Nerve Growth Factor Is Involved in the Supportive Effect by Bone Marrow–Derived Stromal Cells of the Factor-Dependent Human Cell Line UT-7

By Isabelle Auffray, Sylvie Chevalier, Josy Froger, Brigitte Izac, William Vainchenker, Hughes Gascan, and Laure Coulombel

We previously demonstrated that murine MS-5 and Sl/SI⁴ cell lines induce the proliferation of human factor-dependent UT-7 cells in the absence of normally required human cytokines and also stimulate the differentiation of CD34⁺/CD38⁻LTC-ICs. We report in this study that the effect of MS-5 cells on UT-7 cells can be completely explained by the synergistic action of nerve growth factor (NGF) and stem cell factor (SCF) produced by these murine stromal cells. Purified murine NGF was able to support short-term clone formation and long-term growth of UT-7 cells in suspension cultures as efficiently as rhu-granulocyte-macrophage colony-stimulating factor. NGF action was mediated through the TrkA receptor, in which messenger RNA (mRNA) was easily detected in UT-7 cells by Northern blot. MS-5 cells strongly expressed NGF mRNA in Northern blot, and direct implication of MS-5–derived NGF in the induction of UT-7 cells proliferation was demonstrated in inhibition assays with an anti-NGF monoclonal antibody (MoAb) that neutralized by 94% ± 4.1% (n = 5) UT-7 clone formation. However, NGF did not act alone, and several arguments demonstrated the synergistic action of MS-5–derived SCF: (1) an anti-NGF antibody partially inhibited UT-7 cells clone formation in coculture assays, (2) SCF and NGF synergized in an h²-TdR incorporation assay, and (3) the stimulatory effect of 10⁻¹⁴-concentrated MS-5 supernatant was completely inhibited by an anti-NGF but not by an anti-SCF, and levels of soluble NGF (1.2 ng/mL) detected by enzyme-linked immunosorbent assay in 10⁻¹⁴ supernatant of MS-5 cells cultures were below the biologically active concentrations. In contrast, although MS-5 cells also promoted the differentiation of very primitive CD34⁺/CD38⁻ human stem cells both in colony assays and long-term cultures, we could not incriminate MS-5–derived NGF in the observed effect: an anti-NGF MoAb did not inhibit the synergistic effect of MS-5 cells in colony assays or long-term cultures nor did soluble muNGF duplicate MS-5 effect and survival of CD34⁺/CD38⁻ clonogenic progenitor cells promoted by MS-5 was unaffected by an anti-NGF and was not induced by soluble NGF alone or combined with SCF. In contrast, NGF in synergy with SCF supported the short-term maintenance of high numbers of CD34⁺/CD38⁻ mature erythroid progenitors probably through an indirect mechanism implying macrophages. These results suggest that NGF, in which the primary target cells are outside the hematopoietic system, is present in the marrow environment and might act at some steps of hematopoietic stem cell development. These results also underline that the response of cell lines and normal stem cells to stromal cells is mediated by different pathways.

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ble NGF stimulated long-term growth of UT-7 cells and that proliferation of UT-7 cells induced by the MS-5 cells in coculture was blocked by an antibody neutralizing NGF. Transcription of the NGF gene by the MS-5 cells was demonstrated by Northern blot analysis, and the protein was detected using a sensitive enzyme-linked immunosorbsent assay (ELISA). The strong analogy we previously reported on the response of CD34+ cells in short-term cultures. These results demonstrate that NGF locally produced by stromal cells in the bone marrow environment might be physiologically involved in the regulation of the biological properties of some hematopoietic cells, although it is likely to act through the stimulation of accessory cells.

**MATERIALS AND METHODS**

**Cell cultures.** The factor-dependent UT-7 cell line (kindly provided by N. Komatsu, Jichi Medical School, Tochigi-ken, Japan) was established from the bone marrow of a patient with acute megakaryoblastic leukemia. UT-7 cells were grown in Iscove’s modified Dulbecco's medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS) (Techgen, Les Ulis, France) and 2 ng/mL of recombinant human (hu) granulocyte-macrophage colony-stimulating factor (GM-CSF; Genetics Institute, Cambridge, MA). Cell concentration was maintained between 2 and 5 x 10^6/mL by diluting the cells with fresh medium every 2 to 3 days. Dishes were maintained at 37°C in a humidified atmosphere with 5% CO2. The other factor-dependent cell lines TF-1, M-07e and K153 were maintained in similar conditions.

The murine stromal cells MS-5 (kindly provided by K. Mori, Faculty of Science, Niigata University, Japan) were derived from irradiated Dexter-type long-term marrow culture adherent layers.

