The EMBO Journal vol.10 no.2 pp.343-348, 1991

The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells

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Communicated by A.F.Williams

CD44 is a polymorphic integral membrane protein which recognizes hyaluronate and whose proposed roles encompass lymphocyte activation, matrix adhesion and the attachment of lymphocytes to lymph node high endothelial venules (HEVs). Immunochemical and RNA blot data have supported the existence of two forms of CD44: a hematopoietic form expressed by cells of mesodermal origin (and by some carcinoma cell lines) and an epithelial form weakly expressed by normal epithelium but highly expressed by carcinomas. This report describes the isolation of a cDNA encoding a distinct CD44 polypeptide expressed by epithelial cells. Re-expression of each form of CD44 in a B cell line allowed cells transfected with the hematopoietic but not the epithelial form to bind to viable rat lymph node HEV cells in primary culture.

Key words: CD44/cell adhesion/hyaluronate-bearing cells/membrane protein

Introduction

Recently the study of lymphocyte activation, cell-matrix adhesion, and lymph node homing has converged on a broadly distributed human cell surface glycoprotein known as Pgp-1 (Hughes *et al.*, 1981; Lesley and Trowbridge, 1982), Hermes antigen (Jalkanen *et al.*, 1986), ECMRIII (Carter and Wayner, 1988) and CD44 (Dalchau *et al.*, 1980; Haynes, 1986). Monoclonal antibodies recognizing CD44 have been shown (i) to block adhesion of lymphocytes to lymph node frozen sections (Jalkanen *et al.*, 1986); (ii) to interfere with, or in some circumstances provide ancillary support for, lymphocyte activation (Huet *et al.*, 1989; Shimizu *et al.*, 1989; Denning *et al.*, 1990); (iii) to recognize an extracellular matrix receptor with binding affinity for collagen types I and VI (Carter and Wayner, 1988) and (iv) to interfere with lymphocyte development in long term bone marrow cultures (Miyake *et al.*, 1990a).

Biochemical similarities between CD44 and the mouse lymph node homing receptor recognized by antibody Mel-14 had earlier led to the suggestion that CD44 was the human equivalent of the Mel-14 receptor (Jalkanen *et al.*, 1987). Subsequent analysis by cDNA cloning (Goldstein *et al.*, 1989; Idzerda *et al.*, 1989; Nottenburg *et al.*, 1989; Stamenkovic *et al.*, 1989; Zhou *et al.*, 1989) showed that CD44 is the human equivalent of the mouse Pgp-1 protein (Nottenburg *et al.*, 1989; Stamenkovic *et al.*, 1989; Zhou *et al.*, 1989; and that the human homolog of Mel-14 (Bowen *et al.*, 1989; Siegelman and Weissman, 1989; Tedder *et al.*, 1989) antigen is a previously identified protein known as Leu-8 (Camerini *et al.*, 1989). The hematopoietic form of CD44 has recently been shown to recognize hyaluronate (Aruffo *et al.*, 1990; Miyake *et al.*, 1990b; Lesley *et al.*, 1990), and this recognition appears to account for all of the previously proposed adhesion specificities (Aruffo *et al.*, 1990).

Immunochemical evidence has suggested that a second form of the CD44 molecule may be present on some epithelial cells (Stamenkovic *et al.*, 1989; Picker *et al.*, 1989), and that expression of this form is typically increased by neoplastic transformation (Stamenkovic *et al.*, 1989). In this report we show that the second form of CD44 is a distinct polypeptide bearing an additional extracellular domain interposed proximal to the membrane-spanning domain of the hematopoietic/mesodermal form. Reintroduction of the smaller, but not the larger form into a B cell line expressing neither Leu-8 nor CD44 bestowed binding competence for viable rat lymph node high endothelial cells maintained in primary culture.

Results

Isolation of CD44 cDNA clone

To isolate a cDNA clone encoding the epithelial forms of CD44, a cDNA library prepared from the colon carcinoma line HT29 (D.Simmons and B.Seed, unpublished) was transfected into COS cells by the DEAE-dextran method (Seed and Aruffo, 1987). The cells were pooled 48 h after transfection, incubated with anti-CD44 mAb F-10-44-2 (Dalchau *et al.*, 1980) and panned on dishes coated with goat anti-mouse affinity purified antibody. After several washes, the adherent cells were lysed, and episomal DNA purified and transformed into *E.coli*. After two similar rounds of enrichment following spheroplast fusion (Seed and Aruffo, 1987; Aruffo and Seed, 1987), plasmid DNA recovered from seven out of 10 randomly picked colonies were found to direct the appearance of CD44 determinants on transfected COS cells. All seven of the positive clones bore cDNA inserts of ~2.4 kb. A single isolate was chosen for further analysis.

