RAPID COMMUNICATION

Discovery of Novel Hematopoietic Cell Adhesion Molecules From Human Bone Marrow Stromal Cell Membrane Protein Extracts by a New Cell-Blotting Technique

By Bearelli Seshi

In an attempt to define the role of cell adhesion molecules (CAMs) within the bone marrow (BM) microenvironment in normal hematopoiesis and in leukemia development, a novel cell-blotting technique that involved cell adhesion to protein bands after separation by lithium dodecyl sulfate-polyacrylamide gel electrophoresis (LDS-PAGE) and blotting onto polyvinylidene difluoride (PVDF) membrane has been developed. Human BM stromal cell membrane fractions have been prepared by Dexter-type cultures after cell lysis by sonification and differential centrifugations of the sonification contents. The 20,000 g pellets representing membrane fractions have been solubilized by 2% Triton X-100, 0.575% LDS, and 8 mol/L urea in sequential order. The protein extracts are fractionated by LDS-PAGE and screened for CAMs by the new cell-blotting technique. This led to identification of nine protein bands in lanes containing LDS extracts showing adhesion of KG1a (CD34+ progenitor myeloid) cells. Evidence that the BM proteins exhibiting KG1a cell adhesion are novel CAMs is based on the observations that these proteins, in comparison with known CAMs, specifically VCAM-1, CD54, and CD44, show (1) contrasting detergent-solubility properties, (2) different temperature requirement for mediating cell adhesion function, and (3) markedly distinct electrophoretic mobilities. The various cell types tested, notably KG1a, NALM-6, WIL-2, Ramos, HS-Sultan, K562, JY B lymphoblastoid cells, and T lymphoblasts, showed distinctive patterns of binding to different subsets of BM CAMs. These results demonstrate a new approach to studies of molecular mechanisms that may determine specificity of hematopoietic cellular localization within BM microenvironment and may play an important role in controlling hematopoiesis.

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However, use of whole cells or crude mixtures of proteins as immunogens may present the problem of antigenic competition, the situation in which one antigen can suppress the response to another and consequently antibodies may not form for antigens of interest.13,14 Even if MoAbs are developed, they may not have functional blocking activity, or even if they have the blocking activity, they may not identify the molecule of interest at the biochemical level for the following reason. The conventional technique that has been used for biochemical detection of most of the known CAMs—such as ELAM-1 (endothelial-leukocyte adhesion molecule-1),13 CD54,13 ICAM-2 (intercellular adhesion molecule-2),16 VCAM-1,11 VLA-4,17 CD44,18 and CD6119—is dependent on extraction of cell-surface-labeled molecules by means of nonionic detergents such as NP-40 (nonidet P-40) or Triton X-100 before immunoprecipitation20 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, the standard technique would not be able to identify those CAMs that are not solubilized by Triton X-100 or NP-40.

My laboratory has a long-standing interest in CAMs and has been seeking to develop a general technique for identification of such molecules.21 A novel cell-clotting technique involving lithium dodecyl sulfate (LDS)-PAGE22 and polyvinylidene difluoride (PVDF) blotting membrane23 has recently been developed (manuscript in preparation). With the aim of exploring the existence of such CAMs in BM microenvironment, LDS is similar to SDS in providing more complete solubilization of membrane proteins than Triton X-100 or NP-40, with the added advantage that all steps can be conducted at 4°C, thus avoiding thermal denaturation of proteins. Furthermore, the electrophoretic system uniquely preserves functional adhesion protein complexes and removes detergents from proteins during electrophoresis and subsequent blotting, thereby promoting renaturation of proteins before the cell-adhesion assay (manuscript in preparation). The present communication reports on an array of new hematopoietic CAMs, expressed by BM stromal cells and solubilized by LDS, which may mediate cell adhesion between HPC and BM stroma, not accounted for by known CAMs.21

MATERIALS AND METHODS

Reagents. A sample of recombinant soluble form of VCAM-1 (rs VCAM-1) in phosphate-buffered saline (PBS) was kindly provided by Dr Roy Lobb (Biogen, Inc, Cambridge, MA). It was aliquoted and stored frozen at −40°C.

All cell culture media were purchased from GIBCO-BRL (Grand Island, NY). Hydrocortisone and α-methyl mannoside were obtained from Sigma (St Louis, MO).