MS-5 cells were grown at 37°C, 5% CO2 in alpha-minimal essential medium (α-MEM) supplemented with 10% FCS. MS-5 cells were passaged usually every 2 weeks by diluting the flasks 1:10. SV-40-transformed cells SI/SI4 derivate, derived from fetal livers of SI/SI mouse fetuses that genetically lack the SCF gene were kindly provided by D.A. Williams (Indiana University, Indianapolis). NIH-3T3 and L929 murine fibroblasts were also used. Supernatants were collected from confluent cultures of MS-5 or SU/SI1 cells grown 2 to 7 days in α-MEM with 1% FCS. The supernatants of confluent cultures of MS-5 cells were concentrated 10- to 20-fold (Centriprep concentrator; Amicon, Danvers, MA) and kept frozen at -20°C until used in bioassays or ELISA.

The isolation of CD34+/CD38- and CD34+/CD38+ cells from adult normal bone marrow was achieved as previously described. In brief, marrow cells were isolated from bone fragments collected from patients undergoing hip surgery and were separated on Ficoll-Hypaque. Light density (<1.077 g/mL) cells were incubated 45 min at 4°C in α-MEM supplemented with 5% FCS and 100 μg/mL DNase (labeling medium) with a 1:5 dilution of the phycoerythrin (PE) labeled anti-CD34 monoclonal antibody (MoAb) HPCA2 (Becton Dickinson, San Jose, CA) and the fluorescein isothiocyanate (FITC)-labeled anti-CD38 MoAb (Immunotech, Marseille, France).

Cells were washed once and suspended in labeling medium at a concentration of 4 x 10^6 to 5 x 10^6 cells/mL for separation by cell sorting. Cells were analyzed and sorted on an ODAM ATC 3000 cell sorter (ODAM/Bruker, Wissembourg, France) equipped with an INNOVA70-4 Argon ion laser (Coherent Radiation, Palo Alto, CA) tuned at 488 nm and operating at 500 mW. Cells “negative” with respect to CD34 and expressing high levels of CD34 referred to as CD34+/CD38- cells and the complementary fraction (ie, cells expressing high levels of both CD34 and CD38) were sorted. To increase the purity of the sorting, the “morphological” two-paramater histograms (WALS vs electric measurement of the cell volume) was acquired for the cells falling in the CD38+, and an additional gate was applied to retain well-defined homogeneous population and to reject highly diffusive cells and/or too large objects.

**Assessment of the proliferation of UT-7 cells by colony assays and H2-TdR incorporation.** Incorporation of H2-TdR in UT-7 cells was performed in microtiter 96-well plates using UT-7 cells deprived of rhu-GM-CSF for 24 hours. A total of 25,000 UT-7 cells was incubated in the presence of appropriate concentrations of cytokines. After 48 hours in culture, 1 μCi of [3H]thymidine (Amersham, Buckinghamshire, UK) was added during 6 hours. At the end of the incubation period, cells were procured on filters, and the incorporated radioactivity was counted. Clone formation by UT-7 cells was assessed by plating 1,000 or 5,000 UT-7 cells, previously carefully washed three times to remove residual rhu-GM-CSF, in 1 mL of 0.8% methylcellulose in Iscove’s medium, 10% FCS, and 1% bovine serum albumin as previously described. Colony formation was stimulated by either rhu-GM-CSF, rhu-SCF (Amgen, Thousand Oaks, CA), muNGF purified from submaxillary gland (UBI, Lake Placid, NY), or 10,000 to 50,000 MS-5 or SI/SI1 cells. Plates were incubated at 37°C in an atmosphere supplemented with 5% CO2 and saturated in humidity. Hydrocortisone (10-8 mol/L) was added in some experiments. Colonies containing more than 10 cells were scored between day 5 and 8, and clone size was estimated. The effect of MS-5-derived SCF or NGF on UT-7 clone formation was investigated by performing colony assays with UT-7 cells preincubated 45 minutes with an anti-c-Kit antibody (SR-1) diluted 1:100,000-fold or by adding anti-NGF (MoAb 27/21; Boehringer-Mannheim, Meylan, France) (1 μg/mL) in the assay. The concentration of the anti-NGF antibody required to neutralize NGF activity was defined by plating UT-7 cells in the presence of 10 ng/mL muNGF and increasing concentrations of the antibody and counting the residual clones. Concentrations of 100 ng/mL to 1 μg/mL of antibody were required to block colony formation stimulated by 10 ng/mL of purified muNGF. Control antibodies of the same isotype (IgG1) were systematically included in the assays.

In three experiments, we assessed by flow cytometry (FACSort, Becton Dickinson) potential changes in the phenotype of UT-7 cells induced by NGF. To that purpose, cells grown in NGF or switched for 48 hours from supplementation by GM-CSF to NGF (or reversed) were labeled with MoAbs coupled to PE or FITC and recognizing the following antigens: CD4-PE (Becton Dickinson), CD38-FITC (Immunotech), glycoporphin A-PE (Immunotech), GPLIIIA-FITC (Dako, Glostrup, Denmark), CD34-PE (HPC-A2; Becton Dickinson), and CD11b-FITC (Immunotech).

