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Assembly of pericellular matrices by COS-7 cells transfected with CD44 lymphocyte-homing receptor genes

(hyaluronan/extracellular matrix)

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ABSTRACT **The capacity to assemble and retain a pericellular matrix is correlated with the expression of the cell surface binding sites specific for the extracellular matrix macromolecule hyaluronan. These binding proteins have been termed hyaluronan receptors. The lymphocyte-homing receptor CD44 may have identity with these hyaluronan receptors. To determine whether hyaluronan receptors function independently in this capacity for matrix assembly, mammalian cells were transfected with cDNA encoding the putative hyaluronan receptor CD44. After transfection with CD44 cDNA, COS cells gained the capacity to assemble hyaluronan-dependent pericellular matrices in the presence of exogenously added hyaluronan and proteoglycan. Thus, CD44 receptors do function as matrix-organizing, matrix-anchoring hyaluronan-binding proteins. In addition, the expression of CD44/hyaluronan receptors alone is sufficient to direct this matrix assembly. If matrix assembly is a function of cells *in vivo* that express hyaluronan receptors, this raises interesting possibilities for the role of the receptor in cell migration, when new extracellular matrix environments are encountered.**

Many cell types including chondrocytes (1–5), tumor cells (6, 7), and fibroblasts (1, 4, 8, 9) express cell surface binding sites for the extracellular matrix macromolecule hyaluronan (10). These cell surface binding sites show high affinity and specificity for hyaluronan and have been termed hyaluronan "receptors" (8, 10, 11). Recent reports (12–19) suggest that cell surface hyaluronan receptors are related or identical to the CD44 family of lymphocyte-homing receptors, also known as Pgp-1, H-CAM, ECM-III, Hermes antigen, HUTCH-1, and In(Lu)-related p80. Most of these relationship data are based on immunological cross-reactivity (13, 14), sequence homology to cartilage link protein (12, 18, 19), antibody inhibition of hyaluronan binding (13–15), and reactivity with lymph node high-walled endothelial cells (12–14, 16). Nevertheless, considerable doubt remains as to whether CD44 molecules truly function as cell surface hyaluronan receptors because until now the function of hyaluronan receptors on many cell types has not been well established. We have developed methods (5, 7) that document one important function of hyaluronan receptors—namely, their role in anchoring and organizing carbohydrate-rich pericellular matrices around cells.

In cartilage, hyaluronan is present in a multimolecular complex in which up to 100 chondroitin sulfate-rich proteoglycans are noncovalently bound to a single hyaluronan chain (20). These hyaluronan/proteoglycan aggregates are deposited at high concentrations within the cartilage extracellular matrix and are essential to cartilage physiology (20). On chondrocytes these hyaluronan/proteoglycan aggregates appear to be anchored to the cell surface via interaction with hyaluronan receptors (1, 2, 5). Chondrocytes organize these receptor-bound macromolecules into a carbohydrate-rich pericellular matrix. These matrix halos on cells *in vitro* often extend from the plasma membrane by as much as one cell diameter (7, 21). *In vivo*, this pericellular matrix may function as a template structure to facilitate the organization of intracellular matrix (1, 2, 5, 21).

Other cell types, such as tumor cells derived from malignant carcinomas, also express hyaluronan receptors but lack the capacity to synthesize large amounts of hyaluronan or aggregating proteoglycans. These cells thus lack expression of pericellular matrices such as those present on chondrocytes. However, when purified hyaluronan and aggregating proteoglycan are exogenously added, the tumor cells have the capacity to assemble prominent pericellular matrices similar in size to the chondrocyte matrices (7). Noninvasive tumor cells that do not express significant hyaluronan-binding activity do not assemble pericellular matrices under similar conditions (7). Therefore, the capacity to assemble pericellular matrix in the presence of an appropriate mixture of extracellular macromolecules suggests an important function of the cell surface hyaluronan-binding activity on many cell types.

