The construction of an *in vitro* three-dimensional hematopoietic microenvironment for mouse bone marrow cells employing porous carriers

Yoshiaki Tomimori, Mutsumi Takagi* & Toshiomi Yoshida

International Center for Biotechnology, Osaka University, 2-1, Yamada-oka, Suita, Osaka 565-0871, Japan

(* Author for correspondence; E-mail: takagi@icb.osaka-u.ac.jp)

Received 4 November 1999; accepted 29 May 2000

Key words: bone marrow, nonwoven fabric, porous carriers, spatial development, stromal cell

Abstract

Spatial development of mouse bone marrow cells employing porous carriers was investigated in order to design a bioreactor with a three-dimensional hematopoietic microenvironment. Three types of porous carriers were used for examining the spatial development of anchorage-dependent primary stromal cells as feeder cells. Stromal cells were found to spread well at a high density on a polyester nonwoven disc carrier (Fibra cel (FC)) under a scanning electron microscope, while cells on porous cellulose beads (Microcube (MC), 500 μm pore diameter) spread at a low density; cells on another type of cellulose porous beads (CPB, 100 μm pore diameter) were globular. Mouse bone marrow cells were inoculated to dishes containing three types of porous carriers which shared more than 30% of the bottom surface in a dish. The concentration of stromal cells in the well containing FC was lower than that on the other two carriers. However, the weekly output of total hematopoietic cell (suspension cells) increased between day 21 and 28 in the culture using FC while it decreased monotonously in the cultures by use of the other two carriers. The proportion of progenitor cells (BFU-E, CFU-GM) in the total hematopoietic cell population, after showing an initial decrease, increased after 1 week in the culture using FC while the proportion decreased monotonously to zero in the cultures using MC and CPB.

Introduction

Bone marrow is a hemopoietic organ where billions of blood cells are generated. All the hemopoietic cells, of different lineages, are derived from the same precursor, the pluripotent hemopoietic stem cell. Human hematopoietic stem and progenitor cells harvested from bone marrow and umbilical cord blood offers potential in a number of clinical applications including transplantation (Broxmeyer, 1989, 1992; Emerson, 1996). The *in vitro* expansion of these stem and progenitor cells is an attractive and useful method for hematopoietic reconstitution in recipients. However, there was few report about the successful development of such a *in vitro* expansion system.

Marrow stromal cells promote and regulate stem cell self-renewal, commitment, differentiation and proliferation through their secreted extracellu-

lar matrices and cytokine environment (the hemopoietic microenvironment) (Knospe, 1993). Stromal-mediated hemopoiesis has been studied *in vitro* using Dexter's culture system in tissue culture flasks (Dexter, 1977). In this system, the stromal cells form an adherent layer to which the hemopoietic cells are loosely attached. This contact between the stromal cells and hemopoietic cells is essential for hemopoiesis *in vitro*, although there have been some reports on the proliferation of bone marrow progenitor cells without stromal cells, in the presence of excess of cytokines, which are expensive, in the culture (LaJuppa, 1997a; Zandstra, 1997). However, the contact in Dexter's culture system is two-dimensional while the cells in the bone marrow *in vivo* contact each other in three-dimension configuration; this is possibly a reason for the hemopoiesis in Dexter's system to be almost limited to the granulocyte lineage (Wang, 1992). Recently, it was reported

that a three-dimensional hemopoiesis culture system employing porous carriers could allow hemopoiesis of cells of other lineages too (Wang, 1992, 1995), these reports, however, did not refer to the stromal cell development on these carriers.

The construction of an *in vitro* three-dimensional hematopoietic microenvironment should be based on the selection of appropriate porous carrier which would permit stromal cell adhesion. We previously investigated the adhesion and growth of the stromal cell line, SR-4987, on three kinds of porous carriers; namely, middle pore-size beads (CPB), large pore-size cubes (MC), and nonwoven fabrics disks (FC), and showed that the morphology of the attached cells strongly depended on the type of porous carrier (Takagi, 1999). However, it still remains unclear as to how the morphology of stromal cells affects their hematopoiesis-supporting activity.

