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Studies on the interaction between hyaluronan and a rat colon cancer cell line

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Binding studies with ^{125}I-Tyr labelled hyaluronan (HA) on a cultured rat colon cancer cell line were performed to characterize the association of HA to tumour cells *in vitro*. Results show a specific and saturable binding (K_d = 1.36 nM) which indicates the presence of an HA binding receptor on the tumour cells. There is a specific constant increase of cell-associated HA over time, which indicates that HA is specifically taken up by the cells through endocytosis. The binding of ^{125}I-Tyr labelled HA was more effectively inhibited by unlabelled HA of high MW in relation to low MW species of the polysaccharide indicating that the receptor binds HA of high MW with greater affinity than low MW species. In competition experiments, the HA-binding could not be inhibited by other polysaccharides such as chondroitin sulphate and heparin. Nor could ligands for scavenger receptors and antibodies directed towards ICAM-1, CD 44 and RHAMM (Receptor for HA Mediated Motility) significantly inhibit the association of HA to tumour cells.

***Keywords*: Hyaluronic acid, binding, receptors, tumours, polysaccharides, scavenger receptor ligands**

Introduction

Hyaluronan (HA), is a high molecular weight (MW) polysaccharide built from alternating units of glucuronic acid and *N*-acetyl glucosamine, [D-glucuronic acid ($\beta 1 > 3$) *N*-acetyl-D-glucosamine ($\beta 1 > 4$)]$_n$. It belongs to the family glycosaminoglycans which constitutes the meshwork in connective tissue. It however differs from the others in this family as it is not covalently associated with a peptide but the molecule is just one long sugar-chain. HA is an abundant component of extra-cellular matrix carrying out a range of structural functions, being a main structural component in soft connective tissue such as skin, cartilage, vitreous body, joint fluid and the umbilical cord [1]. Because of its charged residues it can associate with many molecules of water. Hence it occupies a much greater space than what is expected from its already very large molecular mass (up to millions of Da).

Cells can bind and respond to HA through cell-surface binding proteins. A number of cells have been reported to bind HA specifically and some of the binding proteins have been identified. RHAMM (Receptor for HA Mediated Motility) has been found on *ras*-transformed cells. These cells also show an increased production of HA which, together with other extracellular glycosaminoglycans such as heparin, bind to RHAMM and are believed to be required for the motility of the transformed cells [2, 3]. HA also binds to the lymphocyte homing receptors in the CD 44 family [2].

Over the past years, our laboratory has investigated the specific binding of HA to liver endothelial cells (LEC) *in vivo* and *in vitro*. Binding studies have shown that HA association to LEC can be inhibited by chondroitin sulphate [4–6] and the non-endogeneous polysaccharide dextran sulphate [7]. This indicates that LEC express receptors that recognize negatively charged polysaccharides in a similar manner to scavenger receptors [8]. Several HA binding proteins on LEC have been described [7, 9]. One of these described binding proteins with a MW of 85–100 kDa, has been identified as ICAM-1 [10]. Investigations have also been done on corneal endothelial cells, mast cell-tumours, synovial cells from rheumatoid arthritis patients and skin from psoriasis patients. In all these examples ICAM-1 is a strong candidate as the HA binding protein because HA seems to block antibody recognition of ICAM-1 [7].

HA is normally rapidly cleared from the circulation, primarily by endothelial cells of the liver via receptor mediated endocytosis [11]. *In vivo* experiments on rats have shown that injected ^{125}I-labelled HA is predominantly found in liver tissue, but also in spleen and kidney, when the rat is killed and the organs are investigated for radioactivity [12]. A specific uptake of HA from the circulation has also been found in mouse mast cell tumours [13].

The objective of the present study is to further investigate and characterize the HA binding to tumour cells. If tumour

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cells bind HA differently from cells normally engaged in HA metabolism, these differences could be utilized to design efficient targeted chemotherapy using HA.

Materials and methods

Cells

LEC were isolated after collagenase perfusion of rat liver and cultivated as previously described [12].

A colon cancer cell line, obtained from a nitrosoguanidine induced tumour in Wistar rats (NGW), was cultured at 37 °C in an F-10 medium (containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 $\mu g\,ml^{-1}$ gentamicin). Before the start of the experiments the cells were incubated for 1 h in a medium with no FCS to ensure that HA within the serum would not interfere with the results.

