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An *in vitro* method to study the effects of hematopoietic regulators during immune and blood cell development

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Abbreviations: BM, Bone marrow; CLP, common lymphoid progenitors; CMP, common myeloid progenitors; HSCs, hematopoietic stem cells; MSCs, mesenchymal stem cells; NK-A, neurokinin-A; NK1, neurokinin-1; NK2, neurokinin-2; SDF-1α , Stromal cell-derived factor 1α; SP, Substance P.

ABSTRACT

In adults, hematopoiesis occurs in bone marrow (BM) through a complex process with differentiation of hematopoietic stem cells (HSCs) to immune and blood cells. Human HSCs and their progenitors express CD34. Methods on hematopoietic regulation are presented to show the effects of the chemokine, stromal-derived growth factor (SDF)-1α and the neuropeptide, substance P (SP). SDF-1α production in BM stroma causes interactions with HSCs, thereby retaining the HSCs in regions close to the endosteum, at low oxygen. Small changes in SDF-1α levels stimulate HSC functions through direct and indirect mechanisms. The indirect method occurs by SP production, which stimulates $CD34^+$ cells, supported by ligand-binding studies, long-term culture-initiating cell assays for HSC functions, and clonogenic assays for myeloid progenitors. These methods can be applied to study other hematopoietic regulators.

INTRODUCTION

Hematopoiesis is the process by which hematopoietic stem cells (HSCs) self-renew to form blood and immune cells of the body. In the healthy adult, the major site of hematopoiesis is the bone marrow (BM) (1,2). HSCs differentiate into common myeloid progenitor (CMP) or common lymphoid progenitor (CLP). CMLs differentiate into multiple lineages to produce erythrocytes, monocytes, megakaryocytes, dendritic cells and granulocytes (3). CLPs generate lymphoid cells, T- and B cells, natural killer cells and dendritic cells (4,5). In contrast, dysfunctional BM shows extra-medullary hematopoiesis, such as in spleen (6). HSCs are mainly found in areas closest to the endosteum of BM in the region of lowest oxygen level (7). The BM is also host to another stem cell, mesenchymal stem cells (MSCs), which is located surrounding blood vessels and in contact with trabeculae (8). MSCs are functionally linked to HSCs since the supporting stromal cells for the latter are generated from MSCs (9,10).

Hematopoiesis is regulated by a complex system involving soluble factors such as cytokines, chemokines, neuropeptides and neurotransmitters (11). Extracellular matrix proteins (ECM) are also involved in hematopoietic regulation and are not mutually exclusive of cytokines (11,12). There is no direct link between families of regulator with functions since the BM microenvironment could influence hematopoietic outcome (11,12). Thus, hematopoietic regulators involve a complex network involving cellular interactions in a network of soluble factors and ECM. This study focuses on a method to study two regulators of hematopoiesis, stromal-derived growth factor (SDF)-1α and the preprotachykinin I gene, also referred to as *Tac1*. SDF-1α belongs to the CXC chemokines family, and interacts with the CXCR-4 receptor that belongs to the family of G-protein, seven

transmembrane receptors (13). SDF-1α is produced by BM stromal cells and could be released or membrane-bound (14,15). CXCR4 receptors are expressed on HSCs, making them poised to form complexes with stromal cells through interactions with membrane-bound SDF-1α (14,15). Disruption of the SDF-1α-CXCR4 complex could cause mobilization of HSCs from BM into the periphery (14). SDF-1α levels show a pattern of gradient changes across the BM (16). Gradient changes in SDF-1α across the BM facilitate mobilization of HSCs in and out of the BM (14). In addition to its mobilization role, SDF-1α has a major role in hematopoiesis at all levels of maturity within the hematopoietic lineages (17,18).

