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Morphology expression and proliferation of human osteoblasts on bioactive glass scaffolds

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Bioactive glass was designed as a scaffold to be used for bone reconstruction or regeneration. Bioactive glass scaffold with pore sizes ranging from in 100 μm to 400 μm in diameter was fabricated by the sol-gel method, and the biocompatibility evaluation of bioactive glass scaffolds was also performed by culture *in vitro* models. Cells cultured in the extracts of bioactive glass appeared to show normal morphology. The scaffolds supported osteoblast growth and induced differentiation within the 21 day culture period. Confocal laser scanning microscopy demonstrated a normal cell distribution and proliferation on porous biomaterials. Osteoblasts attached and proliferated on the scaffold as demonstrated by scanning electron microscopy (SEM). Nodule formation and multilayer structures were observed on the scaffold surface and in the pores of the glass. The relationship between seeding density and viability of human osteoblasts cultured on the porous bioactive glass were measured.

Key words: *bioactive glass scaffold; morphology; proliferation; human osteoblasts; biomaterials*

1. Introduction

Bioactive glass and ceramic materials have been given a lot of attention as candidates for implant materials since they possess certain highly desirable characteristics for some clinical applications of human skeleton substitution, heart values renewing, dental crown repairing [1, 2]. An ideal scaffold for bone tissue engineering applications should fulfil several criteria. First, the scaffold should be biocompatible (non toxic) and act as a three-dimensional (3D) template for *in vitro* and *in vivo* bone growth [3]. It therefore must consist of an interconnected macroporous network with a modal interconnected pore diameter of at least 100 μm to allow cell migration, bone

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ingrowth and eventually vascularization [4, 5]. The scaffold material should be the one that promotes cell adhesion and activity and ideally stimulates osteogenesis at the genetic level [6] so that a tissue engineered construct can be grown *in vitro*, ready for implantation. This construct should have mechanical properties matching those of the host bone. The scaffold should bond to the host bone, creating a stable interface and the scaffold should then resorb at the same rate as the bone is regenerated, with non-toxic degradation products.

Bioactive glass material used to repair and substitute human hard tissue is a new branch of material field and is to be gradually noticed in the field of medicine and clinic. Bioactive glass was chosen as a scaffold material because bioactive glasses bond to bone and stimulate bone growth (osteoinduction) [6]. Bioactive glass may meet biological requirements for its composition and microstructure should be similar to those of hard tissue of human beings. Even though the mechanical performance of bioactive glass cannot be matched to human hard tissue (bone and tooth), bioactive glass can be used as scaffolds to allow cell growth and be helpful to promote bone reconstruction or regeneration in the way of tissue engineering. In many cases, mechanical properties are much less important compared with biological features [7]. For example, due to excellent mechanical properties, metallic implants or prostheses are used to connect broken bones. However, stress that must be transformed by bone is impaired by the metallic implants, consequently, there is no stress to stimulate tissue growth at the broken bone ends, and the bone reconstruction would stop soon. On the other hand, if biodegradable implants such as bioactive glass are used as scaffolds, cellular tissue can grow into the pores in the scaffolds and partial stress will pass through the broken ends which may result in a stimulation action to accelerate bone reconstruction. In the present paper, biodegradable bioactive glass scaffolds were fabricated and modulated by cell culture before the scaffolds are moved into human body [8]. The initial response of human osteoblasts to bioactive glass scaffolds was investigated. Some biological performances related to both material feature and cell characters are revealed in this study.

2. Experimental

Fabrication of bioactive glass scaffolds and their characterization. The composition of porous glass is: SiO_2 (60 mol %), CaO (35 mol %), and P_2O_5 (5 mol %). First, bioactive glass powder was prepared by the sol-gel method [9]. Sol was prepared from tetraethylorthosilicate (TEOS), deionized water as a solvent, hydrochloric acid as a catalyst, and calcium nitrate and TEP as CaO and P_2O_5 precursors. The synthesis was carried out at a low pH causing a spontaneous gelation owing to hydrolysis of TEOS and subsequent condensation of formed Si–OH groups. The sol was kept 3 days at room temperature, to allow the hydrolysis and polycondensation reactions, until the gel was formed. For aging, the gel was heated at 60 °C for 3 days. The dried gel was heated at 160 °C for 2 days, then was ground for 8 h. Then the bioactive glass powder

was blended with foams and polyvinyl solution to obtain slurry, which was soaked by a porous organic material [10]. Finally, the specimen was sintered at 700 °C for 3 h. Pore size in specimen was controlled by pore size in the porous organic material.