**Assessment of hematopoietic progenitor cells by colony assays and long-term cultures.** The effect of NGF on CD34+/CD38+ cells was tested both in clonogenic assays and in long-term cocultures. CD34+/CD38+ clonogenic hematopoietic progenitors, mature and immature burst-forming unit-erythroid (mBFU-E and iBFU-E, respectively), colony-forming unit granulocyte-macrophage (CFU-GM), and colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) were quantified as previously described using methylcellulose colony assays. The assays were supplemented with rhu-erythropoietin (Epo-2 U/mL; Amgen), rhu-interleukin-3 (IL-3, 100 ng/mL)
U(M; Immunex Corp, Seattle, WA), rhu-G-CSF (10 ng/mL; Roger Bellon), rhu-GM-CSF, and rhu-IL-6 (100 U/mL; Genetics Institute). MS-5 cells were also added to the assay (15,000 to 20,000/mL) to stimulate the development of mixed colonies and high proliferative potential progenitors as previously described. Plates were incubated at 37°C in an air atmosphere supplemented with CO₂ and saturated with humidity. Progenitors were scored at days 15 to 16 (immature BFU-E, CFU-GM) and 22-24 (CFU-GEMM) using previously detailed criteria. Dishes supplemented with MS-5 cells were scored at a later time (day 30).

The effect of muNGF or anti-NGF on colony formation by primitive CD34⁺/CD38⁻ cells was tested as described above for UT-7 cells. In experiments where the effect of NGF on cell survival was assessed, CD34⁺/CD38⁻ cells were first plated in methylcellulose in the presence of muNGF alone or with SCF or MS-5 cells alone. The addition of cytokines (SCF, IL-3, Epo) was delayed until day 15, and colonies generated by progenitors that survived until that time were scored at day 30.

Long-term cultures were established by incubating 40 CD34⁺/CD38⁻ cells in 96-well plates precoated with murine stromal MS-5 cells (6,000 cells per well in 100 μL) as previously described. Long-term culture medium included α-MEM supplemented with 12.5% FCS, 12.5% horse serum (HyClone Laboratories, Logan, UT), 10⁻⁴ mol/L 2,2'-mercaptoethanol. Hydrocortisone (10⁻⁶ mol/L) was added in some cultures. To test the effect of NGF, muNGF (10⁻⁵ ng/mL) was added to wells initiated in the presence of hydrocortisone. Conversely, an anti-NGF antibody (1 μg/mL) was added to some wells initiated without hydrocortisone. Cultures were fed every week by half medium change (no cell depletion). After 5 weeks in culture (33°C, 5% CO₂), the progenitor content of each well was assessed in colony assays as described above. At least 6 to 10 different wells were examined per each time point and each culture condition.

The effect of NGF, SCF, and the combination of both was also tested in short-term liquid cultures. CD34⁺/CD38⁻ and CD34⁺/CD38⁻ cells (3,000 per well) were incubated in 96-well plates in methylcellulose colony assays as described above to assess the content of each well in clonogenic progenitors. Northern blot analysis of mRNA encoding NGF and TrkA in stromal cells and UT-7 cells. Total cellular RNA was isolated by the guanidinium thiocyanate method. Ten micrograms each of total cellular RNA extracted from the stromal cell lines MS-5, SI/SI4, L929, and NIH3T3 and from UT-7 cells (both cells grown in GM-CSF and NGF were tested) were size-fractionated in 1.2% agarose-formaldehyde gels and then transferred to nylon membranes (Hybond N; Amersham). Immobilized RNAs were hybridized with 32P-labeled probe with the 32P-dCTP (Amersham), using the Rediprime DNA-labeling system (Amersham). Probes used were a 1,200-pb BamHI fragment encoding the 5'GGCAGCCTGCTC'TCTCATC3', respectively, a 600-pb fragment prepared from recombinant mouse NGF by ELISA. Supernatants collected from confluent MS-5, SI/SI4 stromal cells, NIH-3T3, and L929 (control) cells layers grown 48 hours in 5 mL of α-MEM with 1% FCS were analyzed for their content in NGF by ELISA. Briefly, MoAb anti-NGF (27/21) was coated in 100 mmol/L carbonate buffer, pH 9.6, at a final concentration of 1 μg/mL. After saturation and washes, samples were added and incubated overnight at 4°C. Because NGF is present in solution as a dimer, the detection was performed by using the same antibody labeled with peroxidase and added at a concentration of 0.1 mg/mL for 6 hours. Visualization was performed at 570 mmol/L by using chloropornol red galactopyranoside as substrate. The limit of sensitivity was 10 pg/mL of NGF. Media were used either unconcentrated or after 10- to 20-fold concentration (Centricon concentrator, Amicon).

