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Behavior of fetal rat osteoblasts cultured in vitro on the DP-bioactive glass substratum

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Abstract

It has been shown that new bone grew readily into the macropores of the DP-bioactive glass with chemical composition of Na_2O 8.4%, CaO 40%, P_2O_5 12% and SiO_2 39.6% in weight ratio. The bioactive glass would gradually dissolve in the in vivo environment, which would then be progressively replaced by the regenerated bone. Since the material was biodegradable, we wished to know whether the degradable product of DP-bioactive glass would be harmful to the osteoblast. This report described a dynamic analysis of early behavior of fetal rat osteoblasts cultured on a DP-bioactive glass substratum and expressed how the osteoblast attached onto the DP-bioactive glass substratum.

Osteoblasts were isolated according to the method of Boonekamp with some modifications. Samples for morphological studies were seeded with a cell density of approximately 1×10^4 cells ml^{-1}, where 1×10^4 cells (1 ml) was added in each well and cultured for 8 h. Samples for cell population and rate of cell growth were seeded with a higher cell density of approximately 5×10^4 cells ml^{-1}, where 1 ml of cells was added in each well and cultured up to 6 days. During culturing, the six-well dishes were kept in an incubator (with 95% air humidity and 5% CO_2) at 37°C. During the culturing period, half of the medium was replaced every 72 h. After culturing for a period of time, the substrata were carefully taken out from the culturing well dish. Both substrata and well dishes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h, rinsed with PBS (3×5 min) and dehydrated in a graded ethanol series. Optical microscopy and scanning electronic microscopy (SEM) were used for examination. Alkaline phosphotase stain was utilized for cell phenotype identification. Soda–lime glass and well dish ground (free-substratum) were included in the experiment as controlled groups.

At the initial stage, the cell density of the free-substratum group was much lower than that of the DP-bioactive glass and soda–lime glass group. This was because there were more calcium ions on the free surface of DP-bioactive glass and soda–lime glass. In contrast, there were no free calcium ions on the surface of the free-substratum group. This group had to absorb calcium ions from the cultural medium to its surface at the initial stage. We speculate that an incubation time is needed to take up calcium from the medium, which might be the reason for the lower cell population in the initial 12 h for the free-substratum group.

The process of adhesion of osteoblasts in suspension to DP-bioactive glass substratum involved the following steps: (1) calcium ion and serum proteins were absorbed onto the DP-bioactive glass substratum to form a protein template or fibronectin; (2) rounded cells then contacted the substratum and cell mitosis could also be observed elsewhere; (3) attachment of the osteoblasts to the substratum; and (4) spreading of the osteoblasts on the substratum. The fact that osteoblasts were stained with alkaline phosphate shows that the osteoblast did not transform into other types of cells. This observation indicated that the DP-bioactive glass, although constantly releasing calcium ions into the medium, would not inhibit the osteoblast growth and would not cause morphological transformation.

Keywords: Bioactive glass; Osteoblast; Cell culture; Biodegradation

1. Introduction

An important problem in orthopedic surgery is the choice between a transplanted viable tissue and an implanted synthetic material. Since rejection phenomena and the risk of viral transmission are frequent, transplantation of living tissues by means of allogenic or xenogenic grafts represents only a minor part of reconstructive surgery [1–3]. Research on bone-substitute materials has been developed to overcome this problem. However, we have to face the limitations of these synthetic materials. Direct binding with the in situ tissue, and bone growth inside the prosthetic material should be anticipated in due time [2–5].

Calcium phosphate ceramics and glass ceramics have received much attention as potential bone implant materials

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because they can form a direct bond with bone tissue. It is well known that chemical reactions occurring on the ceramic surface play important roles in the bonding mechanism. In previous reports, DP-bioactive glass with chemical composition of Na_2O 8.4%, CaO 40%, P_2O_5 12% and SiO_2 39.6% in weight ratio has been shown to possess a suitable mechanical strength for orthopedic applications [6]. The material can also directly bond to the bone tissue [7]. The results of the quantitative assessment and histological evaluation gave significant evidence that the new bone grows readily into the macropores of the materials. The implant gradually dissolves in the in vivo environment, and is progressively replaced by the regenerated bone [6,7]. The bioresorbable behavior of the DP-bioactive glass could be considered to occur through a two-step process. The first step was the extracellular dissolution of the implant. The second step was the digestion of particles and migration of the particles. The developed material will be phased out in the physio-chemical environment and subsequently replaced by the regenerated bone tissue. It is believed to have a great potential in orthopedic applications in the near future. Since the material is a biodegradable material, we wished to estimate whether the degraded product of the bioglass is harmful to the osteoblast or not.

