Changes in Hematopoiesis-Supporting Ability of C3H10T1/2 Mouse Embryo Fibroblasts During Differentiation

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To investigate the functional change of stromal cells along with differentiation, we used a differentiation-inducible mouse embryo fibroblast cell line, C3H10T1/2 (10T1/2). Stably determined preadipocyte and myoblast cell lines were established after a brief exposure of 10T1/2 cells to 5-azacytidine. These cell lines terminally differentiated into adipocytes and myotubes, respectively, under appropriate conditions. The hematopoiesis-supporting ability of each 10T1/2-derived cell line was examined by coculture with FACS-sorted murine hematopoietic stem cells (Thy-1+ c-kit+ Lin-). The number of granulocyte-macrophage progenitors (CFU-GM) was slightly reduced after 7 days of culture with parent 10T1/2 fibroblasts, whereas a marked increase in CFU-GM number was observed when the cells were cultured on preadipocytes. Mature adipocytes and myogenically determined cell lines, on the other hand, did not support CFU-GM growth. Further, Northern analysis showed that the preadipocyte cell line acquired the ability to produce a significant amount of stem cell factor (SCF), interleukin-6 (IL-6), leukemia inhibitory factor, and macrophage colony-stimulating factor mRNAs in response to IL-1 or lipopolysaccharide stimulation. Terminal adipocytic differentiation resulted in reduced ability to express these cytokine mRNAs. Similarly, highest IL-6 activity was detected in the supernatant of preadipocyte culture, whereas adipocytes did not secrete IL-6 even after IL-1 stimulation. Interestingly, hematopoiesis-nonsupporting myoblasts and myotubes also expressed abundant SCF mRNA, suggesting that SCF, per se, may not be sufficient for stem cell growth and survival. The 10T1/2-derived cell lines could provide a valuable tool to aid in the analysis of stromal cell development and the search for novel stromal factors.

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Assays for hematopoiesis-supporting ability of 10T1/2-derived cell lines. To prepare a stromal cell layer, 6- or 24-well cluster dishes (Costar) coated with collagen type I-C (Nitta Gelatin, Osaka, Japan) were used. One week before the coculture, A54 and M1601 subclones were cultured under the conversion-inducing conditions described above. Two days before the coculture, parent 10T1/2, A54, and M1601 cells were seeded at a confluent cell density. All the 10T1/2-derived cells were irradiated with 15 Gy just before coculture to prevent excess proliferation and deterioration of culture conditions. Bone marrow cells, obtained from the femurs of DBA/2 mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan), were suspended in IMDM/10% FCS and the nonadherent fraction was separated twice by incubation in a 100-mm dish (Costar) at 37°C for 1 hour. The nonadherent bone marrow cells were seeded onto preestablished 10T1/2-derived subclones in 6-well dishes. The coculture was performed at 37°C in conditions of 95% humidity and 5% CO₂ in air. Viable floating cells were counted with a hemocytometer at 2-day intervals.

A highly enriched stem cell fraction was isolated from the bone marrow cells of 6-week-old C57BL/6 (THY1.2, Ly5.2) mice (Clea Japan Inc, Tokyo, Japan) by fluorescence-activated cell sorter (FACS). The lineage markers used were B220 (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5), CD4 (GK1.5), CD8 (53-6.72), and TER119 (erythroid lineage marker). These antibodies were coupled to biotin by the standard method. Fluorescein isothiocyanate (FITC)-conjugated Thy1.2 was purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). A purified anti-

![Fig 1. Photomicrographs of 10T1/2-derived cell lines: (A) parent 10T1/2 fibroblasts; (B) clone A54 preadipocytes; (C) clone A54 adipocytes; (D) clone M1601 myoblasts; (E) clone M1601 myotubes.](image-url)
c-kit-encoded molecule MoAb^{19} (ACK-2, provided by Dr S-I Nishikawa, University of Kumamoto, Kumamoto, Japan) was conjugated with allophycocyanin (APC) for staining as described^{18}. Bone marrow cells were incubated with ACK-2–APC, FITC-conjugated Thy1.2 and biotinylated antibodies against lineage markers, washed with staining medium (phosphate-buffered saline [PBS] supplemented with 3% FCS and 0.01% sodium azide), then the cells were incubated with phycoerythrin (PE)-coupled streptavidin. After the final wash, the cells were resuspended in staining medium containing 2 \mu g/mL 7-aminoactinomycin D (7-AAMD). Four-color flow cytometry analysis and cell sorting were performed on a FACStar^{58} (Becton Dickinson) equipped with a 488-nm argon laser and a 599-nm dye laser. For control, unstained cells or cells stained with APC-conjugated and biotinylated MoAbs against irrelevant antigen were used. Compensation was adjusted using CaliBRITE (Becton Dickinson) for FITC and PE channels, and a mixture of cells stained only with APC-conjugated or Texas-red-labeled antibody for Texas-red and APC channels. Computer-assisted data analysis of results was performed on a MicroVAX computer (Digital Equipment Corp. Maynard, MA) with FACS/DESK software (version 1.8) made available through the FACS development group at Stanford University.

