IDENTIFICATION OF THE HYALURONAN RECEPTOR

FOR ENDOCYTOSIS (HARE)

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Rat liver sinusoidal endothelial cells (LECs) express two hyaluronan (HA) receptors, of 175 kDa and 300 kDa, responsible for the endocytic clearance of HA. We have characterized eight monoclonal antibodies (mAbs) raised against the 175 kDa HA receptor partially purified from rat LECs. These mAbs also crossreact with the 300 kDa HA receptor. The 175 kDa HA receptor is a single protein, whereas the 300 kDa species contains three subunits, α, β, and γ at 260, 230 and 97 kDa, respectively (Zhou, et al. J. Biol. Chem. 274, 33831-33834, 1999). The 97 kDa subunit was not recognized by any of the mAbs in Western blots. Based on their crossreactivity with these mAbs, the 175, 230 and 260 kDa proteins appear to be related. Two of the mAbs inhibit 125I-HA binding and endocytosis by LECs at 37°C. All of these results confirm that the mAbs recognize the bone fide LEC HA receptor. Indirect immunofluorescence shows high protein expression in liver sinusoids, the venous sinuses of the red pulp in spleen and the medullary sinuses of lymph nodes. Because the tissue distribution for this endocytic HA receptor is not unique to liver, we propose the name HARE (HA receptor for endocytosis).
HA\(^1\) is an important and often abundant extracellular matrix component of all tissues, in particular cartilage, skin and vitreous humor (Evered and Whelan, 1989). HA plays a key role in development, morphogenesis, and differentiation, in cell adhesion and proliferation, and in inflammation and wound healing (Evered and Whelan, 1989; Toole, 1997; Knudson and Knudson, 1993; Laurent and Fraser, 1992). In humans the total body turnover of HA is several grams per day (Evered and Whelan, 1989). Although local turnover of HA occurs in avascular tissues, particularly cartilage (Hua et al. 1993, Aguiar et al. 1999), two major clearance systems are responsible for HA degradation and removal in the body (Laurent and Fraser, 1992). The first is the lymphatic system, which accounts for \(~85\%\) of the HA turnover, and the second is in the liver, which accounts for the other \(~15\%\) of the total body HA turnover. Throughout the body, HA is continuously synthesized and degraded in almost all tissues. At the same time, chondroitin sulfate and other glycosaminoglycans are also released from the cleavage of proteoglycans, especially aggregating proteoglycans associated with HA. Large native HA molecules \(~10^7\) Da) are partially degraded to large fragments \(~10^6\) Da) that are released from the matrix and enter the lymphatic system, flowing to lymph nodes.

The lymph nodes completely degrade the majority of HA \(~85\%) by unknown mechanisms. Neither the responsible cell type, the receptor involved, nor the location in lymph nodes at which HA uptake and degradation occurs has been determined. The remaining HA \(~15\%) that escapes degradation in the lymph nodes ultimately flows into the blood at the thoracic duct. Since HA is an exceptionally viscous polysaccharide in solution, it would be deleterious for the blood concentration of HA, even at relatively low molecular weight, to increase. Clearance of this circulating HA and the other glycosaminoglycan degradation
fragments is presumably important for normal health (Evered and Whelan, 1989; Laurent and Fraser, 1992). Elevated serum HA levels are associated with a variety of diseases and pathological conditions such as liver cirrhosis, rheumatoid arthritis, psoriasis, scleroderma, fibromyalgia and some cancers (Yamada et al., 1998; Lai et al., 1998; Yaron et al. 1997).

LECs in vertebrate liver express a very active, recycling endocytic receptor that removes these extracellular matrix-derived fragments of HA and other glycosaminoglycans, including chondroitin sulfate, from the blood (Laurent and Fraser, 1992; DeBleser et al., 1994; Raja et al., 1988; McGary et al., 1989, McGary et al., 1993). ICAM-1, a 90 kDa protein also known as CD54 (Hayflick et al. 1998), was previously misidentified as the LEC HARE (Forsberg and Gustafson, 1991; McCourt et al., 1994). These workers attempted to purify the HA receptor without the use of an assay to measure HA-binding activity. The claim that the HA receptor had been purified was subsequently acknowledged to be an artifact due to the nonspecific binding of ICAM-1 to the HA affinity resin (McCourt and Gustafson 1997, McCourt et al., 1999). In any case, since ICAM-1 is not a coated pit targeted endocytic receptor, it could not be the true HA receptor in LECs.

Using a photoaffinity derivative of HA (Yannariello-Brown et al., 1992) and a novel ligand blot assay with 125I-HA (Yannariello-Brown et al., 1997), we previously identified two specific HA-binding proteins of 175 kDa and 300 kDa in isolated rat LECs as candidates for the HA receptor. We recently reported the first purification of these two HA receptors using a specific mAb (Zhou, et al. 1999). Based on SDS-PAGE, after reduction of disulfide bonds, the 175 kDa HA receptor is a single protein, whereas the 300 kDa species contains three subunits, α, β, and γ at 260, 230 and 97 kDa, respectively. In this report we characterize a panel of mAbs against the 175 kDa HA receptor and demonstrate for the first time the tissue and cellular
localization of the two HA receptor species with these unique and specific reagents.
MATERIAL AND METHODS

