A Heparan Sulfate-containing Fraction of Bone Marrow Stroma Induces Maturation of HL-60 Cells in Vitro


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ABSTRACT

 Constituents of the bone marrow microenvironment have the capacity to influence both normal and malignant hematopoietic cell behavior. For example, HL-60 human promyelocytic leukemia cells in vitro display a more mature phenotype when grown on a bone marrow stroma-derived matrix. To elucidate which component(s) of the stromal matrix is capable of modulating HL-60 cell phenotype, matrices were treated with a variety of chemicals and enzymes prior to being used in the differentiation assay. Treatment of matrices with collagenase, pronase, chondroitinase, or chloroform/methanol/ether could not abolish the differentiation-promoting activity of bone marrow stroma. In contrast, the activity was destroyed by alkaline treatment (0.5 M NaOH for 18 h) or heparin/heparitinase enzymes. Heparin added to cultures increased maturation of HL-60 cells as determined by esterase production, Fc rosette formation, and morphological appearance. Other stromal components such as laminin, fibronectin, collagen I, collagen IV, or chondroitin sulfate did not alter the HL-60 leukemia cell phenotype. Stromata-derived matrix material which labeled with 35S sulfate and eluted on a DEAE ion-exchange column as a high ionic fraction in 1.5 M LiCl and 7.5% sodium dodecyl sulfate contained the active fraction. A heparan sulfate proteoglycan component isolated by polyacrylamide-agarose gel electrophoresis induced a more mature HL-60 phenotype, and digestion with heparinase/heparitinase in the presence of protease inhibitors abrogated the effects on HL-60 phenotype. We conclude that a heparan sulfate-associated fraction of the bone marrow matrix plays a key role in the regulation of leukemic cell maturation.

INTRODUCTION

The extracellular matrix, including collagen, glycosaminoglycan/proteoglycan, elastin, and glycoproteins, such as fibronectin and laminin (1), can influence the differentiation process of a variety of cell types (2, 3). Although malignant cells characteristically have lost a great deal of regulation, they also retain some dependence on their microenvironment (4, 5). One example of cell-matrix interaction is the influence of the bone marrow microenvironment on both normal (6) and malignant hematopoietic cell development (7).

The role of specific extracellular matrix components in regulating hematopoiesis remains unclear. Inhibition of collagen secretion by bone marrow stromal cells results in a reduction of hematopoietic progenitor cell production (8). However, collagen subtypes are ubiquitous and unlikely to account for tissue-specific stimulation of hematopoiesis that the bone marrow provides (8). Fibronectin has also been associated with developing hematopoietic colonies (9) and promotes the adhesion and differentiation of erythroid precursors (10, 11). Hemoscerin, a matrix protein found specifically in the bone marrow, promotes adhesion of cells of the granulocytic lineage (12). Spooncer et al. (13) reported that β-D-xylosides stimulate glycosaminoglycan production in bone marrow cultures with a concomitant increase in hematopoietic cell production. Changes in glycosaminoglycans in murine spleen and bone marrow during erythropoietic stimulation and suppression have also been described (14, 15). Exogenous glycosaminoglycans added to in vitro assays can inhibit erythroid colony formation (16), and enzymatic removal of glycosaminoglycans from leukocytes and bone marrow stromal cells by hyaluronidase or heparitinase interferes with leukocyte-stromal adhesive relationships (17). Recently, it has been reported that human hematopoietic blast colony-forming cells specifically bind bone marrow heparan sulfate and that this matrix proteoglycan may contribute to localizing hematopoietic stem cells to hematopoietic tissue (18). It has also been suggested that glycosaminoglycans produced by stromal cells can regulate hematopoiesis by binding hematopoietic growth and differentiation factors (19, 20).