In vitro colony assay. The number of granulocyte-macrophage progenitors (CFU-GM) was measured before and after coculture with stromal cells by methylcellulose culture, according to the method of Iscove et al.,^{21} with slight modification. Because hematopoietic stem and progenitor cells adhere to stromal cells, the total cells in the culture were obtained by trypsinization of adherent layers. Cells were washed with IMDM/10% FCS and resuspended in \alpha minimum essential medium containing 30% FCS, 1% bovine serum albumin (BSA), 1.2% methylcellulose, 10^{-4} mol/L 2-mercaptoethanol, 500 U/mL murine interleukin-3 (IL-3) (Kirin Brewery Co, Tokyo, Japan), and 100 ng/mL recombinant murine stem cell factor (SCF) (Kirin). Culture plates were incubated at 37°C in a humidified atmosphere of 5% CO2. Colonies were counted in triplicate after 7 days of culture, using an inverted microscope.

RNA extraction and Northern blot analysis. Parent and adipogenically or myogenically determined 10T1/2 cells were prepared. After the medium was replaced with fresh IMDM/10% FCS, confluent cells were stimulated with 10 ng/mL human recombinant IL-1 (Otsuka Pharmaceutical Co, Tokushima, Japan), 10 mg/mL hypopolysaccharide (LPS) (Sigma), 10 ng/mL 1,2-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma), 10 mg/mL LPS plus 10 ng/mL of TPA, or 15 Gy of irradiation. After 6 hours of incubation, RNA was extracted from untreated and stimulated cells by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method.^{22} For Northern blot analysis, 20 \mu g of total RNA was denatured and fractionated on a 1.2% agarose gel containing formaldehyde, transferred to a nylon filter (Nytran, Schleicher and Schuell, Keene, NH), and hybridized with \alphaP-labeled probes in 6 X SSPE containing 50% formamide at 42°C. The murine SCF and adipsin cDNAs were cloned by a reverse transcriptase-polymerase chain reaction (RT-PCR) method.^{23} Other cDNA probes were provided by a number of investigators: murine granulocyte colony-stimulating factor (G-CSF) cDNA from S. Nagata (Osaka Bioscience Institute, Osaka, Japan); murine IL-6 and IL-7 cDNAs from T. Sudo (Biomaterial Institute, Osaka, Japan); murine leukemia inhibitory factor (LIF) cDNA from T. Kunisada and S.4. Nishikawa (University of Kumamoto, Kumamoto, Japan); murine granulocyte-macrophage colony-stimulating factor (GM-CSF) cDNA from A. Miyajima (DNAX Research Institute of Molecular and Cellular Biology Inc, Palo Alto, CA); murine macrophage colony-stimulating factor (M-CSF) cDNA from S. Clark (Genetics Institute, Cambridge, MA); MyoD cDNA from H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle, WA); and human transforming growth factor \beta (TGF-\beta) cDNA from F. Miyagawa (University of Tokyo, Tokyo, Japan). The cDNA probes were radiolabeled with \alpha-[^{32}P]-dCTP by primer extension with a multiprime DNA labeling kit (Amersham Corp, Arlington Heights, IL), according to the manufacturer’s specifications. Following overnight hybridization, filters were washed three times in 3X SSC/0.1% sodium dodecyl sulfate (SDS) at 42°C, twice in 0.1X SSC/0.1% SDS at 42°C, and then autoradiographed with Fuji RX film (Fuji Photo Film Co, Kanagawa, Japan).
Rehybridization with different cDNA probes was successively performed using the same filter.