Questions remain, however. Are hyaluronan receptors alone responsible for matrix assembly? Can putative hyaluronan receptors, such as CD44, function independently in this capacity? In this study, mammalian cells were transfected with plasmids containing cDNA encoding CD44. We found that cells that express cell surface CD44 assembled pericellular matrices with exogenously added hyaluronan and proteoglycan. Thus, CD44 receptors do act as matrix-organizing/matrix-anchoring functional hyaluronan receptors.

MATERIALS AND METHODS

Transfection of Cells. Mammalian cells (simian virus 40-transformed African Green monkey kidney cell line COS-7, subline M6) were transfected with cDNA encoding the hematopoietic form of CD44 (CD44-H), with cDNA encoding the epithelial form of CD44 (CD44-E) or with the parental vector (CDM8) without an inserted sequence. The CDM8 vector has been used previously for expression of human CD2 on the surface of COS cells (22, 23). The isoforms CD44-H and CD44-E (16, 24) are used to denote a 80- to 90-kDa product derived from hematopoietic cell lines (CD44-H) and an alternatively spliced [enlarged by the addition of ≈400 base pairs (bp)] product derived from a colon carcinoma cell line (CD44-E). Transfectants were prepared by treating nearly confluent COS-7 cells with 2.0 μg of pCD44-H (18), pCD44-E (16), or pCDM8 (23) per ml by using diethylaminoethyldextran as a facilitator (22). After 4.5 hr, cells were treated for 2.0 min with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS). Sixteen hr after transfection, cells were treated with 0.25% trypsin for 10 min and replated at the same cell density.

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Four days after transfection, the cells were detached with 2.0 mM EDTA in PBS, followed by two washes in PBS alone. One aliquot of cells was plated into a 6-well, flat-bottom culture plate (six 35-mm wells; Falcon) for immediate incubation with exogenous macromolecules. Another aliquot of cells was plated into 35-mm dishes and allowed to attach overnight before addition of exogenous macromolecules. A third aliquot of cells were fixed in 1% glutaraldehyde in PBS (pH 7.2) for 5 min at room temperature. Fixative was quenched by two subsequent washes with PBS containing 5.0 mg of bovine serum albumin per ml. This fixation condition preserves hyaluronan binding activity (6, 7) and thereby "freezes" the CD44 receptors at the time of maximal cell surface expression.

Immunolocalization of CD44 on Transfected Cells. Two commercial anti-human CD44 antibodies [BU-52, The Binding Site (San Diego); and J173, AMAC (Westbrook, ME)] were used to document the cell surface expression of CD44 receptors. Glutaraldehyde-fixed transfected COS-7 cells in suspension were incubated for 2 hr with either BU-52 or J173 anti-CD44 antibody diluted 1:10,000 in PBS containing 1% bovine serum albumin, followed by a 1-hr incubation with a peroxidase-labeled goat anti-mouse second antibody (Pierce). Diaminobenzidine was used as the chromogen.

Matrix Assembly with Exogenous Macromolecules. Taken from each transfection group, aliquots containing 1×10^4 freshly EDTA-released cells, glutaraldehyde-fixed cells, or cells plated for 12 hr after EDTA release were incubated at 37°C in 2.0 ml of serum-free Dulbecco's modified Eagle's medium containing 3.0 mg of aggregating proteoglycan monomer [purified from rat chondrosarcoma tumor (7)] and 15 μg of hyaluronan (Sigma, grade I) per ml. Glutaraldehyde-fixed cells were incubated with exogenous macromolecules for 12 hr; freshly plated or 12 hr-attached live cells were similarly incubated for 3 hr. It was determined previously in our laboratory that 3 hr was the minimum time necessary for matrix assembly (5, 7). The glutaraldehyde-fixed cells were kept in suspension with gentle rotation during their incubation with exogenous macromolecules. In some experiments, matrices formed by exogenous hyaluronan and proteoglycan were subjected to subsequent treatment with *Streptomyces* hyaluronidase (Sigma type IX) at 1 unit/ml for 15 min at 37°C. In other experiments, cells were pretreated at 37°C for 10 min with 0.25% trypsin (Sigma), rinsed twice in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum, and then rinsed twice in serum-free medium prior to the addition of exogenous hyaluronan and proteoglycan.