HA

One per cent sodium hyaluronate was obtained from Hyal Pharmaceutical Co, Toronto, Canada. The molecular weight distribution of the HA was determined by chromatography on a calibrated column of Sephacryl HR with porosities of 400, 1000 and 2000 (Pharmacia, Uppsala, Sweden) in 0.25 M NaCl, 0.05% chlorbutanol [4, 12]. The HA content in each fraction was monitored by each determination of the absorbance at 214 nm. The HA had a mean molecular weight of 400–500 kDa. In order to detect the amount of HA bound to the cells HA was labelled with ^{125}I-tyrosine according to Gustafson *et al.* [12]. ^{125}I-Tyr labelled HA will be referred to as HA*. Radioactivity was measured by gamma-counting on a Packard Auto Gamma gamma-counter.

HA of higher MW (Healon®, MW 4×10^6 Da) was obtained from Pharmacia Pharmaceuticals, Uppsala, Sweden.

Oligosaccharides of HA were prepared by digestion in 0.25 M sodium acetate (pH 5.2) with testicular hyaluronidase (Leo, Hälsingborg, Sweden; 1600 IU g^{-1} HA) for 24 h according to Forsberg and Gustafson [9]. Before chromatography on a calibrated column [14] of Sephacryl-S-300 (Pharmacia, Uppsala, Sweden), the digested material was boiled for 15 min in order to inactivate the enzyme. The chromatographed fractions with molecular weights between 4000 and 10 000 Da were collected. The oligosaccharide content in each fraction was measured by the carbazole method [15] using glucuronolactone as standard and then concentrated to 10 $g\,l^{-1}$ by ultrafiltration through a Diaflo (YM 2) membrane.

Potential inhibitors of HA binding

In competition experiments polysaccharides and other molecules were tested as potential inhibitors of the HA binding. Polysulphated hyaluronate (HAPS) was a kind gift from Pharmacia Pharmaceuticals, Uppsala, Sweden. Chondroitin sulphate A (CS) from bovine trachea was obtained from Sigma chemical company, St. Louis, USA (product No. 8529). Dextran sulphate (DxS) with a MW of approximately 500 000 Da was from Pharmacia Biotech, Uppsala, Sweden (Code No. 17-0340-01). Dextran (Dx) with a MW of approximately 500 000 Da was from Pharmacia Biotech, Uppsala, Sweden (Code No. 17-0320-01). Poly I was from Sigma Chemical Company, St Louis, USA (product No. 4154). A K5-polysaccharide was a kind gift from Dr Kerstin Lidholt, University of Uppsala, Sweden. Formylated albumin was a kind gift from Dr Jukka Melko, University of Uppsala, Sweden. Heparin from intestinal mucosa and purified by repeated precipitation with cetylpyridinium chloride [16] was a kind gift from Professor Ulf Lindahl, University of Uppsala, Sweden.

Antibodies

Mouse monoclonal antibody to rat ICAM 1 (Clone No. 1A29 supernatant) and CD 44 (Clone No. MRC 0X-50 supernatant) were obtained from Serotec and diluted 1 : 200 for the immunofluorescence stainings. Specific radioassay (HA 50: Pharmacia, Uppsala, Sweden) on the ICAM 1 and CD 44 antibody media showed that they contained 115 and 346 ng HA per ml respectively. Therefore control media containing HA of the same concentrations were made for the inhibition experiments and used at the same dilutions (1 : 2 and 1 : 4 for ICAM-1 and CD 44, respectively) as the antibody media as a control of inhibition due to HA in the supernatants. A mouse monoclonal antibody against rat RHAMM (3T3-5) was used in the immunofluorescence experiments at a 1 : 50 dilution. A rabbit polyclonal antisera to a peptide sequence in RHAMM known to block cell motility was used in the inhibition experiments. The 'anti-peptide' antibody was diluted 1 : 300 giving a concentration of HA in the medium insufficient to cause inhibition of labelled HA to the cells. The antibodies directed towards RHAMM were kind gifts from Professor Eva Turley, University of Manitoba, Winnipeg. FITC conjugated secondary anti-mouse IgG from horse was obtained from Vector Laboratories and used at a dilution of 1 : 25.

