PAPER

Effect of Hypoxia on the Hematopoietic and Immune Modulator Preprotachykinin-I

Dennis P. Quinlan, Jr, MD; Pranela Rameshwar, PhD; Jing Qian; Paul B. Maloof; Alicia M. Mohr, MD; Carl J. Hauser, MD; David H. Livingston, MD

Objectives: To determine the effect of hypoxia on bone marrow mononuclear cells (BMMCs) and their ability to proliferate into granulocyte-macrophage colony-forming units (CFU-GMs) and erythroid burst-forming units (BFU-Es) and to determine the role of the neuroimmune and hematopoietic mediator, substance P.

Design: Controlled in vitro study.

Setting: University research laboratory.

Materials: Bone marrow aspirates were obtained from the posterior iliac crests of healthy volunteers after obtaining informed consent.

Interventions: The BMMCs were divided into the following groups: (1) normoxia, (2) two hours of hypoxia, and (3) six hours of hypoxia. Additional BMMCs were purified before the period of hypoxia, while others were incubated with neurokinin (NK) receptor antagonists. In other experiments, bone marrow stroma was grown to confluence and randomized to the following groups: (1) normoxia, (2) hypoxia, (3) normoxia and interleukin (IL) 1, and (4) hypoxia and IL-1. All groups were cultured for 2, 6, 12, or 24 hours.

Main Outcome Measures: The formation of CFU-GMs and BFU-Es was measured after 10 to 14 days of incubation of the BMMCs. The messenger RNA of the preprotachykinin-I (*PPT-I*) gene and the *NK-1* and *NK-2* receptors was detected by using semiquantitative reverse transcriptase–polymerase chain reaction or Northern blot analysis on bone marrow stroma. The immunoreactivity of substance P in bone marrow stroma was measured by competitive enzyme-linked immunosorbent assay.

Results: Hypoxia resulted in a 110% increase in the number of CFU-GMs and a 78% increase in the number of BFU-E colonies at 6 hours (both $P<.05$). Elimination of the stromal elements by purification abrogated the increase in colony formation to nonhypoxic levels. Hypoxia induced *PPT-I* gene expression at 24 hours; however, no *PPT-I* expression was found in the hypoxic group incubated with IL-1. The receptor, *NK-1*, was found to be equal in both hypoxic groups; *NK-2* was found to have a 4-fold increase in the hypoxia and IL-1 group over the hypoxia alone group and normoxia and IL-1 group. The levels of substance P immunoreactivity were found to be similar in all groups. Incubation of BMMCs with NK receptor antagonists to *NK-1* alone or *NK-1* and *NK-2* decreased the number of CFU-GM and BFU-E colonies similar to the level in controls.

Conclusions: These results indicate that hypoxia has a role in the proliferation and control of CFU-GMs and BFU-Es. This control seems to be mediated through the bone marrow stroma and modulated by NK receptors and induction of *PPT-I*. The neuropeptide, substance P, probably has a role but is clearly not the only mediator involved.

Arch Surg. 1998;133:1328-1334

From the Department of Surgery, Division of Trauma (Drs Quinlan, Mohr, Hauser, and Livingston), and the Department of Medicine, Division of Hematology (Dr Rameshwar and Messrs Qian and Maloof), University of Medicine and Dentistry of New Jersey–New Jersey Medical School, Newark.

THE IMMUNE and hematopoietic systems communicate with the central nervous system through a series of complex bidirectional communications mediated by soluble factors. These include neuropeptides, neurotransmitters, and cytokines that are produced by cells in each system.[1-3] The bone marrow, thymus, and secondary lymphoid organs are innervated, allowing direct anatomical communication between the nervous system and the immune and hematopoietic systems.[4,5] The neurotransmitter, substance P, is a mediator of the neuroimmune/hematopoietic axis, and experimental evidence indicates that substance P is not only released from sensory nerve fibers, but also is produced locally by the cells in the target lymphoid tissue.[6,7] Substance P receptors have been demonstrated on immune, hematopoietic, and stromal bone marrow cells.[2]

Severe injury, such as burns, shock, and abdominal trauma, has been shown to cause bone marrow failure and is thought to be one of the facets of altered immune function after severe trauma.[8-11] Gamelli et al[12] showed a reduction in the number of bone marrow granulocyte-macrophage pro-

MATERIALS AND METHODS

REAGENTS AND CYTOKINES

Substance P and diethyl pyrocarbonate were purchased from Sigma Chemical Co, St Louis, Mo. CP-96,345-1, an *NK-1* receptor antagonist, was provided by Pfizer Inc, Groton, Conn. SR 48968, an *NK-2* receptor antagonist, was provided by Sanofi Recherche, Montpellier Cedex, France.

Hoffman-La Roche, Nutley, NJ, provided recombinant human IL-1α. Recombinant human GM-CSF, recombinant human erythropoietin, and human IL-3 were provided by the immunology department of the Genetics Institute, Cambridge, Mass.

BONE MARROW MONONUCLEAR CELL PREPARATION

Bone marrow aspirates were obtained from the posterior iliac crests of healthy volunteers, after obtaining informed consent. Aspirates were placed into preservative-free heparin, and low-density bone marrow mononuclear cells (BMMCs) were separated by using a density gradient (Ficoll-Hypaque; Pharmacia LKB Biotechnology, Piscataway, NJ).

Stroma-depleted BMMCs were prepared by passing cells through a column of nylon wool as described previously.[19] Briefly, BMMCs were incubated in nylon wool columns for 1 hour at 37°C in a 5% carbon dioxide incubator. Cells were retrieved in RPMI 1640 with a 10% solution of fetal calf serum (FCS).