Materials  RCA-I-agarose gel was purchased from EY laboratories, Inc. Tris, SDS, ammonium persulfate, N, N’-methylenebisacrylamide, and SDS-PAGE molecular weight standards were from Bio-Rad. Na$^{125}$I was from Amersham Corp. NP-40 was from CalBiochem. HA (human umbilical cord) from Sigma, was purified as described previously (Raja et al., 1984). $^{125}$I-HA was prepared using Iodogen (Pierce) and a unique hexylamine derivative of HA, modified only at the reducing end to contain an iodinatable hydroxyphenyl group as described previously (Raja et al., 1984). Typical specific activities of the radiolabeled HA were 2 x 10$^8$ DPM per nmol oligosaccharide (~3 x 10$^6$ DPM/µg). Nitrocellulose membranes were from Schleicher & Schuell. Acrylamide and urea were from U.S. Biochemical Corp. p-Nitrophenylphosphate was from Kirkegaard & Perry Laboratories. N-glycosidase F (EC 3.5.1.52) and all other chemicals, which were reagent grade, were from Sigma. TBS contains 20 mM Tris-HCl, pH 7.0, 150 mM NaCl.

Preparation of LECs and LEC membranes  Male Sprague Dawley rats were from Harlan, Indianapolis, IN. LECs were isolated by a modified collagenase perfusion procedure (Oka and Weigel, 1987; Yannariello-Brown and Weigel, 1992), followed by differential centrifugation and then discontinuous Percoll gradient fractionation. The final LECs, were collected from the 25/50% interface and washed 3-times with PBS at 4°C, and are >95% pure. For preparation of membranes (Yannariello-Brown and Weigel, 1992), the cells were hypotonically swollen, homogenized, and centrifuged at 1000xg. The supernatant was then centrifuged at 100,000xg to obtain the total membrane fraction, which was stored frozen at -80°C with no loss in HARE activity.
LEC culture and assay for HA endocytosis. The LECs were collected from the first PBS wash after Percoll gradient fractionation. They were washed twice with RPMI-1640 (GIBCO) containing penicillin/streptomycin (100 units each) and 2mM glutamine and plated at 1.5-2 x 10^6 cells/ml on fibronectin-coated (50 µg/ml) 24-well tissue culture plates for endocytosis experiments or on glass coverslips for microscopy. After incubation at 37°C for 2 h in a 5% CO2 atmosphere, the cells were washed 3 times with PBS, once with RPMI-1640 and put back in RPMI-1640 without serum if they were to be used immediately or with 2% (v/v) heat inactivated bovine serum if they were to be cultured overnight before use. Endocytosis assays were performed as described previously using 125I-HA (McGary et al. 1989). Cultured LECs were washed and incubated for 60 min at 37°C with 125I-HA (2 µg/ml) in MEM medium containing 5 µg/ml of an affinity-purified antibody, isolated from ascites fluid using Protein-G-Sepharose or immobilized mannan binding protein (Pierce) in the case of #159. The plates were then chilled on ice, the media were aspirated, the wells were washed 3 times and the cells were solubilized in 0.3 N NaOH. Radioactivity and protein content were determined for each of the samples. Specific uptake, the fraction of endocytosed ligand that is competed with excess unlabeled HA, is at least 90% in this assay.

Monoclonal antibody production. The starting antigen for mAb production was a highly purified fraction of the 175HARE. The preparation of membrane extracts and RCA-1 lectin chromatography to partially purify the 175HARE and 300HARE activities has been described recently (Zhou et al., 1999). After testing a battery of nine lectin resins for their ability to bind HARE, RCA-I lectin was chosen because HARE binding was quantitative, easily displaced with lactose and a significant purification from other membrane proteins was obtained. A further
advantage of lectin chromatography as a purification step is the ability to elute the LEC HARE in a relatively small volume. The eluted HARE was then subjected to nonreducing continuous elution SDS-PAGE using a Bio-Rad Model 491 Prep Cell at 22°C and a flow rate of 0.8 ml/min to separate individual proteins (Fig. 1). The running and elution buffers were 0.1% SDS, 25 mM Tris-190 mM Glycine, pH 6.8. The fractions were screened using the 125I-HA ligand blot assay and the nitrocellulose membrane was stained with copper phthalocyanine tetrasulfonic acid tetrasodium salt after the ligand blot assay to compare protein content and concentration in various fractions.

The eluted 175 kDa fractions were pooled, concentrated, and used to immunize 4 mice. Two mice were each immunized with 20 ug of purified nonreduced protein and two each with 20 ug of purified protein that was first reduced with 10 mM DTT and then alkylated with 50 mM iodoacetamide. The mice were boosted twice at two-week intervals and blood was drawn from tail veins 6 weeks after the first immunization. The sera were tested for specific Ab by ELISA. Standard procedures (Harlow and Lane, 1988) were followed for cell fusion and limited dilution cloning. Briefly, 10^8 immunized mouse spleen cells were mixed with 2x10^7 SP 2/0-Ag 14 myeloma cells and fused in the presence of 50% polyethylene glycol (MW=1500). The hybrid cells were seeded in 96-well flat-bottom plates in HAT selection medium. The hybridoma supernatants were screened using an ELISA assay with the 175 kDa antigen. Consistently positive hybridoma clones were used to produce ascites fluid as described by Harlow and Lane (1988). Antibody isotypes (idio) were determined using the ISO Strip kit from Boehringer Mannheim.