Tris-(hydroxymethyl)aminomethane, ATP (adenosine triphosphate), CHAPS (3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate), EGTA, PMSF (phenylmethylsulfonyl fluoride), and leupeptin were purchased from Sigma; 0.5 mmol/L EDTA was from Bio-Lab (Cleveland, OH); Triton X-100 and ultrapure urea were from Bio-Rad (Hercules, CA); ultrapure LDS was from Sigma (Schwarz/Mann Biotech, Cleveland, OH); glyceral was from Pharmacia LKB (Piscataway, NJ); DTT (dithiothreitol) was from Fisher Biootech (Fisher Scientific, Fair Lawn, NJ); and PBS-CMF (PBS without calcium and magnesium) and PBS(+) (PBS containing calcium and magnesium) were from GIBCO-BRL.

Anti-VCAM-1 mouse MoAb (MoAb 4B9) was purchased from Genzyme (Cambridge, MA), and anti-CD54 (clone 84H10) and anti-CD44 (clone J-173) were obtained from AMAC, Inc (Westbrook, ME). In view of endogenous alkaline phosphatase in stromal cell membrane extracts, an immunoperoxidase method, instead of immunoalkaline phosphatase, was used. Horseradish peroxidase-conjugated rabbit antismouse IgG was purchased from Cappel (Organon Teknika, Durham, NC) and 3-aminoo-9-ethyl-carbazole (AEC) was from Vector (Burlingame, CA).

Cell culture. Dwyer-type stromal cell cultures were grown as described24,25 from BM samples obtained from normal adult donors with informed consent. The stromal cell cultures were set up using CD34+ subset of BM light density cells obtained after an immunofluorescence column separation,26 platting approximately 33 to 35 × 106 cells per 7-75 flask (75-cm² flask). The culture medium consisted of McCoy’s 5A medium with HEPES/12.5% FBS/12.5% horse serum/1 μmol/L hydrocortisone/1% penicillin/streptomycin. In about 2 weeks, when monolayers had grown to confluency, two flasks of primary cultures derived from a given donor were trypsinized and passage into six flasks. After another 2 weeks, monolayer cultures were harvested for protein extraction as described below. Human leukemic cell lines, ie, KG1a (CD34+ myeloid progenitor), NALM-6 (B-lymphoid progenitor), JY (B lymphoblastoid), Ramos (Burkitt’s B-lymphoid), HS-Sultan (plasmacytoma), and K562 (erythroleukemia) were maintained in RPMI 1640/95% FBS/1% penicillin/streptomycin. WI-2 (mature B-lymphoid) human leukemic cells were maintained in Iscove’s medium/9% FBS/1% penicillin/streptomycin. JY B-lymphoblastoid cell line was kindly provided by Dr John Looney (University of Rochester) who obtained it from Dr Julia Greenstein (Dana-Farber Cancer Institute, Boston, MA). NALM-6 cells were obtained from Dr Tucker LeBien (University of Minnesota, Minneapolis) and maintained in our laboratory. All other cell lines are available from the American Type Culture Collection (ATCC) repository (Rockville, MD). B lymphoblasts were derived by culturing peripheral blood mononuclear cells obtained from a normal adult donor in presence of concanavalin A (2.5 μg/mL RPMI 1640/10% FBS/1% penicillin/streptomycin) for approximately 70 hours.27 Before their use as target cells, a sample was stained and verified microscopically for blast transformation. Remaining cells were washed twice in PBS(+) containing 5 mmol/L α-methyl mannoside to neutralize any residual Con A and then twice in plain PBS(+) without α-methyl mannoside.

Cell lysis and preparation of the membrane fraction. An alkaline buffer consisting of 100 mmol/L KCl/5 mmol/L MgCl(1 mmol/L ATP)/25 mmol/L Tris-HCl, pH 9.6 was used as the cell lysis buffer.28 Medium from visibly confluent monolayer cultures (usually a batch of six T-75 flasks) was poured off; 10 mL of cold PBS-CMF was added to each flask; the monolayers were scraped using a rubber policeman; and fragments were collected into a 50-mL centrifuge tube using a 10-mL pipet. Any remaining fragments were recovered by adding an extra 10 mL of PBS-CMF.