Nucleotide sequence of the cDNA

Restriction enzyme analysis of the cDNA insert of the CD44E clone showed that the coding sequence was enlarged relative to the previously identified clone by the addition of ~400 bp. DNA sequence analysis showed that the CD44E cDNA was identical to the hematopoietic CD44H cDNA, with three exceptions: a three amino acid (Ala-Thr-Arg) deletion at positions 221-223; an additional extracellular domain of 135 amino acids, spanning residues 221-355, containing one potential *N*-linked glycosylation site (Asn-Xaa-Ser/Thr); a glycine residue substitution for a glutamic acid residue at position 371, and an additional Ser-Gly motif

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   1 GGGAGACCCAAGCTTCTAGAGATCCCTCGACCTCGAGATCCATTGTGCTCTAAAGAGCGGACCCCAGCCTCTGCCAGGTTCGGTCCGCCATCCTCGTCCCGTCCTCCGCCGGCCCCTGCC

 121 CCGCGCCCAGGGATCCTCCAGCTCCTTTCGCCCGCGCCCTCCGTTCGCTCCGGACACCATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCA
   1                                                           M  D  K  F  W  W  H  A  A  W  G  L  C  L  V  P  L  S  L  A  Q

 241 GATCGATTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTCGGACGGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCC
  22 I  D  L  N  I  T  C  R  F  A  G  V  F  H  V  E  K  N  G  R  Y  S  I  S  R  T  E  A  A  D  L  C  K  A  F  N  S  T  L  P
           ---CHO---                                                                                          ---CHO---
 361 CACAATGGCCCAGATGGAGAAAGCTCTGAGCATCGGATTTGAGACCTGCAGGTATGGGTTCATAGAAGGGCATGTGGTGATTCCCCGGATCCACCCCAACTCCATCTGTGCAGCAAACAA
  62 T  M  A  Q  M  E  K  A  L  S  I  G  F  E  T  C  R  Y  G  F  I  E  G  H  V  V  I  P  R  I  H  P  N  S  I  C  A  A  N  N
                                                                                                                     ---CH
 481 CACAGGGGTGTACATCCTCACATACAACACCTCCCAGTATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCCAATGCCTTTGATGG
 102 T  G  V  Y  I  L  T  Y  N  T  S  Q  Y  D  T  Y  C  F  N  A  S  A  P  P  E  E  D  C  T  S  V  T  D  L  P  N  A  F  D  G
     O---                    ---CHO---                    ---CHO---
 601 ACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGGAGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGG
 142 P  I  T  I  T  I  V  N  R  D  G  T  R  Y  V  Q  K  G  E  Y  R  T  N  P  E  D  I  Y  P  S  N  P  T  D  D  D  V  S  S  G
                                                                                                                   *****
 721 CTCCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACATCTTTTACACCTTTTCTACTGTACACCCCATCCCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGAATCCCTCG
 182 S  S  S  E  R  S  S  T  S  G  G  Y  I  F  Y  T  F  S  T  V  H  P  I  P  D  E  D  S  P  W  I  T  D  S  T  D  R  I  P  R
                               ******                                                                                  ==
 841 TACCAATATGGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAAGATTTGGACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTC
 222 T  N  M  D  S  S  H  S  T  T  L  Q  P  T  A  N  P  N  T  G  L  V  E  D  L  D  R  T  G  P  L  S  M  T  T  Q  Q  S  N  S
     ========================================================================================================================
 961 TCAGAGCTTCTCTACATCACATGAAGGCTTGGAAGAAGATAAAGACCATCCAACAACTTCTACTCTGACATCAAGCAATAGGAATGATGTCACAGGTGGAAGAAGAGACCCAAATCATTC
 262 Q  S  F  S  T  S  H  E  G  L  E  E  D  K  D  H  P  T  T  S  T  L  T  S  S  N  R  N  D  V  T  G  G  R  R  D  P  N  H  S
     ===============================================================================================================---CHO--
1081 TGAAGGCTCAACTCATTTACTGGAAGGTTATACCTCTCATTACCCACACACGAAGGAAAGCAGGACCTTCATCCCAGTGACCTCAGCTAAGACTGGGTCCTTTGGAGTTACTGCAGTTAC
 302 E  G  S  T  H  L  L  E  G  Y  T  S  H  Y  P  H  T  K  E  S  R  T  F  I  P  V  T  S  A  K  T  G  S  F  G  V  T  A  V  T
     ========================================================================================================================
1201 TGTTGGAGATTCCAACTCTAATGTCAATCGTTCCTTATCAGGAGACCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAG
 342 V  G  D  S  N  S  N  V  N  R  S  L  S  G  D  Q  D  T  F  H  P  S  G  G  S  H  T  T  H  G  S  E  S  D  G  H  S  H  G  S
     ===================================================******                      ******
1321 TCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGT
 382 Q  E  G  G  A  N  T  T  S  G  P  I  R  T  P  Q  I  P  E  W  L  I  I  L  A  S  L  L  A  L  A  L  I  L  A  V  C  I  A  V
                    ---CHO--  ******                                          ----------------------------------------------
1441 CAACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGT
 422 N  S  R  R  R  C  G  Q  K  K  K  L  V  I  N  S  G  N  G  A  V  E  D  R  K  P  S  G  L  N  G  E  A  S  K  S  Q  E  M  V
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1561 GCATTTGGTGAACAAGGAGTCGTCAGAAACTCCAGACCAGTTTATGACAGCTGATGAGACAAGGAACCTGCAGAATGTGGACATGAAGATTGGGGTGTAACACCTACACCATTATCTTGG
 462 H  L  V  N  K  E  S  S  E  T  P  D  Q  F  M  T  A  D  E  T  R  N  L  Q  N  V  D  M  K  I  G  V  *