ELISA for quantitation of anti-HARE activity. The 96-well ELISA plates were coated
using a 100 µl of 10 µg/ml purified 175 kDa protein at 4°C overnight and then blocked with 1%
BSA-TBS for 1 h at room temperature. Pre-immune or immune serum or hybridoma supernatant (100 µl) was added to each well. The plate was incubated for 2 h at room
temperature, washed 6 times with 0.05% Tween 20-TBS, and the secondary reagent, a mixture
of goat anti-mouse IgG, IgM, and IgA alkaline phosphatase conjugates (100 µl, ~ 2 µg/ml each)
was added. The plate was incubated for 2 h at room temperature and washed 5 times for 5 min
each with 0.05% Tween 20-TBS and 100 µl of freshly prepared p-nitrophenylphosphate
substrate solution (as prepared using the Phosphatase substrate System Kit from Kirkegaard &
Perry Laboratories) was added to each well. After incubation for 30-60 min at 37°C, 100 µl of
5% EDTA solution was added to the wells to stop the reaction. The plate was then read at 405
nm using a Spectra Max 340 (Molecular Devices).

Immunofluorescence. For analysis of rat tissues liver, spleen, brain, heart, kidney,
muscle, lung, intestine, and lymph node were minced on ice and fixed in 4% formaldehyde
overnight at room temperature. Tissue sections (5 µm) were prepared by the Department of
Pathology, University of Oklahoma Health Science Center. Sections were then dewaxed 5 times
for 5 min each with xylene, followed by 5 washes for 5 min each with alcohol at room
temperature. The tissue was treated with 0.05% trypsin, 0.53 mM EDTA for 30 min at room
temperature, washed with TBS and then blocked with 10% goat serum in TBS at 4°C overnight.
A 1:250 dilution of individual ascites fluid or mixtures of ascites (100 µl) was added and the
tissue slide was incubated for 2 h at room temperature. The slide was washed 5 times for 5 min
each with TBS at room temperature. Goat anti-mouse IgG-Rhodamine Red conjugate (10
µg/ml) was then added and the incubation continued for 1 h at room temperature. The tissue slide
was washed 5 times for 5 min each with TBS and a solution of SlowFade from Molecular Probes was added. A cover slip was overlaid, the edge sealed with finger nail polish, and the slides were viewed by fluorescence microscopy using a Nikon Diaphot 300 or by confocal microscopy using a LEICA TCS NT. For analysis of cultured LECs, the cells were cultured overnight on glass coverslips, fixed in 4% formaldehyde in PBS for 20 min at 23°C and then permeabilized with 0.1% Triton X-100. Nonspecific binding was minimized by incubating the cells for 1-2 h in TBS containing 10% goat serum and 1% (w/v) bovine serum albumin. The cells were then washed, incubated for 1 h at 23°C with 5 µg/ml ascites from the indicated mAbs or normal mouse serum, washed, stained and processed as above with goat anti-mouse IgG conjugated to rhodamine red.

**Ligand blot assay.** Protein samples were solubilized in SDS sample buffer: 16 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, and 0.01% bromophenol blue (Laemmli, 1970) without reducing agent. Cell or membrane samples were sonicated on ice for 10-20 s. After SDS-PAGE, the gel was electrotransferred to a 0.1 µm nitrocellulose membrane for 2 h at 24 V at 4°C using 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol and 0.01% SDS. The nitrocellulose was treated with TBS, 0.1% Tween 20 at 4°C for 2 h and then incubated with 2 µg/ml 125I-HA in TBS without or with a 150-fold excess of HA (as competitor) to assess total or nonspecific binding, respectively. The membrane was washed 5-times (5 min each) with 0.05% Tween 20 in TBS, dried and the 125I-HA bound to protein was detected by autoradiography with Kodak BioMax film. Nonspecific binding in this assay is typically <5% (Yannariello-Brown et al., 1996; Yannariello-Brown et al., 1997).

**Deglycosylation of HARE with N-glycopeptidase F.** Purified HARE (1.17 µg) was
heated with 0.5% SDS at 90°C for 3 min. Samples (22 µl) were chilled on ice for 4 min, and then 0.5 M Tris-HCl, pH 7.2 was added to a final concentration of 10 mM. One-half unit of N-glycopeptidase F (Tarentino, 1985) and distilled water were added to give a final volume of 25 µl. The samples were incubated at 37°C overnight, 9 µl of 4-fold concentrated SDS sample buffer was added and they were heated for 3 min at 90°C. The samples were subjected to SDS-PAGE and Western blotting.

*General.* Protein content was determined by the method of Bradford (Bradford, 1976) using BSA as a standard. Receptor protein content was assessed after precipitation with 5% trichloroacetic acid to remove detergent. SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970). Western blotting procedures were performed essentially as described by Burnette (Burnette, 1981). 125I radioactivity was measured using a Packard Auto-Gamma Counting system. Digital images from confocal microscopy were processed in Excel. Other digital images obtained by scanning 35mm film, blots or autoradiograms with a Hewlett Packard Photosmart S20 or Scanjet 4C were processed using OmniPage Pro (Caere) followed by importing .jpg files into Corel Paint and then Corel Draw, v8.0.

**RESULTS**

We previously found that two very active and specific HA-binding proteins can be detected in LECs by ligand blotting using 125I-HA (Yannariello-Brown *et al.*, 1997). The 175HARE and 300HARE are readily detected after renaturation with Tween 20. These two HARE species show the same specificity, with a panel of polyanionic competitors, as observed
for intact LECs (Raja et al., 1988). The HA-binding activity at 175 kDa and 300 kDa in the ligand blot corresponds perfectly to the previous identification of the HARE on intact LECs by the use of an HA photoaffinity derivative. This HA probe specifically photo-labeled proteins of 175 kDa and 300 kDa (Yannariello-Brown et al., 1992). One utility of the ligand blot assay is that one can use any detergent to solubilize the membranes, since the HARE activity can be renatured after SDS-PAGE and electrotransfer.

