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 SI/SI¹ 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 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-c–e–kit 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 10⁻⁵-concentrated MS-5 supernatant was completely inhibited by an anti-c–e–k but not by an anti-NGF, and levels of soluble NGF (1.2 ng/mL) detected by enzyme-linked immunosorbent assay in 10⁻⁵ 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 implicating 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.

© 1996 by The American Society of Hematology.
proliferation of NGF-stimulated long-term growth. Demonstrated by Northern blot analysis, and the protein was detected using a sensitive enzyme-linked immunosorbent assay (ELISA). The strong analogy we previously reported using a sensitive enzyme-linked immunosorbent assay (ELISA) prompted us to hypothesize that NGF could also be a major contributor in the regulation of primitive progenitors by stromal cells.

Even though we could not demonstrate any contribution of NGF in the supportive effect of MS-5 on CD34+/CD38- neither in long-term culture assays nor in clonogenic assays, there was a striking effect of NGF in combination with SCF on the recovery of CD34+/CD38- mature erythroid progenitors 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 X 10^5 and 5 X 10^5 cells/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-O7e, 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 flask 1:10. SV-40-transformed cells SIS, 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). NIIH3T3 and L929 murine fibroblasts were also used. Supernatants were collected from confluent cultures of MS-5 or SIS1 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-CD38 monoclonal antibody (MoAb) (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 10^5 to 5 X 10^5 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 CD38 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-parameter histograms (WALS v electric measurement of the cell volume) was acquired for the cells falling in the CD38+, and an additional gate was applied to retain a 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 5'-Tdr incorporation.** Incorporation of 5'-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 [6-3H]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 rhs-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 SIS1 cells. Plates were incubated at 37°C in an air 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)7 diluted 1:100,000-fold or by adding anti-NGF (MoAb 272/21; 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 clone 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 FACS 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), GPIIIa-FITC (Dako, Glostrup, Denmark), CD34-PE (HPC-A2; Becton Dickinson), and CD11b-FITC (Immunotech).

**Assessment of hematopoietic progenitor cells by colony assay and long-term cultures.** The effect of NGF on CD34+/CD38- 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 BFU-E, respectively), colony-forming unit granulocyte-macrophage (CFU-GM), and colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte (CFU-GE-MK) were quantified as previously described using methylcellulose colony assays.8 The assays were supplemented with rh-erythropoietin (Epo-2 U/mL; Amgen), rh-interleukin-3 (IL-3, 100
U/mL; 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 cocultures 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-mercaptopethanol. 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 to 6 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⁻ cells (3,000 per well) were incubated in 96-well plates in 100 μL of α-MEM with 20% FCS with either no cytokines, 20 ng/mL muNGF, or a combination of both. After 5 to 10 days, nucleated cells were counted in each well and plated 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 guanidium thiocyanate method. Ten micrograms each of total cellular RNA extracted from the stromal cell lines MS-5, SISI, 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 (Hybden N; Amersham). Immobilized RNAs were hybridized with 3²P-labeled probe with the 3²P-deCTP (Amersham), using the Redigene DNA-labeling system (Amersham). Probes used were a 1,200-pb BamHI/EcoRI fragment containing sequences for the tyrosine kinase domain of NGF and TrkA proto-oncogene, a 408-pb fragment prepared from reverse transcription polymerase chain reaction (RT-PCR) products with oligonucleotides specific of murine NGF (oligonucleotides sense and antisense were 5'TCACACCTGTTGGCTTGC3' and 5'GGGAGGCTGCTTCCATC3', respectively), a 600-pb Psi I fragment encoding the 5' half portion of murine β-actin, and a pRGAHPD 13 plasmid containing the 1,300 pb of rat GAPDH insert. Hybridizations were performed in a solution containing 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 5% SPE, 5% Denhardt's solution, and 0.5 mg/mL denatured salmon sperm DNA at 42°C overnight. Filters were washed to final stringency of 1× SPE, 0.5% SDS for 1 hour at 55°C and autoradiographed. The specificity of the NGF PCR probe was demonstrated by restriction enzyme mapping of the NGF PCR products and subsequent hybridization with internal oligonucleotides. The relative intensities of the bands on the autoradiogram on the Northern blot were quantified by a light scanning densitometer.

