In Vitro Stimulatory Effect of Substance P on Hematopoiesis

By Pranela Rameshwar, Doina Ganea, and Pedro Gascón

The neuropeptide Substance P (SP) is widely distributed in the peripheral nervous system. Its biologic effects have been extensively studied in the immune system. However, even though the bone marrow (BM) is innervated with SP-immunoreactive fibers and some of its cells not only express SP receptors (T and B cells, endothelial cells, and macrophages) but also produce SP (macrophages, eosinophils, and endothelial cells), the effects of SP on hematopoiesis are scanty. Furthermore, SP induces the production of hematopoietic growth factors (HGFs) (interleukin-1 [IL-1], IL-6, and tumor necrosis factor α) from human monocytes. In this study, we have found a potent in vitro stimulatory effect of SP (10⁻⁸ to 10⁻¹² mol/L) on hematopoiesis for both erythroid and granulocytic progenitors in short-term methylcellulose BM cultures. SP alone, in the absence of exogeneous HGFs, is able to sustain hematopoiesis in vitro. This stimulatory effect of SP is: (1) mostly mediated by the adherent cells; (2) completely abrogated by two SP receptor (SP-R) antagonists; and (3) partially reduced by anti–IL-1, IL-3, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Furthermore, it appears that the stimulatory effect of SP may be mediated by IL-3 and GM-CSF because we have also found that SP induces the release of these two cytokines from BM mononuclear cells. Considering that the SP effect occurs at concentrations as low as 10⁻¹¹ mol/L, and via a specific receptor, it appears that SP may play a physiologic role in regulating hematopoiesis, at least partially through the adherent BM cells and the release of HGFs, and may place SP, a neuropeptide, in a new category of hematopoietic regulators.

SUBSTANCE P (SP) is a mammalian undecapeptide that belongs to a family of structurally related peptides known as tachykinins, and is widely distributed in the central nervous system and other areas of the peripheral nervous system. Some of SP’s biologic responses include motor control, contraction of smooth muscle, salivation, micturition, vasodilation, and sensory perception.

Other effects of SP relate to interactions between the peptidergic fibers with immunocompetent cells through specific SP receptors (SP-Rs) (neurokinin-Rs). Three types of neurokinin-Rs (NK-Rs) have been described: NK-1R, NK-2R, and NK-3R. Pharmacologically, the binding affinity of SP to the NK-1R is two to three orders higher than the other tachykinins (NKA or NKB).

Immunocompetent cells (macrophages, T and B cells) and cells that are similar to those that constitute the bone marrow’s (BM’s) stroma (macrophages and endothelial cells) express SP-Rs. Also, stroma-like cells are capable of producing SP: macrophages and endothelial cells. Furthermore, endothelial cells and fibroblasts have been shown to proliferate in response to SP’s stimulation.

Immunologically, SP has been reported to be involved in several inflammatory responses such as direct stimulation of lymphocytes and regulation of tissue repair via enhanced proliferation of fibroblasts and endothelial cells. SP also enhances phagocytosis in both macrophages and neutrophils, stimulates monocytes and T-cell-mediated chemotaxis, and enhances the production of Ig from B cells.

There have been a few reports in which the nervous and hematopoietic systems have been linked: (1) Neuropeptide Y, another neurotransmitter, has been found in rat megakaryocytes; (2) in vitro, SP synergizes with macrophage colony-stimulating factor (M-CSF) in murine myelopoiesis; (3) in vitro, nerve growth factor (NGF) enhances granulocyte-macrophage colony-stimulating factor (GM-CSF) effect on human basophilic cell differentiation and induces the release of inflammatory mediators from matured basophils; and (4) a cholinergic agonist enhances responsiveness of BM progenitor (CD34+ cells) to GM-CSF.

To our knowledge, there has been no report regarding an effect of SP on hematopoiesis in humans.