**Development of mAbs to the 175HARE.** Because we previously identified an HA-binding protein of 85 kDa in hepatocytes (Frost et al. 1990) and because histones avidly bind HA as well (Yannariello-Brown et al., 1996), our purification scheme for the 175HARE began with LECs prepared by collagenase perfusion of rat liver, rather than with whole liver. The final partial purification step for preparing the 175HARE antigen for mAb production was preparative 1-D SDS-PAGE, which separated the 175HARE and 300HARE and gave fractions of discrete masses that still retained HA-binding activity (Fig. 1). The eluted 175 kDa fractions were pooled, concentrated, and used to immunize mice as the nonreduced protein or after being reduced and alkylated. We isolated multiple clones of 11 different hybridomas that were consistently positive in ELISA screens with the nonreduced or reduced 175 kDa antigen.

**Interaction of mAbs with the 175HARE after detergent solubilization or SDS-PAGE.**

MAbs were prepared from ascites fluid produced by the hybridomas and tested for their ability to immunoprecipitate the active 175HARE (Fig. 2) and for their reactivity with a 175 kDa species in Western blots (Figs. 3 and 4). The former ability shows that a mAb recognizes the correct protein, the *bone fide* 175HARE. Three of the original 11 mAbs isolated (numbers 117, 141 and 497) were not against the 175HARE, since they have a different Western pattern (Fig. 3, lanes 9-11) and do not immunoprecipitate HARE (Fig. 2, lanes 2, 7 and 11). This result was expected,
since the starting antigen was not pure. These negative clones were useful as negative controls. Except for mAb-159 (IgM) and mAb-30 (IgG\textsubscript{2b}) all of the HARE-specific mAbs are IgG\textsubscript{1}.

Eight mAbs were obtained that recognize both the rat LEC 175HARE and 300HARE in Western blots after either nonreducing (Fig. 3) or reducing (Fig. 4) SDS-PAGE. Three mAbs, (numbers 54, 159 and 174) recognize both reduced HAREs in Western blots. Most of the mAbs raised against the nonreduced 175HARE no longer react with either HARE species after reduction (Figs. 3 and 4). The exceptions are mAb-159 and mAb-174, which recognize both the 175HARE and 300HARE proteins in Western blots, whether they are reduced (Fig. 4) or nonreduced (Fig. 3). MAb-54 recognizes only the reduced HAREs. (Figs. 3 and 4, lanes 3).

Four of the mAbs also immunoprecipitate both proteins from LEC extracts (Fig. 2). Surprisingly, all mAbs that bind to the 175HARE species, the original antigen, also recognize the 300HARE species. However, as described below, the 300 kDa species is not a dimer of the 175 kDa protein and does not contain a 175 kDa subunit. That eight of eight mAbs raised against the 175HARE cross-react with the 300HARE, suggests that the two proteins share one or more common epitopes. Ongoing studies support this preliminary conclusion\textsuperscript{2}. Initial studies also suggest that the 175HARE may be derived from the 300HARE by proteolytic processing.

Using lectin and mAb-30 affinity chromatography, the two LEC HAREs have recently been purified to homogeneity and partially characterized (Zhou, et al., 1999). Nonreducing SDS-PAGE analysis indicates that these two proteins comprise >99% of the final purified HARE. In addition, the 175HARE and 300HARE that are affinity purified by mAb-30 are recognized by the other 7 mAbs. The liver 175HARE and 300HARE are purified in an apparent mole ratio of 2:1.
To determine if either protein contains disulfide-bonded subunits that are recognized by the mAbs, the 175HARE and 300HARE were analyzed by SDS-PAGE and immunoblotting with or without reduction with β-mercaptoethanol (Fig. 5). The reduced 175HARE yields no other protein species (Zhou, et al., 1999), but the apparent size of the protein increases to ~185 kDa (Fig. 5A, lane 3). After reduction, the 300HARE gives rise to three protein species with Mrs of 97, 230 and 260 kDa (Fig. 5A, lane 4). Based on Coomassie and silver staining, and apparent size, the molar ratio of the three protein components of the 300HARE is 1:1:1. The reduced 175HARE protein (at ~185 kDa) and the two large subunits of the 300HARE complex at 260 kDa and 230 kDa are all recognized by the three mAbs that bind the reduced proteins (Fig 5B, lanes 3 and 4). Interestingly, the ~97 kDa subunit of the 300HARE is the only HARE protein not recognized by any of the mAbs raised against the reduced or nonreduced 175HARE.

After deglycosylation with endo-F, both HARE species still react with the mAbs. During digestion with endo-F, the 175HARE is converted to a single ~150 kDa species, suggesting that up to 10 typical N-linked oligosaccharides may be present. The 300HARE was also reduced in apparent size after enzyme treatment. De-N-glycosylated 175HARE and 300HARE are still able to react with the individual mAbs (not shown) or a mixture of all the mAbs (Fig. 5B, lanes 2 and 4). Both de-N-glycosylated HAREs are also still capable of specific $^{125}$I-HA binding (Zhou, et al., 1999). Therefore, N-linked oligosaccharides do not appear necessary for HA binding and do not comprise a part of the epitopes recognized by the mAbs developed here.

