Hemonectin Mediates Adhesion of Engrafted Murine Progenitors to a Clonal Bone Marrow Stromal Cell Line From *Sl/Sl*^{d} Mice

By Pervin Anklesaria, Joel S. Greenberger, T.J. Fitzgerald, Brent Sullenbarger, Max Wicha, and Alan Campbell

Mutant *Sl/Sl*^{d} 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 *Sl/Sl*^{d} stromal cells or cloned stromal cell lines derived from them to form cobblestone-islands and proliferate. The role of hemonectin, a marrow-specific adhesion protein in the defective hematopoiesis of the *Sl/Sl*^{d} mice, was studied. Indirect immunoperoxidase staining of marrow in situ from *Sl/Sl*^{d} mice showed little specific staining while specific staining was seen in a pericellular distribution in marrow from +/+ mice. Hemonectin expression in several cloned stromal cell lines from *Sl/Sl*^{d} mice was compared by immunoblotting with that in cloned stromal cell lines from normal +/+ littermates. Cell line Sl^{d}3, which has the least hematopoiesis supportive capacity in vitro, showed no detectable hemonectin by immunoblotting, while Sl^{d}1 and Sl^{d}2 showed detectable but greatly reduced amounts compared with normal +/+ 2.4, GBl/6, and D2XRII. Confluent cultures incubated with purified hemonectin 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 (39.15 ± 3.6/dish; 16.3 ± 3.1/dish, respectively), compared with untreated Sl^{d}3 cultures (cobblestone-islands 8.1 ± 3.6/dish; CFU-s forming progenitors 8.8 ± 0.05/dish). Hemonectin-mediated progenitor cell binding to the Sl^{d}3 stromal cells was specifically inhibited by antihemonectin but not by preimmune serum. These data support the role of hemonectin in early progenitor-stromal cell interactions.

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STROMAL CELLS, their extracellular matrix (ECM) proteins, and secreted cytokines create an "inductive microenvironment" within the bone marrow (BM) that influences adhesion, proliferation, and differentiation of hematopoietic stem cells.[1,2] Cell surface proteins expressed by marrow progenitor cells have been shown to act as homing receptors for sugar molecules on the supportive marrow stromal cells.[3] The ligand(s) for these receptors have yet to be defined. Hemonectin a 60-Kd protein associated specifically with the BM may be a putative ligand for these receptors. It is immunologically distinct from other matrix proteins and acts as an attachment molecule for cells of the granulocyte lineage[4] as well as CFC-GM and BFC-e forming progenitors. This attachment has been shown to be developmentally regulated.[5] However, the role of hemonectin in mediating adhesion of stem cells to stroma has not been studied.

Sl/Sl^d mice express a genetic mutation that is manifested by severe anemia and neutropenia, associated with an intrinsic defect in the marrow microenvironment.[6-9] Primary explants of marrow stromal cultures and cloned stromal cell lines (Sl^{d}1, Sl^{d}2, and Sl^{d}3) established from *Sl/Sl*^{d} mice reproduce this microenvironmental defect in vitro and exhibit a significantly reduced capacity to support stem cell adhesion and proliferation compared with cell lines established from +/+ littermates (+/+ 2.4 and +/+ 1.0).[10-13] The cloned stromal cell lines from +/+ and *Sl/Sl*^{d} long-term BM cultures (LTBMCs) were morphologically similar and no differences in their extracellular matrix proteins or cell surface proteoglycans have been detected.[14,15] In contrast, indirect immunoperoxidase studies of in situ marrow from *Sl/Sl*^{d} mice showed reduced levels of hemonectin, as compared with marrow from +/+ littermates. In addition, cloned marrow stromal cell lines established from LTBMCs of *Sl/Sl*^{d} mice showed reduced levels of hemonectin protein.

In the present studies we used cloned stromal cell lines from *Sl/Sl*^{d} mouse LTBMCs to investigate the role of hemonectin in mediating adhesion of stem cells to stromal cells. One stromal cell line Sl^{d}3 incubated with purified hemonectin demonstrated a significant increase in sustained adhesion of added enriched hematopoietic progenitor cells, an effect that was inhibited by specific antihemonectin antiserum. These results indicate that hemonectin is involved in the adhesion of stem cells to stromal cells.

MATERIALS AND METHODS

Mice. *Sl/Sl*^{d} and normal littermate mice (+/+) were obtained from Jackson Labs, Bar Harbor, ME.

Cell lines. Derivation and characterization of clonal BM stromal cell lines +/+ 2.4, Sl^{d}1, Sl^{d}2, Sl^{d}3, and D2XRII has been described.[10,13] The Bl/6embC fibroblast cell line was obtained from Dr Stuart Aaronson, National Cancer Institute, Bethesda MD.

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 (wt/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 antihemonectin antibody,[4] followed by peroxidase conjugated goat anti-guinea pig secondary antibody, then developed using aminoethylcarbamasol and counterstained with Mayers hematoxylin.[16] Under these conditions the reaction product appears red

From the Department of Radiation Oncology, University of Massachusetts Medical School, Worcester; the Division of Hematology/Oncology, University of Michigan; and the Division of Hematology Oncology, V.A. Medical Center, Ann Arbor, MI.