We decided to investigate the effect of SP on hematopoiesis based on the following: (1) BM is an innervated organ and contains SP-immunoreactive fibers (SP-Rs) through specific SP receptors (SP-Rs) antagonists; and (3) partially reduced by anti–IL-1, IL-3, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Furthermore, it appears that the stimulatory effect of SP may be mediated by IL-3 and GM-CSF because we have also found that SP induces the release of these two cytokines from BM mononuclear cells. Considering that the SP effect occurs at concentrations as low as 10⁻¹¹ mol/L, and via a specific receptor, it appears that SP may play a physiologic role in regulating hematopoiesis, at least partially through the adherent BM cells and the release of HGFs, and may place SP, a neuropeptide, in a new category of hematopoietic regulators.

MATERIALS AND METHODS

Cytokines and antibodies. Monoclonal antibodies (MoAbs) (anti-CD3, -CD4, -CD5, -CD8, and -CD14) were generously provided by Dr Steven Clark (Genetics Institute, Cambridge, MA).

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Cell preparation. BM aspirates were taken from the posterior iliac crest from healthy volunteers, after obtaining informed consent.
Aspirates were placed into preservative-free heparin and low-density BM mononuclear cells (BMMNCs) were separated by Ficoll-Hypaque density gradient (Pharcia LKB Biotechnology, Piscataway, NJ). BMMNCs were depleted of T cells by negative selection as previously described\textsuperscript{16} with antibodies to CD3, CD4, CD5, and CD8. After depletion, less than 2% of BMMNCs stained positive for CD3, as determined by fluorescence-activated cell sorting (FACS). BMMNCs were depleted of adherent cells by two-step separation (2 hours, \( x < 1 \)) to plastic surface. The resulting nonadherent cells were less than 2% nonspecific esterase positive and less than 1% expressed CD14 antigen, as determined by FACS analysis.

Mouse anti-human CD34 (Amac) was added to BMMNCs resuspended at \( 5 \times 10^6/\)mL in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum (FCS) (Hyclone, Logan, UT), then incubated at room temperature for 10 minutes, followed by 20 minutes of incubation at 4°C. Cells were washed three times with RPMI 1640 containing 2% FCS. Dynabeads M-450 sheep antinouse IgG (0.6 mg/mL) (Dynal Inc, Great Neck, NY) was added to washed cells and incubated at 4°C for 4 hours while gently shaking. Cells coupled to the Dynabeads were selected by magnetic separation, then washed (three times). FACS analysis showed that 99% of the magnetically separated cells were positive for CD34, whereas 20% were positive for CD33. This was determined with mouse anti-human CD34 (IgG; Becton Dickinson, San Jose, CA) and phycoerythrin-conjugated secondary rat antinouse IgG, (Becton Dickinson). CD33 was determined with fluorescein-conjugated mouse anti-human CD33 (IgG\textsubscript{2}; Coulter Immunology, Hialeah, FL). Double-labeling studies with anti-CD33 and -CD34 showed that 22% of CD34 cells coexpressed CD33. Control antibodies include mouse IgG\textsubscript{2} and IgG\textsubscript{2}. Before FACS analysis, cells were incubated overnight at 37°C for detachment of magnetic beads.

