Original Research

Novel three-dimensional long-term bone marrow culture system using polymer particles with grafted epoxy-polymer-chains supports the proliferation and differentiation of hematopoietic stem cells

Yukio Hirabayashi[1,2], Yoshihiro Hatta[1], Jin Takeuchi[1], Isao Tsuboi[2], Tomonori Harada[2], Kentaro Ono[3], Wilhelm Robert Glomm[4], Masahiro Yasuda[3] and Shin Aizawa[2]

[1]Department of Internal Medicine; [2]Department of Functional Morphology, Nihon University School of Medicine, 30-1 Oyaguchikamicho, Itabashi-ku, Tokyo 173-8610; [3]Department of Chemical Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan; [4]Department of Chemical Engineering, Norwegian University of Science and Technology, Sem Sælands vei 4, Trondheim N-7491, Norway
Corresponding author: Tomonori Harada. Email: harada.tomonori@nihon-u.ac.jp

Abstract

Hematopoiesis occurs in the bone marrow, where primitive hematopoietic cells proliferate and differentiate in close association with a three-dimensional (3D) hematopoietic microenvironment composed of stromal cells. We examined the hematopoietic supportive ability of stromal cells in a 3D culture system using polymer particles with grafted epoxy polymer chains. Umbilical cord blood-derived $CD34^+$ cells were co-cultivated with MS-5 stromal cells. They formed a 3D structure in the culture dish in the presence of particles, and the total numbers of cells and the numbers of hematopoietic progenitor cells, including colony-forming unit (CFU)-Mix, CFU-granulocyte-macrophage, CFU-megakaryocyte and burst-forming unit-erythroid, were measured every seven days. The hematopoietic supportive activity of the 3D culture containing polymer particles and stromal cells was superior to that of 2D culture, and allowed the expansion and maintenance of hematopoietic progenitor cells for more than 12 weeks. Various types of hematopoietic cells, including granulocytes, macrophages and megakaryocytes at different maturation stages, appeared in the 3D culture, suggesting that the $CD34^+$ cells were able to differentiate into a range of blood cell types. Morphological examination showed that MS-5 stromal cells grew on the surface of the particles and bridged the gaps between them to form a 3D structure. Hematopoietic cells slipped into the 3D layer and proliferated within it, relying on the presence of the MS-5 cells. These results suggest that this 3D culture system using polymer particles reproduced the hematopoietic phenomenon *in vitro*, and might thus provide a new tool for investigating hematopoietic stem cell–stromal cell interactions.

Keywords: hematopoiesis, 3D long-term bone marrow culture, stromal cell, hematopoietic microenvironment, polymer particles

Experimental Biology and Medicine 2011; **236**: 1342–1350. DOI: 10.1258/ebm.2011.011075

Introduction

Hematopoietic stem cells (HSCs) can self-renew and produce progenitors that are committed to differentiate into single lineages, leading to the production of a wide range of blood cell types, including erythrocytes, leukocytes, lymphocytes and platelets. Earlier studies demonstrated that the proliferation and differentiation of HSCs is regulated by the bone marrow microenvironment.[1–3] Stromal cells, as distinct from hematopoietic cells, are an essential component of this microenvironment and are necessary for the long-term maintenance of HSCs *in vitro*.[4,5] Several stromal cell lines that possess a hematopoietic supportive function *in vitro* have been cloned from murine and human bone marrow, and used to study the nature of the interactions between HSCs and stromal cells.[6–10] These studies have found that stromal cells regulate the proliferation and differentiation of HSCs through the production of diffusible hematopoietic regulatory factors and extracellular matrix, and through physical cell–cell interactions involving adhesion molecules and

gap junction-mediated cell communication.[11–18] However, the ability of stromal cells to support the expansion of HSCs and to maintain their self-renewal potential has generally been investigated in long-term, two-dimensional (2D) bone marrow culture systems, and most of the reports have shown a decline in HSCs within 4–8 weeks in culture.[19–21] One reason is that the hematopoietic cells slip, and proliferate in the space between the stromal layer and the bottom of the dish, resulting in the peeling-off of the stromal cell sheet and subsequent loss of the stromal cell influence. Moreover, hematopoiesis *in vivo* takes place in the bone marrow, where hematopoietic cells proliferate and differentiate in close association with a three-dimensional (3D) hematopoietic microenvironment, the so-called hematopoietic niche, composed of stromal cells. HSCs are found in spaces within the bone marrow, relying on cell–cell interactions and the local milieu to determine their immediate fate.[22]

Thus, various types of 3D culture systems have been used to prolong the cultivation of HSCs, including the use of ethylene terephthalate (PET) woven mesh,[23] fibronectin-conjugated PET mesh,[24] porous polyvinyl formal resin[25,26] and polyacrylamide hydrogel cell culture scaffold.[27] These 3D culture systems have been shown to induce the growth and differentiation of mesenchymal bone marrow stromal cells,[28,29] and to support their active cytokine production.[30] However, none of these studies demonstrated the maintenance of HSCs in culture for more than four weeks, because of the peeling-off of stromal cells from the 3D support surface. Moreover, their visual observation using microscopy was not easy, because solid and light-impermeable materials were often used.

We recently developed novel polymer particles with grafted epoxy polymer chains for use in establishing a new 3D cell cultivation system.[31] The particles, composed of base polymer particles and grafted polymer chains, were used as a support for cell immobilization. The base polymer particles were synthesized by suspension polymerization of acrylic monomer and 2,2′-azobis[*N*-(2-propenyl)-2-methylpropionamide] (APMPA), and the epoxy polymer chain was extended from the particle surface by graft polymerization. These particles have the advantage of being easily designed, in terms of the size of the particle, length and number of chains, and the composition of the base polymer network. Previous research[31] showed that fibroblast (MS-5), epidermal (HeLa) and osteoblast (MC3T3E1) cell lines adhered to, and grew on the surface of particles, as well as on cells, using tissue culture dishes. Cells were located in particle gaps, forming a complex composed of cells and polymer particles. MS-5 cells formed 3D cell bridges that allowed the growth of cells without the need for adherence to the substrate.[31]

The unique fibroblast cell line, MS-5, was established from irradiated mouse bone marrow stromal cells, and has been shown to support both murine and human HSC proliferation and differentiation *in vitro*.[19,32–34] It seems likely that the 3D cell bridges formed by MS-5 cells in the 3D cell cultivation system[31] might overcome the peeling problem associated with previous 3D and 2D systems, allowing long-term maintenance of HSCs *in vitro*. We, therefore, examined the hematopoietic-supportive ability of MS-5 cells, as a source of stromal cells, in a 3D culture system using polymer particles, and investigated the kinetics, morphology and differentiation of human umbilical cord blood (CB) $CD34^+$ cells co-cultured using this 3D system.

