Spatial development of the cultivation of a bone marrow stromal cell line in porous carriers

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Abstract

The spatial development of the cultivation of a bone marrow stromal cell line (SR-4987) in porous carriers was investigated in order to construct a three-dimensional hematopoietic culture system. Low-rate continuous agitation, 20 rpm, was an appropriate method to achieve initial adhesion of cells onto a cellulose porous beads (CPB, 100 μm pore diameter) in a spinner bottle, compared with other methods such as centrifugation and intermittent agitation. Cell growth with continuous agitation at 70 rpm after initial cell adhesion was not inferior to that at 20 rpm. A 2- and 10-fold increase in the inoculum cell concentration for CPB and another type of porous cellulose beads (Microcube (MC), 500 μm pore diameter) resulted in a 1.2- and 2-fold increase in final cell concentration, respectively. Cells attached to the MC beads and a polyester nonwoven disc (Fibra-cell (FC)) could grow and spread well on the carriers and a fibroblast-like shape was observed under scanning electron microscopy while the cells on CPB were globular. The flatness and inner surface area of these carriers may be the reason for the differences in cell morphology.

Introduction

Bone marrow is a hemopoietic organ where billions of blood cells are generated. All hemopoietic cells, with different lineage, are derived from the same precursor, pluripotent hemopoietic stem cells. Marrow stromal cells promote and regulate stem cell self-renewal, commitment, differentiation, and proliferation through their secreted extracellular matrices and cytokine environment (the hemopoietic microenvironment) (Knospe et al., 1993). Stromal-mediated hemopoiesis has been studied *in vitro* using Dexter's culture system in tissue culture flask (Dexter et al., 1997). In this system, the stromal cells form an adherent layer to which the hemopoietic cells loosely attach.

This contact between the stromal cells and the hemopoietic cells is essential for hemopoiesis *in vitro*, although there have been some reports about the proliferation of bone marrow progenitor cells without stromal cells, employing an excessive addition of expensive cytokines to the culture (Laluppa et al., 1997; Zandstra et al., 1997). However, the contact in Dex-

ter's culture system is two dimensional while the contact in bone marrow *in vivo* is three dimensional and that is considered to be a reason why the hemopoiesis in Dexter's system is limited to macrophage and granulocyte lineage and ceases after long-term cultivation (from several weeks to several months). Recently, it was reported that a three-dimensional hemopoiesis culture system employing porous carriers could allow hemopoiesis to last longer and produce cells of other lineages (Wang and Wu, 1992; Wang et al., 1995).

However, several different kinds of stromal cells such as fibroblasts and adipocytes are included in bone marrow and Dexter's culture system, which could make the control of the hemopoiesis culture process complicated and difficult. Recently, some clonal stromal cell lines have been established (Sutherland et al., 1991; Okada et al., 1992; Pessina et al., 1992; Nakano, 1995; Nishino et al., 1995) and each cell line is characterized by which hemopoietic cell lineage is supported and which cytokines are produced. A hemopoietic microenvironment composed of a porous carrier and a stromal cell line is expected to

realize-controlled long-term hemopoiesis. However, there have been few reports concerning the adhesion and growth of fibroblast cells on porous carriers while there has been much research with cells which are more globular such as endothelial like cells.

Consequently, we investigated the adhesion and growth of a fibroblast stromal cell line in porous carriers.

Materials and methods

Cell and medium

A stromal cell line SR-4987 producing M-CSF, established from a long-term murine bone marrow culture (Pessina et al., 1992) was cultivated with McCoy's 5A medium (Dainihonsei-yaku Co. Ltd., Japan) supplemented with 10% new-born bovine serum (Gibco, Japan), 5,000 U l⁻¹ penicillin and 5 mg l⁻¹ streptomycin (Sigma, MO., U.S.A.).