Hematopoietic progenitor cultures. Total, T-cell-depleted, or adherent-cell-depleted BMMNCs (\( 1 \times 10^6/\)mL) were plated in duplicate for erythroid and myeloid progenitor cells in methylcellulose as previously described.\textsuperscript{19} Cells were cultured in the presence of various concentrations of SP (Peninsula Laboratories, Belmont, CA) and/or various concentrations of two SP-R antagonists, spantide (Peninsula Laboratories) or CP-96,345-1 (gift from Dr Michael Snider, Pfizer Inc, Groton, CT). Granulocyte-macrophage colonies (CFU-GM) were either supplemented with suboptimal (2.6 U/mL) or optimal (28 U/mL) concentration of rhGM-CSF, or cultured in the absence of exogenous rhGM-CSF. Early (BFU-E) and late erythroid (CFU-E) colonies were either supplemented with 2 U of hIL-3 and 2 U of rhEpo, or supplemented only with 2 U of rhEpo. Total BMMNCs were also cultured with SP (\( 10^{-10} \) mol/L) and either of the following neutralizing polyclonal antibodies or in combination: anti-hIL-3, anti-hGM-CSF, anti-hIL-1\textalpha, anti-hIL-1\beta, or anti-hIL-6. Neutralizing concentrations of each antibody were determined by adding various concentrations of antibody to the BM culture in the presence of the optimal SP concentration (\( 10^{-10} \) mol/L). Point of immune complex equivalence was determined to be the lowest point at which the stimulatory effect of SP was reversed. The following are the neutralizing amounts of each antibody used in each culture: anti-hIL-1\textalpha (2 \( \mu \)g/ml), anti-hIL-1\beta (2 \( \mu \)g/ml), anti-hIL-3 (0.5 \( \mu \)g/ml), anti-hIL-6 (2 \( \mu \)g/ml), and anti-hGM-CSF (2 \( \mu \)g/ml). For all cultures, controls included cells cultured without SP.

Biologic assay for IL-3 and GM-CSF. BMMNCs (\( 1 \times 10^6/\)mL) were cultured for 7 days in the presence of various concentrations of SP in RPMI 1640 containing 10% FCS. Supernatants (SNs) were clarified of cells and debris by centrifugation (4,000g for 5 minutes), and then assayed for the presence of biologically active IL-3 and GM-CSF in the SNs. This was determined based on the ability of the SNs to support the growth of the IL-3/GM-CSF-dependent cell line, M-07e.\textsuperscript{18} This clone was provided by Wendy C. Lum (Department of Biochemistry, St Jude Children’s Research Hospital, Memphis, TN). SNs (0.1 mL) in triplicate were serially diluted in medium containing 5% FCS (assay medium) in microtiter wells (Falcon 3072; Becton Dickinson Labware, Lincoln Park, NJ). For each assay, a standard curve was established using serial dilutions of rhIL-3 (50 ng/mL) or rhGM-CSF (1 ng/mL). M-07e cells (2 \( \times 10^6/\)mL) resuspended in 0.1 mL assay medium, were added to each well. After 48 hours, wells were pulsed for 6 hours with 1 \( \mu \)Ci tritiated thymidine ([\( ^3\text{H} \)]Tdr, 2 Ci/mmol; New England Nuclear, Boston, MA). Cells were harvested onto glass fiber filter with a PHD cell harvester (Cambridge Technology, Watertown, MA) and [\( ^3\text{H} \)]Tdr incorporation was determined by liquid scintillation counting. One proliferative cytokine unit was equivalent to one-half maximal growth of the cells as determined by [\( ^3\text{H} \)]Tdr incorporation in the standard curve.

Western blots for IL-3 and GM-CSF. Western blots were performed as previously described.\textsuperscript{19} Protease inhibitors: phenylmethylsulfonyl fluoride (1 mmol/L), leupeptin (5 mmol/L), chymostatin (1 \( \mu \)mol/mL), and (3-[3-Cholamidopropyl]-dimethylammonio)-2-hydroxy-1-propanesulfonate (0.1 mmol/mL) (all protease inhibitors purchased from Sigma) were added to cell-free SNs obtained from 7-day SP-stimulated BMMNCs. SNs were then concentrated 10\( ^X \) with a Centricron 10 microconcentrator (Amicon, Beverly, MA). Proteins were electrophoretically separated on two different 16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In one gel, a lane was loaded with standard hIL-3 (10 ng), whereas the standard lane of the other gel was loaded with hGM-CSF (10 ng). Separated proteins were transblotted to Immobilon PVDF transfer membrane (Millipore, Bedford, MA) for 45 minutes at a constant current of 400 mA and 50 V. Protein-bound membranes were blocked with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) (United States Biochemical, Cleveland, OH) and then incubated overnight at room temperature with either rabbit anti-hIL-3 (5 \( \mu \)g) or goat anti-hGM-CSF (5 \( \mu \)g). Excess antibodies were washed three times (15 minutes each) with 2% BSA in PBS. Immune-complexed membranes were incubated for 1 hour with the appropriate secondary antibodies conjugated with alkaline phosphatase. Color was developed with BCIP/NBT phosphatase substrate system (Kirkgaard & Perry Laboratories Inc, Gaithersburg, MD). For all gels, the molecular weights (MW) of the developed bands were compared with prestained low MW standards (Diversified Biotech, Newton Center, MA).