Consequently, we investigated the hematopoiesis-supporting activity of murine (as a model of human) primary stromal cells attached to three kinds of porous carriers and compared the hematopoietic activity in a three-dimensional culture system employing the most suited porous carrier with that in two-dimensional Dexter's culture.

Materials and methods

Cell and medium

The mouse bone marrow cells were flushed from the femur and tibia of (8–12)-week-old Balb/C male mice into the medium using a 25-gauge needle. Bone marrow cells from different mice were pooled to provide a uniform inoculum. After the cell debris was separated by sedimentation, the number of nucleated cells was determined using the dye-exclusion method (trypan blue) employing a hemacytometer and an inverted microscope.

The medium used for Dexter's culture was McCoy's 5A medium (Gibco; Grand Island, NY, USA) supplemented with 1 mM sodium pyruvate (Gibco), 1 aliquot (in the same concentration as in Eagle's MEM) MEM vitamins (Gibco), 0.4 aliquot MEM amino acids (Gibco), 2 mM L-glutamine (Gibco), 450 mg l⁻¹ NaHCO₃ (Sigma), 1 × 10⁻³ mM hydrocortisone (Sigma), 12.5% FBS (Stem Cell Technologies Inc., British Columbia, Canada), 12.5% horse serum (Stem Cell Technologies), 80 U ml⁻¹ penicillin (Gibco) and 80 µg ml⁻¹ streptomycin (Gibco).

Porous carriers

Three types of porous carriers; namely, medium pore-sized beads, large pore-sized cubes, and non-woven disks, were employed in this study. Their characteristics were as follows; cellulose porous beads (CPB, Asahi Chemical Industry Co. Ltd., Japan) with average bead diameter of 500 µm, pore diameter of 100 µm and surface area of 2.8 m² g⁻¹ measured using the BET method and positively charged with a DEAE group (1.03 meq g⁻¹) (Takagi, 1994, 1997); cellulose microcubes (MC, Biomaterial Co. Ltd., Japan) with average bead diameter of 1 mm, pore diameter of 500 µm and surface area of 6.4 m² g⁻¹ measured using the BET method and positively charged with a polyethyleneimine group (0.3 meq g⁻¹) (Terashima, 1994); nonwoven polyester fiber disk, Fibra-cel (FC, NBS Co. Ltd., USA) disk diameter of 6 mm, thickness of 0.7 mm, surface area of 0.12 m² g⁻¹, 6.5 cm²/disk and positively charged using plasma electric discharge (Kompier, 1991). These carriers were suspended in PBS, autoclaved and the supernatant was replaced with the medium before cultivation.

Cultivation with porous carriers

Each porous carrier was placed in a 24 multiwell for suspension culture (1.8 cm², MS-8024R, Sumitomo Bakelite Co., Tokyo, Japan). The amount of carriers in a well was set to 2.5 mg for CPB and 10 mg for MC so as to cover the floor of the well, and 2 pieces (10.8 mg) of FC were placed in a well. One milliliter of medium containing 2 to 8 × 10⁶ bone marrow cells was introduced into the well and incubated in a humidified incubator containing 5% CO₂, controlled at a temperature of 33 °C. Half the volume of the culture was removed from each well after vigorous pipetting to suspend hematopoietic cells and the same volume of fresh medium was added, at regular intervals of 7 days. The analytical methods for the harvested cells are described later. Briefly, two wells having the same culture condition were destroyed at each culture time for the measurement of stromal cell number as described later and the average value was shown in Figure 1. On the other hand, suspension cells more than 1.5 × 10⁵ cells were harvested from several numbers of wells weekly as mentioned above. The pooled cells were used for the measurement of suspended cell number and CFU assay in two wells as described later. The average of these two CFU counts was shown in Figure 4. All cultures were performed 2

Figure 1. Growth of stromal cells following bone marrow cell cultivation in porous carriers. A: CPB, B: Microcube (MC), C: Fibra cell (FC). The densities of the inoculum of bone marrow cells were 2×10^6 (●), 5×10^6 (▲), and 8×10^6 cells ml^{-1} (■), respectively. Cell concentration was measured by nuclei staining method.

times and the same growth tendency was observed in both.