Hexasaccharides of hyaluronan were prepared as described (5, 7). In one set of experiments, hyaluronan hexasaccharides (125 μg/ml) were added to cells at the time of addition of hyaluronan and proteoglycan. In another set of experiments, fully established pericellular matrices were subjected to subsequent incubation for 2 hr with hyaluronan hexasaccharides (125 μg/ml) to determine whether the newly assembled matrix could be displaced by hyaluronan hexasaccharides.

To determine whether matrix assembly could be inhibited immunologically, transfected COS-7 cells were also incubated with exogenous hyaluronan and proteoglycan in the presence of 0.2–10 μg of anti-CD44 antibodies per ml [BU-52 and J173 (anti-human CD44) or KM201 (anti-mouse Pgp-1; American Type Culture Collection)].

Visualization of the Pericellular Matrix. Pericellular matrices were visualized morphologically by using a particle-exclusion assay (21, 25). After incubation with exogenous macromolecules, the glutaraldehyde-fixed transfected COS-7 cells were transferred to six-well, flat-bottom tissue culture plates (Falcon), and the cells were "splatted" onto the substratum by centrifugation at 500 × *g* for 15 min in an Omifuge RT (Baxter Scientific, McGaw Park, IL) microtiter plate holder. The supernatant medium was removed and replaced with a 0.75-ml suspension of formalin-fixed erythrocytes (10^8 per ml) in PBS containing 0.1% bovine serum albumin (21). The particles were allowed to settle for ≈10 min. The cells were then observed and photographed with an inverted phase-contrast microscope. For matrix visualization on live cells—either cells directly incubated for 3 hr with exogenous macromolecules or cells allowed to attach overnight followed by incubation with exogenous macromolecules—the medium was removed and replaced with a suspension of fixed erythrocytes as described above.

The presence of pericellular matrices was quantified by morphometric analysis (21) with a Summa Sketch Plus digitizing graphics tablet (Summagraphics, Seymour, CT) interfaced with a microcomputer using a SIGMA SCAN software program (Jandel, Corte Madera, CA). Ratios of the area delineated by the perimeter of the pericellular matrix/area delineated by the cell plasma membrane (termed "matrix/cell ratios") were calculated. Cells with large pericellular matrices have values that approach 3.0, whereas cells devoid of matrix have values that approach 1.0.

RESULTS

To further define the role of CD44-related receptor proteins as functional hyaluronan receptors, COS cells were trans-

