Hemonecint Mediates Adhesion of Engrafted Murine Progenitors to a Clonal Bone Marrow Stromal Cell Line From \textit{S\textsuperscript{l}/S\textsuperscript{l}'\textit{}} Mice

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Mutant \textit{S\textsuperscript{l}/S\textsuperscript{l}'\textit{}} mice exhibit decreased marrow hematopoiesis. The defect is known to reside in the marrow microenvironment of these animals, which is reproduced in vitro by primary marrow explants as well as by cloned marrow stromal cell lines. Bone marrow progenitor cells are incapable of adhering to primary \textit{S\textsuperscript{l}/S\textsuperscript{l}'} stromal cells or cloned stromal cell lines derived from them to form cobblestone-islands and proliferate. The role of hemonecint, a marrow-specific adhesion protein in the defective hematopoiesis of the \textit{S\textsuperscript{l}/S\textsuperscript{l}'\textit{}} mice, was studied. Indirect immunoperoxidase staining of marrow in situ from \textit{S\textsuperscript{l}/S\textsuperscript{l}'} mice showed little specific staining while specific staining was seen in a pericellular distribution in marrow from +/+ mice. Hemonecint expression in several cloned stromal cell lines from \textit{S\textsuperscript{l}/S\textsuperscript{l}'} was compared with immunoblotting with that in cloned stromal cell lines from normal +/+ littersmates. Cell line \textit{S\textsuperscript{l}'3}, which has the least hematopoietic supportive capacity in vitro, showed no detectable hemonecint by immunoblotting, while \textit{S\textsuperscript{l}'1} and \textit{S\textsuperscript{l}'2} showed detectable but greatly reduced amounts compared with normal +/+ 2.4, GB1/6, and D2XRII. Confluent cultures incubated with purified hemonecint and engrafted with enriched progenitors showed a significant increase in the cumulative number of cobblestone-islands and day 14 spleen colony-forming units (CFU-s) forming progenitors (9.15 ± 3.6/dish; 16.3 ± 3.1/dish, respectively), compared with untreated \textit{S\textsuperscript{l}'3} cultures (cobblestone-islands 8.1 ± 3.6/dish; CFU-s forming progenitors 8.8 ± 0.05/dish). Hemonecint-mediated progenitor cell binding to the \textit{S\textsuperscript{l}'3} stromal cells was specifically inhibited by antimemonecint but not by preimmune serum. These data support the role of hemonecint in early progenitor-stromal cell interactions.

\textbf{MATERIALS AND METHODS}

\textit{Mice.} \textit{S\textsuperscript{l}/S\textsuperscript{l}'} and normal littermate mice (+/+) were obtained from Jackson Labs, Bar Harbor, ME.

\textit{Cell lines.} Derivation and characterization of clonal BM stromal cell lines +/+ 2.4, \textit{S\textsuperscript{l}'1}, \textit{S\textsuperscript{l}'2}, \textit{S\textsuperscript{l}'3}, and D2XRII has been described.\textsuperscript{10,13} The BI/sembC fibroblast cell line was obtained from Dr. Stuart Aaronson, National Cancer Institute, Bethesda MD.