Although it was not clear which parameter was appropriate to compare these three carriers, several kinds of parameters were calculated as follows; concentration (2.5, 10, 10.8 mg carrier ml^{-1} medium), apparent volume (0.13, 0.15, 0.04 ml carrier ml^{-1} medium) and ratio of surface area of well bottom shared by carriers (100, 100, 31%), respectively, for CPB, MC, and FC. Due to this unclearness, three inoculum densities were tested for each carrier as mentioned later.

Dexter's culture

One milliliter of medium containing 2×10^6 cells was introduced into a 24 multiwell for adhesion culture (MS-8024, Sumitomo Bakelite Co.) and incubated in a humidified incubator containing 5% CO_2 , controlled at a temperature of 33 °C. Half the volume of the culture was removed from each well after gentle pipetting to suspend the hematopoietic cells and the same volume of fresh medium was added at regular intervals of 7 days following a standard protocol of Dexter's culture (Wang, 1992). The methods for analysis of the

harvested cells are described later. All cultures were performed 2 times and the same growth tendency was observed in both.

Colony forming unit (CFU) assay

The cells collected weekly from Dexter's culture were washed and resuspended in the assay medium (Methocult GF M3434, Stem Cell Technologies) which was composed of Iscove's modified Dulbecco medium, 0.9% methylcellulose, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 15% FBS, 1% BSA, 3 U ml^{-1} human erythropoietin, 10 $\mu\text{g ml}^{-1}$ bovine pancreatic insulin, 200 $\mu\text{g ml}^{-1}$ transferrin, 10 ng ml^{-1} mouse IL-3, 10 ng ml^{-1} human IL-6 and 50 ng ml^{-1} mouse SCF. Two plates (35-mm, Corning), each containing 1.1 ml of the assay medium with 5×10^4 cells and one plate containing only 3 ml of sterilized water were placed in a dish (100-mm, Corning) and incubated at 37 °C in a humidified incubator containing 5% CO_2 . The colonies of CFU-GM and BFU-E were scored under an inverted microscope on day 7.

Figure 2. Scanning electron microscopic observation of primary stromal cells cultured on porous carriers. (A, B) CPB; (C, D) MC; (E, F) FC.

Figure 3. Time-course of suspension cell output during bone marrow cell cultivation on porous carriers. (A) CPB; (B) MC, (C) FC. Symbols are the same as for Figure 1.

Morphological analysis

Cells, about 20 000 cells, collected from culture were resuspended in 0.1 ml medium for slide preparation, centrifuged at 500 rpm for 5 min in a cytocentrifuge machine (Cytospin 3, Shandon, Pittsburgh, PA), and stained with Wright's stain (Wako Pure Chemicals, Osaka).

General analysis

The total concentration of hematopoietic cells was determined by the trypan blue method. Stromal cell concentration was determined using the nuclei staining method (Sanford, 1950). Briefly, after the supernatant of the culture containing suspended hematopoietic cells was removed, the residual precipitate containing the carriers was washed twice with 5 ml PBS, filled up to the original volume with the nuclei staining solution (citrate, 21 g l⁻¹; crystal violet, 1 g l⁻¹) and more than 200 nuclei were counted in a hemacytometer after 1 day. Scanning electron microscopic (SEM) examination of the stromal cells attached to carriers was performed after washing with PBS and fixation with glutaraldehyde (2% in PBS).

Results

Effect of inoculum cell concentration on stromal cell growth

In order to study the growth of primary stromal cells on porous carriers, bone marrow cells were cultured on three types of porous carriers at three inoculum cell densities ($2, 5, 8 \times 10^6$ cells ml⁻¹).

