent MC3T3-E1 cells were 1.94×10^4 cell $\cdot$ ml^{-1}), 2.22×10^4 cell $\cdot$ ml^{-1}, 1.81×10^4 cell $\cdot$ ml^{-1}, and 1.94×10^{-1} cell $\cdot$ ml^{-1} for ceramic HAp, type-I layer, type-II layer, and type-III layer respectively (Fig. 9). The largest number of adherent MC3T3-E1 cells was obtained on the type-I layer where the relative number of adherent cells on the type-I layer versus ceramic HAp was 114%. On the other hand, the number of adherent MC3T3-E1 cells was decreased on the type-II layer. The number of adherent cells on the type-III layer was equal to that on the ceramic HAp (Fig. 9 (d)). The type-I layer had a significant difference compared with HAp substrate and type-II layer ($\rho < 0.05$).

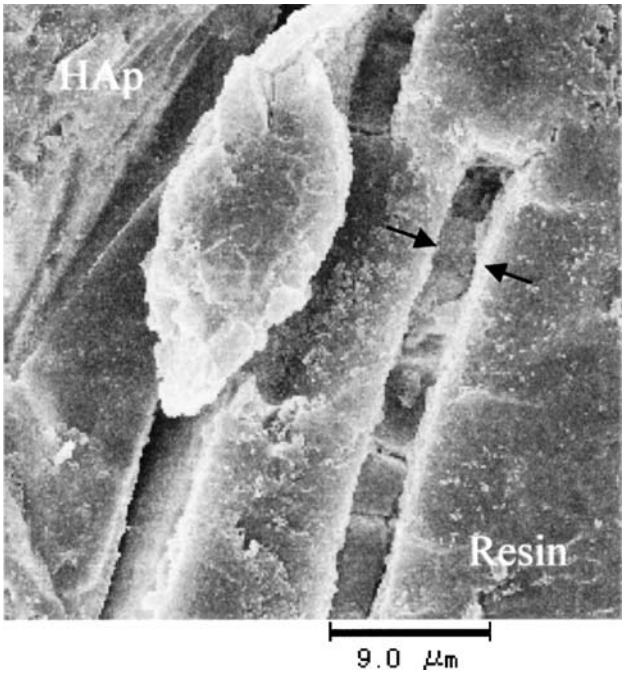

Fig. 7 Photograph of the cross section of type-II layer (bone-like apatite without serum protein) formed in α-MEM for 3 days. The layer was peeled off the ceramic HAp when a resin was poured, and then it was fixed in the hardening resin. Thickness of the layer shown between arrows was 2.8 μm.

Figure 10 shows the morphology of MC3T3-E1 cells adhering to each surface. On the ceramic HAp, the adherent MC3T3-E1 cells were observed to have polygonal shape (Fig. 10 (a)). A remarkable difference in the morphology of adherent MC3T3-E1 cells was observed on the type-I and type-II layer. All of the adherent MC3T3-E1 cells exhibited a flat shape on the type-I layer (Fig. 10 (b)). On the other hand, cells of spherical shape were observed on the type-II layer (Fig. 10 (c)). These spherical cells easily came off the surface, and the remaining cells on the surface were showing in this photo. For the bone-like apatite covered with serum protein (type-III layer), the morphology of the adherent MC3T3-E1 cells was polygonal-shaped, similar to that on the ceramic HAp (Fig. 10 (d)).

The vestige of the adherent MC3T3-E1 cells was confirmed on the type-I layer. Figure 11 shows the surface of the type-I layer before and after cell adhesion. The morphology of the type-I layer was changed by the cell adhesion.

4. Discussion

The bone-like apatite containing serum protein was formed on the ceramic HAp by soaking in culture medium with 10% FBS for 3 days. The soaking time was decided from a previous report, and it was adequate for activation of MC3T3-E1