PREPARATION OF BONE MARROW STROMA

The bone marrow cells, 10^7, were resuspended into stromal medium and placed into 25-cm^2 tissue culture flasks. The stromal medium consisted of the following: α-minimal essential medium (α-MEM, Life Technologies, Grand Island, NY); a 12.5% solution of FCS (Hyclone Laboratories, Logan, Utah); a 12.5% solution of horse serum (Hyclone Laboratories); a 10^{-7}-mol/L concentration of hydrocortisone (Sigma Chemical Co); a 10^{-4}-mol/L concentration of 2-mercaptoethanol (Sigma Chemical Co), and a 1.6-mmol/L concentration of glutamine (Cellgro; Mediatech, Herndon, Va). Cultures were incubated at 33°C. At day 3, nonadherent mononuclear cells were separated by using Ficoll-Hypaque (Pharmacia) density gradient and replaced into the flasks. Cultures were reincubated with weekly replacement of 50% stromal medium until confluence.

HYPOXIA AND STROMAL STIMULATION

Nonadherent cells were removed from confluent bone marrow stroma. Media were replenished with 3 mL of serum-free α-minimal essential medium, supplemented with insulin-transferrin-selenium-A (Life Technologies). All stromal cultures were then studied for *PPT-I, NK-1,* and *NK-2* induction under the following conditions: group 1, normoxic stroma alone, the control group; group 2, hypoxic stroma alone; group 3, normoxic stroma, stimulated with 25-ng/mL IL-1α; and group 4, hypoxic stroma, stimulated with 25-ng/mL IL-1α. The IL-1α was chosen because it is a known inducer of *PPT-I* and *NK-1*.[15]

The stromal cultures were subjected to hypoxia by placing flasks of confluent stromal cultures or BMMCs in a modular incubator that was flushed with a mixture of 5% carbon dioxide and 95% nitrogen (PaO_2, 30-43 mm Hg) for 5 minutes[13] and sealed. Chambers were then transferred to a 37°C incubator and incubated for 24 hours. Following hypoxia, the stromal cultures were further incubated for 6 hours at 37°C in a mixture of 5% carbon dioxide and 95% oxygen. After this period of incubation, RNA was extracted for molecular analyses.

The BMMCs were placed in the hypoxia chamber while in RPMI 1640 with a 10% solution of FCS and incubated for 2 or 6 hours. Once incubated, they were immediately transferred to colony assays.

HEMATOPOIETIC PROGENITOR CULTURES

The BMMCs were separated by using the Ficoll-Hypaque density gradient from bone marrow aspirates. The BMMCs were then subjected to 24-hour hypoxia as described for the bone marrow stroma. Following exposure to hypoxia, more than 95% of the BMMCs were viable as noted by using a trypan blue exclusion procedure. The cells were washed once and immediately plated for hematopoietic progenitors, erythroid burst-forming units (BFU-Es), and granulocyte-macrophage colony-forming units (CFU-GMs).

All BMMCs (5×10^4) were plated in duplicate for BFU-E and CFU-GM cultures in a methylcellulose matrix. The cultures contained a 30% solution of FCS, a 2% solution of bovine serum albumin, Iscoves, 2-mercaptoethanol (2×10^{-4}), and glutamine. The BFU-E cultures were supplemented with 3 U/mL of erythropoietin and 3 U/mL of recombinant human IL-3. The CFU-GM cultures were supplemented with 3 U/mL of recombinant human GM-CSF. Plates were incubated at 37°C in 5% carbon dioxide for 10 days (CFU-GM) or 15 days (BFU-E).

Continued on next page

genitor cells in animals with a burn infection. Mohr et al[13] demonstrated that hypoxia can depress the production of stimulatory myelopoietic cytokines.

Substance P is a member of the neurokinin family that includes neurokinin (NK)–A and NK-B. Substance P is derived from the preprotachykinin-I (*PPT-I*) gene, which contains 7 exons that can be spliced into 4 transcripts, each of which can encode substance P. Neurokinins can bind to 1 of 3 cloned receptors, *NK-1*, *NK-2,* or *NK-3*. Substance P preferentially binds to *NK-1*.

Substance P has been shown to be a positive hematopoietic modulator. Rameshwar et al[14] showed that in vitro, substance P at physiologic concentrations (10^{-9} to 10^{-11} mol/L) exhibited a stimulatory effect on granulocytic-monocytic and erythroid progenitors. These enhancing effects did not require exogenous growth factors (granulocyte-macrophage colony-stimulating factor [GM-CSF] for granulocytic-monocytic or interleukin [IL] 3 for erythroid), suggesting that substance P could be inducing relevant hematopoietic cytokines. Indeed, subsequent studies indicated that incubation with substance P resulted in the production of direct- and indirect-acting hematopoietic cytokines, IL-1, IL-3, IL-6, GM-CSF, and *c-kit* ligand.[15]

The results of studies of the effects of hypoxia on *PPT-I* expression have been conflicting and limited to

EXTRACTION AND MEASUREMENT OF SUBSTANCE P IMMUNOREACTIVITY

The stromal cultures were subjected to hypoxia with or without IL-1α stimulation for steady state mRNA analyses described previously. Time-course experiments in our laboratory have determined 72-hour posthypoxia incubation as the optimum time for the induction of substance P immunoreactivity. Consistent with other studies performed in our laboratory by Rameshwar et al,[20] we have observed that released substance P immunoreactivity is cell-associated. We therefore extracted cell-associated proteins by sonicating the stromal cells in a 0.01-mol/L concentration of acetic acid. Samples were centrifuged for 15 minutes at 10 000*g*, and cell-free extracts were stored at −70°C until ready to be assayed.