Contents from 2 culture flasks were transferred to one 50-mL centrifuge tube and were washed by centrifugation for 10 minutes at 500g (1,500 rpm, Beckman TJ-6, TH-4 rotor; Beckman Instruments, Palo Alto, CA). Supernatants were discarded and the pellets were suspended in approximately 2 mL of lysis buffer and pooled into one 15-mL centrifuge tube. An extra 6 mL of lysis buffer was added and washed by centrifugation was repeated. Finally, a cell pellet equivalent of one T-75 flask of stromal cells was resuspended in the 15-mL centrifuge tube in 1 mL of pre chilled lysis buffer. It proved necessary to lyse cells in a Branson 250 sonifier (Branson Ultrasonics, Danbury, CT) because they could not be lysed by gentler methods, for example, suspending in a hypotonic buffer. To minimize generation of heat and prevent any foam during sonication, the sample tube was immersed in ice/salt/water bath at least up to the sample level. At a microtip setting of 6, two bursts of sonification, each of 10 seconds’ duration, were applied with an interval of 1 to 2 minutes between them. Sonification contents were spun at 600g
in a refrigerated centrifuge at 4°C for 10 minutes (Sorvall RT6000B; Du Pont, Wilmington, DE). The 600g pellet containing nuclear material and any unbroken cells was discarded. The supernatant (~6 mL) was distributed into two or three 12×75-mm polypropylene tubes (Sarstedt, Newton, NC) and centrifuged at 20,000g for 20 minutes at 4°C in a Sorvall centrifuge (RC-5; Superspeed Refrigerated Centrifuge). The 20,000g supernatants comprising of the cytosol proteins were discarded and the pellets encompassing the membrane fraction were saved as P-20s.

Electron microscopy of P-20 pellets. P-20 pellets were prepared as described above from monolayers of stromal cells that were harvested by mechanical scraping. They were fixed in 2.5% glutaraldehyde in Millonig’s buffer for 3 hours, washed with the same buffer for 2×15 minutes, and postfixed in 1% osmium tetroxide for 30 minutes, dehydrated through graded alcohols, and propylene oxide, and embedded in Medcast resin (Ted Pella, Inc, Redding, CA). Ultrathin sections of pellets, stained with uranyl acetate and lead citrate, were examined in a Hitachi H-7100 electron microscope (Hitachi, Danbury, CT).

Sequential extraction of membrane proteins from P-20 pellets. The P-20 membrane pellets (prepared from monolayers after mechanical scraping, and involving no trypsinization) were sequentially solubilized in a series of three extraction buffers containing different detergents. The buffers consisted of (in sequential order): 2% (wt/vol) Triton X-100 in PBS-CMF; 0.575% (wt/vol) LDS/20% glycerol in 125 mmol/L Tris-HCl, pH 6.8; and 8 mol/L urea /2% CHAPS / 25 mmol/L DTT in 50 mmol/L Tris-HCl, pH 7.5. All three extraction buffers contained 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, and 10 μg leupeptin/mL as protease inhibitors. A P-20 pellet in a 12×75-mm polypropylene tube (Sarstedt) was dispersed in an extraction buffer (250 μL per pellet equivalent of one T-75 flask) by drawing through graded syringe needles (20G, 25G, and 27G, usually 3× with each needle) and gentle vortexing; the pellets were pooled into one 12×75-mm tube containing a mini stir bar; then the tube was placed in a beaker that in turn was placed on a magnetic stirrer for 1 hour in the cold room, providing continuous gentle mixing. At the end of incubation, the contents were centrifuged at 20,000g in a Sorvall centrifuge for 20 minutes at 4°C. The 20,000g supernatant was aliquoted and saved as Triton X-100 extract at ~40°C, and the remaining pellet was suspended in the next extraction buffer and the procedure was repeated. By the end of this protocol, the P-20 membrane pellets almost entirely solubilized. The protein extracts were aliquoted and stored frozen at ~40°C. Protein extracts were fractionated by LDS-PAGE as described below.

Sufficient membrane proteins are obtained by this technique using cells grown from a single donor to allow multiple cell binding assays to be performed. Starting with a BM sample from a single donor, six T-75 flasks of first-passage cultures of stromal cell monolayers are grown in about 3 to 5 weeks. They generate ~1.2 mL of LDS-solubilized membrane proteins (after accounting for losses), yielding ~20 aliquots of 60 μL each. Each aliquot, after diluting 1:2 with Tris-HCl buffer, provides a sample of 120 μL for loading at least three lanes, each with 35 μL. Thus, 20 aliquots, obtained from six flasks derived from a single donor, provide sufficient material to run at least 60 lanes and detect cell binding, demonstrating the feasibility of the present method. The technique has provided reproducible results using stromal cells derived from several different donors.

