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Behaviors of Osteoblast-like Cell (MC3T3-E1) on Collagen Grafted Porous Poly L-lactic Acid (PLLA) Membranes with Various Pore Sizes

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Biodegradable three-dimensional porous poly L-lactic acid (PLLA) membranes were applied to culture of mouse calvaria osteoblasts (MC 3T3-E1) to examine the effects of scaffold pore size on cell attachment, proliferation and functions of the cells. PLLA scaffolds have pore sizes in the range of 100-200 μm, 200-300 μm, 300-425 μm and 425-500 μm with a thickness of 1.0 mm. The polymer membranes supported the attachment and the proliferation of seeded osteoblasts, and there was no significant difference in the attachment and the proliferation among the porous PLLA membranes. The differentiation markers of osteoblasts (alkaline phosphatase activity, collagenous protein synthesis and mineralization) increased significantly over the time when the cells were cultured on the porous PLLA membrane with a pore size of 200-300 μm. Collagenous protein produced by osteoblasts on polymer scaffold was mainly composed of type I collagen as proved sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In this study, type I atelocollagen was grafted onto porous PLLA membranes as a biocompatible factor. Osteoblast-like cells displayed an elongated shape and oriented around the pores of type I atelocollagen grafted porous PLLA membranes. The pore size over the range tested was an important parameter for cell function, but did not affect the attachment and the proliferation of MC 3T3-E1. This study suggests that the topography of scaffold should be considered for the transplantation of autogenous osteoblasts to regenerate bone tissue.

Key words: Porous PLLA membrane; Pore size; Osteoblast-like cell (MC 3T3-E1)

INTRODUCTION

Development of a three-dimensional tissue equivalent to bone *in vitro* requires a support to induce adsorption of extracellular matrix, cell adhesion, proliferation, and osteoblastic function (deposition of a collagen matrix and mineralization). The interactions of bone cells with synthetic materials are the subject of intensive studies because of the critical need for materials that help the regeneration of bone tissue.[1-3] An effort to regenerate bone must consider the biological activity of the scaffold and surrounding milieu. In the body the extracellular matrix influences development, polarity, and behavior of anchorage-dependent cells. Furthermore an optimal polymer should provide support but at the same time slowly be degraded, relinquishing space for the formation of new bone by osteoblasts.[1, 4] In addition polymers have to fulfill additional material requirements necessary for biocompatibility, relatively high cell adhesion, cell growth, and retention of osteoblast's function.

Ideal characteristics for a tissue-grafted matrix include spatial and compositional properties that attract and guide the activity of reparative cells. Poly(α-hydroxy ester) is currently being investigated as materials for regeneration of several tissues including cartilage, bone, liver, and intestine.[1-2, 5-6] Poly L-lactic acid (PLLA) is one of the strongest poly (α-hydroxy esters),[4] and is used in the form of screws and plates for internal bone fixation.[3] Synthetic biodegradable polymers can provide temporary scaffold for transplanted cells and allow the cells to secrete extracellular matrix (ECM), enabling a completely natural tissue replacement to occur. The scaffolds should

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be osteoconductive so that osteoblasts attach and migrate on them, and they should be mechanically strong to maintain their structural integrity during culture.[7] Bone graft substitute such as PLLA must be porous allowing tissue ingrowth, and stabilization, and have average pore size of 200-400 μm.[8]

Using the porous PLLA membranes with various pore size as a basis to manipulate substrate topography, we have investigated the effect of pore size on the spatial organization and differentiated functions of surface-localized cultured osteoblast-like cells (MC 3T3-E1) on type I atelocollagen grafted porous PLLA membranes with various pore sizes.

Materials and Method

Preparation of porous PLLA membranes with various pore sizes

PLLA porous membranes were prepared by a solvent-casting particulate-leaching technique.[9] PLLA (MW: 100,000, Polyscience Inc, PA. 18976, USA) was dissolved in chloroform (Sigma C-5312, St. Louis, USA) as the final concentration of 12% (w/w). Sieved NaCl particles, which have pore sizes of 100<d<200 μm, 200<d<300 μm, 300<d<425 μm, and 425<d<500 μm (d: diameter), were added in a 12% PLLA solution and the vortexed dispersion was casted in a glass Petri dish. The solvent was allowed to evaporate from the covered Petri dish over 48 hrs. The resulting PLLA/salt composite membranes were immersed in distilled water on a shaker for 48 hrs to leach out the salt.