Scanning electron microscopy (SEM) on gold-coated specimen was used to examine the morphological and textural features of the sample, using an accelerating voltage of 15 kV. X-ray diffraction (XRD) trace was obtained of the sample using a Philips PW1700 series automated XRD spectrometer, using a step scanning method with CuK_α radiation in Θ–2Θ scans and grazing incidence 2Θ scans. Macropore size distributions were determined by intrusion mercury porosimetry (Poresizer 9320, Micromeritics, U.S.A.). The mesopore diameter distribution was calculated by the BJH method [11] applied to the desorption curves, and micropore analysis was determined by the Horvath and Kawazoe method [12].

Proliferation and morphology of cell cultured in the extract. The cytotoxicity of the scaffold was assessed by preparing aqueous extracts according to the recommended method of International Standards Organisation (ISO) [13]. Stroma osteoblastic cells were obtained from the marrow of young adult male. The tissue culture plastic polystyrene was used as non-toxic negative control material and tin-stabilised (tributyltin) polyvinylchloride was used as the positive toxic control material. Before preparing the aqueous extracts, the bioactive glass discs were sterilized in an autoclave. The control material discs were immersed in 70% (volume fraction) ethanol for 1 h prior to treatment with PBS containing 100 units/cm^3 penicilin and 100 μg/cm^3 streptomycin for 1 h. Then all the discs were immersed in the extracting media for preparation of the extracts. The extract media were Dulbecco's modification of eagles medium (DMEM) supplemented with 10% foetal calf serum (FCS), 50 units/cm^3 penicillin and 50 μg/cm^3 streptomycin for cells. The ratio of the volume of the extractant to the surface area of the material was 1 cm^3/cm^2. The extraction process was carried out in a water bath at 37 °C in 75 cm^3 flasks which were shaken at a speed of 60–65 rev/min. After extracting for 48 h, the extracts were passed through a 0.22 μm filter, then stored at –20 °C. Cell attachment and cell morphology were observed under the reverse microscope.

Cell culture and proliferation on the scaffolds. For cell culture studies, scaffold samples were cut to 8×8×4 mm^3 and fixed to 24-well polystyrene culture plates using 2% agar (to prevent floating). The entire well plates were then sterilized under ultraviolet (UV) light for 1 h. The samples were then incubated in DMEM, supplemented with 1% (v/v) penicillin/streptomycin, at 37 °C for 72 h (preconditioning). Cell seeding was performed by addition of a concentrated cell suspension in a drop-wise manner to ensure cell loading directly onto the foams. After 10 days, other unattached cells were removed from the flasks by repeatedly washing with PBS. With confluent monolayers reached, cells were enzymatically lifted from the flasks by trypsin.

Aliquots of 50 mm^3 of cell suspensions with cell seeding densities of 5×10^3, 1×10^4, 5×10^4, 1×10^5, 1×10^6 and $5\times10^6/cm^2$ were seeded on the top surface area of the bioactive glass scaffolds (specimen) which were mounted in the 24 well polystyrene culture plats, respectively. The scaffolds were left undisturbed in an incubator for 45 min to allow the cells to attach to the scaffolds，after which time an additional 2 cm^3 culture medium containing 20 mm^3 dexamethasone was added to each well. Medium was changed every 2 days. At time intervals of 1, 2, 3, 4, 5, 6 weeks, cells in the scaffolds were washed three times by PBS，and deattached by trypsin solution. Cell numbers were determined both by heamocytometer counter and by a fluorometric quantification of DNA by an assay adapted from West. Cells were visualized in the confocal laser scanning microscope (CLSM) studied by staining the sample with ethdium and phalloidin.

For SEM observation, the specimens cultured for 21 days were dehydrated in a graded series of methanol prior to critical-point drying. The disks with the cultured cells were mounted on the aluminium stubs and coated with gold in a sputtering apparatus, and finally the specimens were examined at 15 kV under a scanning electron microscope.