LDS-PAGE, electrophoretic protein blotting, Coomassie Blue staining of gels, and Coomassie Blue staining of protein blots. These procedures were performed as described in detail (manuscript in preparation). Briefly, BM stromal cell membrane protein extracts, and rs-VCAM-1 were fractionated by LDS-PAGE using “native” 4% to 12% discontinuous gradient mini gels and electrophoretically transferred to PVDF. Necessary precautions were observed during electrophoresis and blotting to maintain cold conditions and avoid thermal denaturation of proteins. Protein gels and blots were stained by Coomassie Blue (CB) as described.

Cell adhesion assay on protein blots; propidium iodide (PI) staining and hematoxylin staining of cell bands on PVDF. Nonspecific binding sites on PVDF blots were blocked by incubating in 5% BSA/PBS(+) for 1 hour at room temperature, followed by overnight incubation in 1% BSA/PBS(+) in the cold room (4°C). Cell adhesion was assayed both at 4°C and 37°C (ie, in a standard tissue culture incubator). Target cells were washed once in PBS-CMF by centrifugation. Cells were resuspended at 1.5 to 3×10^6 mL in 30 mL of 1% BSA/PBS(+) or 5% FBS/RPMI 1640, depending on adhesion

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Fig 1. Electron microphotograph of BM stromal cell 20,000g pellet (P-20) showing plasma membrane vesicles. Confluent monolayers of BM stromal cells were harvested and a stromal cell membrane pellet (P-20) was prepared as described and submitted for standard electron microscopic study. This is a representative picture (original magnification ×45,000) showing numerous vesicles consistent with plasma membranes, occasional mitochondria, and small dense particles morphologically consistent with glycogen particles. In other data not shown, remnants of Golgi apparatus, scarce granular endoplasmic reticulum, lysosome-like dense bodies, and occasional lipid droplets consistent with adipocyte differentiation typical of stromal cells are also present.
assay temperature at 4°C or 37°C, respectively. Notably, to avoid nonspecific adherence of cells to the background at 37°C, FBS was substituted for BSA in cell incubation medium and washing media (except for KG1a, which showed minimal nonspecific binding even in the presence of BSA). Before adding cells, blots were washed 3 × 5 minutes using 0.5% BSA/PBS(+), or 5% FBS/PBS(+) and then cell incubations were performed in plexiglass adhesion assay chambers at 4°C or 37°C for 90 minutes or 60 minutes, respectively. After cell incubation, blots at 4°C were washed 3 × 5 minutes using cold 0.5% BSA/PBS(+), and blots at 37°C were washed 3 × 5 minutes using 5% FBS/PBS(+). Prewarmed to 37°C and incubating during washes in a 37°C oven. Cell blots were fixed in 10% neutral buffered formalin for 2 hours at room temperature to provide sufficient fixation on the membrane and allow stains to enter the cells. Cell bands were developed by PI staining or hematoxylin staining. Adherent cells were visualized using a fluorescence microscope equipped with epi-illumination and a UV excitation filter. Cell bands stained by hematoxylin were visualized using standard transmission light microscope.

Western blotting. The protocol for blocking of nonspecific binding sites on PVDF was the same as that used for cell adhesion assay on protein blots as given above and as described in detail elsewhere (manuscript in preparation). Immunoperoxidase staining was performed using AEC as substrate.

RESULTS AND DISCUSSION

Preparation, electrophoretic separation, and blotting of stromal cell membrane protein extracts. As the electron microscope in Fig 1 shows, the 20,000g pellets (P-20s) representing BM stromal cell membrane fractions were enriched in plasma membrane vesicles. The presence of plasma membrane components was confirmed by detection of alkaline phosphatase, a plasma membrane marker enzyme, in Triton X-100 extracts as well as LDS extract of P-20 pellets following LDS-PAGE, PVDF blotting, and treating the blot with substrate for alkaline phosphatase (data not shown). The P-20 preparations were sequentially solubilized by different detergents including Triton X-100, LDS, and urea/DTT/CHAPS in sequential order until the pellet almost completely disappeared. The 20,000g supernatants containing solubilized membrane proteins were fractionated by LDS-PAGE using 4% to 12% discontinuous gradient mini gels. As shown in Fig 2A, excellent resolution of proteins was obtained. As judged by banding pattern, much more than half of total amount of proteins appears to have been solubilized by Triton X-100 before LDS extraction. This strategy significantly enriched proteins that are soluble in LDS but insoluble in Triton X-100. Consequently, the LDS extract showed relatively fewer but more prominent protein bands. Urea/DTT/CHAPS was used to solubilize any remaining proteins after Triton X-100 and LDS extractions. Using cells grown from a single donor, sufficient membrane proteins can be obtained by this technique to allow multiple cell adhesion assays to be performed. The results reported here have been reproduced using stromal cells derived from multiple different donors.

