CD44 and its Ligand Hyaluronate Mediate Rolling under Physiologic Flow: A Novel Lymphocyte–Endothelial Cell Primary Adhesion Pathway

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Summary

The extravasation of leukocytes from the blood into tissues occurs as a multistep process: an initial transient interaction ("rolling"), generally thought to be mediated by the selectin family of adhesion molecules, followed by firm adhesion, usually mediated by integrins. Using a parallel plate flow chamber designed to approximate physiologic flow in postcapillary venules, we have characterized a rolling interaction between lymphoid cells and adherent primary and cultured endothelial cells that is not selectin mediated. Studies using blocking monoclonal antibodies indicate that this novel interaction is mediated by CD44. Abrogation of the rolling interaction could be specifically achieved using both soluble hyaluronate (HA) and treatment of the adherent cells with HA-reactive substances, indicating that HA is the ligand supporting this rolling interaction. Some B and T cell lines, as well as normal lymphocytes, either constitutively exhibit rolling or can be induced to do so by phorbol ester or in vivo antigen activation. These studies indicate that CD44 and its principal ligand hyaluronate represent another receptor/carbohydrate ligand pair mediating a novel activation-dependent pathway of lymphocyte/endothelial cell adhesion.

The venules in which circulating leukocytes must engage the vessel wall are subject to high shear stresses, resulting in the requirement for specialized adhesion mechanisms to ensure that arrest and extravasation of leukocytes can occur appropriately. It has become clear that the specificity and regulation of adhesion results from various types of adhesion receptors acting in sequential fashion. The major steps of adhesion, described primarily for neutrophils, have included a primary event, during which leukocytes are engaged by the vessel wall and then retarded by a repeated transient interaction, followed by release. This results in a stuttered traversing of the length of the vessel known as "rolling," which is followed by a secondary firm adhesion of the rolling cells and subsequent transmigration (1, 2). Primary adhesion has been attributed in different systems to the interaction between the lectin domain of members of the selectin family and their carbohydrate ligands on endothelial cells (EC)\(^1\), whereas secondary (firm) adhesion is due to heterodimeric \(\beta_2\) integrins interacting with their EC ligands, members of the immunoglobulin gene superfamily, primarily intracellular adhesion molecule (ICAM) 1 and ICAM-2 (for review see reference 3). The primary (rolling) interaction does not require activation of the selectin molecule and is transient, releasable, and immediately repeatable, whereas the secondary (firm) adhesion event requires activation of integrin molecules and is not readily reversible.

Although much of this multistep model of adhesion under shear forces was established based on neutrophil–EC interactions, it has been less clear whether these rules are equally applicable to the even more complex extravasation physiology of lymphocytes. Lymphocytes have been shown to roll both in vivo and in vitro shear stress flow assays, and in many instances this primary adhesion is selectin dependent (4–7). Furthermore, \(\beta_2\), as well as other integrins not characterized in neutrophils (i.e., \(\alpha_4\beta_1\) and \(\alpha_4\beta_7\)) participate in secondary lymphocyte adhesion (8–10). However, data pointing toward additional molecular complexity of lymphocyte extravasation mechanisms have recently emerged. For example, \(\alpha_4\beta_1\) and \(\alpha_4\beta_7\) integrins on lymphocytes have been shown to mediate primary as well as secondary interactions (11), and primary adhesion of human T cells has been shown to use as yet uncharacterized molecules (7).

Because mechanisms of lymphocyte trafficking must accommodate the additional complexities of a myriad of dis-

\(^1\)Abbreviations used in this paper: bPG, bovine proteoglycan; CS-A, chondroitin sulfate A; DCF, Ca\(^{2+}\)Mg\(^{2+}\)-free HBSS; EC, endothelial cell; FITC, fluorescein-conjugated HA; GoRlg, goat anti-rat Ig; HA, hyaluronate; HS, heparin sulfate; ICAM, intracellular adhesion molecule.

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tinct, sometimes tissue-specific, homing behaviors, it would not be surprising if other ligand pairs participated in lymphocyte interactions with endothelium. Here, using a parallel plate flow chamber designed to approximate postcapillary physiologic flow, we have characterized a novel rolling interaction between lymphoid cells and adherent cultured EC which is not selectin or α4-integrin mediated. Our studies indicate that this novel interaction has as its basis a distinct protein–carbohydrate ligand interaction, namely, the interaction between the proteoglycan core and cartilage link protein family member CD44 and its principal ligand, hyaluronate (HA). Phorbol ester activation, known to induce HA binding, also induces rolling behavior in several CD44-bearing lymphoid cell lines as well as in normal lymphocytes, which do not otherwise exhibit this activity, indicating CD44 may function in lymphocyte extravasation as an activation-dependent primary adhesion molecule. These results further our understanding of functional roles for CD44 and its interaction with HA, help clarify the previously proposed but poorly defined role of CD44 in lymphocyte homing, and suggest that CD44 might selectively participate in the well-described enhanced homing of activated lymphocytes to sites of inflammation.

