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Accessible hyaluronan receptors identical to ICAM-1 in mouse mast-cell tumours

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Immunohistochemical studies of the hyaluronan (HA)-receptor (R), originally found on liver endothelial cells (LEC) and related to the intercellular adhesion molecule 1 (ICAM-1), showed that polyclonal antibodies against HARLEC (HA receptor on LEC) also stain structures in mouse mastocytomas, mainly vessels. To test if intravenously administered HA might target the tumour receptors *in vivo*, mice carrying an inoculated mastocytoma in one hind leg muscle were injected in the tail vein with ^{125}I-tyrosine (T)-labelled HA and killed 75 min after injection when organs and tissues were checked for radioactivity. When doses exceeding the binding capacity of the liver were injected, a significant increase in radioactivity (up to five-fold) within the tumour tissue was found. The weight adjusted difference between control and tumour tissue was greater for smaller tumours, probably due to necrosis in the larger. HA-staining of tumours from animals receiving ^{125}I-T-HA, showed HA in areas that also stained weakly for ICAM-1 using monoclonal antibodies. ICAM-1 staining was dramatically increased after hyaluronidase treatment of the sections, indicating that the HA is bound to these receptors and thereby blocks antibody recognition.

Keywords: hyaluronan, hyaluronic acid, radioactive, receptors, endothelial cells, ICAM-1, mouse, tumours, targeting, *in vivo*

Abbreviations: ICAM-1, intercellular adhesion molecule 1; HA, hyaluronan; HARLEC, hyaluronan receptor on liver endothelial cells; MW, molecular weight.

Introduction

The polysaccharide hyaluronan (hyaluronic acid; HA) is rapidly cleared from the circulation, primarily by endothelial cells of the liver via receptor-mediated endocytosis [1]. The receptor for HA on these cells has been characterized and purified from rat liver endothelial cells (LEC) and polyclonal antibodies produced [2]. The antibodies raised against the HA receptor on LEC (named HARLEC) and used in this study, have been shown to inhibit HA binding to LEC and LEC membranes [2]. Previous immunohistochemical studies using the antibody have shown that the receptor is present in sinusoids in the liver, spleen, and lymph nodes, and the capillaries in the small intestine [3]. The 90–100 kDa protein has a pI of around 6.7 and binds specifically to immobilized HA [2–4], and contains amino acid sequences identical to intercellular adhesion molecule 1 (ICAM-1) [4]. Human corneal endothelial cells (CEC), previously shown to have receptors for HA with similar characteristics as HARLEC [5], are specifically stained by anti-HARLEC [6]. The CEC staining is inhibited if the corneas are treated with HA prior to staining, while the staining intensity is increased by hyaluronidase treatment [6]. The tissue distribution of HARLEC corresponds well with tissues participating in the clearance of HA [1, 3, 5, 6].

Other cell-surface 'receptors' for HA have been described [7] and include the lymphocyte homing receptor CD44 and a receptor for HA-mediated motility of fibroblasts (RHAMM). Many tumours have been reported to be HA enriched [8–10] and HA binding sites on cells derived from some tumours have been described [8]. When HA-binding proteins from chondrosarcomas were characterized by affinity chromatography on immobilized HA, the major HA-binding protein was found to have a molecular weight (MW) of around 100 kDa [11].

The present work was initiated in order to determine if receptors similar to HARLEC/ICAM-1 are present within mouse mastocytomas and if accessible HA binding sites are present in tumour tissue *in vivo*.

Materials and methods

Polysaccharides

The HA used for labelling and uptake- and turnover-studies was extracted from avian tissue and supplied by Hyal

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Pharmaceutical Corporation (HPC), Toronto, Canada. The molecular weight distribution of the HA was determined by chromatography on a calibrated column of Sephacryl HR with porosities noted as 400, 1000 and 2000 (Pharmacia, Uppsala, Sweden) in 0.25 M NaCl, 0.05% chlorbutanol [12]. The HA content in each fraction was monitored by determination of the absorbance at 214 nm. Radioactivity was measured by gamma-counting on a Packard auto-gamma gamma-counter.