Statistical analysis. The statistical significance was determined using: (1) one-sample \( t \)-test and (2) one-way repeated measures, analysis of variance (ANOVA) followed by Tukey’s multiple comparison at \( P < .0001 \). All results are given as a two-tailed \( P \) value. In the final statistical analysis, 20% was subtracted from each experimental point because of the variation in hematopoietic BM cultures.

RESULTS

Effect of SP on myeloid and erythroid colonies in the presence or absence of exogenous growth factors. The potential role of SP on hematopoiesis was investigated by culturing normal BMMNCs with various concentrations of SP (\( 10^{-6} \) to \( 10^{-13} \) mol/L) in methylcellulose supplemented with suboptimal concentrations of the appropriate growth factors (suboptimal GM-CSF for CFU-GM or IL-3 and Epo for BFU-E and CFU-E). The numbers of CFU-GM, BFU-E, and CFU-E colonies were markedly increased in cultures containing SP (\( 10^{-8} \) to \( 10^{-12} \) mol/L). For BFU-E and CFU-E, a statistical significance was found at SP concentrations from \( 10^{-8} \) to \( 10^{-11} \) mol/L at \( P < .01 \) and \( P < .001 \), respectively. For CFU-GM, a statistical significance was found from \( 10^{-9} \) to \( 10^{-11} \) mol/L SP at \( P < .02 \) (Fig 1). SP did not affect the numbers of CFU-GM obtained with the optimal concentration of GM-CSF (data not shown).
Effect of Substance P on Hematopoiesis

To determine the significance of SP in this stimulatory effect, a similar set of experiments was performed in which SP (10^{-9} to 10^{-11} mol/L) was added to the cultures as the only source of exogenous factor. In these experiments, SP alone was a potent stimulator of myeloid progenitors (Table 1), substituting for exogenous GM-CSF in the production of myeloid colonies. However, SP by itself had no effect on erythroid progenitors (data not shown) unless Epo was present. In the presence of Epo and SP, a potent stimulation for erythroid colonies was observed (Table 1), thus substituting for exogenous IL-3. Statistical analysis comparing the hematopoietic effect of cultures with and without SP were found to be significant at \( P < .001 \) for CFU-E at 10^{-9} mol/L SP and at \( P < .0001 \) for all other cultures (Table 1).

Effects of SP on myeloid and erythroid progenitors in the presence of two SP-R antagonists. It has been reported that SP binds with high affinity to the putative NK-1R and, to a lesser extent, to the NK-2 and NK-3Rs. Therefore, we tried to block the stimulatory effect of SP by culturing BMMNCs in the presence of SP and two different SP-R antagonists, SP, at two optimal concentrations (Fig 1), 10^{-9} mol/L and 10^{-10} mol/L, was cultured with various concentrations of [D-Arg^1, D-Trp^2, Leu^3]SP (spantide), a pan-neurokinin receptor antagonist, or CP-96,345-1, a nonprotein antagonist that specifically binds to the NK-1 receptor. The stimulatory effect of SP, when cultured in the presence of various concentrations of either spantide or CP-96,345-1, was reduced to control levels in a dose-dependent manner in cultures of all progenitor colonies (Fig 2). The SP-R antagonists by themselves showed no effect in cultures (Fig 2).