1681 AAAGAAACAACGTTGGAAACATAACCATTACAGGGGAGCTGGGACACTTAACAGATGCAATGTGCTACTGATTGTTTCATTTCGAATCTATAATAGCATAAAATTTTCTACTCTTTTTGT

1801 TTTTTGTGTTTTGTTCTTTAAAGTCAGGTCCAATTTGTAAAAACAGCATTGCTTTCTGAAATTAGGGCCCAATTAATAATCAGCAAGAATTTTGATCGTTTCAGTTCCCCACTTGGAGGC

1921 CTTTCATCCCTCGGGTGTGCTATGGATGGCTTCTAACAAAAACCTACCACATAGTTATTCCTGATCGCCAACCTTGCCCCCCACCAGCTAAGGACATTTCCAGGGTTAATAGGGCCTGGT

2041 CCTGGGAGGAAATTTGAATGGGTCATTTTGCCCTTCCATTAGCCTAATCCCTGGGCATTGCTTTCCACTGAGGTTGGGGGTTGGGGTGTACTAGTTACACATCTTCAACAGACCCCCTCT

2161 AGAAATTTTTCAGATGCTTCTGGGAGACACCCAAAGGGTAAGTCTATTTATCTGTAGTAAACTATTTATCTGTGTTTTTGAAATATTAAACCCTGGATCAGTCCTTTTATTCAGTATAAT

2281 TTTTTAAAGTTACTTTGTCAGAGGCACAAAAAGGGTTTAAACTGATTCATAATAAATATCTGTACCTTCTTCGAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and derived amino acid sequences. Potential *N*-linked glycosylation sites are marked −CHO−; potential glycosaminoglycan attachment sites and the transmembrane domain are underlined. The epithelial-specific extracellular domain is double underlined.

which may represent an attachment site for chondroitin sulfate (Figure 1). The mature protein would comprise 473 residues with a predicted M_r of 51.3 kd. Comparison of the protein sequence encoded by the epithelial cell specific domain showed no close relationship to sequences reported in existing databases.

RNA blot analysis

Previous RNA blot analyses had shown that CD44 transcript patterns were cell-type dimorphic, with three major species of 1.6, 2.2 and 5.0 kb detected in RNA isolated from a variety of hematopoietic cell lines and similar species of about 2.0, 2.6, and 5.4 kb detected in RNA isolated from some but not all epithelial tumor (carcinoma) cell lines (Stamenkovic *et al.*, 1989). Several primary carcinomas expressed all six major transcripts as well as variable minor transcripts of 3.6 and 4.6 kb (Figure 2), whereas a mesothelioma (mesoderm-derived epithelial cell tumor) specimen and activated lymphocytes expressed only the three transcripts common to hematopoietic cell lines. To determine whether the transcript pattern reflected the inclusion of the additional segment present in the epithelial CD44 cDNA, RNA blot were hybridized with a probe specific for the epithelial domain. Comparison of the resulting pattern with the pattern generated by the full length cDNA showed that the higher but not the lower molecular weight isoforms were selectively recognized by the epithelial domain-specific probe (Figure 2).