Labelling of HA

The HA was labelled with DL-tyrosine (Sigma Chemical Company, St Louis, USA) as previously described [13], after CNBr-activation of the polysaccharide by the method of Glabe *et al.* [14]. Briefly, 15 mg HA was activated at pH 11 by 8 mg CNBr for 5 min. The activated polysaccharide was separated from the reaction mixture on a small column of Sephadex G25 (PD 10, Pharmacia, Uppsala, Sweden) equilibrated with 0.2 M borate buffer pH 8.0. The activated HA was incubated overnight with 1 mg tyrosine (T) (Sigma Chemical Company, St Louis, USA). The T bound to HA (T-HA) was separated from unbound T on a PD 10 column equilibrated with phosphate buffered saline (pH 7.5) (PBS), containing NaCl (8 g l^{-1}), KCl (0.2 g l^{-1}), KH_2PO_4 (0.2 g l^{-1}) and Na_2HPO_4 (1.15 g l^{-1}).

A part of the T-HA was iodinated with ^{125}I by placing 100 µg of T-HA together with 0.5 mCi ^{125}I in a small glass tube covered with a film of 10 µg 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma Chemical Company, St Louis, USA). Unincorporated ^{125}I was removed on a PD 10 column equilibrated with PBS and the iodinated T-HA (^{125}I-T-HA) stored at 5°C. The specific radioactivity was usually 1500–5000 dpm ng^{-1}.

The ^{125}I-T-HA kept a high molecular weight-profile upon gel filtration chromatography with a mean MW of around 0.5×10^6 Da, and was found to be cleared from the circulation with the kinetics and organ distribution reported for biosynthetically labelled HA of high MW. The ^{125}I-labelled polysaccharide was also taken up by isolated rat liver endothelial cells both *in vivo* and *in vitro*, indicating that the labelling does not interfere with the binding to specific cell-surface receptors found on these cells [1, 2].

Immunostaining

All staining was made with the ABC-elite method [Vectastain® Elite ABC (Vector Laboratories, Burlingame, CA, USA)]. Frozen sections of 6 µm were prepared and mounted on glass slides, coated with Vectabond (Vector Laboratories). To remove unspecific binding to serum proteins, the antiserum to HARLEC as well as the preimmune serum was adsorbed on a Sepharose® 4B gel (Pharmacia) coupled with rat serum proteins (6 mg protein per ml gel). Coupling was performed as described by the manufacturer (*Affinity Chromatography; Principles and Methods*, by Pharmacia LKB Biotechnology). The serum and the gel were mixed in equal volumes and incubated for 6 h at 4°C.

The monoclonal antibody to mouse ICAM-1 (clone KAT 1) was from Seikagaku Corporation, Tokyo, Japan, and produced by rat cells.

The frozen sections were fixed in cold methanol for 10 min and dried for 10 min before washing in phosphate buffered saline (PBS). Peroxidase was blocked in 0.3% H_2O_2 in methanol, and the sections were once more washed in PBS. To block endogenous biotin and biotin-binding activity, an avidin/biotin blocking kit from Vector laboratories was used. The sections were then incubated for 30 min in PBS containing 4% goat serum (HARLEC staining) or 4% rabbit serum (monoclonal KAT 1 staining). The HARLEC antiserum, was diluted 1:50 in 4% goat serum in PBS, and incubated for 1 h. The monoclonal antibody to ICAM-1 was diluted 1:50 in 4% rabbit serum and incubated for 3 h. After washing in PBS, the sections were incubated with the second goat anti-rabbit or rabbit anti-rat antibody (Vector Laboratories) diluted 1:200 in PBS for 30 min.

The staining for HA was performed essentially as described previously by Madsen *et al.* [15], using biotinylated hyaluronan binding proteins from cartilage (b-HABP), but without CPC treatment, on frozen sections treated with methanol and blocked for endogenous biotin binding activity with an avidin/biotin blocking kit (Vector Laboratories).

After incubation with the second antibody, or b-HABP, the sections were washed and incubated with the ABC-Elite-complex (Vectastain^R, Elite ABC). To develop the colour, peroxidase substrate kits (Vector Laboratories) containing 3,3 diaminobenzidine or 3-amino-9-ethylcarbazole were used, and the sections were incubated in the mixture for 5–10 min. After washing, the sections were counterstained in Mayers Haematoxylin for 1.5 min. The glass slides were mounted in Kaisers glycerol-gelatin (Merck).

Hyaluronidase treatment of sections

After methanol fixation and washing in PBS the sections were incubated with 5 U ml^{-1} of streptomyces hyaluronidase (Amano Pharmaceutical Co., Ltd, Japan), 1.8 µg ml^{-1} pepstatin (Sigma), 1.8 mM EDTA (Merck), 1.8 µg ml^{-1} soybean trypsin inhibitor (Sigma), 2.0 mM iodo acetic acid (Sigma), 0.18 mM E-amino-n-caproic acid (Sigma) and 9.0 mM benzamide (Sigma) for 2 h at 37°C. The regular staining protocol was then followed.