Materials and Methods

Chemicals and Reagents. PMA, rooster comb hyaluronate (HA), chondroitin sulfate A (CS-A), heparin sulfate (HS), and neuraminidase were purchased from Sigma Chemical Co. (St. Louis, MO) and streptococcal hyaluronidase was purchased from ICN (Irvine, CA). Hyluronidase and neuraminidase were used for 1 h at 37°C at final concentrations of 20 TRU/ml and 1 U/ml, respectively. Ionomycin was purchased from Calbiochem Corp. (La Jolla, CA). Fluorescein-conjugated HA (Fl-HA) was the generous gift of J. Lesley (Salk Institute, La Jolla, CA). Biotinylated bovine proteoglycan (bPG) extracted from bovine nasal cartilage was kindly provided by C. Underhill (Georgetown University School of Medicine, Washington, DC).

Cell Culture and Antibodies. The murine LN endothelial lines SVEC4-10 (provided by K.A. O’Connell, Johns Hopkins University School of Medicine, Baltimore, MD) (12) and TME-3H3 (provided by A. Hamann, Department of Immunology, Medizinischen Klinik, Germany) (13) are SV40-transformed lines. The murine T cell line SAKRTLS12 was the gift of P. Kincade (OMRF, Oklahoma City, OK) and J. Lesley. Murine T cell lines and B cell hybridomas were maintained in DMEM or RPMI 1640 with high glucose, 15% FCS plus pyruvate, and glutamine. Activation of T cell lines and normal mouse lymphocytes was accomplished by adding 1 ng/ml PMA plus 500 ng/ml ionomycin to the tissue culture medium and incubating for 18–24 h at 37°C.

Primary LN EC cultures were made by pooling cervical and axial nodes from three animals, as described (14, 15). Organs were minced, rinsed to remove lymphocytes, treated with collagenase for 30 min at 37°C, and plated on 35-mm culture dishes in supplemented IMDM (20% FCS). After confluence, cells were used directly or after a single passage to fresh plates. FACScan analysis indicated that >99% of the population stained positive for the known endothelial markers MECA-32, CD31, and endoglin, and negative for the macrophage marker MAC-1. Human umbilical vein EC were prepared and kindly provided by N. Oppenheimer-Marks (University of Texas Southwestern Medical Center [UTSWMC]).

The following mAbs were used in these studies: anti-CD44 antibodies KM201, KM81 (16), IM7 (17) (American Type Culture Collection, Rockville, MD), and RAWB14, provided by J. Lesley (18); anti-α4 selectin 10A10 and anti-E-selectin 9A9, the gift of B.A. Woltzky (Hoffmann-LaRoche Inc., Nutley, NJ); anti-VCAM antibodies MK1.9.2 and MK2.7.1, the anti–ICAM-1 antibody YN-1, provided by P. Thorpe (UTSWMC); anti–ICAM-2 3C4, provided by A. Takashima (UTSWMC); anti-α4 integrin R1.2, anti–MAC-1 M1/70, anti–parallel node addressin MECA 79, and anti–mucosal addressin MECA367 (19); anti-endoglin and MECA32 (20), provided by E. Butcher (Stanford University, Stanford, CA); anti–CD31 (21) and anti-sLeα HECA452; anti-LFA-1 β2 M17/4 (PharMingen, San Diego, CA), anti-Thyl.1 (Bioproducts for Science, Inc., Indianapolis, IN); anti-CD45R0 MB23G (American Type Culture Collection). FITC- and PE-conjugated goat anti-rat immunoglobulin (G~xl~Ig) were purchased from Cappel Laboratories, Cochranville, PA, and streptavidin–PE was obtained from CALTAG Laboratories, South San Francisco, CA.

In Vivo Activation of Murine LN Cells. 8–10-wk-old C57Bl/6 mice were injected in the hind footpad and at the base of the tail with 25 μl of CFA emulsified 1:1 with 25 μl of PBS. Two additional injections in the base of the tail were given at weekly intervals, and draining inguinal LN as well as distal control nodes were harvested 3 d after the last injection.

FACS Analysis. 5 × 106 cells were stained with primary antibody, Fl-HA, or bPG–biotin in 100 μl PBS/5% FCS for 20 min on ice and then washed with 1 ml of PBS/5% FCS. Fluorochrome-labeled secondary antibody or streptavidin was added for 20 min, and cells were again washed before analysis. Data were collected on a FACScan® analytical instrument (Becton Dickinson, San Jose, CA) and analyzed using Lysys II (Becton Dickinson) software.

Adhesion Assay under Flow Conditions. Physiological flow conditions were produced using a parallel plate flow chamber as previously described (22). Briefly, flow occurs over a 35-mm tissue culture dish (Corning Inc., Corning, NY) containing either an adherent cell monolayer or a coating of substrate. The culture dish and a plexiglass chamber are held 1.27 × 10−2 cm apart by a silicon gasket cut to form two flow chambers, each 0.6 cm wide. The wall shear stress for this geometry can be calculated as previously described (22). Unless otherwise noted, experiments were carried out at a wall shear stress of 2.0 dynes/cm².