Detection of NGF and SCF in medium conditioned by MS-5 cells by ELISA. Supernatants collected from confluent MS-5, SISI 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 chlorophenol 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 (Centrprep 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 (Genecins Institute) was used as tracer antibody at a final concentration of 1 μg/mL. After an overnight incubation, peroxidase-labeled goat antirat 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 murine SCF, kindly provided by Dr Turner (Genecins 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 or on one or yet unidentified identities 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 SISI 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 SISI 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 < 0.01) (data not shown). Both results are very reminiscent of
our previous observations that the differentiation of CD34+/CD38– primitive progenitor marrow cells in coculture with MS-5 cells was compromised by the addition of hydrocortisone.13

Unexpectedly, among various cytokines tested for their ability to support the proliferation of UT-7 cells, we found that murine NGF, purified from submaxillary glands, promoted the formation of 519 ± 63 colonies of up to 100 cells per 5,000 UT-7 cells plated (mean ± SEM of 31 different experiments). Analysis of the response of UT-7 cells to increasing concentrations of NGF indicated that maximum number of colonies was observed for 10 ng/mL. When the response of UT-7 cells was assessed by a short-term (48 hours) $^{3}H$-TdR incorporation assay, significant incorporation was first detected for NGF concentrations of 5 ng/mL and was maximal at 10 ng/mL muNGF (Fig 1). Maximal $^{3}H$-TdR incorporation values were, however, three times lower than those obtained with UT-7 cells stimulated by 50 ng/mL rhu-SCF (Fig 1). Both mu-NGF and rhu-SCF synergized in the different bioassays; thus, 1 ng/mL of mu-NGF increased the response of UT-7 cells to 5 and 10 ng/mL of rhu-SCF by a factor of 2 and 1.5, respectively. Addition of 1 ng of SCF and 1 ng of NGF together led to significant $^{3}H$-TdR incorporation, whereas each cytokine alone used at the same concentration led to background incorporation (Fig 1). Of note, we verified that rmu-SCF was as efficient as rhu-SCF in stimulating the proliferation of UT-7 cells alone or in synergy with NGF (data not shown). Neureotrophin-3 (NT-3), another member of the neurotrophin family, did not support the proliferation of UT-7 cells (data not shown).

In addition to its effect on short-term proliferation of UT-7 cells, muNGF alone could support long-term proliferation of UT-7 cells, and these cells were grown continuously in muNGF–(10 ng/mL) containing medium for more than 6 months with a doubling time of 32 hours, identical to the growth rate observed when cells were stimulated by rhu-GM-CSF.

In three experiments, the phenotype of UT-7 cells grown for a long period in NGF was compared with that of cells grown in rhu-GM-CSF or switched for 48 hours from GM-CSF- to NGF-supplemented medium (or reversed). Analysis by flow cytometry did not reveal consistent changes in the phenotype (data not shown).

As expected from the results of the functional experiments mentioned above, a band corresponding to a mRNA species of 3,200 bp expected for the TrkA receptor message was detected by Northern blot in UT-7 cells. TrkA message was detected in UT-7 cells cultured during several months in the presence of either 10 ng/mL of muNGF (Fig 2, lane B), 2 ng/mL of rhu-GM-CSF (Fig 2, lane C), and also in cells deprived of cytokines during 2 days (Fig 2, lane D) or cocultured with MS-5 stromal cells for 2 (Fig 2, lane E) or 7 days (Fig 2, lane F).

Altogether, these results demonstrate that muNGF can support clone formation and long-term amplification of UT-7 cells through the TrkA receptor, an effect previously reported exclusively with rhu-IL-3, rhu-GM-CSF, or rhu-Epo. These results, together with the known complete biological cross-reactivity of NGF between mouse and humans, and the known secretion of NGF by fibroblasts33 prompted us to investigate whether MS-5 and SI/SI4 stromal cells produced NGF.