Proteins were transferred to PVDF electrophoretically. Most bands seen on gel are represented on the blot as well (Fig 2A v Fig 2B). The transfer buffer consisted of standard Tris-glycine buffer (pH 8.7) containing no LDS or SDS (Fig 1C and D). The transfer buffer consisted of standard Tris-glycine buffer (pH 8.7) containing no LDS or SDS (Fig 1C and D). The transfer buffer consisted of standard Tris-glycine buffer (pH 8.7) containing no LDS or SDS (Fig 1C and D).

**Fig 2.** Cell adhesion to novel BM stromal cell membrane proteins fractionated by LDS-PAGE and blotted onto PVDF membrane. Stromal cell membrane proteins were prepared as described. Samples are as follows: lane 1, membrane proteins extracted by 2% Triton X-100 (lipid rich material floating at the top of the supernatant, seen only with BM stromal cells, but not fibroblasts. This is consistent with adipocyte differentiation of stromal cells.); lane 2, membrane proteins extracted as in lane 1 but representing the clear portion of supernatant; lane 3, membrane proteins extracted by 0.575% LDS; lane 4, membrane proteins extracted by 8 mol/L urea/25 mmol/L DTT/2% CHAPS. Final samples contained 0.2875% LDS and 10% (or 20%) glycerol in 125 mmol/L (or 62.5 mmol/L) Tris-HCl, pH 6.8, loaded with 35 μL/lane. (A) CB staining of the gel. (B) CB staining of the protein blot on PVDF membrane. (C) PI staining of NALM-6 cells adherent to protein blot on PVDF at 4°C, and viewed under a long-wave UV light. (D) PI staining of KG1a cells adherent to protein blot on PVDF at 4°C, and viewed under a long-wave UV light. As judged by comparison to gel (A, lane 3) and protein blot (B, lane 3), the thick cell band in gel bands (C, lane 3) and (D, lane 3) probably represents three overlapping bands. That this thick cell band is indeed composed of three separate cell bands may be better appreciated by noting a shift in the pattern of bands in this area when binding of different cell types is examined (see Fig 8).
Fig 3. Photomicrographs of cells adherent to BM stromal cell membrane protein bands on PVDF membrane, stained by hematoxylin. (A) Correlation between low-power microscopic view of a cell band, cell blot, and protein blot. (Top left) CB staining of the protein blot on PVDF membrane, representing a replica of the LDS-extracted proteins. (Top right) Hematoxylin staining of myeloid progenitor (KG1a) cells adherent to protein bands on PVDF membrane. Note that some proteins are more adhesive than others as reflected by some protein bands appearing weak on protein blot and strong on cell blot and vice versa. (Bottom) Low-power microscopic view of a band of cells confirming a positive band caused by cell binding (original magnification x 5). (B) KG1a cells viewed at a higher magnification (original magnification x 25). Note that some cells appear round and densely stained whereas most other cells appear flat and spread out, suggesting spreading of some cells on the protein substrate.

was not quantitative, as determined by staining of gels following transfer (not shown).

Cell adhesion to human BM stromal cell membrane protein bands on PVDF. The LDS-extracted membrane protein sample showed six protein bands by 4% to 12% gel that are associated with lymphoid progenitor (NALM-6) cell adhesion (Fig 2, A through C, lane 3). Apparently the same bands are also exhibiting adhesion to the progenitor myeloid (KG1a) cells (Fig 2D, lane 3). The split bands (upper portion of lane 3) are distinctive and indicate the specificity of the adhesion interactions in this new assay. The lack of adhesion of cells to the background of the blot and to additional multiple protein bands visible by CB staining further underscores the specificity of the cell binding observed. Positive bands were confirmed as being due to cell binding by microscopic visualization (Figs 3 and 4). One protein band extracted by Triton X-100 selectively binds the lymphoid (NALM-6) cells (Fig 2C, lanes 1 and 2). The stromal cell proteins solubilized by urea/DTT/CHAPS (Fig 2, C and D, lane 4) show no cell adhesion. The negative results with urea/DTT/CHAPS-soluble fraction may be due to absence or denaturation of any adhesion molecules remaining in the membrane fraction after removal of proteins solubilized by Triton X-100 and LDS. For purpose of discussion, CAMs reported here will
henceforth be referred to as BM CAMs, although their occurrence on other cell types has not been studied and excluded.