RT-PCR for G-CSF and macrophage inflammatory protein 1α (MIP-1α) mRNA. The expression of murine G-CSF and MIP-1α mRNAs by 10T1/2-derived cell lines was studied by a RT-PCR method, using 1 µg total RNA with a Gene Amp kit (Perkin Elmer Cetus, Norwalk, CT), according to the manufacturer's specifications. As a control, β-actin mRNA expression was examined. The oligonucleotide primers used were: G-CSF 5' primer, 5'-CTG GAG CAA GTG AAG AAG AT-3'; 3' primer, 5'-CA OAT GGT GGT GCC AAA G-3'; MIP-1α 5' primer, 5'-TGA CAA GCT TAC CCT CTG TCA-3'; 3' primer, 5'-TCC AAG ACT CTC AGG CAA TCA-3'; β-actin 5' primer 5'-GTG GGC CGC TCT AGG CAC CAA-3'; and 3' primer, 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3'. The predicted RT-PCR products were 302 bp for G-CSF mRNA, 323 bp for MIP-1α mRNA, and 540 bp for β-actin mRNA.25-27 The PCR products were subjected to 3% NuSieve (FMC Bio Products, Rockland, ME) agarose gel electrophoresis with ethidium bromide and visualized with ultraviolet illumination. Southern blot analysis was performed using nylon membrane (Nytran, Schlieren and Schuell). After prehybridization, DNA blots were hybridized with a 32P-labeled G-CSF cDNA probe in 6 × SSC/0.1% SDS at 65°C overnight, then washed twice with 3 × SSC/0.1% SDS at 65°C, 0.1 × SSC/0.1% SDS at 65°C, and exposed to radiographic film.

Measurement of IL-6 and G-CSF concentrations in culture supernatants of 10T1/2-derived cell lines. After confluent 10T1/2-derived cells were prepared, culture medium was replaced with fresh IMDM/10% FCS and incubation was continued for additional 4 days in the presence or absence of IL-1. The culture supernatants were assayed for IL-6 and G-CSF activities using factor-dependent cell lines. Biological activity of IL-6 was estimated by IL-6–dependent MH60 cell proliferation assay.38 The MH60 cells were adjusted to 5 × 10⁴ cells/200 µL in 96-well microtiter plates (Becton Dickinson), and cultured in the presence of serially diluted test samples for 60 hours. G-CSF activity was similarly estimated using DA-IN cells, which is a subclone of IL-3–dependent DA-1 and exhibits G-CSF–dependent growth (manuscript in preparation). DA-1N cells were washed, adjusted to 1 × 10⁵/100 µL/well, and cultured with test samples for 48 hours. The cell growth was determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Dojin, Kumamoto, Japan) method.39 IL-6 and G-CSF concentrations in test samples were calculated by superimposing them on the standard curves.

RESULTS

Establishment of adipogenically or myogenically determined cell lines from 10T1/2 cells. Adipogenic and myogenic determination of 10T1/2 cells was observed in about 3.1% and 1.2% of cells, respectively. Clone A54 was selected as the representative preadipocyte cell line, ie, the cell line that had the ability to differentiate into adipocytes under appropriate conditions (Fig 1). Differentiated cells of clone A54 expressed abundant mRNA for adipin, an adipocyte-specific proteinase (Fig 2), and almost 100% of A54-derived adipocytes had fat droplets, which was confirmed by oil red-O staining.40 Clone M1601 was the representative myogenically determined cell line. Apparent myotube formation was observed when M1601 cells were
cultured in IMDM containing 2% horse serum (Fig 1). Myo-D gene expression was observed only in myoblasts and myotubes (Fig 2). Further, α-actin mRNA, a muscle differentiation marker, was selectively expressed in terminally differentiated myotubes.27