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Address reprint requests to Pervin Anklesaria, PhD, Radiation Oncology, University of Massachusetts Medical Center, 55 Lake Ave N, Worcester, MA 01655.

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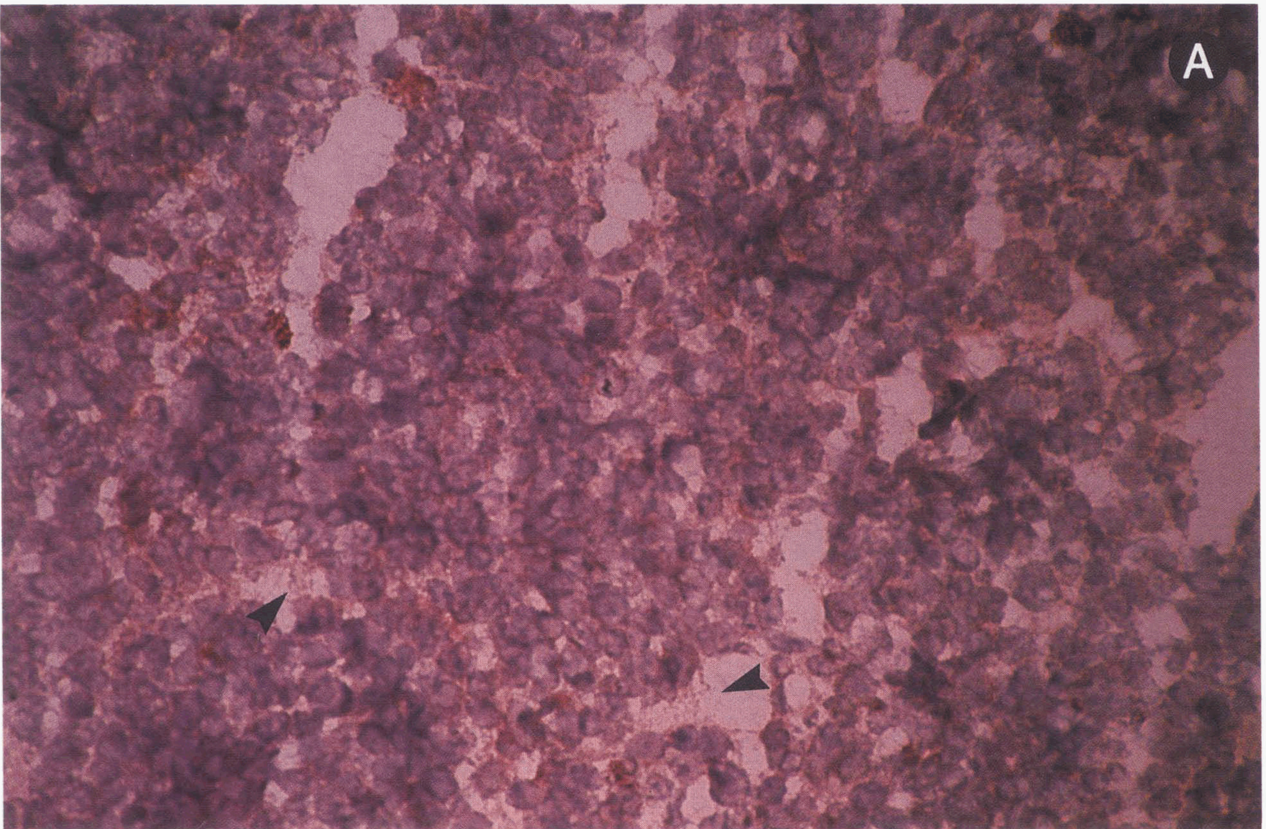