MAb-174 specifically blocks HA binding in the ligand blot assay and blocks the endocytosis of $^{125}$I-HA by LECs in culture. Endocytosis and accumulation of $^{125}$I-HA at 37°C by cultured LECs was completely inhibited by mAb-174 (Fig. 6). Only one other mAb (#235)
had any appreciable affect on HA endocytosis, consistently causing partial (~50%) inhibition of
125I-HA endocytosis. None of the other anti-175HARE mAbs, nor the three negative control
mAbs affected HA uptake (not shown). These results confirm unequivocally that our eight anti-
175HARE mAbs are specific for the bone fide HARE present in LECs. Interestingly, mAb-174
also blocked HA binding to both the 175HARE and 300HARE in the ligand blot assay (Fig. 7A).
At 5 µg/ml, mAb-174 blocked 70% and 50% of the specific 125I-HA binding to the 175HARE
and 300HARE bands, respectively (Fig. 7B). The same concentration of mouse IgG or the other
mAbs (not shown) showed no inhibition. Since mAb-174 completely blocks HA uptake by
LECs and also recognizes both proteins in Western blots, then both the 175 kDa and 300 kDa
proteins could be independent HAREs capable of mediating HA binding and endocytosis. It is
important to note, that although mAb-174 inhibits 125I-HA binding and endocytosis by LECs at
37°C, it is unable to block HA binding to live LECs at 4°C2. There is no detectable endocytosis
of HA by LECs at 4°C (McGary et al, 1989)

Tissue distribution and immunolocalization of HARE. Western blot analysis of various rat
tissues indicated that the 175HARE and 300HARE proteins are highly expressed in spleen as
well as liver (Fig. 8A). The other tissues tested (brain, lung, heart, muscle, kidney, and intestine)
showed no significant reactivity with a mixture of all eight anti-HARE mAbs. As with the liver,
each mAb showed the same pattern of reactivity, recognizing both HARE species in the spleen
(not shown). Both HARE species isolated from spleen were also active in the 125I-HA ligand
blot assay (Fig. 8B) verifying that these receptors specifically bind HA.

Confocal indirect immunofluorescence demonstrated that the HARE proteins are localized
to the sinusoids in liver (Fig. 9) as expected. No staining was observed in the parenchymal cells.
Confirming this cellular distribution, the protein is not expressed in isolated hepatocytes in culture (not shown), but is strongly expressed in purified, cultured LECs (Fig. 10). HARE is present in LECs in a pattern typical for an endocytic, recycling receptor (Mellman, 1996); it is at the cell surface, in pericellular vesicles (presumably endosomes) and in the perinuclear region, presumably in ER and Golgi. In rat spleen, the HARE proteins are present in the venous sinuses of the red pulp (Fig. 11A-D). No significant staining was observed in the germinal centers or white pulp of the splenic nodules. In rat lymph nodes, HARE is localized to the medullary sinuses (Fig. 12). It is not present in the spheroid nodules, their germinal centers or in medullary cords. Vascular endothelial cells were not stained in any of the tissues examined.

**DISCUSSION**

After its discovery >65 years ago (Meyer and Palmer, 1934), HA was shown to be a common, ubiquitous, component of ECMs in vertebrates. HA is a linear unbranched alternating polymer composed of only two monosaccharides; $\beta(1,4)$-N-acetyl-D-glucosamine and $\beta(1,3)$-D-glucuronic acid. HA is not sulfated, not covalently attached to a protein core, and is typically 100-700 times the size of other GAG chains attached to proteoglycans. The molecular weight of native HA can exceed $7 \times 10^6$. The physicochemical and rheologic properties of HA, particularly its viscoelasticity, are ideally suited for its role in specialized ECMs of skin, cartilage, and fluids such as in the vitreous humor of eye and synovium of joints.

Previously, most investigators believed that the physiological function of HA in the ECM was only structural or physical. Now, however, HA is recognized as a pharmacologically active, signaling molecule, as well as an ECM structural component. Despite its simple structure, HA influences a wide range of cell functions and behaviors, including cell migration, differentiation,
and phagocytosis (Knudson and Knudson, 1993; Laurent and Fraser, 1992; Turley, 1992). HA is implicated as an important molecule in morphogenesis (Toole, 1997; Gakunga et al., 1997; Iocona, et al., 1998), wound healing (Laurent et al. 1998; Burd et al., 1991; Weigel et al., 1986), angiogenesis (Deed et al. 1997; Rahmanian et al.1997), and metastasis. For example, HA is required for ductal branching morphogenesis in the prostate (Gakunga et al. 1997) and normal tissue differentiation depends on the presence of ECM proteoglycans, such as aggrecan and perlecan, that form large aggregates with HA (Vertel et al. 1994; Handler et al.1997).

It is now accepted that some cells respond to HA of different size. Angiogenesis, in particular, is stimulated by small, but not large, HA (West, et al. 1985, Deed et al. 1997) and activated macrophages are induced to express a large number of genes in response to small, but not large, HA (Horton et al. 1998). Similarly, only small, not large, HA induces NO synthase expression in LECs and Kupffer cells, but not hepatocytes or stellate cells (Rockey et al.1998). Small HA fragments stimulate signal cascades in these various cell types through specific cell surface receptors.

