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Stroma-dependent Maintenance of Cytokine Responsive Hematopoietic Progenitor Cells Derived from Long-term Bone Marrow Culture

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ABSTRACT. **Hematopoietic cells maintained for long periods on primary cultures of bone marrow stromal cells formed cobblestone colonies (Dexter's long-term bone marrow culture, LTBC). These stably maintained hematopoietic cells (for 4 months) were transferred to a coculture on an established spleen stromal cell line (MSS62), and maintained under stromal cell layer, where they retained their invasive ability in the restricted space between the stromal cell layer and culture substratum (DFC culture). DFC contained lineage-negative (Lin^-), c-Kit^+, Sca-1^+ cells and spontaneously produced Mac-1^+, Gr-1^+ cells. DFC could not grow in the absence of MSS62 stromal cells, although, GM-CSF, IL-3, or IL-7 stimulated its growth. Production of granulocyte and monocytic cells was maintained by GM-CSF or IL-3 while it was decreased by IL-7. RT-PCR analysis showed that the IL-7 responsive cell population expressed early lymphoid markers (Ikaros, Pax-5, Oct-2, Rag-1, TdT, IL-7R and Iμ), while lacking expression of receptors for G-CSF (G-CSFR) and for M-CSF (M-CSFR), or myeloperoxidase (MPO). These results suggested that DFC simultaneously contained lymphoid-committed progenitors and myeloid-committed progenitors, and that cytokines may expand their responding progenitor cells under the influence of signals provided by the stromal cells. Such a stromal cell-dependent culture system may be useful to analyze the switching mechanism from constitutive to inducible hematopoiesis *in vitro*.**

Key words: stromal cell/cobblestone/hematopoietic progenitor

Dexter's long-term bone marrow culture (LTBC) allows a balance to be maintained between self-renewal and differentiation of hematopoietic progenitor cells, with the progenitor cells being thought to be regulated by cellular interaction with the stromal cells (Dexter *et al.*, 1976; Toksoz *et al.*, 1980). While many defined cytokines are shown to be required for the rapid expansion of the progenitor cells (inductive hematopoiesis), they were not sufficient factors for constitutive hematopoiesis (Wineman *et al.*, 1996; Verfaille, 1993; Levesque *et al.*, 1996; Iguchi *et al.*, 1997). Stromal cell-dependent hematopoiesis is characterized by slow growth and formation of cobblestone area in LTBC and reflected the constitutive hematopoiesis which occurred in bone marrow (Ploemacher and Brons, 1989; Ploemacher *et al.*, 1993). Constitutive hematopoiesis may require additional factors secreted from the stromal cells, extracellular matrices and adhesion molecules, as observed in LTBC (Levesque, 1996; Zuckerman and Wicha, 1983; Paul *et al.*, 1991; Koenigsmann *et al.*, 1992). Addition of cytokines to LTBC stimulated expansion of the respective progenitors in culture, thus LTBC may be appropriate for studying the switching mechanism from constitutive (stromal cell-dependent) to inductive (cytokine-promoted) hematopoiesis. However, detailed cellular and molecular analysis is difficult because LTBC contains mixtures of a variety of stromal cells (Dexter *et al.*, 1984; Gimble, 1990) and heterogeneous hematopoietic cells. If the clonal hematopoietic stem cells or their progenitor cells could be maintained on a single type of stromal cells, the facilitated cellular and molecular analysis of the switching mechanism may be easier. Thus, in the present study, we tried to maintain the stromal cell-dependent hematopoietic cells without cytokine addition, and obtained a reproducible culture we called DFC. Since the DFC could be maintained by replacing the mixed

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Abbreviations: GM-CSF, granulocyte macrophage-colony stimulating factor; IL-3, interleukin-3; IL-7, interleukin-7; G-CSF, granulocyte-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; RT-PCR, reverse transcription-polymerase chain reaction; DTT, dithiothreitol; M-MLV, Moloney-mouse leukemia virus.

stromal cell population of LTBC with the previously established spleen stromal cell line (MSS62), the properties of growth and differentiation of DFC on stromal cells and under cytokine-promoted condition were examined.