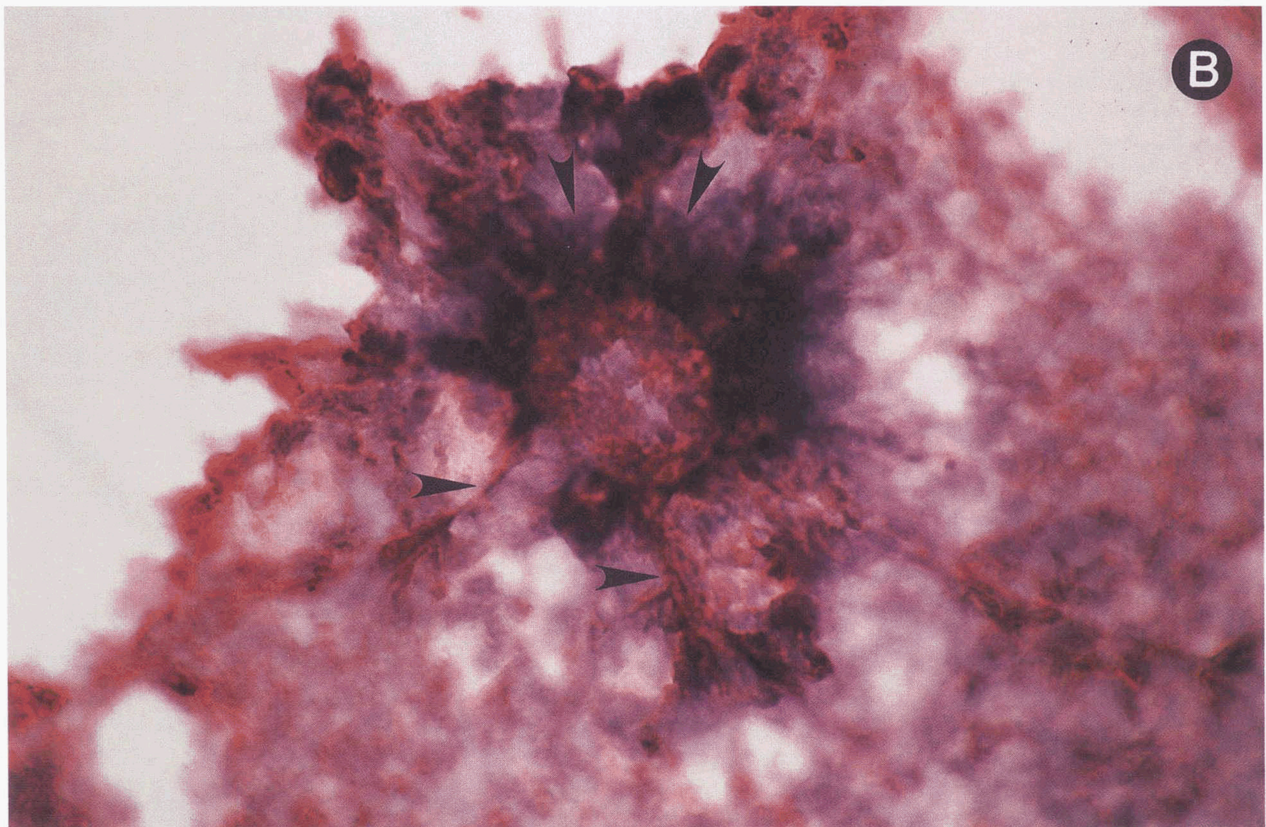
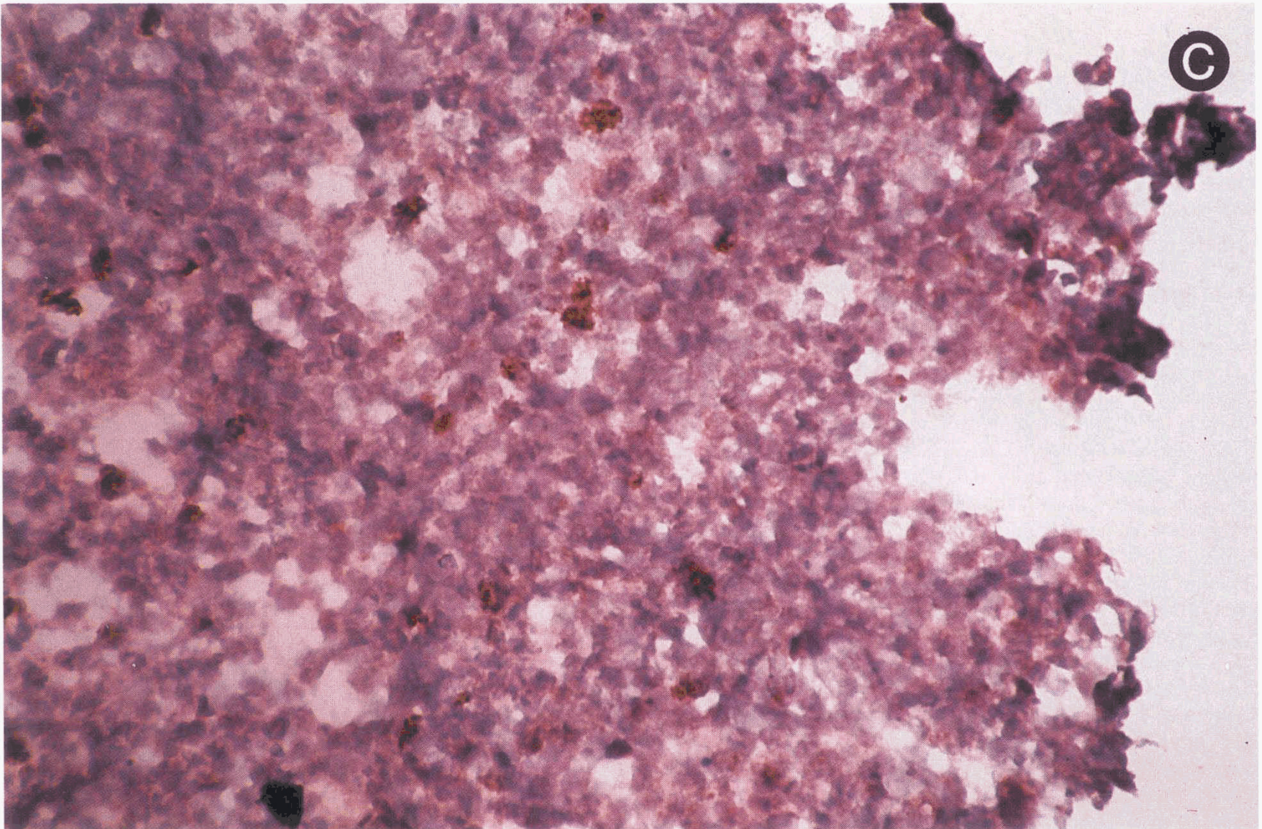