\textit{Immunohistochemical staining in situ.} Animals were killed and femurs immediately removed and frozen on dry ice. The frozen marrow was then carefully dissected en bloc from longitudinally split femurs and snap frozen in optimum cutting temperature (OCT) in liquid nitrogen. Marrow was not allowed to thaw, thus maintaining native architecture. Eight-micron cryostat sections were cut, fixed with 2% paraformaldehyde (w/vol.) in phosphate-buffered saline (PBS) for 20 minutes, washed five times with 0.1 mol/L glycine in PBS, and incubated with 1:100 dilution of polyclonal antimemonecint antibody,\textsuperscript{4} followed by peroxidase conjugated goat anti-guinea pig secondary antibody, then developed using aminoethylcarbamosul and counterstained with Mayers hematoxylin.\textsuperscript{11} Under these conditions the reaction product appears red.
1. Immunohistochemical localization of hemonectin in situ. S1/S2 or normal +/+ littermate BM was snap frozen and cryostat sections stained by the immunoperoxidase technique as described in Materials and Methods. (A) S1/S2 marrow stained with antihemonectin. Faintly discernible extracellular staining is seen, indicated by arrows. (B) Higher power view of +/+ marrow stained with antihemonectin. A venous sinus is seen in cross section. Antiheamonecin-stained reticular processes extend outward from the adventicium (arrows), which is partly surrounded by hematopoetic cells, identified by hematoxylin stained nuclei (arrow, original magnification ×800).
Fig 1. (Cont'd) (C) S/Lmarrow control section stained with anti-hemorrhage in the presence of soluble antigen. Little or no extracellular staining is seen (original magnification ×500). Some focal cytoplasmic staining is seen resulting from incompletely suppressed endogenous peroxidase. (D) +/+ Marrow control section stained with anti-hemorrhage in the presence of soluble antigen. Extracellular staining seen in (B) is inhibited by the presence of antigen, indicating specificity (original magnification ×500).
and nuclei blue. Other controls included deletion of primary antibody and depletion of both primary and secondary antibodies.

**Immunoblot analysis of stromal cell lysates using specific hemonectin antiserum.** Each indicated stromal cell line was grown to confluence, washed three times in serum-free media, and dissolved in 0.05 M Tris buffer pH 7.4 containing 1% wt/vol deoxycholate, 0.5% (vol/vol) NP-40, 0.15 M NaCl, leupeptin 1 μg/mL, Tryptsin inhibitor 10 μg/mL. Equal micromgram quantities of lysates were then run on a 10% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose. The blots were then developed using 1:1000 dilution of antibody against hemonectin as serum and goat anti-guinea pig peroxidase-linked secondary antibody.

**In vitro hematopoietic progenitor cell engraftment to hemonectin-coated stromal cell lines.** Confluent cultures of a clonal stromal cell line SPF3 (35-mm dish; Falcon, NJ) were incubated with indicated concentrations of chromatographically purified hemonectin in serum-free Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Grand Island, NY) supplemented with 1% bovine serum albumin (BSA; Sigma Chemical Company, St Louis, MO) for 3 hours at 33°C, 5% CO₂. Untreated SPF3 cultures were incubated similarly without hemonectin. After incubation, cultures were rinsed and engraffred with 2 to 3 × 10⁶ enriched progenitors from day 30 to 60 SPF3/LTBMCs and cobblestone-islands, defined as adherent cell foci formed by cocultivated progenitors on stromal cells (more than 25 cells), were counted at indicated times in 70 microscopic fields and the counts were then cumulated for 21 days. Nonadherent cells released into culture medium were harvested weekly, viable cells counted, and cultures replenished with fresh medium. Enriched progenitors plated in the absence of stromal cells, or in plastic dishes coated with hemonectin, were not viable at day 7 and no detectable stromal cells were observed in the dishes.

High proliferative potential colony-forming cells (HPP-CFC) were obtained from BM of SJLPFU-injected C57Bl6 mice (SJL-BM) as described. Adherent cells from SJL BM were removed by passing through a Sephadex G-10 column (Pharmacia LKB, Piscataway, NJ). Each confluent culture of SPF3 cells (35-mm dish, Falcon) either treated with hemonectin (as described above) or untreated were cocultivated with 5 × 10⁴ BM cells. Plastic dishes (35-mm, Falcon) were coated with either 0.1 mL/dish of matrigel (Collaborative Research, Bedford, MA) or 10 μg/dish hemonectin. Cobblestone-islands formed were counted as described above. CPU-s forming progenitors were quantitated by injecting nonadherent cells harvested into lethally irradiated C57Bl6 mice (900 cGy).

**Specificity of hemonectin-mediated progenitor-stromal cell interaction.** Specific antihemonectin IgG was prepared as described. Antihemonectin was added every 48 hours at 25, 50, and 100 μg/dish to SPF3 cultures coated with hemonectin (10 μg/dish). Cultures were then cocultivated with progenitor cells and cobblestone-islands counted on indicated days as described above.