Materials and methods

Materials

2,2′-azobis(isobutyronitrile), APMPA, dipotassium hydrogen phosphate, 25 wt% of glutaraldehyde aqueous solution, glycidyl methacrylate (GMA), hydrochloric acid, methacrylic acid (MA), methanol, methyl methacrylate, 36 wt% of paraformaldehyde aqueous solution, saccharose, sodium dihydrogen phosphate and trypan blue were purchased from Wako Pure Chemical Co (Osaka, Japan). Cresol red, poly(vinylpyrolidone) K-90, sodium hydroxide and toluene were purchased from Nacalai Tesque (Kyoto, Japan). Pentaerythritol triacrylate was purchased from Sigma-Aldrich Co (St Louis, MO, USA). The monoclonal antibodies used in this study, anti-CD41a (HIP8) and phycoerythrin (PE)-conjugated-anti-CD34 (HPCA-2), were purchased from Becton Dickinson (San Jose, CA, USA), and polyclonal rabbit anti-mouse immunoglobulins/horseradish peroxidase were purchased from DakoCytomation (Glostrup, Denmark).

Preparation of cell support with grafted polymer chains

Polymer particles with grafted epoxy polymer chains were prepared as described previously.[31] For graft polymerization, GMA and MA were used at a ratio of 4:1 (w/w), and the resulting particles were washed in a funnel with 40 times volume of distilled water and methanol. These polymer particles are subsequently referred to as G-02 polymer particles.

The particle diameter distribution was measured using a laser particle diameter analyzer, Microtrac FRA (Microtrac Inc, Montgomery, PA, USA), and 200–300-μm particles were used in this study. The amount of epoxy group in the polymer particles was measured using the hydrochloric acid-dioxane method,[35] and particles with more than 1.03×10^{-3} μmol/g-particle of epoxy group were used.[31]

MS-5 stromal cells

The cloned murine stromal cell line, MS-5, was maintained in 7 mL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), penicillin (50 U/mL; Gibco BRL, Grand Island, NY, USA) and streptomycin (100 μg/mL; Gibco BRL) in 25-cm^2 flasks (Falcon 3013; Becton Dickinson). The cells were cultured in a humidified incubator at 37°C and 5% CO_2, and were subcultured at a split ratio of 1:4 every 7 d, using 0.25% trypsin plus 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), and maintained as described.

Collection of human umbilical CB and isolation of $CD34^+$ cells

Umbilical CB was obtained from full-term deliveries after receiving informed consent, according to a protocol approved by the ethics committee of Nihon University School of Medicine and Tokyo Cord Blood Bank. CB was collected in bags containing heparin and processed within 24 h. After separation over Ficoll Isopaque (1.077 g/mL), CB low-density mononuclear cells (CBMNCs) were washed and suspended in IMDM supplemented with 10% FCS.

$CD34^+$ cells were isolated from CBMNCs by positive immunomagnetic cell separation method (Dynal CD34 progenitor cell selection system; Dynal Biotech Inc, Lake Success, NY, USA), as indicated by the manufacturer. A total of 1×10^8 of CBMNCs was incubated with 100μL of Dynabeads M-450 CD34 (4×10^7 beads) for 30 min at 4°C. Rosetted target $CD34^+$ cells were washed with IMDM twice, then Dynabeads were released using 100 μL of DETACHaBEAD CD34 (Dynal Biotech Inc). Separated $CD34^+$ cells were washed with IMDM twice, and their purity was determined by flow cytometry (CytoACE-150; Japan Spectroscopic, Tokyo, Japan) using an antibody against human CD34. More than 96% of the collected cells were found to be $CD34^+$, and more than 98% of cells were viable, as indicated by trypan blue dye exclusion. $CD34^+$ cells were used as a source of HSCs.

3D co-culture system

First, $5–10 \times 10^5$ MS-5 cells were added to 4 mL of IMDM supplemented with 10% FCS in the presence of $1–5 \times 10^4$ G-02 polymer particles in 14 mL of polypropylene in a round-bottomed tube (Falcon 2006; Becton Dickinson). The mixture was incubated in a humidified incubator at 37°C and 5% CO_2 for 24 h, transferred into 35-mm plastic dishes (Falcon 3046; Becton Dickinson), and incubated in a humidified incubator at 37°C and 5% CO_2. Once cells were immobilized on the surface of the particles, they grew and remained attached to the particles, and formed bridges between the particles. The supernatant was replaced with fresh growth medium every seven days. After 2–3 weeks, when the MS-5 cells had grown and formed a 3D layer in the culture dish, $CD34^+$ cells (1×10^4) suspended in 4 mL of the growth medium were inoculated onto the MS-5 cells. Three milliliters of supernatant with hematopoietic cells was subsequently replaced with fresh growth medium every seven days and the culture was continued. An aliquot of cells was harvested and assayed to determine the total number of cells and the numbers of hematopoietic progenitor cells (colony forming unit [CFU]-Mix, CFU-granulocyte-macrophage [GM], CFU-megakaryocyte [Meg] and burst-forming unit-erythroid [BFU-E]). The harvested cells were mainly floating mature cells and loosely adherent cells. Control cultures included MS-5 cells grown in dishes without G-02 particles, and $CD34^+$ cells inoculated onto MS-5 cells in traditional 2D cultures. The morphology of the proliferated hematopoietic cells that formed blood island-like organizations in 3D culture was studied after staining with hematoxylin and eosin.

In some experiments, cells in the adherent layer were harvested and the number of hematopoietic progenitor and $CD34^+$ cells was examined at two and four weeks after cultivation. To obtain adherent cells from 2D and 3D cultures, first, supernatant including floating cells were removed, and the adherent layer was washed with PBS once. Adherent cells were treated with 0.25% trypsin plus 0.02% EDTA in PBS at 37°C, then stromal cells (MS-5 cells) and hematopoietic cells ($CD34^+$ derived cells) were harvested together. Cells were washed with IMDM supplemented with 10% FCS and the number of cells was counted. An aliquot of cells was assayed to determine the number of hematopoietic progenitor cells (CFU-Mix, CFU-GM and BFU-E). $CD34^+$ cells in the adherent layer were detected using flow cytometry and immunohistological examination.

Slide preparation and staining for differential counting

The harvested cells from the supernatant and adherent layer were counted using a hemocytometer, and viable cells were distinguished by trypan blue dye exclusion. Slides were prepared by cytospinning 6×10^4 cells in 200 mL IMDM onto a slide. The slides were then air-dried and stained with Wright-Giemsa stain, esterase stain (α-naphthyl acetate) or immunohistological stain using anti-human-CD41a and anti-human-CD34 antibodies.