Uptake studies in vivo

Male (A/Sn × Leaden) F_1 mice were inoculated with transplantable mastocytoma [16] in one hind leg muscle 7–11 d before the experiments. The animals were anaesthetized with pentobarbital (45 mg per kg body weight) and received an

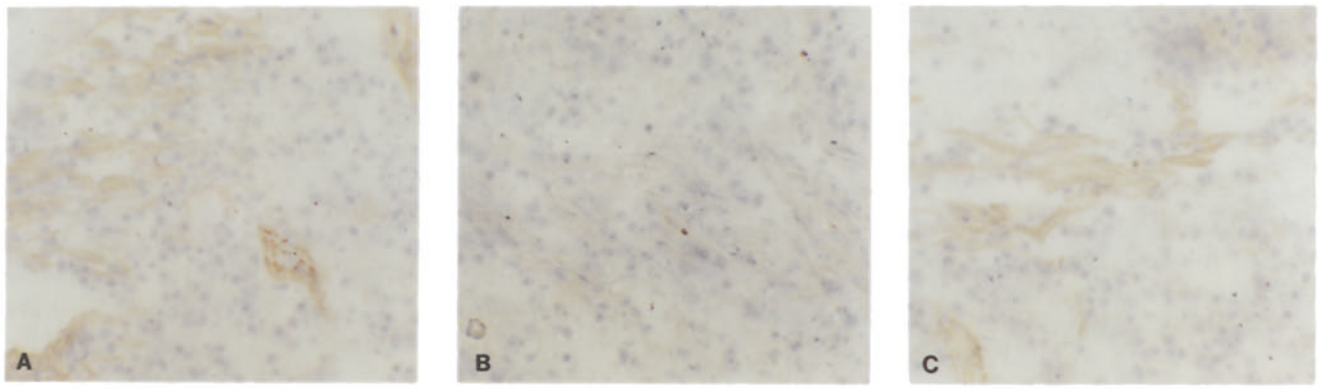


Figure 1. Immunohistochemical staining of mastocytomas. Frozen sections of mouse mastocytomas were stained for receptors using antibodies directed against the hyaluronan receptor on rat liver endothelial cells (HARLEC). (a) and (c) show staining produced by anti-HARLEC, (b) is a control staining using preimmune antibodies.

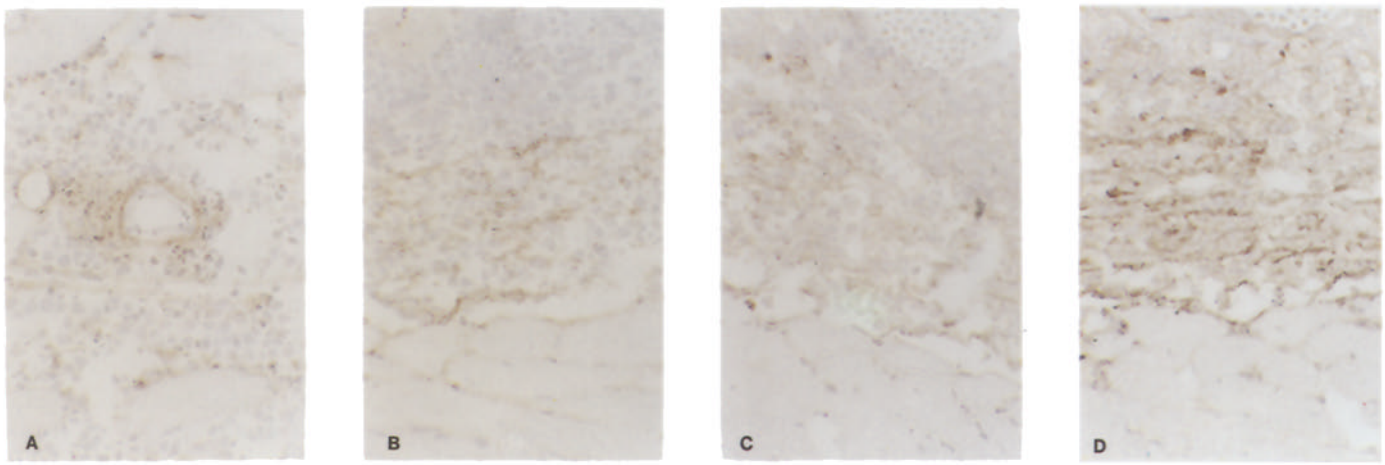
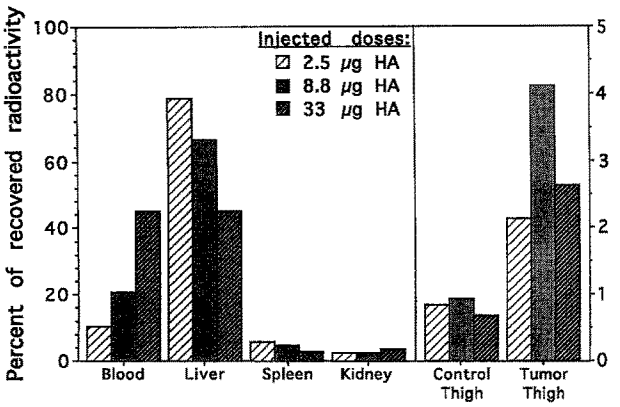