We have recently demonstrated that the extracellular matrix produced by human bone marrow stromal cells can induce maturation of HL-60 human promyelocytic leukemia cells, a cell line that has been extensively used to study leukocyte maturation (21). Approximately 20% of cells adhere to bone marrow matrix, with the remainder appearing to be in close association (22). Forty % of HL-60 cells grown on matrix are able to induce Fc rosettes, a parameter of phagocytic leukocyte maturation, and 86% display nonspecific esterase activity, a marker of monocyte/macrophage-like differentiation (21). These phenotypic changes are reversible and dependent on the continued presence of the matrix molecules (21). The studies described in this report examine the role of the various matrix components in the regulation of HL-60 promyelocytic leukemia cell maturation.

MATERIALS AND METHODS

Chemicals. Collagenase, chondroitin ABC lyase, heparinase, fibronectin, heparin (H-3125), and chondroitin sulfate were purchased from Sigma Chemical Company (St. Louis, MO). Heparitinase and [35S]-sulfate were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). EN2HANCE was purchased from New England Nuclear (Wilmington, DE). Pronase was supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN). DEAE-Sephal was obtained from Pharmacia (Uppsala, Sweden) and DEAE ion-exchange paper from Bio-Rad (Richmond, CA).

Degradation of Bone Marrow Matrices. A bone marrow stromal cell line previously established in this laboratory (21) was grown to confluence in 25-cm2 tissue culture flasks containing RPMI 1640 supplemented with 15% heat-inactivated fetal calf serum and 50 μg/ml gentamicin (GIBCO, Grand Island, NY). After 6 days in stationary culture, the adherent layer was washed with PBS and exposed to 1.0% Triton X-100.

1 The abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; CME, chloroform/methanol/ether; DTT, dithiothreitol; GM-CSF, granulocyte-macrophage colony-stimulating factor.
X-100 (vol/vol) and subsequently washed with PBS to remove remaining nuclei and cytoskeleton elements (22). This material was discarded. The matrix was removed from the flask by exposure to 0.2% SDS at 37°C for 1 h, followed by extensive water washes. This material was precipitated with 4 volumes of 100% ethanol at −20°C for 6 h and centrifuged at 10,000 x g for 20 min. The precipitate was resuspended in water and treated with one of the following enzymes: 1 mg collagenase in RPMI (37°C, overnight), 0.5 units chondroitin ABC lyase (37°C, 6 h) (23), 0.03 IU heparinase (34°C, 1 h) (24) and 0.03 IU heparinase (43°C, 1 h) (24), or 1 mg pronase in PBS (37°C, overnight). Samples were then boiled to inactive the various enzymes. The solubilized matrix was also treated with 0.5 M NaOH for 18 h, neutralized, and dialyzed against water overnight. Other preparations of solubilized matrices were exposed to C:M:E (2:2:1) for 2 h. Boiled or dialyzed control matrices and matrices treated with enzyme buffers alone were also prepared. Each sample was then air dried on tissue culture dishes of similar surface area leaving a visible coating. Adding the material to suspension cultures of HL-60 had similar results to drying.

HL-60 Differentiation. To assess the effect of degradation of the various matrix components on HL-60 maturation, HL-60 cells were seeded at a density of 1 x 10⁶ cells in 10 ml media/fetal calf serum onto the various treated and control matrices. After 72 h in culture, cells in suspension were collected, and attached cells were detached by exposure to 2 mM EDTA for 3 min at 37°C, followed by gentle pipetting. Cell numbers were determined using a model ZF Coulter particle counter (Hialeah, FL). As previously reported, approximately 20% of HL-60 cells adhere to untreated matrix (21). Attachment to the other substrata was similar.

Suspension and attached cells were combined, and morphological maturation was assessed using Wright-Giemsa staining and nonspecific esterase staining (25). Induction of Fc rosettes was measured by a modification of the method of Bianco et al. (26).

Defined Matrices. HL-60 maturation was also measured in culture flasks coated with 40 µg/cm² purified collagen I from bovine skin (27), collagen IV (28), or laminin, both from the Engelbreth-Holm-Swarm tumor (29). Allies of human plasma fibronectin substrata have previously been reported (21). Heparin or chondroitin sulfate (40 µg/cm²) was added to the media of some cultures. Uronic acid content of the solubilized matrix was determined by the carbazole method of Bitter and Muir (30).