Progenitor cell colony assay

CFU-Mix, CFU-GM and BFU-E harvested from the cultures were assayed using a Methocult GF H4434V kit (Stem Cell Technologies, Vancouver, BC, Canada), according to the manufacturer's protocols. Cells were cultured in a humidified incubator at 37°C and 5% CO_2, and the colonies were counted using a dissecting microscope. CFU-Mix, CFU-GM and BFU-E were counted 14 d after plating of the cells.

CFU-Meg was assayed using a MegaCult-C kit (Stem Cell Technologies) according to the manufacturer's protocols. After a 14-d culture, a spacer was placed onto the culture chamber slide, a thick filter card was placed on top and liquid allowed to soak the card. The thick filter card was removed as soon as it became fully saturated, and the slide was placed into fixative solution (cold acetone) for five minutes, and allowed to air-dry for 10 min. Then, the culture was stained with anti-human-CD41a antibody, and CD41a-positive colonies were counted as CFU-Meg.

Scanning electron microscopy

Samples were fixed in 2% glutaraldehyde, 4% paraformaldehyde in 0.1 mol/L cacodylate buffer solution (PBS, pH 7.4) overnight, post-fixed in 1% osmium tetroxide in 0.1 mol/L PBS for two hours, dehydrated through graded ethanols and embedded in Quetol-812 (Nisshin EM Corp, Tokyo, Japan). Ultrathin sections were cut with a diamond knife on an ultramicrotome (Ultracut UCT; Leica, Wien, Austria). The thin sections were stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (TEM-1200EX; JEOL, Tokyo, Japan).

Statistical analysis

The results are expressed as the mean $\pm$ SD of triplicate experiments. Differences between the means were determined using analysis of variance, and $P < 0.05$ was considered significant.

Results

Growth and formation of 3D structure of MS-5 cells in culture with G-02 particles

A schematic illustration of G-02 particles with numerous grafted polymer chains with epoxy and carboxyl groups on the surface is shown in Figure 1a. MS-5 cells adhered easily to the particles, then grew and bridged the gaps between particles (Figure 1b). After 2–3 weeks, when the MS-5 cells had grown and formed a 3D layer in the culture dish, human umbilical CB-derived $CD34^+$ cells (1×10^4), suspended in 4 mL of growth medium, were inoculated onto the MS-5 cells. The $CD34^+$ cells quickly slipped into the 3D layer of MS-5 cells (Figures 2a and 2b). The $CD34^+$ cells then proliferated and differentiated within the 3D layer. The proliferated hematopoietic cells formed blood island-like organizations in 3D culture, while differentiated cells separated from the layer and migrated to the supernatant (Figure 2c). Figure 3 shows the proliferation of hematopoietic ($CD34^+$) cells in 3D culture with MS-5 cells observed by scanning electron microscopy two weeks after inoculation. MS-5 cells are seen on the surface of the particles and bridging the gaps between particles, forming a 3D structure, while hematopoietic cells slipped into the 3D layer and proliferated there, attached to the MS-5 cells.

Fluctuations in the numbers of hematopoietic cells in culture

Three milliliters of supernatant with hematopoietic cells was replaced with fresh growth medium every seven days, and the culture was continued. An aliquot of cells in supernatant was harvested and assayed to determine the total numbers of cells, and the numbers of CFU-Mix, CFU-GM and BFU-E. The changes in the total cell numbers in the cultures are

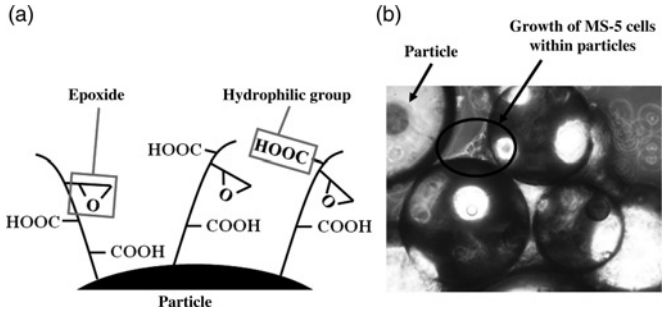

Figure 1 Polymer particles with grafted epoxy polymer chains. Schematic diagram of G-02 particles with numerous grafted polymer chains with epoxy and carboxyl groups on the surface (a). MS-5 cells bound easily to the particles, then grew and bridged the gaps between particles (b) to form a three-dimensional structure in culture

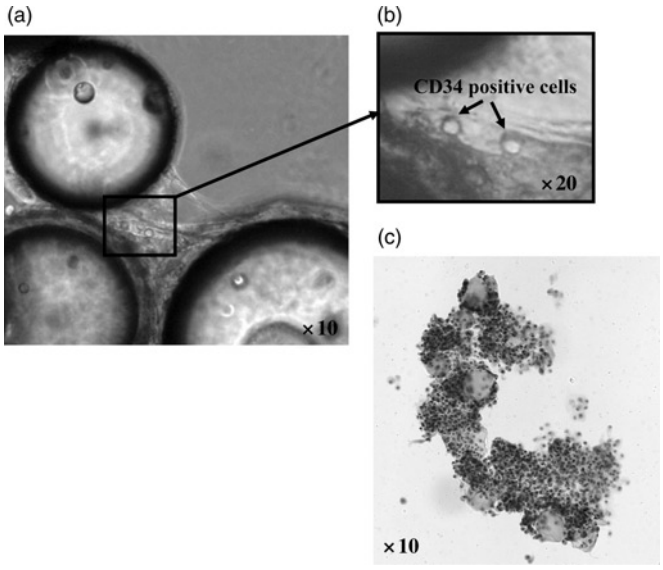

Figure 2 Homing and growth of $CD34^+$ cells in culture with G-02 particles immobilized with MS-5 cells. $CD34^+$ cells (1×10^4) derived from human umbilical cord blood were inoculated onto the MS-5 cells. Phase-contrast micrographs showed that $CD34^+$ cells quickly slipped into the three-dimensional (3D) layer constructed with MS-5 cells (a, b). Morphological observation (hematoxylin and eosin staining) showed that the proliferated hematopoietic cells formed a blood island-like organization in 3D culture (c)