Role of the BM’s adherent cells and T cells in SP’s stimulation of erythroid and myeloid colonies. In this study, SP was able to replace the in vitro requirement for exogenous HGFs in hematopoiesis (Table 1). Because the BM’s adherent cells and T cells are rich sources of HGFs, the next set of experiments were designed to determine whether this stim-
ultulatory effect was mediated through these two populations of cells. BMMNCs were depleted of adherent cells, then cultured with the two optimal SP concentrations (10^{-10} to 10^{-9} mol/L) (Fig 1) in the presence or absence of exogenous growth factors. In both cases, SP-supplemented cultures showed less stimulation of BFU-E, CFU-E, and CFU-GM than controls (Table 1 and Fig 1). Even though the stimulatory effect was less, SP alone was still able to sustain hematopoiesis from BMMNCs depleted of adherent cells by partially replacing exogenous growth factors (Table 2).

T-cell-depleted BMMNCs, cultured in the presence of SP (10^{-9} to 10^{-10} mol/L), produced no significant changes in the percent of BFU-E and CFU-GM compared with total BMMNCs except for BFU-E, where, at the optimal SP concentration (10^{-10} mol/L), a 34% decrease was observed compared with total BMMNCs (Fig 3).

To further determine the role of accessory cells, CD34-positive cells were cultured in the presence of SP (10^{-10} to 10^{-9} mol/L) for erythroid and myeloid colonies in three experiments. For CFU-E and CFU-GM, at both SP concentrations, there was a 25% decrease in the number of colonies, whereas for BFU-E there was a 28% increase at 10^{-10} mol/L SP.

Stimulatory effect of SP on hematopoiesis via growth factors. In this study we have found that SP is able to substitute for exogenous growth factors necessary for in vitro BM cultures (Table 1) and, because SP is known to induce the release of various HGFs from immune cells, we next cultured BMMNCs in the presence of SP (10^{-10} mol/L) and various antibodies to known HGFs (Fig 4). Antibodies to IL-1 and IL-6 were added because it has been reported that SP induces the release of IL-1 and IL-6 from human monocytes, cells known to constitute the BM's stroma. Antibodies to IL-3 and GM-CSF were added because SP is capable of replacing these two growth factors in our BM cultures. Furthermore, in parallel studies, we have found that SP induces the release of IL-3 and GM-CSF from BMMNCs in liquid cultures (Fig 5).

Antibodies to either IL-1 or IL-6 had no effect on SP's enhancement of erythroid or myeloid colonies. However, in the presence of anti-IL-3 or anti-GM-CSF, the enhancing effects of SP on the numbers of BFU-Es and CFU-Es were reduced by 30% and 34%, respectively. For CFU-GM cultures, anti-IL-3 and anti-GM-CSF blocked the stimulatory effects of SP by 64% and 67%, respectively. A combination of all four antibodies decreased SP's stimulation of CFU-GM by 83% (Fig 4). The decreased colony formation was not caused by nonspecific suppression by IgG because equivalent concentrations of murine nonimmune IgG did not suppress colony formation (number of colonies equivalent to cell control, data not shown).

Release of IL-3 and GM-CSF from BMMNCs. We studied the release of IL-3 and GM-CSF from BMMNCs based on our findings: (1) SP is capable of replacing these two HGFs in BM cultures (Table 1) and (2) neutralizing anti-IL-3 and GM-CSF reduce the number of colonies in cultures supplemented with SP alone (Fig 4). The release of these two growth factors from SNs obtained from SP-stimulated (10^{-6} to 10^{-13} mol/L) BMMNCs was determined in a proliferative assay with the IL-3/GM-CSF--dependent M-07e cell line. The proliferation of the M-07e cells increased 2- to 75-fold above cell background with the optimal SP concentration at 10^{-11} mol/L (Fig 5).