On all the porous carriers examined, stromal cell concentration on day 7 (176 h) was higher and confluence was reached earlier as inoculum cell density increased (Figure 1). Regardless of the type of porous carriers, stromal cells reached final concentrations on day 14 (333 h) and 21 (509 h) in cultures at inoculum density of 8×10^6 cells ml⁻¹ and 2.5×10^6 cells ml⁻¹, respectively. However, the final cell concentration for different inoculum cell densities were almost similar within the cultures using same kind of porous carrier. The stromal cell concentrations on day 7 not only per one ml medium but also one mg carrier was the highest for the culture on CPB and lowest for FC among the three types of porous carriers examined. The ratio of stromal cell concentrations on day 7 for CPB, MC and FC was approximately 8:3:1. The or-

Figure 4. Progenitor cell content in bone marrow cultures on porous carriers. (A) CPB; (B) MC; (C) FC. BFU-E (○) and CFU-GM (□) were determined by CFU assay in the culture at the inoculum density of 2×10^6 cells ml^{-1} .

der of the porous carriers for final cell concentration was the same as that for stromal cell concentration on day 7 and the ratio of the final cell concentrations on CPB, MC and FC was approximately 2.5:2:1. Thus, the differences in final cell concentration between carriers was markedly smaller than those in stromal cell concentration on day 7.

Comparison of morphology of stromal cells adhered to each porous carriers

In order to investigate the stromal cell morphology, the stromal cells on the three types of porous carriers were observed under a scanning electron microscope after removal of hematopoietic cells. Almost all stromal cells on CPB were globular (Figures 2A, B). Although stromal cells on MC spread a little compared with those on CPB, their cell density inside the pores seemed not to be so high (Figure 2C, D). On the other hand, stromal cells in the pores of FC spread well (Figure 2F) and their density was markedly higher than that in the pores of MC. Moreover, stromal cells on FC formed several numbers of cell layers arranged in three dimension (Figure 2E).

Hematopoietic cell maintenance in three-dimensional cultures on different types of porous carriers

Half of the hematopoietic cells were harvested weekly from the bone marrow cell culture employing three kinds of porous carriers at three inoculum cell concentrations and the data are shown in Figure 3. Suspension cell output decreased dramatically during the first 2 (0–333 h) (for CPB and MC) or 3 weeks (0–509 h) (for FC). Beside the output on day 7 (176 h), the suspension cell outputs from the cultures at higher inoculum densities were not necessarily higher than those at lower inoculum densities. Although the output did not increase in the cultures using CPB and MC, the suspension cell output started to increase in the culture using FC from day 21 (509 h).

Progenitor contents in the suspension cell output were analyzed for the cultures at the inoculum densities of 2×10^6 cells ml^{-1} , in which the ratio of suspension cell output to inoculum cell densities was the highest (Figure 4). Progenitors contents in freshly isolated bone marrow cells was 33 (BFU-E) and 45 (CFU-GM)/ 5×10^4 suspension cells. The progenitors contents in the cultures on CPB and MC decreased with culture time and reached to 0 on day 21 (509 h)

Figure 5. Mature cell ratio in bone marrow cell cultures. A: Dexter's culture, B: Three-dimensional culture on the porous carrier FC. Lymphocyte (L), erythrocyte (E), macrophage (M) and granulocyte (G) were analyzed by Wright's staining.

and 28 (673 h), respectively. On the other hand, while progenitor cells contents in the culture on FC decreased during the initial 7 days (176 h), it started to increase on day 7 and was maintained at a high level comparable to the initial level until day 21 to 28.