summarized in Figure 4. The total cell number in 2D culture with MS-5 cells increased and was maintained up to four weeks after inoculation, but gradually decreased and disappeared at eight weeks. In contrast, the total cell number in 3D culture continued to increase and was maintained for more than eight weeks after inoculation ($P < 0.05$ with respect to 2D culture). Significantly increased numbers of hematopoietic progenitors (CFUs) were also observed in 3D culture, compared with 2D culture (Figure 5). When $CD34^+$ cells were cultured in the presence of G-02 particles, but without MS-5 cells, the number of hematopoietic cells was increased after one week of culture, but quickly decreased and disappeared within four weeks, suggesting that MS-5 cells are necessary for the proliferation of hematopoietic cells in culture. The numbers of progenitor cells including CFU-Mix, CFU-GM and BFU-E are shown in Figure 6. The levels of each type of progenitor were higher in 3D than in 2D culture up to 12 weeks after inoculation. Interestingly, morphological examination of cytospin preparations showed that various types of hematopoietic cells, including blast-like cells, granulocytes, macrophages and megakaryocytes at different maturation stages, appeared in the 3D culture medium after one, two and four weeks of culture (Figure 7). In contrast to 3D culture, foamy cells quickly appeared in the 2D culture and culture without MS-5 cells after two weeks, and most cells in the supernatant were found as foamy cells after four weeks in culture (Figure 7). These foamy cells were positive for esterase (α-naphthyl acetate) stain (Figure 7) and have phagocytic activity (date not shown), suggesting macrophage-like characteristics. Immunocytological staining indicated that the megakaryocytes present in the 3D culture were positive for anti-CD41a antibody (Figure 7). In fact, the numbers of CFU-Meg in 3D culture were also increased when compared

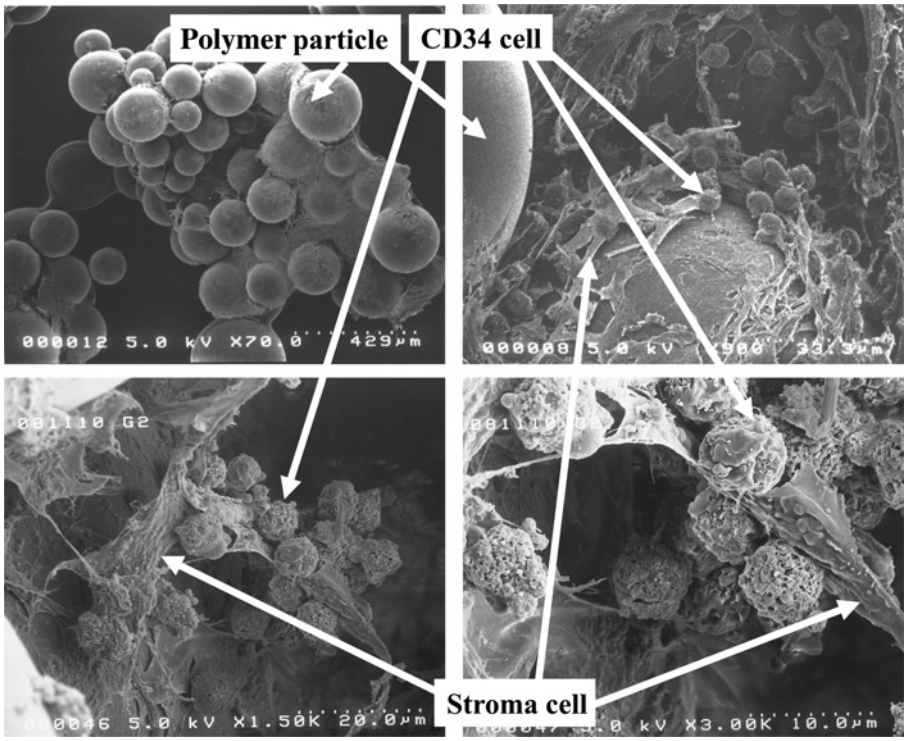

Figure 3 Morphological observation of the three-dimensional (3D) co-culture system by scanning electron microscopy. Proliferation of $CD34^+$ cells in 3D culture with G-02 particle-immobilized MS-5 cells was observed by scanning electron microscopy two weeks after inoculation. MS-5 cells grew on the surface of the particles and bridged the gaps between particles, forming a 3D structure. The hematopoietic cells slipped into the 3D layer and proliferated, adhered to the MS-5 cells

with 2D culture at one, two, four and eight weeks of culture (Table 1). These findings suggest that $CD34^+$ cells were able to differentiate into various types of blood cells in this 3D culture system.

Further, the number of total and hematopoietic progenitor cells, and the presence of $CD34^+$ cells in the adherent layer were studied. Adherent cells including stromal cells (MS-5 cells) and hematopoietic cells (originated $CD34^+$ cells) were obtained by trypsin-EDTA treatment at two and four weeks after cultivation. No significantly difference of the total cell numbers in the adherent layer between 2D

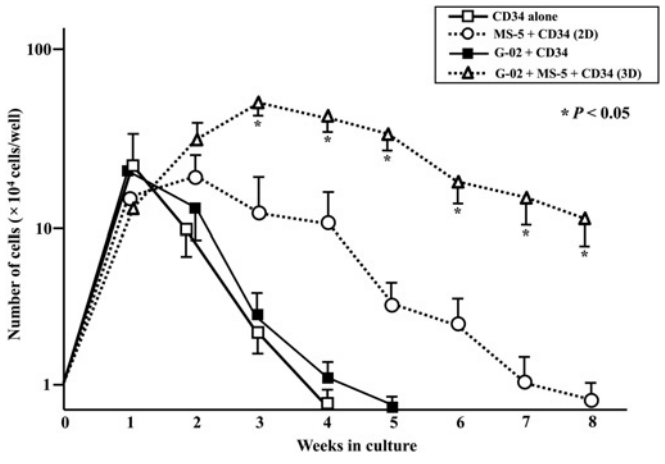

Figure 4 Changes in the total cell numbers in supernatant of cultures. $CD34^+$ cells (1×10^4) were inoculated onto the MS-5 cells, supernatant with cells was replaced with fresh growth medium every seven days and the total cell number was counted. Two different wells were prepared for each point, and experiments were performed in triplicate. The results are expressed as the mean ± SD. *Significant difference ($P < 0.05$) with respect to two-dimensional culture

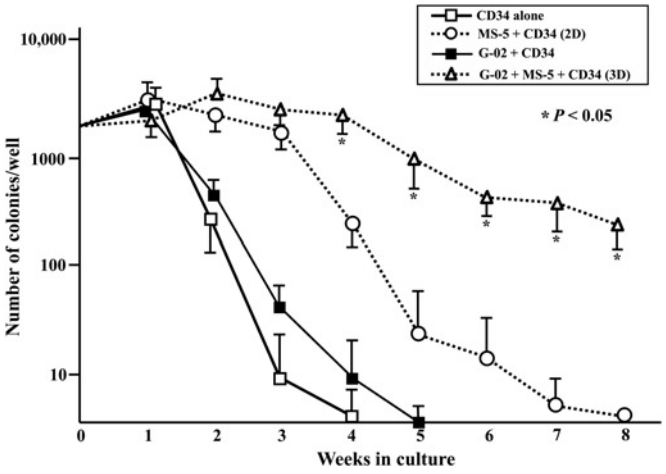

Figure 5 Changes in the numbers of hematopoietic progenitor cells in culture. $CD34^+$ cells (1×10^4; usually containing 3000–4000 progenitors) were inoculated onto the MS-5 cells, supernatant with hematopoietic cells was replaced with fresh growth medium every seven days, and the total numbers of hematopoietic progenitor cells, including CFU-Mix, CFU-GM and BFU-E, were counted. The numbers of the sum of all progenitors (CFU-Mix + CFU-GM + BFU-E) are represented in this figure. Two different wells were prepared for each point, and experiments were performed in triplicate. The results are expressed as the mean ± SD. *Significant difference ($P < 0.05$) with respect to two-dimensional culture. CFU, colony-forming unit; GM, granulocyte-macrophage; BFU-E, burst-forming unit-erythroid

