Generation of functional clonal cell lines from human bone marrow stroma

(colony-stimulating activity/burst-promoting activity/hematopoiesis *in vitro*/DNA transfection)

KENICHI HARIGAYA* AND HIROSHI HANDA†

*Department of Pathology, Keio University School of Medicine, 35 Shinanomachi Shinjuku-ku, and †The Institute of Medical Science, University of Tokyo, Shiroganedai Minato-ku, Tokyo, Japan

ABSTRACT   Five clonal human bone marrow stromal cell lines were isolated from the adherent cell populations in long-term liquid cultures after transfection with the recombinant plasmid pSV3gpt. All the cell-line feeder layers and their conditioned media stimulated the proliferation of committed granulomonocytic stem cells (CFUc) from human bone marrow. The size and number of early erythroid stem cell (BFUe)-derived colonies were significantly increased when in the presence of 10% conditioned medium from the cell lines. Furthermore, a substantial number of mixed colonies with erythroid components were observed in the cultures in the presence of erythropoietin and conditioned medium. These findings suggest that the human bone marrow stromal cell lines obtained after transfection with pSV3gpt may be extremely useful in identifying the hematopoietic factors derived from the hematopoietic microenvironment and in analyzing their mechanism of action.

Bone marrow stromal cells are known to play a critical role in the proliferation and differentiation of hematopoietic stem cells (1, 2). The establishment of clonal marrow stromal cell lines, therefore, permits direct inquiry into a variety of hematopoietic stromal functions. Several cloned cell lines of murine marrow stromal cells producing the factors that modulate the proliferation and differentiation of murine hematopoietic stem cells have been established (3–5), but few clonal cell lines have been isolated from human bone marrow stroma. This paper describes the generation of five functional clonal cell lines after transfection of stromal cells of human long-term liquid cultures of bone marrow cells with the recombinant plasmid pSV3gpt (6).

MATERIALS AND METHODS

Establishment of Clonal Human Marrow Stromal Cell Lines. To obtain clonal human marrow stromal cell lines, adherent cell populations in 6-week-old long-term cultures derived from human bone marrow (7) were transfected with recombinant plasmid pSV3gpt DNA, containing the coding sequence of the early region of simian virus 40 (SV40). The cultures were established from a piece of rib taken from a 48-year-old hematologically normal male patient who underwent lung surgery. Twenty-four hours after passaging the adherent cells of the bone marrow cultures at 10^6 cells per 100-mm plate, the cells were washed once with McCoy's 5a modified medium (GIBCO), fresh McCoy's 5a medium supplemented with 10% fetal bovine serum (Reheis, Tokyo) was added, and the cells were exposed to a calcium-phosphate precipitate containing pSV3gpt DNA (10 μg/ml) added according to the method of Graham and Van der Eb (8). After incubation for 10–12 hr at 37°C, the cells were washed and

kept at a temperature of 37°C for 3 days in McCoy's 5a medium supplemented with 20% fetal bovine serum. The transfected cells then were treated with EDTA/trypsin (GIBCO), and 10^2–10^3 cells were dispersed for cloning on 100-mm plates in McCoy's 5a medium supplemented with 20% fetal bovine serum (9). The medium was changed twice a week. Approximately 2 months after transfection, the colonies were large enough to be subjected to a second cloning. After the second cloning, the cells were passaged once every 10–14 days at a split ratio of 1:4.

Examination of Characteristics of Established Cell Lines. Each cell line was examined for the presence of SV40 large tumor (T) antigen by indirect immunofluorescence with rabbit polyclonal anti-T antigen and fluorescein-conjugated goat anti-rabbit IgG. Each cell line also was examined for the presence of monoclonal antigens for phagocytic mononuclear cells (10) and factor VIII (Dakopatts, Glostrup, Denmark). The ability of the cells to convert to adipocytes was tested by exposing them to 1 nM–1 μM hydrocortisone hemisuccinate (Upjohn) or insulin (1–10 μg/ml; Sigma) for 5 weeks after day 6 of culture.