SVEC4-10, TME-3H3, and primary LN EC were plated and used at confluence. Soluble HA and CS-A were plated at 2.5 mg/ml in PBS, 1 ml/plate, and incubated overnight at 4°C on a rocking platform, and then blocked with 1 ml FCS at 4°C for 1 h before use. After equilibration of flow with medium alone, lymphoid cells were resuspended at a concentration of 3 × 106 cells/ml in RPMI 1640 equilibrated to 37°C and pulled continuously across the flow chambers. For Ca2+Mg2+-free experiments, lymphoid cells and endothelial monolayers were washed three times in HBSS without Ca2+Mg2+ (DCF), and lymphoid cells were further incubated in DCF for 30 min, washed, and applied to the assay. For blocking studies, antibodies were either added at saturating concentrations to the cell suspension before flow or added to the inlet solution once the cells had already begun to perfuse into the chamber. HA-blocking studies were performed by adding soluble HA to the cell suspension before flow. Interaction of lymphoid cells with the substrate was monitored by the use of an in-
Results

Primary Adhesion (Rolling) of a Murine T Cell Line, BW5147, on a Murine LN EC Line. Using a parallel plate flow chamber (22), a murine clonal T cell line, BW5147, was perfused over confluent monolayers of the SV40-transformed LN endothelial line SVEC4-10 at a wall shear stress of 2.0 dynes/cm². This shear stress is considered to be within the physiologic range found in venules and some large blood vessels (23-25), and has been shown to discriminate selectin- and integrin-mediated interactions (7). Under these conditions, the interaction observed was predominantly that of rolling (Fig. 1 A), which was manifested by >97% of the interacting cells. Less than 3% of the interacting cells were observed to firmly adhere, and these also showed a rolling interaction preceding arrest. If flow was stopped and then restarted after allowing cells to settle on the endothelial monolayer, essentially the entire population began to roll, indicating that these clonal lymphoid cells were homogeneous with respect to the capacity to engage in the rolling interaction. In addition, cells interacted with the EC substrate over a range of wall shear stresses (Fig. 1 A). Throughout this range, 95-100% of the interacting cells exhibited rolling (Fig. 1 A), suggesting that the rolling interaction is a property intrinsic to this lymphoid cell on the substrate rather than to a particular condition of flow. At 2.0 dynes/cm², the lymphoid cells were observed to roll at an average velocity of 12.9 ± 6.4 (SD) μm/s. The distribution of velocities within a population of ~200 cells from three separate experiments was determined and is shown in Fig. 1 B; 79% of the interacting cells rolled at velocities between 5.0 and 20.0 μm/s. These velocities and shear stress responses are comparable to those observed between the selectin family of adhesion molecules and their endothelial carbohydrate ligands (2, 22, 24, 26-28). Furthermore, the level of tethering (defined as the number of cells undergoing initial contact in the first 20 s after commencing flow [29]) was determined to be ~50% higher over a range of comparable shear stresses than that reported with the most efficient lymphoid cells in other systems (29, 30) (data not shown).

Independence of BW5147/EC Primary Adhesion from Selectin-Ligand Interactions. Because the phenomenon of leukocyte rolling has been primarily attributed to the selectin family of adhesion molecules, both BW5147 and SVEC4-10 were analyzed for selectin expression. Neither cell line expressed detectable L-selectin, P-selectin, or E-selectin, as measured by cell surface staining (data not shown). In addition, Northern blot analysis for RNA shows no L-selectin transcripts in BW5147 (data not shown). The cells lacked detectable MAC-1, LFA-1 β2, or α4 integrins, but demonstrated high levels of surface CD44 (data not shown). Therefore, CD44 appears to be the major known adhesion molecule borne by this BW cell line. By additional FACS® analysis (data not shown), SVEC4-10 appears to be devoid of the EC adhesion molecules VCAM-1 (the counterligand for α4 integrins), ICAM-1 and -2 (the known endothelial β2 integrin ligands), peripheral node addressins (a set of high-affinity L-selectin ligands expressed by high endothelial venules), and the mucosal addressin MadCAM-1 (the primary ligand for α4β7-integrin). However, CD44 was also expressed on this endothelial line.

Selectin-ligand interactions are known to be Ca²⁺-dependent by virtue of the Ca²⁺-dependent NH₂-terminal number of adherent cells was determined. Results of total adherent cells are from a representative experiment (C). (B) Velocity distribution of rolling BW5147 cells at a wall shear stress of 2.0 dynes/cm². Rolling was analyzed as described in Materials and Methods, and the frequency of cells within each velocity range was calculated as a percentage of the total number of cells evaluated (~200).
lectin domains of selectins (31, 32). Performing the flow assay in Ca\(^{2+}\)-Mg\(^{2+}\)-free (DCF) medium (Fig. 2 A) or in EDTA (0.25 mM) had no effect on BW5147 rolling. Likewise, neuraminidase treatment of the BW5147 cells, known to abrogate selectin-mediated interactions with their carbohydrate ligands, showed essentially no effect on the rolling interaction (Fig. 2 A). In addition, anti-sLe\(^\alpha\) antibodies showed no staining of the endothelial monolayer (data not shown). Lastly, EL4hi, a variant of the T cell line EL4, which has been selected for high levels of expression of L-selectin, showed no ability to roll or interact in this system (Table 1). The cumulative data indicate that this interaction is not selectin mediated.

