Response of Simian Virus 40 (SV40)–transformed, Cultured Human Marrow Stromal Cells to Hematopoietic Growth Factors

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Abstract

The response of marrow stromal cells transformed with wild-type simian virus 40 to recombinant growth factors was examined. When transformed stromal cells were plated in semisolid medium without the addition of growth factors, only 0.4% of cells formed colonies while with the addition of recombinant factors such as interleukin 1 (IL-1) or tumor necrosis factor (TNF), up to 10% of the cells formed colonies. Colonies were individually plucked and cell lines were developed that could be analyzed for expression of growth factors. The data show that unstimulated marrow stromal cells lines produced no detectable colony-stimulating activity. However, cell lines derived from "autonomously growing colonies" and from colonies grown with T cell–conditioned medium, with IL-1α or β, or with TNFα produced colony-stimulating activity and transcripts for granulocyte/macrophage-colony-stimulating factor (CSF), granulocyte-CSF, and IL-1β. A novel feature of the cell lines derived from colonies was that the production of growth factors was constitutive and persisted in excess of 4 m.

Introduction

Hematopoietic cell proliferation, differentiation, and survival in vitro (1, 2), and probably in vivo (3, 4) are dependent on the presence of specific hematopoietic growth factors (5). In long-term marrow cultures, the survival and proliferation of hematopoietic stem cells are dependent on the presence of an adherent population of diverse mesenchymal cells termed "stromal cells" (6).

It is now generally accepted that stromal cells can produce relevant hematopoietic growth factors when appropriately stimulated. Despite early data in favor of the absence of colony-stimulating activity in unstimulated stromal cells from murine long-term cultures (6), more recent studies suggest low level basal production of colony-stimulating activity by adherent cells from long-term cultures (7, 8) and from cloned murine stromal cell lines (9–11). In addition, RNA transcripts for macrophage (M)[1]-colony-stimulating factor (CSF) were found in cultured stromal cells (12), and granulocyte/macrophage (GM)-CSF activity (13) and transcripts (14) have been reported in stimulated murine stromal cell lines. Although murine stromal cells can support IL-3–dependent cell lines, no evidence for production of IL-3 or its message has been found (15).

Human stromal cells, related mesenchymal cells such as endothelial cells and fibroblasts, and macrophages which are resident cells of the hematopoietic microenvironment transcribe granulocyte (G)-CSF and GM-CSF when stimulated by the inflammatory mediators IL-1 or tumor necrosis factor (TNF) in vitro (16–25).

Human stromal cells are a heterogeneous population consisting of endothelial-like cells, fibroblastic cells, and macrophages (reviewed in Singer et al. [26]). Attempts to reproducibly clone stromal cells from human long-term marrow cultures have been generally unsuccessful. In order to develop clonable stromal cells, we transformed long-term marrow culture adherent layers with simian virus 40 (SV40) (27), a technique which has been used to immortalize many types of non-hematopoietic cells (28–30). The transformed cell lines retain many of the properties of the originating cells. When stromal cells were exposed to SV40, they integrated the viral genome and expressed the SV40 T-antigen (27). Several passages after exposure to SV40, the cell lines began to grow rapidly and became clonable.

A unique feature of SV40–transformed stromal cell lines is the obligate production of "round" as well as stromal-like cells even in rigorously cloned cell lines (27). Some round cells express antigens associated with hematopoietic cells such as T200 (31) whereas the adherent cells produce interstitial and basal lamina collagens and express smooth muscle-type actin, an epitope associated with nontransformed stromal cells (27, 32). When the transformed stromal cell lines were treated with trypsin to create single cell suspensions and cultured in semisolid medium in the presence of PHA-stimulated T cell–conditioned medium (PHA-LCM), up to 10% of the cells plated formed colonies of large blastlike cells (27). In the absence of a source of colony-stimulating activity, only rare colonies were formed. When colonies were individually plucked with a fine pipette and placed in suspension culture, cell lines were developed that resembled the original transformed stromal cell lines. Using stromal cell lines from patients heterozygous for glucose-6-phosphatase dehydrogenase, cell lines derived from individual colonies were shown to be true clones (27 and unpublished data). With this system, it was therefore possible to develop clonal cell lines from colony-forming cells responsive to specific growth factors.

The present study used growth of transformed stromal cells, as colonies, to identify and clone cells that either were capable of spontaneous anchorage-independent growth or that could be stimulated to form colonies by recombinant hematopoietic growth factors. After expansion of the clones into cell

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1. *Abbreviations used in this paper:* CSF, colony-stimulating factor; GM, granulocyte/macrophage; LCM, lymphocyte-conditioned medium.

lines, they were compared to the parent cell lines. The data indicate that several hematopoietic growth factors can promote anchorage-independent growth by transformed stromal cells. A novel finding was that all cell lines derived from colonies were constitutive producers of several hematopoietic growth factors whether the colonies grew spontaneously or were stimulated by exogenous growth factors. Moreover, growth factor production was maintained at high levels for as long as the cell lines could be passaged.