Western blotting. Western blotting of stromal cell membrane protein extracts using MoAbs to VCAM-1, CD54, and CD44 have identified all three molecules in the Triton X-100 extracts (Fig S, A through C) but not the LDS extract. This is consistent with the observation that these molecules (eg, VCAM-1) can be detected on intact stromal cells by immunofluorescence, and can be immunoprecipitated from Triton X-100 lysates of these cells. The electrophoretic mobilities of the known CAMs are notably different from those of the BM CAMs, pointing to novelty of the BM CAMs.

VCAM-1, CD54, and CD44, although identified by Western blotting in Triton extracts, did not exhibit cell binding. That the lack of binding was because of quantitatively small amounts of protein below the sensitivity of the assay rather than denaturation was shown by testing the ability of this cell blotting system to preserve adhesion function of a known cell membrane adhesion protein, recombinant soluble VCAM-1 (rs VCAM-1).

Cell adhesion to purified protein of VCAM-1. rs VCAM-1 was electrophoresed by LDS-PAGE and electrophoblotted along with BM stromal cell membrane protein extracts. As judged by electrophoretic mobilities, the BM CAMs are quite distinct from VCAM-1 (Fig 6). Cell adhesion assay at 37°C showed binding of Burkitt's lymphoma (Ramos) cells to VCAM-1 band (Fig 6C), as can be expected from earlier
Fig 5. Western blotting analysis of BM stromal cell membrane proteins using antibodies to known CAMs and immunoperoxidase staining. (A) Using MoAb to VCAM-1. Samples are as described in Fig 2 legend (lane 1, Triton X-100 extract [lipid-rich fraction]; lane 2, Triton X-100 extract [clear supernatant]; lane 3, LDS extract; lane 4, urea extract). Note that VCAM-1 is present in Triton extracts (lanes 1 and 2) but not in LDS extract (lane 3) or urea extract (lane 4). For comparison, locations of BM CAMs solubilized by LDS and identified by cell adhesion using the new cell-blotting technique are diagrammatically shown, numbered 1 through 9. (B) Using MoAb to CD44. Samples are as described in (A). Note that CD44 is predominantly solubilized by Triton X-100 (lanes 1 and 2), and trace amount of CD44 not solubilized by Triton X-100 is extracted by LDS and seen in lane 3. The diffuse band pattern of CD44 is also noteworthy and is consistent with its molecular heterogeneity. (C) Using MoAb to CD54 (ICAM-1). Samples differ slightly from those in (A) and (B) and are as follows: lane 1, whole cell lysate of K562 as a positive control for CD54 prepared using 0.5% Triton X-100; lane 2, BM stromal cell membrane proteins extracted by 2% Triton X-100 (lipid-rich material floating at top of supernatant); lane 3, membrane proteins extracted as in lane 2 but representing the clear portion of supernatant; lane 4, membrane proteins extracted by 0.575% LDS; lane 5, membrane proteins extracted by 8 mol/L urea/25 mmol/L DTT/2% CHAPS. Note that the CD54 band is exclusively present in Triton extracts and not seen in LDS or urea extracts, and that the position of the band is identical to that of the positive control. The appearance of trace positivity with a BM CAM in the LDS extract (lane 4) migrating in a different position from the control (protein band no. 7) probably represents cross-reactivity and may signify some relationship of the BM CAM to the Ig superfamily.

This suggests that the present system, comprising of LDS-PAGE, electroblotting, and other steps, does indeed retain cell adhesion function of VCAM-1. As shown in Fig 7, the new cell-blotting assay is highly specific and sensitive. The specificity is demonstrated by near-complete blockade of NALM-6 cell adhesion to VCAM-1 by an anti–VCAM-1 MoAb. Sensitivity of the assay approached concentrations as low as 0.3 μg of VCAM-1 loaded in a lane, bearing in mind that sensitivity is dependent on multiple factors including adhesion protein itself, cell type, and cell adhesion assay temperature. VCAM-1, CD54, and CD44, although detectable by Western blot in the Triton X-100 extract, are probably present below the sensitivity level of the assay, because all known adhesion proteins that have been tested have retained their cell adhesion function by the present system. It should be remembered that the stromal cells that were used in the present study represented uninduced stroma, which express low levels of VCAM-1.11 It may be necessary to induce stroma with cytokines before harvesting to detect certain molecules in stromal cell extracts by cell blotting.