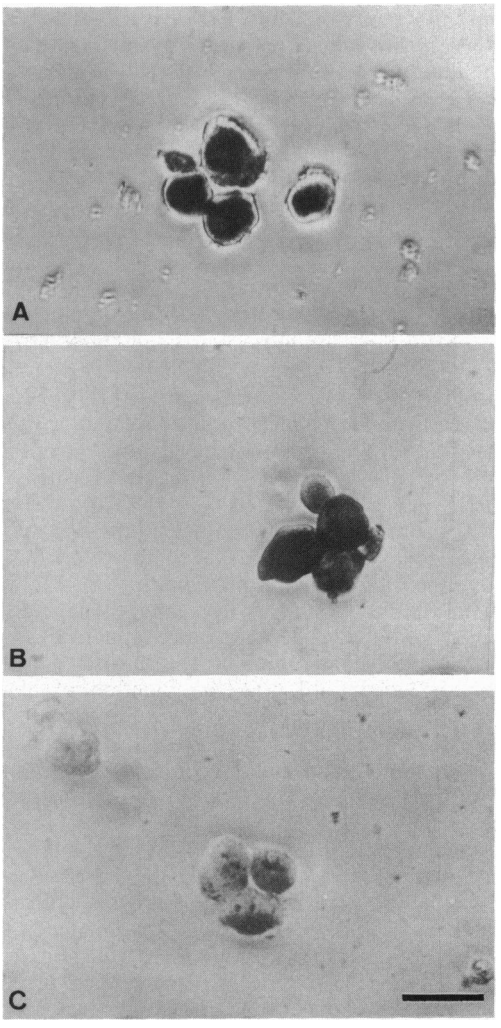

FIG. 1. Immunolocalization of CD44 on transfected COS-7 cells. Cells transfected with CD44-H cDNA (*A*) and *CD44-E* cDNA (*B*) exhibit immunoreactivity for the CD44 epitope recognized by the BU-52 antibody, whereas the cells transfected with the parental CDM8 vector (*C*) do not exhibit immunoreactivity. (Bar = 50 μm.)

fected with cDNA encoding CD44-H or CD44-E. The CDM8 vector used for these transfections effected a high but transient expression of CD44 (22, 23). In this study, the transfected cells were assayed as live cells on day 4 or 5 after transfection or, as glutaraldehyde-fixed cells, were "frozen" at a period of maximal expression on day 4. The fixed cells were used to provide a time-independent, homogeneous population of cells for multiple repetitions of assays. In earlier studies we have found no differences between live or glutaraldehyde-fixed cells in their capacity to assembly hyaluronan-rich pericellular matrices (5, 7). Two anti-human CD44 antibodies, BU-52 and J173, were used to document the cell surface expression of CD44 receptors. Both CD44-H- and CD44-E-expressing transfectants showed immunoreactivity with both antibodies. Fig. 1 *A* and *B* show strong indirect immunoperoxidase staining on the CD44-H- and CD44-E-expressing transfectants for CD44 epitope with the BU-52 antibody. Control cells, transfected with the parental *CDM8* vector without inserts, were negative under the same conditions (Fig. 1*C*).

The transfected cells then were incubated with hyaluronan and aggregating proteoglycan under conditions described in our earlier studies as being optimal for matrix assembly (5, 7). Both live (Fig. 2 *A* and *B*) and glutaraldehyde-fixed COS-7 cells (Fig. 3*A*) transfected with CD44-H cDNA and incubated in the presence of exogenously added hyaluronan and aggregating proteoglycan organized these components into prominent pericellular matrices equivalent in size to those formed around embryonic or chondrosarcoma chondrocytes (5, 21). The slight dissymmetry of the fixed cell matrices was due to centrifugation effects. The fact that this gel-like matrix remained intact during centrifugation nonetheless suggests a highly stable interaction between these macromolecules and the cell surface. The freshly EDTA-released cells (Fig. 2*A*) became weakly attached to their substratum after 3 hr of incubation but did not undergo cell spreading at this early time point. Nonetheless, matrix assembly occurred even as the cells were attaching to the dish. Since matrix assembly was observed on the fixed cells in suspension as well as cells in various stages of cell spreading, pericellular matrix assembly appears independent of the state of cell interaction with a substratum.

Interestingly, live or glutaraldehyde-fixed COS-7 cells transfected with cDNA encoding the other isoform of CD44, CD44-E (Fig. 2*E* and Fig. 3*B*, respectively) also had the capacity to assemble prominent pericellular matrices in the presence of exogenously added hyaluronan and proteoglycan.

Live or fixed COS-7 cells transfected with the control CDM8 vector did not assemble pericellular matrices under similar conditions (Fig. 2*F* and Fig. 3*C*, respectively). In addition, the parental nontransfected COS-7 cells also did not assemble matrices in the presence or absence of exogenously added macromolecules (data not shown). Therefore, in this system expression of CD44 was essential for matrix assembly. Pretreatment of CD44-H- or CD44-E-expressing cells with 0.25% trypsin for 10 min, to remove cell surface proteins, eliminated the capacity of these cells to assemble pericellular matrices (Table 1).