Biocompatibility testing in vitro often involves the detection of cell damage and death, i.e. cytoxicity [8]. The International Standards Organization recommends test methods to assess biocompatibility and mutagenicity tests. Whilst such screening is useful to detect overt adverse effects of a test material, other less dramatic expressions of incompatibility may be overlooked. For instance, the rate of growth, proliferation and differentiation of cells on a material may be dependent on successful initial attachment and spreading of the cells on the surface of the substratum [9,10]. In this respect, the initial and short-term responses of cells to an implant material in vitro may provide valuable indicators of the long-term biocompatibility in vivo [9–11]. By the way, as the bioactive glass demonstrated better bonding to bone, it is thought that the bioactive layer might stimulate osteoblasts in their bone forming behavior. In vivo, it appeared that a positive correlation existed between the formation of a bioactive layer and the appearance of collagen in the extracellular matrix. An investigation into the behavior of osteoblasts in close contact with bioactive glasses will give more understanding of the bonding-to-bone properties of the bioactive glass.

To eliminate the complexities normally present in in vivo studies, we chose a simple in vitro model using fetal rat osteoblast. The purpose of this study was to examine the cell behavior of fetal rat osteoblasts cultured upon the DP-bioactive glass substratum, and to investigate the proposed stimulating effects of DP-bioglass on osteoblast. This report describes a dynamic analysis of the early behavior of fetal rat osteoblasts cultured on a DP-bioglass substratum, and on a soda–lime glass substrate used as the contrast group. We used a combination of time-lapse video microscopy and scanning microscopy to monitor the behavior of dispersed cells upon initial contact and attachment to a DP-bioglass substratum. This has been facilitated by the inherent semi-transparency of the materials selected, which permits visual observation of the living cells. A sequence of cell phenotypes can be distinguished, each reflecting the behavioral activity at that time. Morphometric analysis of cell populations as they pass through this sequence has been used to assess the short-term cellular response to the substratum.

2. Materials and methods

2.1. Materials preparation

The glass used in the study was based on the DP-bioglass of Na_2O–CaO–SiO_2–P_2O_5 system. A batch mixture of nominal composition of Na_2O 8.4%, CaO 40%, P_2O_5 12% and SiO_2 39.6% in weight ratio was melted in a platinum crucible at 1400°C for 1 h. The melted glass was poured into 0°C ice–water to quench it as glass frit. The glass frit was pulverized into grains of about 5 μm using a Spex 8000 alumina ball mill. The glass powder was pressed into a disc of 6 mm in diameter and 5 mm in thickness under a hydrostatic pressure of 270 MPa. The glass powder compact was placed on a platinum sheet and heated to 810°C at a rate of 5°C min^{-1} in an SiC heated furnace. After 2 h soaking time, it was allowed to cool in the furnace. The sintered DP-bioglass discs were fixed with Mikroskopie (Merck, Germany) on a plane parallel dish and ground with water-cooled silicon carbide, followed by polishing with cerium oxide paste. During polishing water–ethanol mixtures, from 50–50% up to pure ethanol, were used to prevent the bioactive glass from undesirable corrosion by water. All glass samples were cleaned first by rising with xylene, and followed by a 50–50% acetone–ethanol mixture and then three times with pure ethanol. We obtained thin translucent disks with a smooth and clean surface (6 mm in diameter and 0.5 mm in thickness) [6,9].

Soda–lime glass was prepared with the same procedure as for the DP-bioactive glass. Sawing, grinding, polishing and cleaning were performed as described above, resulting in disks with the same dimensions.