Microscopic characterization

After 24 hrs-fixation, surface and cross-section morphology of porous PLLA membranes with pore sizes of 100<d<200 μm, 200<d<300 μm, 300<d<425 μm, and 425<d<500 μm (d: diameter) were observed by a scanning electron microscope (SEM, Hitach S-800, Tokyo, Japan)

Type I atelocollagen grafting onto the ozone treated porous PLLA membranes

Type I atelocollagen was prepared as previously described,[10] and grafting them onto PLLA membrane foams was processed by ozone oxidation as previously described.[11] After ozone treatment at 60 V for 60 min, the membrane was immediately immersed in 0.5% type I atelocollagen solution in a glass tube. Mohr's salt [$FeSO_4(NH_4)_2SO_46H_2O$] was added to the solution to decompose the peroxides, and coupling was allowed to proceed at 35 °C and pH 8.0.[12] The non-grafted type I atelocollagen was removed by washing thoroughly with distilled water, and type I atelocollagen grafted PLLA membranes were dried in a vacuum oven at 25 °C.

Cell culture

Mouse calvaria osteoblasts (MC 3T3-E1) were maintained in α-modified essential medium (α-MEM) supplemented with 5% FBS, penicillin (100 U/ml), and streptomycin (100 g/ml) in tissue culture flasks in a CO_2 incubator (5% CO_2, 95% humidity) at 37 °C. Confluent monolayers were propagated by trypsinization (0.25% trypsin, 0.02% EDTA) and replating at 1:2 dilution. For experiments, osteoblasts were used in the third to fifth passages.

Cell attachment

Each specimen (1.2 cm^2) of type I atelocollagen grafted porous PLLA membranes treated by ozone at 60 V for 60 min was thoroughly washed by phosphate-buffered saline (PBS) solution and held to tissue culture plate (48 well). 2.0×10^4 osteoblasts were seeded on the specimens, and cultured for 4 hrs in α-MEM supplemented with 5% FBS. Unattached cells were removed by washing with PBS, and the attached cells were separated from the substrate by incubation in 100 μl of 0.25 w/v trypsin solution for 10 min at 37 °C, and 400 μl of media was added in later. After centrifugation, cells were placed in fresh medium. An aliquot of the resulting cell suspension was counted by using a Neubauer haemocytometer (Sigma Z35, 962-9, St. Louis, Mo., U.S.A.)..

Cell proliferation

Type I atelocollagen grafted porous PLLA membranes were fixed onto the bottom of tissue culture plates (48 well) by an inert, silicone-based, vacuum grease to prevent them from floating in the growth media. 2.0×10^4 MC 3T3-E1 were plated on specimens or the Tissue Culture Polystyrene (TCPS) well bottom in α-MEM supplemented with 5% FBS, and the medium was changed every 24 hrs during incubation in a CO_2 incubator. Cell proliferation on each specimen was determined after 1, 3, 5, 7, and 9 days. To remove unattached cells, specimens were gently washed by phosphate buffered saline (PBS) solution. The attached cells were separated from the substrate by incubation in 100 μl of 0.25 w/v trypsin solution for 10 min at 37 °C, and 400 μl of media was added in later. After centrifugation, cells were placed in fresh medium. An aliquot of the resulting cell suspension was counted by using a Neubauer haemocytometer (Sigma Z35, 962-9, St. Louis, Mo., U.S.A.).