Tac1 encodes peptides belonging to the tachykinin family of peptides (19). Tachykinins' presence in BM could be from the innervation in BM as neurotransmitters and from endogenous sources (20,21). The major Tac1 peptides are substance P (SP) and neurokinin-A (NK-A) (22). Substance P induces the production of cytokines with stimulatory effects on hematopoiesis whereas neurokinin-A acts in opposing manner (Table 1). SP and NK-A bind the neurokinin (NK) receptors with varying affinities (23). Three NK receptors have been described, NK1-NK3 (24). This family of receptors belongs to the 7-transmembrane G-protein coupled receptor family (25). SP shows preference for NK1 and NKA demonstrates high affinity binding to NK2 (23). NK receptors are expressed on hematopoietic progenitors and differentiated immune cells (20). The hematopoietic effects mediated by NK1 and NK2 are confounded by crosstalk between these receptors, and fragments of *Tac1* peptides that bind NK1 (22).

We have reported *Tac1* expression by SDF-1α in the BM stroma (19). This production was important in SP-mediated effects on hematopoiesis (19). The hematopoietic effects were examined as the levels of primitive and mature hematopoietic progenitors by long-term culture-initiating cell assay and clonogenic assay (19). Here we describe detailed methods to investigate hematopoiesis through secondary regulators and the incorporation of stromal cells.

MATERIALS AND METHODS

Reagents and cytokines

α-Minimum Essential Medium (α-MEM), glutamine, hydrocortisone, SP, and phthalic acid dially ester were purchased from Sigma (St. Louis, MO). Fetal calf sera (FCS) and horse sera (HS) were purchased from Hyclone Laboratories (Logan, UT). Recombinant human SDF-1α was purchased from R&D Systems, prolyl-4-hydroxylase mAb from Dako Cytomation, PE-anti-CD14 from BD Pharmingen. Recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) was provided by the Immunology Department of Genetics Institute (Cambridge, MA). ^{125}I-Tyr^{8}-SP (2200 Ci/mmol/L) was purchased from Perkin Elmer (Billerica, MA). Dimethyl phthalate was purchased from Fisher Scientific, Pittsburgh, PA. Dynabead M-450 CD34 was purchased from DynaI Inc. (Lake Success, NY). PE-anti-CD34 was purchased from Becton Dickinson, San Jose, CA and Tri-color-anti-CD45 from Caltag Laboratories (Burlingame, CA).

Primary bone marrow cells

BM aspirates were obtained from the posterior iliac crest of healthy volunteers following the guidelines made by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey (Newark, NJ). BM aspirates were obtained from healthy donors between the ages of 18-25 years.

Clonogenic assays for granulocyte-macrophage colony-forming units (CFU-GM)

Mononuclear cells (BMNCs) were isolated from BM aspirates by Ficoll-Hypaque density gradient and then assayed for CFU-GMs in sera free culture as described (26). 105 mononuclear cells/ml were plated in methylcellulose with different concentrations of Substance P (SP) and 3 U/mL rhGM-CSF. Colonies with more >20 cells were counted at day 10.

Preparation of BM Stroma

107 nucleated cells from BM Aspirates were added to 25 cm^2 tissue culture flasks (Falcon 3109) in stromal media (α-MEM with 20% FCS) and incubated at 37°C for 3 days. Mononuclear cells were then separated by Ficoll-Hypaque density gradient and replated in fresh stromal medium. Cells were incubated until confluency with weekly replacement of 50% stromal medium. At confluency, the trypsin-sensitive adherent cells were passaged at least 5 times before being used for experiments. Flow Cytometry studies indicated stromal cells were negative for CD14 and positive for prolyl 4-hydroxylase.

Modified long-term culture-initiating cell (LTC-IC) culture

LTC-IC assays were performed as described (19). Stromal cells were cultured in 25-cm^2 flasks and at confluence, were γ-irradiated with 150 Gy delivered by a cesium source. After 16 h, media were replaced with 5 ml of fresh media containing 107 BMNCs/flask. At weekly intervals, 50% of culture media were replaced. The non-adherent cells were studied at various intervals in clonogenic assays for CFU-GM, described above.