Methods

Subjects. Long-term marrow cultures were generated from marrow aspirates taken from normal marrow donors after written informed consent was obtained under protocols approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Long-term marrow cultures. Long-term marrow cultures were grown from marrow buffy coat cells (2×10^6/ml) using McCoy's 5A complete medium containing 10^{-6} M hydrocortisone, 12.5% fetal calf serum, and 12.5% horse serum as previously described (27).

Transformation with SV40. SV40 transformation of stromal cells in long-term marrow cultures was performed as described (27). Briefly, confluent adherent layers from normal long-term marrow cultures were trypsinized, passaged, and wild-type SV40 (SV40/vero 7: Meloy Laboratories, Springfield, VA) was added at 10 particles per cell. After 1–3 wk, growth became vigorous and the large T antigen was expressed (33). The cultures were maintained in McCoy's 5A solution with 10% fetal calf serum and were passaged weekly.

Colony assays and development of cell lines from colonies. Transformed stromal cells growing as adherent cell lines were treated with trypsin (1:250; Difco Laboratories, Inc., Detroit, MI) in EDTA for 5–10 min, washed, and passaged through a 25-gauge needle a sufficient number of times to prepare single-cell suspensions. The cells were plated in semisolid medium consisting of 20% fetal calf serum, 1.25% methylcellulose (Eastman Kodak Co., Rochester, NY) in Iscove's medium with 10^{-6} M 2-βmercaptoethanol, and a test-stimulating substance. The test substances included medium alone, PHA-LCM (34), and the following recombinant human growth factors: IL-1α and β (35, 36), IL-3 (37), B-CSF (IL-4) (38), and GM-CSF (39) (kindly donated by Immunex Corp., Seattle, WA), tumor necrosis factor-alpha (TNFα [40], Genentech, South San Francisco, CA), IL-2 (41, Hoffman La Roche Inc., Nutley, NJ), M-CSF (42, lot A422, kindly donated by Cetus Corp., Emeryville, CA), and G-CSF (43, Amgen Corp., Thousand Oaks, CA). Dose-response experiments were performed for each factor and the concentration stimulating the greatest number of colonies was used subsequently.

In order to derive cell lines consisting entirely of progeny of colony-forming cells, individual colonies were plucked with a finely drawn pipette, placed in 96-well microtiter dishes, and serially passaged until sufficient numbers of cells were available to characterize. Conditioned medium from these colony-derived cell lines and the parent cell lines was saved and assayed for colony-stimulating activity as described below. After several passages in suspension culture, the ability of the colony-derived cell lines to form colonies spontaneously was tested and compared to that of the continuously passaged parent cell lines.

Assay of colony-stimulating activity. Bone marrow mononuclear cells from normal donors were depleted of adherent cells by incubation in plastic petri dishes for 12 h at 37°C. The cells were then plated at a concentration of 5×10^4/ml in medium consisting of 1.25% methylcellulose, 10% fetal calf serum, 1% deionized bovine serum albumin, and 10^{-6} M, β-mercaptoethanol. To assay burst-promoting activity, 0.5 U/ml of semipurified erythropoietin (BC Cancer Center, Vancouver, BC) was added to the mixture. Test-conditioned media (20% vol/vol) or medium alone were added. Colonies of over 50 cells were counted using an inverted microscope after 14 d of incubation. Colonies of GM and erythroid bursts were counted separately on the same culture dishes.

Northern blot analysis for transcripts of growth factors. RNA was extracted from unstimulated SV40–transformed cell lines, from cell lines derived from autonomously growing colonies, or from colonies stimulated with PHA-LCM or with recombinant human growth factors (44). Poly-A+ RNA was selected over an oligo-dT column (45). 10 μg of poly-A+ RNA was run in a 1.0% denaturing agarose-formaldehyde gel. This was transferred to nitrocellulose and baked (45). The resulting northern blots were hybridized under stringent conditions in Ullrich solution (46) with 40-mer probes for:

M-CSF (5'TGATACTCCTGCTCGCCAGGA-GACAGACCAACAACAGCAG3');

G-CSF (5'TCTTCCTCACTTGCTCTAAGC-ACTTGAGCAGGAAGCTCTG3');

GM-CSF (5'TTCAGGAGACGCCGGGCC-TCCTGGATGGCATTCACATGCT3');

and

IL-3 (5'AGTTAACCCAGCTTGTCTT-CAAGGACGTTGTCTGGGTCAT3'),

which were end-labeled with [γ^{32}P]chATP using polynucleotide kinase. These probes were specific for their respective transcripts (17–19). Full-length fragments for GM-CSF and IL-1β (kindly donated by Immunex Corp.) and TNFα (kindly donated by Genentech Corp.) and a 1.8-kb fragment for procollagen Iα (47) were labeled by nick translation and hybridized under stringent conditions (45).

Electron microscopy. For electron microscopy, cells were harvested after exposure to trypsin and placed in 3% glutaraldehyde for 2 h at 4°C and processed by standard techniques.