Fig 1. Immunohistochemical localization of hemonectin in situ. *Sl/Sl*^{d} or normal +/+ littermate BM was snap frozen and cryostat sections stained by the immunoperoxidase technique as described in Materials and Methods. (A) *Sl/Sl*^{d} marrow stained with antihemonectin. Faintly discernible extracellular staining is seen, indicated by arrows. (B) Higher power view of +/+ marrow stained with antihemonectin. A venous sinus is seen in cross section. Antihemonectin-stained reticular processes extend outward from the adventicium (arrows), which is partly surrounded by hematopoietic cells, identified by hematoxylin stained nuclei (arrow, original magnification ×800).



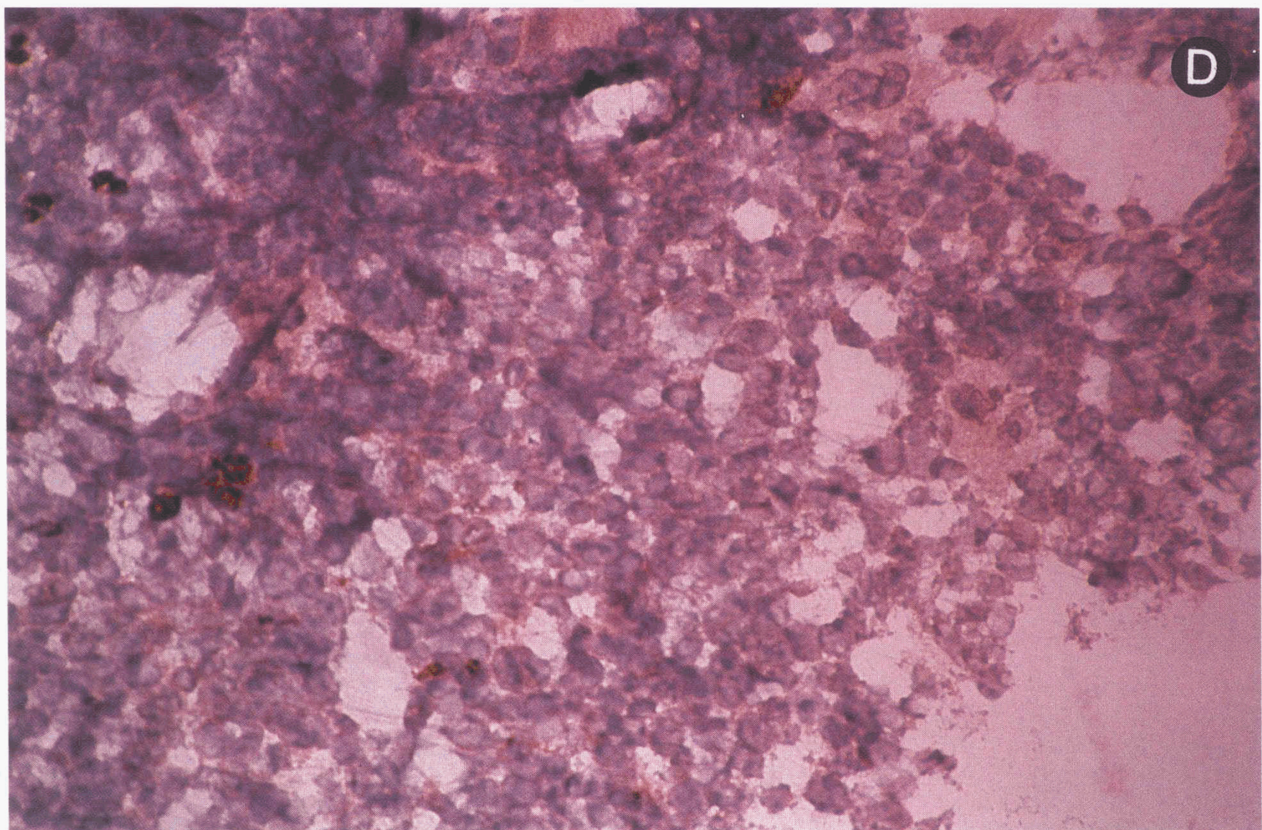


Fig 1. (Cont'd) (C) *Sl/Sl*[d] marrow control section stained with antihemonectin in the presence of soluble antigen. Little or no extracellular staining is seen (original magnification ×500). Some focal cytoplasmic staining is seen resulting from incompletely suppressed endogenous peroxidase. (D) +/+ Marrow control section stained with antihemonectin 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 deletion of both primary and secondary antibodies.