Porous carriers

Three kinds of porous carriers; middle pore-size beads, large pore-size cubes, and nonwoven disks, were employed in this study. Their respective characteristics were as follows; cellulose porous beads: (CPB, Asahi Chemical Industry Co., Ltd., Japan) with average bead diameter (500 μ m), pore diameter (100 μ m), surface area (2.8 m² g⁻¹) measured using BET method and positively charged with a DEAE group (1.03 meq g⁻¹) (Takagi et al., 1994a; Takagi et al., 1997); the cellulose Micro-cube®: (MC, Biomaterial Co., Ltd., Japan) with average bead diameter (1 mm), pore diameter (500 μ m), surface area (6.4 m² g⁻¹) measured using the BET method and positively charged with a polyethyleneimine group (0.3 meq g⁻¹) (Terashima et al., 1994); and a polyester Fibracell®: (FC, NBS Co, Ltd., U.S.A.) with disk diameter (6 mm), thickness (0.7 mm), surface area (0.12 m² g⁻¹, 6.5 m²/disk) and positively charged using the plasma electric discharge (Kompier et al., 1991). These carriers were suspended in PBS, autoclaved and the supernatant was replaced with medium before cultivation.

Cultivation

The following cell adhesion procedure to carriers including inoculum cell concentration was decided by reference to our previous studies in which human

embryo lung cells (fibroblast) were attached to surface type microcarriers (Cytodex 1) (Takagi et al., 1994b) and hepatocyte cell lines were cultured with porous carriers (Takagi et al., 1997). Also, inoculum size was adjusted based on weights of carriers rather than surface area measured BET method, because the measured area should contain very small pores inside which cell can not enter. Ten milliliters of medium in a spinner bottle (100 mL; Shibata Hario Co., Tokyo) was inoculated with $0.9\text{--}1.5 \times 10^7$ cells together with CPB (36 mg) or MC (78 mg). Also, 1 mL of the medium in a well (6-well plate, 9.4 cm², Corning Coster Japan Co. Ltd.) was inoculated with 5×10^5 cells together with 2 pieces of FC (10.8 mg). All were incubated at 37°C in 5% CO₂ atmosphere and agitated intermittently, 8 times for 10 sec at intervals of 15 min, at 20 rpm (for CPB and MC) or manually (for FC). The cultures with CPB and MC were incubated without agitation for a further 4 h, then 20 mL of the growth medium was added and then continuously agitated at 70 rpm. Measuring glucose concentration for each sample immediately, the culture medium was replaced with fresh medium 2 to 5 times before glucose depletion.

Analysis

Cell concentration was determined using the nuclei staining method (Sanford et al., 1950). Briefly, after the supernatant of culture sample was removed, the residual precipitation containing carriers was filled up to the original volume using the nuclei staining solution (citrate, 21 g/l, crystal violet, 1 g/l) and more than 200 nuclei were counted with hemacytometer after 1 day. Glucose in the culture was measured with the glucose-oxidase peroxidase method, using in auto-analyzer (Biochemistry Analyzer 2700; YSI Inc., Ohio, U.S.A.). Scanning electron microscope (SEM) observation was performed to cells attached to carriers, after fixation with glutaraldehyde (2% in PBS).

Results

Attachment and growth conditions of cells on CPB

Cultivation of adhesion-dependent cells was composed of two processes, namely, the cell adhesion to the substrate and the cell proliferation on the substrate.

Figure 1. Comparison of three methods for the cell adhesion to porous carriers. The intermittent agitation (○), continuous agitation at 20 rpm (●) and centrifuge (□) methods were employed during the cell adhesion process to CPB (1.2 g l^{-1}) for initial 6 h. All cultures were agitated at 70 rpm during later growth process from 6 h.

Agitation was one of the most important operational variables affecting the plating efficiency for the former process and the cell growth rate for the latter process. The effect of the agitation rate on each process was investigated using CPB as a porous carrier.

The following two methods for cell adhesion during the first 6 h were compared to the intermittent agitation method mentioned above. The culture was agitated continuously at 20 rpm for 6 h (the continuous agitation method). The mixture of cells and carriers was centrifuged 5 times at 1000 rpm for 1 min each and settled in a spinner bottle for 5.5 h (the centrifuge method). After 6 h of cell adhesion onto CPB employing these methods, all cultures were agitated at 70 rpm. Cell growth was observed even during the adhesion process of the continuous agitation method (Figure 1), whereas a rapid decrease in cell concentration from 6.1 to $4.3 \times 10^5 \text{ cells ml}^{-1}$ and a 20 h lag phase were observed for the methods utilizing centrifugation and intermittent agitation, respectively. These results revealed that the shear rate yielded from 20 rpm agitation did not disturb cell adhesion and that continuous agitation improved plating efficiency. Consequently, continuous agitation at 20 rpm was confirmed as appropriate for the cell adhesion process.

