SHORT COMMUNICATION

Cross-linked collagen gel spheres as a useful carrier for cell culture of MC 3 T 3-E 1 clonal osteogenic cells

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Introduction

Biochemists have been recently focusing their attention upon the local factors that regulate the growth and differentiation of animal cells. In the case of bone cells, bone-derived growth factor¹⁾, bone morphogenetic protein²⁾, skeletal growth factor³⁾ and differentiation factor⁴⁾ were isolated and partially characterized. These regulating factors are believed to be synthesized by osteoblasts, and secreted into bone matrix. However, purification and characterization of these factors from bone matrix is generally difficult mainly due to their low quantities in the bone tissue.

The goal of this study is to establish a large-scale cell culture of osteoblasts to isolate these factors from the culture media and to obtain detailed information about the action mechanism of these factors on the formation and resorption of bone. So far, no one has succeeded in the large-scale cell culture of osteoblasts. The main reason for this fact might be that there has been no suitable carrier of osteoblasts.

Osteoblasts are anchorage-dependent cells and need some solid phase for the growth and expression of their phenotypes. In order to expand the surface of the solid phase, van Wezel developed a microcarrier cell culture, in which he used positively charged dextran with an average diameter of 150 μ m as a carrier of fibroblasts⁵⁾. This proved to be useful for large-scale cultures.

In the present study, we introduced collagen gel spheres for the first time as a carrier of osteoblasts and succeeded in demonstrating that collagen gel spheres are a suitable carrier for osteoblasts.

Materials and Methods

Collagen gel spheres (Collagen Microsphere) were provided by Koken Co. (Tokyo). These spheres with an average diameter of 0.5 mm were made of reconstituted collagen from bovine skin. The spherical form of the gel was obtained by suspending soluble collagen in toluene at 5 °C and then incubating it at 37 °C. The collagen gel spheres cross-linked with 0.5% hexamethylenediisocyanate.

Cell culture: The MC3T3-E1 clonal osteogenic cells were isolated from newborn C57/BL/6 mouse calvaria^{6,7)} and were provided by Dr. Kodama H. (Nihonzenyaku Co. Fukushima). The MC3T3-E1 cells were grown in α modification of Eagle's minimal essential medium (Irvine scientific, Santa Ana, CA) supplemented with 10 % fetal calf serum and 60 μ g/ml of kanamycin sulfate. Cells were maintained at 37 °C in a fully-humidified atmosphere of 5 % CO₂ in air. These cells were verified to produce a bone-like structure with calcified nodules upon being cultured 25 days after confluency on conventional plastic dishes. The collagen spheres were placed in culture medium for 24 hours before use. Approximately 1×10^6 cells were inoculated in 60-mm plastic dishes which contained 200-300 collagen gel spheres and

Fig. 1 Cross-section of osteogenic cell layers developed on the collagen gel spheres. The cells were cultured for 70 days after inoculation. Stained with haematoxyline-eosin. Note that cells piled up and made seven to eight layers. $\times 80$.

2 ml of culture medium. The culture medium was changed every two days. Collagen gel spheres were harvested on the 28th and 42nd days after confluency.

Measurement of alkaline phosphatase activity: After collagen spheres were washed with calcium, magnesium-free physiological saline, alkaline phosphatase activity was measured by the Kind-King method⁹⁾.

DNA content: DNA content was measured by the method of Hinegardner⁹⁾.

Detection of newly synthesized protein with fluorography: Collagen gel spheres were cultured with medium containing [³H]-proline for 24 hours (0.1 mCi/dish) on the 28th and 42nd days after confluency. The fraction which contained cell and matrix was harvested and was homogenized in 0.5 M EDTA/1 M NaCl/0.05 M Tris-HCl (pH 7.4). The cell-matrix homogenate was analyzed by SDS polyacrylamide gel (7.5 % and 12.5 %) electrophoresis¹⁰⁾. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue and fluorographed¹¹⁾.

Results and Discussion

Osteoblasts attached and elongated on the surface of collagen spheres with a fibroblast-like shape after 24 hours. Cells continued to divide on spheres and covered the entire surface at the 7 to 9th day. At 15 to 20th days, cells have changed their shape from a fibroblast-like form to a polygonal one.

Fig. 2 Electron microscopy of intercellular matrix on collagen gel spheres which were cultured for 70 days. Hydroxyapatite crystals were precipitated (indicated by arrows) on and between collagen fibers.

Fig. 3 Alkaline phosphatase activity of osteoblasts cultured on a plastic dish (Control) and on the collagen gel spheres (Collagen Gel).

After confluency, the cells on spheres took on a multi-layer configuration and formed a seven-or eight-cell-layered structure (Fig. 1).

Cell number per bead reached to average 2,100 at 28 days, and this value corresponded to 3.2×10^7 cells/ml. Considering that the cell density in the conventional monolayer

Fig. 4 Fluorograms of the proteins extracted from the cell-matrix fraction cultured on a conventional plastic dish (a) and from collagen gel sphere culture (b). Note the similarity of the patterns found in the two systems. (A) 28 days after confluence. (B) 42 days after confluence. α_1 and α_2 indicate α_1 chain and α_2 chain of type 1 collagen, respectively.

culture system is usually maximum 2 to 5×10^6 cells/ m^2 ⁽¹²⁾, in this study our results indicated that collagen gel culture is a useful system for large-scale culture.

With electron microscope observations, precipitation of hydroxyapatite crystals was found in the intercellular matrix (Fig. 2). These crystals might be derived from matrix vesicles, since we have already observed that these cells produced numerous calcification nodules via matrix vesicle formation in conventional plastic dish cultures.

The level of alkaline phosphatase activity per μg of DNA in the cell-matrix fraction on the collagen gel spheres was similar to that found in cultures on plastic dishes (Fig. 3). In order to confirm that the cells cultured on collagen spheres could express an osteo-

genic phenotype, we compared the fluorography pattern of the proteins synthesized by cells on gel spheres with that of cells which had already formed calcified nodules on conventional plastic dishes. We found that the fluorographic patterns in the cell matrices were very similar to each other (Fig. 4). These results indicated that osteoblasts could grow, divide and maintain their osteoblastic phenotype on collagen gel spheres. Therefore, collagen gel spheres are considered to offer a good environment for osteoblasts to express their intrinsic phenotype, and to produce normal intercellular matrices.

This study showed for the first time that osteogenic cells can grow and differentiate to express their intrinsic phenotype on collagen gel spheres. Now we are trying to provide three-dimensional culture conditions on the collagen gel spheres so that osteoblasts could more effectively express their phenotypes to produce a bone-like structure. (This study was supported by the Suhara Memorial Foundation in 1986).

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