Expression of the epithelial form of CD44 in COS cells allowed the immunoprecipitation of a predominant cell

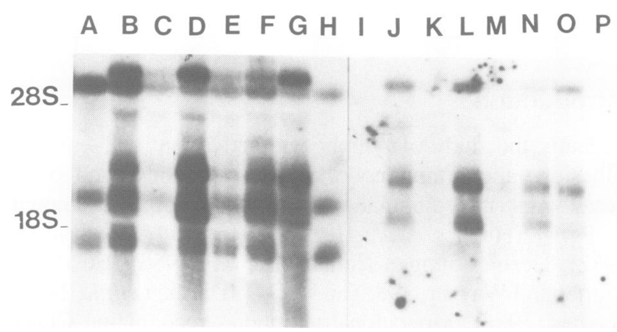

Fig. 2. CD44 transcripts in primary tumors of mesenchymal and epithelial origin, in normal epithelium, and in lymphocytes. The RNA blot was hybridized with a probe containing sequences common to both cDNA clones in lanes A−H. The same filter was hybridized to a probe derived from the epithelial-specific extracellular domain in lanes I−P. Lanes A and I, mesothelioma (mesenchymal tumor); lanes B and J, colon adenocarcinoma sample 1; lanes C and K, normal colonic epithelium sample 1; lanes D and L, colon adenocarcinoma sample 2; lanes E and M, normal colonic epithelium sample 2; lanes F and N, esophageal carcinoma, lanes G and O, thyroid carcinoma; lanes H and P, lymphokine activated T lymphocytes.

surface form of ~ 130 kd, while parallel immunoprecipitation of the hematopoietic form showed a species of molecular mass ~ 80 kd (Figure 3). Concordant biochemical and RNA blot analysis of two colon carcinoma cell lines, one bearing only the epithelial cell transcript pattern and the other principally the hematopoietic cell pattern, showed that the presence of the higher molecular weight RNAs corresponded

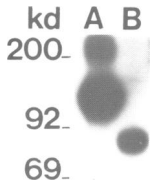

Fig. 3. Immunoprecipitations of CD44 from: lane A, CD44E-transfected COS; lane B, CD44H-transfected COS.

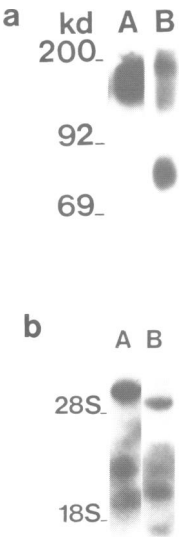

Fig. 4. (**a**) Immunoprecipitations of CD44 from: lane A, colon carcinoma SW948; lane B, colon carcinoma SW480. (**b**) CD44 transcripts in colon carcinoma lines: lane A, SW948; lane B SW480.

to a large (~160 kd) form of CD44, whereas the lower molecular weight RNAs corresponded to a smaller (~80 kd) form of CD44 and variably represented higher molecular weight forms (Figure 4 and data not shown)

Establishment of rat peripheral lymph node primary cultures

To help establish the biological significance of CD44 expression by lymphocytes, an alternative system for measuring lymphocyte adhesion was sought. In recent years Ager (1987) and subsequently Ise *et al.* (1988) have described culture conditions which allow the selective outgrowth of cells exhibiting the characteristic sulfate anabolism and cell surface phenotype of lymph node high epithelial cells *in situ*. Following the procedures prescribed by Ise and coworkers, we have established primary cultures of lymph node stromal cells. By morphological, immunochemical, and biochemical criteria, these cultures appear identical to those described by Ager (1987) and Ise *et al.* (1988). In contrast to the pattern of adhesion seen by lymphocyte and human lymphoid cell lines *in vivo*, binding of cells to the rat HEV cultures was found not to correlate with the presence of Leu-8, but rather with the presence of CD44 (Aruffo *et al.*, 1990). To examine the behavior of the two different forms of CD44 in this system, stable transfectants expressing the hematopoietic or epithelial forms of CD44 were established from the Burkitt lymphoma cell line Namalwa, which lacks both Leu-8 and CD44. Flow cytometric analysis (Figure 5) demonstrated that the transfectant cell lines showed identical surface CD44 expression and identical forward light scatter profiles (a measure of cell size, and hence of cell surface area).

Binding of the parental and transfectant cell lines was measured on viable rat node-derived cells in culture, typically at reduced temperature to allow the clearest definition of specific interaction. Quantitative binding analysis showed that transfectants bearing the hematopoietic, but not the epithelial form of CD44 bound to the rat HEV-derived cell lines (Figure 6). To confirm that binding was mediated by hyaluronate (Aruffo *et al.*, 1990), HEV cells were incubated with 50 μg/ml of hyaluronate or treated with streptomyces hyaluronidase (1 μg/ml) prior to performing the binding assays. Both types of treatment blocked adhesion (Figure 6). Binding was partially dependent on the presence of calcium but not magnesium in the incubation buffer, was unaffected by the presence or absence of sodium azide, and was abolished by glutaraldehyde pretreatment of the target cells (Figure 7). Inclusion of monoclonal antibodies P1, P2 (Pals *et al.*, 1989) as ascites at a dilution of 1:100, or Hermes-3 (Jalkanen *et al.*, 1986) at a concentration 50 μg/ml in the binding assay had no effect on the fraction of cells specifically bound. However a 1:100 dilution of a mouse polyclonal anti-CD44 antiserum was found to specifically inhibit binding (Figure 6).