Immunoblot analysis of stromal cell lysates using specific antihemonectin antiserum. Each indicated stromal cell line was grown to confluence, washed three times in serum-free media, and dissolved in 0.05 mol/L Tris buffer pH 7.4 containing 1% wt/vol deoxycholate, 0.5% (vol/vol) NP-40, 0.15 mol/L NaCl, leupeptin 1 μg/uL, Trypsin inhibitor 10 μg/L. Equal microgram quantities of lysates were then run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose.[17,18] The blots were then developed using 1:1000 dilution of antihemonectin antiserum prepared as described[4] 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 Sl^d3 (35-mm dish; Falcon, NJ) were incubated with indicated concentrations of chromatographically purified hemonectin[5] 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% Co_2. Untreated Sl^d3 cultures were incubated similarly without hemonectin. After incubation, cultures were rinsed and engrafted with 2 to 3 × 10^6 enriched progenitors from day 40 to 60 C57Bl/6 LTBMCs.[10,19] Cobblestone-islands, defined as adherent cell foci formed by cocultivated progenitors on stromal cells[9] (more than 25 cells), were counted at indicated times in 70 microscopic fields and the counts were then cumulated for 21 days.[10] 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 5FU-injected C57Bl/6 mice (5FU-BM) as described.[20] Adherent cells from 5FU BM were removed by passing through a Sephadex G-10 column (Pharmacia LKB, Piscataway, NJ).[21] Each confluent culture of Sl^d3 cells (35-mm dish, Falcon) either treated with hemonectin (as described above) or untreated were cocultivated with 5 × 10^4 BM cells. Plastic dishes (35-mm, Falcon) were coated with either 0.1 mL/dish of matrigel[22] (Collaborative Research, Bedford, MA) or 10 μg/dish hemonectin. Cobblestone-islands formed were counted as described above. CFU-s forming progenitors were quantitated by injecting nonadherent cells harvested into lethally irradiated C57Bl/6 mice (900 cCy).[23]

Specificity of hemonectin-mediated progenitor-stromal cell interaction. Specific antihemonectin IgG was prepared as described.[4] Antihemonectin was added every 48 hours at 25, 50, and 100 μg/dish to Sl^d3 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 Sl/Sl^d and +/+ littermates. To compare expression of hemonectin in Sl/Sl^d 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 Sl/Sl^d 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 Sl/Sl^d 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 marrow stromal cell lines. Although LTBMCs established from Sl/Sl^d mice may lack some cellular components of intact marrow, both primary stromal cell explants and cloned BM stromal cell lines (Sl^d1, Sl^d2, and Sl^d3) demonstrate significantly reduced sustained adhesion and proliferation of hematopoietic progenitor cells as compared with cloned cell lines from +/+ LTBMCs.[10,13] Immunostaining of primary marrow cultures from Sl/Sl^d and +/+ mice with specific antihemonectin antibody confirmed the in situ staining pattern. In the Sl/Sl^d-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 Sl/Sl^d mice. Immunoblotting of cell extracts from the Sl^d3 cell line showed no detectable hemonectin, while extracts from Sl^d1 and Sl^d2 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, D2XRII, and Bl/6embC[10,13,19] 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 Sl^d3 cells was observed by indirect immunostaining[14] (data not shown). Thus, both in situ and in vitro, hemonectin in marrow stromal cells was greatly decreased in Sl/Sl^d 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 Sl^d3 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.[24,25] Multilineage and committed progenitors are then released for several weeks from these cobblestone-islands.[24] The ability of stromal cell lines 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.[24,25] The Sl^d3 stromal cell line has been shown to support less than 1% of engrafted hematopoietic progenitors as compared with the +/+ 2.4 cell line.[10,13] We next tested the ability of chromatographically purified hemonectin[5] to improve the hematopoiesis-supportive ability of the Sl^d3 cell line by quantitating progenitor cell adhesion to these stromal cells. For these experiments we first tested the ability of the Sl^d3 cells to bind exogenous hemonectin. Immunoblot analysis of pro-