Figure 5. Mastocytomas stained for hyaluronan and ICAM-1 75 min after injection of ^{125}I-T-HYA. (a) and (b) staining for hyaluronan using biotinylated HA binding proteins from cartilage. (c) Immuno-staining of the same area as in (b) for ICAM-1. (d) Immuno-staining of the same area as in (b) and (c) for ICAM-1 after hyaluronidase treatment. Plates (b), (c) and (d) are serial sections.



injection in the tail vein of 20–1500 μg ^{125}I-T-HA (5–15 ×

Figure 2. The recovery of radioactivity in different organs of mice carrying a mastocytoma in one thigh 75 min after an intravenous injection of three different amounts of ^{125}I-T-HYA.

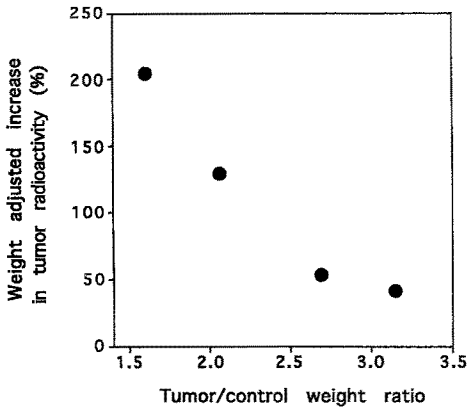


Figure 3. The relationship between increase in tumour-associated radioactivity in relation to control 75 min after an intravenous injection of ^{125}I-T-HYA and the size of the tumour (represented by the tumour:control weight ratio).

10^6 cpm) and unlabelled HA in 0.05–0.2 ml 0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.4.

After 75 min the animals were killed. Liver, lungs, kidneys, heart, spleen, hind leg muscles and in some instances skin, fat, intestines and urine were assayed for radioactivity. The data were processed using a Macintosh SE/30® or a Macintosh IIsi® computer (Apple Computer Inc., Cupertino, CA, USA). Statistical analysis was performed using Statworks® (version 1.1, Cricket Software, Malvern, PA, USA). The graphs were constructed using the Cricket Graph® program (version 1.3, Cricket software, Malvern, PA, USA) and Canvas (version 3.0.2., Deneba Systems Inc, Miami, FL, USA).

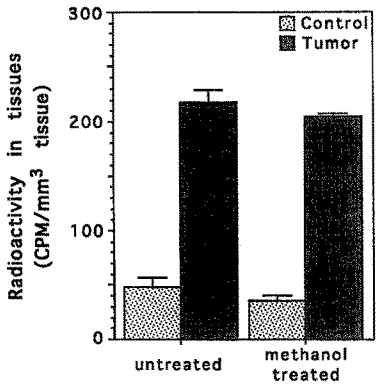


Figure 4. Radioactivity in sections of tumour and control tissue before and after methanol treatment. Results are mean values and error bars represent the standard deviation of the data ($n = 6$).

Results

When tissue sections from mouse mastocytomas were stained for the presence of HARLEC it was found that the staining was localized mainly to round vessel-like structures (Fig. 1a) and in stretches (Fig. 1c) rich in collagen, as seen by the Van Gieson staining (not shown). Some scattered receptor-staining was also seen over the tumour tissue. The staining was specific, as preimmune antibodies gave no staining (Fig. 1b), and was only very slightly enhanced by hyaluronidase treatment (not shown).