Inhibition of BW5147–EC Rolling Interaction by Treatment with CD44-specific mAbs. To determine the role of CD44 in this interaction, the anti-CD44 Abs KM201, KM81, and IM7 were tested for their effects on BW5147 rolling. As seen in Fig. 2 A, all of these antibodies completely and specifically inhibited the BW5147–SVEC4-10 rolling interaction when BW5147 cells were preincubated with antibody, washed, and then applied to the assay. A fourth anti-CD44 antibody (IRAWB14), which does not inhibit CD44–HA interactions (18), did not inhibit rolling. Two control antibodies, anti-Thy1.1 and anti–H-2K\(^d\), which recognize cell surface markers expressed at high levels on BW5147, also showed no significant effect on rolling. Since, in continuous flow, both initial contact (tethering) as well as subsequent rolling are required to observe the interaction, it was of interest to assess the effect of anti-CD44 antibody on rolling in isolation. Therefore, flow was initiated and antibody was introduced after cell rolling was in progress. All of the cells in the field were followed to the end of the chamber over a 7-min period after the introduction of antibody. It can be seen in Fig. 2 B that anti-CD44 mAb, but not anti-Thy1.1, resulted in the loss of cells interacting with the monolayer surface over time, although initial cell–cell contact (tethering) had already been established. When flow was stopped in the presence of blocking antibody and cells were allowed to settle, the reinitiation of flow resulted in the immediate “washing away” of the settled cells, without any indication of even transient interaction. Taken together, these results suggest that both initial contact as well as subsequent rolling is mediated by CD44.

Because SVEC4-10 also expresses CD44, it was important to determine whether endothelial CD44 contributed to this interaction as well. Saturating levels of anti-CD44 antibodies were added to the endothelial monolayers, allowed to incubate for 15 min, and rinsed to remove excess antibody immediately before initiation of flow of BW5147. Primary adhesion was not affected by pretreatment of SVEC4-10 with any of the blocking antibodies KM201, KM81, or IM7 (Fig. 2 A), and FACS analysis after the assay showed antibody still bound to the EC (data not shown). These results suggest that it is the CD44 on the lymphoid cell that has the primary role in this adhesive interaction. In further support of this, whereas both BW5147 and SVEC4-10 are CD44\(^+\), only BW5147 binds HA (data not shown).

Mediation of CD44-dependent Rolling Occurs via the CD44 Ligand Hyaluronan. Because HA is one of the best-studied ligands for CD44, and HA has been reported previously to be expressed on small vessel vascular endothelium (15), we examined its potential involvement in this rolling interaction. bPG has been shown to bind to HA with very high affinity (33). A biotinylated form of bPG was used to determine levels of surface HA on SVEC4-10, and these cells do bear significant amounts of HA on their surface (Fig. 3). This bPG reactivity is blocked by soluble HA and is removed by hyaluronidase treatment of the cells before staining. Significantly, bPG incubation with SVEC4-10 cells

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**Figure 2.** Inhibition of rolling of BW5147 cells on SVEC4-10 monolayers in the parallel plate flow assay. (A) Reagents used for treatment of the lymphoid (upper) or endothelial (lower) cells are indicated. Performing the assay in Ca\(^{2+}\)-Mg\(^{2+}\)-free HBSS (DCF medium) and treatment of BW5147 with hyaluronidase or neuraminidase had no effect on the rolling interaction. Antibody treatments were performed by incubation of either BW5147 cells or SVEC4-10 cells with anti-CD44 or control mAb and washing before addition of BW5147 cells to the feed solution. BW5147 cells were treated with anti-CD44 mAbs KM201, KM81, IM7, and IRAWB14, or with control antibodies Thyl.1 or H-2K\(^d\). SVEC4-10 cells were treated with anti-CD44 antibodies or an irrelevant isotype control antibody M1/70 (anti–MAC-1) by 30-min incubation before assay with no effect on rolling. However, treatment of SVEC4-10 with bPG inhibited rolling. In all cases, excess reagent was removed by washing cells before the flow assay. (B) Antibody to CD44 or Thy1.1 was added to the feed solution after achieving steady-state rolling of BW5147. Rolling cells were monitored for up to 7 min for visible effects by antibody and were seen to release from the surface after the addition of IM7, whereas anti-Thy1.1 had no significant effect.
Table 1. Rolling of Murine Cell Lines in Parallel Plate Flow Assays Correlates with CD44 Surface Expression and Fluoresceinated HA Binding

<table>
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<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>CD44*</th>
<th>Fl-HA*</th>
<th>SVEC HA Roll²</th>
<th>HA Roll²</th>
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<tbody>
<tr>
<td>BW5147</td>
<td>—</td>
<td>86</td>
<td>69</td>
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<td>+</td>
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<tr>
<td>TK23</td>
<td>—</td>
<td>58</td>
<td>148</td>
<td>+</td>
<td>+</td>
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<td>42</td>
<td>+</td>
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<tr>
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<td>225</td>
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<td>+</td>
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*CD44 expression (IM7/GaRlig-PE) and Fl-HA binding are given as the mean fluorescence intensity.
‡+, >25 cells/mm² per minute; −, <4 cells/mm² per minute. Rolling was specifically inhibited by anti-CD44 mAb KM201 (>95%) but not significantly affected by irrelevant control antibody (<14%).