2.2. Cell culturing method

Osteoblasts were isolated according to the method of Boonekamp with some modifications [11–13]. Briefly, calvaria from 20 day old rat embryos were excised aseptically. The calvaria were incubated for 2×10 min at 37°C with 4 mM EDTA in phosphate buffered saline (PBS). After rinsing the calvaria for 3×5 min with PBS, they were incubated for 10 min with collagenase (1 mg ml^{-1} PBS) at 37°C. The cell suspension obtained was discarded as it contained periosteal fibroblasts, still present after removal of the periostea. Then osteoblasts were isolated by further collagenase treatment for 2×30 min. The supernatant was centrifuged for 5 min at 1500 rpm. The pellet obtained was resuspended in culture

medium: minimum essential medium (α-MEM), containing 5% inactivated fetal calf serum, 1 mg ml^{-1} glucose and 90 μg ml^{-1} gentamycin. The sterilized substructures were placed in six-well culture dishes. Samples for morphological studies were seeded with a cell density of approximately 1×10^4 cells ml^{-1}, where 1×10^4 cells (1 ml) was added in each well and cultured for 8 h. Samples for cell population and rate of cell growth were seeded with a higher cell density of approximately 5×10^4 cells ml^{-1}, where 1 ml of cells was added in each well and cultured up to 6 days. During culturing, the six-well dishes were kept in an incubator (with 95% air humidity and 5% CO_2) at 37°C. During the culturing period, half of the medium was replaced every 72 h.

2.3. Preparation for optical microscopy and SEM

After culturing for a period of time, the substrata were carefully taken out of the culturing well dish. Both substrata and well dishes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h, rinsed with PBS (3×5 min) and dehydrated in a graded ethanol series. Substrata were then dehydrated by critical point drying and were followed by gold sputtering [11–14]. The samples were examined at 15 kV in a JEOL JAX-840A apparatus. The well dishes were stained (Sttute method) with hematoxylin and eosin stain for population observation. The alkaline phosphate stain was used to examine whether the osteoblasts transformed into fibroblasts or other tumor cells or not [15].

3. Results and discussion

3.1. The cell population observation

After culturing for a period of time, osteoblasts were washed out, centrifuged, collected and finally homogeneously mixed with PBS to 1 ml. 200 μl of the mixed solution was taken out and injected into cytometer for cell population calculation. In the experiment soda–lime glass was used as the contrast group, and free-substratum, i.e. no implant materials included in the culturing dishes, was used as the control group. Fig. 1 showed the summary of cell population and culture time where osteoblasts were co-cultured with DP-bioactive glass, soda–lime glass, and free-substratum, respectively. The cell population increased with the culturing time for all three groups. However, the DP-bioactive glass group has higher cell population all the time, compared with those of the soda–lime and free-substratum groups. The cell density even attained 4.8×10^5 cells ml^{-1} for the DP-bioactive glass group after culturing for 6 days. The soda–lime glass group and free-substratum group showed no significant difference in cell population during the experimental period.

After the initial 12 h of the experiment, the free-substratum group had a lower cell population, and increased thereafter with the culturing time. After culturing for 6 days, the cell population of the free-substratum group was about 2.3×10^5 cells ml^{-1}. The osteoblast growth of the soda–lime glass group had the same tendency as DP-bioactive glass group but the cell population was much lower than that of the DP-bioactive glass group. The cell population for the soda–lime glass group was approximately 3.1×10^5 cells ml^{-1} after 6 days of culture.

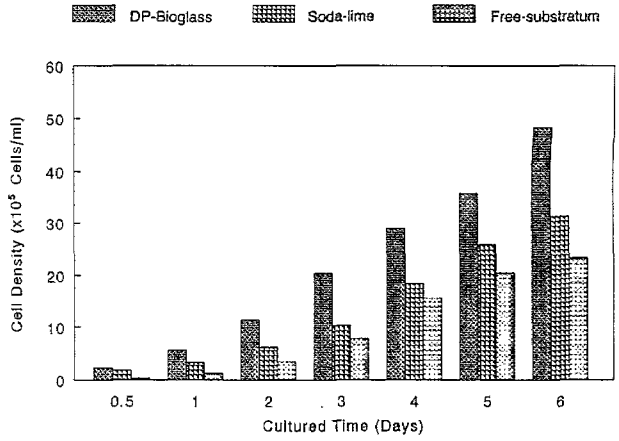

Fig. 1. Summary of cell population and culture time where osteoblasts were co-cultured with DP-bioactive glass, soda–lime glass, and free-substratum, respectively.