Results

Colony formation

When SV40–transformed stromal cells were cultured in semisolid medium in the presence of PHA-stimulated T cell–conditioned medium or various recombinant growth factors, up to 10% of the plated cells formed colonies consisting of up to several hundred, large, undifferentiated, blastlike cells. In control cultures without added growth factors, 0.4% of the plated cells formed colonies. Statistically significant stimulation of colony growth was found, in order of decreasing activity with IL-1, TNF, IL-2, IL-3, and GM-CSF (Table I). M-CSF, IL-4, and G-CSF did not stimulate colony growth. A representative dose-response curve (for IL-1α) is shown in Fig. 1.

Development of colony-derived cell lines

In order to obtain stromal cell lines derived from cells that formed spontaneous colonies and cell lines from colony-forming cells that had responded to a specific growth factor, colonies were individually plucked from the culture dishes, placed in suspension culture, and passaged until sufficient cells were available for further studies. Within 24 h in suspension culture, the cells became adherent to plastic and numerous elongated cells interspersed with adherent "round" cells began to grow from the colony. Within 1 wk, the cells grew to confluence and were passaged (Fig. 2). The colony-derived cell lines consistently produced more 'round' cells than did the parent cell lines (Fig. 3).

When examined by electron microscopy, colonies grown in PHA-LCM consisted of undifferentiated "hematopoietic-like" cells (Fig. 4). Electron microscopy of a cell line from

Table I. Effect of Growth Factors on Colony Formation by SV40-transformed Stromal Cell Lines

Stimulant	Concentration	Plating efficiency	SD	*n*
	U/ml	%		
None	—	0.4	0.4	20
PHA-LCM (%)	20%	6.3*	1.8	20
IL-1α	10	7.1*	1.0	20
IL-1β	10	6.4*	1.5	20
TNFα	1,000	3.7*	0.6	20
IL-2	100	2.4*	0.8	8
IL-3	100	2.2*	0.8	6
GM-CSF	1,000	1.8*	0.6	8
IL-4 (BSF-1)	100	1.0	0.7	4
G-CSF	1,000	0.5	0.1	2
M-CSF	1,000	0.2	0.1	3

SV-40-transformed stromal cells between passages 6 and 18 from independently derived cell lines were grown until confluent, harvested with trypsin, and plated in semisolid medium with the growth factors listed above. *n*, number of independent experiments.
* $P < 0.01$ when compared to the cloning efficiency of cells plated without stimulation (autonomous colonies).

these colonies disclosed the presence of a population of cells with diameters less than 20 μm whereas in the parent cell lines, all cells were greater than 27 μm in diameter. The smaller cells in the colony-derived cell lines contained short segments of rough endoplasmic reticulum and a more irregular nuclear contour when compared to larger cells in the parent stromal cell lines.

To examine whether cells grown as colonies expressed protein products associated with stromal cells, suspensions were prepared directly from pooled colonies grown with T cell–conditioned medium and examined for the presence of actin and vimentin filaments with monoclonal antibodies CGA-7, HHF, and 43βE8 (27, 32). In contrast to what was observed in suspension culture, no fibrillar material was seen in the colony-derived cells (27). However, when the colonies were placed in suspension culture and allowed to become adherent over 48 h, approximately 70% of the stromal-like cells reexpressed both actin and vimentin (27).

To assess the effect of growth factors that stimulate colony formation by stromal cells on the expression of collagen 1, a major extracellular protein synthesized by normal and transformed stromal cells (26, 27), the effect of IL-1α and TNFα, two potent stimulators of colony formation, was tested. Striking downregulation of collagen type 1 RNA transcripts was found in stromal cells stimulated with IL-1 or TNF compared to unstimulated cells (data not shown). This suggests that growth factors that promote anchorage-independent growth decrease the expression of structural matrix molecules normally produced by adherent stromal cells. As shown in Fig. 5, downregulation of collagen I expression was also seen in cell lines derived from autonomously growing colonies when compared to the parent cell lines. This experiment was reproduced using three independently derived stromal cell lines.

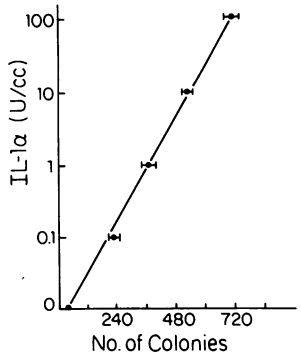

Figure 1. Dose-response curve showing the effect of increasing concentrations of recombinant IL-1α on colony formation by SV40-transformed stromal cells.

When cell lines derived from colonies were recultured in semisolid medium in the absence of added growth factors, the frequency of autonomous colony formation was substantially higher than that of the parent cell lines (Table II). This increased frequency of autonomous colony formation persisted for six passages. The acquisition of relative autonomy for anchorage-independent growth suggested the possibility that the colony-derived cell lines might be obligate producers of growth factors and thus capable of autocrine stimulation.