Isolation of $CD34^+$ BM cells

$CD34^+$ cells were positively selected from BMNC with an isolation kit (Dynabeads M-450 CD34) purchased from DynaI Inc. (Lake Success, NY) with a 2-step method as described. BMNC (10^7-10^8) were washed twice and resuspended in 1 ml of cold isolation buffer (Ca^{2+}/Mg^{2+} - free PBS with 2% BSA). 10^8 Dynabeads M-450 CD34 were incubated with the cell suspension at 4°C for 30 min using gentle agitation at 5 min-intervals. Dynabeads were then magnetically selected with Dyna1 MPC and then washed (3x) with isolation buffer. Cells were uncoupled from the magnetic beads by incubating at 37°C for 15 min with equal volumes of isolation buffer and DETACHaBEAD CD34 liquid. Cells were double labeled with PE-conjugated anti-CD34 and Tri-color-conjugated anti-CD45. The process was repeated and the final population was >90% positive for CD34 and CD45. Since endothelial cells also express CD34, the inclusion of this cell subset in the selected cell population was determined using anti-von Willebrand Factor. Less than 1% of selected cells were positive for vWF.

Binding Assay

Varying concentrations (3-200 mmol/L) of ^{125}I-Tyr^8-SP (2200 Ci/mmol/L) were added to 1ml of $CD34^+$ cells and resuspended at 10^6/mL. Tubes were rotated at 4°C with a Rotating Wheel Two-way Mixer (Robbins Scientific, Sunnyvale, CA) for 4 hours in order to allow for binding. After this period of incubation, cells were layered in siliconized microcentrifuge tubes on 0.5 mL phthalate oils (90 parts dimethyl phthalate and 10 parts phthalic acid dially ester) and then centrifuged at 10,000 g for 1 min at 4°C. The cells were incubated for 4 h, which falls within the range required for equilibrium that was established in time course binding assays ranging from 1 to 12 h. The radioactivity was measured in the pellet and supernatant fractions in order to determine the amount of bound and unbound ligand, respectively. Radioactivity was determined using the Beckman gamma counter (Beckman Instruments, Irvine, CA). Triplicate binding were performed for each donor and concentration of ligand. The McPherson's modification of the Radioligand Binding Analysis software was used to analyze the data.

Results and Discussion

The role of Tac1 peptides as mediators within the neural-immune-hematopoietic axis has been widely studied (20,21).The hematopoietic effects of SP and NKA are mostly mediated through their interaction with NK1 and NK2, respectively (27,28). However, the effects of these peptides are not only direct, but indirectly through the production of cytokines and other hematopoietic regulations in BM resident cells (Table 1).

Experimentally, studies can be done to show effects of *Tac1* peptides on hematopoiesis, at an early stage by LTC-IC assays (27-33). In addition to the early phase, SP and NKA also affect hematopoietic progenitors at the myeloid level, which was studied by clonogenic assays for CFU-GM (Table 1). The effects of tachykinins are not only mediated through cytokine production, but could also occur directly. This premise is based on the ability of SP to bind $CD34^+$ BM cells at dissociation constant of 70 nM/L on a cell subset that includes both early and late human progenitors (Fig. 1).The methods described in this technique paper could be applied in studies to investigate the roles of cytokines in the network developed by *Tac1* in BM stromal cells.

Fig. 2 summarizes how the methods could be applied to study the effects of SDF-1α on *Tac1* expression, and the consequence to hematopoiesis. SDF-1α is expressed as baseline in stromal cells. Upon stimulation by exogenous SDF-1α, which could be produced by other accessory cells, and also by stroma, *Tac1* expression is upregulated. This could lead to the production of SP, which could mediate direct stimulation on HSCs (Fig. 1), or indirectly by inducing the production of other cytokines in cells within BM microenvironment. These cytokines could stimulate hematopoiesis. To address questions similar to the network described in Fig. 2, experimental studies could be developed by clonogenic assays, LTC-IC assays and cytokine quantitation and mechanistic studies on cause-effect relationship could be done by knockdown and expression approaches (19).The effects of SP on the stimulation of primitive HSCs and progenitors have been attributed to the induction of specific hematopoietic growth factors including IL-3, IL-6, GM-CSF, SCF, flt3/flk2 (29). Similarly, NK-A, induces the production of specific cytokines with hematopoietic suppressive effects (30). We have reported the production of TGF-β and MIP-1α by NK-A in stromal cells (30). Together, the positive and negative effects of the tachykinins lead to an understanding of hematopoietic homeostasis.