Fibronectins are thought to be involved in a wide variety of cellular activities [16,17]. The simplest unifying interpretation of these findings was that the fibronectins function as adhesive proteins that bind cells to other cells or to the substratum. This conclusion is based on experiments in vitro utilizing model systems of cellular adhesion, in which purified fibronectin is found to increase the adhesion of cells to cells or to the substratum on which they are grown. Serum factors are required for the attachment of cells to collagen-coated dishes or for cells to attach and spread on tissue culture dishes. These cell attachment and cell spreading factors are now supposed to be connected with serum fibronectin (cold insoluble globulin). Such serum factors can bind directly to collagen or to tissue cultural dishes in the absence of cells. The fibronectin-mediated attachment of cells to collagen or spreading onto plastic substratum requires divalent cations such as calcium and the expenditure of cellular metabolic energy. The calcium ion could form a chalet or complex compound to connect the material surface and fibronectin (or protein template), where cells could be attracted to the template for attachment and spreading [16–18].

In the initial stage, the cell density of the free-substratum group was much lower than that of the DP-bioactive glass and soda–lime glass groups. This was because that there were more calcium ions on the free surface of DP-bioactive glass and soda–lime glass. In contrast, there was no free calcium ion on the surface of the free-substratum group. This group had to absorb calcium ions from the cultural medium to its surface in the initial stage. We speculate that an incubation time is needed to take up calcium from the medium, which might be the reason for the lower cell population in the initial 12 h for the free-substratum group. The calcium ion concen-

tration in the cultural medium for the three groups will be discussed in the next section.

3.2. Calcium ion concentration

As mentioned in the previous section, calcium ions may play an important role in the formation of fibronectin and a protein template during the culture periods. The ion is also a required element for the ossification process because it is needed to reconstitute the mineral structure of regeneration bone. The ion was supposed to have a positive effect on the growth of the osteoblast. These two reasons led us to measure the calcium ion concentration in the culture medium. The measurement of calcium ion concentration in the culture medium was achieved by ICP–Auger electron spectroscopy.

Fig. 2 shows the calcium concentration in the culture medium in which the three test groups were soaked for 0.5–6 days. For the free-substratum group, the ion concentration decreased slightly in the initial 12 h and then maintained a constant value. We speculate that the free-substratum group had no calcium ion on the well ground material. The calcium ion in the medium had to be absorbed to the well ground to form a protein template or fibronectin that caused the decrease in ion concentration in the initial stage.

The ion concentration was not much changed in the experimental period for the soda–lime glass group. Soda–lime glass was thought to undergo only a small amount of dissolution in the medium and was supposed to have some of free calcium ion on its surface. It is not necessary for the soda–lime glass to absorb calcium ion from the medium onto its free surface, which was why the calcium ion concentration in the medium remained in a steady-state condition all the time.

In the DP-bioactive glass group, the calcium concentration in the medium was much higher than for the soda–lime glass and free-substratum groups. DP-bioactive glass has been shown to gradually dissolve in the physiological environment. The calcium ion would be constantly released from the DP-bioactive glass, causing the calcium concentration in the medium to increase with the cultural time. After 3 days, the concentration reached a plateau because the calcium concentration has been saturated in the medium. Since the calcium ion had a positive effect on the osteoblast growth, the high calcium concentration in the medium for the DP-bioactive glass group also led to a higher cell population which was in agreement with the observation in Section 3.1.

It has become increasingly manifest that minor or trace elements in a biological cell play important roles in various biological processes including metabolism, cell growth, gene expression, and cancer growth [19–21]. The same question should be raised: what would be the effects of calcium content on morphological and functional changes of the osteoblast? We prepared a series of solutions with different calcium contents in a range of 0–20 $\mu g\ ml^{-1}$, and then co-cultured them with the 1×10^5 osteoblasts for 2 days. The result are shown in Fig. 3. It reveals that the cell numbers increase with the calcium content and the control group (solution without calcium or 0 $\mu g\ ml^{-1}$) has a lowest value. It is possible that the calcium ion is beneficial to the growth of the osteoblast. Fig. 4(a) shows the morphology of osteoblast co-cultured with the solution of 10 $\mu g\ ml^{-1}$ calcium content for 1 day. The osteoblasts show proliferation to confluence. However, the osteoblasts cultured with controlled solution just attach on the well ground and show no sign of confluence (Fig. 4(b)).