also blocked the rolling BW5147 (Fig. 2 A). The same reagent was used to stain BW5147 cells, and a slight shift in fluorescence of less than twofold over background was seen (data not shown). However, hyaluronidase treatment of the BW5147 cells before flow assay had no effect on rolling (Fig. 2 A), again suggesting that it is primarily CD44 expressed on BW5147 interacting with hyaluronate expressed on the EC that mediates this adhesion.

To directly address the role of HA in CD44-mediated rolling, BW5147 was infused into the flow chamber over a 35-mm tissue culture dish that had been coated with 2.5 mg/ml soluble HA, HS, or CS-A. Cells passed across the HA-coated dishes rolled in numbers comparable to those seen on SVEC4-10 plates, whereas control substances did not support rolling (Fig. 4). Titration of the HA coating solution from 10 mg/ml to as low as 0.1 mg/ml supported BW5147 rolling without significant changes in the number of cells/mm² per minute or rolling velocities (data not shown). Treatment of the HA-coated plates with hyaluronidase but not neuraminidase abrogated the rolling, as did pretreatment of plates with soluble bPG, confirming the specificity of this cell–ligand interaction (Fig. 4). The HA rolling interaction could be completely and specifically inhibited by the use of the HA–blocking anti-CD44 mAbs KM201 and KM81 (Fig. 4). IM7 also inhibited the rolling interaction, although not completely (Fig. 4), consistent with previous reports (34). A control anti-Thyl.1 antibody had no significant effect on rolling.

In separate experiments on SVEC4-10 monolayers, either soluble HA or CS-A was added to the BW5147 suspension at varying concentrations. Introduction of soluble HA immediately resulted in inhibition of rolling, with 50% inhibition seen at 10 µg/ml of soluble HA and complete inhibition at 250 µg/ml (data not shown). Soluble CS-A had no effect on rolling.

Other Murine Lymphoid and Endothelial Cells Participate in CD44-mediated Rolling. To determine whether similar rolling activity is exhibited by other murine cell lines, a panel of lymphoid cells was tested for ability to roll on SVEC4-10. As summarized in Table 1, the results demonstrate that only those cells expressing CD44 display this behavior and that the ability to roll on HA-coated plates correlates precisely with the ability to roll on SVEC4-10 cells. Two T cell lines, TK23 and VL3, and two B cell hybridomas, MelD54 and MB23G2, express CD44 and display constitutive rolling behavior. Thus, a variety of T and B cell lines exhibit the rolling interaction. However, CD44 expression alone is not sufficient for rolling behavior, since the SAKRTTL12 and EL4 cell lines express similar high levels of cell surface CD44, yet do not show constitutive rolling behavior (see below).

Two additional EC substrates were also tested for participation in CD44-mediated lymphocyte rolling. TME-3H3, an SV40-transformed EC line (13), showed staining with bPG ~5–10-fold over background (data not shown). These cells supported rolling in numbers comparable to that seen with SVEC4-10 (40 cells/mm² per minute at 2.0 dynes/cm²), and the rolling was completely and specifically abolished by anti-CD44 mAbs and completely inhabitable with
Figure 4. Rolling of BW5147 cells on glycosaminoglycan-coated dishes in parallel plate flow assays is mediated by CD44-HA interaction. (A) Assay dishes were coated overnight with 2.5 mg/ml CS-A, HS, or HA, and BW5147 cells were flowed under conditions of shear stress (2.0 dynes/cm²). (B) Assay dishes coated with 2.5 mg/ml HA were treated with hyaluronidase, neuraminidase, or bPG before application of lymphoid cells. Both hyaluronidase and bPG treatment significantly inhibited rolling, whereas neuraminidase had no effect. (C) BW5147 cells were incubated with mAbs to CD44 or Thy1.1 before introduction into the feed solution and initiation of flow. All three anti-CD44 antibodies, but not anti-Thy1.1, inhibited BW5147 rolling.

Figure 5. Rolling of BW5147 cells on other EC substrates. TME-3H3 EC and BALB/c primary LN EC cultures were plated in culture dishes as described and used in the flow assay. BW5147 cells rolled in significant numbers across both kinds of cell monolayer. Rolling on both EC types was specifically inhibited by anti-CD44 antibodies and by soluble HA, but not by anti-Thy1.1.

Figure 6. Phorbol ester activation of murine T cell lines can induce rolling on HA-coated dishes. The T cell lines C6VL and SAKRTLS12 were incubated for 18 h with PMA plus ionomycin or for 20 min with the activating anti-CD44 antibody IRAWB14. Cells were introduced into the feed solution with or without treatment. Prior incubation of lymphoid cells with PMA plus ionomycin, but not with IRAWB14, resulted in the induction of rolling of treated cells on HA plates.
than that induced by PMA activation. Induction by means of this antibody alone was not sufficient to cause the cells to roll (Fig. 6). Thus, there appears to be a threshold for rolling behavior that is higher than that required for soluble HA binding alone, or the induction for soluble HA binding is qualitatively different than that required for rolling.