Growth factor production by stromal cell lines

Bioassay. The amount of colony-stimulating activity produced by transformed stromal cell lines and colony-derived cell lines was tested by evaluating the effect of conditioned medium on growth of erythroid and granulocyte/macrophage colonies from adherent cell-depleted, mononuclear bone marrow cells (Table III). Conditioned media from several passages of each of the colony-derived cell lines were tested. As shown in Table III, unstimulated transformed stromal cell lines did not have significant GM colony-stimulating activity or erythroid burst-promoting activity. In contrast, conditioned media from colony-derived cell lines regardless of whether they were derived from autonomous colonies or from colonies stimulated by recombinant growth factors were consitutive producers of both burst-promoting activity and GM colony-stimulating activity. Persistently high levels of colony-stimulating activity were detectable for up to 16 passages after the cell lines were established. In contrast, when suspension cultures of SV40-transformed stromal cells were stimulated with the same growth factors for 5 d, washed, and recultured, colony-stimulating activity was detectable in conditioned media only for 1–2 wk. For example, 224±19 colony-forming unit–GM-derived colonies/10^5 marrow mononuclear cells were grown in the presence of conditioned medium from a transformed stromal cell line that had been stimulated for 5 d 1 wk earlier with PHA-LCM. (No PHA-LCM was present in the conditioned medium since the stromal cells had been washed and passaged before the conditioned medium was collected.) Conditioned medium collected from the stromal cell line two weeks after exposure to PHA-LCM stimulated 50±15 GM colonies while by the third week, only 13±5 colonies were grown.

RNA studies. Data from the bioassay of colony-stimulating activity suggested that those stromal cells that formed colonies produced hematopoietic growth factor(s). To identify these factors, poly-A+ RNA was selected 14 wk after a stromal cell line was derived from colonies stimulated by PHA-stimulated T cell–conditioned medium. After electrophoresis, Northern blots were hybridized to cDNA probes as shown in Fig. 6. G-CSF, GM-CSF, IL-1β, and TNFα transcripts were expressed

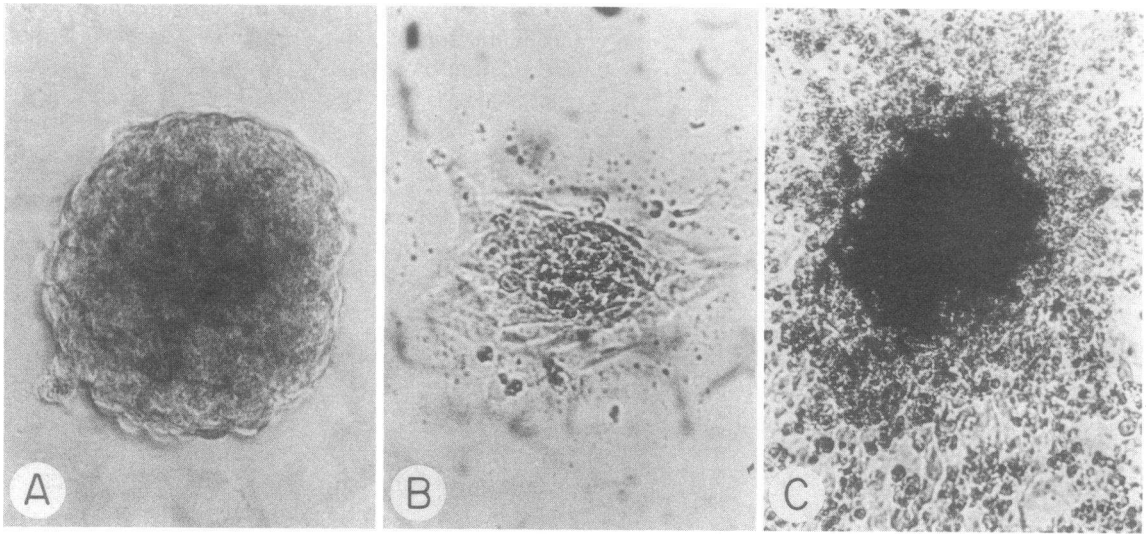

Figure 2. Stromal cell colonies were individually plucked from semisolid medium and then placed in microtiter wells. (*A*) A freshly plucked colony. (*B*) After 24 h of culture when the cells have begun to adhere to the plastic and become fibrosblastoid. (*C*) After 1 wk of culture showing a mixed population of round and elongated cells.

by the colony-derived cell line while the unstimulated parent cell line expressed only a trace amount of IL-1β. Both the parent cell lines and the colony-derived cell lines expressed M-CSF transcripts; neither hybridized with a probe to IL-3.

To determine which growth factors in PHA-stimulated T cell–conditioned medium might induce both colony formation and growth factor synthesis, whole cellular RNA was extracted from additional cell lines derived by expanding colonies grown with IL-3, TNFα, IL-1β, and TNFα+IL-1β. In each case, IL-1β and GM-CSF transcripts were readily detectable on Northern blots. In the parent cell lines, no GM-CSF transcripts were seen but trace amounts of IL-1β were sometimes found (data not shown).