Preparation of NGW cells for the experiments

The NGW cells were cultivated in F-10 medium in 175 ml culture flasks. Twenty-four hours before an experiment the medium was removed and the cells were subjected to 5 ml EDTA/Trypsin solution for 5 min to loosen the cells from the flask. The free floating cells in suspension were transferred to a test tube and the test tube was centrifuged 45 s at $490 \times \mathbf{g}$. The EDTA/Trypsin-supernatant was removed and the cells in the bottom of the tube were resuspended in 10 ml of the medium. The concentration of cells in the medium was determined by counting in a Bürker-chamber. The cell suspension was then further diluted to 100 000–150 000 cells per ml and seeded in 24 well plates (area 2 cm^2 per well) coated with fibronectin as previously described for

LEC [9, 17]. 1.5 ml cell suspension was transferred to each dish on the cultivating plate. The cultivating plates were kept at 37 °C under standard cultivating conditions over night. One hour before the start of the experiment the medium was replaced with a medium without serum to ensure that any HA in the serum would not interfere with the results.

Binding experiments

HA*, and in competition experiments unlabelled polysaccharides (or other potential inhibitors), were added to a medium containing glutamine (2 mM) and gentamicin (50 μg ml^{-1}) to make test-media. Three hundred μl test medium was added to each 2 cm^2 well containing between 50 000 and 150 000 cells. The cells were incubated for 70–240 min at either 7 °C or 37 °C in standard cultivating conditions. After the termination of the incubations, the medium was removed and the cells washed three times in PBS. The cells were removed from the bottom of the dishes with 300 μl lysis buffer (20 mM Hepes pH 7.4, 10% Glycerol, 1% Triton) in each dish for 15 min. The lysed cells from each dish were transferred into Ellermann tubes and analysed for radioactivity in a gamma-counter as previously described [17].

Immunofluorescence study

Monolayer cultures of about 200 000 NGW cells were prepared and incubated over night in F-10 medium with 10% fetal calf serum on fibronectin-coated coverslips in 35 mm dishes. Nonspecific binding was blocked by incubating the cultures for 15 min with 3% normal horse serum in PBS containing $CaCl_2$ (0.1 g l^{-1}) and $MgCl_2$ (0.1 g l^{-1}) at room temperature. The cultures were then incubated for 45 min with the primary antibodies. After washing three times with 3% normal horse serum the cells were incubated for 45 min with the FITC-conjugated secondary antibody. After washing in PBS containing calcium and magnesium, the coverslips were mounted on glass slides in Fluoromount G (Southern Biotechnology Ltd, Birmingham, USA). The slides were viewed in a Nikon Labophot microscope equipped with epi-illumination and photographed on Kodak Tmax 400 ASA black and white film for paper prints.

Data processing

The data were processed using a Macintosh SE/30®, Macintosh IIsi® or Macintosh 7200 computer (Apple Computer Inc, Cupertino, CA, USA). The graphs were constructed using the Cricket Graph® program (version 1.3, Cricket Software, Malvern, PA, USA.) and Canvas (version 3.5., Deneba Systems Inc, Miami, Fl, USA). Statistical analyses (Student's *t*-test) were performed using Statworks® (version 1.1, Cricket Software, Malvern, PA, USA).

Results

When the association of HA* to LEC and NGW cells was studied in competition experiments with or without a 50-fold excess of unlabelled CS or DxS, it was found that although the binding to NGW cells was significant, the effective inhibition of HA* binding by CS and DxS seen in experiments with LEC could not be observed in experiments with the NGW cells (Figure 1). This indicated that the binding of HA* to this cancer cell-line is more specific than the binding to LEC. In order to further investigate the specificity, a series of experiments was set up where the NGW cells were subjected to increasing concentrations of potential inhibitors in competition with 1 μg ml^{-1} HA*. The amount of bound HA* remained essentially constant in the presence of the tested molecules (Figure 2). None of the molecules tested seem to inhibit the binding of HA to the tumour cells to any great extent. There was a slight increase in the uptake of HA in the presence of HAPS and poly I but this was probably due to nonspecific interactions of the polysaccharides with the cells and to the added molecules. At a CS concentration of 50 μg ml^{-1} a small inhibition was seen (Figures 1 and 2). To test if CS inhibits significantly at concentrations above 50 μg ml^{-1}, further inhibition experiments with CS were performed. A statistically significant inhibition was seen at 200 μg ml^{-1} (not shown). To determine if this inhibition was due to HA contamination of the CS preparation, an HA assay (HA-50, Pharmacia, Uppsala, Sweden) was carried out on the CS preparation. The batch was found to contain 1.9 ng HA per μg CS, corresponding to 0.4 μg HA per ml at 200 μg ml^{-1} CS. To determine if the HA in the CS preparation is responsible for the diminished HA* association to the cells at high CS concentrations,