Mouse SCF was also measured in an ELISA assay; purified goat immune IgG directed against mouse SCF (R&D Systems, Minneapolis, MN) were coated overnight as described above at a concentration of 10 μg/mL in 100 mmol/L carbonate buffer, pH 8.6. After washing and a saturation step with 100 mol/L Tris, 20% sucrose, pH 7.8, the samples were added to the wells in duplicate for a 6-hour incubation at 37°C. MoAb directed against mouse SCF (Genecis Institute) was used as tracer antibody at a final concentration of 1 μg/mL. After an overnight incubation, peroxidase-labeled goat antirabbit antibody (Dako) was added at a 1:2,000 dilution for a 4-hour incubation step. ABTS (Sigma, l'Isle d'Abeau Chesnes, France) was used as substrate, and the reading was carried out at 405 nm. ELISA calibration was performed by using recombinant purified mouse SCF, kindly provided by Dr Turner (Genecis Institute); the limit of sensitivity was 10 pg/mL.

RESULTS

Human factor-dependent UT-7 cells respond to muNGF. To determine whether MS-5-derived growth promoting activity was accounted for by a known cytokine crossing barrier species, we tested the effect of a panel of different cytokines on clone formation by UT-7 cells. Most experiments were performed with UT-7 cells grown in rhu-GM-CSF. Clone formation by UT-7 cells normally depends on the addition of exogenous human cytokines, such as rhu-GM-CSF, rhu-IL-3, or rhu-Epo on or as yet unidentified activities produced by murine stromal cells MS-5, as we recently demonstrated. Thus, in 33 experiments, 2 ng/mL of rhu-GM-CSF stimulated the formation of 2,010 ± 153 UT-7 colonies for 5,000 plated cells (40.2% ± 3% cloning efficiency) at day 8, when no clone was detected in the absence of growth factor (Table 1). UT-7 cells cloning efficiency in the presence of either MS-5 or SI/SI4 murine stromal cell lines was 5.5% ± 0.7% (n = 35) and 5.1% ± 1.5% (n = 4), respectively. The size of the clones (50-100 cells per colony) induced by the MS-5 or the SI/SI4 cells was below the size of the clones observed with rhu-GM-CSF (500 to 1,000 cells per colony). Interestingly, the supportive effect of MS-5 cells on clone formation by UT-7 cells was suppressed by 67% ± 11% when 10⁻⁶ mol/L hydrocortisone was added to the methylcellulose medium (Table 1). Addition of hydrocortisone also suppressed UT-7 cells proliferation induced by MS-5 cells in liquid coculture by 60% ± 7% (n = 5, P < .01) (data not shown). Both results are very reminiscent of...
null
28s
18s
18s

Fig 2. Northern blot hybridization analysis of trkA receptor mRNA in UT-7 cells. Total mRNA of each cell type was isolated by the gua-nidium thyocyanate method, and 10 μg was loaded per lane. Transcripts of trkA were analyzed in UT-7 cells grown in different conditions: stimulation by NGF (lane B) or rhu-GM-CSF (lane C), deprived of cytokines (Lane D) or cocultured with MS-5 cells (lane E) or 7 (lane F) days. mRNA extracted from PC12 cells was used as a positive control. Blots were hybridized with probes for the trkA and for GAPDH to estimate the amount of RNA in each lane.

whereas TF-1 cells did in agreement with our previous study (data not shown).

Murine stromal cells MS-5 and SI/SI⁴ cells produce NGF. We first examined by Northern blot analysis whether MS-5 and SI/SI⁴ cells transcribed the gene encoding NGF (Fig 3). The probe was obtained through RT-PCR products amplified with synthetic primers specific for mouse β-NGF. As shown in Fig 3, a band corresponding to the 1,300-bp NGF transcript was detected in total RNA extracted from mouse fibroblasts L929 cells used as a positive control, from MS-5, and from SI/SI⁴ stromal cells and was of equal intensity. A band of lower intensity was present in gels with RNA from NIH3T3 fibroblasts. Interestingly, when Northern blots were performed with RNA extracted from MS-5 cells grown in medium supplemented with 10⁻⁶ mol/L hydrocortisone (Fig 3), the intensity of the bands corresponding to the NGF RNA was decreased at least twofold, as estimated by densitometry.

In a second series of experiments, to determine whether MS-5–derived NGF was released in a soluble form, we measured the amounts of NGF protein in the supernatants of MS-5, SI/SI⁴, NIH-3T3, and L929 cell cultures using a sensitive ELISA. As shown in Table 2, 3 to 8 ng/mL of NGF was measured in medium conditioned by the L929 cell line and concentrated 10-fold. In contrast, NGF was undetectable in the supernatant from MS-5 cultures when used unconcentrated, and levels ranged from 0 to 3.5 ng/mL in four different samples concentrated 10-fold. Similar values (0.6 to 0.9 ng/mL) were detected in two different samples of 10-fold concentrated medium from SI/SI⁴ cells. Interestingly, and in keeping with the results of the Northern blots (see above), concentrations of NGF were found to be 2-fold lower in supernatants from MS-5 and SI/SI⁴ cultures treated with hydrocortisone (0.1 and 0.25 ng/mL, respectively).