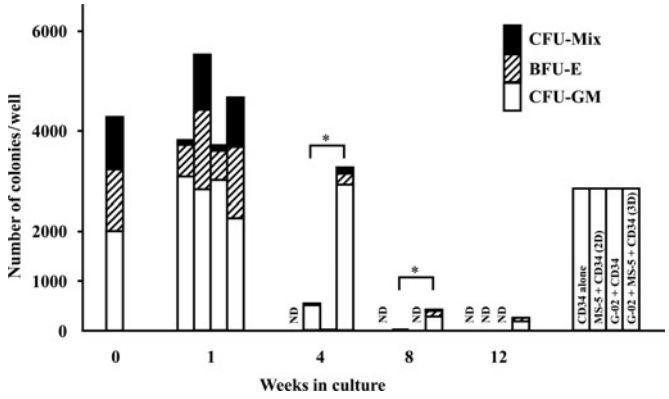

Figure 6 Changes in the numbers of each hematopoietic progenitor cell type in culture. Numbers of CFU-Mix, CFU-GM and BFU-E at one, four, eight and 12 weeks in culture were expressed. Progenitor cells (colony-forming cells) were not detected at weeks 8 and 12 in cultures of $CD34^+$ cells alone, or in cultures with G-02 + $CD34^+$ cells, but without MS-5 cells. *Significant difference ($P < 0.05$) with respect to two-dimensional culture. CFU, colony-forming unit; GM, granulocyte-macrophage; BFU-E, burst-forming unit-erythroid; ND, not detected

Table 1 Number of colony-forming unit-megakaryocyte (CFU-Meg) in supernatant

	Number of CFU-Meg/well	
	MS-5 + CD34 (2D culture)	MS-5 + G-02 + CD34 (3D culture)
Week 0 (start)	358 ± 34	
1 week	143 ± 42	495 ± 55
2 week	96 ± 24	404 ± 23
4 week	13 ± 5	244 ± 31
8 week	0	22 ± 6

Cells in supernatant were collected and the number of CFU-Meg was assayed
The results are expressed as the mean ± SD

and 3D culture was observed; however, Figure 8 shows that the numbers of progenitor cells (CFU-GM, BFU-E and CFU-Mix) in the adherent layer of 3D culture were extremely higher than that of cells in 2D culture ($P < 0.05$). The presence of $CD34^+$ cell was assayed by flow cytometry and immunohistological examination at four weeks after cultivation (Figure 9). Flow cytometry showed that 0.6% and 5.8% of cells among the total adherent layer cells were positive for CD34 in 2D and 3D cultures, respectively (Figure 9a). The percentage of $CD34^+$ in 3D culture was low (5.8%); however, absolute number (1.96×10^5/well) was 20-fold higher as compared with the beginning of the culture (1.0×10^4/well). Immunohistological examination also showed the presence of $CD34^+$ cells in the adherent layer of 3D culture (Figure 9b).

Discussion

Hematopoiesis takes place in the bone marrow *in vivo*, where hematopoietic cells occur in intimate association with distinctive stromal cell elements. The results of several previous studies have suggested that the stromal cell microenvironment plays an important role in hematopoiesis,[1–3] and Schofield[36] proposed the concept of a niche as a specialized microenvironment housing HSCs. A variety of *in vitro* co-culture experiments with stromal and hematopoietic cells have demonstrated that the niche requires both the anatomical and functional dimensions of the stromal cells for the regulation of stem cell quiescence, self-renewal and differentiation. Indeed, while a Dexter-type culture system allows the *in vitro* reproduction of hematopoiesis in terms of the proliferation and differentiation of HSCs, their long-term survival and self-renewal ability are often exhausted within 4–8 weeks in culture.[19–21] Instead of using a traditional 2D culture, recent studies demonstrating the role of 3D topography in hematopoiesis have attracted more interest, and a number of 3D culture systems have been designed to facilitate cell–cell and cell–matrix interactions in an effort to mimic the *in vivo* bone marrow structure.[23–27]

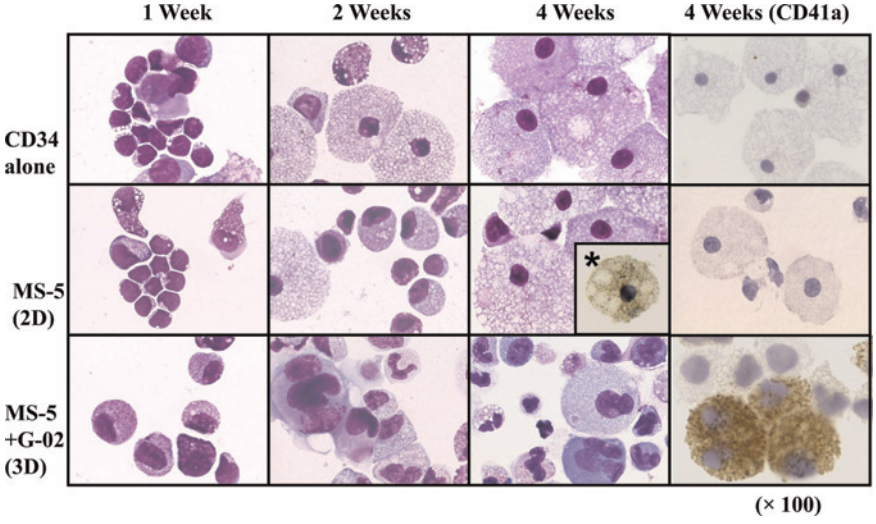

Figure 7 Morphological changes in hematopoietic cells in culture. Slides were prepared by cytospinning, air drying and staining with Wright-Giemsa stain, esterase stain (α-naphthyl acetate) or immunohistological stain using anti-human-CD41a antibody. *Esterase stain (α-naphthyl acetate) of foamy cell. Original magnification: ×100