The presence of pericellular matrices was quantified by morphometric analysis; Table 1 shows the matrix/cell ratios. The combination of hyaluronan and proteoglycan added to fixed cells expressing CD44-H or CD44-E resulted in matrix/cell ratios of 2.55 or 2.35, respectively (e.g., cells depicted in Fig. 3 *A* and *B*). Addition of equivalent concentrations of

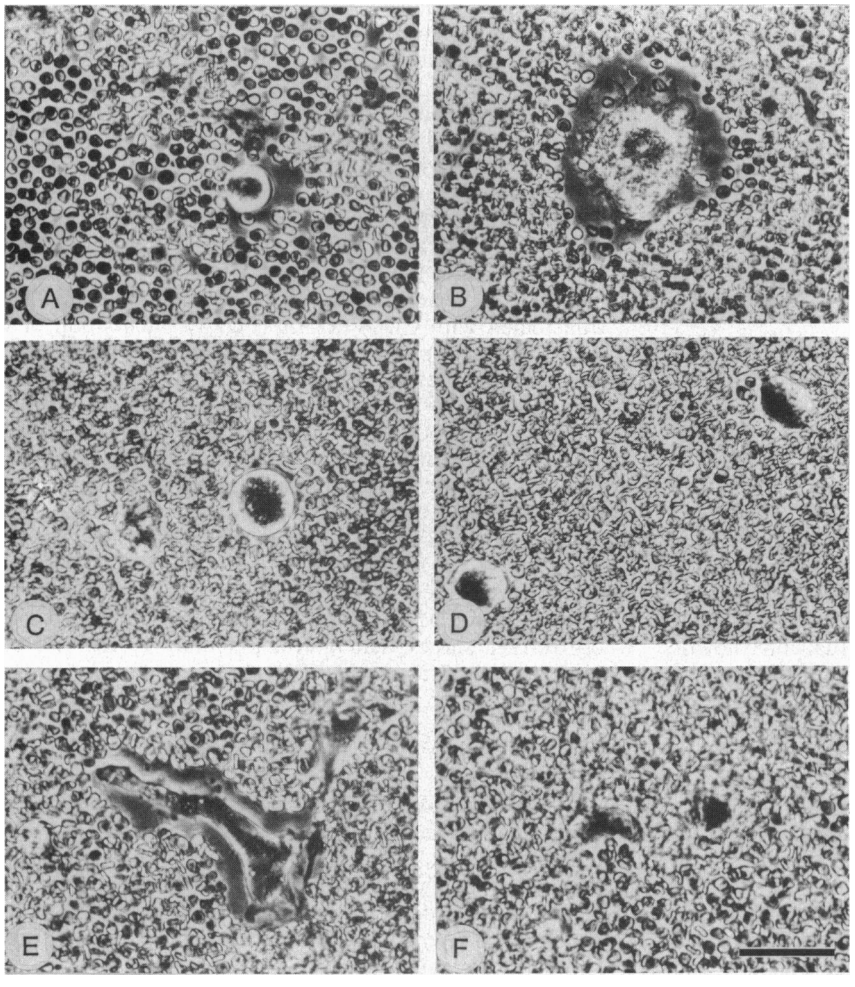

FIG. 2. Matrix assembly on live COS-7 cells transfected with two isoforms of CD44 cDNA. Cells were incubated in medium containing hyaluronan and aggregating proteoglycan for 3 hr, either directly after being released from monolayer culture by treatment with EDTA (*A*) or after being allowed to attach overnight (*B–F*). Cells transfected with CD44-H cDNA (*A* and *B*) exhibited significant pericellular matrices. Matrices formed by 3-hr incubation on these cells were displaced by 15 min with *Streptomyces* hyaluronidase (*C*) or subsequent incubation of the cells for 2 hr with hyaluronan hexasaccharides (*D*). Cells transfected with CD44-E cDNA (*E*) also exhibited prominent pericellular matrices, whereas cells transfected with the control CDM8 vector (*F*) did not assemble pericellular matrices. (Bar = 50 μm.)