The results of the calcium ion concentration measurement were quite coincident with that of Section 3.1. The cell population of the free-substratum group was much lower than those of the other two groups in the initial 12 h, because there was an incubation time for calcium accumulation to form a calcium complex to connect the protein template and fibronectins. The DP-bioactive glass group demonstrated a higher cell population because of the constant release of calcium ions from the material playing a positive role in the cell growth.

In addition, we observed osteoblasts co-cultured with DP-bioactive glass for 2 days. The osteoblasts were well attached, reached confluence, and fused in parallel with each other. The osteoblasts stained with alkaline phosphate showed that the osteoblast did not transform into other types of cells after culturing for 2 weeks (Fig. 5). The observation reflected that the DP-bioactive glass, although constantly releasing calcium

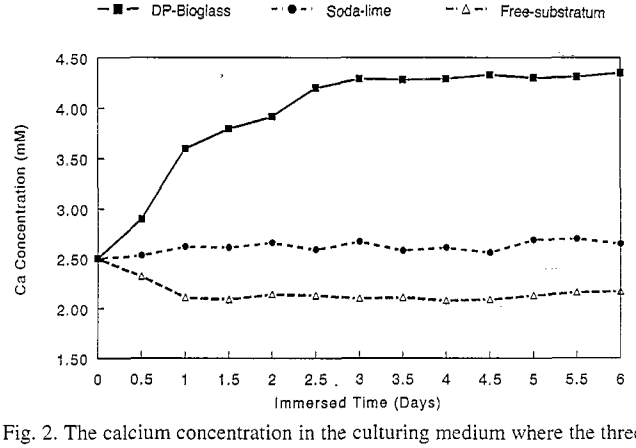

Fig. 2. The calcium concentration in the culturing medium where the three test groups were soaked for 0.5–6 days.

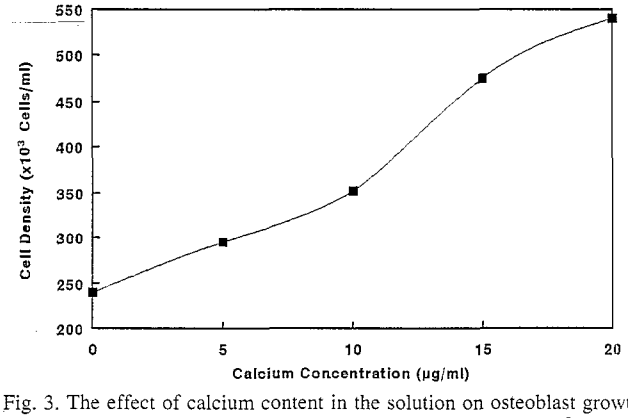

Fig. 3. The effect of calcium content in the solution on osteoblast growth after being cultured for 2 days.

Fig. 4. (a) The morphology of osteoblasts cultured with a solution of calcium content 10 μg ml^{-1} for 2 days. The osteoblasts proliferated to confluence. (b) Osteoblasts just attached on the well ground and showed no sign of confluence having occurred after culturing with controlled solution for 2 days.

Fig. 5. Osteoblasts stained with alkaline phosphate showing that the osteoblast did not transfer into other types of cells after being cultured with DP-bioactive glass for 2 weeks.

ion into the medium, would not inhibit the osteoblast growth and would not cause the morphological transformation.