Specific adhesion of the transfectants generally could not be demonstrated with other cells in culture. The sole exception was the hamster fibroblast line BHK, which bound both hematopoietic and epithelial transfectant cells, the latter less avidly than the former (data not shown).

The hematopoietic and epithelial CD44 isoforms show similar glycosaminoglycan substitutions

Immunoprecipitation of the hematopoietic form of CD44 yields 90 and 180 kd species (Figure 8, Jalkanen *et al.*, 1988; Stamenkovic *et al.*, 1989) and it has proposed that the 180 kd species arises by chrondroitin sulfate substitution of the 90 kd form (Jalkanen *et al.*, 1988). CD44 immunoprecipitates from stable transfectants bearing the epithelial isoform revealed a single 130 kd species without the associated higher molecular weight variants seen in comparable immunoprecipitates of transfectants bearing the hematopoietic form (Figure 8). Both stable transfectants were subjected to digestion with enzymes which cleave different glycosaminoglycans. The size of both isoforms was reduced by chondroitin A−C lyase and keratanase but was unaffected by heparitinase or hyaluronidase (Figure 8). Chondroitin lyase reduced the molecular weight of the hematopoietic form more than did keratanase, while both enzymes had a roughly equal effect on the epithelial form. Both 90 kd and 180 kd variants of hematopoietic CD44 were susceptible to digestion, with the higher molecular weight species losing twice the molecular mass lost by the lower molecular weight species in each case (data not shown). The 180 kd form therefore does not appear to derive from glycosaminoglycan substitution of the 90 kd form. The ratio of molecular weights of the two forms would argue for the larger form being a dimer of the smaller form. However, the higher molecular weight form persists under reducing conditions and

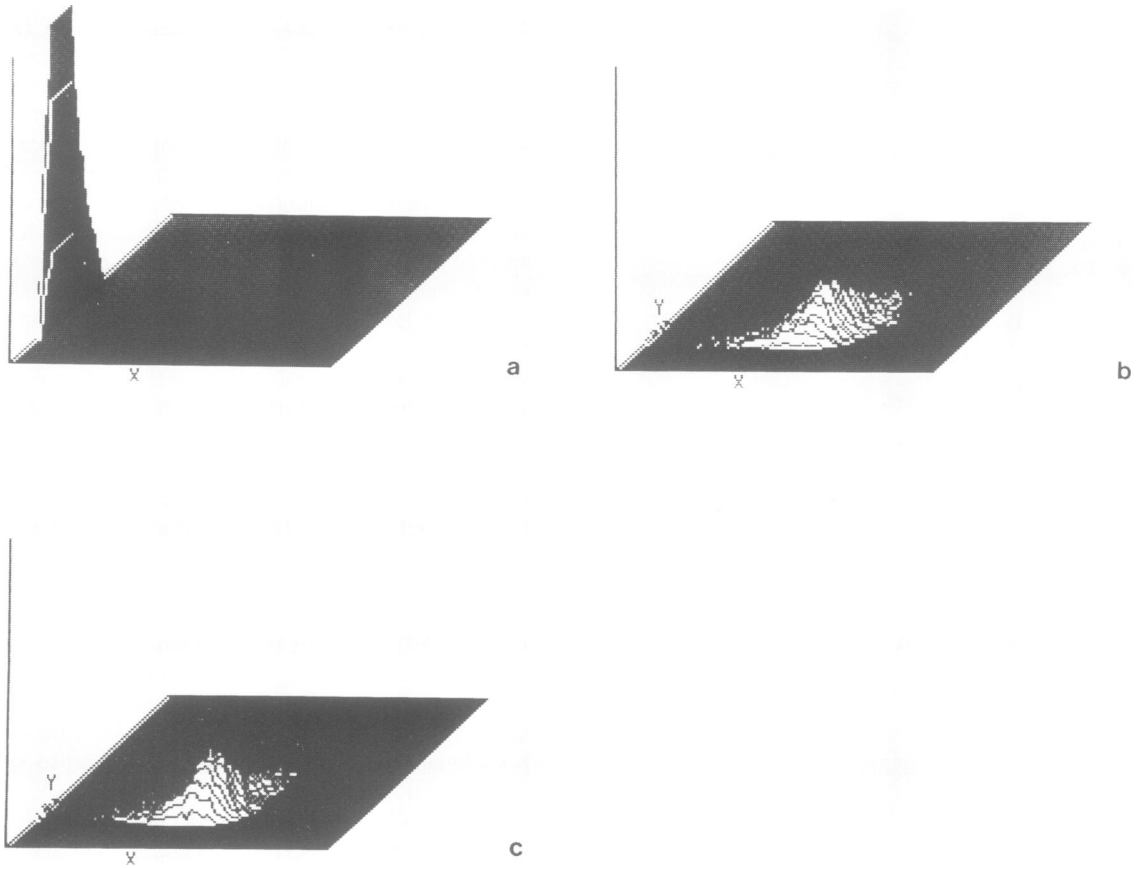

Fig. 5. FACS analysis of CD44H and CD44E expression in Namalwa transfectants. (**a**) Namalwa cells treated with anti-CD44 mAb F10-44-2 and affinity purified FITC-conjugated goat antibody to mouse IgG. (**b**) CD44H-Namalwa transfectant similarly stained with F10-44-2. (**c**) CD44E-Namalwa transfectant stained with F10-44-2.