Data from the bioassay suggested that colony-derived cell lines from autonomous colonies produced colony-stimulating activity while the parent cell lines did not ($P < 0.0001$; Table III). To verify this observation, cell lines derived from autonomous colonies were examined for growth factor RNA expression. Expression of GM-CSF (Fig. 7) and IL-1β (Fig. 8) was upregulated in the cell lines derived from autonomous colonies when compared to the unstimulated parent cell line. These experiments were repeated on 10 independently cloned

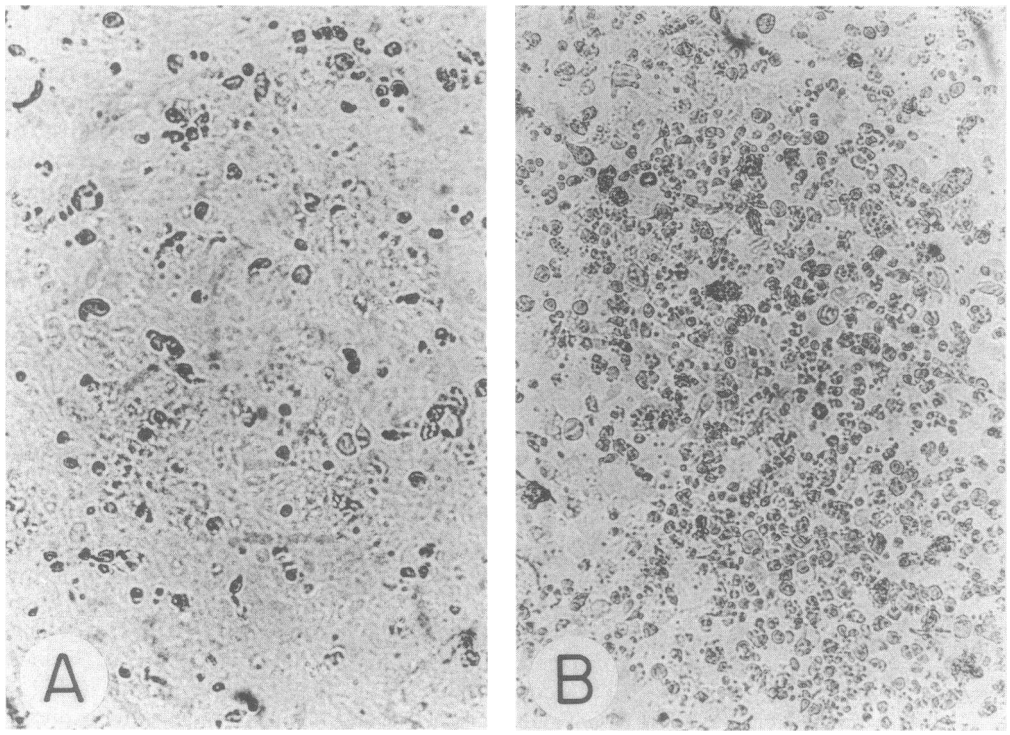

Figure 3. Suspension culture of (*A*) parent SV40–transformed stromal cell line and (*B*) a cell line derived from a single IL-1α–stimulated colony. The colony-derived cell lines, regardless of initial stimulus, produced more round cells than the parent cell lines.