Alkaline phosphatase activity

Porous PLLA membranes were fixed to the bottom of

tissue culture plates (48 well) by an inert vacuum grease. 2.0×10^4 osteoblasts were plated and cultured on specimens or the 48 well plate in α-MEM supplemented with 5% FBS, and the medium was changed every 24 hrs during incubation in a CO_2 incubator. ALPase activity on each specimen was determined after 1, 3, 5, 7, and 9 days. To remove unattached cells, specimens were gently washed by PBS solution. Each specimen was incubated with 100 μl of a ρ-nitrophenyl phosphate solution (Diagnostic Kit 245, Sigma, St. Louis, Mo., U.S.A.) at 37 °C for 30 min. The production of ρ-nitrophenol in the presence of ALPase was measured by monitoring light absorbance by the solution at 405 nm using ELISA reader (Spectra Max 340, Molecular Device Inc., Ca., U.S.A.). The ALPase activity represented nanomoles of ρ-nitrophenol releases per one specimen after 30 min of incubation at 37 °C.[13]

Mineralization

The bone-like nodule formation was observed using alizarin red-S histochemical staining and morphologic examination of osteoblast-like cells cultured on type I atelocollagen grafted porous PLLA membranes. MC3T3-E1 was cultured on each specimen in α-MEM supplemented with 5% FBS for 12 days. Cultured osteoblasts and matrix were fixed on ice for 30 min with 100% (v/v) methanol and subsequently air-dried. The fixed cells and matrix were washed with PBS, and stained with 0.02% toluidine blue O (Sigma T-3260, St. Louis, U.S.A.) for 10 min prior to alizarin red-S staining. The stained cells and matrix were washed with 0.1 M borate buffer, pH 4.0, and stained with 0.5% (w/v) alizarin red-S (Sigma A-5533, St. Louis, U.S.A.) for 15 min. The stained matrix was washed with borate buffer and distilled water. Finally, the stained matrix was air dried and photographed.

Characterization of type I collagen synthesized by cells

MC 3T3-E1 was allowed to attach for 4 hrs in α-MEM containing 5% FBS, and then was cultured on porous PLLA membranes. After the culture, the proteins were extracted by treatment with 0.5 M acetic acid containing pepsin (2800 unit/mg, P-7013, Sigma co., St Louis, Mo., U.S.A.) for 4 hrs at room temperature with shaking. The protein-extract solutions were transferred to 1.5 ml microcentrifuge tubes along with 2.0 M NaCl. The tubes were incubated at 4 °C with shaking for 90 min, and then centrifuged 13,000 rpm for 15 min at 4 °C. The precipitates were suspended again in 0.5 M acetic acid. The extracts were analyzed using sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE). Loading buffer [50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 0.01% (w/v) bromophenol blue] and type I collagen (Sigma C-3511, St Louis, U.S.A.) solution (5 mg/ml in 0.5 M acetic acid) as the marker were prepared. 8% running gels overlaid with 5% stacking gel, and then the gel was