conclusion data as to its nature could not be obtained. Taken together, these observations suggest that the hematopoietic and epithelial CD44 isoforms are both substituted with glycosaminoglycans.

Discussion

In this work we have shown that two isoforms of CD44 can be found on cells of epithelial or hematopoietic/mesodermal origin. Previous characterization of CD44 has focused on the hematopoietic/mesodermal form, leaving the possibly separate attributes of the epithelial form unexamined. We find here that by at least one criterion, the hyaluronate-mediated adhesion of cells to high endothelial cells, the two forms are distinct. Surprisingly, the larger form fails to bind effectively to node-derived cells. The binding defect cannot be easily ascribed to substantial differences in glycosaminoglycan substitution and is more likely attributed to an interference with binding mediated by the presence of the additional peptide sequences. Whether this interference is the result of a conformational change in the binding domain or the inability of the molecule to form aggregates which may be necessary for hyaluronate binding remains to be determined.

The role of lymphocyte CD44 in tissue recirculation is uncertain in part because no direct *in vivo* experiments have documented a necessity for the molecule in homing. The recent discovery that CD44 is the principal cell surface receptor for hyaluronate (Aruffo *et al.*, 1990; Miyake *et al.*, 1990b; Lesley *et al.*, 1990) suggests that CD44 primarily mediates functions other than homing, such as cell motility, division, adhesion to extracellular matrix and adhesion to stromal cells. CD44 may have an accessory function in lymphocyte adhesion to HEV cells by helping stabilize interactions between more specific homing receptors and their ligands, but its affinity for hyaluronate suggests a more prominent role in regulating lymphocyte migration in tissues subsequent to diapedesis. The later role may help explain the observed difference in adhesion promoted by the two isoforms. Although both lymphocytes and epithelial cells interact with extracellular matrix, they have very different requirements. Epithelial cells are mostly sessile and subject to contact inhibition, while lymphocytes are motile, and contact-unregulated.

A similarly polymorphic matrix adhesion protein of epithelial cells, syndecan, expresses one isoform on cells in simple (unlaminated) epithelium, and another isoform on cells in stratified epithelium (Sanderson and Bernfield, 1988). It has been suggested that the different syndecan isoforms (which differ in glycosaminoglycan substitution) are required to provide cell−matrix interactions appropriate to the epithelial architecture. The much broader tissue distribution of CD44 suggests that it promotes a type of adhesion which is needed or exploited by a wide variety of cells. Because epithelial cell expression of CD44 appears to be coordinated

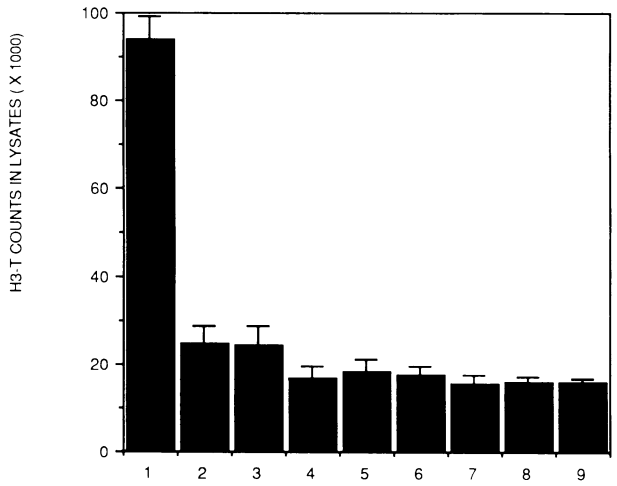

Fig. 6. Binding of CD44 transfectants to lymph node stromal cells. Binding of transfected and untransfected Namalwa cells was quantified by measurement of the [^{3}H]thymidine content of adherent cells. Lanes 1–3: Adhesion to untreated HEV cells; Lane 1: CD44H cells, lane 2: CD44E cells; lane 3: Namalwa control cells; Lanes 4–6: adhesion to HEV cells in the presence of 50 μg/ml of hyaluronate: lane 4: CD44H cells; lane 5: CD44E cells; lane 6: Namalwa control cells; Lanes 7–9: adhesion to HEV cells pre-treated with *Streptomyces* hyaluronidase (1 μg/ml): Lane 7: CD44H cells; Lane 8: CD44E cells; lane 9: Namalwa control cells.