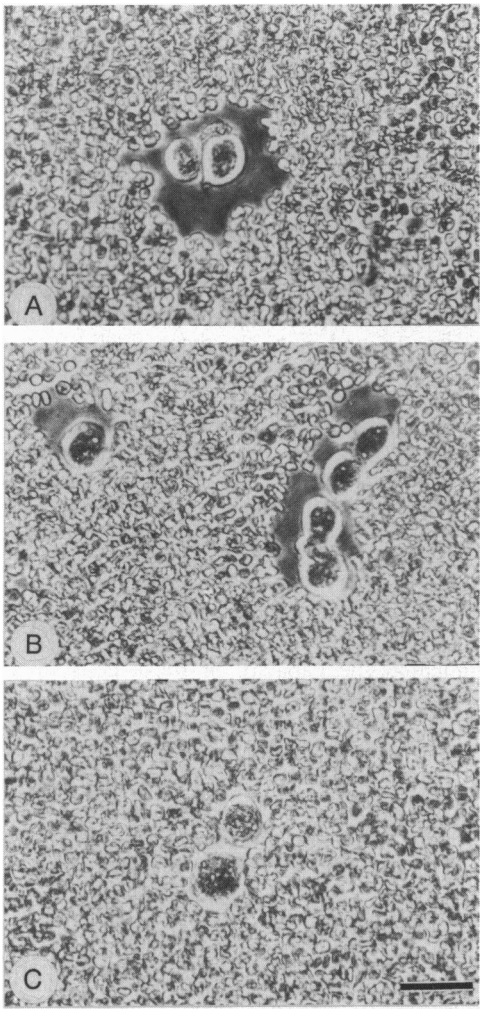

FIG. 3. Matrix assembly on glutaraldehyde-fixed COS-7 cells transfected with two isoforms of CD44 cDNA. Cells were incubated in medium containing hyaluronan and aggregating proteoglycan; matrix assembly was observed at 12 hr. Cells transfected with CD44-H cDNA (*A*) and CD44-E cDNA (*B*) exhibited significant pericellular matrices. Cells transfected with the control CDM8 vector (*C*) did not assemble pericellular matrices. (Bar = 50 μm.)

hyaluronan without proteoglycan or proteoglycan without hyaluronan to fixed cells expressing CD44-H or CD44-E or to fixed CMD8 control-vector-transfected cells did not result in pericellular matrix assembly. These experiments rule out possibilities such as exogenous proteoglycan interacting directly with CD44 molecules or other cell membrane components by some unknown mechanism. Pericellular matrices assembled on fixed or live CD44-H-expressing cells were removed by incubation of these cells with a dilute solution of *Streptomyces* hyaluronidase (Table 1 and Fig. 2*C*, respectively). The same results were obtained by treatment of fixed CD44-E cells (Table 1) and live CD44-E cells (data not shown). This finding demonstrates that the hyaluronan polysaccharide provides the underlying support for these matrices.

Addition of excess hyaluronan hexasaccharides inhibited matrix assembly in the presence of exogenous hyaluronan plus proteoglycan on both fixed CD44-H- and CD44-E-expressing cells (Table 1). These small oligosaccharides act as competitive inhibitors of hyaluronan/hyaluronan–receptor interactions and serve to define binding to the receptor proteins (6, 11). Hyaluronan hexasaccharides, while blocking hyaluronan binding to hyaluronan receptors, do not compete with the binding of hyaluronan to other proteins such as proteoglycans, link protein, hyaluronectin, glial

Table 1. Morphometric analysis of pericellular matrices on transfected COS-7 cells expressing CD44-H or CD44-E