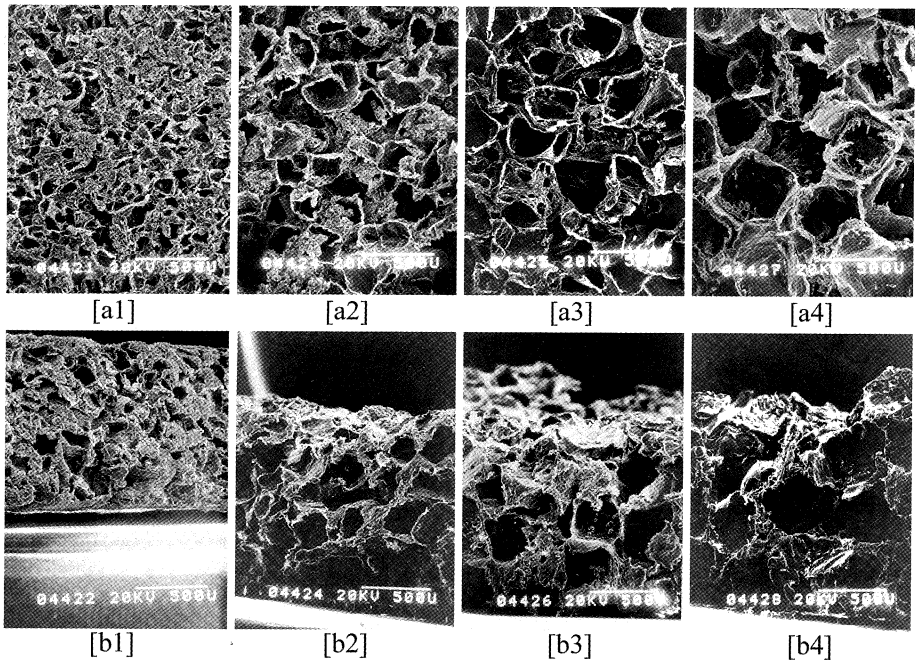

Figure 1. The surface and the cross-section morphology of porous PLLA membranes (SEM, (×50). (a1) 100-200 μm PLLA surface, (a2) 200-300 μm PLLA surface, (a3) 300-425 μm PLLA surface, (a4) 425-500 μm PLLA surface, (a2) 100-200 μm PLLA cross-section, (b2) 200-300 μm PLLA cross-section, (c2) 300-425 μm PLLA cross-section, (d2) 425-500 μm PLLA cross-section.

run at 80 V until the dye front ran off the gel. The gel was removed from the glass plates and briefly washed in distilled water. The gel was stained by Coomassie Brilliant Blue R250 (Sigma B-0149, St Louis, U.S.A.) on a shaker at room temperature. After completion of staining, gel was briefly rinsed in distilled water and photographed by CSC camera. The intensities of the bands were quantified by densitometry and normalized relative to the levels of type I collagen.

Results

Microscopic characterization of porous PLLA membranes

Porous PLLA membranes were prepared by a solvent-casting particulate-leaching technique. The surface and cross-section morphology of porous PLLA membranes was observed by SEM. As shown in figure 1, the salt particle size affected the pore size distribution of porous PLLA membranes. PLLA scaffolds have pore sizes in the range of 100-200 μm, 200-300 μm, 300-425 μm and 425-500 μm with a thickness of 1.0 mm.

Cellular behavior on porous PLLA membranes

Cell attachment onto the surface of type I atelocollagen grafted porous PLLA membranes was performed to study the interaction of grafted surface with osteoblast-like cell. The density of attached cells per surface area of 1.2 cm^2 was determined by cell counting after incubation for 4 hrs. As shown in figure 2, there was no significant difference among porous PLLA membranes in MC 3T3-E1 attachment ($P>0.05$). The number of attached cells was 9400±892, 9450±622, 8583±1068, and 8333±1080 cells on porous PLLA membranes with 100-200 μm, 200-300 μm, 300-425 μm, and 425-500 μm of pore size, respectively.

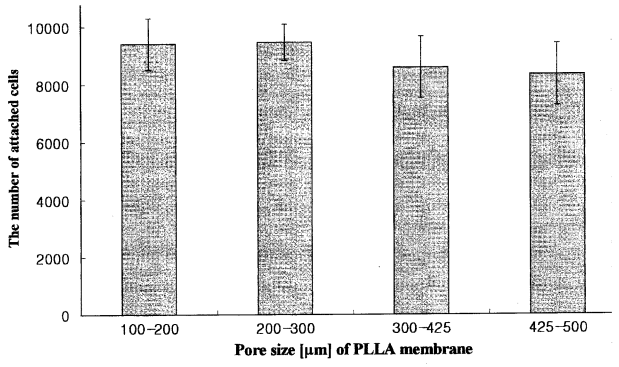

Figure 2. Attachment of osteoblast-like cells (MC 3T3-E1) onto type I atelocollagen grafted porous PLLA membranes: 100-200 : 100-200 μm PLLA, 200-300 : 200-300 μm PLLA, 300-425 : 300-425 μm PLLA, 425-500 : 425-500 μm PLLA. Each point represents the mean±SEM cells in 6 specimens.

MC 3T3-E1 proliferation was observed for 1, 3, 5, 7, and 9 days. As shown in figure 3, there was no significant difference among type I atelocollagen grafted porous

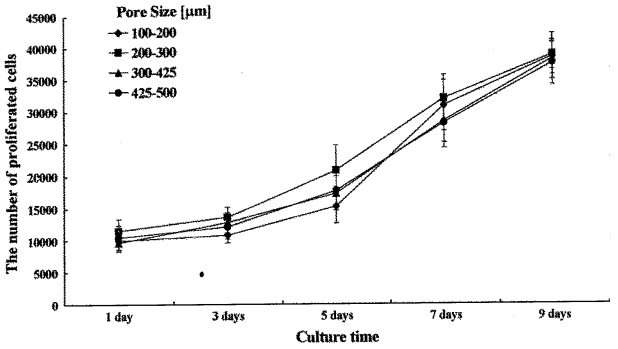

Figure 3. Osteoblast-like cell (MC 3T3-E1) growth on type I atelocollagen grafted porous PLLA membranes for 9 days. (◆): 100-200 μm PLLA, (■): 200-300 μm PLLA, (▲): 300-425 μm, (●): 425-500 μm PLLA. Each point represents the mean ±SEM cells in 6 specimens.