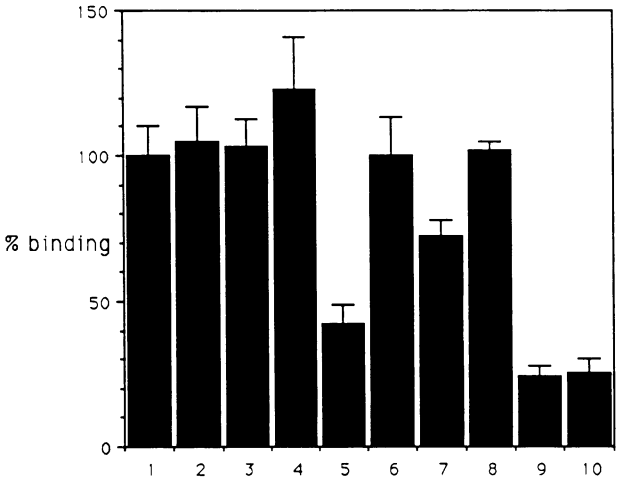

Fig. 7. Binding of CD44H transfectants to lymph node HEV cells under various conditions. Binding is expressed as the percentage of counts measured for CD44H cells bound to HEV cells in media alone. CD44H cell binding was assessed under the following conditions: Lane 1, media alone; lanes 2–5, in the presence of: lane 2, mAb P1; lane 3 mAb P2, lane 4, mAb Hermes 3; lane 5, polyclonal anti-CD44 antiserum; lanes 6–9 in the presence of: lane, $CaCl_2$ only; lane 7, $MgCl_2$ only; lane 8, sodium azide; lane 9, prefixation of stromal cells with glutaraldehyde; lane 10, Namalwa cells (negative control).

with proliferative status, it may prove fruitful to explore whether CD44 coordination has functional consequences for cells outside the immune system.

Materials and methods

Immunoprecipitations

Cells from the colon carcinoma lines SW480 and SW948 or CD44-transfected COS cells were surface-labeled with ^{125}I and lysed in a Tris buffered saline solution containing 20 mM iodoacetamide, 1 mM PMSF, and 1% NP-40. After preclearing with goat anti-mouse immunoglobulin beads (Cappel, Malvern, PA, USA) the lysates were treated with

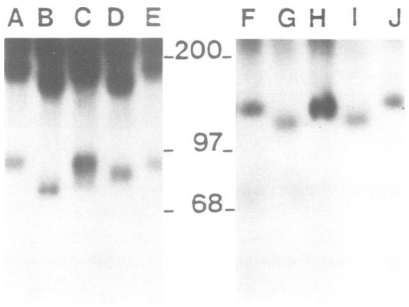

Fig. 8. Chondroitin lyase, keratanase, heparitinase and hyaluronidase digestion of lysates derived from CD44H (hematopoietic form) and CD44E (epithelial form) transfectants. Lanes A–E, CD44H transfectants treated with: lane A, media; lane B, chondroitin A–C lyase; lane C, hyaluronidase; lane D, keratanase; lane E, heparitinase; lanes F–J, CD44E transfectants treated with: lane F, media; lane G, chondroitin A–C lyase; lane H, hyaluronidase; lane I, keratanase; lane J, heparitinase. Molecular weights are shown in kd.

J173 or F-10-44-2 mAb and protein A–Sepharose CL-4B (Sigma, St. Louis, MO, USA). The beads were washed, eluted, and electrophoresed on a 6% acrylamide gel under reducing conditions.

Chondroitin lyase digestion was performed on CD44H and CD44E lysates in 1% SDS, 100 mM Tris pH 6.8, and 0.1 M β-mercaptoethanol. After diluting the eluates with 1–5 volumes of PBS, chondroitin lyase AC (ICN, Lisle, IL, USA) was added to 0.5 U/ml and incubated at 37°C for 1 h. Keratanase (0.1 U/ml), heparitinase (0.01 U/ml) and hyaluronidase (0.1 U/ml) (ICN, Lisle, IL, USA) digestions of CD44H and CD44E lysates were performed in PBS, at a pH of 7.4, 7.0 and 7.4 respectively at 37°C for 1 h.

RNA blot hybridization

Total RNA was prepared from cell lines or tissue specimens obtained at surgery by the guanidinium thiocyanate/cesium chloride method; 20 μg of RNA were loaded per lane on 1% agarose gels, electrophoresed, transferred onto nylon filters and hybridized with ^{32}P-labeled CD44 probes. The washed filters were subjected to autoradiography for 12–48 h. A probe corresponding to the epithelial form-specific extracellular domain was prepared by subjecting the cDNA clone to *Hin*fI endonuclease digestion. A 358 bp fragment was separated on a 1.5% Nu sieve agarose gel, purified, and ^{32}P-labeled by random priming.