Hematopoiesis-supporting abilities of 10T1/2-derived cell lines. To investigate whether adipogenic and myogenic determination of 10T1/2 fibroblasts and their terminal differentiation influence the ability to support hematopoiesis in vitro, murine bone marrow hematopoietic cells were cocultured with parent 10T1/2 cells, A54 preadipocytes, terminally differentiated A54 adipocytes, M1601 myoblasts, or M1601-derived myotubes. When nonadherent fractions of normal bone marrow cells were cultured on 10T1/2-derived cells, the number of viable nonadherent bone marrow cells was maintained for 10 days and the efficiency of CFU-GM growth was not significantly different among these, except for the culture on A54-derived adipocytes (data not shown). To avoid the influence of contaminated accessory cells in the nonadherent bone marrow cell fraction, which may affect hematopoiesis in vitro, we used a highly enriched stem cell population17 in subsequent experiments. The FACS-sorted Thy-1+ c-kit+ Lin- cell fraction (Fig 3) of normal murine bone marrow cells contained 95 ± 26 CFU-GM per 300 cells (Fig 4). This stem cell fraction (300 cells/well) was cultured on 10T1/2-derived cell lines for 7 days, and the changes in CFU-GM number were examined. The number of CFU-GM slightly decreased (59 ± 13/well) during the culture on parent 10T1/2 cells. On the other hand, significant expansion of CFU-GM (887 ± 128/well) was observed when they were cultured on A54 preadipocytes. In this coculture, hematopoietic cells formed a discrete cobblestone area underneath the preadipocytes. Interestingly, A54 cells completely abolished the ability to support hematopoiesis in vitro after terminal adipocytic differentiation. The myogenically determined M1601 cell line also lost hematopoiesis-supporting ability at both the myoblast and myotube stage.

The 10T1/2-derived cell lines were not appropriate for long-term culture, because these cells were irradiated before coculture and it was difficult to maintain the culture conditions. Moreover, A54 preadipocytes had a tendency of spontaneous differentiation in some degree into adipocytes when they were cultured under confluent conditions for a long time.

SCF mRNA expression in 10T1/2-derived subclones. SCF mRNA expression was studied by Northern blot analysis (Fig 5). Although SCF mRNA was detected in all types of 10T1/2-derived cells, there was considerable variation in the amount. Parent 10T1/2 cells expressed little, if any, SCF mRNA even after stimulation with various agents. On the other hand, SCF mRNA expression by A54 preadipocytes was markedly augmented by stimulation with irradiation, LPS, TPA, and LPS plus TPA. Such inducible expression of SCF mRNA almost disappeared at the adipocyte stage. Unexpectedly, M1601 myoblasts and myotubes constitutively expressed a large amount of SCF mRNA.
Expression of various cytokine mRNAs in 10T1/2-derived cell lines. The same blots as those described in Fig 2 were rehybridized with murine IL-6, murine LIF, and murine M-CSF cDNAs.

Expression of other cytokines. The expression of various cytokine genes in 10T1/2-derived cell lines was examined by rehybridization of the same filters as those described above (Fig 6). IL-6 mRNA was not detected in parent 10T1/2 cells or A54 adipocytes even after stimulation. On the other hand, A54 preadipocytes expressed a small amount of IL-6 mRNA constitutively, and the level was much increased on stimulation with IL-1, TPA, and LPS/TPA. Moreover, IL-6 activity in culture supernatants was determined using IL-6–dependent cell line MH60 (Fig 7). IL-6 was most efficiently produced at the preadipocyte stage.
stage, and the concentration was increased on IL-1 stimulation. Interestingly, A54 mature adipocytes did not secrete IL-6 even after IL-1 treatment. A similar expression pattern was observed for LIF and M-CSF mRNAs. A54 preadipocytes expressed LIF and M-CSF mRNAs in an IL-1-inducible manner, and terminally differentiated adipocytes lost the ability to express these mRNAs. M1601 myoblasts and myotubes expressed a substantial amount of IL-6 mRNA when stimulated with IL-1 and LPS, but IL-6 activity in the culture supernatant was relatively low. The expression levels of LIF and M-CSF mRNAs in these myogenically determined cells were very low.

G-CSF, GM-CSF, and IL-7 mRNAs were barely detected in these cells by Northern blot analysis. Using the RT-PCR method, a significant amount of G-CSF mRNA was detected in A54 preadipocytes and adipocytes in response to IL-1 stimulation, whereas parent 10T1/2 cells and M1601 myoblasts and myotubes expressed little, if any, G-CSF mRNA, even after IL-1 stimulation (Fig 8). Similarly, G-CSF activity was detected in the culture supernatants of A54 preadipocytes and adipocytes when these cells were stimulated with IL-1 (Fig 9). Myogenically determined cells did not produce G-CSF, which was compatible with the result of RT-PCR. With regard to negative regulators of hematopoiesis, Northern analysis showed that all of the parent and 1OT1/2-derived cells equally expressed TGF-β mRNA. On the other hand, MIP-1α mRNA was not detected in these cells by RT-PCR analysis (data not shown).