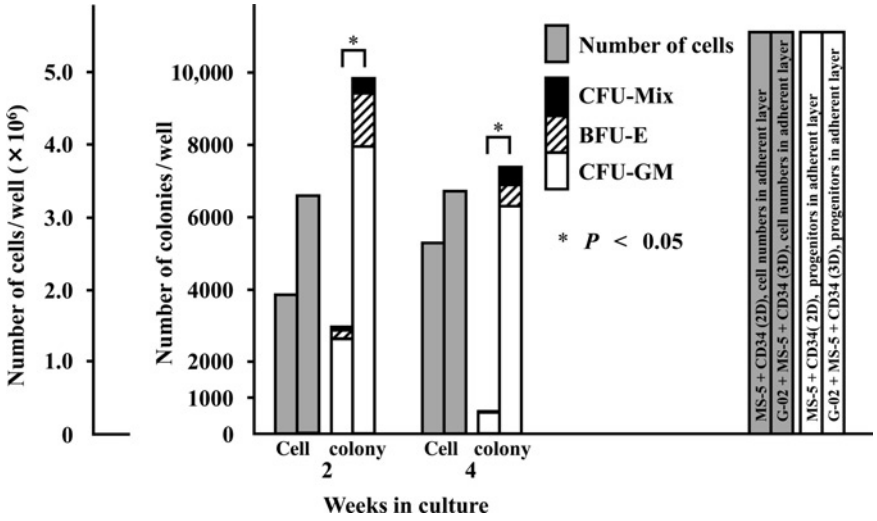

Figure 8 Changes in the numbers of total cells and each hematopoietic progenitor cell type in the adherent layer. Numbers of total and progenitor cells in the adherent layer at two and four weeks of culture were expressed. Adherent cells were treated with 0.25% trypsin plus 0.02% ethylenediaminetetraacetic acid in phosphate-buffered saline, and MS-5 and hematopoietic cells ($CD34^+$ derived cells) were harvested together. An aliquot of cells was assayed to determine the total cell number and the numbers of hematopoietic progenitor cells (CFU-Mix, CFU-GM and BFU-E). *Significant difference ($P < 0.05$) with respect to two-dimensional culture. CFU, colony-forming unit; GM, granulocyte-macrophage; BFU-E, burst-forming unit-erythroid

In this study, we established a novel 3D culture system using polymer particles with grafted epoxy polymer chains for cell immobilization.[31] These polymer particles require no chemical modification, and the cells adhere on addition of the cell suspension. The benefit of this particle is the easy design of the base polymer and grafted chain, in terms of particle size, the length and number of chains, and the composition of the base polymer network. A previous study showed that the grafted epoxy chain was necessary for MS-5 cell adhesion and growth, and that the optimal amount of epoxy groups was 1.03×10^{-3} mol/g-particle. These polymer particles also supported the growth and 3D structure of various types of adherent cells.[31]

Cloned MS-5 cells were used as stromal cells in the current study. They adhered to the surface of the particles, then grew and formed bridges between particles to produce a 3D layer in the culture dish. Inoculated $CD34^+$ cells quickly slipped into the 3D layer of MS-5 cells, and subsequently proliferated and differentiated in the culture. Both the proliferation and differentiation were maintained for significantly longer in the 3D system, compared with the traditional 2D culture system. Zhang *et al.*[23] reported the expansion of $CD34^+$ human HSCs (891-fold increase

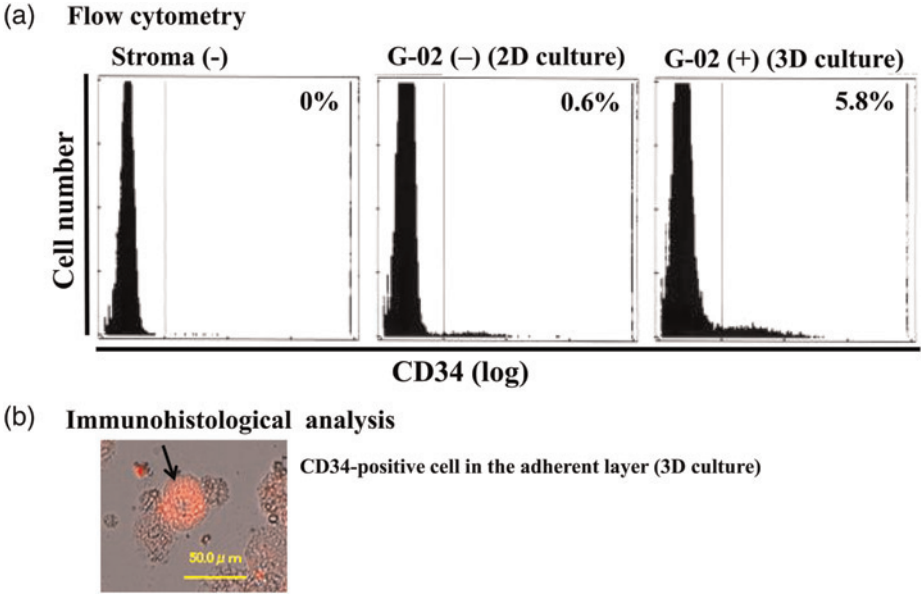

Figure 9 Detection of $CD34^+$ cells in the adherent layer by flow cytometry and immunohistological examination. Adherent cells were treated with 0.25% trypsin plus 0.02% ethylenediaminetetraacetic acid, and MS-5 and hematopoietic cells ($CD34^+$ derived cells) were harvested together. The presence of $CD34^+$ cells among these cells was analyzed by flow cytometry (a) and immunohistological (b) examination. (b) Original magnification: ×100

in total hematopoietic cells) in a 3D HSC-mesenchymal stem cell co-culture system using PET woven mesh, but the exogenous addition of hematopoietic growth factors (stem cell factor [SCF], Flt-3 ligand, thrombopoietin and interleukin [IL]-3) was required. The successful prolongation of stem cell proliferation and differentiation observed in our culture system did not require the addition of any biological agents, such as hematopoietic growth factors. In some experiments, adherent cells were collected by trypsin-EDTA treatment, and increased numbers of $CD34^+$ cells and progenitors (CFU-Mix, BFU-E and CFU-GM) were found in 3D culture (Figures 8 and 9). It has been reported that $CD34^+$ cells can differentiate into not only hematopoietic cells but also mesenchymal cells; however, these results suggest that $CD34^+$ cells and hematopoietic progenitors were well reproduced in the 3D culture system, and 3D structure with stromal cells may provide proper conditions for proliferation and differentiation of HSCs.