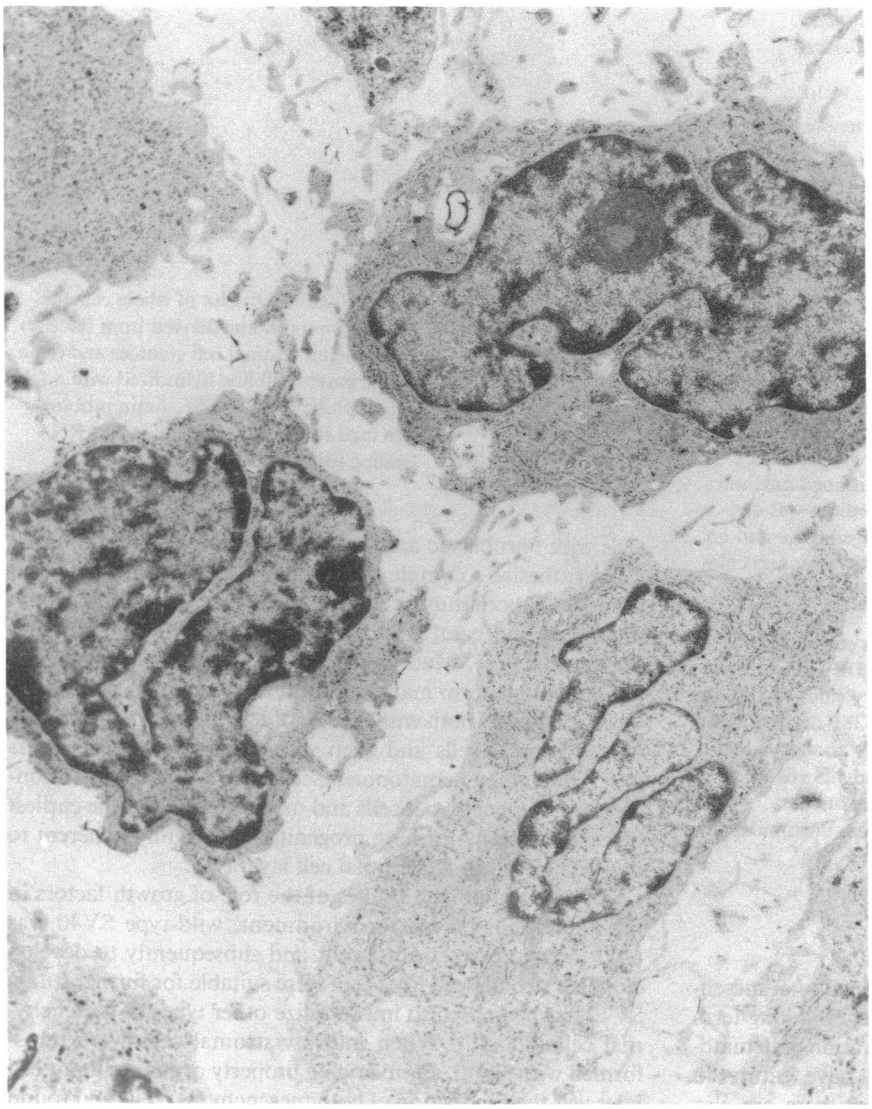

Figure 4. Electron microscopic view (×3,000) of a colony-derived stromal cell line stimulated with PHA-LCM.

cell lines derived from colonies grown from three different transformed stromal cell lines. In each case, mRNA for GM-CSF and IL-1β was heavily expressed but was not detectable in the parental cell lines.

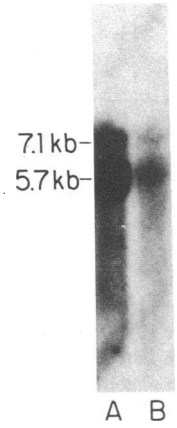

Figure 5. Northern blot probed with a 1.8-kb cDNA probe to procollagen type I. (*A*) Parent transformed stromal cell line; (*B*) a cell line derived from autonomously growing colonies. Hybridization bands are seen at 7.1 and 5.7 kb. The gels were also probed with a cDNA for actin to demonstrate equivalent loading of lanes (data not shown).

Table II. Autonomous Colony Formation by SV40–transformed Stromal Cell Lines Derived from Colonies Stimulated by Various Growth Factors

Cell line	Plating efficiency	*n*
	%	
Parent	0.4±0.4	20
Spontaneous*	2.2±0.2	3
IL-1β*	1.8±0.2	3
IL-1α*	2.2±0.2	3
TNFα*	2.0±0.4	4
TNFα + IL-1α*	2.0±0.1	7

* Cell lines derived from individual colonies that either grew spontaneously or were stimulated by the various recombinant growth factors were harvested with trypsin and cultured in semisolid medium without added growth factors. When the cloning efficiencies of the colony-derived cell lines plated without growth factors were compared to that of the parent cell lines, the differences were highly significant ($P < 0.001$; Student *t* test).

Table III. Colony-stimulating Activity in Conditioned Medium from Transformed Stromal Cell Lines Derived from Pooled Colonies

Stromal cell stimulus	Percentage of maximal CFU-GM growth			Percentage of maximal BFU-E growth		
	Mean	SD	*n*	Mean	SD	*n*
Autonomous	60*	28	18	83	—	1
PHA-LCM	93*	29	69	67	26	21
IL-1α	107*	33	27	124	20	2
IL-1β	133*	10	4	—	—	—
TNFα	89*	32	21	86	29	2
TNFα + IL-1α	69*	33	29	—	—	—

Monocyte-depleted marrow mononuclear cells were cultured at a concentration of 10^5/ml with 20% by volume conditioned medium from the various cell lines. The test conditioned medium was obtained after the colony-derived cell lines were passaged twice and no longer contained the original stimulating material. Background colonies, generally fewer than 20 GM and 15 erythroid burst-forming units (BFU-E) dish, were subtracted from each test data point. Conditioned medium from the parent stromal cell lines stimulated 13±9% (n = 18) of CFU-GM and 10±11 (n = 3) of BFU-E stimulated by PHA-LCM which was assigned an arbitrary value of 100%. The mean number of colony-forming unit (CFU)-GM–derived and BFU-E–derived colonies grown with PHA-LCM/10^5 monocyte-depleted mononuclear cells were 152±53 (n = 18) and 121±67 (n = 12), respectively. n, number of independent experiments.
* $P < 0.0001$ compared to conditioned medium from unstimulated stromal cell lines.

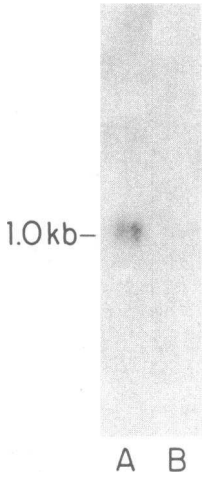

Figure 7. Northern blot of whole cellular RNA from a cell line derived from (lane *A*) spontaneous stromal cell colonies and (lane *B*) the parent cell line hybridized with a full-length probe for GM-CSF. Actin probing was used to demonstrate equivalent RNA loading (data not shown).