**RESULTS**

**Immunohistochemical localization of hemonectin in situ in mutant SJ/LPt and +/- littermates.** To compare expression of hemonectin in SJ/LPt mutant mice and their normal littermates, immunohistochemical studies using polyclonal antihemonectin antibody were performed. Frozen sections of BM were prepared to preserve the ECM, the anatomic relationships of stromal cells, and their reticular processes. The data showed little specific staining of SJ/LPt ECM (Fig 1A). In contrast, hemonectin was detected throughout the intercellular areas of the marrow from +/- littermates. Large reticular adventitial cells surrounding venous sinusoids were strongly positive in +/- marrow, and were often associated with developing hematopoietic cells (Fig 1B). These stromal cells were smaller and less apparent in SJ/LPt marrow. Control marrow sections stained with specific antihemonectin antibody in the presence of purified antigen were negative (Fig 1C and D), as were sections stained with secondary antibody alone (data not shown).

**Expression of hemonectin in cloned narrow stromal cell lines.** Although LTBMCs established from SJ/LPt mice may lack some cellular components of intact marrow, both primary stromal cell explants and cloned BM stromal cell lines (SJ/LPt, SJ/LP, and SJ/LPt3) demonstrate significantly reduced sustained adhesion and proliferation of hematopoietic progenitor cells as compared with cloned cell lines from +/- LTBMCs. Immunostaining of primary marrow cultures from SJ/LPt and +/- mice with specific antihemonectin antibody confirmed the in situ staining pattern. In the SJ/LPt3-derived primary marrow stromal cultures, 22% of the cells expressed hemonectin compared with 78.5% of stromal cells in similarly established +/- cultures (data not shown). We next studied hemonectin expression in cloned stromal cell lines from SJ/LPt mice. Immunoblotting of cell extracts from the SPF3 cell line showed no detectable hemonectin, while extracts from SPF1 and SPF2 showed greatly reduced but detectable levels compared with +/- 2.4, a cloned stromal cell line from +/- littermates (Fig 2B). Immunoblotting of cell extracts from several hematopoiesis-supportive stromal cell lines GBl/6, +/- 2.4, D2xrI1, and B1/6embC10 showed a 60-kD band using specific antihemonectin serum (Fig 2A). Similar absence of hemonectin but not other matrix proteins, such as fibronectin, laminin, collagen I, collagen IV, and ICAM-I, in SJ/LPt3 cells was observed by indirect immunostaining (data not shown). Thus, both in situ and in vitro, hemonectin in narrow stromal cells was greatly decreased in SJ/LPt mice. The data suggest a direct correlation between the ability of a stromal cell line to support hematopoiesis and expression of hemonectin.

**Adhesion of and support of HPP-CFC to SPF3 stromal cell line is enhanced by hemonectin.** Hematopoietic progenitors, when cocultivated with clonal stromal cell lines, bind and proliferate to form discrete foci of hematopoietic activity called cobblestone-islands. Multilineage and committed progenitors are then released for several weeks from these cobblestone-islands. The ability of stromal cells to support hematopoiesis was quantitated by the number of cobblestone-islands formed and the number of hematopoietic progenitors recovered in the culture supernatant over several weeks after coculture. The SPF3 stromal cell line has been shown to support less than 1% of engrafted hematopoietic progenitors as compared with the +/- 2.4 cell line. We next tested the ability of chromatographically purified hemonectin to improve the hematopoiesis-supportive ability of the SPF3 cell line by quantitating progenitor cell adhesion to these stromal cells. For these experiments we first tested the ability of the SPF3 cells to bind exogenous hemonectin. Immunoblot analysis of pro-
HEMONECTIN MEDIATES HPPCFC ADHESION TO STROMA

Fig 2. Immunoblot analysis of stromal cell lysates. (A) Comparison of hemonectin expression by five different stromal cell lines. BL/6emb C, D2XRRI, GBI/6, and +/+ 2.4 all support hematopoietic progenitors while SI°3 does not. (B) Comparison of stromal cell lines +/+ 2.4 derived from the normal littermate with three different cloned stromal cell lines derived from SI/SIT LTBMC. Twice the microgram quantity of protein was loaded in each lane of this gel to maximize detection of small quantities of hemonectin in the SI°3 cell lines. SI°1 and SI°2 show slightly more hemonectin expression than SI°3, but considerably less than the normal +/+ 2.4 cells. (C) SI°3 and GBI/6 cells after incubation with 10 μg purified hemonectin and cell extracts immunoblotted as above.