**Activated Murine Lymphocytes Exhibit CD44-mediated Rolling.** The phenomenon of CD44-mediated rolling on endothelium has not been previously described. To establish a potential role for CD44-mediated rolling in physiologic lymphocyte homing, we next assessed the ability of activated and nonactivated BALB/c spleen and LN cells to interact with HA-coated surfaces in the parallel plate flow assay (Fig. 7). Since CD44 expression on normal lymphocytes can be upregulated in vitro by treatment with phorbol ester (36), we treated lymphocytes and splenocytes as described above for testing on HA-coated surfaces. Activated and nonactivated cells were stained with F1-HA plus anti-CD44-PE at the time of assay. Consistent with activation, both the forward and side light scatter profiles of the lymphoid populations were increased (data not shown), indicating a significant blast population (57 vs. 8% of live cells for LN cells and 36 vs. 11% for spleen cells). Although significant numbers of BALB/c lymphocytes are CD44+ without activa-

![Figure 7](image-url)

**Figure 7.** CD44-HA-dependent LN and spleen cell rolling after activation in vitro and in vivo. BALB/c LN (A) and spleen cells (B) were stained with F1-HA plus IM7/GrR1g-PE immediately after removal from the animals or after 20-h incubation with PMA plus ionomycin. Increased staining of the CD44+ populations with R-HA is seen in the in vitro-activated cell populations. (C) Rolling of unactivated and in vitro-activated LN and spleen cells on HA-coated plates. Rolling was only seen in the activated cell populations and was specifically inhibited by treatment with anti-CD44 antibody (IM7) or with soluble HA. Anti-CD45Rb had no effect on rolling of activated lymphocytes. (D) Rolling of in vivo-activated LN cells on HA- and SVEC4-10-coated plates. Rolling was seen on both substrates and was inhibited by treatment of cells with anti-CD44 antibody.
tion, consistent with previous reports for this mouse strain (36, 37), a large CD44⁺/HA-binding double-positive population was not found among resting cells, but was present after activation of both LN and spleen cells (Fig. 7, A and B). Whereas <1% of resting LN cells and 4% of resting splenocytes were CD44⁺/HA-binding, 23 and 34%, respectively, of the activated cell populations stained for both markers. Using the freshly isolated unactivated spleen and LN cells, rolling was almost undetectable. In contrast, both the activated LN and spleen cells rolled in significant numbers (Fig. 7 C); as many as 28 cells/mm² per minute were seen to roll in some experiments. Blocking of activated lymphocyte rolling was equally efficient with anti-CD44 (IM7) or soluble HA, whereas anti-CD45Rb, recognizing the T-200 antigen generally present on peripheral T cells, had no significant effect (Fig. 7 C).

Because of the flow dynamics and the limited efficiency with which interacting cells can effectively access the flat plate surface, only a fraction of potentially interacting cells passing through the laminar flow chamber actually have an opportunity to interact with the substrate. Therefore, to obtain an estimate of the proportion of cells capable of rolling in the activated LN population, flow was interrupted and activated lymphocytes were allowed to settle. We found that 26% (± 10.5%) of the activated LN population and 19% (± 6%) of the activated spleen population rolled when flow was reinitiated and that this was CD44 dependent, consistent with the proportion of cells induced to increased CD44-HA binding (Fig. 7 A). As a further approach to determine the proportion of cells capable of undergoing tethering, activated LN cells were allowed to adhere to HA-coated wells, which were gently rotated under shear conditions (6). Under these conditions, a similar fraction of adherent cells (18 ± 4% vs. 2% for unactivated cells) was obtained, and this was completely inhibitable by anti-CD44 mAbs. These results indicate that this CD44-HA adhesion event can occur in normal cell populations under conditions of cell activation.

To further establish the physiologic relevance of the CD44-mediated interaction, we sought to examine whether rolling activity could be detected in lymphocyte populations activated in vivo. Inguinal LN draining a site of complete adjuvant-induced inflammation at the base of the tail were removed 3 d after the last of three weekly challenges. FACS® analysis showed an increased percentage of CD44-HA-binding cells in these nodes (3–6.5%), compared with <1% in control LN distal to the injection site from the same animals. When analyzed for CD44-mediated rolling on both HA and SVEC4-10, small but significant numbers of cells exhibited rolling behavior (Fig. 7 D). As with in vitro activated lymphocytes, rolling was effectively ablated by anti-CD44 mAbs.