Discussion

Marrow stromal cells are a heterogeneous collection of mesenchymal cells which grow as an adherent cell layer in long-term marrow cultures and are necessary for proliferation of hematopoietic cells (7). Many cells within the stroma have features in common with smooth muscle cells; however there are also cells with fibroblastic and endothelial characteristics. Stromal cells synthesize a complex proteoglycan matrix similar to that found in smooth muscle cultures (48). They also produce both interstitial and basal lamina collagens, thrombospondin, and fibronectin (26). Macrophages are interspersed within the stroma of long-term marrow cultures. Hematopoietic cell proliferation occurs both within the extracellular matrix produced by the stromal cells and deep within the stromal cell layers (48). Developing hematopoietic cells are seen in intimate contact with mesenchymal cells and macrophages and the earliest detectable colony-forming progenitors are tightly adherent to or actually within the stromal cell layer (49).

In order to pursue studies of the role of growth factors in the hematopoietic microenvironment, wild-type SV40 was used to transform stromal cells and subsequently to develop clonable stromal cell lines that were suitable for further study. SV40 has been used to immortalize other types of mesenchymal cells (28–30). When marrow stromal cells were transformed with SV40, an invariable property of the resultant cell lines was the production of both mesenchymal cells and round

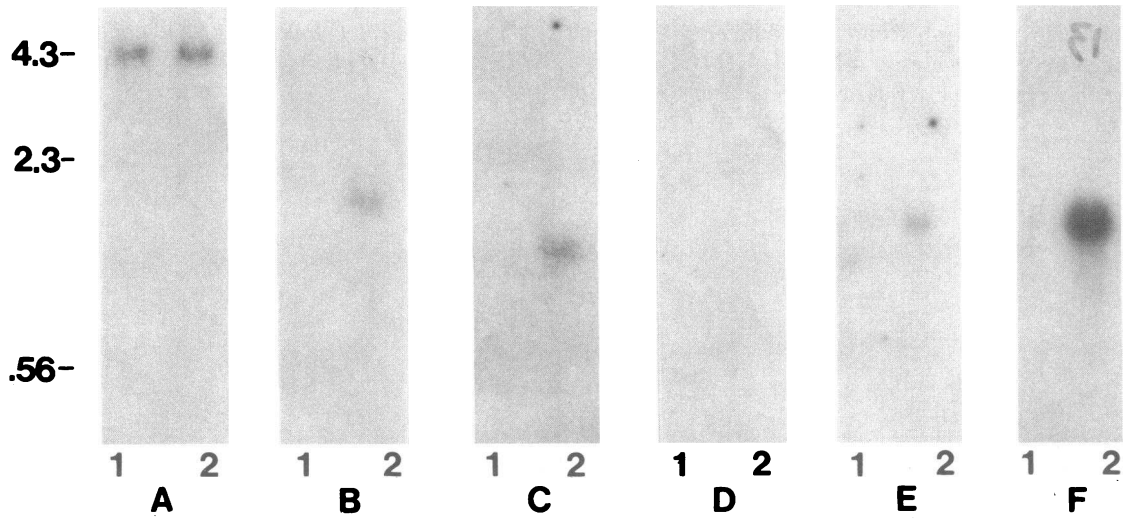

Figure 6. Northern blot analysis of a colony-derived cell line stimulated with PHA-stimulated T cell–conditioned medium. The RNA was harvested 14 wk after the colony was individually plucked and placed in suspension culture. No further stimulation was employed. Lane *1* contains mRNA from the parent cell line and lane *2* is from the colony-derived cell line. The blot was sequentially hybridized with oligonucleotide probes to: (*A*) M-CSF; (*B*) G-CSF; (*C*) GM-CSF; (*D*) IL-3; (*E*) TNFα; (*F*) IL-1β. Actin probing was used to demonstrate loading equivalence.

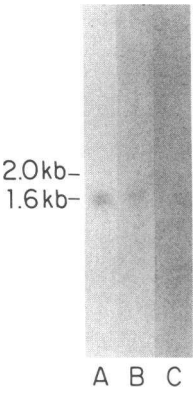

Figure 8. Northern blot of whole cellular RNA from (lane *A*) a stromal cell line derived from colonies stimulated by activated T cell–conditioned medium, (lane *B*) a cell line derived from spontaneous growing colonies, and (lane *C*) the parent cell line which was grown only in suspension culture hybridized with a full-length probe for IL-1β. Actin probing was used to demonstrate equivalent RNA loading (data not shown).

cells (27). While the stromal-like cells had similar expression of cytoskeletal filaments to nontransformed stromal cells (32), the round cells did not express actin or vimentin but did react with monoclonal antibodies to certain hematopoietic determinants (27). When hematopoietic growth factors were added to transformed stromal cells plated in semisolid medium, up to 10% of the cells formed colonies of undifferentiated blastlike cells.