The amounts of either IL-3 or GM-CSF present in the SNs were determined by culturing the M-07e cells in the presence

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<th>Table 2. Hematopoietic Progenitors Cultured From BMMNCs Depleted of Adherent Cells</th>
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<td>SP (mol/L)</td>
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<tr>
<td>CFU-GM (n = 3)</td>
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<td>BFU-E (n = 3)</td>
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BMMNCs were depleted of adherent cells and cultured in the presence of two optimal concentrations of SP without exogenous growth factors as described in Materials and Methods (hIL-3 and hGM-CSF). Epo (2 U) was added to erythroid cultures. Results are expressed as X ± SD. Controls represent total BMMNCs cultured with exogenous growth factors: rhEpo and hIL-3 for BFU-E and CFU-E, and rhGM-CSF for CFU-GM.
of various neutralizing anti–GM-CSF and/or anti–IL-3. In the presence of anti–IL-3, M-07e cells proliferated more than in the presence of anti–GM-CSF (Fig 5). In the presence of both antibodies, the proliferation of the M-07e cells was reduced to background levels.

The M-07e cells have been previously shown to respond minimally to c-kit ligand and to synergize with IL-3 and GM-CSF to enhance the proliferation of the M-07e cells. In addition to the ability of anti–IL-3 and GM-CSF to block the proliferation of the M-07e, the presence of these two cytokines in the SNs was immunologically confirmed with Western blots. Bands were observed for both IL-3 (Fig 6A) and GM-CSF (Fig 6B) from SNs that had high biologic activity in the M-07e proliferation assay (Fig 6, lane 4), compared with the control SNs that showed low activity (Fig 6, lane 3).

DISCUSSION

We have found a significant SP-mediated enhancement of both erythroid and myeloid colonies by cultures supplemented with exogenous growth factors (Fig 1). However, at high SP concentrations, there was a reduction of the stimulatory effects of SP (Fig 1). This reduction of colony formation may be caused by desensitization of the G-protein that has been shown to be coupled to the three NK-Rs. This is plausible because the NK-Rs have been previously shown to be desensitized after repeated application of their respective ligand. The amino and carboxyl terminal of the three NK-Rs show a pattern similar to the other members of the G protein-coupled receptors. The amino terminal possess potential N-glycosylation sites whereas the carboxyl terminal possess serine and threonine residues as possible phosphorylation sites that may be responsible for the desensitization process.

In addition, the increase in colony formation was also observed even in the absence of exogenous GM-CSF (CFU-GM) or IL-3 (BFU-E and CFU-E) (Table 1). However, SP did not substitute for Epo in erythroid cultures (authors' personal observation, June 1992).

The hematopoietic effects were mediated by an NK-R based on the abrogation of these effects to baseline levels by two NK-R antagonists. First, by a pan-neurokinin receptor antagonist, pan tide, and second by an NK-1R-specific antagonist, CP-96,345. These findings indicate that the hematopoietic effect of SP is mainly mediated by the high affinity SP-R (NK-1R) (Fig 2).

The heterogeneity of the BM, in terms of the vast amounts of different cell types provides a limitation in the elucidation of the cells responsible for the SP effect. In this study, we separated the BM cells into the adherent-depleted and T-cell-depleted populations. We have observed a marked reduction in the ability of SP to stimulate colony formation in adherent-depleted BMMNCs cultured without exogenous growth factors (Table 2), thus implicating the adherent cells as a main mediator in the stimulatory effect of SP. T-cell-depleted cultures did not significantly change the stimulatory effects on the late erythroid colonies (CFU-E) and on CFU-GM. In contrast, the stimulatory effect on BFU-E was reduced (Fig 3), implicating that the indirect effect on T cells may be on the more primitive BM progenitors. In addition, based on (1) the ability of SP to sustain in vitro hematopoiesis without the addition of exogenous HGFs and (2) the reduced stimulatory effect of SP in the presence of neutralizing antibodies to known HGFs (Fig 4), it appears that SP exerts its effects on hematopoiesis mainly via the release of HGFs.
Fig 4. BMMNCs (1 x 10^3) cultured with SP (10^-10 mol/L) and various neutralizing antibodies: murine anti-hGM-CSF, rabbit anti-IL-3, anti-hIL-1α and β, and goat anti-hIL-6. Neutralizing concentrations of each antibody were determined as described in Materials and Methods. BMMNCs were cultured as described in Materials and Methods. Controls included the same concentration of cells cultured with medium alone. Results are expressed as mean (±SD) of three different experiments.