Materials and Methods

Long term bone marrow cell culture

Mouse femoral bone marrow was obtained from 10- to 15-week-old C57BL/6 mice and cultured in Fischer's medium (Sigma, St. Louis, MO, USA) supplemented with 20% horse serum (HS) and 10^{-7} mol/L hydrocortisone. Cultures were maintained until many hematopoietic colonies were produced at 33°C for 3 to 4 weeks. To prepare cobblestone area-forming hematopoietic cells, cultures were treated with 0.5% collagenase in DMEM (Dulbecco's MEM, GibcoBRL, Grand Island, NY, USA) followed by gentle pipetting. Cells harvested from the adherent layer of long-term bone marrow cultures were precultured to separate hematopoietic cells from stromal cells for 2 hr in culture medium at 37°C. Hematopoietic cells were collected in suspension after a preculture, and stromal cells were adhered onto a plastic culture substratum. The harvested hematopoietic cells were passaged onto 15 Gy irradiated primary bone marrow culture independently prepared with 10% fetal bovine serum (FBS), 10% HS, and without hydrocortisone. Hematopoietic cells obtained from an adherent layer were maintained for 4 months with 10% FBS and 10% HS containing Fischer's medium on a stromal cell layer of primary bone marrow culture by serial passages every 10 days. After 4 months, hematopoietic cells (DFC) were passaged on a monolayer of stromal cell line, MSS62, established from newborn spleen (Yanai *et al.*, 1989) with 0.1 mM 2-mercaptoethanol, 10% HS and 10% FBS containing Fischer's medium. MSS62 was maintained in RITC 80-7 (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 2% FBS, 10 μg/mL transferrin, 1 μg/mL insulin, and 10 ng/mL epidermal growth factor (EGF, recombinant; generously supplied by Wakunaga Pharmaceutical Co., Ltd., Tokyo, Japan).

Cytokines

Recombinant murine G-CSF, GM-CSF, IL-3 and IL-6 were kindly provided by KIRIN (Tokyo, Japan). Recombinant murine IL-7 was purchased from R&D Systems (Minneapolis, MN, USA).

Flowcytometry

For immunofluorescence staining, DFC cells were separated from MSS62 stromal cells, washed and 1×10^6 cells were incubated with the first antibody in 0.2% BSA-PBS for 30 minutes on ice. Monoclonal antibodies specific to lineage markers were used: fluorescein isotiocyanete (FITC) conjugated anti-mouse Gr-1, Mac-1, B220, TER119, CD4 and CD8 antibodies (Pharmingen, San Diego, CA), phycoerythrin (PE) conjugated anti-mouse Sca-1 (Pharmingen), and biotinylated anti c-Kit monoclonal antibody (ACK-2; generously supplied by Dr. S.-I. Nishikawa, Kyoto University). The stained cells were washed with 0.2% BSA-PBS, and incubated with Streptavidin APC (Becton-Dickinson, San Jose, CA) for 30 minutes on ice as the second antibody for c-Kit staining. After washing, cells were analyzed by FACStar PLUS (Becton-Dickinson) and Cellquest software (Becton-Dickinson).

May-Giemsa stain

For morphological observation, cell suspension ($5–6\times10^4$ cells) was plated on a slide by Cytospin (Shandon, Cheshire, UK). After fixation with May-Grunwald solution, the sample was stained with Giemsa solution.

RT-PCR analysis

Expression of myeloid and B lymphocyte lineage specific genes was examined by RT-PCR in DFC cells. The total RNA of DFC cells ($1–2\times10^6$ cells) was extracted by the acid phenol procedure using ISOGEN (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) according to the manufacturer's protocol. Five μg of total RNA was incubated with random primer (15 μg/mL PROMEGA, Madison, WI), M-MLV reverse transcriptase (40 U/μg total RNA) in 50 mM Tris HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 1 mM dNTPs at 37°C for 60 min. The RT reaction was terminated by heat inactivation (70°C, 10 min). PCR amplification was performed using the cDNA products with 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM dNTPs, 0.5 U of AmpliTaq Gold™ (Perkin Elmer, Branchburg, NJ) and 1 μM of specific primer set for each gene (Matsuda *et al*., 1999; Yanai *et al*., 1999) for 25 cycles consisting of successive incubation at 94°C (30 sec), 55°C (30 sec), 72°C (90 sec). PCR products were electrophoresed by 2% agarose gel, and visualized by ethidium bromide staining.