The present study was undertaken to examine the response to and synthesis of hematopoietic growth factors by transformed stromal cell lines. Although SV40–transformed stromal cells proliferate in the absence of growth factors under serum-free conditions, only rare cells are capable of anchorage independent growth in semisolid medium without the addition of exogenous growth factors (27). Using colony formation as a measure of growth stimulation, the proliferative response of stromal cells to factors such as GM-CSF, IL-2, and IL-3 thought to act only on hematopoioetic cells is striking. Further studies are needed to determine if transformed stromal cells bear specific receptors for these growth factors.

When cell lines derived from either spontaneously growing or growth factor stimulated colonies were subsequently plated in semisolid medium without exogenous growth factors, approximately 2% of the cells formed spontaneous colonies whereas only 0.4% of cells in the unstimulated parent cell lines formed colonies without added growth factors (Table II). Although it is possible that these spontaneous colonies grew in response to low levels of growth factors present in the fetal calf serum used in these experiments and thus were not truly autonomous, it was probable that spontaneous colony-forming cells were autostimulatory. To test this hypothesis, cell lines were derived from individual autonomous and growth factor-stimulated colonies and tested for GM colony-stimulating activity and for growth factor mRNAs.

Conditioned medium from unstimulated SV40–transformed stromal cells had little or no hematopoietic colony-stimulating activity. However, after stimulation of the stromal cells in suspension culture with IL-1α or β, T cell–conditioned medium, or with TNFα, colony-stimulating activity was produced. However, the activity diminished to control levels after 1–2 wk. In contrast, when cell lines originating from colonies were developed from individually harvested colonies stimulated by the same growth factors, constitutive production of colony-stimulating activity was observed for at least 16 passages without a significant decline in activity. These data suggest that unless colony-stimulating activity–producing cells were cloned by growing them as colonies, they were overgrown by non–colony-stimulating activity–producing cells in suspension culture.

Cell lines derived from autonomous colonies produced colony-stimulating activity that stimulated significantly more GM colonies and erythroid bursts than did conditioned media from unstimulated, transformed stromal cells: (60±28% of the GM colonies and 83% of the erythroid burst-forming units stimulated by T cell–conditioned medium compared to 13±9% ($P < 0.0001$) and 10±11, respectively). The percent of transformed stromal cells that formed colonies was enhanced by several growth factors (see Table I). Conditioned medium from cell lines started from colonies grown in IL-1α or β stimulated significantly more colony-forming unit–GM growth from normal bone marrows than did cell conditioned medium from cell lines derived from autonomous colonies. These data suggest that all transformed stromal cells capable of colony growth are constitutive producers of colony-stimulating activity and that the level of colony-stimulating activity expression can be further amplified by growth factors such as IL-1.

Analysis of RNA confirmed the findings of the colony-stimulating activity bioassay. For as long as tested (six passages), cell lines derived from spontaneous colonies expressed higher levels of GM-CSF and IL-1β transcripts than did the parent cell lines (Figs. 7 and 8) suggesting that constitutive, autocrine secretion of GM-CSF, IL-1β, or perhaps additional growth factors was responsible for autonomous colony formation. In both the colony-stimulating activity bioassay and on Northern blot analysis, IL-1 and TNF were potent stimuli for further upregulation of GM-CSF.

We hypothesize that both the round cells in suspension culture of the transformed stromal cells and autonomous colonies are produced in response to autocrine stimulation by GM-CSF, IL-1β, or other growth modulatory molecules produced by cells present in low frequency. Since the frequency of cells forming colonies increases approximately 20-fold in response to IL-1 and each of the colonies gives rise to a cell line with apparently permanent upregulation of growth factor expression, growth factors can dramatically amplify their expression in this system.

The relevance of the present findings using SV40 transformed stromal cells to growth factor responses of nontransformed stromal cells is suggested by similarities of the present findings with other studies. For example, like the transformed stromal cells, both murine and human stromal cells only produce GM-CSF when stimulated by IL-1 (50, 51). In addition, other component cells of long-term marrow culture adherent layers including fibroblasts (19, 23, 24) and endothelial cells (17, 52) produce GM-CSF and G-CSF when stimulated by IL-1 or TNF. However, as also found with unstimulated SV40–transformed stromal cells, hematopoietic growth factors other than M-CSF were produced at low levels or were not detectable in resting stromal cells or cultures of component cell types. Nevertheless, local concentrations of growth factors may be relatively high since they are concentrated by the extracellular matrix produced by stromal cells (53). In the SV40–transformed cultures, only by developing cell lines from the low frequency of cells that produced autonomous colonies was it possible to determine that small numbers of transformed stromal cells autonomously upregulate growth factor expression. To determine if low frequency upregulation of growth factor expression occurs in nontransformed stromal

cells is technically difficult because of their lack of clonability. Nevertheless, the present observations on the control of growth factor gene expression in SV40–transformed stromal cells may be relevant to mechanisms of gene control in non-transformed stromal cells and may lead to a greater understanding of physiologic regulation of growth factor gene expression.

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