Production of Hematopoietic Factors in the Established Lines. The effect of the feeder layer or conditioned medium from each clone on the proliferation of human hematopoietic stem cells was examined. First, to investigate the production of colony-stimulating activity (CSA), 5 × 10^4 cells of each cell line were inoculated into 35-mm tissue culture dishes (Lux) as feeder layers 3 days prior to the inoculation of the target bone marrow cells. The feeder layers were overlaid with double-layered agar containing 10^5 nucleated bone marrow cells in the upper layer as described (3), and their effects on the proliferation of committed granulomonocytic stem cells (CFUc) were examined. Human placental conditioned medium (11) was used as a stimulator for the positive control cultures. Cytochemical examination of the colonies was performed according to the method of Li et al. (12).

The production of the factor or factors responsible for the proliferation and differentiation of committed early erythrocytic stem cells (BFUe) was investigated in these established lines. Conditioned medium from each clonal cell line was added to dishes of the methylcellulose culture of BFUe described by Murphy and Sullivan (13), modified by substituting Iscove's medium (Boehringer Mannheim) for α-medium. The dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 14–16 days of culture, colonies containing cells producing hemoglobin were scored by using a Nikon TMD inverted microscope (Nihon Kogaku, Tokyo). Then individual colonies were taken from the dish with a micropipette, and the cells were counted by a Coulter electronic particle counter. The remaining samples were

Abbreviations: BFUe, early erythroid stem cells; CFUc, committed granulomonocytic stem cells; CFUmix, multipotent stem cells; G, granulocyte; M, macrophage; CSA, colony-stimulating activity; SV40, simian virus 40; T antigen, large tumor antigen.
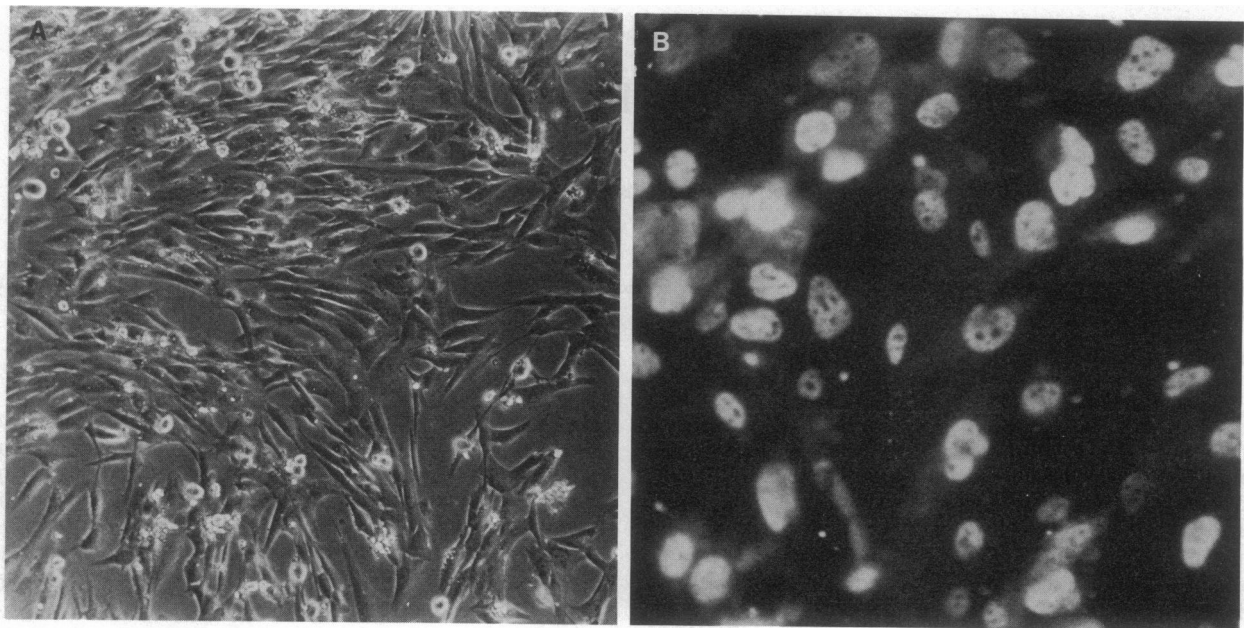
FIG. 1.   Cytologic characteristics. (A) Cell line KM-102, viewed with an inverted microscope, exhibits fibroblastic appearance and round cells in the process of mitosis. ($\times$130.) (B) SV40 T antigen in nuclei of cells from cell line KM-102, visualized by indirect immunofluorescence. ($\times$350.)