A neural source of SP within the BM’s cavity is possible because the BM is a richly innervated organ. The peptidergic innervation (immunoreactive pan-tachykinin) of the BM, a primary lymphoid organ, has been shown in vascular and nonvascular locations of the BM of rats and guinea pigs. Potential targets for SP within the BM include SP-R expressing accessory cells found within the marrow’s microenvironment (T and B cells, mast cells, and macrophages).

Non-neural sources of SP include eosinophils and cells similar to those that constitute the BM’s stroma: endothelial cells and macrophages. Because stromal cells produce most of the humoral (HGFs) components of the marrow’s microenvironment that provides the right milieu for sustained hematopoiesis, it is also possible that the effects of SP may be indirect via stromal cells such as fibroblasts, a rich source of HGFs. SP has been shown to stimulate the proliferation of fibroblasts and it shares amino acid homology with the brain-derived acid fibroblast growth factor. The stromal macrophage may also be involved because it has been found that SP acts as a costimulant with M-CSF to induce clonal proliferation of murine marrow cells and in our studies removal of adherent cells resulted in marked reduction in the number of progenitor colonies (Fig 3).

The remaining stimulatory activity after BM depletion of either adherent or T-cell depletion may be explained by the effect of SP on residual stroma-forming cells such as fibroblast and endothelial cells and, to a much lesser extent, nonadherent cells other than T cells. This is a plausible idea considering that fibroblasts, macrophages, endothelial cells, and T cells are capable of producing different HGFs such as IL-2, IL-3, and GM-CSF by the action of SP (Fig 5). Possible involvement by T cells and other accessory cells has also been suggested by reduced colony formation with neutralizing antibodies to IL-3 and GM-CSF (Fig 4), both capable of being produced by T cells. However, our results show that the main in vitro effect of SP on hematopoiesis is via adherent cells and not via T cells because removal of the latter had little impact on the total number of colonies. Furthermore, cultures of CD34-positive cells in the presence of SP resulted in a reduction of the stimulatory effect on BFU-E and appear to suppress colony formation for CFU-E and CFU-GM compared to the stimulatory effect observed with total BMMNCs (Fig 3). The suppression observed may be explained by SP’s additional ability to function as a differentiation factor. This is possible because it had been reported that in the absence of accessory cells, SP acts as a differentiation cofactor for B cells. It is also possible that an SP-induced negative factor may predominate in the absence of accessory cells.

Release of HGFs is essential in providing signals for stem cells and blood cell progenitors to proliferate and differentiate. The effect of SP on hematopoietic progenitors appears to be mediated by a specific receptor and it is observed at concentrations as low as 10^-12 mol/L, suggesting a potential physiologic role for SP on the regulation of hematopoiesis. These findings not only suggest a place for SP, a neuropeptide, in a new category of hematopoietic regulators, but it may well implicate a regulatory effect of the nervous system, via neuropeptides, on blood formation.

Fig 5. BMMNCs (1 x 10^3/ml) were cultured with SP (10^-10 to 10^-13 mol/L) for 7 days (inset). SNs were assayed in the presence or absence (inset) of neutralizing anti-IL-3 (II), anti-GM-CSF (III), or anti-IL-3 and anti-GM-CSF (III) in a proliferative assay with the M-07e cell line as described in Materials and Methods.
Fig 6. Western blot analysis of SNs from SP-stimulated BMMNCs. Concentrated SNs were separated on 16% SDS-PAGE (see Materials and Methods). Transblotted membranes were incubated with either anti-IL-3 (A) or anti-GM-CSF (B). Lane 1, 5 μL prestained low MW standard; lane 2, standard IL-3 (A) or standard GM-CSF (B); lane 3, 10^{-9} mol/L SP; lane 4, 10^{-11} mol/L SP.

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