These low (<1 ng/mL) concentrations of NGF did not alter the proliferation of UT-7 cells measured by clone formation or H²-TdR incorporation (Fig 1). It was therefore surprising to observe a significant number of clones (1,153, mean of two experiments) and thymidine incorporation by UT-7 cells in the presence of conditioned medium from MS-

<table>
<thead>
<tr>
<th>Stromal Cells</th>
<th>Fold Concentration</th>
<th>n</th>
<th>NGF (ng/mL)*</th>
<th>CPM (% of max)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L929</td>
<td>10</td>
<td>2</td>
<td>4; 3.6</td>
<td>37; 72</td>
</tr>
<tr>
<td>MS-5</td>
<td>1</td>
<td>2</td>
<td>0.1; 0.1</td>
<td>0, 0</td>
</tr>
<tr>
<td>SI/SI⁴</td>
<td>10</td>
<td>4</td>
<td>1.3; 3.5; 0; 0</td>
<td>11; 29; 71; 24</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
<td>12</td>
</tr>
</tbody>
</table>

Supernatants were collected from confluent cultures of the different stromal cells and concentrated as indicated. Supernatants were analyzed either fresh or after they have been frozen at −20°C. The proliferative effect of these supernatants of UT-7 cells as assessed by H²-TdR incorporation as described. Each number in column 4 (NGF concentration) refers to the result obtained in one experiment.

* NGF concentration is expressed in ng/mL of concentrated supernatant as measured by ELISA in each experiment.

† Data obtained from H²-TdR incorporation experiments. In each experiment the number of CPM obtained when UT-7 cells were stimulated by the supernatant of stromal cells was divided by the number of CPM obtained when cells were stimulated by GM-CSF × 100.
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5 cells (final concentration 20% to 50%). Two sets of data indicated that the activity present in the supernatant was SCF. First, concentrated supernatant from the SI/SI^4 cell line was devoid of activity stimulating thymidine incorporation in UT-7 cells (Table 2). Second, in three experiments, addition of an anti-c-kit antibody (SR-1) blocked the incorporation of H^3-TdR in UT-7 cells induced by MS-5 supernatant by 96.3% ± 1.9% (n = 3). Addition of an anti-NGF led to a low nonsignificant inhibition as compared with the control IgG (data not shown). The amount of SCF measured by an ELISA specific for murine SCF was undetectable in unconcentrated MS-5 conditioned medium and found to be 1 to 3 ng/mL in 10×-concentrated medium from MS-5 cultures (equivalent to 100 to 300 pg/mL in unconcentrated medium). These numbers were not significantly decreased in supernatants from hydrocortisone-treated cultures (data not shown).

Both SCF and NGF produced by MS-5 cells are responsible for the proliferation of human UT-7 cells. As soluble muNGF promoted the proliferation of UT-7 cells and because MS-5 cells (and SI/SI^4 cells) expressed strongly the mRNA encoding NGF but released only low amounts in the supernatant, we investigated the role of this cytokine in a coculture assay. To that purpose, in five experiments, we measured clone formation by UT-7 cells plated in colony assays with MS-5 in the presence or in the absence of 1 μg/mL of an MoAb neutralizing mouse NGF. As shown on Fig 4, addition of 1 μg/mL of an anti-NGF MoAb inhibited by 84.7% ± 4.1% (n = 5) the formation of UT-7 cells clones induced by MS-5, whereas a control isotype-matched antibody had very little effect (17.8% ± 9.4% inhibition). The anti-NGF similarly inhibited UT-7 cells clone formation induced by SI/SI^4 cells (Fig 4). These results strongly suggested that MS-5- and SI/SI^4-derived NGF was at least partly responsible for the effect of both stromal cell types on UT-7 cells. Interestingly, this contrasted with the lack of inhibitory effect of the anti-NGF MoAb on the activity present in MS-5 supernatant (see above). However, the observation that an anti-c-kit antibody also partially blocked the action of MS-5 cells (49.1% ± 8.2% inhibition of clone formation) indicated that both SCF and NGF were involved in UT-7 cell proliferation, which fitted with the above-described synergy between both molecules (Fig 1). The level of inhibition of clone formation observed with combined anti-NGF and anti-c-kit was not higher than that observed with the anti-NGF alone (data not shown). In two experiments, we found that irradiated human marrow-derived adherent cells stimulated the formation of high numbers of UT-7 clones in methylcellulose, but this activity was inhibited neither by an anti-c-kit nor by an anti-NGF (data not shown).