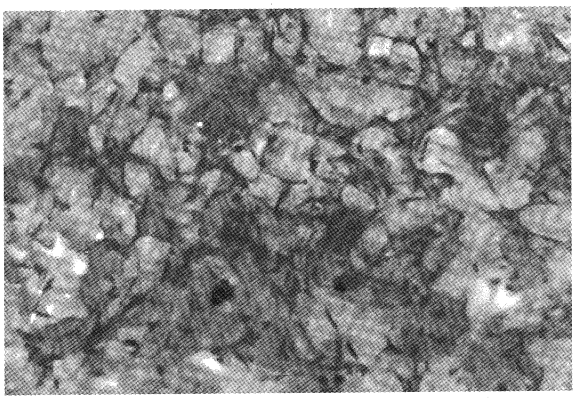

Figure 4. Morphological characterization of osteoblast-like cells (MC 3T3-E1) cultured on type I atelocollagen grafted porous PLLA membrane.

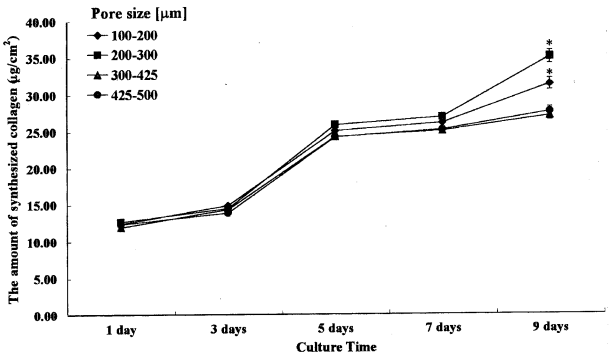

Figure 5. Type I collagen synthesis by osteoblast-like cells (MC 3T3-E1) cultured on type I atelocollagen grafted porous PLLA membranes for 9 days. (◆): 100-200 μm PLLA, (■): 200-300 μm PLLA, (▲): 300-425 μm PLLA, (●): 425-500 μm PLLA: Each point represents the mean±SEM μg/cm^2 in 6 specimens. A statistically significant ($P<0.05$) increase in 100-200 μm PLLA and 200-300 μm PLLA group was observed after 9 days of culture.

PLLA membranes in MC 3T3-E1 growth ($P > 0.05$).

Collagenous protein synthesized by MC 3T3-E1 cultured on porous PLLA membranes for 1, 3, 5, 7, and 9 days was investigated. As shown in figure 5, the collagenous protein synthesized by MC 3T3-E1 on the porous PLLA membrane with 100-200 μm and 200-300 μm of pore size was slightly higher ($P<0.05$), which were 31.25 ±0.79 and 34.95±0.89 μg/cm^2, than on other porous PLLA membranes in 9 days. Compared to porous PLLA membranes with 100-200 μm and 200-300 μm of pore size, the collagenous protein synthesized on the porous PLLA membrane with 300-425 μm and 425-500 μm of pore sizes were 27.00±0.56 and 27.55±0.64. SDS-PAGE was used to characterize the proteins produced by MC 3T3-E1 on porous PLLA membranes. (Figure 6) The gel for matrix extracted from the culture on porous PLLA membranes showed characteristic bands of α1 (I), α2 (I) and α1 (III) chain of type I collagen.[14] Other higher-molecular-weight bands (arrows) were not characterized but considered to be uncoiled collagen molecules.