Discussion

Primary and secondary adhesion of leukocytes under conditions of physiologic flow have been attributed to selectins and integrins and their respective ligands on vascular endothelium; other receptors have not been implicated to date. In this study, we present in vitro experiments suggesting that another pair of ligands can mediate lymphocyte rolling on endothelial monolayers. In this system, clonal lymphoid B and T cell lines, as well as activated normal lymphocytes, roll on LN-derived EC as a result of the interaction between CD44 and its carbohydrate ligand hyaluronate. The response of adhesion to varying wall shear stress and rolling velocities suggests that this CD44-mediated rolling would most likely operate, as do selectins, at postcapillary venular wall shear stresses (1–4 dynes/cm²), but not at arteriolar or arterial stresses (>8 dynes/cm²). Although similar in character to selectin interactions with their carbohydrate ligands, our experiments effectively rule out a role for the known selectins and suggest that CD44 mediates an alternative primary leukocyte-EC adhesion pathway.

The association of CD44 with mechanisms of lymphocyte homing originated from observations that antigens recognized by certain mAbs against human CD44 (38) were similar in some properties to gp90ME § (39–41), the murine LN-specific homing receptor L-selectin. Some of these anti-CD44 antibodies were also reported to interfere with binding of lymphocytes to the high endothelial venules of secondary lymphoid tissues in frozen sections. Since the homologue of L-selectin exists in humans, with similar distribution and function to that described in mice (42–44), the role that CD44 plays in normal lymphocyte trafficking to secondary lymphoid tissues has remained unresolved. Nonetheless, it has been shown that interactions of CD44 with its major ligand hyaluronate can mediate static binding of lymphocytes to cultured EC lines in both mice and humans (15, 16, 18). The physiologic function of this interaction has not been defined. Our findings indicating a role for CD44 in primary adhesion under flow strengthens the possibility that this molecule participates in determining patterns of lymphocyte traffic. However, as we discuss below, we consider the evidence as favoring the likelihood that this interaction primarily manifests at tertiary extralymphoid sites of inflammation rather than secondary lymphoid tissues.

CD44 constitutes a widely distributed set of cell surface glycoproteins that has been studied in numerous systems, with the described roles for CD44 ranging from extracellular matrix binding, lymphocyte homing, and activation, to lymphopoiesis and metastasis (for review see reference 45). CD44 has been identified on most hematopoietic cells as well as nonhematolymphoid elements and, biochemically, has been shown to display great molecular heterogeneity owing both to variegated posttranslational modification and alternative exon use. Primary molecular analysis predicted a protein bearing homology to proteoglycan core and cartilage link proteins, which are known to interact with the glycosaminoglycan hyaluronate, a major component of the extracellular matrix. Although CD44 has been shown to bind other ligands, it is well established that HA is indeed a major ligand for CD44. The physiological role of the CD44-HA interaction has not yet been clarified.
Our results indicate that the cognate ligand for CD44 EC in the described rolling interaction is also hyaluronate (Figs. 3 and 4). In addition, the major properties of the rolling interaction can be duplicated on HA-coated plates (Fig. 4), and there is a direct relationship for all cells tested between endothelial and HA plate rolling (Table 1). Average rolling velocities on HA-coated plates (13.0 ± 3.5 µm/s) over a broad range of HA concentrations are similar to those found on EC, suggesting that the ligand that is limiting in this behavior is the amount and/or activity of cell surface CD44 capable of binding HA rather than the HA disaccharide polymer. Thus, the observed rolling interaction is not effectively different on various endothelial monolayers and over a wide range of concentrations of HA plates, likely overlapping what is relevant in vivo. These observations add to the selectins and their carbohydrate ligands another receptor–carbohydrate pair, CD44 and HA, capable of mediating primary adhesion and rolling under shear forces.

HA binding is not generally a constitutive property of CD44; rather, there appears to be activation dependence of CD44 for this characteristic to manifest (34). The mechanism by which CD44 becomes “activated” to bind HA is not known, although structural and posttranslational alterations of CD44 abound, and their relationship to HA binding is controversial (45, 46). We tested several cell lines of both B and T lineage and demonstrated constitutive high-level HA binding as well as rolling (Table 1). In addition, we show that other lymphoid lines as well as normal lymphocytes can be “activated” to CD44-mediated rolling. In our experiments, PMA and ionomycin treatment of both SAKRTLS12 and C6VL caused increases in FI–HA binding and resulted in conversion to a rolling phenotype. The increased FI–HA–binding levels on SAKRTLS12 (Table 1) did not lead to comparable elevated levels of rolling (Fig. 6). This may indicate that CD44 density is also an important factor in determining interactions under shear force, or may simple reflect that the CD44–HA interaction has different features in static assays on immobilized HA versus binding to soluble HA (45). Alternatively, it is possible that the cell surface distribution of CD44 is different on different cell lines; for example, perhaps CD44 on C6VL is more accessible distributed or clustered on microvilli than on SAKR. Consistent with previous reports showing noninducibility to HA binding of some CD44–expressing cell lines (35), some of our CD44+ cells also could not be induced to roll (Table 1).