Effects of MS-5-derived NGF on the differentiation of human CD34^+/CD38^- clonogenic progenitor cells and LTC-IC. We previously reported that stromal MS-5 cells stimulate the cloning efficiency of very primitive human CD34^+/CD38^- stem cells in short-term semisolid assays and the differentiation of CD34^+/CD38^- LTC-IC in the absence of hydrocortisone, whereas addition of this hormone dramatically suppressed this latter stimulatory effect. This behavior was strikingly similar to that of UT-7 cells, which prompted us to examine the contribution of MS-5- and SI/SI^4-derived muNGF to the differentiation of CD34^+/CD38^- cells (Fig 5) nor did it change the size of the colonies,
Table 3. Survival of CD34+/CD38- Hematopoietic Progenitors in the Presence of SCF, NGF, and MS-5 Cells

<table>
<thead>
<tr>
<th>Additive at Day 0</th>
<th>n</th>
<th>mBFU-E</th>
<th>iBFU-E</th>
<th>CFU-GEMM</th>
<th>CFU-GM</th>
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<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCF</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCF + NGF</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>NGF</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MS-5</td>
<td>6</td>
<td>8 ± 5</td>
<td>32 ± 4</td>
<td>49 ± 5</td>
<td>53 ± 13</td>
</tr>
<tr>
<td>MS-5 + anti-NGF</td>
<td>3</td>
<td>20 ± 10</td>
<td>34 ± 15</td>
<td>54 ± 4</td>
<td>60 ± 23</td>
</tr>
<tr>
<td>MS-5 + SCF</td>
<td>3</td>
<td>14 ± 12</td>
<td>60 ± 25</td>
<td>93 ± 7</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>MS-5 + NGF</td>
<td>1</td>
<td>21</td>
<td>17</td>
<td>ND</td>
<td>50</td>
</tr>
</tbody>
</table>

A total of 1,000 CD34+/CD38- cells were plated in methylcellulose in the presence of soluble recombinant cytokines or MS-5 cells as indicated in the left column. At day 14, rhSCF (50 ng/mL), rh-IL-3 (100 U/mL), and rh-Epo (2 U/mL) were added and colonies scored after an additional 14 days at 37°C. Control dishes were included where CD34+/CD38- cells were plated with SCF, IL-3, and Epo from day 0. Numbers represent the proportion of colonies observed in each condition as compared to colonies scored in the control dishes. n = number of number of experiments.

whereas addition of MS-5 cells had a major effect. A trend toward higher number of colonies was apparent when 100 ng/mL NGF was used. mu-NGF also failed to stimulate colony formation by human marrow CD34+ progenitor cells in combination with either SCF, IL-3, and Epo (erythroid conditions) or with G-CSF and GM-CSF (granulocytic conditions), even when suboptimal concentrations of SCF (5 ng/mL) and IL-3 (20 U/mL) were used (data not shown).

DISCUSSION

Understanding mechanisms by which stromal cells regulate biological properties of normal primitive hematopoietic progenitor cells requires a definition of reproducible readouts

Table 4. Effect of Addition of NGF or Anti-NGF Antibody on the Differentiation of CD34+/CD38- LTC-I Culture at Limiting Dilutions on MS-5 Cells

<table>
<thead>
<tr>
<th>Cells per Well</th>
<th>Additive</th>
<th>No. of Wells</th>
<th>No. Positive Wells*</th>
<th>No. Progenitors per Positive Wells</th>
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<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>HC</td>
<td>10</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>HC + NGF</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>142 ± 17</td>
</tr>
<tr>
<td>40</td>
<td>Anti-NGF</td>
<td>10</td>
<td>10</td>
<td>168 ± 26</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>HC</td>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>HC + NGF</td>
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<td>0</td>
<td>10</td>
<td>10</td>
<td>137 ± 15</td>
</tr>
<tr>
<td>35</td>
<td>Anti-NGF</td>
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<td>6</td>
<td>169 ± 19</td>
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</tbody>
</table>

Long-term cultures were initiated with 35 (experiment 2) or 40 (experiment 1) CD34+/CD38- cells in 96-well plates containing confluent pre-established MS-5 cells. Hydrocortisone (HC) was added each week at 10⁻⁴ M/mL in some wells together with NGF at 10 ng/mL. Wells were individually sacrificed after 5 weeks of culture and the content of each well was plated in methylcellulose assays supplemented by rhSCF (50 ng/mL), rh-IL-3 (100 U/mL), rh-Epo (2 U/mL), and rh-G-CSF (10 ng/mL). Progenitors were scored after 10-20 days as described in Materials and Methods.