made into smears and Wright/Giemsa stained, and the types of colonies were morphologically determined. To determine the capacity of BFUe to survive a period of culture in the absence of erythropoietin concentrations sufficient for colony formation, a simple experiment was done: 5×10^4 human bone marrow cells were seeded in methylcellulose medium with serum in the presence or absence of the conditioned media, and erythropoietin (2 units/ml) was added to the cultures after a period of delay.

Ammonium Sulfate Fractionation of Conditioned Media. Precipitates of conditioned medium (100 ml) at 0–50% and 50–80% saturation with ammonium sulfate were collected by centrifugation at 10,000 $\times$ *g* for 20 min. Each precipitate was dissolved in 20 ml of 5 mM sodium phosphate buffer at pH 7.5 and dialyzed against the same buffer to remove residual ammonium sulfate. Supernatant at 80% saturation with ammonium sulfate was concentrated 5-fold by ultrafiltration (Amicon apparatus with PM-10 membrane) and dialyzed

against 5 mM sodium phosphate buffer at pH 7.5. Then each solution was adjusted to 50 ml with the same buffer, sterilized by filtration, and assayed for hematopoietic factors.

RESULTS

Five clonal stromal cell lines were isolated from the transfected cells. These clonal cells were designated KM-101–105 and went through more than 40 passages following isolation. All the clonal cells were fibrocytic in appearance (Fig. 1A).

Table 1.   Clonal stromal cell lines from human bone marrow stimulate the formation of CFUc-derived colonies

Cell line	No. of colonies (mean $\pm$ SD, $n = 4$)		Type of colonies, % of total		
	Feeder layer	10% CM	G/M	G	M
KM-101	104.0 $\pm$ 12.7	86.5 $\pm$ 9.2	26.5	71.5	2.0
KM-102	96.3 $\pm$ 6.9	99.5 $\pm$ 8.1	47.5	37.5	15.0
KM-103	87.3 $\pm$ 3.8	93.0 $\pm$ 5.7	39.5	51.5	9.0
KM-104	93.5 $\pm$ 6.4	86.0 $\pm$ 17.3	30.0	65.5	4.5
KM-105	91.0 $\pm$ 11.3	103.8 $\pm$ 13.7	30.5	63.0	6.5

The number of colonies formed per 10^5 human bone marrow nucleated cells stimulated with either a feeder layer or 10% conditioned medium (CM) from each cell line was ascertained at day 14 by CFUc assays, with discrete aggregates of 50 or more cells being scored as colonies. Control CFUc cultures produced 61 $\pm$ 4 cells (mean $\pm$ SD, $n = 4$) per 10^5 bone marrow nucleated cells when 10% placental conditioned medium was added. With use of a Nikon microscope ($\times$90), the colonies were classified by type in dried agar pellicles stained by the method of Li *et al.* (12), combining α-naphthyl butyrate esterase and naphthol AS-D chloro-acetate esterase. Two feeder-layer-stimulated cultures were used for determination of colony-type, and the figures shown represent the mean percentages of colonies thus counted.
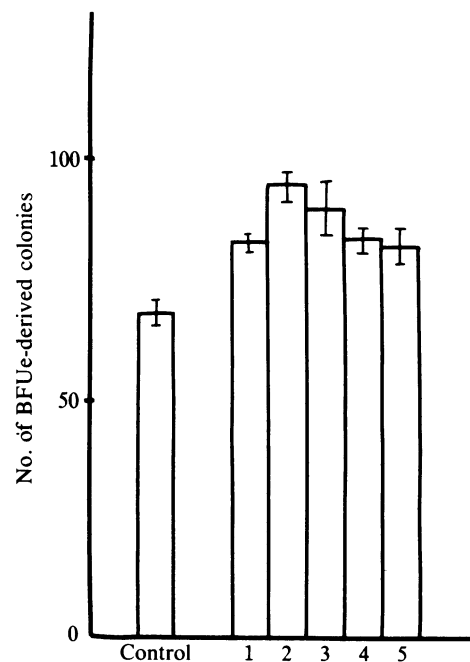