Competitive enzyme-linked immunosorbent assay determined the substance P immunoreactivity levels as described.[15] Samples were assayed in triplicate as undiluted and in 3 serial dilutions. Bound anti–substance P was detected by incubating for 1 hour with optimum alkaline phosphatase–conjugated goat antirabbit IgG, 150 ng/mL (Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md). The color was developed with a phosphatase substrate (Sigma 104, Sigma Chemical Co), diluted in a buffer (a 0.5-mmol/L concentration of diethonalamine, a 0.5-mmol/L concentration of magnesium chloride, and a 0.02% solution of sodium azide). A standard curve was developed with absorbance at 405 nm vs serial dilutions of standard substance P, assayed in duplicate and ranging from 100 to 0.08 ng/mL. The optimal concentrations of each reagent were predetermined by using an enzyme-linked immunosorbent assay.

SEMIQUANTITATIVE REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION FOR *PPT-I* AND *NK-1*

Total RNA was extracted from bone marrow stroma. Total RNA, 1 µg, was reverse transcribed in a total volume of 25 µL for 1 hour at 42°C. The reaction was terminated by using a temperature of 94°C for 5 minutes.

The polymerase chain reaction (PCR) for *PPT-I* and *NK-1* was performed in 50 µL of reaction mixture with 4 ng and 40 ng, respectively, of complementary DNA (cDNA) obtained from stroma. The PCR mixture contained the following concentrations: 20 mmol/L of tromethamine (pH 8.4), 10 mmol/L of potassium chloride, 2 mmol/L of magnesium chloride, 0.8 mmol/L of deoxynucleotide triphosphate, 0.4 µmol/L of each primer, and 2.5 U *Taq* DNA Polymerase (Life Technologies). Samples were overlaid with oil and amplified in the linear ranges for *PPT-I, NK-1,* and *NK-2* (35, 25, and 35 cycles, respectively) and glyceraldehyde 3-phosphate dehydrogenase (25 cycles) by using a DNA thermal cycler (DNA Thermal Cycler 480, Perkin Elmer-Cetus, Norwalk, Conn). The cycling profile for each was 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Each sample was subjected to a final extension at 72°C for 7 minutes. The PCR products (15 µL) were resolved on 1.5% agarose gel containing ethidium bromide, and the bands were compared with a 1-kilobase (kb) DNA ladder, λ DNA/*Hin*d III fragments, or a low DNA mass ladder. All DNA standards were purchased from Life Technologies.

NORTHERN BLOT ANALYSIS

The bone marrow stroma was stimulated with IL-1 (25 ng/mL), hypoxia, or both for 2, 6, 12, or 24 hours. At 6 hours thereafter, all RNA was extracted, and 10 µg was electrophoretically separated on 1.2% agarose. The RNA standards (0.24-9.5 kb and 0.16-1.77 kb) were used for size comparison. The RNA was transferred to nylon membranes and mobilized by UV cross-linking and baked for 1 hour at 80°C. Membranes were hybridized with deoxyadenosine triphosphate labeled with phosphorus 32 (α-^{32}P) human β-*PPT-I* cDNA. The α-^{32}P was in a concentration of 3000 Ci/mmol and was obtained from Dupont/New England Nuclear, Boston, Mass. The probe was labeled with a random primer kit (Prime-IT II; Stratagene, La Jolla, Calif). Membranes were exposed to storage phosphor screen (Molecular Dynamics, Sunnyvale, Calif) for 24 hours and then scanned on a phosphoimager (Phosphor Imager, Molecular Dynamics). A second rehybridization with a deoxyadenosine triphosphate, α-^{32}P–labeled 28S ribosomal (rRNA) cDNA probe was performed with the same membrane. Before rehybridization, membranes were stripped by boiling for 5 minutes. Ethidium bromide visualization of rRNA and/or hybridization with the 28S rRNA cDNA probe was used as a loading control and for normalization. The total areas of hybridization were determined by using densitometric software (ImageQuant; Molecular Dynamics).

STATISTICAL ANALYSIS

All data are expressed as mean ± SD. Statistical analysis was performed using analysis of variance and the Tukey-Kramer multiple comparisons test. A *P* value of less than .05 was considered significant.

nontrauma models. Ringstedt et al[16] showed no increase in the levels of *PPT-A* messenger RNA (mRNA) in the rat brain following hypoxia that occurred immediately after birth. Johnson et al[17] reported a severalfold decrease in striatal substance P–like immunoreactivity in rat brains subjected to hypoxia and ischemia. Substance P immunoreactivity has been found to be increased in the carotid bodies of rats after long-term hypoxia.[18] With the recognition of the neuroimmune/hematopoietic axis and the fact that the bone marrow is the primary lymphoid organ in adults, we studied the effects of hypoxia on hematopoiesis and one of its major mediators, substance P.