Table 1: Correlations between Tac1 peptide-mediated hematopoietic effects and cytokine production.

Tachykinins	Hematopoietic effects	Hematopoietic regulators	Reference
Neurokinin A (NKA)	↓	Suppressive	31
Substance P (SP)	↑	Broad-acting; Stimulatory	32

↓: Decrease hematopoietic activity
↑: Increase hematopoietic activity

The general hematopoietic effects of the two major *Tac1* peptides, substance P and neurokinin-A. Each peptide induces the production of specific cytokines in BM-resident cells. The cytokines correlates with hematopoietic effects mediated by the peptide.

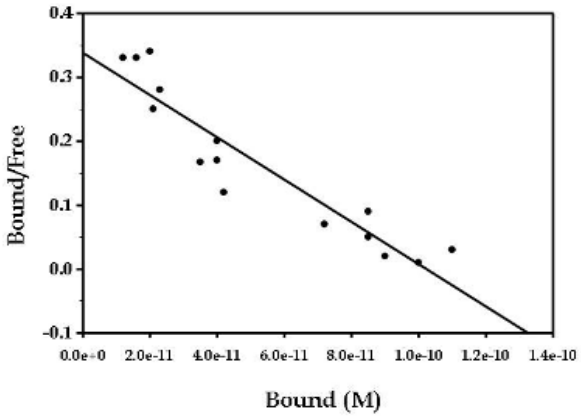

Fig. 1: Scatchard analysis of SP-binding sites on CD34⁺ cells. We performed ligand-binding studies with ^{125}I-Tyr-SP to confirm SP is capable of binding to CD34$^+$ cells and also to determine the binding kinetics of SP on these cells.

Additionally, SDF-1α is shown to be involved in hematopoiesis (19). In BM, SDF-1α follows a gradient pattern with the highest levels close to the stromal compartment (32). CXCR4, expressed on HSCs mediates retention in BM (32). Changes in SDF-1α in BM could cause increased hematopoietic activity and also mobilization of HSCs. The molecules involved in this process could be identified using the methods described as the basis for questions.

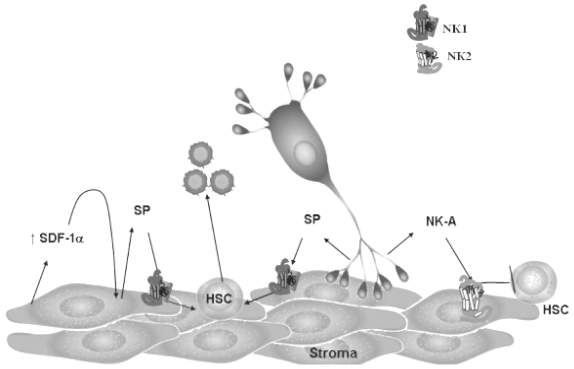

Fig. 2: The effects of SP, NK-A and SDF-1α on hematopoiesis. BM stroma is innervated by peptidergic fibers, which can release two neuropeptides, SP and NK-A. SP interacts with BM Stroma via a specific receptor, NK1, which induces growth factors that stimulate hematopoiesis. NKA can also be produced by peptidergic fibers, which interact with NK2 on BM Stroma, which causes inhibition of hematopoiesis. High levels of SDF-1α stimulate BM Stroma to induced SP production, which stimulates hematopoiesis.

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PROTOCOLS

Perform all procedures under sterile conditions.