Results

LTBC of hematopoietic cells

LTBC was conducted by Dexter's protocol, and during the first 4 weeks of culture many hematopoietic cells were released into the culture medium while maintaining of the cobblestone areas formed under the stromal cells. As the steady-state phase, the release of the hematopoietic cells lasted for almost 12 weeks. To passage cobblestone area-forming hematopoietic progenitor cells onto the independently established bone marrow stromal cell layer, cultures were treated by collagenase followed by preincubation to remove nonhematopoietic cells. Only stroma-dependent hematopoietic cells were passaged onto stromal cell feeders made from LTBC. After 12 weeks (8 passages), the cultures reached a steady state where most hematopoietic cells were maintained under stromal cell layers by forming cobblestone areas; this culture was designated as DFC culture. On the LTBC-derived stromal cell layer, DFC cells exhibited uneven distribution caused by heterogeneity of stromal cell population. When we investigated whether established stromal cell lines of hematopoietic tissues could support maintenance of DFC, we found that spleen stromal cell line,

MSS62, has the ability to maintain DFC. MSS62 was previously established from newborn mice spleen and shown to support rapid expansion of erythroid progenitor cells (Yanai *et al.*, 1989). Morphology (Fig. 1) and the expression of markers on DFC cells were analyzed by flow cytometry. DFC contained granulocytes, monocytes, macrophages, and blastic cells. The cultures were constantly maintained with cobblestone area formation for several months (about 10 passages), and hematopoietic cells grew slowly (Fig. 2; generation time=2.6 days). The properties of the cell population maintained on MSS62 stromal cells showed Gr-1$^+$ (2.8%), Mac-1$^+$ (5.6%), or Lin$^-$ (Gr-1$^-$, Mac-1$^-$, B220$^-$, TER119$^-$; 91.0%), and 8.9% of Lin$^-$ was Sca-1$^+$/c-Kit$^+$ (Fig. 3).

Effect of cytokines on growth and differentiation of DFC

The effect of cytokines on growth and differentiation of DFC was examined. Addition of GM-CSF, IL-3 and IL-7 had a strong stimulatory effect on growth after one week, while G-CSF and IL-6 showed no effect (Fig. 4). The population doubling time of DFC became faster and reached 0.50, 0.42, and 0.43 days of the control with the addition of GM-CSF, IL-3, and IL-7, respectively. These stimulatory effects were only observed in the coculture with MSS62 stromal cells; if DFC cells were cultured in the absence of stromal cells, none of these cytokines supported maintenance of DFC. Addition of GM-CSF or IL-3 showed that cells were adhering to the top of stromal cells and shedding into culture medium, whereas with the addition of IL-7, cells tended to form cobblestone areas underneath the stromal cell layers. Accompanying these differences, the morphologies, Giemsa staining (Fig. 5), and flowcytometrical analysis of DFC cells (Fig. 6) showed that IL-3 or GM-CSF stimulated growth expansion of the myeloid cell lineages such as monocytes or granulocytes, but that the proportions of Lin$^+$ and c-Kit$^+$ cells were essentially the same as those on MSS62 stromal cells without cytokines. On the contrary, IL-7 seemed to promote growth of blastic cells, and myeloid cells were scarcely observed after 7 days. RT-PCR analysis for expression of hematopoietic cell lineage markers (Fig. 7) showed that IL-7 promoted expansion of the early B lymphoid progenitors. While B220, a pan-B-cell marker, was not expressed even with the addition of IL-7, expression of many lymphocyte-specific markers, such as genes for Pax-5, Ikaros, Rag-1, TdT and IL-7 receptor, and Iμ, a sterile transcript of germline IgG heavy chain, were enhanced in IL-7 stimulated culture suggesting the expansion of lymphoid-committed progenitor cells. In addition, the cells in IL-7 stimulated culture showed decrease of gene expressions for receptors of GM-CSF, G-CSF and M-CSF, and also showed the myeloid specific genes MPO, CD11b and CD18 (Fig. 8), suggesting the decrease of myeloid-committed progenitor cells. These results indicated that MSS62 cells retained DFC as immature cells and spontaneously in-