RESULTS

EFFECTS OF HYPOXIA ON BONE MARROW PROGENITORS

At 2 hours of hypoxia, there was no significant change in colony formation (**Figure 1**). However, after 6 hours, the results showed a significant increase in CFU-GMs and BFU-Es ($P<.05$), (Figure 1). We also observed that 6 hours of hypoxia resulted in a greater percentage of colonies with more than 30 cells (**Figure 2**). An increase in fibroblast cell–like proliferation also was noted at 2 and 6 hours of hypoxia com-

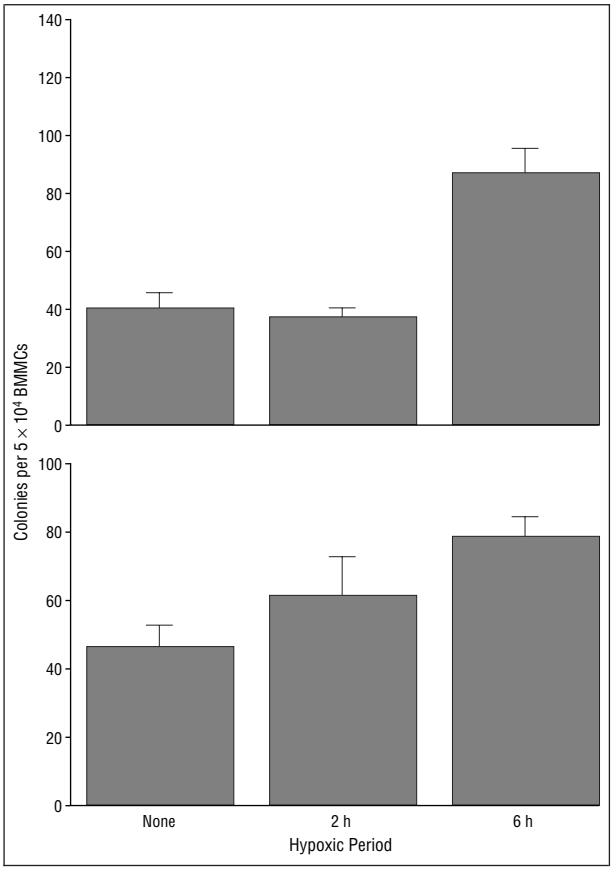

Figure 1. *Effects of hypoxia on bone marrow progenitor colonies. Bone marrow mononuclear cells (BMMCs) were exposed to hypoxia for 2 or 6 hours. Colonies were enumerated at day 10 and day 15 for granulocyte-macrophage colony-forming units (top) and erythroid burst-forming units (bottom), respectively. The results are expressed as the mean of 6 experiments (see the "Materials and Methods" section); error bars indicate the SD.*

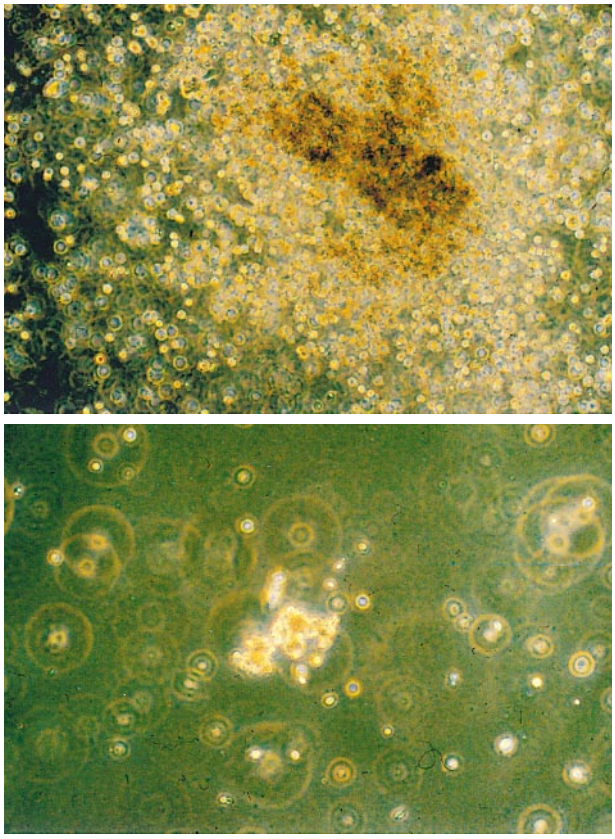

Figure 2. *Relative size of progenitor colonies in bone marrow mononuclear cells (BMMCs) subjected to hypoxia. The BMMC clonogenic assays were performed as described for Figure 1, and are described in the "Hematopoietic Progenitor Cultures" subsection of the "Materials and Methods" section. Representative colonies are shown for BMMCs exposed to hypoxia (top) and normoxia (bottom).*

pared with the normoxia control. These results indicate that hypoxia significantly increases the growth of CFU-GMs and BFU-Es compared with normoxic BMMCs, and hypoxia seems to be a myelopoietic stimulus.

EFFECTS OF BONE MARROW STROMA IN THE HYPOXIA-MEDIATED INCREASE IN GROWTH OF CFU-GM AND BFU-E CULTURES

The stromal cells were depleted from BMMCs by passing through nylon wool, and cells were then subjected to 6 hours of hypoxia. Stromal depletion decreased the growth of CFU-GMs and BFU-Es in normoxic controls, and it completely abrogated the hypoxia-mediated increase in colony growth in both cell lineages ($P<.05$) (**Figure 3**). Since this separation technique results in a minimum loss of $CD34^+$ cells,[19] the results indicate that the bone marrow stroma is necessary for and has a role in the increase in the growth of CFU-GMs and BFU-Es that is caused by hypoxia.