MC 3T3-E1 grown on the type I atelocollagen grafted porous PLLA membrane with 200-300 μm of pore size expressed greatly higher ALPase activity ($P<0.001$), which were 267.8±48.5, 263.1±61.0 and 519.4±105.9 nmole/min/mg, than ALPase activity on the culture of other porous PLLA membranes in 5, 7 and 9 days of the experiment. Compared to porous PLLA membrane with 200-300 μm of pore size, MC 3T3-E1 grown on porous PLLA membranes with 100-200 μm, 300-425 μm and 425-500 μm had relatively low ALPase activity, which were 103.3±17.7, 73.5±21.9 and 91.8±20.1 nmole/min/mg, in 5 days. However, MC 3T3-E1 grown on PLLA membrane with 100-200 μm of pore size expressed slightly higher ALPase activity ($P<0.05$), which were

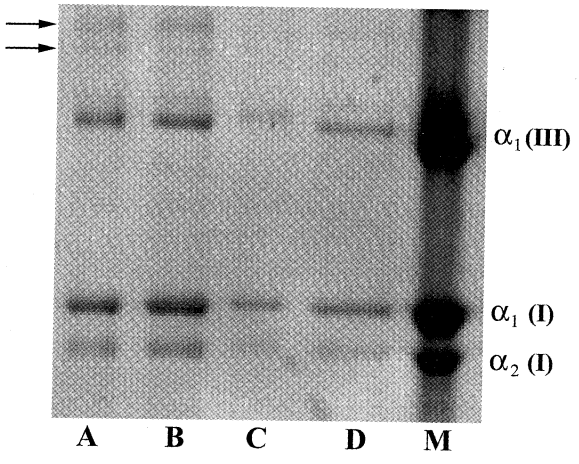

Figure 6. Characterization (SDS-PAGE) of type I collagen produced by osteoblast-like cells cultured on type I atelocollagen grafted porous PLLA membranes after 9 days of culture. (A): 100-200 μm PLLA, (B): 200-300 μm PLLA, (C): 300-425 μm PLLA, (D): 425-500 μm PLLA, (M) Type I collagen (Sigma) as control marker

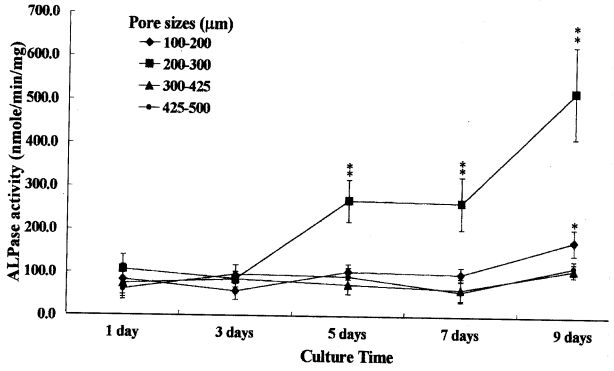

Figure 7. Alkaline phosphatase activity of osteoblast-like cells (MC3T3-E1) cultured on type I atelocollagen grafted porous PLLA membranes for 9 days. (◆): 100-200 μm PLLA, (■): 200-300 μm PLLA, (▲): 300-425 μm PLLA, (●): 425-500 μm PLLA: Each point represents the mean±SEM nmole/min/mg in 6 specimens. A statistically significant ($P<0.001$) increase in 200-300 μm PLLA group was observed after 5 days of culture. (*: $P < 0.05$, **: $P < 0.001$)