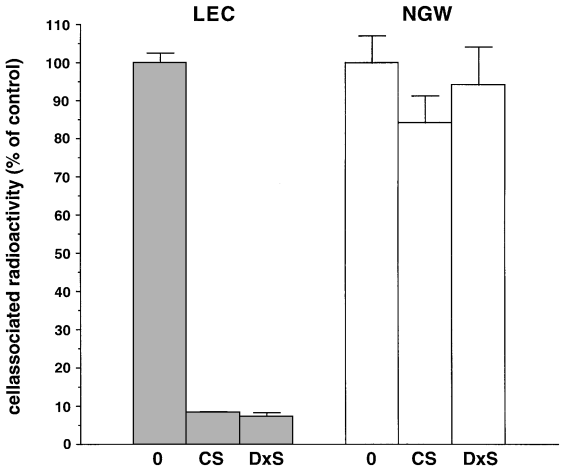

Figure 1. The cell association of ^{125}I-Tyr labelled HA (1 μg ml^{-1}) to liver endothelial cells (filled bars) or NGW cells (open bars) in the absence (0) or presence of 50 μg ml^{-1} chondroitin sulphate (CS) or 50 μg ml^{-1} dextran sulphate (DxS). Cell associated HA was determined after 70 min at 37 °C. Results are mean values ± SD of three determinations. See Materials and methods for details.

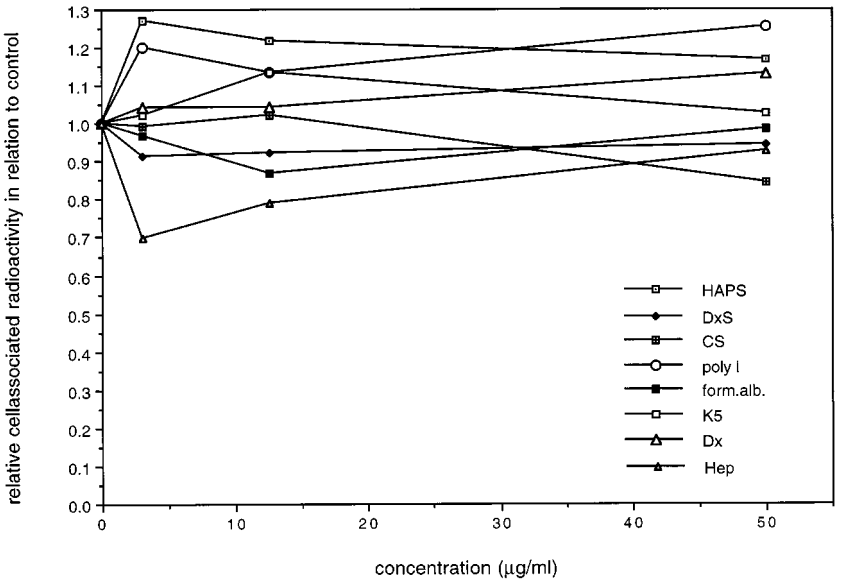

Figure 2. The relative cell association of ^{125}I-Tyr labelled HA at indicated concentrations of polysaccharides and other tested molecules. Cell association was determined after 70 min at 37 °C. Cell association 1.0 is the radioactivity for the 1 μg ml^{-1} HA* control. Results are mean values of triple determinations. See Materials and methods for details.