Known HA-binding proteins, or hyaladherins (Toole, 1990), can be put into four categories: enzymes, ECM components, cell surface receptors and soluble plasma or intracellular molecules. Several types of cell surface HA receptors have been characterized including CD44, RHAMM, ICAM-1, and the endocytic receptor in LECs. Based on the nonexclusive tissue distribution of this latter endocytic HA receptor, it should no longer be called the liver (or LEC) HA receptor. We, therefore, propose the more general name HARE, HA Receptor for Endocytosis. The HARE proteins identified here are distinct from all of the previously identified cell surface receptors for HA, which do not appear to mediate endocytosis of HA via the coated pit pathway. A liver scavenger receptor able to bind and internalize HA has also been reported.
(McCourt, et al. 1999). It remains to be determined how this latter HA receptor and the HARE reported here are related.

CD44 is a large diverse family of transmembrane glycoproteins found in lymphocytes, epithelial and endothelial cells, hemopoietic cells and in many cancer cells (Lesley et al. 1993). CD44 has structural homology to cartilage link proteins, and some forms of CD44, although not all, bind HA (Bajorath et al. 1998). The ability of CD44 on chondrocytes to internalize HA by a receptor mediated process has been well documented (Hua et al. 1993, Aguiar et al. 1999). Based on the relatively slow kinetics of CD44-mediated HA uptake and the ability of bound HA to modulate CD44 turnover in chondrocytes, the endocytosis of HA in these cells appears to be fundamentally different than the HA endocytosis mediated by rapidly recycling HARE in LECs (McGary et al. 1989, McGary et al. 1993).

RHAMM is a cell surface (Turley et al. 1993) and intracellular (Hofmann et al. 1998) protein that mediates a cell migration response in the presence of HA. ICAM-1 is a cell surface adhesion molecule that binds HA (Hayflick et al. 1998). ICAM-1 was incorrectly identified as the LEC endocytic HA receptor (McCourt et al. 1994). This report, although later explained as an artifact (McCourt et al., 1997) has not been formally withdrawn and subsequent reports (Gustafson et al. 1995; Fuxe et al., 1996) based on these erroneous findings may still confuse the field.

HA is being used in an increasing number of clinical applications, due to its physical properties and nonimmunogenicity. For example, sterile solutions of pure, pyrogen-free, high molecular weight HA are used in ophthalmological surgeries worldwide (Goa and Benfield, 1994). HA is ideally suited for this use, since it is a natural ocular component, and its physical properties keep the eyeball from collapsing. Many patients with osteoarthritis or rheumatoid
arthritis now receive intra-articular injections of HA and experience significant improvement (Pelletier and Martel-Pelletier, 1993). In arthritic joints HA suppresses the release of proinflammatory mediators by leukocytes, attenuates the nociceptive response and has a direct analgesic effect (Ghosh, 1994). HA has also been used to heal perforated tympanic membranes and restore hearing (Laurent et al., 1988). HA has been used topically to reduce postoperative pericardial adhesions and as an aerosol to prevent elastase-mediated injury in pulmonary emphysema (Cantor et al. 1998). HA is also used as a vehicle for drug delivery (Illum et al. 1994). Because of the wide use of HA in medical applications, it is important that we understand the biological effects of HA and how its synthesis and degradation are regulated in humans.

HA turnover and metabolism in mammals, including humans, is well understood at the whole body and organ level based on numerous elegant studies by Laurent and Fraser and their coworkers (Laurent and Fraser, 1992). Mice (Fraser et al. 1983) and rabbits (Fraser et al. 1981) rapidly remove injected $^3$H-HA from the blood and concentrate it in liver and, to a lesser extent, in spleen and lymph nodes. Based on these earlier studies and our present results, we conclude that the same HARE found in liver is present and functional in spleen and lymph node.

Studies of HA turnover and metabolism in mammals at the cell and molecular level have focused primarily on the HARE found in liver, and studies on HA turnover in skin (Tammi et al., 1994) and cartilage (Morales and Hascall 1989, Hua et al. 1993, Aguiar et al. 1999). Liver sinusoidal endothelial cells in vertebrates are multi-purpose scavengers that are specialized to remove circulating degradation products released from ECMs throughout the body (DeBleser et al. 1994). These degraded ECM components include chondroitin sulfate, collagen α-chain, laminin, HA and the C- and N-terminal propeptides of procollagen. HA is continuously synthesized and secreted by fibroblasts, keratinocytes, chondrocytes and other specialized cells in
ECMs throughout the body. Despite the daily turnover of gram quantities of HA, the two HA clearance systems in lymph and liver keep the normal steady-state concentration of HA in blood very low (i.e. 10-100 ng/ml).

Blood HA levels could rise due either to increased HA turnover in the body, exceeding the capacity of LECs to remove it, or to compromised function of HARE in LECs. For example, elevated serum HA is a marker for liver fibrosis in hepatitis C virus-associated chronic liver disease (Yamad et al. 1998). There is also an increased ascitic level of HA in liver cirrhosis due to increased HA synthesis by peritoneal cells and decreased uptake by LECs (Lai, et al. 1998). Blood HA levels are elevated in a variety of diseases, such as fibromyalgia (Yaron et al., 1997).

Banerji et al. (1999) recently discovered a lymph-specific homologue of CD44 that is able to bind HA. This lymph HA receptor, designated LYVE-1, is localized to the luminal face of lymph vessel walls in the lymphatic system. It is not present on blood vessels. Like CD44, LYVE-1 is not a recycling endocytic receptor designed for the continuous and efficient internalization of HA. Together the two lymphatic HA receptors, HARE and LYVE-1, would create an effective mechanism to mediate the removal of HA from lymph, especially if the HA levels in the entering lymph varied substantially. LYVE-1 could bind HA from afferent lymph and create a reservoir of sequestered HA. Although HA bound to LYVE-1 would not be internalized and degraded, it would be removed from circulating lymph. When this bound HA dissociates and enters the sinusoids of the lymph node, it would then be bound and endocytosed by HARE. HARE would always be clearing HA from lymph but if the HA concentration saturated this system, the reservoir capability of LYVE-1 would minimize the amount of HA that escaped the node in efferent lymph and entered the blood.