FIG. 2.   Number of BFUe-derived colonies (mean $\pm$ SD, $n = 4$) in methylcellulose cultures with or without 10% conditioned medium from each marrow stromal cell line. The number of colonies was ascertained at day 14; discrete aggregates or clusters of 64 or more hemoglobin-containing cells were scored as BFUe-derived colonies. Controls were usual BFUe cultures without conditioned medium, and 1–5 represent cultures containing 10% conditioned medium from KM-101, KM-102, KM-103, KM-104, or KM-105, respectively.

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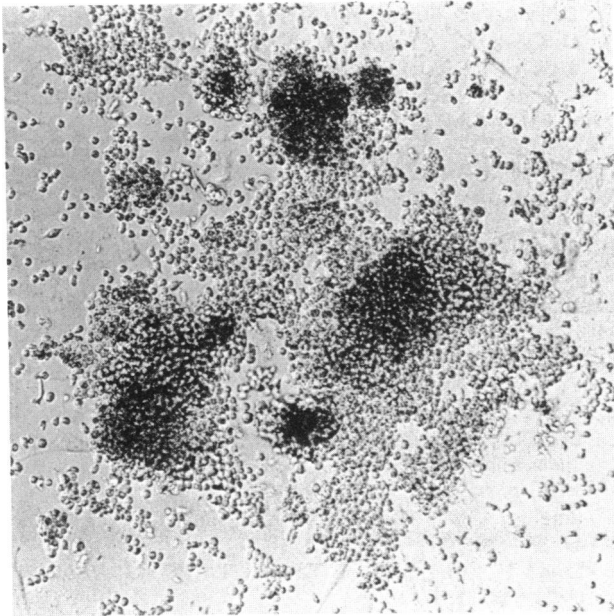Proc. Natl. Acad. Sci. USA 82 (1985)   3479

FIG. 3. Representative mixed colony in methylcellulose culture medium containing 10% KM-102 conditioned medium and erythropoietin (2 units/ml), photographed *in situ*. The dark portions of the colony are densely packed (orange-red color) hemoglobin-containing cells, and the more flattened peripheral areas are myelocytic cells and macrophages. (×130.)

They did not exhibit conversion to adipocytes even when they were exposed to hydrocortisone (1 nM–1 μM) hemisuccinate or insulin (1–10 μg/ml) for 5 weeks after subconfluence. These clonal cell lines did not show any phagocytic mononuclear cell antigen or factor VIII-associated antigen. The presence of SV40 T antigen in the nuclei of each cell line was revealed by indirect immunofluorescence staining (Fig. 1B).

The CFUc cultures containing feeder layers produced significantly more colonies than did the cultures containing human placental conditioned medium (Table 1). The addition of human placental conditioned medium to cultures containing feeder layers did not enhance the effects of the feeder layers. Colonies stimulated by feeder layers exhibited a variety of morphologies; cytochemical examination revealed that they comprised mixed granulocyte/macrophage (G/M), granulocyte (G), and macrophage (M) colonies. The conditioned media of the cell lines stimulated the proliferation and differentiation of G/M-CFUc, G-CFUc, and M-CFUc as much as did the feeder layers.

The number of BFUe-derived colonies increased by 20–40% when 10% conditioned media were added to the cultures for BFUe ($P < 0.05$) (Fig. 2). The number of cells in each BFUe-derived colony increased by 200% in the cultures containing 10% of each conditioned medium compared with that in the control BFUe culture ($P < 0.05$). Two to 11