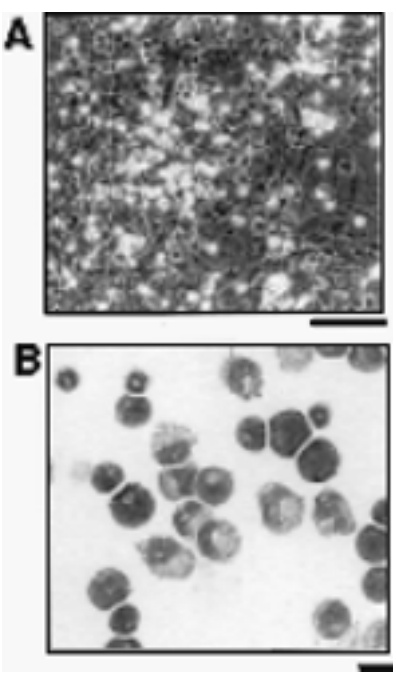

Fig. 1. DFC cells on MSS62 stromal cells. DFC cells were maintained on an MSS62 layer containing phase-contrast nonrefractile cells and adhering phase-contrast bright cells (A). Bar indicates 100 μm in A. They were separated from the MSS62 layer by preculture and cytocentrifuged. Cells were stained with May-Grunwald-Giemsa (B). Bar indicates 10 μm in B.

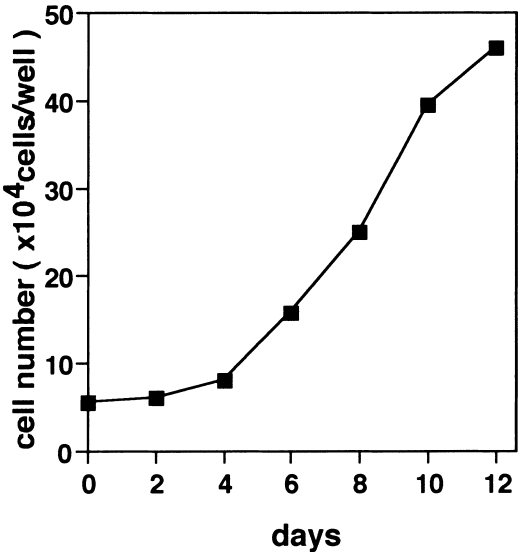

Fig. 2. Growth of DFC. Cells were plated on MSS62 feeder cell layer in 6-well culture plates. Cell numbers were counted after preincubation to separate DFC from MSS62 and are the average of 2 wells.