Total protein determination of cells cultured on materials. To investigate cell proliferation and colonization rate on the materials, osteoblastic cells were cultured on the materials placed in 24 well plates with cell seeding densities of $5\times10^4/cm^2$. After seeding 1 h, 1.5 cm^3 culture medium was added to each well and the samples were incubated as before. At 24 h intervals the medium in the each row (4 wells) was removed and the cells washed with PBS to remove all the traces of the culture medium. On the 8th day, the final row was taken, and the cell protein in all the wells was solubilised using 1 cm^3 0.5mol/dm^3 NaCl and total protein content determination by the modified Lowry assay. Briefly, to each well, 100 mm^3 of 0.1 M NaOH was added and the plates were then covered with mylar film. The plates were swirled gently over night before 50 mm^3 of the cell lysates were transferred to a new 24 well plate together with bovine serum albumin (50 mm^3 of 0.063–1.0 μg/cm^3) as a standard. 200 mm^3 of the solution containing 50 cm^3 of 2% Na_2CO_3 dissolved in 0.1 M NaOH mixed with 0.5 cm^3 of 1% $CuSO_4$ and 0.5 cm^3 of 2% Na-K-tartrate was added to each well and the plates were swirled for exactly 10 min. 50 mm^3 of the Fohlin–Ciocalteu phenol reagent (diluted 1:5) was then added to each well and the plates were again left to swirl for 30 min. Absorbance was monitored at 690 nm using a Labsystems iEMS Reader MF. For comparison, a control group of cells was cultured in the 24 well plates (made of polystyrene) without materials.

Statistical analysis. All measurements were collected and expressed as mean standard deviations. Single factor analysis of variance was employed to assess the statistical significance of results for all biological experiments.

3. Results

3.1 Materials characterization

The XRD study of the bioactive glass confirms that the material is amorphous (Fig. 1) because no diffraction maxima are observed and only a broad band for 2Θ between 10° and 50° is detected for the gel bioactive glass.