The present results show that the two HA clearance systems in lymphatic tissues and liver
are mediated by the same HA receptor, HARE. Further molecular analysis at the cDNA/mRNA level will be required to determine if there is a single HARE or a family of closely related proteins and genes. Nonetheless, our results support the conclusion that mammals express an endocytic HA receptor in the sinusoids of liver and lymphatic tissues, which is a localization well suited to keep the systemic levels of non-ECM-associated HA very low. Liver, spleen and lymph node clearly express large amounts of HARE for this purpose. Rat LECs contain ~ 2-4 \times 10^5 HA binding sites per cell, a typical number for a recycling, endocytic receptor system (Raja et al. 1988). More sensitive methods, such as RT-PCR, will be required to determine whether other tissues express substantially lower, but significant, levels of HARE.

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FOOTNOTES

1. Abbreviations. ECM, extracellular matrix; HA, hyaluronic acid, hyaluronate, hyaluronan; HARE, HA receptor for endocytosis; ICAM-1, intercellular adhesion molecule-1; LECs, sinusoidal liver endothelial cells; Tris, trishydroxymethylamino methane; TBS, tris-buffered saline.
REFERENCES


FIGURE LEGENDS

**Figure 1.** Partial purification of the 175HARE from rat LECs by preparative SDS-PAGE. LEC membranes were solubilized in 2% (v/v) NP-40 and the extract fractionated over RCA-I-Sepharose. HARE was eluted with lactose, concentrated and run on a preparative 5% polyacrylamide gel set up for continuous elution as described in Experimental Procedures. Collected fractions (0.5 ml/tube) containing HARE were found using a dot blot assay and a crude polyclonal Ab. Positive fractions were then subjected to SDS-PAGE, and electotransfer and then either ligand blotted with $^{125}$I-HA followed by autoradiography (A) or stained with CPTS (B) to identify the 175HARE (open arrows) and the 300HARE (closed arrows). The increasing $M_r$ of the 175HARE and 300HARE may be due in part to heterogeneous size of these glycoproteins. Fractions 30-38 with very active 175HARE were pooled for mAb production.

**Figure 2.** Immunoprecipitation of the 175HARE and 300HARE from LEC extracts by a panel of 175HARE-mAbs. Abs from ascites produced from each hybridoma were bound to Protein-G-Sepharose, except for #159 which was bound to rabbit anti-mouse-IgM-Sepharose. After washing 5 times with TBS containing 0.1% Tween 20 for 5 min each, the resin was incubated
with NP-40 extracts of LEC membranes at 4°C for 2 h. The resin was then washed as above, eluted with 1x Laemmli sample buffer and the eluate was subjected to SDS-PAGE and ligand blotting. The solid and open arrows indicate the positions of the 300HARE and 175HARE, respectively. Resin with no mAb (lane 1) or with the negative control mAbs (141, 117 and 497) did not immunoprecipitate HARE. Of the Western-positive mAbs (see Figs 3 and 4), four showed roughly equal ability to purify both HAREs; numbers 235, 467, 28 and 30. Some results with numbers 154 and 174 suggested a preference for the 300HARE, but this result has not been reproducible.

**Figure 3. Reactivity of a panel of 175HARE-mAbs in Western analysis after nonreducing SDS-PAGE of LEC extracts.** Ascites from 11 hybridoma clones, that were positive in ELISA screens with the 175HARE antigen, were screened (at a 1:1,000 dilution) for reactivity with lysates of rat LECs. Seven of these clones showed strong reactivity with proteins at both 175 and 300 kDa (lanes 1-8, except lane 3). Clone 54 only recognizes the reduced protein (Fig. 4). Three clones gave very different patterns (lanes 9-11) and do not recognize the 175HARE antigen. R and N show mouse antisera raised against reduced (R) or nonreduced (N) 175HARE antigen. The solid and open arrows indicate the positions of the 300HARE and 175HARE, respectively.

**Figure 4. Reactivity of a panel of anti-175HARE mAbs in Western analysis after reducing SDS-PAGE of LEC extracts.** Only mAbs 54 (lane 3) and 159 (lane 5) show strong reactivity, which is identical, with the reduced 175HARE and 300HARE proteins. The solid and open arrows indicate the positions of the nonreduced 300HARE and 175HARE, respectively. MAb-174, which also blocks HA binding (Fig 6), shows weaker reactivity with the reduced 175HARE.
and the 260 kDa subunit of the 300HARE (lane 6). The other mAbs, including those positive for the nonreduced proteins, are not reactive.

Figure 5. Effect of reduction or deglycosylation on the reactivity of 175HARE-mAbs with affinity purified HARE. A. The 175HARE and 300HARE proteins were immunoaffinity-purified using mAb-30 (Zhou et al., 1999) and analyzed by SDS-PAGE, without (lane 1) and with (lanes 2, 3 and 4) reduction using β-mercaptoethanol, followed by silver staining. The nonreduced 175HARE and 300HARE bands, separated as in lane 1, were excised, reduced and reanalyzed by SDS-PAGE (lanes 3 and 4). The 175HARE gives a single ~185 kDa species (solid arrowhead b) after reduction (lane 3), whereas the 300HARE gives rise to three subunits, designated α, β, γ at about 260, 230 and 97 kDa, respectively (lane 4). The 185 kDa protein is not seen in the 300HARE complex. The solid and open arrows indicate the positions of the nonreduced 300HARE and 175HARE, respectively. Panel B shows the reactivity of the HARE proteins, either reduced or treated with endoglycosidase F as indicated, with a mixture of all eight mAbs (5 µg/ml each) against the 175HARE. After reduction, only the 97 kDa γ subunit of the 300HARE is not recognized. Nonreduced 300HARE or 175HARE are indicated by the solid or open arrows, respectively.