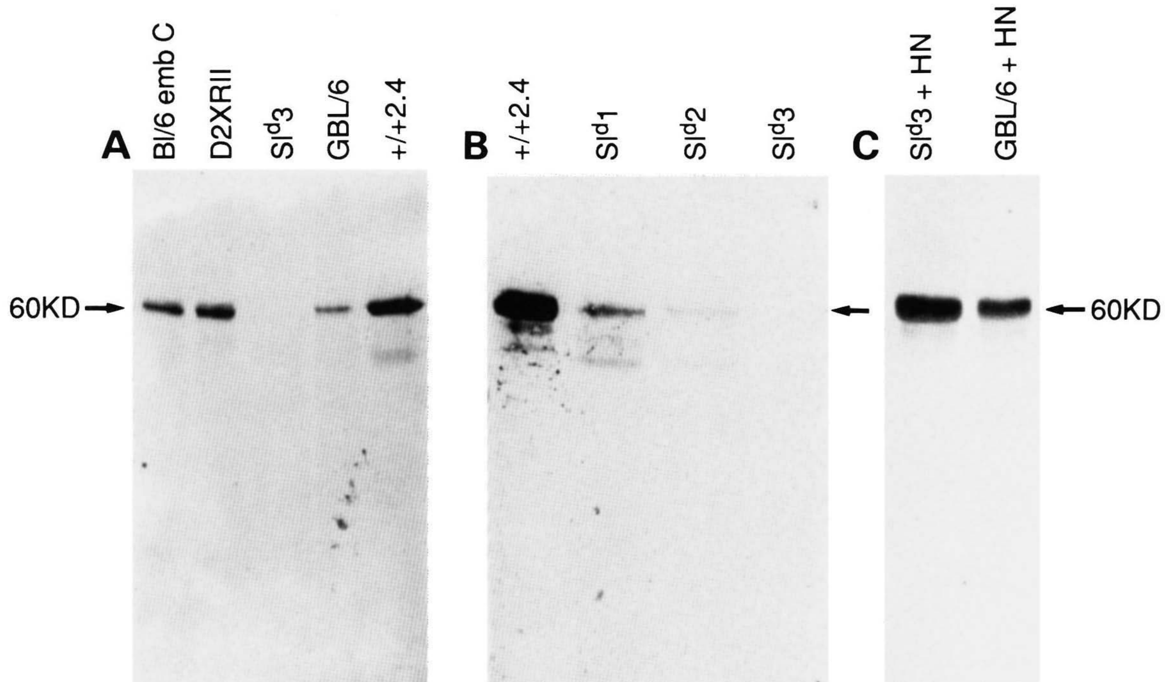


Fig 2. Immunoblot analysis of stromal cell lysates. (A) Comparison of hemonectin expression by five different stromal cell lines. BL/6emb C, D2XRII, GBl/6, and +/+ 2.4 all support hematopoietic progenitors while Sl^{d}3 does not. (B) Comparison of stromal cell lines +/+ 2.4 derived from the normal littermate with three different cloned stromal cell lines derived from *Sl/Sl^d* 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 Sl^{d}3 cell lines. Sl^{d}1 and Sl^{d}2 show slightly more hemonectin expression than Sl^{d}3, but considerably less than the normal +/+ 2.4 cells. (C) Sl^{d}3 and GBl/6 cells after incubation with 10 μg purified hemonectin and cell extracts immunoblotted as above.

teins extracted from Sl^{d}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 Sl^{d}3 cells (Fig 2B). This result establishes the ability of the Sl^{d}3 stromal cells to bind exogenously added hemonectin.

Increasing concentrations of hemonectin (1 μg to 10 μg) added to confluent cultures of the Sl^{d}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 Sl^{d}3 cells was next compared with that of +/+ 2.4 stromal cell line. Hemonectin (10 μg/dish) was added weekly to confluent Sl^{d}3 or +/+ 2.4 cultures. Cobblestone-islands formed by progenitor cells on hemonectin-coated Sl^{d}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 Sl^{d}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 Sl^{d}3 dishes coated with hemonectin (55.8 ± 13.3 × 10^4/dish) compared to control Sl^{d}3 dishes (21.57 ± 4.5 × 10^4/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 × 10^4/dish) compared with untreated +/+ 2.4 cells (cobblestone-areas, 286.0 ± 24.1/dish; nonadherent cells, 137.5 ± 47.2 × 10^4/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.[20] Because cells of the granulocyte lineage have been shown to bind to hemonectin,[4] 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.[20] Accessory cells were depleted by passing cells through a Sephadex G-10 column.[20] Sl^{d}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 Sl^{d}3 cell line (8.1 ± 3.6/dish; Table 1). No detectable viable cells were harvested from plastic dishes coated with hemonectin or Matrigel[21] (Table 1).

Hemonectin-mediated progenitor cell adhesion to Sl^{d}3 cells is inhibited by specific antihemonectin antiserum. The specificity of hemonectin mediated progenitor cell binding to Sl^{d}3 stromal cells was further confirmed by adding different concentrations of specific antihemonectin antiserum.[4] The antiserum has been shown to be specifically against he-