Proteins extracted from SI°3 cells incubated with hemonectin showed a single 60-Kd protein (Fig 2C), similar to that detected in normal stromal cell lines. This 60-Kd band was not detected in untreated SI°3 cells (Fig 2B). This result establishes the ability of the SI°3 stromal cells to bind exogenously added hemonectin.

Increasing concentrations of hemonectin (1 μg to 10 μg) added to confluent cultures of the SI°3 stromal cell line resulted in a linear increase in the number of cobblestone-islands formed by a fixed number of added hematopoietic progenitor cells (Fig 3A). There was also an increase in the number of viable cells released into the culture supernatant (Fig 3B).

The hematopoiesis-supportive capacity of hemonectin-coated SI°3 cells was next compared with that of +/+ 2.4 stromal cell line. Hemonectin (10 μg/dish) was added weekly to confluent SI°3 or +/+ 2.4 cultures. Cobblestone-islands formed by progenitor cells on hemonectin-coated SI°3 dishes (307.9 ± 49.4/dish) was comparable in numbers and size to those formed on +/+ 2.4 cell line (286 ± 24.1/dish) and significantly higher than those formed on untreated SI°3 controls (94.3 ± 16.2/dish; P < .01; Figs 4A and 5B). There was also an increase in the number of viable, nonadherent cells released into culture supernatant from SI°3 dishes coated with hemonectin (55.8 ± 13.3 × 10⁴/dish) compared to control SI°3 dishes (21.57 ± 4.5 × 10⁴/dish; Fig 4B). Addition of hemonectin to the +/+ 2.4 cell line did not increase formation of cobblestone-islands (282.5 ± 15.6/dish) or nonadherent viable cells (140.5 ± 68.7 × 10⁴/dish) compared with untreated +/+ 2.4 cells (cobblestone-areas, 286.0 ± 24.1/dish; nonadherent cells, 137.5 ± 47.2 × 10⁴/dish).

Cells recovered from the nonadherent phase of murine LTBMCs contain relatively large numbers of multilineage and committed hematopoietic progenitors, and terminally differentiated cells; but only a small number of early stem cells also called HPP-CFC. The HPP-CFCs are generally considered to have a greater proportion of day 14 CFU-s forming primitive stem cells. Because cells of the granulocyte lineage have been shown to bind to hemonectin, it is possible that the above results could be explained by hemonectin-mediated adhesion of granulocyte-lineage committed progenitor cells rather than the HPP-CFC. To rule out this possibility, a population of BM cells enriched for HPP-CFC were obtained by 5-FU pretreatment of C57Bl/6 mice. Accessory cells were depleted by passing cells through a Sephadex G-10 column. SI°3 cell line incubated with hemonectin supported twice the number of day 14 CFU-s forming progenitors (16.3 ± 3.1/dish) as compared with untreated SI°3 cell line (8.1 ± 3.6/dish; Table 1). No detectable viable cells were harvested from plastic dishes coated with hemonectin or Matrigel (Table 1).

Hemonectin-mediated progenitor cell adhesion to SI°3 cells is inhibited by specific antihemonectin antiserum. The specificity of hemonectin mediated progenitor cell binding to SI°3 stromal cells was further confirmed by adding different concentrations of specific antihemonectin antiserum. The antiserum has been shown to be specifically against he-
hemonectin and did not crossreact with other matrix proteins. Addition of 25, 50, and 100 μg/dish of specific antihemonectin IgG to hemonectin-treated S13 cultures inhibited progenitor cell binding and cobblestone-island formation by 6%, 51%, and 63%, respectively (Fig 5A through D). Preimmune IgG (100 μg/dish) added to hemonectin-treated S13 cultures had no effect on the size or number of cobblestone-islands formed as compared with hemonectin-treated S13 cultures (Fig 5E). Similarly, addition of 100 μg/dish of specific antihemonectin IgG but not preimmune IgG to normal +/- 2.4 cultures inhibited progenitor cell binding by 75% (data not shown).