Figure 6. Endocytosis of HA by LECs at 37°C is inhibited completely by mAb-174. Cultured primary rat LECs were washed and incubated for 60 min at 37°C with 125I-HA (2 µg/ml) in MEM medium containing 5 µg/ml of purified antibody from each of the 11 indicated hybridomas. The data (i.e. the mean of triplicates ± SD) are expressed as a percent of the specific HA uptake
(DPM/mg protein) in the absence of any antibody. The 100% value represents the uptake of ~5% of the initial 125 I-HA.

Figure 7. MAb-174 inhibits 125I-HA binding to both the 175HARE and 300HARE in the ligand blot assay. A. Affinity purified HARE samples were subjected to SDS-PAGE, electrotransfer to nitrocellulose and blocking as described in methods. Nitrocellulose strips were then incubated with TBST containing nothing (lane 9), 50 µg/ml nonlabeled HA (lane 10), or 0.5, 1, 5 or 10 µg/ml mAb-174 (in lanes 1-4, respectively) or nonimmune mouse IgG (lanes 5-8 respectively) at room temperature for 90 min. 125I-HA was then added to a concentration of 0.06 µg/ml and the samples were processed for ligand blot activity and autoradiography. The level of specific binding in the ligand blot assay is >90% (Yannariello-Brown et al., 1996). B. The autoradiograph was scanned to quantitate the band densities for 125I-HA binding to the 175HARE (open bars) and 300HARE (closed bars) with and without excess HA or with 5 µg/ml control or mAb-174 IgG.

Figure 8. Western blot analysis of HARE tissue distribution. Crude NP-40 extracts were prepared from homogenates of the indicated rat tissues, and HARE proteins were immunopurified using mAb-235 conjugated to Sepharose. Samples (~100 µg protein) were analyzed by nonreducing SDS-PAGE on a 5% gel, transferred to nitrocellulose membrane, and incubated with 125I-HA for the ligand blot assay. Autoradiographic analysis (panel A) showed bands at 300 and 175 kDa (solid and open arrows, respectively) in spleen and liver, indicating the presence of both HARE species. After the ligand blot, Western blot analysis was performed on
the same membrane (panel B) using a mixture of mAbs 159, 467, 174, and 235 (25 mg/ml ascites). Both HARE bands were present at 300 and 175 kDa in spleen and liver. The nonspecific band (indicated by the solid circles), present in all lanes at ~150 kDa, was due to mouse antibodies co-eluted with the HARE during immunopurification.

**Figure 9. Confocal immunofluorescent localization of HARE in liver.** Rat liver sections were incubated with a mixture of the eight anti-175HARE mAbs (A, B, and D) or normal mouse serum (C) and stained with anti-mouse IgG conjugated to Rhodamine Red as described in Methods. The sections were analyzed by confocal microscopy using Kr lasers (A and C). The phase contrast image in panel B is the same field as shown in panel A (arrows highlight sinusoidal borders). The bars in Panels A-C are 10 µm. Panel D is a nonconfocal fluorescence micrograph at a lower magnification (400x). CV indicates a central vein. Arrowheads indicate sinusoids among columns of hepatocytes.

**Figure 10. Immunofluorescent localization of HARE in cultured LECs.** LECs were cultured overnight on glass coverslips, fixed and incubated with (B and C) or without (A) 0.1% Triton X-100 to permeabilize the cells. The cells were then treated, as described in Experimental Procedures, with a mixture of ascites fluid from the eight anti-175HARE mAbs (A and B) or normal mouse serum (C) and stained with goat anti-mouse IgG conjugated to rhodamine red. Over 90% of the cells were positive for HARE and showed similar staining patterns. The bar represents 5 µm.
Figure 11. Immunofluorescent localization of HARE in spleen. Sections of rat spleen were incubated with a mixture of eight anti-175HARE mAbs (A, B, C and D) or normal mouse serum (E), washed and stained with goat anti-mouse IgG conjugated to Rhodamine Red. The confocal fluorescent and phase contrast images, respectively in A and B, show the same field. The bars in Panels A and B are 10 µm. The arrows indicate sinuses lined with positive cells. Panels C, D, and E were viewed by normal fluorescence microscopy at magnifications of 200x, 400x, and 400x, respectively. The splenic nodules (SN) were unstained.

Figure 12. Immunofluorescent localization of HARE in lymph node. Sections of rat mesenteric lymph node were incubated with a mixture of the eight anti-175HARE mAbs (A, B, and C) or normal mouse serum (D, E and F), washed and stained with goat anti-mouse IgG conjugated to Rhodamine Red. The confocal fluorescent and phase contrast images, respectively in A and B, and in D and E, show the same fields. Panels C and F show the merged fluorescent and confocal images, respectively, of A plus B and D plus E. The bars are 20 µm and all panels are at the same magnification. A germinal center (G), sinusoids (S) and medullary cords (C) are indicated in panels A and C.