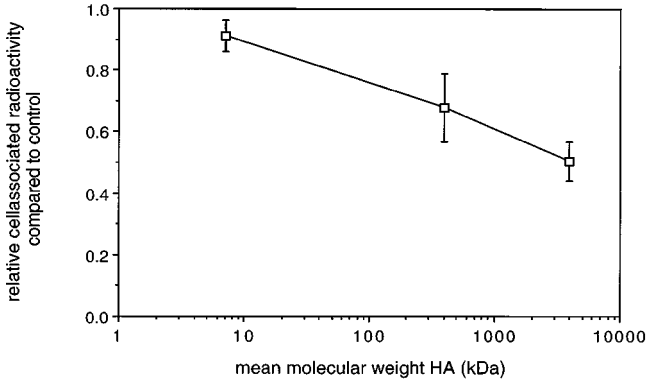

Figure 3. The cell association of ^{125}I-Tyr labelled HA at different MWs of unlabelled HA at a concentration 0.4 μg ml^{-1}. Cell association was determined after 70 min at 37 °C. Each point is the average of three different experiments ± SD. See Materials and methods for details. At an HA MW of 4×10^6 Da and 4×10^5 Da a significant ($p = 0.001$ and $p = 0.011$ respectively) inhibition of the HA* association to the cells is seen. Note that the MW is on a logarithmic scale.

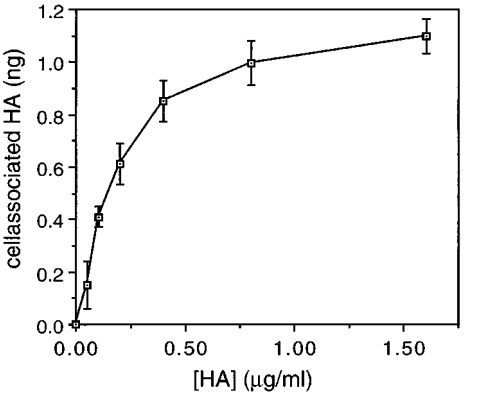

Figure 4. The cell association of ^{125}I-Tyr labelled HA at indicated concentrations. The data were obtained after 70 min at 37 °C and non-specific uptake has been subtracted. Each point is the average of double experiments. Error bars represent the range. See Materials and methods for details.

competition experiments were performed at an HA concentration of 0.4 μg ml^{-1}. As the MW of HA in the CS stock was not known, the competition experiments were performed with HAs of different molecular weights (Figure 3). Using HA with a molecular weight of 4×10^6 Da ($p = 0.001$) and 4×10^5 Da ($p = 0.011$) a significant inhibition was seen. With the HA of lowest MW a smaller inhibition was observed (Figure 3).

Characterization of the HA/receptor interaction was carried out through saturation experiments at HA* concentrations between 0.05 and 1.6 μg ml^{-1}. A set of double controls with 50 μg ml^{-1} unlabelled HA was carried out to account for nonspecific binding and uptake. HA binds specifically to the cells and the flattening out of the curve (Figure 4) indicates that it is a saturable binding mediated by a receptor.

Since the binding was carried out at 37 °C, uptake of HA into the cells might have occurred and therefore similar experiments were carried out at 7 °C where no active uptake occurs (Figure 5). Here a problem occurred as at this low temperature some cells easily lost contact with the bottom of the wells. This is probably the reason behind the large variation between the individual results. To obtain a reliable estimate of affinity and receptor number, four different

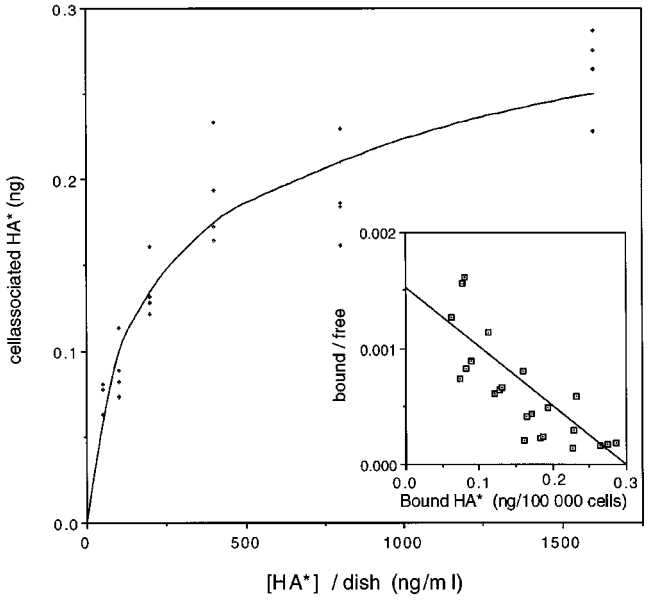

Figure 5. The cell association of ^{125}I-Tyr labelled HA at indicated concentrations. The data were obtained after 70 min at 7 °C and nonspecific uptake has been subtracted. At each concentration the four individual results are shown. The line represents an imaginary saturation curve. The same data, presented as a Scatchard plot [20], are inserted into the figure. See Materials and methods for details.