Results

Extracellular matrix produced by human bone marrow stromal cells promotes the maturation of HL-60 promyelocytic leukemia as evidenced by cell morphology, esterase activity, and formation of Fc rosettes (21). In an attempt to determine which components of this matrix were promoting maturation, the matrix was treated with a variety of degradation procedures and evaluated for activity. Cells grown on a bone marrow stroma-derived matrix treated with pronase, collagenase, chondroitin ABC lyase, or C:M:E had morphology similar to cells on untreated matrix with reduced nuclear-cytoplasmic ratio, irregular cytoplasmic borders, and cytoplasmic vacuoles. In contrast, treatment of the human bone marrow stromal cell-derived matrix with alkali or with heparinase/heparitinase produced material which failed to promote morphological maturation. Thus, Wright-Giemsa staining of cells grown in culture flasks with alkali-treated or heparinase/heparitinase-treated matrix demonstrated morphology similar to cells on plastic. Maturation of cells was also monitored under various conditions using a cytochemical reaction for nonspecific acid esterase. Cells grown on plastic, alkali-treated matrix, or heparinase/heparitinase-treated matrix showed no nonspecific acid esterase activity, although cells on untreated matrix or matrix treated with various other enzymes or C:M:E were positive, indicating no loss of matrix differentiation ability with these treatments. All slides were coded and read by independent observers. As can be seen in Fig. 1, the influence of bone marrow stroma-derived matrix on the ability of HL-60 cells to form Fc rosettes, another parameter of differentiation, was also sensitive to alkali or heparinase/heparitinase.

To assess the influence of individual extracellular matrix components on HL-60 cell phenotype, HL-60 cells were grown in the presence of collagen, laminin, or glycosaminoglycan, and maturation was assessed. As seen in Table 1, heparin induced differentiation of these cells as monitored by esterase activity in 42% of cells compared to 86% which was previously reported for the unfractionated bone marrow matrix. In contrast, only 7% of cells growing in suspension in plastic culture flasks demonstrated esterase activity. These heparin-treated cells also displayed changes in morphology, including irregular cytoplasmic borders and cytoplasmic vacuoles (Fig. 2c). Cells grown in the presence of chondroitin sulfate, collagen, or laminin did not display any similar change in esterase activity, although cells on laminin had some morphological changes in the cytoplasm. Also, approximately 40% of HL-60 cells grown in the presence of heparin induced rosette formation, similar to previous reports for bone marrow matrix (21). Cells from cultures incubated with collagen, laminin, or chondroitin sulfate did not form rosettes to any greater degree than control cells.

Since both the degradation and defined-matrices studies suggested that the heparin/heparan sulfate component of the ma-

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confluent stromal cells were grown in medium supplemented with 40 µCi/ml [35S]sulfate for 4 days. The Triton X-100/SDS-derived matrix was washed with 1.5 M LiCl in 0.05 M Tris/ HCl-7 M urea-10 mM DTT, pH 6.8, and eluted on a DEAE-Sephacel column in 7.5% SDS in 1.5 M LiCl and the same buffer. The inability of 1.5 M LiCl with no SDS to elute the radiolabeled material from the column appears to be due to a phospholipid component of the macromolecule, since phospholipase C-treated material is eluted in 1.5 M LiCl. The structure of this molecule is currently being investigated. When HL-60 cells were cultured on substrata coated with the DEAE-separated [35S]sulfate-labeled matrix fraction, 42% demonstrated esterase activity and 56% formed Fc rosettes. Changes in HL-60 cell morphology similar to those previously described on the complete matrix were observed (Fig. 2b).