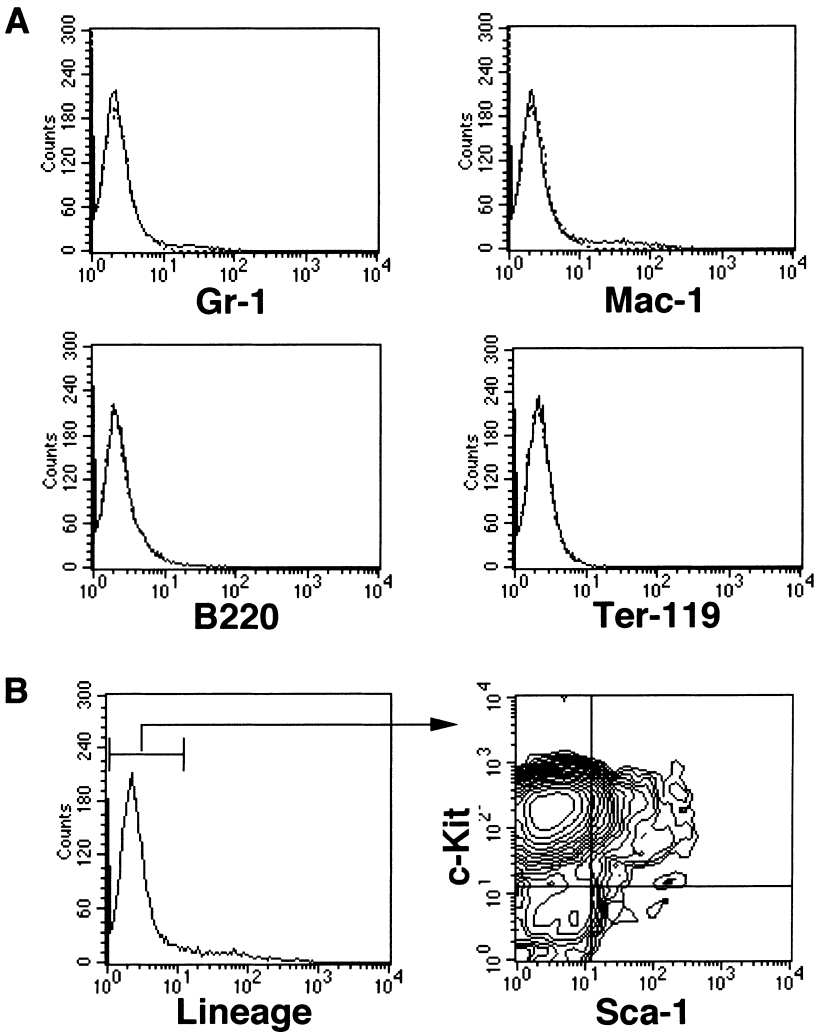

Fig. 3. Flowcytometrical analysis of DFC cells. Expression of surface markers on DFC separated from MSS62. (A) Expression of lineage specific markers (Gr-1 for granulocytes, Mac-1 for monocytes, B220 for B cells, and TER119 for erythroblasts). (B) Expression of Sca-1 and c-Kit among Lin^- population.

duced myeloid cell lineages, and that IL-3 and GM-CSF stimulated growth expansion with the same population balance in expression of surface antigen, while IL-7 selectively stimulated expansion of the B-lymphoid cell lineage of DFC. Addition of erythropoietin to the culture did not induce any erythroid differentiation (data not shown), which is consistent with a lack of erythropoietin receptor gene expression. Although M-CSF receptor and G-CSF receptor were expressed in DFC, neither cytokine showed a stimulatory effect.

Discussion

In the present study we developed a new culture system for stromal cell-dependent primitive hematopoietic cells using established stromal cells, starting from Dexter's LTBC. We previously established a stromal cell-dependent primitive cell line of B-lymphocyte lineage (Matsuda *et al.*, 1999) and a primitive hematopoietic cell line (Yanai *et al.*, 1999) using established bone marrow stromal cell lines selected for their high cobblestone area forming ability (Okuyama *et al.*, 1995). Different stromal cells may fix different phenotypes of established hematopoietic cell lines (Koguma *et al.*, 1998; Spooncer and Dexter, 1996; Itoh *et al.*, 1996). Bone marrow stroma consisted of different types of mesenchymal cells such as preadipocytes, endothelial cells and fibroblastoid cells, as observed in the Dexter type LTBC (Piersma *et al.*, 1985; Grigoriadis *et al.*, 1988; Okuyama *et al.*, 1995), and may have different supporting activity for the hematopoietic stem cells and their progenitors (Kameoka *et al.*, 1995). We therefore attempted to culture the hematopoietic cells on the primary cultured bone marrow stromal cell layers, expecting that the stroma-dependent primitive hematopoietic cells may be different from previously obtained stro-

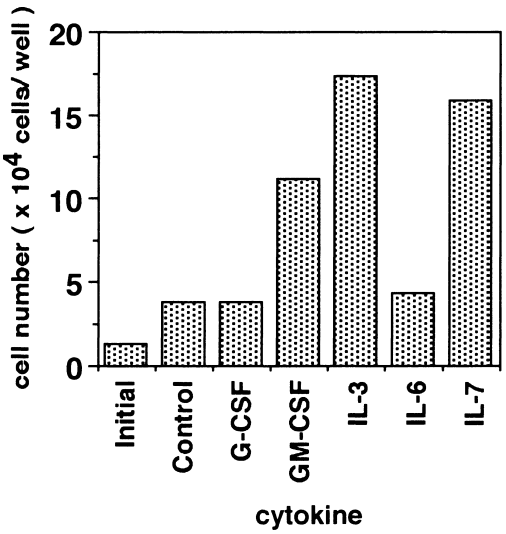

Fig. 4. Effect of several cytokines on MSS62-dependent DFC. IL-3 (10 ng/mL), IL-6 (1 ng/mL), IL-7 (5 ng/mL), G-CSF (10 ng/mL), or GM-CSF (10 ng/mL) was added to DFC on MSS62 for 7 days. Cell numbers were counted after preincubation to separate DFC from MSS62.