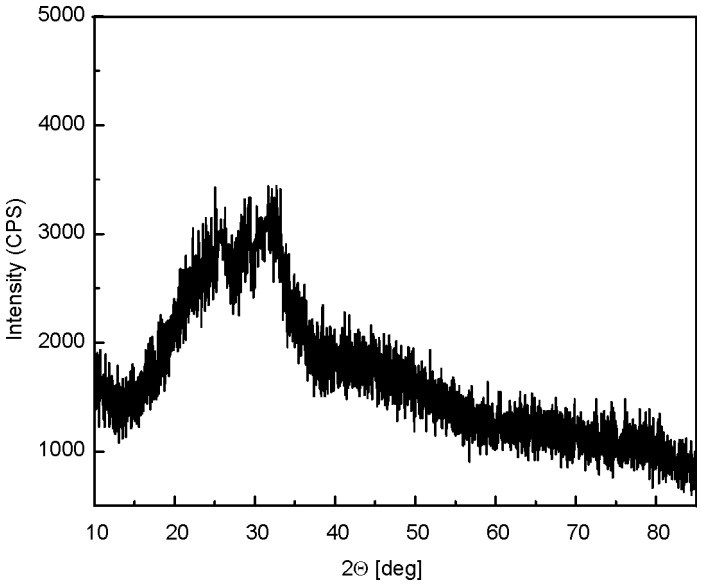

Fig. 1. XRD of bioactive glass

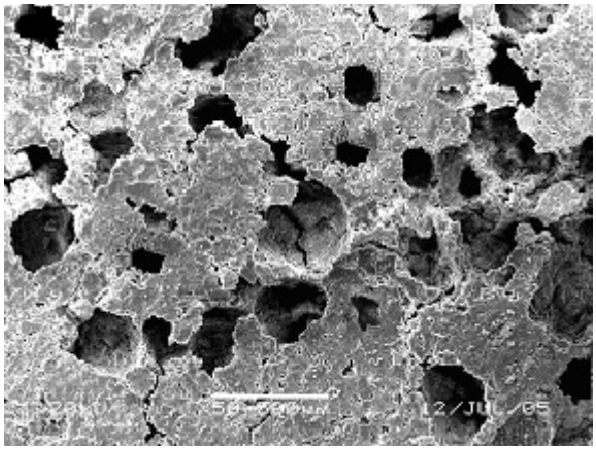

Fig. 2. SEM of bioactive glass scaffold obtained by the sol-gel method

SEM in Fig. 2 shows typical macroporous structure of the bioactive glass scaffolds with pore size ranged within 100–400 μm, which is 10–20 diameters of human osteoblast. The average pore size diameter is 55.75 μm and the pore volume is 0.435 cm^3/g measured by low pressure mercury intrusion porosimetry. Porous glass has a narrow and small pore size distribution in mesoporous scale with diameters in the range of 2–50 nm. The micropores in samples are mostly between 0.5 and 1 nm and the distributions are almost identical. Macropore size in scaffold is very important because cell growth in pores requires enough nutrition and metabolism. Too small pore size will limit cellular metabolism and also limit the cells to move into the pores. For cell growth in the pores, cell number must be sufficient because the environmental condition must be met for cell proliferation which depends on cell communication each other. The lower the cell density, the less information, the lower proliferation rate [14].

3.2. Cell morphology cultured in the extracts

Figure 3 shows the morphological observation of osteoblastic cells cultured in the extracts and the control. Cells appeared to show normal morphology in both the negative control (Fig. 3b) and extracts (Fig. 3c) with cell sizes ranging from 10 to 20 μm in diameter. Cells attached to the culture plate, and cells round in shape were in state of

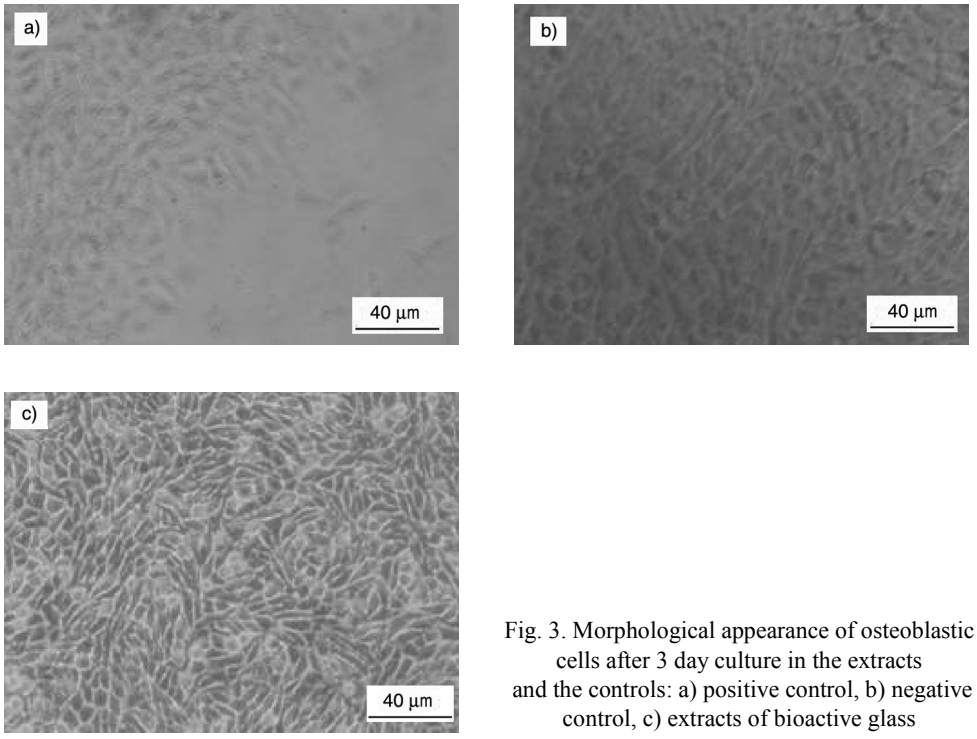

Fig. 3. Morphological appearance of osteoblastic cells after 3 day culture in the extracts and the controls: a) positive control, b) negative control, c) extracts of bioactive glass

synthesis of DNA (cell mitosis) which indicated that cells had strong generation abilities. However, it is apparent that the number of cells was increased in glass extract more than that in negative control. This indicated that bioactive glass extracts can promote cell growth and differentiation. After 3 day exposure to the positive control, almost all of the cells died and detached of the bottom of the culture plate (Fig. 3a). These results showed no toxicity was present in bioactive glass materials, and the positive control was severely toxic and caused a marked detachment and death in cell culture.