DISCUSSION

The S1/S1 mutant mouse provides an excellent model system with which to dissect the complexity of the BM microenvironment. The data indicate that hemonectin expression is reduced in the S1/S1 BM stroma. Addition of purified hemonectin to one of the cloned marrow stromal cell lines established from these mice in vitro increases the ability of the stromal cell line to bind engrafted progenitors and improves its hematopoietic support capacity. Hemonectin did not mediate adhesion of HPP-CFC to plastic, indicating that hemonectin interacts with other stromal cell surface growth factors or ECM proteins to stabilize HPP-CFC-stromal cell binding. This stable interaction may increase the sensitivity of the stem cells to membrane associated growth factors. Cooperation between adhesion protein (LFA1–ICAM-I) complexes and the T-cell receptor has been recently demonstrated to increase the sensitivity of T cells for antigen recognition. In the marrow microenvironment, although we cannot entirely exclude the possibility that the S1 defect is complex at the protein level, other studies did not show major quantitative differences in expression of fibronectin, laminin, collagen I, III, or IV, or glycosaminoglycans between S1/S1 and +/- 2.4 stromal cells. Furthermore, no difference in the ability of membrane-associated proteoglycans to sequester growth factors and present them in a biologically active form was observed.

Similarly, no significant differences in the ECM protein expression between primary stromal cell explants of S1/S1 and +/+ mice LTBMCs have been demonstrated. In contrast, differences in the matrix from the skins of S1/S1 and +/- embryos was detected.

It is interesting to note that the abnormal hematopoiesis of S1/S1 mice shows an unexplained disparity between the two major hematopoietic organs, BM and spleen. BM hematopoiesis is reduced, while the total number of multilineage and committed myeloid progenitors in spleen are relatively unaffected in the basal unirradiated state. The distinctive tissue distribution of hemonectin, present in BM but absent in spleen, suggests a basis for this finding. The finding that hematopoietic stem cell homing is inhibited by galactosyl and/or mannosyl residues to the BM but not to the spleen further suggests that mechanism of stem cell adhesion to BM and spleen stroma is different. In addition, the effect of the S1 mutation is not uniform in all hematopoietic organs, with BM hematopoiesis being reduced and spleen hematopoiesis being relatively unaffected.
Hemonec tin mediates HPP-CFC adhesion to stroma.

Fig 5. Specificity of hemonec tin-mediated progenitor-stromal cell interaction. (A) Numbers of cobblestone-islands formed by nonadherent progenitors from C57B1/6 LTBMCs (40 to 60 days old) on Sld3 dishes either treated with 10 pg/dish of hemonec tin ( △ ), or untreated Sld3 dishes (x). Nonadherent progenitors cocultivated with hemonec tin-treated Sld3 cultures were treated every 48 hours with either 100 pg/dish preimmune IgG (O) or 25 pg/dish ( ▲ ), 50 pg/dish (■) or 100 pg/dish (■) of specific antihemonec tin IgG. Results were cumulated for 8 days and expressed as mean ± SD of two experiments with three dishes per group per experiment. The photomicrographs taken from the same experiment on day 8 after cocultivation represent cobblestone-islands formed on (B) untreated Sld3 cultures, (C) hemonec tin-treated Sld3 cultures, (D) hemonec tin and antihemonec tin IgG (100 pg/dish) treated Sld3 cultures, and (E) Sld3 cultures treated with hemonec tin and preimmune IgG (100 pg/dish). Original magnification ×400.