Comparison of mature hematopoietic cells in the three-dimensional culture on FC with that in conventional Dexter's culture

In order to compare the ratio of mature cells between a three-dimensional hematopoietic cell culture and a two-dimensional one, mature cell ratio was analyzed for both culture on FC and Dexter's culture. The macrophage percentage was maintained at 30 to 50% from day 21 to 35 in the culture on FC, while it decreased to approximately 10% on day 21 and lower than 5% on days 28 and 35 in Dexter's culture (Figure 5). Although erythrocytes were not observed at all after day 28 in Dexter's culture, 5 and 8% of erythrocytes was found on day 28 and 35 in the culture on FC.

Discussion

Surface area of porous carriers per unit culture volume could be calculated based on the BET measurement data supplied by each producer as 70 (CPB), 640 (MC), and 13 (FC) $\text{cm}^2 \text{ml}^{-1}$, respectively. However, surface area measured by BET method should contain also too small pores for cell adhesion. Consequently, these physical data was not discussed in correlation with culture results in this study.

Although some culture materials were reported to be toxic for hematopoietic progenitor cells (LaIuppa, 1997b), the porous carriers tested here, CPB and MC, were considered not to be more toxic for hematopoietic cells compared with FC because concentrations of stromal and hematopoietic cells in the culture with CPB or MC were higher or almost the same as these with FC (Figures 1 and 3). Moreover, the decreasing rates of progenitor cell contents during 1st week in the culture with CPB and MC were smaller than that in the culture with FC (Figure 4).

Although stromal cell concentration on day 7 (176 h) strongly depended on the type of porous carrier and that in the culture on CPB was approximately 10 times that in the culture on FC, there were no

significant differences in the final cell concentrations between the different carriers (Figure 1). FC occupied only 30% of the floor of the well, while CPB and MC covered the floor of the well completely. This could be the reason for the large differences in the stromal cell concentration on day 7 between the carriers. The stromal cell concentration could probably be improved by increasing the floor area of a well containing FC, since a well can contain only 2 pieces of the disk-shaped FC with a diameter of 6 mm.

Final cell concentration did not depend on the inoculum cell density (Figure 1). This may be attributable to the maximum cell concentration being limited by the inner surface area of the each porous carriers because there was no depletion of glucose and glutamine and the surface aeration capacity without agitation was calculated to be high enough to supply dissolved oxygen to cells of these concentrations (Takagi, 1994).

In our previous study on the adhesion and growth of the stromal cell line, SR-4987, on the three types of porous carriers examined in this study, cells on CPB, MC, FC were either globular, spread a little, spread very well showing a needle-like shape, respectively. Consequently, we considered that globular cells observed on CPB in this study (Figures 2A, B) was not hematopoietic cells but stromal cells. Moreover, similar morphologies were observed when primary stromal cells purified by plate-adhesion method were cultured in these carriers (data not shown). It was also considered to be possible that different stromal cell types (e.g., endothelial cells, adventitial reticular cells, macrophages, etc.) adhered differentially to the different carriers. However, the differences in primary stromal cell morphology observed under the scanning electron microscope between the three types of porous carriers were almost similar to those observed for the stromal cell line, SR-4987 although primary stromal cells did not show needle-like morphology on FC. Consequently, the morphological difference observed in this study was considered to due to not different stromal cell types but simple morphology change of same kind of stromal cells by the difference in carrier characters.

Although volumetric concentration of stromal cells based on culture volume was lower in the culture with FC compared with those in the cultures with CPB and MC (Figure 1), apparently higher density of stromal cells was observed under SEM for the culture with FC. The volumetric concentration of stromal cells based on carrier volume in the culture with FC can be higher than those with CPB and MC, because FC oc-

cupied only 30% of the floor of the well as mentioned above. It was also supposed that the higher adhesion strength of stromal cells onto FC compared with those to CPB and MC may result in higher density of stromal cells after washing and fixation procedure for SEM observation.