3.3. CLSM and SEM observation

As a result observed by CLSM (Fig. 4), after stained with ethdium and phalloidin, human osteoblast cells with the nucleus in the colour of red grew well on the surface of materials. Cells were observed to attach and spread on the scaffold structures. Cell morphology image showed elongated ellipses, with long processes extending from the cell body. In porous materials, cells grew along the walls of the pores and penetrated down to the bottom of many pores. There were some changes in the cell morphology depending on the shape of the pore wall.

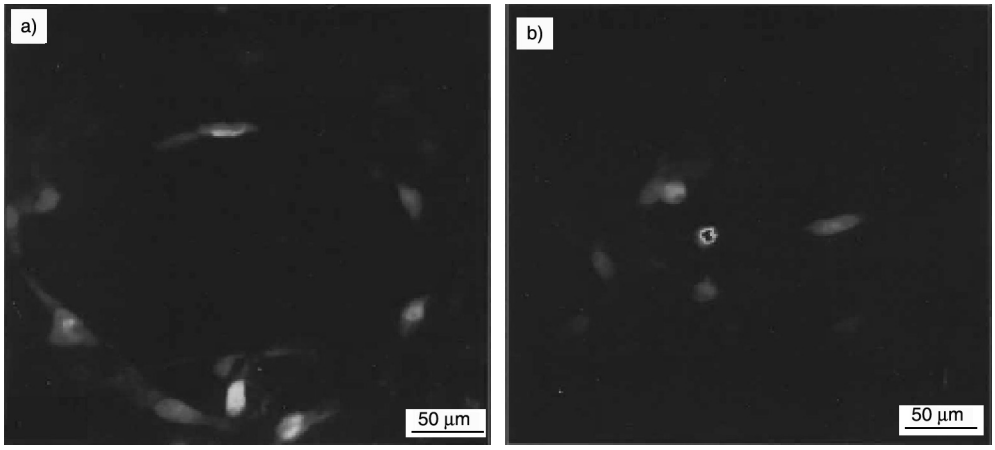

Fig. 4. CLSM images of cells in pores: a) 20 µm from the surface, b) 40µm from the surface

Mineralized nodule formation is basically considered to be the final result of differentiation and function of the osteoblasts. Figure 5 shows the osteoblasts morphology on the bioactive glass scaffold, where cells have been cultured for 21 days. Cells formed multilayer structures on surfaces of the materials (Fig. 5a), and nodule structures where mineralization occurs can be observed. The nodules were connected to the cell surface by extracellular fibres. The same multilayer structures and nodules were formed in scaffold pore, and cells attach and spread as shown in Fig. 5b. We found many nodules of various sizes and shapes on the cell multilayer. Cells surrounded collagenous matrix, matrix vesicles, and minerals. The rough surface of the porous

material may result in an uneven distribution of cells. Also some cells may migrate into and grow in the deep pores. Therefore, the cell distribution on the porous material surface is different from place to place.

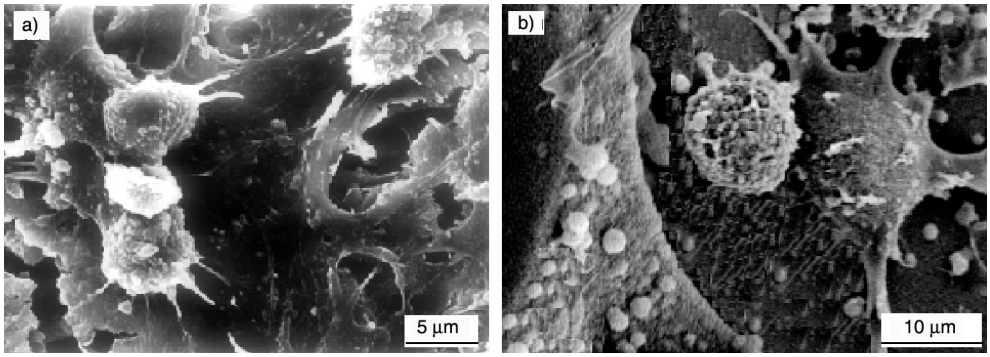

Fig. 5. SEM of human osteoblasts cultured on bioactive glass scaffold surface (a) and within a pore (b) for 21 days