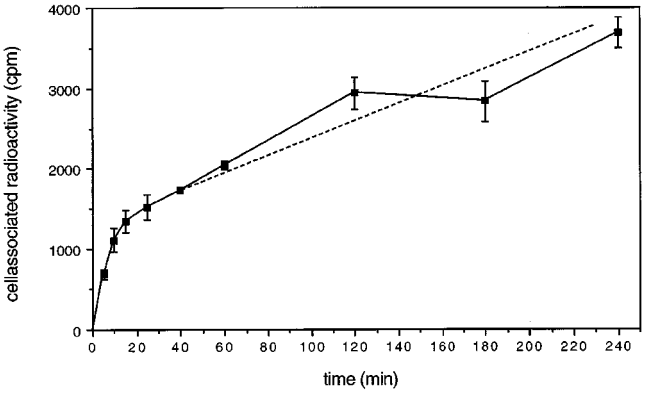

Figure 6. The cell association of ^{125}I-Tyr labelled HA over time at a concentration 1 μg ml^{-1}. Incubations were carried out at 37 °C and terminated at 5, 10, 15, 25, 40, 60, 120, 180 and 240 min. Each point is the mean of three tests and SD is shown as error bars. Nonspecific association was determined with 50 μg ml^{-1} unlabelled HA at 5, 15, 60, and 240 min and has been subtracted. The dashed line represents an assumed uptake at a constant rate.

experiments were performed. By Scatchard plot analyses of the binding data it was found that approximately 4000 molecules can bind to each cell with a K_d of 1.4 nM. Mean MW of HA was taken as 450 000 Da.

HA-binding studies over time were carried out at 37 °C to determine if there is uptake of HA into the cells. During the first 20 min there is a fast increase in HA*-binding (see Figure 6). Between 20 and 40 min the curve levels out, indicating that a saturation of most binding sites has been reached. As there is a continued, but less pronounced, increase in specific cell associated radioactivity after this point, it looks as if there is an uptake of HA into the cells.

Inhibition experiments with receptor antibodies were carried out to determine if the HA-binding to NGW cells can be inhibited by antibodies directed to some of the earlier described HA-binding receptors. Antibodies directed towards CD 44, ICAM-1 and RHAMM were found to specifically stain cultured NGW cells when studied by immunofluorescence (Figure 7), indicating that these molecules are expressed on the surface of the cells. As a specific radioassay (HA 50, Pharmacia, Uppsala, Sweden) on the CD 44 and ICAM-1 antibody media had shown that they contained HA (346 and 115 ng ml^{-1} respectively), two control media with HA concentrations of 346 and 115 ng ml^{-1} were made and used as a control of inhibitory binding activity due to HA in the antibody solutions. Neither CD 44, ICAM-1 nor RHAMM antibodies were able to affect the HA binding significantly (Figure 8). Nor did the small amounts of HA in the CD 44 and ICAM-1 antibody solutions have any significant effect on the association of HA*.

Discussion

The presence of saturable binding sites for ^{125}I-Tyr labelled HA on the NGW tumour cells and the effective inhibition of this binding by unlabelled HA but not by any other tested molecule at high concentrations (Figures 1–5 and 8), indicate that these cells carry specific receptors for HA. As there is a time dependent increase in HA* association to NGW-cells at 37 °C even when nonspecific uptake has been accounted for (Figure 6), it seems as if the cancer cells take up HA specifically and not only through pinocytosis. Experiments to determine if the receptor can be upregulated by cytokines and if HA is degraded intracellularly by NGW cells are underway.