After the cell adhesion process at 20 rpm, cultures were agitated at 20 and 70 rpm, respectively. The cell growth rate and maximum cell concentration were almost identical at both agitation rates (Figure 2). It was confirmed that cell adhesion could withstand a shear

Figure 2. Comparison of cell growth at the agitation rates of 20 and 70 rpm. Cells were attached to CPB (1.2 g l^{-1}) using the continuous agitation method (20 rpm) for the initial 6 h and the cultures were then agitated at agitation rates of 20 (●) and 70 (○) rpm, respectively.

rate of 70 rpm during cell growth and that the shear rate is not the limiting factor for cell growth.

Effect of inoculum cell concentration on the final cell concentration

To confirm the effect of inoculum cell concentration on the final cell concentration, 5 and $10 \times 10^5 \text{ cells ml}^{-1}$ and 3.2 and $32 \times 10^5 \text{ cells ml}^{-1}$ were inoculated for culture with CPB and MC, respectively. More wide gap between the high and low inoculum concentrations was employed with MC than that with CPB in order to get more clear difference between the final cell concentrations. Cell concentration achieved in the culture using CPB with higher inoculum concentration reached 1.25 times ($1.56 \times 10^6 \text{ cells ml}^{-1}$) of that with a lower concentration inoculum ($1.25 \times 10^6 \text{ cells ml}^{-1}$) (Figure 3). That difference was clearly observed because each maximum cell concentration was maintained constant for several days. For the cultures with MC, the maximum cell concentration achieved with the higher inoculum concentration was 2.4 times ($2.2 \times 10^6 \text{ cells ml}^{-1}$) that achieved with the lower inoculum concentration ($9.0 \times 10^5 \text{ cells ml}^{-1}$) (Figure 4). A higher inoculum cell concentration resulted in a higher final cell concentration with both carriers, whereas the ratio of maximum cell concentration of the culture with the higher inoculum concentration to that of the culture with the lower inoculum concentration (1.25 for CPB and 2.4 for MC) was apparently

Figure 3. Effect of inoculum cell concentration on cell growth in the culture with CPB. Inoculum cell concentrations of 5 (○) and 10 (●), $\times 10^5$ cells/ml were employed respectively for the culture with CPB (1.2 g l^{-1}).

lower than the ratio of inoculum cell concentration (2 for CPB and 10 for MC). The glucose consumption rate in the stationary phase of the culture with higher inoculum concentration was maintained, in contrast to that of the culture with lower inoculum concentration. Consequently, high inoculum cell concentration was effective in increasing the final cell concentration in porous carriers.

Comparison of the adhesive cell morphology between three types of porous carriers

Cells cultured using FC achieved a high cell concentration (Figure 5) and all cultures exhibited active glucose consumption even in the stationary phase. In order to study the effects of the porous carrier type on cell morphology, cells shown in Figures 3, 4 and 5 with lower inoculum concentrations were observed under SEM at the stationary culture phase (Figure 6).

Figure 4. Effect of inoculum cell concentration on cell growth in the culture with MC. Inoculum cell concentrations of 3.2 (○) and 32 (●) $\times 10^5$ cells ml^{-1} were employed respectively for the culture with MC (2.6 g l^{-1}).

Many cells were observed to be attached to the CPB and almost all the cells on the CPB were globular. The cells attached to MC exhibited high density and were well spread, not only on the inner surface but also between two walls, showing typical fibroblast-like morphology as observed on the culture-dish surface. In contrast, cells attached to FC were dense on some fibers and exhibited a thin, needle-like morphology, which was markedly different from the morphology of cells on CPB and MC; also there were many fibers bearing no cells.