* A well was considered positive when at least one clonogenic progenitor was detected after 5 weeks in culture. Numbers represent the mean ± SEM of the numbers of progenitors counted in 610 wells.
Table 5. Effect of NGF, SCF, and the Combination of NGF + SCF on CD34+/CD38+ or CD34+/CD38- Cells Cultured 5 Days in Stromal-Free Liquid Culture

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Cytokines</th>
<th>Nucleated Cells (×1,000/Exp1)</th>
<th>Progenitor Cells per Well</th>
<th>BFU-E Exp1</th>
<th>BFU-E Exp2</th>
<th>CFU-GM Exp1</th>
<th>CFU-GM Exp2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+/CD38+</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>32</td>
<td>72</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>rhSCF</td>
<td>7</td>
<td>5</td>
<td>28</td>
<td>60</td>
<td>174</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td>mu-NGF</td>
<td>2.5</td>
<td>3</td>
<td>12</td>
<td>72</td>
<td>76</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>rhuSCF +</td>
<td>10</td>
<td>8</td>
<td>330</td>
<td>440</td>
<td>206</td>
<td>592</td>
</tr>
<tr>
<td></td>
<td>mu-NGF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD34+/CD38-</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>rhSCF</td>
<td>ND</td>
<td>2</td>
<td>3</td>
<td>12</td>
<td>45</td>
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<tr>
<td></td>
<td>mu-NGF</td>
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<td>1.5</td>
<td>8</td>
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<tr>
<td></td>
<td>rhuSCF +</td>
<td>ND</td>
<td>7</td>
<td>22</td>
<td>23</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mu-NGF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

A total of 3,000 CD34+/CD38+ or CD34+/CD38- human marrow cells were cultured in 96-well plates in α-MEM with 20% FCS and either no cytokines or 20 ng/mL rhu-SCF, 50 ng/mL mu-NGF, or a combination of 20 ng/mL rhu-SCF + 50 ng/mL mu-NGF. After 5 and 10 days at 37°C, 5% CO2, all cells from one well were removed, counted, and plated in colony assays supplemented with recombinant human cytokines (SCF, IL-3, G-CSF, and Epo) and 30,000 MS-5 cells. Results from two experiments are shown.

that can be easily measured. We attempted such an approach with normal CD34+/CD38- cells in limiting dilution assays and reported the synergistic action of murine stromal cells MS-5 on the differentiation of CD34+/CD38- LTC-IC and its downregulation by hydrocortisone. In a search of a more easily workable model to screen stromal-derived activities, we reported that MS-5 cells could induce in the factor-dependent UT-7 cell line a proliferative response equivalent to that induced by the human cytokines used to sustain long-term growth of these cells. More recent experiments reported here also document the downregulation of this activity by hydrocortisone. It was therefore tempting to speculate that screening stromal-derived activities with UT-7 cells as the initial targets will lead us to stromal molecules also active in the regulation of normal hematopoietic progenitors.

It has become evident from recent work with knock-out mice or in vitro assays that growth factors, whose target cells are not primarily hematopoietic, such as bFGF or HGF, have a broader spectrum of mitogenic activity than initially described and contribute to the regulation of hematopoiesis. We now provide evidence that NGF, a member of the neurotrophin family, also belongs to this group. As is shown in this study, this cytokine is produced in a biologically active state by marrow-derived stromal cells and is partly responsible for the mitogenic activity of these stromal cells on a hematopoietic factor-dependent cell line. This is based on the observation that murine NGF was as efficient as rhu-GM-CSF, rhu-IL-3, and rhu-Epo in sustaining continuous proliferation of UT-7 cells. Second, NGF transcripts were abundant in MS-5 cells, and the NGF protein was detected in the supernatant of these murine stromal lines.

Finally, blockade of the MS-5-derived NGF activity by an anti-NGF MoAb abolished the proliferation of UT-7 cells in the coculture assay. Indirect evidence is also provided by the observation that MS-5-derived proliferative activity on UT-7 cells is abolished by hydrocortisone, which downregulates the expression of NGF as it does for most cytokines. However, hydrocortisone does not seem to affect SCF production by MS-5 as shown by the similar amounts of SCF released in the supernatants of hydrocortisone-treated and intact MS-5 cultures and by previous findings using RT-PCR strategy showing that hydrocortisone modified neither quantitatively nor qualitatively SCF transcripts in MS-5 cells and other stromal cells.

The amount of NGF detected in the concentrated supernatant of MS-5 cultures was less than 1 ng/mL, which is below the threshold of 5 ng/mL required to trigger thymidine incorporation in UT-7 cells. It was therefore highly likely that in the coculture, NGF was acting in association with another molecule. When used as soluble purified molecules in a UT-7 cells thymidine incorporation assay, both NGF and either mur- or rhu-SCF synergized. That SCF was potentiating the action of NGF in the MS-5 coculture assays was further suggested by the results of inhibitory experiments using an anti-c-kit antibody that partially inhibited the response of UT-7 cells to MS-5 in coculture. The anti-c-kit MoAb also completely neutralized the activity present in MS-5-conditioned medium, confirming that SCF was released in MS-5 supernatant. This was in agreement with the lack of stimulatory activity on UT-7 cells in the supernatant of 5/Sl/cells, which also transcribe the NGF gene but release <1 ng/mL NGF in the supernatant.