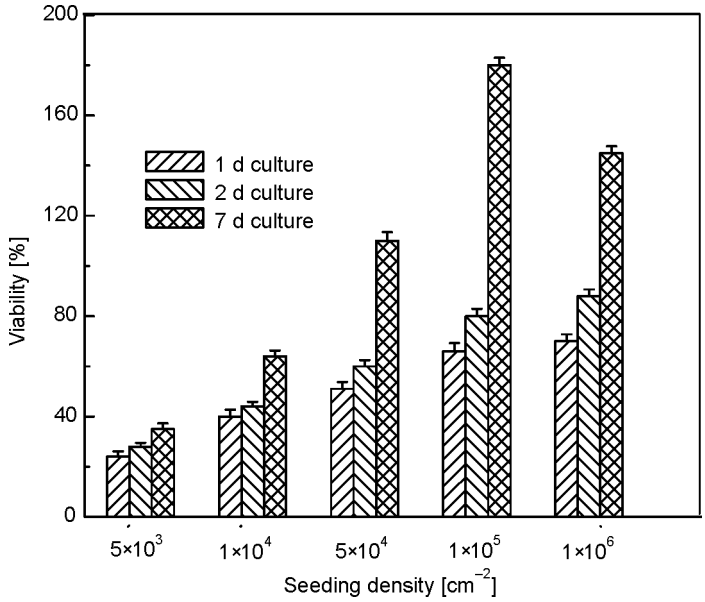

Fig. 6. Viability of human osteoblasts cultured on a bioactive glass

Cell proliferation at the surface of bioactive glass was measured by determining the increase in DNA content up to 7 days (Fig. 6). The proliferation results indicate a correlation between culture time and cell proliferation. Cell viability increased with the increase of culture time, there were statistically significant differences in cell viability between 7 day culture and fresh ones when seeding density exceeded $1\times10^4/cm^2$. On the other hand, cell viability was significantly influenced by seeding den-

sity, as shown in Fig. 6. For cell healthy growth, seeding density at least of $1\times10^4/cm^2$ is required; otherwise, cells in pores will die due to less information among cells.

3.4. Total protein determination of cells culture on materials

Proliferation of the human osteoblasts was measured quantitatively in terms of protein content on the materials (Fig. 7). Our results showed that total protein increased gradually in all cultures from day 1 up to day 6, then decreased, thereafter increased again after day 8. During the early stages of culture (days 1-4), no differences was noted between the bioactive culture and the control. However, on day 6 the total protein was significantly higher in the bioactive culture when compared to control cultures. From the results of protein assay, osteoblasts proliferation is more rapid on bioactive glass than on polystyrene, which indicates bioactive glass has the stimulating effects to promote cell proliferation rate.

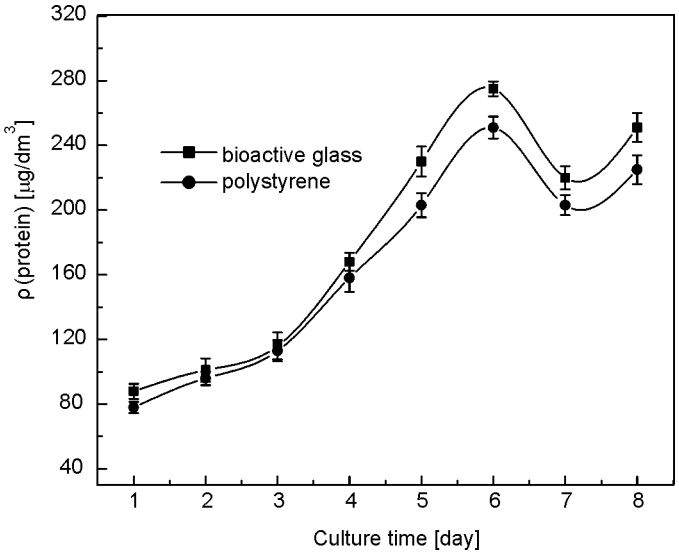

Fig. 7. Proliferation in terms of total protein of cells on bioactive glass compared with the cell culture on polystyrene culture plates

4. Discussion

An ideal scaffold would be the one that mimics the extracellular matrix of the tissue that is to be replaced so that it can act as a template in three dimensions onto which cells attach, multiply, migrate and function. The biological characteristics of bioactive glass based materials when used as implants depend on many factors such as

material composition, microstructure, porosity, and pore structure (morphology and size) [10].