In competition experiments the binding of HA to the tumour cell receptors could not be inhibited by any of the polysaccharides or potential inhibitors tested (Figures 1 and 2). In some cases, especially when adding poly I and polysulphated HA, a slight increase in binding could be observed (Figure 2). It is possible that these molecules activate the cells to express more receptors under the conditions used. The inhibition observed at high concentrations of CS could, after further experiments, be explained by small amounts of high MW HA present in the CS preparation. The MW of the HA is likely to be in the higher MW range as the preparation is obtained from connective tissue.

The HA association to NGW cells could not be inhibited by CD 44, ICAM-1 or RHAMM antibodies (Figure 8) despite the fact that immunofluorescence studies indicate expression of these receptors on the cell surface (Figure 7). Even though this indicates that the colon cancer cells HA

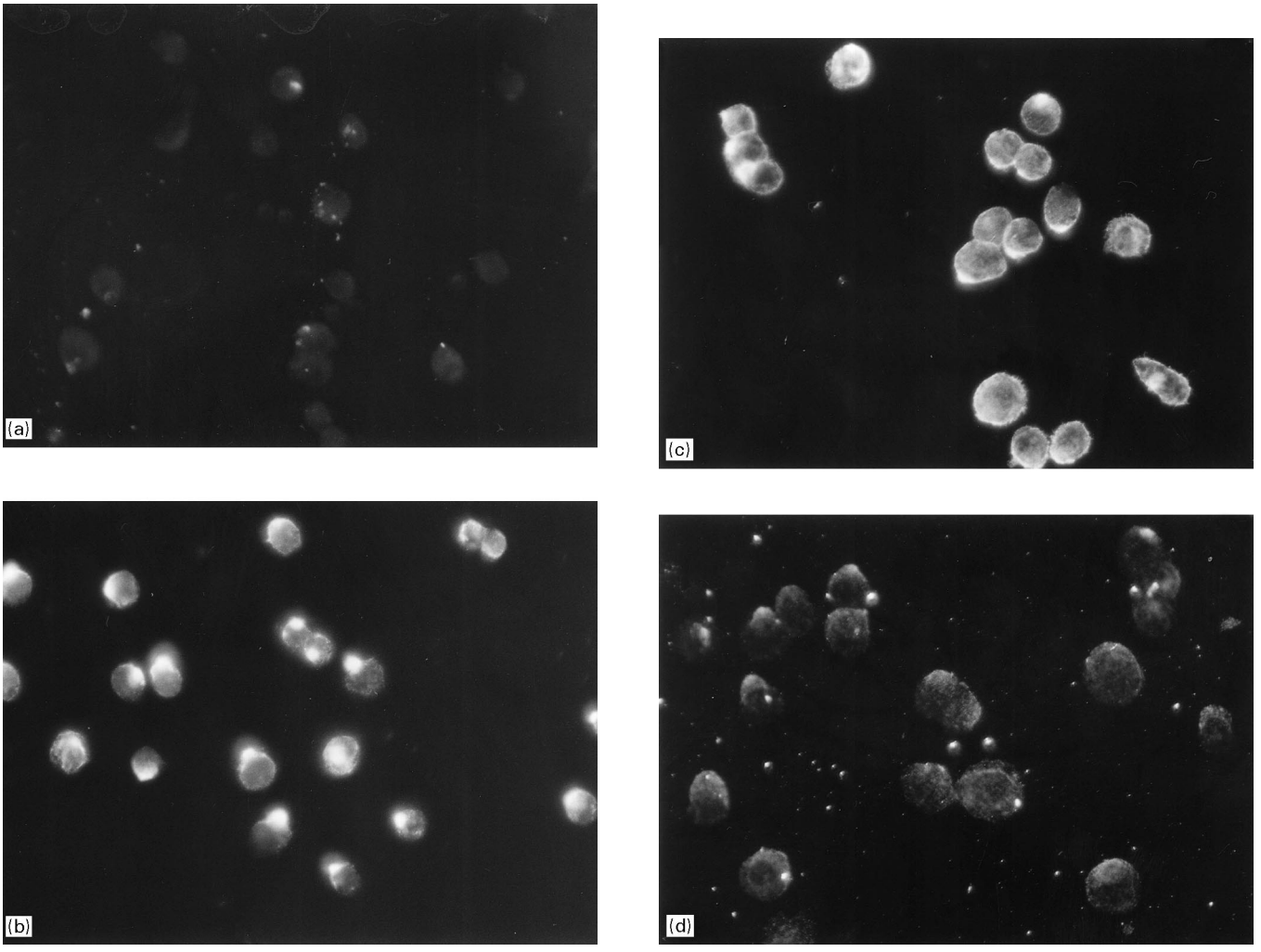

Figure 7. Visualization of possible HA binding receptors on NGW cells cultured on glass slides. After incubation with normal horse serum (a), monoclonal antibodies to rat ICAM-1 (b), monoclonal antibodies to rat CD 44 (c) and monoclonal antibodies to rat RHAMM (d), the cultures were stained using fluorescent horse anti-mouse IgG. Although some nonspecific spots can be observed, especially in RHAMM specific staining of cells can be seen for ICAM-1, CD 44 and RHAMM. See Materials and methods for details.

receptor differs from other known HA binding proteins, the lack of inhibition does not exclude the possibility that one or more of these receptors are involved in HA binding as the antibody binding site might be different from the HA binding site. However, the same ICAM-1 antibodies used in this study have been shown to reduce HA binding to LEC by about 50% [19]. The antibody to CD 44 slightly stimulated binding in relation to control. The observed increase in HA binding might be due to some stimulatory effect of the CD 44 antibodies. However, it cannot be ruled out that other factors in the tissue culture supernatant are responsible for the increased binding. This stimulatory effect also shows the complexity of antibody manipulation and that other studies are needed in order to characterize the binding structures.