The discrepancy between a very low concentration of soluble NGF despite a high rate of transcription of the NGF gene as assessed by Northern blot and the presence of active NGF at the cell surface as shown by inhibition of UT-7 cell proliferation by an anti-NGF MoAb suggested that MS-5-derived NGF was expressed as a transmembrane molecule or bound to MS-5 cell surface or extracellular matrix or associated with a third partner. Many mesenchyme regulators, such as bFGF, transforming growth factor beta, and hematopoietic cytokines, remain closely associated with their producer cells either by binding to proteoglycans or their sulfate glycosaminoglycans or because they act as transmembrane proteins. Biological activity of these cytokines appears often enhanced when presented by local components of the environment as compared with their soluble counterparts.

Even though neurotrophin-6, a member of the neurotrophin family, exists exclusively as an heparin-sulfate-bound cytokine, such molecular forms have not been reported for NGF. Although the mouse NGF gene includes two alternative splices leading to multiple RNA species, only the 1.3-kb transcript was detected in MS-5 cells as in murine and human fibroblasts.

Evidence has accumulated recently that underlines the potential physiological importance of mesenchymal regulators such as bFGF, HGF, or others in hematopoiesis. Particularly relevant to our results is also the observation that several cytokines of the IL-6 family are shared by cells of the...
hematopoietic and the nervous system. NGF does not belong to those but has been known for a long time to activate mature cells engaged in inflammatory or immune responses such as lymphocytes, monocytes, and mast cells by promoting functions such as mast cell degranulation and basophil release of inflammatory mediators. Stimulation by NGF of mast cells differentiation has also been reported, but little is known, however, on the action of NGF on other hematopoietic progenitor cells. Two studies have shown that NGF could stimulate colony-forming cells from mouse bone marrow in synergy with other hematopoietic cytokines. A direct action of NGF on the progenitor cells was not demonstrated; however, and in one study, the action of NGF was T-cell dependent. In our study also, the dramatic effect of NGF and SCF on the recovery of erythroid progenitor cells after 5 days in liquid culture is likely to be indirect and mediated through the release of cytokines by macrophages, which are known target cells for NGF.

We published previously that MS-5 cells added to colony assays strikingly enhanced the cloning efficiency of primitive CD34+/CD38- BFU-E, CFU-GEMM, and CFU-GM human marrow cells in synergy with SCF, IL-3, and Epo and supported LTC-IC differentiation during a 5-week coculture period. Furthermore, this synergistic activity was abolished in cultures supplemented with hydrocortisone when both UT-7 cells and normal CD34+/CD38- progenitor cells were used as target cells. In view of our findings that NGF produced by both MS-5 and STI/STI1 stromal cells was a major contributor in the support of UT-7 cells proliferation by these cells, it was tempting to speculate that NGF was also involved in the effect of MS-5 on CD34+/CD38- cells. This was also logical in view of the physiological action of NGF in the nervous system as this cytokine primarily acts by maintaining survival of neurons during development but has little proliferative effect. Until now, we failed to detect any effect of NGF, used alone or in combination with various cytokines, on early CD34+/CD38- clonogenic progenitors or LTC-ICs. Thus, NGF, neither alone nor combined with SCF substituted for MS-5 activity in colony or LTC-IC assays, and the anti-NGF antibody was without effect. Survival of early clonogenic cells was also not primarily dependent on NGF. These conclusions diverge from previous studies suggesting a proliferative effect of NGF in colony assays and seem at odds with our results that NGF promotes the growth (or survival) of late erythroid progenitors in short-term assays. This may be explained either because most of our experiments used the CD34+/CD38- fraction that is depleted of mature erythroid progenitors and contains immature progenitor cells requiring more than one cytokine to proliferate. Both colony and long-term cultures assays initiated with CD34+/CD38- were done at limiting dilutions that minimized cell-cell interactions and therefore might mask the effect of NGF that is most likely indirect through the stimulation of accessory cells such as lymphocytes or macrophages absent from the limiting dilutions and present in assays performed with high concentrations of unfractonated or CD34+/CD38- cells. NGF may also act preferentially on mature progenitor cells of the erythroid lineage that are usually not produced in LTC-IC assays.

Our results therefore suggest that NGF, locally secreted by stromal cells and mature cells of the monocytic and lymphoid lineage, might in turn contribute to the regulation of hematopoietic differentiation through the activation of accessory cells and the release of regulatory molecules. Our results argue against a crucial role for NGF in the support of CD34+/CD38- cells by MS-5 at initial steps, although we cannot rule out that NGF may bind to a fraction of CD34+/CD38- cells and trigger subtle events in these cells, an effect that might be difficult to identify for reasons inherent to stem cells assays that require combination of redundant cytokines to proliferate and complete their differentiation. In contrast to proliferative assays performed with leukemic cell lines or more mature progenitors, which require only one mitogen, a proliferative response triggered by NGF might be very difficult to apprehend in normal stem cells. An alternative experimental approach to further elicit the putative effect of NGF or related neurotrophins in the early steps of hematopoiesis could be the detection and selection of TrkA+CD34+ cells provided that sufficient numbers of receptors are expressed on the cell surface.

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