Condition	Matrix/cell ratio*	
	CD44-H	CD44-E
HA + PG	2.55 ± 0.21	2.35 ± 0.24
HA	1.00 ± 0.02	1.03 ± 0.06
PG	1.00 ± 0.03	1.08 ± 0.05
HA + PG → h'ase†	1.00 ± 0.03	1.01 ± 0.02
HA + PG + HA_6‡	1.02 ± 0.04	1.02 ± 0.04
HA + PG → HA_6§	1.05 ± 0.04	1.00 ± 0.02
trypsin → HA + PG¶	1.03 ± 0.03	1.04 ± 0.02
HA + PG + KM201‖	1.02 ± 0.03	ND

HA, hyaluronan; PG, rat chondrosarcoma aggrecan; ND, not determined.

*Cell images were digitized, and the values are the mean matrix area/cell area ratio ± the 95% confidence range from the mean (n = 50).

†Matrices formed by exogenous HA and PG were subjected to subsequent treatment with *Streptomyces* hyaluronidase (h'ase).

‡Simultaneous addition of HA and PG and HA_6 to cells.

§Matrices formed by exogenous HA and PG were subjected to subsequent incubation for 2 hr with hyaluronan hexasaccharides (HA_6).

¶Cells were pretreated with trypsin prior to the addition of exogenous HA and PG.

‖Live CD44-H-expressing cells, directly after release from monolayers with EDTA, were incubated for 3 hr with HA and PG in the presence of 10 μg of KM201 anti-Pgp-1 antibody per ml.

hyaluronan-binding protein, or the endothelial clearance receptor (11). These results demonstrate that the attachment of the pericellular matrix to the cell surface depends on protein-binding sites that specifically recognize a hexasaccharide sequence of hyaluronan—i.e., functional definition of the hyaluronan receptor. Addition of hyaluronan hexasaccharides to fully established pericellular matrices on fixed or live cells transfected with CD44-H cDNA also resulted in the displacement of the matrix within 2 hr of additional incubation (Table 1 and Fig. 2*D*, respectively). The same displacement occurred with fixed (Table 1) and live (data not shown) cells transfected with CD44-E cDNA. Control CD44-H and CD44-E transfected cells did not shed their matrices following a 2-hr incubation in the presence of medium without hexasaccharides (data not shown).

Although the anti-human CD44 antibodies stained the cells transfected with CD44 cDNA (Fig. 1), incubation of cells with these antibodies did not block pericellular matrix formation in the presence of exogenous hyaluronan and proteoglycan (data not shown). However, coincubation of live cells transfected with CD44-H cDNA with an anti-Pgp-1 antibody, KM201, at the time of addition of exogenous hyaluronan and proteoglycan completely inhibited pericellular matrix assembly (Table 1). Although Pgp-1 is the murine homologue of human CD44, Thomas *et al.* (26) recently reported that KM201 had the capacity to block binding of human CD44 cDNA-transfected human melanoma cells to hyaluronan-coated wells. KM201 inhibition of pericellular matrix assembly further shows that matrix assembly in this system is mediated via interaction of hyaluronan with a CD44 cell surface receptor antigen.

DISCUSSION

COS cells after transfection with cDNA encoding either CD44-E or CD44-H isoforms gain the capacity to assemble hyaluronan-dependent pericellular matrices. These pericellular matrices can be removed by a brief treatment with *Streptomyces* hyaluronidase, an enzyme that specifically degrades hyaluronan. Additionally, pericellular matrices can be removed by displacement after incubation with excess hyaluronan hexasaccharides. These hyaluronan hexasaccha-

rides specifically compete with high molecular weight hyaluronan for binding sites on the cell surface. Trypsin pretreatment of cells abolished the capacity of CD44-expressing transfectants for pericellular matrix assembly. Coincubation of transfected cells with exogenous hyaluronan and proteoglycan in the presence of hyaluronan hexasaccharides or anti-Pgp-1 antibody also prevented matrix assembly. Thus, cell surface CD44 molecules function as hyaluronan receptors that are attachment sites for specific extracellular matrix components. In addition, the expression of CD44/hyaluronan receptors is independently sufficient to direct matrix assembly.