The calculated dissociation constant for HA binding to the NGW cells ($K_d = 1.36$ nM) shows similarity with that on J 774 macrophages ($K_d = 1.5$ nM) [18]. However, in the J 774 experiments an HA with higher MW was used so the K_d cannot be directly compared.

Efforts ought to be directed towards determining as much as possible about the nature of the HA binding structure on cancer cells. It would be informative to do further antibody inhibition experiments with antibodies directed towards scavenger receptors and other possible HA receptors, even though we could not see inhibition of HA-binding with scavenger receptor ligands such as poly I, formylated albumin, CS and DxS.

CS has previously been shown to inhibit HA association to LEC [4–7] but does not affect the HA binding to NGW tumour-cells even at high concentrations. Hence it will be interesting to test if a specific HA association to tumours can be accomplished *in vivo* if the uptake in the liver is simultaneously blocked with CS. If this can be

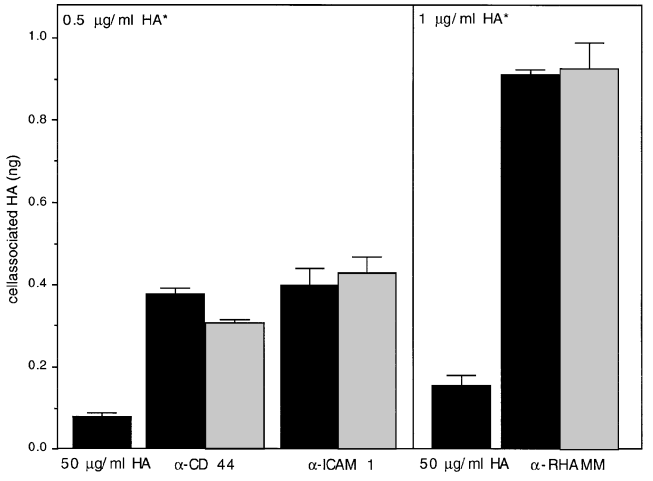

Figure 8. The cell association of ^{125}I-Tyr labelled HA at concentrations of 0.5 or 1 µg ml^{-1}. Cell associated radioactivity was determined after 70 min of incubation at 37 °C. In competition experiments cells were subjected to unlabelled HA (50 µg ml^{-1}) or antibody solutions as well as HA*. Grey bars represent control experiments where cells were incubated with HA* only or, in the CD 44 and ICAM 1 controls, small amounts of HA at a concentration corresponding to HA present in the antibody solutions. Experiments were carried out in quadruplicate and SD is indicated as error bars. Binding of HA* in the presence of antibody to CD 44 was statistically different from control ($p = 0.008$).

achieved it will open up a range of new possibilities in chemotherapy design. By connecting cytotoxic agents to HA and blocking the efficient liver uptake, by *eg* CS, the drug will preferentially come in contact with and attack the tumour and not healthy tissue.

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