INDUCTION OF RELEVANT STIMULATORY HEMATOPOIETIC GROWTH FACTORS IN HYPOXIC BONE MARROW STROMA

Stromal cultures were divided into the aforementioned groups, and the mRNA was examined by using Northern blot analysis and reverse transcriptase–PCR (RT-PCR) at 2, 6, 12, and 24 hours to determine the optimum timing for the induction of *PPT-I*. There was no

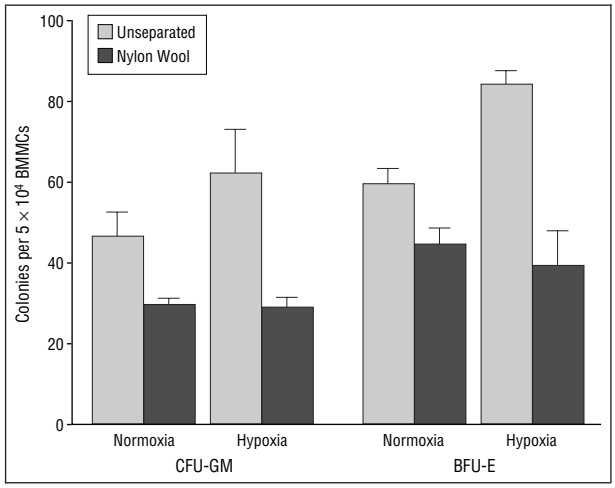

Figure 3. *Effects of bone marrow stromal depletion on erythroid burst-forming units (BFU-Es) and granulocyte-macrophage colony-forming units (CFU-GMs). Stroma-depleted bone marrow mononuclear cells (BMMCs) and untreated cells were subjected to 6 hours of hypoxia and cultured for BFU-Es or CFU-GMs. The control group included normoxic cells that were similarly treated. The results are expressed as the mean of 4 experiments (see the "Materials and Methods" section); error bars indicate the SD. Nylon wool was used to prepare stroma-depleted bone marrow cells.*

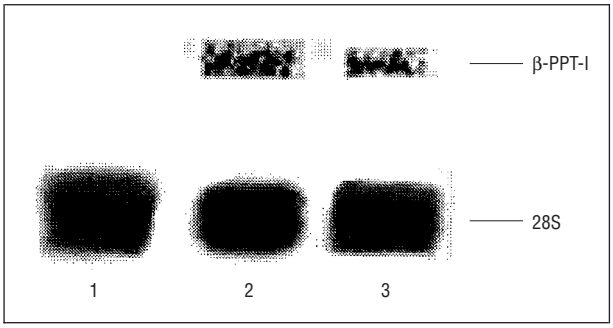

Figure 4. *Northern blot analysis of the β-preprotachykinin-I (β-PPT-I) in hypoxic bone marrow stroma. Lane 1 represents unstimulated normoxic stroma; lane 2, unstimulated hypoxic stroma; and lane 3, normoxic stroma stimulated with interleukin 1α; 28S indicates the deoxyadenosine triphosphate, α-phosphorus 32–labeled 28S ribosomal RNA complementary DNA probe.*

detectable *PPT-I* message in the 2-, 6- and 12-hour groups (data not shown). Compared with unstimulated normoxic stroma (**Figure 4**, lane 1), the steady state *PPT-I* mRNA levels were increased at 24 hours in stroma subjected to hypoxia alone (Figure 4, lane 2) and normoxic stroma stimulated with IL-1α (Figure 4, lane 3). These results, shown by Northern blot analysis for steady state *PPT-I* mRNA, also were observed by using semiquantitative RT-PCR, performed with specific *PPT-I* primers. Furthermore, RT-PCR, a sensitive technique, did not show a visible band for the unstimulated normoxic group. The results, therefore, support our findings that hypoxia could be a mediator of *PPT-I* induction.

Because *PPT-I* induction in bone marrow stroma is sensitive to hypoxia (Figure 4), the next set of experiments was designed to measure the levels of the major *PPT-I* peptide, substance P. Substance P immunoreactivity levels were similar in hypoxic stromal extracts and normoxic stroma stimulated with IL-1α, as shown in the following tabulation.

Group	Substance P Immunoreactivity, ng/mL
Normoxia	2.3 ± 0.6
Hypoxia	148.0 ± 50.0
Normoxia and IL-1	140.0 ± 35.0
Hypoxia and IL-1	110.0 ± 35.0

Of note, the hypoxic stroma stimulated with IL-1α had no further increase in substance P immunoreactivity, and there was a slight decrease in the levels of substance P immunoreactivity, although this decrease was not statistically significant ($P = .58$).

INDUCTION OF *NK-1* AND *NK-2* IN HYPOXIC BONE MARROW STROMA

Consistent with the findings given in other reports,[20] we did not observe constitutive expression of *NK-1*. However, *NK-1* was up-regulated in the 3 experimental groups: hypoxia alone, hypoxia stimulated with IL-1α, and normoxic stroma stimulated with IL-1α. *NK-2*, reported to be constitutively expressed in unstimulated stromal cells,[2] was present in all 3 experimental groups. However, when normalized to glyceraldehyde 3-phosphate dehydrogenase, *NK-2* mRNA levels were decreased 4-fold in the normoxic group stimulated with IL-1α and the hypoxia alone group.

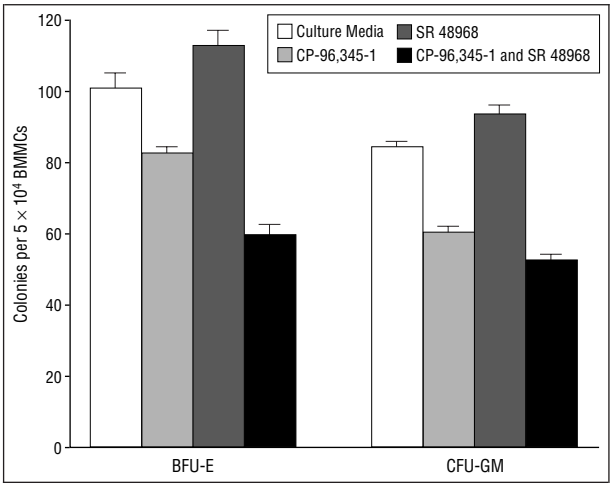

Figure 5. *Effects of neurokinin (NK) receptor antagonists on bone marrow progenitor colonies. The bone marrow mononuclear cells (BMMCs) were exposed to hypoxia and cultured for granulocyte-macrophage colony-forming units (CFU-GMs) or erythroid burst-forming units (BFU-Es) in the presence of the* NK-1 *receptor antagonist (CP-96,345-1, provided by Pfizer Inc, Groton, Conn), the* NK-2 *receptor antagonist (SR 48968, provided by Sanofi Recherche, Montpellier Cedex, France), or both. The results are expressed as the mean of 3 experiments (see the "Methods and Materials" section); error bars indicate the SD.*