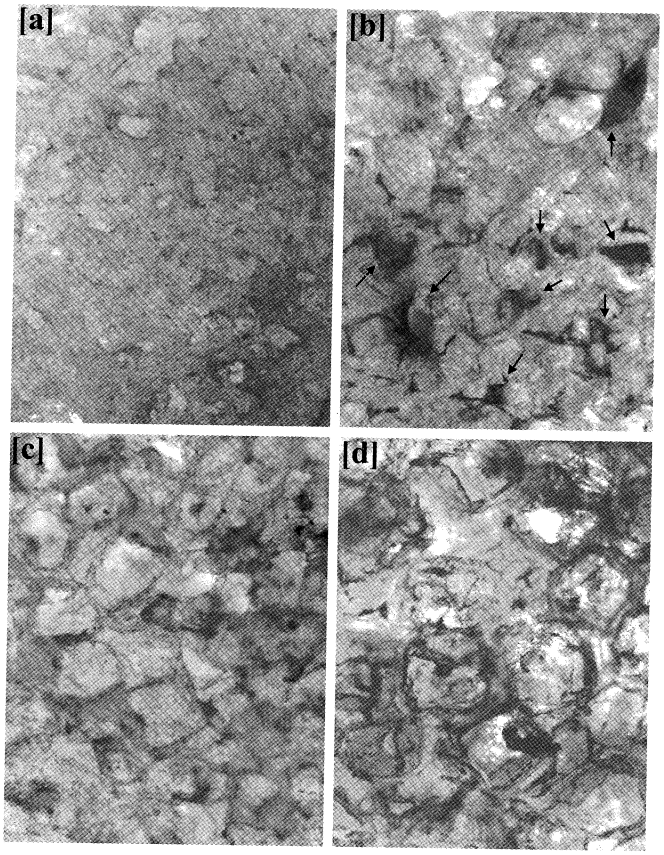

Figure 8. Morphological characterization of the mineral deposition phenotype in osteoblast-like cells (MC3T3-E1) on type I atelocollagen grafted porous PLLA membranes in 12 days (Alizarin red-S staining). (A): 100-200 μm PLLA, (B): 200-300 μm PLLA, (C): 300-425 μm PLLA, (D): 425-500 μm PLLA, Arrow: mineral deposits

175.3±30.2 nmole/min/mg, than those of porous PLLA membranes with 300-425 μm and 425-500 μm, which were 109.5±15.1, and 116.4±16.1 nmole/min/mg cells at 9 days of the experiment. (Figure 7)

The mineralization in MC 3T3-E1 on porous PLLA membranes was determined by Alizarin red-S staining in 12 days. Mineralized nodules were generally formed in culture on the type I atelocollagen grafted porous PLLA membrane with 200-300 μm of pore size. In contrast, mineralized nodule was not yet detected in culture on other porous PLLA membranes in 12 days. (Figure 8)

Discussion

The ideal bone graft is autologous bone graft. However, there is a limit to obtain its mass enough to be required. To avoid this surgical invasion, synthetic materials were used as a bone graft. A matrix as a three-dimensional cell substratum is crucially important to construct a tissue-like structure either *in vivo* or *in vitro*. The ingrowth of tissue into porous biomaterial is dependent on the biocompatibility of the implant material and on the interconnection and dimensions of its pore structure. The capacity of bone tissue in response to mechanical demands has long been recognized but the cellular activity remained poorly understood.[15] In this study, cellular activity in porous PLLA membranes in relation to their pore sizes was investigated in order to establish an optimal pore structure for use as a bone graft substitute.

Collagen grafted porous PLLA membrane might provide suitable support for cell adhesion and growth.[16] The results of this experiment presented that there is no significant difference of cell attachment and proliferation among collagen grafted porous PLLA membranes with different pore sizes. Cell attachment and proliferation were not influenced by the topography such as pore size of collagen grafted porous PLLA membranes.

The approximate stage of osteoblast's differentiation in vitro can be estimated by determining the relative expression levels of marker proteins such as type I collagen and alkaline phosphatase. Alkaline phosphatase is thought to play a primary role in mineralization and has been proposed to be a progression factor in osteoblast differentiation.[13] Osteoblasts (MC 3T3-E1) cultured on PLLA membranes with 100-200 μm and 200-300 μm of pore size showed relatively higher alkaline phosphatase activity than others. Especially, alkaline phosphatase was much highly expressed by osteoblasts cultured on PLLA membranes with 200-300 μm of pore size in 5 days in comparison with that on PLLA membranes with 100-200 μm of pore size.

Type I collagen is predominant matrix protein synthesized by osteoblasts, and occupied to approximately 94% of collagen with small amount of type III and V collagens. As a major component of bone ECM, type I collagen has been reported to elicit increased alkaline phosphatase activity in osteoblastic cells.[17] In this study, ECM proteins, which was deposited by osteoblasts cultured on porous PLLA membranes, were extracted by pepsin treatment, and then characterized by SDS-PAGE. Type I collagen was major protein in ECM proteins synthesized by osteoblasts, proved by the characteristic bands of α1 (I), α2 (I) and α1 (III) type I collagen from the result of SDS-PAGE (Figure 6). And the amount of synthesized type I collagen was higher when cells were cultured on the PLLA membranes with 100-200 μm and 200-300 μm of pore size than others. Expression and ordered accumulation of type I collagen during the matrix maturation periods are required for mineralization and development of the osteoblast phenotype.[13]