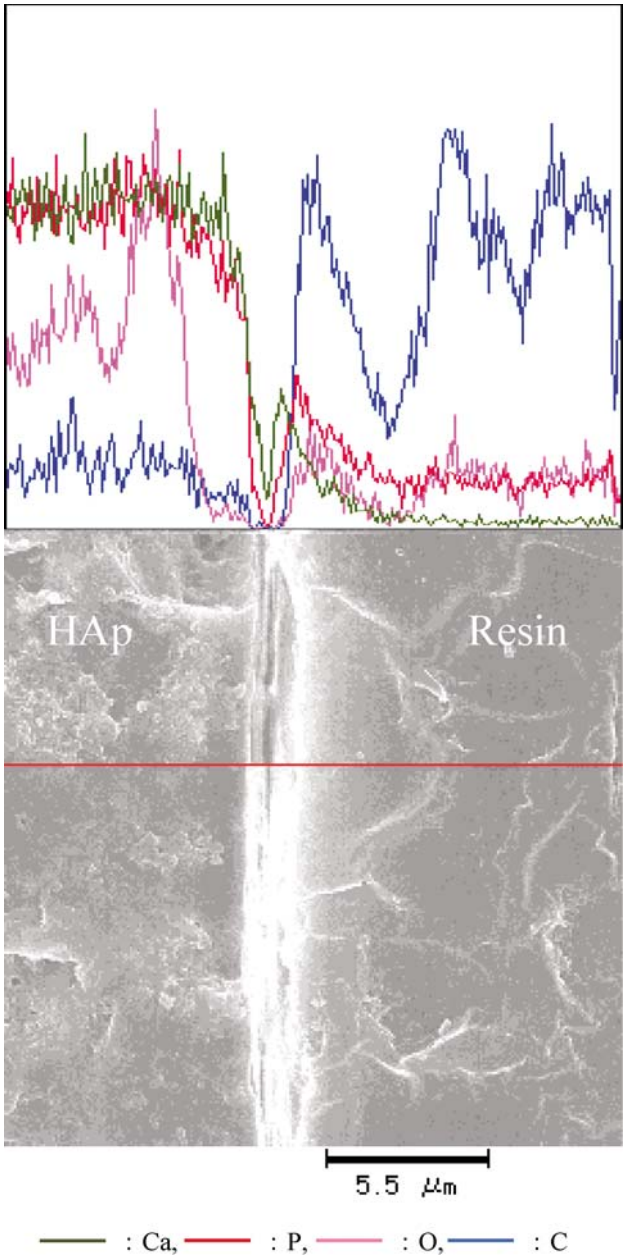

Fig. 8 EDS line spectra of the cross section of type-I layer (bone-like apatite containing serum protein) formed in α-MEM supplemented with 10% FBS. The layer was detected in a crevice between ceramic HAp and the hardening resin, and its thickness was measured to be 500 nm.

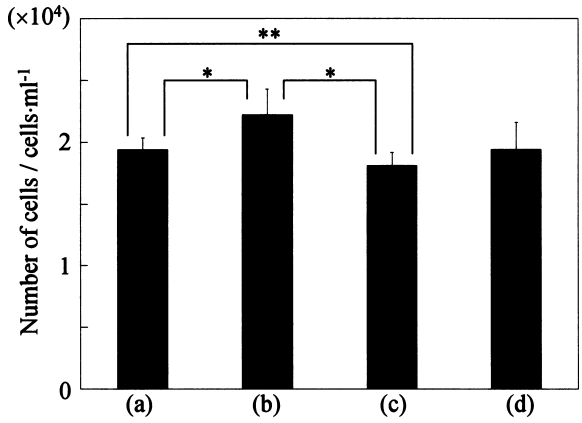

Fig. 9 Number of adherent MC3T3-E1 cells after cultivation for 3 days, on each surface. The ceramic HAp had 1.94×10^4 cells $\cdot$ ml^{-1} adhering (a). The bone-like apatite containing serum protein had 2.22×10^4 cells $\cdot$ ml^{-1} adhering (b). The bone-like apatite had 1.81×10^4 cells $\cdot$ ml^{-1} adhering (c). The bone-like apatite covered with serum protein had 1.94×10^4 cells $\cdot$ ml^{-1} adhering (d). Asterisks indicated the result of statistical evaluation. (*: $\rho < 0.05$, **: $\rho < 0.1$, without *: $\rho > 0.1$)

cells. However, the results on L929 cells were not similar to that of MC3T3-E1 cells. Differences in the morphology and proliferation of L929 cells were not observed between the ceramic HAp and the bone-like apatite containing serum protein.

The proliferation of SK-N-SH cells was increased on the bone-like apatite containing serum protein. SK-N-SH cell promptly connect with the surrounding cells, and then these connected cells form a colony. The adhesion strength between the cell and the substrate is weak. Accordingly, many exfoliations were observed on bone-like apatite containing serum protein because the colony formation was accelerated with increasing number of cells on bone-like apatite containing serum protein. Nevertheless, the remaining cells were well distributed over the bone-like apatite containing serum protein. These results showed a small effect of the bone-like apatite containing serum protein on SK-N-SH cell's growth.

A difference in an effectiveness of the bone-like apatite containing serum protein was discovered in three types of cells. In a previous report, it was suggested that the formation of the bone-like apatite containing serum protein is related to the acceleration of MC3T3-E1 cell's proliferation. Similarly, it was considered that this relationship would be applied to L929 cells and SK-N-SH cells. However, the results of this report do not support the relationship on L929 cells and SK-N-SH cells. It was suggested that the cell behavior after adhesion made a difference to the cell growth on the bone-like apatite containing serum protein. The phagocytosis of osteoblasts on bone tissue or biomaterials has been reported [29]. The vestige of cell adhesion on the bone-like apatite containing serum protein (type-I layer) was clearly observed only on MC3T3-E1 cells. The surface of the vestige of MC3T3-E1 cells seems to be the dissolved type-I layer. It was suggested that the MC3T3-E1 cells dissolved the type-I layer by phagocytosis. Accordingly, only the MC3T3-E1 cell can make practical use of the internal part of the bone-like apatite containing serum protein, which it does by gradually invading the latter.