BM is distinctive among all organs for the multiplicity of cell types and myriad cell-to-cell interactions. It is viewed as the “breeding ground” for all blood cell types. Therefore, adhesion of different hematopoietic cell types was investigated using this system to identify possible lineage-specific adhesion proteins. In addition to KG1a (CD34+ progenitor myeloid) and NALM-6 (progenitor B-lymphoid) human leukemic cell lines, the following cell lines have been studied: JY (B lymphoblastoid), Ramos (Burkitt’s B-lymphoid), WIL-2 (mature B-lymphoid), HS-Sultan (plasmacytoma), K562 (erythroleukemia) human leukemic cell lines, and Con A-induced T lymphoblasts. As shown in Fig 8 and summarized in Table 1, different hematopoietic cells exhibit adhesion to different subsets of BM CAMs as well as some common BM CAMs. Activated T cells, as can be expected, showed minimal or no binding to any BM CAMs, further underscoring the specificity of cell adhesion mediated by these proteins. It is also intriguing that progenitor cells (ie,
KG1a and NALM-6) bound to a greater number of stromal cell protein bands than the more mature cell types (ie, WIL-2 and JY B-lymphoblastoid cells). These findings provide a new avenue for study of molecular mechanisms related to determining specificity to hematopoietic cellular localization within BM microenvironment and of hematopoiesis.

As judged by molecular weight markers shown in Fig 8A and summarized in Table 1, there appear to be several “families” of BM CAMs detected by cell blotting, falling under different molecular weight ranges. LDS has a great affinity for polyacrylamide at 4°C, which may lead to deficiency of LDS in the frontal region of the gel and accelerated migration of low molecular weight (LMW) proteins. Therefore, it is important to bear in mind that the positions of protein bands in the LDS-PAGE gel do not necessarily correspond to the true molecular weights, and may underestimate the molecular weights.

It is also of interest to note that when LDS extracts were fractionated by 4% to 20% continuous gradient gel (instead of 4% to 12% discontinuous gradient gel as described in this report), cell adhesion was observed only in the LMW region, approximately corresponding to protein bands no. 5 through 8 (not shown). As shown by studies using various lectins,
Fig 8. Adhesion of different hematopoietic cells to different subsets of BM stromal cell CAMs solubilized by LDS. (A) BM CAMs as identified by KG1a cell binding either at 4°C or 37°C and correlated with BM protein bands on the gel. Lane 1, LDS extract of stromal cell membrane proteins fractionated by LDS-PAGE and stained by CB; lane 2, PI staining of KG1a cells adherent to protein blot on PVDF at 4°C and viewed under CB; lane 3, hematoxylin staining of KG1a cells adherent to protein blot on PVDF at 37°C; lane M, broad-range molecular-weight markers (Bio-Rad) stained by CB. BM protein bands showing cell adhesion are diagrammatically displayed, numbered 1 through 9. Note that bands 6 through 8 are clearly distinct on CB staining of the gel, but the cell bands in this region appear to be overlapping. The interpretation that these represent three separable adhesive proteins is supported by binding patterns of different cell types, eg, HS Sultan cells bind only to bands 6 and 7 (B, lane 5), JY B cells bind only to bands 7 and 8 (C, lane 4), and WIL-2 cells bind to band 8 alone (B, lane 4). Lane M shows broad-range molecular-weight markers consisting of a mixture of nine proteins ranging from 200 to 6.5 kD. Even though the marker proteins appear to be resolved well and evenly distributed from top to bottom of the lane, there are clearly more than nine bands, some of which most likely represent molecular complexes. (B) Cell adhesion assayed at 4°C. The target cells are as follows: lane 1, NALM-6 cells; lane 2, KG1a (CD34+) cells; lane 3, K562 cells; lane 4, WIL-2 cells; and lane 5, HS-Sultan cells. Cell bands in all lanes were developed by PI stain. (C) Cell adhesion assayed at 37°C. The target cells are as follows: lane 1, KG1a cells; lane 2, HS-Sultan cells; lane 3, Ramos cells; lane 4, JY B lymphoblastoid cells; and lane 5, T lymphoblasts. Cell bands in lanes 1 and 2 were developed by hematoxylin stain, whereas cell bands in lanes 3 through 5 were developed by PI stain. It appears that progenitor cells like NALM-6 and KG1a bind to the greatest number of protein bands; on the other hand, more mature cell types such as WIL-2 and JY B lymphoblastoid cells bind to a minimum number of protein bands—the ones that are common to most cell types. Of further interest are the differences in the binding pattern of HS-Sultan (a plasmacytoma cell line) at 4°C (B, lane 5) versus 37°C (C, lane 2), the significance of which remains to be determined.