Interestingly, morphological examination showed that various types of hematopoietic cells, including blast-like cells, granulocytes, macrophages and megakaryocytes at different maturation stages, appeared in the 3D culture medium after one, two and foour weeks of culture (Figure 7), suggesting that $CD34^+$ cells were able to differentiate into various types of blood cells in this system. In fact, increased numbers of hematopoietic progenitors including CFU-Mix, BFU-E, CFU-GM and CFU-Meg were detected in the 3D culture system (Figures 6 and 8 and Table 1). Sullenbarger *et al.*[27] reported the prolonged and increased production of platelets using 3D polyacrylamide hydrogel cell culture scaffold, while Berthier *et al.*[37] showed the supportive function of MS-5 cells for megakaryocytic differentiation of embryonic stem cells in 2D culture; however, exogenous hematopoietic factors were necessary in both of these studies. Zdzisinska *et al.*[30] reported the enhanced production of IL-11 and hepatocyte growth factor from bone marrow stromal cells in 3D culture with a gelatin sponge scaffold, compared with that in 2D culture. It is possible that the production of cytokines from MS-5 cells was modulated in 3D culture, supporting the differentiation of $CD34^+$ cells into various types of blood cells in this study. Although MS-5 cells originate from the murine bone marrow, they are able to activate the growth of human $CD34^+$ HSCs by producing murine SCF and murine granulocyte colony-stimulating factor.[34] Various cytokines with different combinations have been studied to support the proliferation and differentiation of $CD34^+$ cells *in vitro*, and successful cytokine-mediated expansion has been reported as a means of increasing the number of $CD34^+$ cells.[38–41] Comparative studies to investigate the production of various cytokines by stromal cells in 3D and 2D cultures are now under consideration. The roles of macrophages and endothelial cells, generated from umbilical CB $CD34^+$ cells, in the configuration of the stem cell niche, and the complexities of these cells may also contribute to the hematopoietic phenomenon observed in this 3D culture system. Studies are therefore underway to examine the modulation of cytokine production in both MS-5 and $CD34^+$-derived cells, using murine and human biomarkers, individually.

HSCs, enriched in a fraction of $CD34^+$ cells obtained from each bone marrow, CB and peripheral blood, were found to have different biological characters, such as response to cytokine stimulation.[42,43] In addition, cell preparation for cytokine-mediated proliferation and differentiation would seem to influence the resulting cell population, even though HSCs were obtained from same blood source.[44,45] Thus, comparative studies in a 3D culture system using $CD34^+$ cells originating from bone marrow and peripheral blood are now designed, because these cells are the common source for HSCs.

Although further studies are required to analyze the mechanisms responsible for the supportive activities of MS-5 cells in 3D culture, the results of the current study indicate that a 3D culture system using polymer particles might provide a new tool for investigating the regulatory behaviors of stromal cells *in vitro*.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. YoH, JT, IT, WRM, MY and SA conducted the experiments; KO made polymer particles; and YuH, SA and TH wrote the manuscript.

ACKNOWLEDGEMENTS

This work was supported in part by Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science and Nihon University School of Medicine Alumni Association 60th Anniversary Fund Research Grant (2010).

REFERENCES

1 Wolf NS, Trentin JJ. Hemopoietic colony studies. V. Effect of hemopoietic organ stroma on differentiation of pluripotent stem cells. *J Exp Med* 1968;**127**:205–8
2 Greenberger JS. The hematopoietic microenvironment. *Crit Rev Oncol Hematol* 1991;**11**:65–84
3 Mayani H, Guilbert LJ, Janowska-Wieczorek A. Biology of the hemopoietic microenvironment. *Eur J Haematol* 1992;**49**:225–33
4 Dexter TM, Allen TD, Lajtha LG. Conditions controlling the proliferation of haematopoietic stem cells *in vitro*. *J Cell Physiol* 1977;**91**:335–44
5 Mori KJ, Fujitake H, Okubo H, Dexter TM, Ito Y. Quantitative development of adherent cell colonies in bone marrow cell culture *in vitro*. *Exp Hematol* 1979;**7**:171–6
6 Kodama H, Amagai Y, Koyama H, Sakai S. A new preadipose cell line derived from new born mouse calvaria can promote the proliferation of pluripotent hemopoietic stem cells *in vitro*. *J Cell Physiol* 1982;**112**:89–96
7 Song ZX, Shadduck RK, Innes DJ, Waheed A, Quesenberry PJ. Hematopoietic factor production by a cell line (TC-1) derived from adherent murine marrow cells. *Blood* 1985;**66**:273–8
8 Hunt P, Ribertson D, Weiss D, Rennick D, Lee F, Witte ON. A single bone marrow-derived stromal cell type supports the *in vitro* growth of early lymphoid and myeloid cells. *Cell* 1987;**48**:997–1005
9 Harigaya K, Handa H. Generation of functional clonal cell lines from human bone marrow stroma. *Proc Natl Acad Sci USA* 1985;**82**:3477–80
10 Aizawa S, Yaguchi M, Nakano M, Inokuchi S, Handa H, Toyama K. Establishment of a variety of human bone marrow stromal cell lines by the recombinant SV40-adenovirus vector. *J Cell Physiol* 1991;**148**:245–51
11 Heard J, Fichekson S, Varet B. Role of colony-stimulating activity in murine long-term bone marrow cultures: evidence for its production and consumption by the adherent cells. *Blood* 1982;**59**:761–7
12 Aizawa S, Yaguchi M, Nakano M, Toyama K, Inokuchi S, Imai T, Yasuda M, Nabeshima R, Handa H. Hemopoietic supportive function of human bone marrow stromal cell lines established by a recombinant SV40-adenovirus vector. *Exp Hematol* 1994;**22**:482–7