Bioactive glasses are amorphous silica based materials that are biocompatible, bioactive, osteoconductive, and even osteoproductive [15]. Their bone bonding ability has been attributed to the formation of a hydroxycarbonate apatite (HCA) layer in the surface of the glass on contact with body fluid [16]. The composition and structure of the HCA layer is similar to that of bone mineral, hence a strong bond can be formed. An important discovery for the development of regenerative medicine is that dissolution products from bioactive glasses exert a genetic control over osteoblast (bone progenitor cells) cell cycle and rapid expression of genes that regulate osteogenesis and the production of growth factors [17, 18]. Silicon has been found to be a major contributor to the mineralization of bone and gene activation, which leads to the substitution of silicon for calcium into synthetic hydroxyapatite. *In vivo* results showed that bone ingrowth in silicon substituted HA granules was significantly greater than that into phase pure HA granules [16].

In the present investigation, an excellent biocompatibility of bioactive glass has been demonstrated by the *in vitro* cell growth on the material. Normal morphology of cells was retained after culture on material, and the cells were able to attach and grow on the material surface as well in deep pores within the materials. Primary human osteoblasts were observed to attach, spread, proliferate and form mineralized nodules when cultured on bioactive glass materials. Extensive nodule formation on the scaffolds was observed by SEM.

CLSM is an important method in many areas of biological and medical research. By confocal scanning, the out-of-focus-blur can be eliminated. The illumination is restricted to a single point and almost all the light emission from regions above and below the focal plane of a CLSM is physically prevented. A clear image can be obtained. Compared with SEM observation, CLSM is more favourable to observe cells in situ and to investigate the cell biological components and cell substructure by using staining. Specially, by CLSM the cells on different layers behind the first layer can be clearly observed and their shape measured. Therefore, the cells attached to the pore wall in the porous material could be scanned step by step down to the deep pore and numbers of cells at precise depths throughout the substratum could be estimated. A difficulty in our research was that neither CLSM nor SEM is able to observe cells which have moved into the lateral pores within the material.

During bone regeneration by autogenous osteoblast sterilized from stem marrow cells, the scaffolding materials used in this approach must allow the attachment of osteblasts because they are anchorage-dependent cells that require a supportive matrix in order to survive and proliferate. This approach shows that osteoblast can attach to the bioactive glass scaffolds. Subsequently, human osteoblasts populated the constructs by the proliferation of the transplanted cells and the migration of cells into the construct from the surrounding tissue while the bioactive glass gradually degrades or

partly degrades. Eventually, the construct is filled with calcified extracellular matrix secreted by the osteoblasts and is devoid of the synthetic biodegradable bioactive glass.

Cell proliferation on the materials was assessed by measuring total protein content by the Lowry method. Previous studies [17, 18] have shown that measurement of the total cell protein in proliferating cell cultures correlates well with cell numbers throughout the log phase of growth. As the osteoblasts approach confluence, they produce extracellular matrix on the materials. This is composed predominantly of type I collagen and, although it will contribute to the overall protein content of the cultures, it will not be detected by the Lowry assay. This method detects tyrosine, trytophan, and phenylalanine amino acid residues on protein molecules, whereas collagen, being mainly composed of glycine, praline and hydroxyproline, is not detected. The protocol used for the Lowry assay ensures that only attached cells are measured, and it is assumed that to remain attached to the materials the cells must be viable. Thus, the protein content is equivalent to the number of viable cells.

Cell viability was significantly influenced by seeding density. We failed to culture cells at low seeding densities on porous materials if the material had not been immersed in culture medium. We believe that there are two factors producing this effect. One is related with the cells themselves. Growing cells need to communicate with each other, and at low density due to lack of communication, cell colonization in deep pores would be impaired. The other factor results from the material surface. Higher bioactivity of material surface will induce acute inflammation by metabolism and secretion will impair after cells phagocyte the materials.

5. Conclusions

Bioactive glass scaffolds support the attachment, proliferation and mineralized nodule formation of human primary osteoblasts. It provides an appropriate environment for the proliferation and differentiation of osteoblasts and allows the ingrowth of vascular tissue to ensure the survival of the transplanted cells. During short culture term, cells displayed normal morphology features and migrated into deep pore. Multilayer structures and nodules were formed on the scaffold surface and in the pores of the glass. The result of total protein assay reviews that bioactive glass has the stimulating effects to promote cell proliferation rate. This scaffold therefore has potential in bone tissue regeneration.

Acknowledgements

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