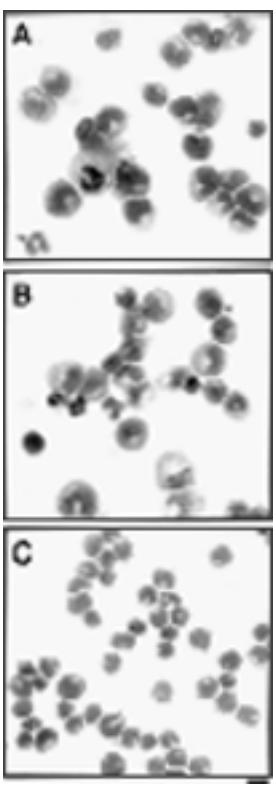

Fig. 5. Growth stimulated hematopoietic cells by GM-CSF, IL-3, or IL-7. Each cytokine was added for 7 days. GM-CSF (A), IL-3 (B), and IL-7 supplemented culture (C). Hematopoietic cells growing on MSS62 cells were harvested and stained with May-Grunwald-Giemsa. Bar indicates 10 μm.

mal cell-dependent hematopoietic cell lines using the clonal bone marrow stromal cell lines as feeders. We obtained DFC, which was maintained on a previously established stromal cell line from newborn mouse spleen (MSS62), in place of mixed stromal cells of LTBC. DFC was a mixture of granulocytes (Gr-1$^+$), macrophages (Mac-1$^+$), and Lin$^-$/c-Kit$^+$ primitive progenitor cells, and grew slowly depending on the stromal cells. On MSS62 stromal cells, the majority of DFC (91%) remained as the progenitor cells and a minor population of the cells spontaneously differentiated into either granulocytes or macrophages. The stromal cell-dependent maintenance of the primitive progenitors observed on MSS62 cells may be similar to the constitutive hematopoiesis, and the sufficient signals required for the constitutive hematopoiesis may be supplied by MSS62 stromal cells. It is of interest to determine how these cells can be maintained by the factors produced by MSS62 stromal cells.

On MSS62 stromal cells, addition of cytokines selectively produced the differentiated cell populations from DFC. IL-3 or GM-CSF expanded the cell population that expressed Mac-1, Gr-1 and several myeloid specific genes, while IL-7 expanded the cell population that expressed lymphoid lineage-specific transcription factors and IL-7 receptor subunit on the stromal cells. MSS62 may support two lineages of the committed hematopoietic progenitor cells in DFC to a similar extent without inducing their differentiation, while maintaining a slow proliferation frequency. Signals from the cytokine receptors may be stimulated by those being supplied from the stromal cells, because the action of

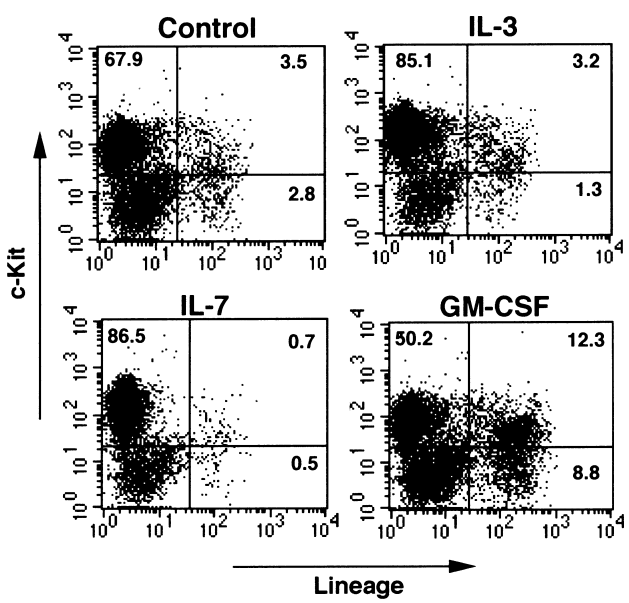

Fig. 6. Flowcytometrical analysis of cytokine stimulated DFC. Expression of surface markers on DFC separated from MSS62 after 7 days culture with cytokines. Expression of c-Kit and lineage markers (cocktail of Gr-1, Mac-1, CD4, CD8, TER119, and B220) was observed. Numbers in quadrant indicate % of total cells.