Autoradiography of a polyacrylamide-agarose gel of the ion-exchange-separated matrix is shown in Fig. 3. Less than 10% of the counts remain at the top of the gel (band 1) and in addition there are 2 areas (2 and 3) of radioactivity which migrate faster than a reference rat chondrosarcoma proteoglycan but slower than chondroitin sulfate chains. The material from the 3 areas was transferred to DEAE paper, eluted, and individually tested for activity on HL-60 cells. Only band 2 displayed an effect on HL-60 phenotype. Fifty-five % of HL-60 cells grown on this substrata were able to form rosettes and 38% displayed esterase activity. HL-60 cells exposed to material from band 2 also appeared morphologically more mature than cells on plastic with irregular cytoplasmic borders and cytoplasmic vacuoles. Cells grown on material from regions 1 or 3 did not demonstrate esterase activity or rosette formation to any greater extent than cells on plastic.

Autoradiography of an SDS-polyacrylamide gel electrophoresed under reducing conditions of the band 2 active material from Fig. 3 is shown in Fig. 4, lane A. Lane A contains a wide band of radioactivity at the top of the gel. Such heterogeneity is common with proteoglycans due to differential glycosylation. This material is susceptible to heparinase/heparitinase digestion in the presence of protease inhibitors as demonstrated in lane B. The material at the bottom of lane A may represent proteoglycan fragments formed during processing of the band 2 material and faster eluting digested material is present in lane B.

EFFECT OF HEPARAN SULFATE ON HL-60 CELLS

Table 1 Influence of substrate on HL-60 cell phenotype

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Esterase* (%)</th>
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<tbody>
<tr>
<td>Chondroitin sulfate</td>
<td>2平</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>2平</td>
</tr>
<tr>
<td>Type IV collagen</td>
<td>15</td>
</tr>
<tr>
<td>Heparin</td>
<td>42平</td>
</tr>
<tr>
<td>Laminin</td>
<td>15平</td>
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* HL-60 cells were collected 72 h after seeding and were assayed. Mean of duplicate flasks in a minimum of 2 experiments with 200 cells scored by two independent observers per flask. SEM < 5%.

Fig. 2. Photomicrograph of HL-60 cells under various culture conditions showing the morphological appearance of HL-60 cells grown on uncoated plastic (a), bone marrow-derived heparin/heparan sulfate-coated flasks (b), and in the presence of commercial heparin (c) 72 h after seeding. Slides were prepared by using a Shandon-Elliott cytopsin centrifuge (Wright-Giemsa, x 1200; bar, 50 µm).

Fig. 3. Autoradiography of the polyacrylamide-agarose gel electrophoresis of the ion-exchange separated [35S]sulfate-labeled human bone marrow matrix. RCPG, rat chondrosarcoma proteoglycan; CS, chondroitin sulfate.

Fig. 4. Autoradiography of an SDS-polyacrylamide gel electrophoresed under reducing conditions of the band 2 active material from Fig. 3 is shown in lane A. Lane A contains a wide band of radioactivity at the top of the gel. Such heterogeneity is common with proteoglycans due to differential glycosylation. This material is susceptible to heparinase/heparitinase digestion in the presence of protease inhibitors as demonstrated in lane B. The material at the bottom of lane A may represent proteoglycan fragments formed during processing of the band 2 material and faster eluting digested material is present in lane B.


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B. HL-60 cells grown on aliquots of the lane B material appear morphologically similar to cells grown on plastic and demonstrate no greater ability to form rosettes than cells grown on plastic. In contrast, treatment of this proteoglycan-enriched material with chondroitin ABC lyase or pronase does not abrogate the effects on HL-60 phenotype in that rosette formation and esterase activity are 100% of cells grown on undigested material.