These gel systems are quite distinct (manuscript in preparation). The 4% to 12% discontinuous gradient gel tends to favor or preserve multimeric complexes whereas 4% to 20% continuous gradient gel tends to dissociate and resolve them into monomeric units. Based on such observations, the high molecular weight (HMW) protein bands in LDS lane of 4% to 12% gel exhibiting cell adhesion may represent functional complexes of polypeptides that became dissociated in the 4% to 20% gel and lost their cell adhesive function. In contrast, the LMW proteins in LDS lane of 4% to 12% gel (ie, protein bands no. 5 through 8, Fig 8A and Table 1) may represent monomeric proteins that retain their adhesive function even under dissociating conditions of 4% to 20% gel electrophoresis. In light of such observations, the nine protein bands exhibiting cell adhesion could correspond to more or less than nine distinct proteins.

The cell adhesion assay temperature also showed a striking effect on cell binding to stromal cell membrane proteins. Protein bands no. 1 and 2 (Fig 8A, lane 3, and Table 1) and protein band no. 9 (Fig 8C, lanes 1 and 3, and Table 1) showed cell adhesion only at 37°C. The BM CAMs exhibiting cell adhesion at 4°C are unlikely to be integrins because cell adhesion involving integrins is mostly observed at 37°C. For similar reasons, the BM CAMs (protein bands no. 3 through 8) are unlikely to be extracellular matrix (ECM) proteins because ECM proteins, which mediate cell adhesion via cell surface integrin receptors, failed to exhibit cell binding at 4°C by cell blotting (manuscript in preparation).

That the BM proteins showing cell adhesion are novel CAMs is predicated on the following: (1) known CAMs, specifically VCAM-1, CD54, and CD44, have been identified by Western blotting in Triton extracts, obtained before LDS extraction, but not in LDS extracts; (2) the BM CAMs could be extracted only by LDS, whereas most known CAMs
are solubilized by Triton or NP-40 as well; (3) the electrophoretic mobilities of the novel BM CAMs as detected by cell blot are markedly different from those of known CAMs as detectable by Western blot (VCAM-1, CD54, CD44) and by simultaneously running purified protein of one of them (rs VCAM-1) in a parallel lane; and (4) six of the nine BM CAMs show cell adhesion not only at 37°C but also equally well at 4°C, unlike cell adhesion involving integrins that is temperature dependent and observed only at 37°C.

Because the BM CAMs could only be solubilized by LDS, they would escape detection by conventional techniques, ie, cause the cell blotting not only provides a direct approach for identifying and isolation techniques. Thus, cell blotting not only provides a direct approach for identifying CAMs but may also be capable of identifying CAMs not detectable by conventional protein solubilization and isolation techniques.

The new BM CAMs may account for cell adhesion between hematopoietic cells and BM stroma that cannot be ascribed to known CAMs, eg, VCAM-1. Most intriguing of all is that some of the new BM CAMs appear to be restricted to a hematopoietic cell lineage or a stage of differentiation (Table 1). The significance of this finding remains to be determined. Sequencing the BM CAMs and cloning their corresponding genes will be crucial to understanding their relationship to known families of CAMs. The cell-blotting assay would greatly facilitate the generation of MoAbs for functional studies of the hematopoietic microenvironment, because relevant adhesion proteins identified by cell blotting have already been electrophoretically separated. It is possible that one or more of these proteins may be important in maturation or differentiation of normal and neoplastic hematopoietic cell precursors.

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REFERENCES


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<tr>
<th>Table 1. Summary of Characteristics of Hematopoietic Cell Binding at Either 37°C or 4°C to BM CAMs Soluble in LDS but Insoluble in Triton X-100</th>
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<tbody>
<tr>
<td>Protein Band No.</td>
</tr>
<tr>
<td>Approximate molecular weight (kD)</td>
</tr>
<tr>
<td>K562 myeloid progenitor cells</td>
</tr>
<tr>
<td>KSultan plasmacytoma cells</td>
</tr>
<tr>
<td>Ramos (Burkitt’s) B-lymphoid progenitor cells</td>
</tr>
<tr>
<td>JY B-lymphoblastoid cells (EBV-transformed)</td>
</tr>
<tr>
<td>WIL-2 Mature B-lymphoid cells</td>
</tr>
<tr>
<td>Con A-induced T lymphoblasts (activated T cells)</td>
</tr>
</tbody>
</table>

Abbreviations: EBV, Epstein-Barr virus; +, cell adhesion; +/-, trace positivity.


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Discovery of novel hematopoietic cell adhesion molecules from human bone marrow stromal cell membrane protein extracts by a new cell-blotting technique

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