ROLE OF *NK-1* AND *NK-2* IN HYPOXIA-MEDIATED INCREASE OF CFU-GM AND BFU-E CULTURES

Because of the up-regulation of *NK-1* combined with detectable levels of substance P after hypoxia, we designed the next series of experiments to determine whether part of the hematopoietic stimulation we observed after hypoxia actually involved the NK receptors. We used specific *NK-1* and *NK-2* receptor antagonists in the hematopoietic progenitor assays. In 3 separate experiments, we observed 61 ± 14 and 51 ± 11 colonies per plate for BFU-E and CFU-GM, respectively. After exposure to hypoxia, these levels were increased by 67% for BFU-Es and 60% for CFU-GMs (**Figure 5**). We next blocked the *NK-1* receptor with a specific antagonist (CP-96,345-1), thereby allowing signals to be mediated by *NK-2*. We observed a decrease in both types of colonies (BFU-Es, 33%; CFU-GMs, 20%). This observation is consistent with other reports that show that *NK-2* mediated hematopoietic inhibition.[2] Blocking of the *NK-2* receptor with a specific antagonist (SR 48968) resulted in increased colony formation (BFU-Es, 72%; CFU-GMs, 80%). This finding is consistent with those in other reports that showed that *NK-1* mediated progenitor stimulation.[14] In the presence of both antagonists, there was complete inhibition to normoxic levels (Figure 5).

COMMENT

Severe trauma has been found to cause hematopoietic suppression.[8-11] Experimental evidence indicates that the neuroimmune/hematopoietic axis has a role in the modulation of hematopoiesis. We studied the relationship between hypoxia and bone marrow progenitors in vitro. Hypoxia increased the number and the size of the

Statement of Clinical Relevance

Tissue hypoxia is the basic physiologic derangement associated with injury and shock. Because the results in the present study parallel previous studies of whole animal hypoxia performed in our laboratory, we believe this experimental system of in vitro hypoxia adequately mimics the clinical situation. The clinical conundrum highlighted by the results of the present study is that hypoxia, by itself, seems to increase cellular proliferation, whereas, after injury, there is chronic anemia, which is unresponsive to exogenous factors. Furthermore, injury and shock initiate a cascade of neural and hormonal signals. The present study also demonstrates that bone marrow hematopoiesis is modulated by hypoxia and this neuroendocrine response.

CFU-GM and BFU-E colonies, and this increased growth seemed to be modulated by the bone marrow stroma.

The increase in CFU-GM colony size may not be equated with enhanced immune function. One possibility may be that the $CD34^+$ primitive stem cells are propagated to a multipotent but immature stage and, therefore, a nonimmune competent stage of differentiation. Cipolleschi et al[21] showed that cord blood cells subjected to hypoxia included a greater percentage of $CD34^+$ cells. The authors stated that their results suggested that hypoxia may inhibit the differentiation of cord blood cells. Similar mechanisms in bone marrow–derived $CD34^+$ cells are yet to be determined.

The ability of hypoxia to mediate the proliferation of fibroblastlike cells also could lead to bone marrow failure after hypoxia. The stromal architecture is an integral component of the bone marrow microenvironment for supporting hematopoiesis.[22,23] An increase in the proliferation of fibroblasts could disrupt the highly organized network of the stroma, leading to hematopoietic dysregulation. The ability of hypoxia to induce the proliferation of BFU-Es and CFU-GMs depends on the bone marrow stromal cells, as was demonstrated by the reduced number of colonies in the stroma-depleted cultures (Figure 3). Dependence on the stroma for progenitor proliferation reinforces the potential for hypoxia-mediated hematopoietic dysregulation. Since nervous innervation occurs in the stroma, and the cells of the stroma can produce neurotransmitters such as substance P,[1-6] hypoxia could be mediating alterations in the neuroimmune/hematopoietic axis.

We studied whether hypoxia could induce the production of substance P in bone marrow stromal cultures. We determined the local production of substance P in the bone marrow stroma. We also studied the mRNA levels for the 2 natural receptors, *NK-1* and *NK-2*, and for *PPT-I*. Hypoxia resulted in *PPT-I* expression; however, mRNA was not detected in the hypoxic stroma stimulated with IL-1α. It is of interest that this was the group that showed the slight decrease in substance P immunoreactivity levels compared with hypoxia alone (see the tabulation in the "Results" section). Because we found substance P product with no detectable message, we postulate that there may be a prepackaged cellular source of substance P or that there is an increase in the translation of the message that was present. In addition, cytosolic translation of substance P has been demonstrated in cancer cell lines. If this reduction in *PPT-I* and substance P immunoreactivity was sufficient to cause an alteration in hematopoiesis, one could theorize that hypoxia and IL-1α act through a "two-hit" method. As shown in studies by Gamelli et al,[12] Raff et al,[9] and Mohr et al,[13] there is evidence that bone marrow fails to respond to a positive stimulus in the presence of hemorrhage or hypoxia.