It has been demonstrated that resting normal T and B cells, indeed all normal hematopoietic cells that express CD44, do not appreciably bind HA in vitro (45). Normal T cells, but not bone marrow myeloid cells, can be induced to bind HA with the antibody IRAWB14 (18, 36) or in vivo in an allogeneic response (47). Resting B cells can be induced to bind HA after several days culture in IL–5 (48) or in vivo in a chronic graft vs. host reaction (49). Our results indicate that CD44 and HA binding can be induced on T cells from spleen and LN in vitro by PMA/ionomycin (but not PMA alone [18, 36]), and that this treatment results in a population that also exhibits CD44–HA–mediated rolling on EC (Fig. 7 C). We also obtained results demonstrating the in vivo relevance of increased CD44–HA binding and subsequent rolling during the course of an immune response in the LN draining a site of local inflammation (Fig. 7 D). Similar results have also been obtained with spleen cells taken from animals undergoing acute GVHD, wherein T cells have been stimulated across an allogeneic barrier (DeGrendele, H.C., unpublished results). These experiments emphasize that cells with this interactive potential clearly are produced in the course of an immune response, supporting an in vivo role.

Our inability to separate HA binding from rolling (Table 1) by any means is highly suggestive that they both are the result of the CD44–HA interaction. Quantitatively, however, it seems clear that the threshold for detection of soluble HA binding is considerably lower than that for the rolling interaction. This is illustrated by use of IRAWB14, which, consistent with previous reports (18, 36), resulted in three of four cell lines in rapid 2.5–7-fold increases over baseline levels of soluble HA binding; yet in none of these were the increases sufficient for HA-mediated rolling (Table 1). It is likely that this reflects a requirement for increased numbers of CD44–HA interactions to overcome the fluid shear stress forces of physiologic flow. Alternatively, the difference between IRAWB and PMA induction may be the result of redistribution of CD44 to more surface-accessible compartments of the plasma membrane such as microvilli; other potential mechanisms such as phosphorylation events, posttranslational glycosylation, exon usage, or CD44 clustering could also be involved. Although the mechanism remains to be clarified, the observation that CD44 activation can lead to primary adhesion under physiologic flow now suggests one possible role for this induction.

It has long been appreciated that memory/effector and lymphoblast populations engage in discrete tissue-specific homing pathways. In particular, recirculation of these subsets tends to primarily involve extralymphoid (tertiary) tissues, that is, those inflammatory sites at which final effector cells exert their function. Such preferred homing mechanisms of memory/effector subsets have been described for inflamed skin, intestinal lamina propria, and inflamed synovium (for review see reference 50). There is also ample evidence for increased CD44 expression in activated and memory/effector lymphocyte populations (51, 52). Therefore, we would envisage that antigen–specific or other activation signals would be accompanied by increased CD44 expression and “activation” to HA-binding proficiency. Such activated cells could then be released into the circulation to sites of antigen localization in tertiary sites, where HA on endothelium would serve as ligand to initiate adhesion and support extravasation of activated memory/effector lymphocyte subsets. In support of this notion, anti–CD44 mAb treatment of mice prevented lymphocyte extravasation at an inflammatory site of delayed-type hypersensitivity, but had no influence on normal recirculation (53). We have also observed CD44–mediated rolling on HA by both in vitro- and in vivo–activated (but not resting) human lymphocytes (DeGrendele, H.C., unpublished data). Consider-
ing the very wide distribution of both CD44 and HA throughout the organism, the need for stringent regulation of CD44-HA binding might well be anticipated. Regulation of HA on endothelium in response to local pathophysiologic conditions would be one potential control point. In this regard, hyaluronate is found in both small and large vessels (33, 54) and has also been shown to luminal aspects within venules of LN (15). We have confirmed the presence of HA on the luminal aspect of small vessels in frozen sections of LN as well as spleen using the pPG reagent (data not shown). In addition, HA deposition has been shown to be markedly enriched intradermally in the course of delayed type hypersensitivity reactions (55), perhaps providing more efficient CD44-mediated interaction. Thus, we propose that the CD44-HA interaction would serve as part of a mechanism for regulating trafficking of activated effector cells to inflammatory sites, and potentially the dissemination of CD44+ metastatic cells to endothelium at target sites.

The paradigm of the pathway of leukocyte adhesion to endothelium as a multistep cascade determined by a variety of adhesion receptors continues to serve as a useful model, and it has evolved and continues to be refined to accommodate new observations. The findings presented here add new complexities that broaden the concept in significant ways. We show that primary adhesion is not the exclusive province of selectins or integrins, but also of activated CD44, thereby providing a potential physiologic role for the CD44-HA interaction. The ability to rapidly alter binding affinities to affect functional leukocyte-EC interaction has been described for integrins (56, 57) and may indeed be a common property of many leukocyte adhesion molecules. Thus, CD44 can now be added to a growing list of adhesion receptors sharing a constellation of features. However, the CD44 activation, which transpires over hours, is distinct from that of integrin receptors, which requires minutes. The slower induction and relatively prolonged duration of CD44 activation is more in keeping with a role in memory/effector cell homing outlined above. The adhesive behavior described in this system may suggest that such primary rolling is also a common property of receptor-carbohydrate ligand interactions under flow. These observations add to the repertoire of elements that can regulate adhesion under flow and emphasize the diverse array of leukocyte adhesion receptors and their vascular ligands that can participate in the control of leukocyte traffic.

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