Table 1. Support of HPP-CFC by Sld3 Cell Line Incubated With Hemonec tin

<table>
<thead>
<tr>
<th>Enriched Progenitors Cocultivated With</th>
<th>Cobblestone-Islands</th>
<th>CFU-s</th>
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<tbody>
<tr>
<td>Sld3</td>
<td>8.1 ± 3.65</td>
<td>8.85 ± 0.05</td>
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<tr>
<td>Sld3 + hemonec tin</td>
<td>39.15 ± 3.6*</td>
<td>16.3 ± 3.1*</td>
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<tr>
<td>Plastic coated with</td>
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<tr>
<td>Hemonec tin</td>
<td>ND</td>
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<tr>
<td>Plastic alone</td>
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Cobblestone-islands per dish were counted as described in Materials and Methods. CFU-s forming progenitors per dish were quantitated by injecting lethally irradiated C57B1/6 mice (900 cGy) with nonadherent viable cells recovered from untreated Sld3 (number of cells harvested/dish = 6.9 ± 1.4 × 10⁴) and hemonec tin-coated Sld3 dishes (number of cells/dish = 12 ± 2.6 × 10⁴) 2 weeks postengraftment. The number of colonies formed on spleens were counted on day 13. Control irradiated noninjected mice had 1.1 ± 0.34 colonies/spleen. Results are mean ± SD from 8 to 10 dishes per group. For each group 10 mice were injected.

Abbreviations: ND, none detected; NT, not tested as there were no detectable viable cells (less than 10 cells/mL) on day 7 or 14 from any of these groups. No adherent stromal cells were detected in these dishes. *P < .05.

Cell adhesion molecules play a crucial role in embryonic development, tissue organization, inflammatory response, and influence cellular activities such as differentiation and proliferation. Proteoglycans in marrow stromal cells are known to affect stem cell proliferation and differentiation by serving as reservoirs for growth factors. However, the role of other adhesion molecules in the BM stromal cell populations and their corresponding receptors on adjacent stem cells have been poorly defined. Adhesion proteins, especially of the Leu-CAM family, are upregulated by cytokines (interleukin-1) or products of activated macrophages or mast cells such as tumor necrosis factor, histamines, and transforming growth factor β. Hemonec tin may belong to the class of adhesion proteins that are regulated by specific cytokine(s). In normal stromal cell lines, constitutive secretion of specific growth factors may regulate expression of hemonec tin. Lack of or altered cytokine(s) secretion in S/l/Sld mutant stromal cells in vivo or in vitro might directly or indirectly induce abnormalities in hemonec tin expression. Such a role has been predicted for mast cell growth factor (MGF), a new member of the hematopoietic growth factor family that stimulates mast cells and hematopoietic progenitors through the c-kit encoded receptor. The gene for MGF maps near the Sl locus. There is a quantitative difference in the production of this factor by cell lines established from normal and viable S/Sld mice, probably due to minor structural alterations in the mgf gene. It has been suggested that the Sl

Mast cell and macrophage lineages. In the S/l/Sld mice mast cells, granulopoiesis and erythropoiesis are more severely affected than megakaryopoiesis (MK). This pattern correlates with recent data that shows that, although hemonec tin has specificity for morphologically recognizable precursors of the granulocyte lineage, this specificity is bilincal at the progenitor cell level. Erythroid (burst-forming unit-erythroid) and granulocyte (colony-forming unit-granulocyte-macrophage [CFU-GM]) progenitors bind to hemonec tin, but CFU-MK do not.

Proliferation of stem cells in the stroma is an integral step in embryonic development. Hemonec tin may mediate the stroma-endothelial interactions that are involved in these early stages of development. Hemonec tin is also expressed on mast cells and megakaryocytes, which are important regulators of hematopoiesis. The expression of hemonec tin on these cells suggests a role in the regulation of hematopoiesis by mediating interactions between these cells and the stroma. However, the exact role of hemonec tin in the regulation of hematopoiesis and how it interacts with other adhesion molecules remains to be elucidated.
locus encodes more than one protein, a concept originally proposed as a basis for the pleiotropic effects of mutations at this locus, which are expressed in the BM, skin, and gonads. This theory is supported by recent evidence that among homozygous lethal mutants (Sl, Sp, and Sl(he)), in which mgf gene is completely deleted, the timing of embryonic death varies. Such observations could be explained by effects of Sl mutations on flanking genes encoding proteins important for development. Current studies characterizing the hemonectin gene and protein should determine whether expression of the hemonectin gene is regulated by cytokine(s) such as MGF.

REFERENCES

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