The expression of *NK-1* and *NK-2* was studied by using RT-PCR. The *NK-1* message was expressed in response to hypoxia and IL-1α. The *NK-2* message was found to be decreased in the hypoxia group along with the normoxic group stimulated with IL-1α. This finding is consistent with other data that show that *NK-1* is upregulated as *NK-2* is decreased. These data are further supported by the receptor antagonist data that showed a decrease when the *NK-1* and *NK-2* receptor antagonists were added, but the *NK-1* receptor antagonist was effective alone. This shows that the effect of substance P on hypoxic stroma is mediated through the *NK-1* receptor.

The results of the present study demonstrate that neuroimmune effects seem to have a role in the hypoxic regulation of committed bone marrow progenitors. Since shock and central nervous system and other soft tissue trauma have been shown to be potent stimuli for neuroendocrine stimulation and immune failure, the results of the present study promote a better understanding of the role of the neural influences on hematopoiesis. Control of the neuroendocrine response after injury has implications for posttraumatic infections that occur after hypoxia or hemorrhage.

Supported in part by grant HL-57675 from the National Institutes of Health, Bethesda, Md, and grant 31-97 from the Foundation of the University of Medicine and Dentistry of New Jersey, Newark.

Presented at the 18th Annual Meeting of the Surgical Infection Society, New York, NY, May 1, 1998.

We thank Pedro Gascon, MD, Department of Medicine, Division of Hematology, University of Medicine and Dentistry of New Jersey–New Jersey Medical School, for providing the bone marrow stroma.

Corresponding author: David H. Livingston, MD, University Hospital E-245, 150 Bergen St, Newark NJ, 07103.

REFERENCES

1. Savino W, Dardenne M. Immune-neuroendocrine interactions. *Immunol Today.* 1995;16:318-321.
2. Rameshwar P, Poddar A, Gascon P. Hematopoietic regulation mediated by interactions among neurokinins and cytokines. *Leuk Lymphoma.* 1997;28:1-10.
3. Maestroni GJM. Adrenergic regulation of hematopoiesis. *Pharmacol Res.* 1995; 33:249-253.
4. Felten DL, Felten SY, Bellinger DL, et al. Noradrenergic and peptidergic innervation of secondary lymphoid organs: role in experimental rheumatoid arthritis. *Eur J Clin Invest.* 1990;22:37-41.
5. Weihle E, Nohr D, Michel S, et al. Molecular anatomy of the neuro-immune connection. *Int J Neurosci.* 1991;59:1-23.
6. Lorton D, Bellinger DL, Felton SY, et al. Substance P innervation of spleen in rat: nerve fibers associated with lymphocytes and macrophages in specific compartments of the spleen. *Brain Behav Immun.* 1991;5:29-40.

7. Pascual DW, Bost KL. Substance P production by P388D1 macrophages: a possible autocrine function for this neuropeptide. *Immunology.* 1990;71:52-56.
8. Livingston DH, Gentile PS, Malangoni MA. Bone marrow failure after hemorrhagic shock. *Circ Shock.* 1990;30:255-263.
9. Raff G, Livingston DH, Wang MT, Rameshwar P. Hemorrhagic shock abolishes the myelopoietic response to turpentine-induced soft tissue injury. *J Surg Res.* 1995;59:75-79.
10. Peterson V, Hansbrough J, Buerk C, et al. Regulation of granulopoiesis following severe thermal injury. *J Trauma.* 1983;23:19-24.
11. Chaudry IH, Ayala A. Mechanism of increased susceptibility to infection following hemorrhage. *Am J Surg.* 1993;165:59S-67S.
12. Gamelli RL, He L, Lui H. Marrow granulocyte-macrophage progenitor cell response to burn injury as modified by endotoxin and indomethacin. *J Trauma.* 1994;37:339-346.
13. Mohr AM, Upperman JS, Taneja R, et al. Differential effects of hypoxia and endotoxin on the secretion and expression of bone marrow interleukin-1 and interleukin-6. *Shock.* 1997;7:324-331.
14. Rameshwar P, Ganea D, Gascon P. In vitro stimulatory effect of substance P on hematopoiesis. *Blood.* 1993;81:391-398.
15. Rameshwar P, Ganea D, Gascon P. Introduction of IL-3 and granulocyte-macrophage colony-stimulating factor by substance P in bone marrow cells is partly mediated through the release of IL-1 and IL-6. *J Immunol.* 1994;152:4044-4054.
16. Ringstedt T, Tang L, Persson H, et al. Expression of c-fos, tyrosine hydroxylase and neuropeptide mRNA in the rat brain around birth: effects of hypoxia and hypothermia. *Pediatr Res.* 1995;37:15-20.
17. Johnson M, Hanson GR, Gibb JW, et al. Effect of neonatal hypoxia-ischemia on nigrostriatal dopamine receptors and on striatal neuropeptide Y, dynorphin A and substance P concentrations in rats. *Brain Res Rev Dev Brain Res.* 1994;83:109-118.
18. Wang ZZ, Dinger B, Fildone SJ, et al. Changes in tyrosine hydroxylase and substance P immunoreactivity in the cat carotid body following chronic hypoxia and denervation. *Neuroscience.* 1998;83:1273-1281.
19. Dorshkind K, Johnson A, Collins L, et al. Generation of purified stromal cell cultures that support lymphoid and myeloid precursors. *J Immunol Methods.* 1986;89:37-42.
20. Rameshwar P, Poddar A, Zhu G, et al. Receptor induction regulates the synergistic effects of substance P with Il-1 and PDGF on the population of bone marrow fibroblasts. *J Immunol.* 1997;158:3417-3424.
21. Cipolleschi MG, D'Ippolito G, Bernabei PA, et al. Severe hypoxia enhances the formation of erythroid bursts from human cord blood cells and the maintenance of BFU-E in vitro. *Exp Hematol.* 1998;25:1187-1194.
22. Lichtman LA. The ultrastructure of the hematopoietic environment of the marrow: a review. *Exp Hematol.* 1981;9:391-410.
23. Queensberry PJ, McNiece IK, McGrath HE, et al. Stromal regulation of hematopoiesis. *Ann N Y Acad Sci.* 1989;554:116-127.