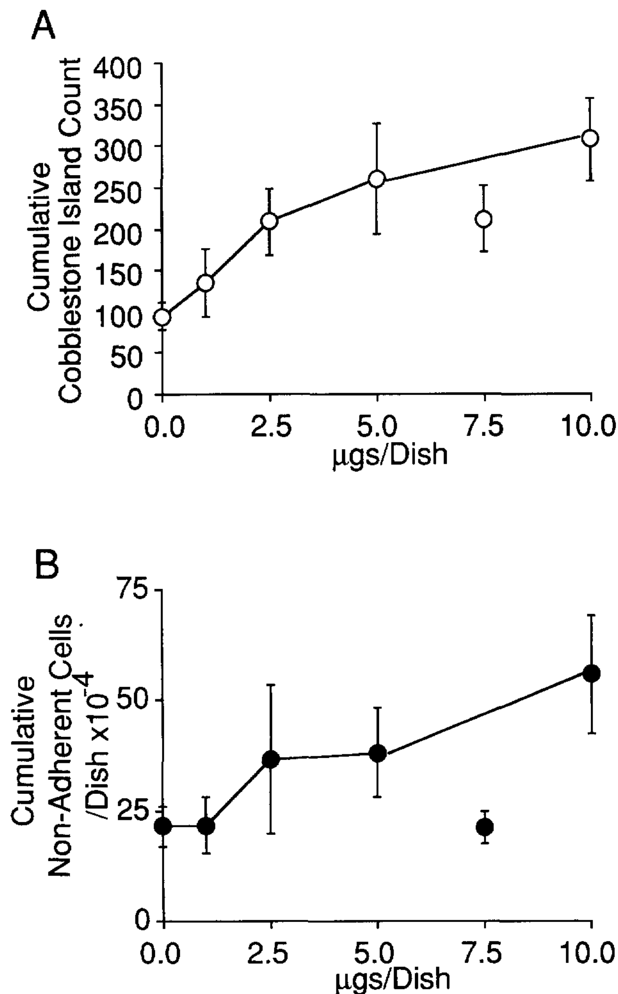


Fig 3. Effect of hemonectin on adhesion of hematopoietic progenitors to Sl^{d}3 stromal cells. Confluent cultures of Sl^{d}3 stromal cells (35-mm dish, Falcon) were incubated with indicated concentrations of purified hemonectin and engrafted with 2 to 3 $\times$ 10^6 enriched progenitors from LTBMCs. Cobblestone-islands (A) and nonadherent viable cells (B) were counted at weekly intervals and results are expressed as mean $\pm$ SEM (six dishes, representing two experiments).

monectin and did not crossreact with other matrix proteins.[4] Addition of 25, 50, and 100 µg/dish of specific antihemonectin IgG to hemonectin-treated Sl^{d}3 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 Sl^{d}3 cultures had no effect on the size or number of cobblestone-islands formed as compared with hemonectin-treated Sl^{d}3 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 *Sl/Sl^d* mouse provides an excellent model system with which to dissect the complexity of the BM microenvironment.[8-12] The data indicate that hemonectin expression is reduced in the *Sl/Sl^d* 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.[26] In the marrow microenvironment, although we cannot entirely exclude the possibility that the *Sl* 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 Sl^{d}3 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.[15] Similarly, no significant differences in the ECM protein expression between primary stromal cell explants of *Sl/Sl^d* and +/+ mice LTBMCs have been demonstrated.[27] In contrast, differences in the matrix from the skins of *Sl/Sl^d* and +/+ embryos was detected.[28]

It is interesting to note that the abnormal hematopoiesis of *Sl/Sl^d* 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.[6,29] The distinctive tissue distribution of hemonectin, present in BM but absent in spleen,[4] 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[3] further suggests that mechanism of stem cell adhesion to BM and spleen stroma is different. In addition, the effect of the *Sl* mutation is not uniform in all hematopoi-

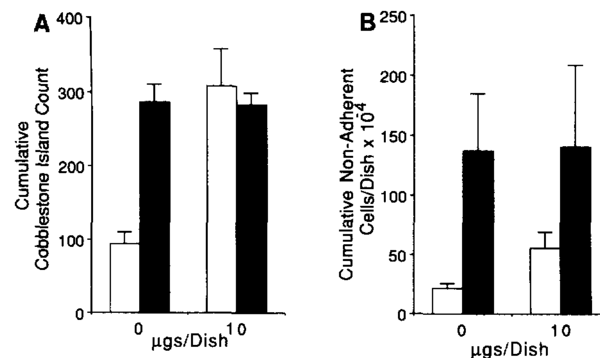


Fig 4. Sustained adhesion of hematopoietic progenitors to Sl^{d}3 stromal cells is mediated by hemonectin. (A) Adhesion and cobblestone-island formation by enriched hematopoietic progenitors on Sl^{d}3 (□) and +/+ 2.4 (■) cells, either treated (10 µg/dish) or untreated (0 µg/dish) with hemonectin. (B) Represents total nonadherent cells per dish harvested from the same set of cultures as in (A). Results are expressed as cumulative mean $\pm$ SEM over a period of 21 days. Data are representative of at least three experiments per group and three dishes per experiment.