**DISCUSSION**

Hematopoiesis in bone marrow is believed to be supported through interaction with stromal cells. Previous studies by other investigators have suggested that preadipocytes, among the various types of stromal cell components, have the ability to maintain hematopoiesis in vitro. In the present study, we used differentiation-inducible 10T1/2 fibroblasts to investigate their functional changes along with differentiation. This 10T1/2-derived cell line system made it possible to analyze stromal cell function systematically, because each cell line was derived from the same parent 10T1/2 cells.

Coculture of a highly enriched hematopoietic stem cell population (Thy-1<sup>+</sup> c-kit<sup>+</sup> Lin<sup>−</sup>) with 10T1/2-derived cell lines showed that hematopoiesis-supporting ability was significantly elevated at the preadipocyte stage. The acquisition of this stromal cell function was not solely related to 5-azacytidine treatment, because myogenic determination was accompanied by apparent reduction in hematopoiesis-supporting ability. Furthermore, terminal adipocytic differentiation resulted in complete abrogation of the ability to support CFU-GM growth, a result that mimics reduced hematopoiesis in fatty bone marrow in vivo. When non-adherent bone marrow cells were cocultured, most of the 10T1/2-derived cell lines but not fully differentiated adipocytes maintained the in vitro short-term hematopoiesis. It remains to be determined whether A54 adipocytes not only lost hematopoiesis-supporting ability but also acquired the nature to suppress hematopoiesis after terminal differentiation. Preliminary experiments showed that A54 adipocytes did not support CFU-GM growth even after the addition of SCF and IL-6 to the culture of stem cell fraction, suggesting the presence of negative growth regulation. On the other hand, the culture supernatant of adipocytes did not contain the activity to inhibit CFU-GM-derived colony formation (data not shown). In this regard, stem cell inhibitor (SCI), which has been shown to be identical to MIP-1α, is of particular interest, because SCI/MIP-1α is known to inhibit stem cell proliferation without affecting colony formation by CFU-GM. However, SCI/MIP-1α mRNA was not detected in our 10T1/2-derived cells. Moreover, the mRNA expression of TGF-β, another putative inhibitor of hematopoiesis, did not change along with differentiation of 10T1/2 cells.

Cytokine gene expression in various 10T1/2-derived cell lines was also studied. Intriguingly, the inducible level of SCF mRNA expression paralleled hematopoiesis-supporting ability in the preadipocyte-adipocyte lineage. Similarly, the abilities to express IL-6, LIF, and M-CSF mRNAs and to secrete IL-6 into the culture supernatants were also elevated at the preadipocyte stage and reduced after terminal adipocytic differentiation. Thus, the changes in cytokine gene expression seemed to be closely associated with the ability to support hematopoiesis in this lineage, suggesting the presence of common mechanisms for regulating these functions along with stromal cell development.

Unexpectedly, the myogenically determined cells, myoblasts and myotubes, also expressed a considerable amount of SCF mRNA. This finding suggests that SCF alone may not be sufficient for the maintenance of hematopoietic stem.
cells and that other stromal cell factors required for optimal stem cell growth may be expressed by preadipocytes, although the presence of SCF is important for stem cell maintenance because the addition of a neutralizing antibody against SCF to the culture reduced the hematopoiesis-supporting ability of preadipocytes. The biological significance of SCF in myogenically determined cells is unknown.

The extracellular matrix (ECM) of bone marrow stromal cells also provides an environment that is suitable for the survival of hematopoietic stem cells. The ECM, which consists of proteoglycans, collagen, and glycoproteins, forms a highly organized network that enables the cells to migrate and interact with each other. Recent studies have emphasized the role of adhesion molecules in hematopoiesis; eg, it has been reported that the addition of CD44 (a cell adhesion molecule) antibody to LTBMC resulted in the cessation of hematopoiesis in vitro. Although the detailed mechanisms are still unclear, cell-to-cell or cell-to-ECM interaction is considered to be important in the support of hematopoiesis. The OT1/2-derived cell lines would be useful to investigate these phenomena. This unique OT1/2 system of stromal cell differentiation could provide a valuable tool for analyzing stromal functions.

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