Transplantation of isolated cells seeded in biodegradable PLLA has been also investigated as a new means of creating biologic substitutes to regenerate, replace, or enhance tissue function. Synthetic polymers have been widely used for a variety of biomedical applications, and are generally designed to be appropriate for their proposed mechanical function, and bioinert. The limiting factors in the use of polymers as implants are lack of tissue compatibility and lack of resistance to biological environment. One approach to enhance the biocompatibility of an implant material is to exploit the normal interaction of cell with their extracellular matrix (ECM) molecules. Therefore, extensive work to develop practical and economical methods for surface modification of polymer has been carried out, and many different biologically functional molecules have been chemically or physically immobilized on polymeric supports.[18-21] For binding biomolecules covalently to an inert solid polymer surface, type I atelocollagen was grafted onto the porous PLLA membranes through ozone oxidation in this study as previously described.[11] The collagen is a major biological macromolecule of ECMs in tissues, and it has successfully been used to produce commercialized biomaterials for a wide range of applications including burn dressings, hemostats, and soft tissue augmentation.[22-24] The successful use of the collagen may be attributed to their low immunogenicity and high capacity of cell adherence.[16]

The ability of bone cells to migrate into the biomaterial and the relationships they establish between their membrane and the biomaterial surface may directly influence their activity. In bone, the β1-integrin subunit is shared by several integrins in osteoblasts.[25, 26] The β1-subunit is involved in adhesion of osteoblast to type I collagen and

is supposed to play a role in osteoblast morphology. As shown in figure 4 and 8, osteoblast-like cells displayed an elongated shape and oriented around the pores of type I atelocollagen grafted porous PLLA membranes. The type I collagen grafted on porous PLLA membrane might appear to influence the orientation of the collagen fibers deposited by cells.[27]

Osteoblasts not only synthesize the bone matrix but mineralize it as well.[28] The bone-like nodule formation (mineralization) was observed by morphologic examination of osteoblasts cultured on porous PLLA membranes. Mineralized nodules were generally formed by osteoblasts on the PLLA membranes with 200-300 μm of pore size after 12 days of culture. This mineralization might be related to the high expression of alkaline phosphatase and type I collagen by osteoblasts.

The results from ALPase activity, mineralization and collagenous protein synthesis indicated that the optimal pore size for differentiation of mouse calvaria osteoblasts was approximately 200-300 μm. Pore size is an important parameter for bone ingrowth into scaffold. Numerous studies have demonstrated the potential for bone growth into porous system. The in vivo studies to dates have concluded that the optimum pore size for bone ingrowth is probably near 200 μm.[29, 30] But the mechanism for cell activity related to pore size has not been understood well and would be studied further. This study using chemically identical but geometrically different cell substrata is the first demonstration that a matrix with a certain geometrical size is most favorable for cell differentiation. A minimum pore diameter of 100 μm has been found to be necessary for penetration of bone into superficial pore layers. Development of osteons inside the pore has been found to take place at diameters of 200 μm.[31]

Current strategies for tissue engineering focus on the extension of cell-matrix basic science principles for the development of implantable matrices that mimic natural tissue. We have previously shown that collagen grafted onto polymer provides a favorable environment for cell attachment and function.[11] This study demonstrates that topography of polymer scaffold such as pore size may be also an important factor for promoting cellular function.

Conclusion

This study has demonstrated that mouse calvaria osteoblasts can be cultured on three-dimensional poly L-lactic acid membranes to form a calcified bone-like tissue in *vitro*. Osteoblast attachment and proliferation were not affected by the pore size of PLLA membranes in the range of 100-500 μm and increased over time for all scaffolds. However, the pore size affected significantly cellular function related to osteoblast maturation. Alkaline phosphatase activity, type I collagen synthesis and mineralization were especially promoted in the culture on PLLA membrane with a pore size of 200-300 μm compared to other pore sizes. Achieving cell culture on the surface of three-dimensional porous scaffold may require topography of scaffold such as pore size.

Acknowledgements

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