The importance of the adhesive protein that was adsorbed within the bone-like apatite was demonstrated. The formation of the bone-like apatite presents an extensive adsorption

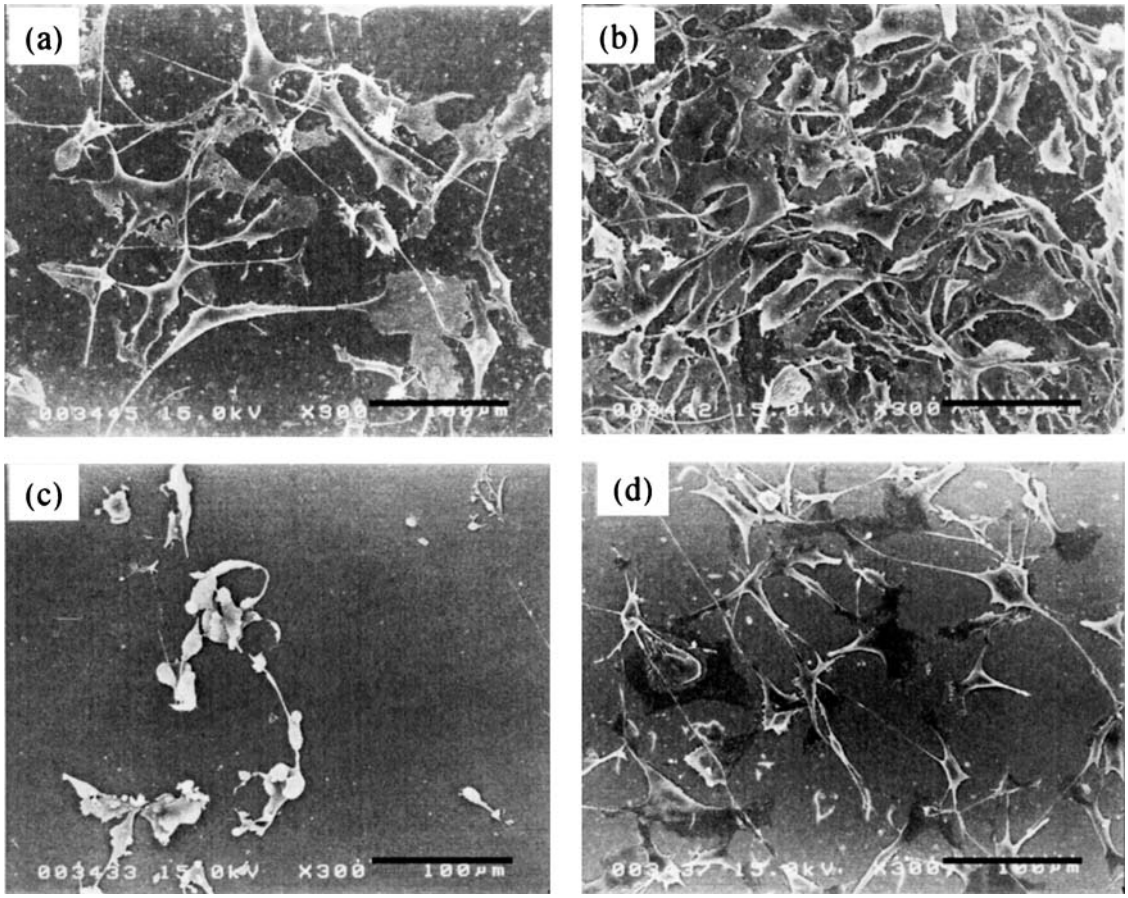

Fig. 10 SEM photographs of the MC3T3-E1 cells adhering to each surface. The adherent cells were evenly distributed and formed junctions on the ceramic HAp (a). The cells adhering to the bone-like apatite containing serum protein had a flat shape, and closely contacted the surrounding cells (b). Spherical adherent cells were observed on the bone-like apatite (c). Adherent cells on the bone-like apatite covered with the serum protein. These cells spread and formed junctions, looking remarkably similar to those on the ceramic HAp (d). (bar: 100 μm.)

