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The Endocytic Hyaluronan Receptor in Rat Liver Sinusoidal Endothelial Cells Is Ca^{+2}-Independent and Distinct From a Ca^{+2}-Dependent Hyaluronan Binding Activity

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Abstract Isolated and cultured rat liver sinusoidal endothelial cells (LECs) retain the ability to specifically bind ^{125}I-hyaluronan (HA) and internalize it using a coated pit pathway [Biochem J, 257:875–884, 1989]. Here we have determined the effect of Ca^{+2} on the binding and endocytosis of HA by LECs. ^{125}I-HA binding to intact LECs at 4°C occurred both in the absence (10 mM EGTA) or the presence of physiologic concentrations of Ca^{+2} (1.8 mM). However, the specific binding of ^{125}I-HA to LECs increased linearly with increasing Ca^{+2} concentrations. After permeabilization with the nonionic detergent digitonin, the Ca^{+2}-independent HA binding activity increased ~743%, while the Ca^{+2}-dependent binding activity was enhanced only ~46%. Therefore, the Ca^{+2}-dependent HA binding activity appears not to be intracellular, whereas the Ca^{+2}-independent HA receptor is found both inside LECs and on the cell surface. When LECs were allowed to endocytose ^{125}I-HA at 37°C in 10 mM EGTA or in 1.8 mM Ca^{+2}, no differences were seen in the extent or rate of endocytosis. When LECs were allowed to endocytose ^{125}I-HA in the presence of 10 mM Ca^{+2}, the amount of cell-associated radioactivity increased approximately 20–50-fold. However, this additional cell-associated ^{125}I-HA was not sensitive to hyperosmolarity and was removed by washing the cells in 10 mM EGTA at 4°C. Therefore, the Ca^{+2}-dependent cell-associated ^{125}I-HA had accumulated on the cell surface and had not been internalized. From these studies we conclude that LECs have at least two types of specific HA binding sites. One, the previously characterized HA receptor, is Ca^{+2}-independent, localized both extracellularly and intracellulary, and mediates the efficient binding and subsequent endocytosis of HA using a coated pit pathway. The other newly recognized HA binding activity is Ca^{+2}-dependent, localized extracellularly, and is not responsible for the endocytosis of HA in rat LECs.

Key words: hyaluronic acid, endocytosis, receptors, calcium ions

Hyaluronic acid (HA) is a non-sulfated member of the glycosaminoglycan (GAG) family [1]. HA is a linear polymer composed of repeating disaccharide units (glucuronic acid and N-acetylglucosamine) and has an MW from $0.2–10 \times 10^6$. A biological role has been proposed for HA during morphogenesis and development [2], cell-cell and cell-substratum adhesion [3], tumorigenesis and metastasis [4], and in maintaining the mechanical stability and resilience of tissues such as cartilage [5]. Although HA is distributed ubiquitously throughout the body, circulating blood levels of HA are normally low to undetectable and HA can only be found as a minor component of the total excreted GAGs in the urine [6,7]. However, the HA levels in blood and/or urine can be substantially elevated during the normal aging process [8,9], in diseases of accelerated aging [9,10], in certain types of cancer [11,12], and in kidney disease [7] and arthritis [13].

The daily total body turnover of HA in humans is estimated to be about 4 g/d [14]. The fact that HA levels in blood and urine are extremely low despite this very large turnover of HA suggests that the clearance mechanisms for HA are extremely efficient and may be very important for normal health. When large HA ($M_r = 1–2 \times 10^6$) is injected intravenously into mammals, it is rapidly cleared from the circulation [15–17]. The site of clearance of HA was

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determined by whole body autoradiography and direct analysis of tissues to be the liver in mice and rabbits [15,16] with significant amounts also taken up by the spleen in rabbits [15]. The HA binding activity was solely associated with the endothelial cells in liver [17].

The HA receptor on rat liver endothelial cells (LECs) has been characterized in detail by us [18,19] and others [17,20]. The receptor is of moderately high affinity ($K_d = 5.8 \times 10^{-8}$ M) and is not specific for HA alone, since binding can be competed by other GAGs such as heparin [19] and chondroitin sulfate [17,19]. The HA receptors in LECs recycle and are responsible for multiple rounds of internalization of HA through a coated pit pathway [19], similar to other Class II receptors [21]. In order to understand the mechanisms regulating LEC HA receptor function, we have been purifying the receptor. During this work, we determined that Ca^{+2} was not required for HA binding to detergent solubilized LEC membrane extracts immobilized onto nitrocellulose paper [22]. However, in the presence of increasing concentrations of Ca^{+2}, a significant increase in specific HA binding was seen. In order to proceed further with the purification, we needed to determine if Ca^{+2} stimulates or is required for HA binding to intact cultured LECs as well as to the LEC extracts and if so, whether the Ca^{+2}-dependent binding activity is mediated by the endocytic HA receptor. Here we show that the binding and subsequent endocytosis of HA in LECs is not dependent on Ca^{+2}. However, LECs have another distinct extracellular specific HA binding activity that is Ca^{+2}-dependent.