It may be a little difficult to flush all of hematopoietic cells out of the carrier pores. However, there was no marked difference in suspension cell output during 1st week between cultures with different carriers and same inoculum densities (Figure 3). So, the data of suspension cell output shown in Figure 3 was considered to show the quantitative difference of hematopoietic cell number grown in each porous carriers. The reason why the suspension cell output from the culture with higher inoculum density was not necessarily higher than those from the culture with lower inoculum density (Figure 3) is not clear. However, it could be supposed that there may be some depletion in medium components or oxygen during first week (0–176 h) in the culture with high inoculum density.

The rapid decrease in suspension cell output during the initial 2 (0–333 h) to 3 (0–509 h) weeks (Figure 3) could be due to the death of mature cells since almost all of the hematopoietic cells in the inoculum were mature cells and their life-span is normally very short (4 to 5 days). The reason why the suspension cell output from the culture using FC was a little lower than that from the other cultures could be the apparently lower stromal cell concentration in the culture on FC compared with the other cultures, using CPB and MC (Figure 1). Moreover, it might be also a reason that some hematopoietic cells might not attach to stromal cells in FC at inoculation because FC occupied only 30% of the floor of the well as mentioned before. This disadvantage of FC was expected to be overcome by increasing the stromal cell concentration with increase of the floor area of a culture well containing FC.

Although the output did not increase in the cultures on CPB and MC, the suspension cell output started to increase in the culture on FC from day 21 (509 h) (Figure 3). While the progenitors contents in the cultures on CPB and MC decreased with culture time, the progenitor cells contents in the culture on FC started to increase on day 7 and was maintained at a high level comparable to the initial level (Figure 4). This might indicate that stromal cells on FC could support the self-renewal of progenitor cells while stromal cells on the other porous carriers namely, CPB and MC, could not do so. The maintenance of progenitor cells contents in the culture on FC could be responsible for

the maintenance of suspension cell output during 4th week (Figure 3).

While more than 95% of mature cells consisted of granulocytes on days 28 and 35 in Dexter's culture, the mature cells in the culture on FC showed more variety including approximately 30% macrophages and 5 to 8% erythrocytes (Figure 5). This result might indicate that hematopoiesis in the culture on FC was more similar to that in the bone marrow compared with that in Dexter's culture. This advantage of the culture using FC is considered to be due to the three-dimensional microenvironment constructed in the FC carrier containing a well-spread and dense stromal cell layer (Figure 2).

There was a report that three-dimensional hematopoietic cell culture employing collagen porous carriers resulted in the production of cells with wider lineages containing erythrocytes (Wang, 1995). However, that report did not refer to the stromal cell development on the carrier and adhesion of stromal cells on the carrier was not shown under SEM observation.

This article clearly demonstrated that the non-woven fabrics carrier, FC, was an appropriate porous carrier among the three types examined here to construct a three-dimensional hematopoietic microenvironment including the well-spread and dense stromal cell layer having a high hematopoiesis-supporting activity. However, further study involving improvement of hematopoiesis-supporting activity of stromal cells in three-dimensional environment and measurement of earlier progenitors such as CFU-GEMM compared with CFU-GM and BFU-E might be necessary to establish more effective expansion system of progenitor or stem cells.

Conclusions

Mouse bone marrow cells were inoculated onto three types of porous carriers. Bone marrow stromal cells were found to spread well at a high density on a polyester nonwoven disc carrier, Fibra cel (FC), compared with other two kinds of carriers. The weekly harvested total hematopoietic cell output increased between day 21 and 28 in the culture using FC while it decreased monotonously in the cultures using the other carriers. The increase of progenitor cells proportion in the total hematopoietic cell population was observed only in the culture with FC. Comparison of the culture using FC with Dexter's culture revealed that the three-dimensional culture using FC resulted in a marked

increase in the proportion of macrophages and erythrocytes among the mature cells. Consequently, FC was proved to be an appropriate porous carrier for the construction of three-dimensional hematopoietic microenvironment with well-spread stromal cells having high hematopoietic supportive activity.

Acknowledgement

We thank Dr Wu, J.H.D. in University of Rochester for his helpful suggestions and technical introduction of bone marrow cell culture.

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