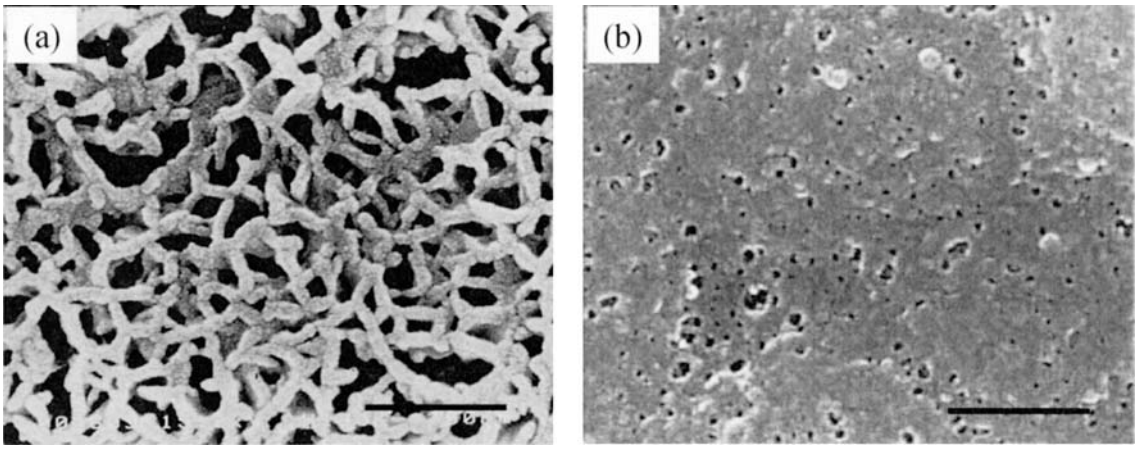

Fig. 11 SEM photographs of the bone-like apatite containing serum protein (type-I layer) before and after MC3T3-E3 cell adhesion. The bone-like apatite containing serum protein was formed in α-MEM supplemented with 10% FBS for 14 days (a). The morphology of the layer had a network structure with long-term formation of the layer. After adhesion of the MC3T3-E1 cells, the network-structure of the layer disappeared (b). (bar: 300 nm).

site for the adhesive serum proteins which, in turn, provide a large quantity of adsorbed serum protein the function of which promotes cell adhesion. The interaction between cytoplasmic constituents such as integrins on the membrane at the site of cell adhesion and serum adhesive protein such as fibronectin and vitronectin has an important role in cell adherence [30–39]. It has been reported that bone-like apatite enhances the selective adsorption of high molecular weight proteins from serum, and that the adsorbed serum protein promotes the subsequent adhesion of osteoblast-like cells [40]. The type-III layer that was bone-like apatite covered with serum protein is, in a sense, an inorganic-organic composite, and the aim of this method was to rapidly obtain a layer with similar behavior to the type-I layer. The thick type-III layer that was composed of bone-like apatite and serum protein was obtained by a separate precipitation, and its morphology and MC3T3-E1 cell's response were not similar to those of the type-I layer. The effect of the type-III layer on cell proliferation was less than that of the type-I layer. It was revealed that cell activation depends on the range of the serum protein adsorption in the bone-like apatite. Finally, it is necessary to distribute the serum protein all over the bone-like apatite in order to provide an active substrate for the MC3T3-E1 cells. Consequently, the bone-like apatite containing serum protein obtained by the coprecipitation of bone-like apatite and serum protein has a pronounced role in the activity of osteoblast-like cells.

5. Conclusion

The relationship between the bone-like apatite deposition and the cell growth was demonstrated on MC3T3-E1 cells in previous report. However, the relationship was not applied to L929 cells and SK-N-SH cells. Although the mechanism of acceleration of the cell proliferation was not demonstrated in this study, it was considered that induced surface charge by the electrical polarization of ceramic HAp directly affected the cell proliferation.

On the other hand, a particular effect of the coprecipitation of bone-like apatite and serum protein on MC3T3-E1 cells was discovered. Bone-like apatite without serum protein was effectively grown on the ceramic HAp, but did not have a good effect on cell growth. On the other hand, a bone-like apatite containing serum protein formed by the concerted reaction between the deposition of bone-like apatite and the adsorption of serum protein was favorable to cell proliferation and adhesion. The formation of the bone-like apatite presents an extensive adsorption site for adhesive serum proteins. MC3T3-E1 cells with phagocytosis can make practical use of the internal part of the bone-like apatite containing serum protein by virtue of their gradual invasion thereof. It is necessary to distribute the serum protein all over the bone-like apatite for providing an active substrate for the MC3T3-E1 cells, Consequently, the bone-like apatite containing serum protein may well be applicable as a biomaterial with exceptional bone tissue compatibility. Moreover, an application of the bone-like apatite containing serum protein as a substrate for bone tissue engineering is possible, because the bone-like apatite containing serum protein can be formed on a material that we can already successfully coat with HAp using the biomimetic method.

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