13 Dormady SP, Bashayan O, Dougherty R, Zhang XM, Basch RS. Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. *J Hematother Stem Cell Res* 2001;**10**:125–40
14 Bodo M, Baroni T, Tabilio A. Haemopoietic and stromal stem cell regulation by extracellular matrix components and growth factors. *J Stem Cells* 2009;**4**:57–69
15 Wagner W, Roderburg C, Wein F, Diehlmann A, Frankhauser M, Schubert R, Eckstein V, Ho AD. Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. *Stem Cells* 2007;**25**:2638–47
16 Wang X, Hisha H, Mizokami T, Cui W, Cui Y, Shi A, Song C, Okazaki S, Li Q, Feng W, Kato J, Ikehara S. Mouse mesenchymal stem cells can support human hematopoiesis both *in vitro* and *in vivo*: the crucial role of neural cell adhesion molecule. *Haematologica* 2010;**95**:884–91
17 Paraguassu-Braga FH, Borojevic R, Bouzas LF, Barcinski MA, Bonomo A. Bone marrow stroma inhibits proliferation and apoptosis in leukemic cells through gap junction-mediated cell communication. *Cell Death Differ* 2003;**10**:1101–8
18 Durig J, Rosenthal C, Halfmeyer K, Wiemann M, Novothny J, Bingmann D, Dührsen U, Schirrmacher K. Intercellular communication between bone marrow stromal cells and CD34+ haematopoietic progenitor cells is mediated by connexin 43-type gap junctions. *Br J Haematol* 2000;**111**:416–25
19 Issaad C, Croisille L, Katz A, Vainchenker W, Coulombel L. A murine stromal cell line allows the proliferation of very primitive human CD34 ++/CD38- progenitor cells in long-term cultures and semisolid assays. *Blood* 1993;**81**:2916–24
20 Loeuillet C, Bernard G, Rémy-Martin JP, Saas P, Hervé P, Douay L, Chalmers D. Distinct hematopoietic support by two human stromal cell lines. *Exp Hematol* 2001;**29**:736–45
21 Sensebe L, Li J, Lilly M, Crittenden C, Herve P, Charbord P, Sinfer JW. Nontransformed colony-derived stromal cell lines from normal human marrows. I. Growth requirement and myelopoiesis supportive ability. *Exp Hematol* 1995;**23**:507–13
22 Adams GB, Scadden DT. The hematopoietic stem cell in its place. *Nat Immunol* 2006;**7**:333–7
23 Zhang Y, Chai C, Jiang XS, Teoh SH, Leong KW. Co-culture of umbilical cord blood CD34+ cells with human mesenchymal stem cells. *Tissue Eng* 2006;**12**:2161–70
24 Feng Q, Chai C, Jiang XS, Leong KW, Mao HQ. Expansion of engrafting human hematopoietic stem/progenitor cells in three-dimensional scaffolds with surface-immobilized fibronectin. *J Biomed Mater Res A* 2006;**15**:781–91
25 Tun T, Miyoshi H, Aung T, Takahashi S, Shimizu R, Kuroha T, Yamamoto M, Ohshima N. Effect of growth factors on *ex vivo* bone marrow cell expansion using three-dimensional matrix support. *Artif Organs* 2002;**26**:333–9
26 Miyoshi TT, Ema H, Nakauchi H, Ohshima N. New type of matrix support for bone marrow cell cultures: *in vitro* culture and *in vivo* transplantation experiments. *ASAIO J* 2000;**46**:522–6
27 Sullenbarger B, Bahng JH, Gruner R, Kotov N, Lasky LC. Prolonged continuous *in vitro* human platelet production using three-dimensional scaffolds. *Exp Hematol* 2009;**37**:101–10
28 Petrenko YA, Petrenko AY, Damshkaln LG, Volkova NA, Lozinsky VI. Growth and adipogenic differentiation of mesenchymal stromal bone marrow cells during culturing in 3D macroporous agarose cryogel sponges. *Bull Exp Biol Med* 2008;**146**:129–32
29 Ishaug-Riley SL, Crane-Kruger GM, Yaszemski MJ, Mikos AG. Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* 1998;**19**:1405–12
30 Zdzisinska B, Rolinski J, Piersiak T, Kandefer-Szerszen M. A comparison of cytokine production in 2-dimensional and 3-dimensional cultures of bone marrow stromal cells of multiple myeloma patients in response to RPMI18226 myeloma cells. *Folia Histochem Cyto* 2009;**47**:69–74
31 Yasuda M, Kunieda H, Ono K, Ogino H, Iwasaki T, Hiramoto M, Glomm WR, Hirabayashi Y, Aizawa S. Adhensive cell cultivation on polymer particles having grafted epoxy polymer chain. *Tissue Cell* 2011;**43**:115–24
32 Itoh K, Tezuka H, Sakoda H, Konno M, Nagata K, Uchiyama T, Uchino H, Mori KJ. Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. *Exp Hematol* 1989;**17**:145–53
33 Suzuki J, Fujita J, Taniguchi S, Sugimoto K, Mori KJ. Characterization of murine hemopoietic-supportive (MS-1 and MS-5) and non-supportive (MS-K) cell lines. *Leukemia* 1992;**6**:452–8
34 Nishi N, Ishikawa R, Inoue H, Nishikawa M, Yoneya T, Kakeda M, Tsumura H, Ohashi H, Mori KJ. *In vitro* long-term culture of human primitive hematopoietic cells supported by murine stromal cell line MS-5. *Leukemia* 1997;**11**:468–73
35 Frederic TW. *Determination of Organic Compounds: Methods and Procedures*. New York: Wiley-Interscience, 1970:207
36 Schofield R. The relationship between the spleen colony-forming cell and the hematopoietic stem cell. *Blood Cells* 1978;**4**:7–25
37 Berthier R, Prandini MH, Schweitzer A, Thevenon D, Martin-Sisteron H, Uzan G. The MS-5 murine stromal cell line and hematopoietic growth factors synergize to support the megakaryocytic differentiation of embryonic stem cells. *Exp Hematol* 1997;**25**:481–90
38 Abboud MR, Xu F, Payne A, Laver J. Effects of recombinant human Steel factor (c-kit ligand) on early cord blood hematopoietic precursors. *Exp Hematol* 1994;**22**:388–92
39 Bertolini F, Lazzari L, Lauri E, Corsini C, Sirchia G. Cord blood-derived hematopoietic progenitor cells retain their potential for *ex vivo* expansion after cryopreservation. *Bone Marrow Transplant* 1994;**14**:347–53
40 Moore MAS, Hoskins I. *Ex vivo* expansion of cord blood-derived stem cells and progenitors. *Blood cells* 1994;**20**:468–81
41 Ruggieri L, Heimfeld S, Broxymeyer HE. Cytokine-dependent *ex vivo* expansion of early subsets of CD34+ cord blood myeloid progenitors is enhanced by cord blood plasma, but expansion of more mature subsets of progenitors is favoured. *Blood Cells* 1994;**20**:436–54
42 De Bruyn C, Delforge A, Lagneaux L, Bron D. Characterization of CD34+ subsets derived from bone marrow, umbilical cord blood and mobilized peripheral blood after stem cell factor and interleukin3 stimulation. *Bone Marrow Transplant* 2000;**25**:377–83
43 Hao QL, Shah AJ, Thiemann FT, Smogorzewska EM, Crooks GM. A functional comparison of CD34 + CD38- cells in cord blood and bone marrow. *Blood* 1995;**86**:3745–53
44 Koutna I, Klabusay M, Koutova V, Krontorad P, Svoboda Z, Kozubek M, Mayer J. Evaluation of $CD34^{+}$-and Lin^{-}-selected cells from peripheral blood stem cell grafts of patient with lymphoma during differentiation in culture *ex vivo* using a cDNA microarray technique. *Exp Hematol* 2006;**34**:832–840
45 Case J, Rice A, Vowels M. Cytokine mediated expansion of human umbilical cord blood CD34+ cells: comparison of the use of partially purified and pure CD34+ target cells. *Hematol Cell Ther* 1997;**39**:193–7

(Received March 4, 2011, Accepted July 20, 2011)