A. Stromal Isolation, 2-step procedure:

Step 1:

1. Get approval for use of human tissues by the Institutional Review Board.
2. Obtain bone marrow (BM) aspirates from healthy donors. The aspirate is obtained in a syringe containing preservative-free heparin at 50 U/ml. The heparin is diluted in any tissue culture media containing 50 U/mL Penicillin and 0.05 mg/mL Streptomycin.
3. Count the nucleated cells in the aspirates.
4. Add ~ 10^7 nucleated BM aspirate cells into a 25 cm^2 tissue culture flasks (Falcon 3109). Adjust total volume to 7 ml of stroma-I media. Stroma-I Media:
   - α-MEM containing 12.5% Fetal Bovine Serum, 12.5% Horse Sera, 0.1% μ
   - Hydrocortisone, 0.1 μM 2-mercaptoethanol, and 1.6mM glutamine
   - Incubate flasks in a 37°C incubator with 5% CO_2.
5. At day 3, remove the non-adherent cells from the flasks and place in a conical tissue culture tube. Combine the cells of each donor. Wash residual red blood cells (RBCs) in the flasks with sera-free media and transfer to the tubes containing the non-adherent cells. The wash media should be at room temperature.
6. Avoid the tissue culture flasks to dry by quickly adding 6 mL stromal media. Return flasks to the culture incubator.
7. Centrifuge tubes containing the non-adherent cells at 500 *g*. If the total volume is ~50 ml, centrifuge for 20-30 min. If 10-20 mL, centrifuge for 10-15 min.
8. Aspirate media.
9. Resuspend pellet in sera-free α-MEM. Determine the total volume to resuspend the cells using the following guide: Pellets from five flasks should be resuspended in 20 mL α-MEM. If the pellet is from more than five flasks, split the cell suspension in multiple 50 mL conical tissue culture tubes.
10. Add equal volumes of Ficoll Hypaque to the bottom of each tube. Centrifuge tubes at room temperature for 25-30 min at 500 *g*.
11. Carefully remove tubes from centrifuge. Aspirate the top layer comprising the suspension media. The next layer of white represents the mononuclear fraction (BMNC). Using a Pasteur pipet aspirate the BMNC and transfer into a clean sterile conical tissue culture tube. This would require taking a small volume of Ficoll Hypaque. Resuspend the cells with 10-20 volumes of sera-free α-MEM.
12. Centrifuge tubes at 500 *g* for 20-30 min at room temperature.
13. Aspirate the media. Resuspend the pellet in stroma-I media. The volume of media will depend on the total numbers of flasks from which the non-adherent cells were obtained. For example, if the starting numbers were 3 flasks, add 3 mL of stromal media.
14. Remove the tissue culture flasks from the incubator.
15. Add 1 mL of cell suspension/flask.
16. Re-incubate flasks. Each week replace 50% of the culture media with fresh stromal media until confluence.

Step 2:

1. Trypsinize confluent stromal cells from step 1 by adding 1-2 mL of 0.05% Trypsin with 0.053 Na-EDTA to each flask. Incubate in a 37°C-incubator for 5 min. Examine flasks with an inverted microscope. If the cells de-adhered, collect the cells and place into a conical tissue culture tube containing α-MEM with 10% FCS. Pool cells from a particular donor. If the cells do not appear de-adhered, tap the flasks and re-examine the flasks. If the cells remain adherent, reincubated the flasks until the cells de-adhered.
2. Pellet cells by centrifuging at 500 *g* for 10-15 min. Collect trypsin-sensitive cells and place in a test tube

containing any type of tissue culture media containing 10% FCS.