The finding of HARLEC immunoreactivity in the mastocytomas without prior hyaluronidase treatment, indicated that exogenous HA delivered to the tumour could possibly bind to the tumour tissue. To test this possibility, mice inoculated in one hind leg muscle with mastocytomas were given ^{125}I-labelled HA intravenously. The association of radioactivity with different organs, including the tumour muscle and the control muscle, was checked 75 min after injection. When tracer dose were given, it was found that almost all of the radioactivity was extracted by the liver and only minor amounts were detected in the tumour and control tissues. However, when the dose exceeded the amount that could be extracted immediately by the liver, a significantly increased uptake in the tumour tissue relative to control tissue was found (Fig. 2). The increase was generally between 50% and 200%, when adjusted for the increase in weight of the tumour tissue, with the difference being more pronounced for smaller tumours with little or no necrosis (Fig. 3).

When tissue sections of a similar size from control and tumour tissues where studied, a five-fold increase in the tumour was found (Fig. 4). Treatment of the sections with methanol only slightly reduced the radioactivity.

Histochemical analysis of frozen sections of tumours from animals receiving intravenous ^{125}I-T-HYA revealed that HA is,

to a high degree, colocalized with HARLEC/ICAM-1 (Fig. 5). By removal of the HA by hyaluronidase, a dramatic increase in ICAM-1 immunoreactivity was noted (compare Fig. 5c and d).

Discussion

When we found HARLEC-immunoreactivity in vessel-like structures of mastocytomas we asked ourselves if these structures could possibly be actively binding HA *in vivo*. The fact that the immunohistochemical staining did not increase significantly following hyaluronidase treatment pointed to a possible uptake of circulating HA and the binding sites not normally being occupied. This is in agreement with what is found for the liver receptors, to which the antibodies used in these studies are directed against, where little effect of hyaluronidase treatment on immunoreactivity is seen, probably because the HA that is bound is rapidly endocytosed with its receptor and the receptors are then recirculated to the surface free of HA. As there is a targeting to the tumour after intravenous administration of the labelled HA (Figs 2 and 3) it is likely that the receptors mediate uptake of the labelled polysaccharide in the tumours also. If the receptors were not to take part in endocytosis they would probably be occupied by endogenous polysaccharide at the time of injection, and targeting with exogenous HA would be blocked.

The increased uptake seen in the tumour was found to be negatively correlated to tumour size ($p < 0.05$, $n = 4$). This is likely to be due to the observed necrotic areas in the larger tumours.

When tissue sections of the tumours were analysed histochemically, it was possible to distinguish in detail between tumour and non-tumour tissue. The increase in radioactivity associated to tumour tissue relative to control (Figs 2 and 3) was then even more pronounced (Fig. 4), probably because irrelevant non-binding structures such as connective tissues could be excluded. The treatment of the sections with methanol did not significantly affect the results, indicating that the major part of the HA present in the tumour has been extracted from the circulation by binding sites (receptors) present in the tumours.

The staining for HA in the tumours of mice receiving an intravenous injection, is most prominent in areas that also stain positive for ICAM-1 (Fig. 5b and c). These areas also show a dramatic increase in ICAM-1 immunoreactivity after hyaluronidase treatment (Fig. 5c and d). This indicates that receptors identical to the ones on the liver endothelial cells and containing amino acid sequences identical to ICAM-1 [4], also actively bind HA in tumours.

The MW of the major HA-binding protein of rat chondrosarcomas [11], is identical to the MW of HARLEC/ICAM-1 [2–4]. It is possible that ICAM-1 is expressed in these tumours, predominantly in the vessels. This could explain why the protein is absent from *in vitro* cultures of chondrosarcoma cells but found in intact tumours [11].

Targeting and binding of HA to endothelial cells expressing ICAM-1 could also explain the inhibition of acute and chronic inflammation seen after systemic administration of HA in animal models [17]. The mechanism could be a competition between HA and leukocytes for ICAM-1, resulting in suppression of leukocyte infiltration into the inflamed tissues.

Our study shows that functional HA-receptors are present in mouse mastocytomas. The receptors are probably involved in endocytosis of HA. After endocytosis the receptors are probably recirculated to the cell membrane as has been shown for the LEC receptors [18], or more receptors are synthesized *de novo*, in order to appear at the cell surface as free receptors.

We believe that further studies of the interaction between HA and ICAM-1 are indicated. Cell-type specific differences in the interaction may be found, and these could possibly be used to specifically target pathological sites expressing this type of HA-receptor.

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