Discussion

Continuous agitation at 20 rpm was appropriate for the cell adhesion process (Figure 1). The minimum agitation rate of 20 rpm for the suspension of microcarriers was employed for continuous agitation because a higher shear rate was supposed to inhibit the

Figure 5. Cell growth in the static culture with FC.

attachment of free cells to microcarriers. We found that oxygen should be limited for the intermittent method because microcarriers and cells settled almost every time during the adhesion process and this could explain partly why the plating efficiency of the continuous agitation method was superior to that in the intermittent agitation method. It would also be expected that the accessibility of cells to the beads using this method was not as satisfactory as the continuous agitation method. Besides the high plating efficiency, the simplicity of operation of the continuous agitation method may be a practical advantage in the cell adhesion process. Cells could adhere and grow inside the porous carriers where the medium flow rate is markedly lower than on the surface of the carriers and this may be one of the reasons why cell adhesion could withstand a shear rate of 70 rpm during cell growth (Figure 2).

An increasing inoculum cell concentration was effective for achieving higher cell density with porous carriers (Figures 3 and 4) and the high cell density in porous carriers may prove to be an advantage for the construction of the hematopoietic microenvironment. This effect of inoculum concentration may be due to the characteristic of possible cell extension within areas from the point of initial cell attachment within a carrier because there was no marked difference of empty carrier number between the cultures with the high and low inoculum concentrations. This effect is not observed in cultures using surface-type microcarriers because cells are then able to move from one carrier to another during agitation. The cells in the cultures (Figures 3 and 4) with higher inoculum concentration actively consumed glucose for cell maintenance, even during the stationary phase. Another advantage, for the culture of bone marrow stromal cells, of this method is longer maintenance of the stromal cell layer supporting the long-term proliferation and differentiation of bone marrow hematopoietic cells. An explanation of why the cell concentrations after the cell adhesion process were apparently lower than respective inoculum cell concentration shown in Figures 3 and 4 is not available.

Cell morphology was markedly different between the cultures with the three carriers (Figure 6). Cells on CPB, MC and FC were globular, spread well, and thin and needle-like, respectively. The electric charges were positive for all the carriers, whereas the charge densities differ slightly. The flatness of the inner surface was greater in the MC than in the CPB due to the fact that the pore diameter of MC was larger than that of CPB. The surface of the FC along the length was the flattest of all the carriers but it was not flat along the circumference. Cells might be able to spread better on a flatter surface. Besides, MC had wider spaces inside the pores for cell to spread compared with CPB because of the different diameters. The length the fibers in FC (longer than 1 mm) was much longer than the cells' diameter (less than $50\ \mu\text{m}$). Consequently, cell morphology depends on the characteristics of the porous carriers, namely flatness and area of inner surface. Further investigation is required to determine which cell morphology provides appropriate support for bone marrow hematopoietic cell cultivation.

The cells were grown in stationary culture in a well when using FC, because homogeneous suspension of FC discs in a spinner flask is difficult. So, the attached-cell distribution onto FC was not uniform (Figure 6).

Figure 6. Electron microscopy observations of cells attached on several kinds of porous carriers. A, CPB; B, MC; C, FC. The lines in the photos indicate scales of 50 μm .

A method for uniform cell adhesion to FC should be studied in the future.

Three-dimensional hematopoietic culture system might need high density of stromal cells on which proliferation and differentiation of high-density hematopoietic cells occur. Stromal cells in high density could support the hematopoiesis not only through direct contact to many hematopoietic cells but also by production of soluble cytokines and increasing its concentration locally inside the pore. Those may lead to the hematopoietic microenvironment. The continuous agitation method was appropriate for the effective initial adhesion of stromal cells to porous carriers and higher inoculum concentration was revealed to be beneficial to get high cell density in porous carriers. Generally, cell morphology affect markedly mammalian cell function. So, it can be supposed that the supportive function for hematopoiesis of well spread stromal cells may be different from that of globular stromal cells. It should be confirmed in future experimentally.

Conclusion

Continuous agitation at a low rate, 20 rpm, was appropriate for the cell adhesion process to porous carriers and cell adhesion could withstand a shear rate of 70 rpm during the cell growth process. High inoculum cell concentration was effective for increasing the final cell concentration in porous carriers. Cell morphology was markedly different between the cultures, with

three carriers; cells on CPB, MC and FC were globular, well spread, and thin and needle-like, respectively. It was revealed that cell morphology depends on the characteristics of the porous carriers, namely flatness and inner surface area. These results should contribute to construct the high functional three-dimensional hematopoietic system with porous carriers and stromal cell lines.

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