DISCUSSION

Extracellular matrix produced by human bone marrow stromal cells can induce a more mature phenotype and inhibit proliferation of HL-60 human promyelocytic leukemia cells in vitro (21). The current studies demonstrate that this activity in the stromal matrix is associated with the presence of heparin/heparan sulfate proteoglycan. It has previously been suggested that glycosaminoglycans/proteoglycans can regulate hematopoiesis by binding hematopoietic cell growth factors, such as GM-CSF (19). Heparan sulfate from mouse bone marrow binds GM-CSF and interleukin 3 and presents them in biologically active form to hematopoietic cells (20). No mRNA for GM-CSF and interleukin 3 was detected in our bone marrow stromal cells4, studies of the effects of recombinant human GM-CSF and G-CSF on HL-60 cells demonstrated no morphological maturation (34), and growth factors such as GM-CSF disassociate from heparin in the presence of a high salt concentration, such as the method used for the ion-exchange chromatography purification (19, 35). Also, the maturation-promoting activity was abolished by heparinase/heparitinase enzyme treatment of the active proteoglycan-enriched fraction in the presence of protease inhibitors. Commercially available heparin from porcine intestinal mucosa demonstrated similar maturation-promoting activity to that of the bone marrow-derived heparin/heparan sulfate proteoglycan-associated fraction. Some differences in activity of bone marrow-derived heparin/heparan sulfate and pig intestinal mucosal heparin, such as the differences in necessary concentrations for activity reported here, may be anticipated, since heparin chains from different sources have differences in sulfation which may contribute to the activity (36). Also, the proteoglycan core protein may allow a local concentration of glycosaminoglycans not present in alkali-treated matrix or free heparin chains. The results suggest that heparin/heparan sulfate is associated with activity that can induce a more mature phenotype in HL-60 cells. Although proteoglycan could act directly, it may also act by inducing HL-60 to produce an autonomous differentiation factor perhaps similar to one produced by HL-60 cells treated with 1,25-dihydroxyvitamin D3 or dimethyl sulfoxide (37). We have previously reported that conditioned media from HL-60 and stromal cell cocultures, but not from stromal cells alone, can alter the phenotype of other HL-60 cells (21). We are currently investigating the mechanism of proteoglycan activity and characterizing the proteoglycan molecule to exclude any possibility of an active bound protein contaminant.

Heparan sulfate is found in the plasma membrane and extracellular matrix of most mammalian cells and, thus, is strategically located to regulate cell-microenvironment interactions (38). Cell membrane heparan sulfate by interacting with polymerized actin may provide a means of communication between the cell and stroma (39). Noordegraaf et al. (40) have suggested that glycosaminoglycan metabolism may be important in the interaction of hematopoietic cells and stromal cells. In their pericellular location, heparin and heparan sulfate control proliferation of a variety of cell types (41–43). Heparan (41) and heparan sulfate (42) inhibit smooth muscle cell proliferation. Heparan sulfate proteoglycan produced from liver plasma membrane can inhibit growth of hepatoma cells in culture (43). Although the exact mechanism of growth inhibition remains unknown, it has been suggested that internalized glycosaminoglycans block the transition of the cell from G0/G1 to S-phase (44). Since heparin and heparan sulfate are distinct polysaccharide structures and heparin is a product of mast cells (38), our bone marrow matrix proteoglycan produced by a stromal cell line will be referred to as heparan sulfate.

We have been interested in the role of glycosaminoglycans/proteoglycans in cell differentiation (45–47). A decrease in cellular glycosaminoglycans is associated with the induction of differentiation of HL-60 human promyelocytic leukemia cells (45). Glycosaminoglycans change during differentiation of a variety of cell types (48, 49). Neuron maturation is accompanied by increased cell surface heparan sulfate (50). Rat sympathetic neurons grown on endothelial cell-derived extracellular matrix form neurites (51). Similar to the present study, the active endothelial matrix factor is susceptible to heparinase digestion (51). Although, in contrast to our results, neurite outgrowth was not promoted by heparin or heparan sulfate glycosaminoglycan, neurite outgrowth was only assayed on surfaces coated with <1 μg/cm². However, the neurite-promoting matrix factor was trypsin sensitive (51).

In summary, a heparan sulfate-associated fraction of human bone marrow matrix can influence myeloid leukemia cell growth and differentiation. Future studies will attempt to more fully characterize these macromolecules.

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