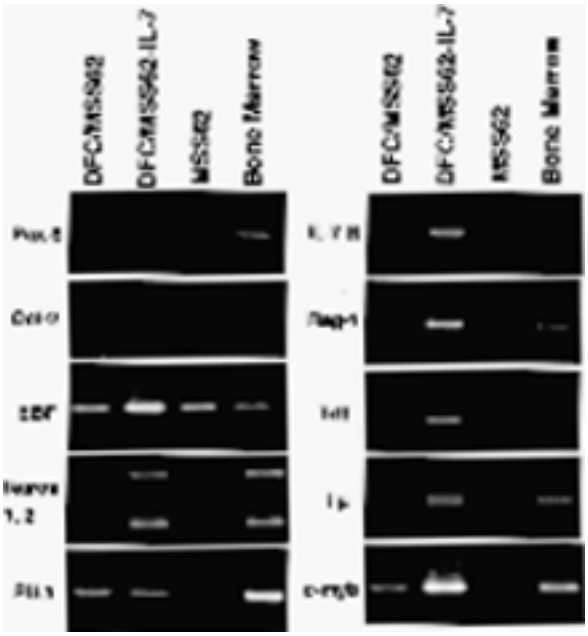

Fig. 7. Expression of B cell specific genes in DFC. Total RNA was prepared from bone marrow, MSS62 stromal cells, IL-7 induced DFC separated from MSS62, and DFC separated from MSS62. RT-PCR products were resolved on 2% agarose gel.

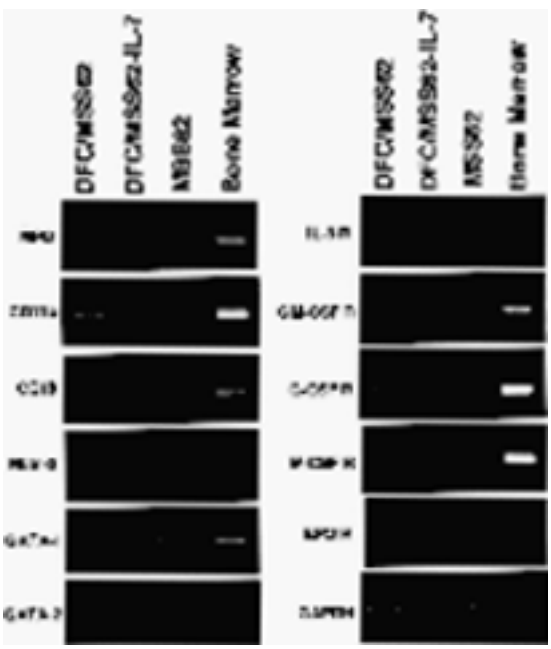

Fig. 8. Expression of hematopoietic genes in DFC. Total RNA was prepared from bone marrow, MSS62 stromal cells, IL-7 induced DFC separated from MSS62, and DFC separated from MSS62. RT-PCR products were resolved on 2% agarose gel.

cytokines was stimulated only in the presence of stromal cells. Cytokines may selectively act to switch the rapid expansion of the two cell populations, similar to inductive hematopoiesis. Constitutive hematopoiesis depending on stromal cell signals retained ability to accept cytokine signals. However, some primitive hematopoietic cell lines have been established from LTBC such as FDCP (Spooncer *et al.*, 1986), 32D (Greenberger *et al.*, 1983), and Myl-D-7 (Itoh *et al.*, 1996) while these can be maintained without stromal support, DFC cells maintained stromal cell dependency and responded to the cytokine signals only when stromal signals were present. Thus, our coculture system provided a constitutive hematopoiesis which could be switched to inductive hematopoiesis, and it is of interest to examine how the stromal factors were costimulated by the action of cytokines.

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