To determine whether response to NGF was a common property of leukemic cell lines, we tested clone formation and $^{3}H$-TdR incorporation of TF-1, M-O7e, and K153 cells, all normally dependent on human cytokines for their proliferation. Neither M-O7e nor K153 cells responded to NGF, but rather grew in rhu-GM-CSF.
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 H^3{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 H^3{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.

and SI/SI^4 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/SI^4 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/SI^4, 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/SI^4 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/SI^4 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 H^3{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-
with MS-5 cells. A total of 5,000 UT-7 cells were plated in methylcellulose assays in the presence of nonimmune IgG1 (■) and IgG2a (□), anti-NGF (●), or anti-c-kit SR-1 MoAb (●). Cultures were supplemented with MS-5 cells alone, SI/SI4 cells, 10 ng/mL muNGF, or 50 ng/mL rhSCF. Each histogram represents the mean ± SEM of the percent inhibition of clone formation observed in three experiments.

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/SI4 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 H3-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/SI4 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/SI4 cells (Fig 4). These results strongly suggested that MS-5- and SI/SI4-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, but this activity was inhibited neither by an anti-c-kit nor by an anti-NGF (data not shown).

In a first series of three experiments, addition of soluble muNGF to the combination of SCF, IL-3, Epo, and G-CSF did not significantly alter the cloning efficiency of CD34+CD38− cells (Fig 5) nor did it change the size of the colonies.

Fig 4. Effect of antibodies neutralizing either murine NGF or the human c-kit receptor on clone formation by UT-7 cells cocultured with MS-5 cells. A total of 5,000 UT-7 cells were plated in methylcellulose assays in the presence of nonimmune IgG1 (■) and IgG2a (□), anti-NGF (●), or anti-c-kit SR-1 MoAb (●). Cultures were supplemented with MS-5 cells alone, SI/SI4 cells, 10 ng/mL muNGF, or 50 ng/mL rhSCF. Each histogram represents the mean ± SEM of the number of colonies counted in three to four experiments.

Fig 5. Effect of muNGF and anti-NGF antibody on colony formation by CD34+CD38− cells plated in methylcellulose assays in the presence or absence of MS-5 cells. A total of 1,000 CD34+CD38− cells were plated in methylcellulose assays in the presence of rhuSCF (50 ng/mL), rhu-IL-3 (100 U/mL), and rhu-Epo (2 U/mL) (△) and either muNGF (10-50 ng/mL) (■), MS-5 cells (20,000 cells/mL) (□), or MS-5 cells added together with an anti-NGF neutralizing MoAb (1 µg/mL) (●). Each histogram represents the mean ± SEM of the number of colonies counted in three to four experiments.
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>
</tr>
</thead>
<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>23</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, rhuSCF (50 ng/mL), rhu-IL-3 (100 U/mL), and rhu-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 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). Conversely, in the same experiments, addition of an anti-NGF antibody did not inhibit the synergistic effect of MS-5 cells on either immature BFU-E, CFU-GEMM, and CFU-GM.

A major effect of NGF is to allow survival of neurons during development, and we next investigated whether NGF would have the same function in hematopoiesis in a modified colony assay based on the delayed addition of SCF, IL-3, and Epo up to 14 days. Addition of SCF, NGF, or both during the first 2 weeks in the absence of additional human cytokines did not support progenitor cell survival, even with NGF concentrations up to 50 ng/mL. In contrast, the presence of MS-5 cells alone during the initial 2-week period allowed 30% to 50% of CD34+/CD38- clonogenic progenitors to survive and form colonies detected 14-25 days after the addition of human cytokines (n = 6) (Table 3). Addition at day 0 of anti-NGF to this assay was without effect on cell survival induced by MS-5 (Table 3).