3.3. The adhesion of cultured osteoblast to DP-bioactive glass substratum

From the SEM observation, a highly invariant sequence of events of osteoblasts attaching on the DP-bioactive glass could be defined. On sedimentation onto the substratum from suspension, osteoblasts exhibited many bleb-like protrusions which were constantly in motion (the intensity of blebbing varied from cell to cell). At the point where attachment to the substratum occurred, blebbing largely ceased and, upon withdrawal of the blebs, the cell acquired a smoother profile. Initial attachment was followed by a spreading stage, during which extension of the cytoplasm along the substratum proceeded with a concomitant flattening of the central mass. The margin of the spreading cell was invariably active. It was characterized by extension and withdrawal of filopodia and lamellipodia. It then gave rise to the appearance of ruffle. Spreading typically took place in a radially symmetric fashion, but where a distinct leading edge became established. The cell then became polarized both in its spreading and in its subsequent locomotion. This general pattern of osteoblast attachment behavior was observable in the soda–lime glass and free-substratum groups, although with important quantitative differences.

A number of behavioral phenotypes were defined in sequence, which exemplify the changes in morphology displayed by an osteoblast as it attached and adhered to a DP-bioactive glass substratum [12–14,22]. The process of adhesion of osteoblasts in suspension to DP-bioactive glass substratum involved the following steps (as shown in Figs. 6–10): (1) calcium ion and serum proteins were absorbed onto the DP-bioactive glass substratum to form a protein template or fibronectin; (2) rounded cells then contacted the substratum and cell mitosis could also be observed elsewhere; (3) attachment of the osteoblasts to the substratum; and (4) spreading of the osteoblasts on the substratum. The attachment and spreading of various cultured cells onto the solid substrates have been studied by optical microscopy. SEM studies revealed more morphological details: the osteoblasts made initial contact with the DP-bioactive glass surface through the radial extension of filopodia followed by cytoplasmic webbing between the filopodia or the formation of footpads which may mediate osteoblasts–DP-bioactive glass substratum adhesion. Finally, cytoskeletal elements within

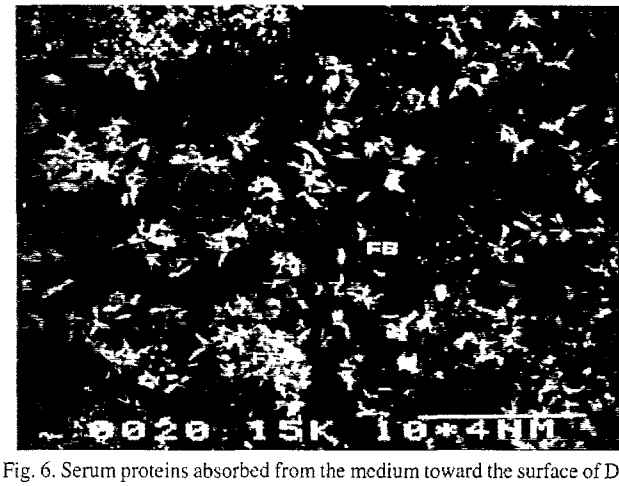

Fig. 6. Serum proteins absorbed from the medium toward the surface of DP-bioactive glass substratum. FB: serum proteins and fibronectin.

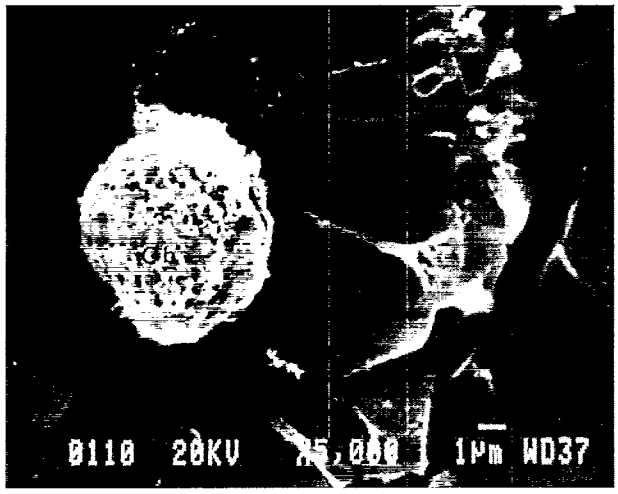

Fig. 7. Thin film of protein template or fibronectin covered onto the surface of DP-bioactive glass substratum. Pt: protein template, Fb: fibronectin, Ob: osteoblast.