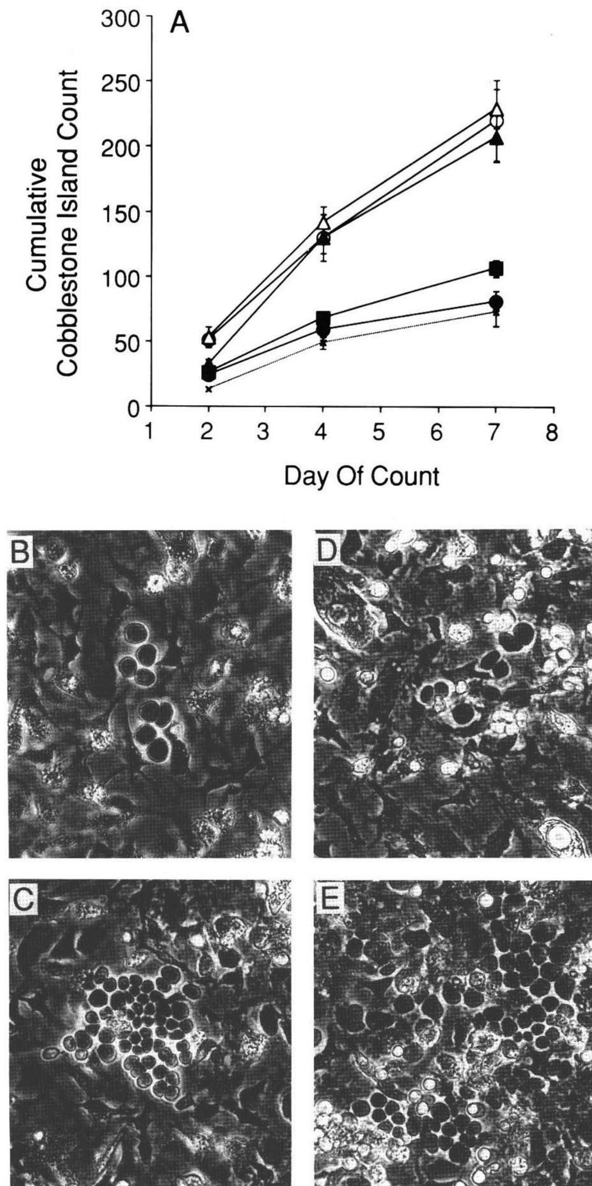


Fig 5. Specificity of hemonectin-mediated progenitor-stromal cell interaction. (A) Numbers of cobblestone-islands formed by nonadherent progenitors from C57Bl/6 LTBMCs (40 to 60 days old) on Sl^{d}3 dishes either treated with 10 μg/dish of hemonectin (△), or untreated Sl^{d}3 dishes (x). Nonadherent progenitors cocultivated with hemonectin-treated Sl^{d}3 cultures were treated every 48 hours with either 100 μg/dish preimmune IgG (○) or 25 μg/dish (▲), 50 μg/dish (■) or 100 μg/dish (●) of specific antihemonectin 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 Sl^{d}3 cultures, (C) hemonectin-treated Sl^{d}3 cultures, (D) hemonectin and antihemonectin IgG (100 μg/dish) treated Sl^{d}3 cultures, and (E) Sl^{d}3 cultures treated with hemonectin and preimmune IgG (100 μg/dish). Original magnification ×400.

etic lineages. In the *Sl/Sl^d* mice mast cells, granulopoiesis and erythropoiesis are more severely affected than megakaryopoiesis (MK).[6,29,30] This pattern correlates with recent data that shows that, although hemonectin has specificity for morphologically recognizable precursors of the granulocyte lineage, this specificity is bilineal at the progenitor cell level. Erythroid (burst-forming unit-erythroid) and granulocyte (colony-forming unit-granulocyte-macrophage [CFU-GM]) progenitors bind to hemonectin, but CFU-MK do not.[5]

Cell adhesion molecules play a crucial role in embryonic development, tissue organization, inflammatory response, and influence cellular activities such as differentiation and proliferation.[31] Proteoglycans in marrow stromal cells are known to affect stem cell proliferation and differentiation by serving as reservoirs for growth factors.[32] 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.[31] Hemonectin 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 hemonectin. Lack of or altered cytokine(s) secretion in *Sl/Sl^d* mutant stromal cells in vivo or in vitro might directly or indirectly induce abnormalities in hemonectin 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.[33] The gene for MGF maps near the *Sl* locus.[33] There is a quantitative difference in the production of this factor by cell lines established from normal and viable *Sl/Sl^d* mice, probably due to minor structural alterations in the *mgf* gene.[33,34] It has been suggested that the *Sl*

Table 1. Support of HPP-CFC by Sl^{d}3 Cell Line Incubated With Hemonectin

Enriched Progenitors Cocultivated With	Cobblestone-Islands	CFU-s
Sl^{d}3	8.1 ± 3.65	8.85 ± 0.05
Sl^{d}3 + hemonectin	39.15 ± 3.6*	16.3 ± 3.1*
Plastic coated with		
Hemonectin	ND	NT
Matrigel	ND	NT
Plastic alone	ND	NT

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 cCy)[20] with nonadherent viable cells recovered from untreated Sl^{d}3 (number of cells harvested/dish =6.9 ± 1.4 × 10^5) and hemonectin-coated Sl^{d}3 dishes (number of cells/dish = 12 ± 2.6 × 10^5) 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.

*$P < .05$.

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.[35] This theory is supported by recent evidence that among homozygous lethal mutants (*Sl*^{j}, *Sl*^{gb}, and *Sl*^{18H}) 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.

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