3. Resuspend cells in Stroma-II media. Add 3 mL of media for cells obtained from each flask. Stroma II Media: α-MEM with 20% heat inactivated FCS.
4. Add 1 ml of cell suspension to 25 cm^2 tissue culture flask. Adjust the volume to 7 ml with fresh Stroma-II media.
5. At cell confluence, repeat cells passages, #1-4 of Step 2.
6. Repeat step 5 four times.
7. Confirm the purity of stromal cells by immunofluorescence using the following antibodies:
   - Anti-CD14 (PE, FITC or any other fluorochrome), expected to be negative.
   - Anti-fibroblasts (non-overlapping fluorochrome, i.e., different from the conjugate used for anti-CD14. For example, if anti-CD14 is conjugated to PE, anti-fibroblasts should be conjugated to any fluorochrome but PE, expected to be positive.

Immunofluorescence Stain:

1. Collect cells in a polypropylene test tube.
2. Spin cells 10 min at 150 rpm.
3. Resuspend cell pellet in 1X PBS to wash
4. Spin 10 min at 150 rpm
5. Aspirate PBS and repeat
6. Resuspend cell pellet in 1-2 mL PBS
7. Add 1°Antibodies (CD14 and anti-fibroblast). The concentration will need to be titrated every time a new vial of antibody is purchased.
8. Incubate 1-2 h, shake every 10 min
9. Wash 2 times with 1X PBS

10. Add 300 μL PBS
11. Read on the Flow Cytometer
    * If CD14 is positive, continue to passage cells since macrophages are insensitive to trypsinization.

B. Clonogenic Assay:

1. Get approval for use of human tissues by the Institutional Review Board.
2. Obtain bone marrow (BM) aspirates from healthy donors. The aspirate is obtained in a syringe containing preservative-free heparin at 50 U/mL. The heparin is diluted in any tissue culture media containing 50 U/mL Penicillin and 0.05 mg/mL Streptomycin.
3. Isolate the mononuclear fraction by Ficoll Hypaque density gradient with the aspirates, diluted 1:1 with sera-free media.
4. Add 1 mL of ~ 10^5 nucleated BM aspirate cells in suspension in 35 mm Nunc suspension dishes.
5. Incubate one week at 37°C.
6. Count colonies with >20 cells.

Preparation of Methylcellulose:

Note: do not use fresh methylcellulose. Freeze at least 2 month prior to use, this helps the methylcellulose to dissolve.

- Materials
- Methylcellulose, 4000 centipose – Fisher Scientific (Cat# M-352)
- 2 L-Erlenmeyer flask
- Large magnetic stir bar (must be large enough to stir methylcellulose at semisolid phase)
- 1.5 L endotoxin-free double distilled water
- Autoclavable measuring cylinder 1L

- Sodium bicarbonate solution (7.5%)

Autoclave:

- 12 g (1.2%) methylcellulose (weigh in beaker then cover with double layer of aluminum foil)
- Erlenmeyer flask with stir bar
- Measuring cylinder, covered with double layer of foil
- 1.5 liters of Endotoxin free double distilled water (Three 500 mL bottles)
  (Autoclave dry and wet items separately)

Methylcellulose (1.2%):

- chill 1 500 mL bottle of water to 4°C
- Prepare 2x Iscove's Media (if Iscove's is not available, use 2x α-MEM)
- Set up a stirring/hot plate under sterile hood. Slowly pour methyl cellulose in 300 mL boiling distilled water. Stir vigorously while pouring.
- Stir just under boiling until the mixture is homogenous.
- Add 200 mL cold distilled water
- Cool to room temperature. Add 500 ml 2x Iscove's (room temp) while stirring
- Add 26.7 mL sodium bicarbonate (7.5%)
- Continue stirring in the cold room for 48 h
- Aliquot in 50 mL tubes, store at -20°C
- Thaw at 4°C prior to use

Long-Term Culture-Initiating Cell (LTC-IC) Assay:

- Place isolated stroma (as described above) in culture dishes with stroma media.
- At confluence, α-irradiated the cells for 150 Gy.
- Leave overnight
- After 16 hours, replace media with 5ml of fresh medium containing 10^7 BM mononuclear cells.
- Add 200 mL cold distilled water
- Weekly replace 50% of culture medium
- At week 6 and 12, take 10^5 of the non-adherent cells for analyses in clonogenic assays for CFU-GM.