DISCUSSION

Roderick A. Barke, MD, Minneapolis, Minn: This is a very interesting paper and a nice presentation. It has been assumed that bone marrow failure contributes by some mechanism to both anemia and immunosuppression following prolonged insults and critical illness. You demonstrate that hypoxia increases granulocyte-macrophage colony-forming units in response to GM-CSF and erythroid burst-forming units in response to erythropoietin and IL-3. Could you please elaborate on your thoughts regarding the effect of hypoxia on proliferation compared to differentiation in this model? You alluded to this but did not discuss in detail. More specifically, does hypoxia actually increase proliferation but impair differentiation in your model?

Secondly, along the same lines, you show from stromal depletion experiments that the bone marrow stimulus in response to hypoxia resides in the stroma. You also suggest that hypoxia increases proliferation of bone marrow fibroblasts. Substance P increases overall secretion of fibroblast matrix metalloproteinases; in fact, it increases expression of a whole cassette of metalloproteinases including stromelysin. This expression may disrupt bone marrow microstructure. Do you have any evidence demonstrating that this in fibroblast proliferation increases matrix metalloproteinases, or any information of the effect of metalloproteinases on differentiation in your model?

You demonstrate that hypoxia increases preprotachykinin-I mRNA. This transcript is similar to POMC (proopiomelanocortin), which we have studied in our laboratory. Expression of POMC and preprotachykinin-I is tissue specific and regulated by a variety of silencer elements in the promoter. These elements act to repress the function of several enhancer elements in the preprotachykinin-I gene. Have you studied gel shifts in the promoter region of this gene to see if binding to these silencer elements has been altered following hypoxemia in an attempt to explain the mechanism of how gene expression of preprotachykinin-I is increased?

Substance P (SP) and neurokinin-A, along with other neurokinins are derived from the preprotachykinin gene through post-translational alternate splicing. Substance P binds preferentially to the *NK-1* receptor, as you mentioned, whereas neurokinin-A binds preferentially to the *NK-2* receptor. Substance P enhances proliferation, as you have demonstrated. Neurokinin-A, however, is inhibitory. Could you speculate on the differential regulation of substance P vs neurokinin-A in your model?

Finally, regarding the clinical significance of this work, you demonstrate these hypoxia-mediated effects at around P-50 or 25-50 mm Hg of oxygen and the synergistic effects are observed at 6 hours. Patients do not usually survive that period of time at such low oxygen levels. Are these effects seen at 90% saturation or a P_{O_2} of 60 mm Hg? How do you connect the profound hypoxemia you are studying with the clinical situation one might observe?

Dr Quinlan: In regard to your questions, all of them have not been done yet and are currently being looked at now. Whether the cells have actually differentiated or just proliferated and whether this fibroblast matrix actually disrupts maturation is under study.

In regard to the promoter, the lab that I am working with is also currently trying to clone the promoter and sequence it. So when we have that available we might be able to look at the promoter.

With regard to the alternate splicing of SP and NK-A, I could speculate that, yes, it would be nice if I could have looked for NK-A to see if I was getting more NK than SP. However, β-PPT is specific for substance P. Whether there is neurokinin-A there, there probably is, but I didn't look for it.

In regard to the Pa_{O_2} of 35 to 50, I did not look at an intermediate Pa_{O_2} of 60. This is an in vitro model, of course, which, as to the best of my ability, has the change in pH and other facets of clinical shock.

Carol Miller-Graziano, PhD, Worcester, Mass: There has been a recently identified non–*NK-1* type or non–*NK-2* type receptor for substance P on macrophage, human macrophage in particular, which supposedly is very involved in growth factor induction in those cells. I therefore wondered if you had any evidence at all in your stromal cells for a similar type of non-NK receptor induction?

Dr Quinlan: No, we do not.

Thomas K. Hunt, MD, San Francisco, Calif: I am now confused. Did you measure the P_{O_2} in these cultures during the exposure? Are you dealing with hypoxia or release from anoxia?

Dr Quinlan: I did measure the Pa_{O_2} and it was between 35 and 50.

Dr Hunt: You are using the word Pa_{O_2} in animals. Could you define Pa_{O_2} for me?

Dr Quinlan: We actually analyzed, using a blood gas analyzer, the medium surrounding the stroma.

Dr Hunt: By Pa_{O_2} you do not mean arterial P_{O_2}?

Dr Quinlan: No.

Dr Hunt: Okay. So the P_{O_2} is 35, and that is an excellent step to have taken. So you are dealing with hypoxia. In that case, your data are very interesting in the sense that quite a few people around this country are looking for a mechanism to sense that P_{O_2} using an oxygen sensor, which implies an oxygen sensor, and you are very clearly out of that system. In other words, you have defined a mechanism now involving hypoxia, which does not involve a sensor, and that is a very interesting accomplishment.