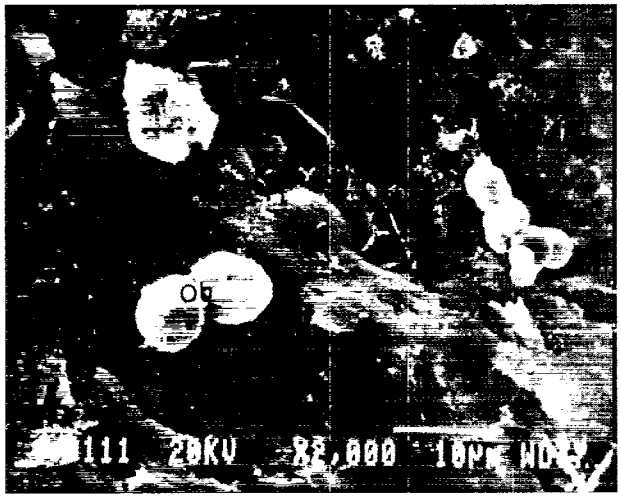

Fig. 8. Rounded cells attached onto the protein template or fibronectin covered on DP-bioactive glass substratum. Ob: osteoblast, Pt: protein template, Fb: fibronectin.

Fig. 9. Cell mitosis observed elsewhere. Ob: osteoblast, Pt: protein template, Fb: fibronectin, Cm: cell mitosis.

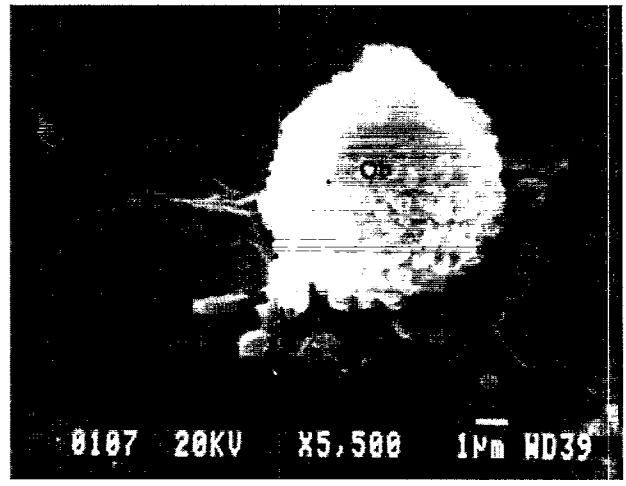

Fig. 10. Osteoblasts attached to and spread on the substratum through the filopodial filaments sprawl on the protein template. Ob: osteoblast, Fl: filopodial filament.

the osteoblasts reorganized, resulting in flattening of the central mass of the osteoblasts.

4. Conclusions

The cell populations increased with the cultured time for all the three test groups. The DP-bioactive glass group had a higher cell population all the time, compared with the soda–lime and free-substratum groups. The cell population even attained 4.8×10^5 cells ml^{-1} after culturing for 6 days. The soda–lime glass and free-substratum groups showed no significant difference in cell population during the experimental period. In the initial 12 h of the experiment, the free-substratum group had a lower cell population, which then increased with the culturing time. The cell growth of the soda–lime glass group had the same tendency as the DP-bioactive glass group but the cell population was much lower than that of the DP–bioactive glass group. The cell population of the free-substratum group was much lower than that of the other two groups in the initial 12 h, because there was an incubation time for calcium accumulation to form a calcium complex which connects the protein template and fibronectins. The DP-bioactive glass group demonstrated a higher cell population because of the constant release of calcium ions from the material, which exerts a positive effect on the cell growth.

The process of adhesion of osteoblasts in suspension to DP-bioactive glass substratum involved the following steps: (1) calcium ion and serum proteins were absorbed onto the DP-bioactive glass substratum to form a protein template or fibronectin; (2) rounded cells then contacted with the substratum and cell mitosis could also be observed elsewhere; (3) attachment of the osteoblasts to the substratum; and (4) spreading of the osteoblasts on the substratum. The osteoblasts were stained with alkaline phosphate, which shows that the osteoblast did not transform into other types of cells. The observation reflected that the DP-bioactive glass, although

constantly releasing calcium ions into the medium, would not inhibit osteoblast growth and would not cause morphological transformation.

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