Our results somewhat conflict with other studies that describe the absence of hyaluronan binding to the CD44-E receptor isoform (24, 26). The previous results were based on the inability of CD44-E cDNA-transfected Namalwa or melanoma cells to bind to hyaluronan-coated plastic wells. Our matrix assembly assay system may be a more sensitive method to detect hyaluronan-binding activity on cells than hyaluronan binding/attachment assays. In other studies in our laboratory, hyaluronan receptors have been detected by this assay on cells that showed positive immunoreactivity but gave negative results with hyaluronan-binding assays (ref. 7 and unpublished data). Alternatively, the capacity to bind hyaluronan may also depend on the pattern of glycosylation of CD44-E expressed in these COS cells compared with Namalwa or melanoma cells (24, 26) and not to differences in primary sequence.

Although the two anti-human CD44 antibodies stained the cells transfected with CD44 cDNA, incubation of cells with these antibodies did not block pericellular matrix formation in the presence of exogenous hyaluronan and proteoglycan. The epitope recognized by the antibodies may thus be sufficiently distant from the hyaluronan-binding domain as to not block binding. Both of these antibodies (BU-52 and J173) have in fact been shown to immunoprecipitate detergent-solubilized CD44 hyaluronan-binding activity but have little to no effect on the direct binding of [^{3}H]hyaluronan to intact cells that express CD44 (13).

CD44/hyaluronan receptors are expressed in a wide variety of tissues (8, 10–12, 17, 27). This distribution usually, but not always, mimics tissue distribution of hyaluronan (8, 27). In some tissues CD44 may function to anchor fibroblasts, epithelia, and hematopoietic cells to the extracellular matrix. It has been demonstrated that CD44 binds other ligands in addition to hyaluronan, such as collagen types I and VI and the endothelial glycoprotein mucosal vascular addressin (17). The binding of one ligand may modulate the binding of CD44 to another ligand. Thus, several mechanisms may be available to cells to regulate CD44 binding together with modulation by glycosylation and alternative splicing. Alternatively, the intercellular interaction of hyaluronan with CD44 receptors may stabilize homotypic or heterotypic cell-to-cell interactions (8, 14). There also is evidence for CD44-cytoskeletal interactions (8, 17) providing for signal transduction via information in matrix composition. Such adhesive multiligand interactions may have widespread importance such as in cell-matrix recognition during cytodifferentiation, in regulation of cell shape, and in cell motility.

In some tumor cells there is an increased level of expression of cell surface CD44, which appears to correlate with the proliferative or differentiation status of the cells (8, 16, 24, 28). For example, we demonstrated that the invasive human bladder carcinoma cell lines HCV-29T and HU-456 exhibit high-affinity, high-specificity cell surface hyaluronan-binding sites, whereas noninvasive human bladder papilloma cells do not exhibit binding activity (7). Expression of unoccupied hyaluronan receptors may allow tumor cells to adhere to and be translocated through hyaluronan-enriched extracellular matrices (6, 9, 26, 29). Another function of hyaluronan receptors on tumor cells may be the support of assembly of hyaluronan-enriched pericellular matrices. The invasive human bladder carcinoma cell lines expressing hyaluronan receptors/CD44 (6, 13) had the capacity to assemble a pericellular matrix by using exogenously added hyaluronan and aggregating proteoglycan (7). These pericellular matrices may function to reduce cell–cell adhesion, provide a matrix cocoon layer of protection from immune surveillance (30), or induce a new structural order on the extracellular matrix. In this way, tumor cells, embryonic cells, or cells participating in wound healing/regeneration may utilize macromolecules within newly encountered extracellular matrix environments to facilitate their migration, growth, or state of differentiation.

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