Stable CD44 transfectants

CD44H and CD44E cDNA clones were inserted into a modified p205 plasmid containing the EBNA-1 gene (Yates *et al.*, 1985; A.Peterson, unpublished). 100 μg of DNA were transfected into the Burkitt lymphoma cell line Namalwa by electroporation at 400 V, 1000 μF. Transfected cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, NY, USA) with 20% fetal bovine serum, aliquoted into 24 well plates at 10^5 cells per well, and allowed to grow for 48 h. Hygromycin B was then added to 500 μg/ml. Hygromycin resistant cells were cloned by limiting dilution and tested for CD44 expression on a FACS.

Lymph node stromal cell cultures

Rat cervical nodes were removed, sectioned into 1 mm slices and rinsed several times in IMDM. The sections were then resuspended in IMDM in the presence of 0.5% collagenase type II (Sigma) and incubated at 37°C for 1 h with gentle rocking. The collagenase treated suspension was filtered through a 100 μm nylon mesh, briefly centrifuged, resuspended in IMDM/20% fetal bovine serum and seeded onto 10 cm culture dishes. 12 h later, the dishes were washed to remove non-adherent cells.

Anti-CD44 anti-serum

A CD44-immunoglobulin fusion protein was derived by ligating the portion of cDNA encoding the extracellular domain of CD44H to sequences encoding human immunoglobulin G constant region (Aruffo *et al.*, 1990). The construct was introduced into the vector CDM7 [a derivative of CDM8 (Seed, 1987) which lacks the polyoma origin of replication], and transfected into COS cells. Supernatants containing the soluble CD44-Ig fusion protein were removed after 10 days of culture, and incubation with 200 μl of packed trisacryl beads overnight. The beads were washed several times with PBS/1% Nonidet-P40 followed by PBS/30 mM NaCl, and the protein was eluted

in 1 ml of 0.1 M acetic acid and neutralized with 50 μl 2 M Tris base. The typical yield was 10–20 ng of protein/μl.

About 2 μg of protein were mixed with 250 μl of Freund's complete adjuvant and injected into mice intraperitoneally. Two boosts with complete and one with incomplete adjuvant were delivered over a three week period. Ascites was collected and tested for reactivity with CD44 transfectants by immunofluorescence at dilutions of 1:100 to 1:1000.

CD44 cell–cell adhesion assays

Lymph node stromal cells were grown to confluence in 24 well plates. 10^7 cells each of CD44H, CD44E stable transfectants and Namalwa or CD1-Namalwa stable transfectants were incubated with 50 μCi/ml [^{3}H]thymidine for 12 h, washed several times in PBS, resuspended in IMDM/10% FBS and evaluated for [^{3}H]thymidine incorporation. 2×10^5 cells were introduced per well and incubated with the confluent stromal cells for 30 min at 4°C with gentle rocking. Wells were washed with IMDM several times, adherent cells were lysed with 0.1% SDS and the [^{3}H]thymidine content of lysates was measured in a scintillation counter. Hyaluronate blocking assays were performed by incubating the HEV cells with 50 μg/ml hyaluronate (Sigma, St. Louis, MO, USA) at 37°C for 1 h. Hyaluronidase treatment was performed by incubating HEV cells with 1 μg/ml *Streptomyces* hyaluronidase (Calbiochem, La Jolla, CA, USA) for 1 h at 37°C in IMDM. Cells were washed in PBS and fresh medium was added for adhesion assays. Antibody blocking assays were carried out by pre-incubating transfectants or Namalwa control cells with monoclonal or polyclonal antibodies for 30 min at 4°C. Monoclonal antibodies were used at a concentration of 1:100 (ascites) or 50 μg/ml (Hermes-3 purified antibody). To determine divalent cation requirements, adhesion assays were performed with or without calcium and magnesium chloride (0.7 mM and 0.5 mM respectively). To determine the effects of glutaraldehyde fixation, stromal cells were fixed in 1% glutaraldehyde for 20 min prior to binding assays. Sodium azide, when used, was adjusted to 0.02%.

Acknowledgements

We thank Eugene Butcher, Carl Figdor, and Barton Haynes for generous gifts of monoclonal antibodies. Alejandro Aruffo is a Fellow, and Ivan Stamenkovic a Special Fellow, of the Leukemia Society of America. Martine Amiot was supported in part by a grant from the Association pour Recherche contre le Cancer. This work was funded by an award from Hoechst AG to the Massachusetts General Hospital.

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Received on March 23, 1990; revised on November 15, 1990

Note added in proof

The sequence data reported here are available from the EMBL/Genbank databases under the accession number X55150.