mixed colonies containing densely packed hemoglobin-containing cells, myelocytic elements, and macrophages (CFU-mix-derived colonies) were formed out of 5×10^4 nucleated bone marrow cells in the presence of each conditioned medium (Fig. 3). No CFUmix-derived colonies were formed in the culture without conditioned medium. During 72 hr of erythropoietin deprivation in the absence of conditioned medium, the number of BFUe-derived colonies decreased by two-thirds, and they were reduced by 90% in the cultures administered erythropoietin on day 6; further erythropoietin deprivation resulted in no BFUe-derived colonies (Table 2). During 72 hr of erythropoietin deprivation, however, the cultures containing 10% conditioned medium from either KM-102 or KM-103 produced about as many large colonies derived from BFUe and CFUmix as did the cultures initiated with 10% in the presence of conditioned medium and erythropoietin. In the cultures with conditioned media, even when erythropoietin was administered after day 6, substantial numbers of colonies derived from BFUe and CFUmix were produced: 11.3 ± 1.1 at day 7 and 9.5 ± 1.5 at day 8. This activity was observed mainly in the supernatant fraction after precipitation of conditioned medium with ammonium sulfate at 80% saturation, whereas CSA was mainly in the precipitate at 50–80% saturation with ammonium sulfate.

DISCUSSION

Bone marrow stromal cells, because of their proximity to hematopoietic cells, are thought to produce hematopoietic regulators. However, no substantial data have been obtained regarding hematopoietic factors derived from human marrow stromal cells. One reason for this is the difficulty of multiple subculturing of the marrow stromal cells and, therefore, the failure to obtain clonal stromal cells. In attempts to grow primary marrow stromal cells from human long-term liquid cultures without DNA transfection, growth became very slow after 7–8 passages, and further subcultures were not successful. Thus, the identification and analysis of hematopoietic factors produced by individual marrow stromal cells was not feasible. Transfection with recombinant DNA containing a viral oncogene has made it possible to establish clonal human marrow stromal cell lines. These cells are neither phagocytic mononuclear cells, nor endothelial cells, nor preadipocytes. They are all fibroblastic in appearance and express nuclear SV40 T antigen. Our studies indicate that these clonal human cell lines actively secrete hematopoietic factors, because the conditioned media from these cell lines stimulated proliferation of G-, M-, and G/M-CFUe, BFUe, and CFUmix. The cell lines also produced a factor(s) that promoted survival of BFUe and CFUmix. The following information suggests that this effect is due to burst-promoting activity (BPA). It has been suggested that BPA resides in a molecule similar to interleukin 3, multi-colony-stimulating factor, P-cell-stimulating factor, and hematopoietic growth factor in the murine system and that it promotes proliferation and survival of BFUe and CFUmix in *in vitro* hematopoiesis

Table 2. Number of colonies derived from BFUe and CFUmix in cultures to which erythropoietin was added on different days of culture

| | No. of BFUe- and CFUmix-derived colonies | | | | | |
	Day 0	Day 3	Day 5	Day 6	Day 7	Day 8
+CM	34.8 ± 2.9 (4.8)	30.5 ± 6.5 (7.5)	21.3 ± 3.6 (3.6)	17.0 ± 1.6 (4.3)	11.3 ± 1.1 (2.3)	9.5 ± 1.5 (1.8)
−CM	24.0 ± 3.5 (0)	8.0 ± 1.0 (0)	6.0 ± 0.7 (0)	2.5 ± 1.7 (0)	0	0

Bone marrow cells (5×10^4) were inoculated into 1 ml of culture medium. Conditioned medium (CM) was supernatant from KM-103. Erythropoietin (2 units/ml) was added on the indicated day in culture. Numbers in parentheses are mean numbers of CFUmix-derived colonies, which are included in mean numbers of colonies derived from BFUe and CFUmix (mean ± SD, $n = 6$).

(14). Our data suggest that clonal stromal cells from human bone marrow produce both BPA and CSA and that these are partially separated by ammonium sulfate fractionation.

It is not known whether SV40 T antigen acts by modulating the expression of the genes coded for hematopoietic factors in the marrow stromal cells or by allowing establishment of clonal stromal cells that normally produce the factors *in vivo*. Nevertheless, transfection with recombinant DNA containing oncogenes, either viral or cellular, into the adherent cells of human bone marrow can be used to generate functional human bone-marrow stromal cell lines. The human stromal cell lines obtained in this manner may be extremely useful in identifying hematopoietic factors derived from the individual cellular elements of the hematopoietic microenvironment and in analyzing their mechanism of action. Furthermore, this method would provide a useful strategy for studying the cellular and molecular mechanisms of human hematopoietic stem-cell differentiation.

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