MS-5 cells used as feeders in long-term cultures of CD34+/CD38- with no exogenously added human growth factors also allowed differentiation of CD34+/CD38- LTC-IC as assessed by the high output of clonogenic progenitors detected after 4 to 6 weeks of culture in the absence of hydrocortisone. Thus, in two experiments, LTC were initiated in 96-well plates with 35 or 40 CD34+/CD38- cells per well (10 wells per cell concentration), and clonogenic progenitors were measured 5 weeks later in each well. A mean of 142 ± 17 and 137 ± 15 progenitors was found per well in experiments 1 and 2, respectively. The weekly addition of anti-NGF in wells established in parallel did not change the progenitor output (168 ± 26 and 169 ± 19, respectively, in experiments 1 and 2). Conversely, addition of soluble NGF (50 ng/mL) to LTC established on MS-5 cells in the absence of hydrocortisone, which dramatically decreased the output of progenitors at week 5 (Table 4), did not restore a progenitor output comparable with that observed in the absence of hydrocortisone.

To detect more subtle effects of NGF that might be masked in the presence of stromal cells, we initiated short-term stroma-free cultures of CD34+/CD38- and CD34+/CD38- cells in the presence of either NGF or SCF alone or a mixture of both. As illustrated in Table 5, in the two experiments performed with CD34+/CD38- cells, there was a dramatic increase (×10) in the number of mature erythroid progenitor cells (CFU-E + mature BFU-E) recovered after 5 days in the wells incubated with SCF and NGF as compared with those with each cytokine alone. A twofold increase in nucleated cells was seen in the presence of both cytokines as compared with each alone (Table 5), and 30% of these were macrophages as determined on May-Grünwald-Giemsa stained cytospheroidal slides. No significant changes were seen in the progenitor output of wells established with CD34+/CD38- cells.

**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-IC Cultured 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>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>HC</td>
<td>10</td>
<td>2</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>1</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>Anti-NGF</td>
<td>10</td>
<td>1</td>
<td>1</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>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>Anti-NGF</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
</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 μg/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 with 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 6-10 wells.
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-IC8 and its downregulation by hydrocortisone.13 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.14 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 thymidine incorporation assay, both NGF and either murine 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 SKY4 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

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>Cytokines</th>
<th>Nucleated Cells (×1,000)</th>
<th>Progenitor Cells per Well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
</tr>
<tr>
<td>CD34+/CD38+</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>rhSCF</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>mu-NGF</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>rhuSCF + mu-NGF</td>
<td>10</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 26 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.

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.13

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 cell thymidine incorporation assay, both NGF and either murine- 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 SKY4 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, 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 SI/SI4 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.

ACKNOWLEDGMENT

We thank Amgen, Genetics Institute, and Immunex for providing cytokines and the surgeons and staff of the operating rooms who provided bone marrow samples. We are also indebted to K. Mori (MS-5 cells), N. Komatsu (UT-7 cells), D. Williams (SI/SI4 cells), and V. Broudy (SR-1 anti-c-kit; University of Washington, Seattle) for sharing their cell lines and reagents with us and to Anne Dubart for her help in the realization of Northern blot and PCR probes.

REFERENCES


14. Verfaillie CM: Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. Blood 82:2045, 1993


41. Snider WD: Functions of the neurotrophins during nervous system development: What the knockouts are teaching us. Cell 77:627, 1994


45. Ohtsuki T, Suzu S, Hatake K, Nagata N, Miura Y, Motoyoshi
K: A proteoglycan form of macrophage colony-stimulating factor that binds to bone-derived collagens and can be extracted from bone matrix. Biochem Biophys Res Commun 190:215, 1993


Nerve growth factor is involved in the supportive effect by bone marrow-derived stromal cells of the factor-dependent human cell line UT-7 [published erratum appears in Blood 1996 Oct 1;88(7):2818]

I Auffray, S Chevalier, J Froger, B Izac, W Vainchenker, H Gascan and L Coulombel