MATERIALS AND METHODS

Materials

Cell culture media and reagents were purchased from Gibco (Grand Island, NY) with the exception of bovine calf serum, which was purchased from Hazelton Research Products Inc. (St. Lenexa, KA) or Flow Laboratories (McLean, VA). $Na^{125}I$ was purchased from Amersham Corp. (10–20 Ci/μg iodine). 1,3,4,6,-Tetrachloro-3α,6α-diphenylglycouril (Iodogen) was from Pierce. Fibronectin was a generous gift of Dr. G. Fuller (University of Alabama, Birmingham, AL). Digitonin was purchased from Kodak Chemical Corp. (Rochester, NY) and 25% (w/v) stock solutions were prepared in dimethylsulfoxide. Bisbenzimide (Hoeschst dye 33258) was from Behring Diagnostics. Collagenase (Type I) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bovine serum albumin (BSA; fraction V) was from Armour Biochemicals (Tarrytown, NY). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Purification of HA and Preparation of Radiolabelled HA

Human umbilical cord HA (from Sigma) was purified by complexation with cetyl pyridinium chloride, fractionation on celite and ethanol precipitation [23]. The purified HA contains $< 0.2\%$ protein and $< 0.1\%$ sulfate by weight. HA oligosaccharides with $M_r \sim 80{,}000$ (~ 400 mer) were obtained from a specific lot supplied by Sigma and were used without further fragmentation. As described previously, HA oligosaccharides, which normally do not have free amino groups, were uniquely modified at the reducing end of the molecule to produce an alkylamine derivative [24]. This modification allows subsequent reaction of the free amine with the Bolton-Hunter reagent to produce a hydroxyphenyl derivative that is then iodinated with $Na^{125}I$ using the Iodogen reagent to achieve routinely a specific activity of 150 cpm/fmol HA [24]. The ^{125}I-HA used in these studies bound to permeable LECs with a $K_d = 1.3 \times 10^{-8}$ M and a $B_{max} =$ 85,000 molecules/cell in the presence of 1.8 mM Ca^{+2}.

Liver Sinusoidal Endothelial Cell Isolation and Culture

Male Sprague-Dawley rats were purchased from Harlan Breeding Laboratories (Houston, TX) and used between 6 and 10 weeks of age. The method of Eriksson et al. [17] was utilized for LEC isolation. Rat livers were collagenase perfused as previously described [25] and the non-parenchymal cell fraction was collected from the first three differential centrifugations. The cells were washed, resuspended in RPMI-1640 containing 2% bovine calf serum (BCS), and further purified by centrifugation through a discontinuous Percoll gradient prepared in phosphate buffered saline [17]. Cells banding at the 25/50% interface were removed, washed in RPMI-1640 containing 20% BCS, penicillin/streptomycin (100 units each), and 2mM glutamine, and then plated onto 24-well tissue culture dishes (13 mm wells) that had been previously treated with 50 μg/ml of fibronectin (0.5 ml) in RPMI-1640. After a 120 min incubation at 37°C, the cell layer was washed 3 times in

phosphate buffered saline and the medium was replaced. After an overnight incubation, the cultures were > 95% endothelial cells as judged by acetylated low density lipoprotein uptake and non-specific esterase staining [18]. This protocol routinely yields ~60–100 × 10^6 LEC/250 g rat. LEC cultures were used after 16–18 h.

Quantitation of the Total and Surface HA Binding Sites in LECs

Measurement of specific ^{125}I-HA binding to cultured LECs was performed as previously described [19] with minor modifications. Cultured LECs were first washed and then incubated serum-free with Eagle's modified minimum essential medium supplemented with 2.4 g/L HEPES (pH 7.4), 0.22 g/L $NaHCO_3$, and 0.15% BSA w/v (Medium 1/BSA) for 60 min at 37°C. The cells were chilled to 4°C, the medium was removed, and ^{125}I-HA at 4 μg/ml was added in Medium 1/BSA containing EGTA or $CaCl_2$ as indicated. The cells were incubated for 120 min at 4°C with gentle swirling. The cell layers were then washed quickly 3 times with 0.5 ml ice-cold Medium 1/BSA and solubilized in 0.4 ml 1.0% deoxycholate. Radioactivity was determined and the same samples were then assayed for DNA content. Total cellular activity (surface plus internal HA binding sites) was measured in the presence of 0.055% digitonin [19,26]. Surface HA binding activity was measured in the absence of detergent. Specific ^{125}I-HA binding was determined by subtracting the radioactivity bound in the presence of a 125-fold excess of non-radioactive underivatized HA (nonspecific binding) from the radioactivity bound in its absence (total binding).

Endocytosis of ^{125}I-HA in Cultured LECs

To measure the uptake of ^{125}I-HA in cultured LECs, the radiolabel was added to a concentration of 2.0 μg/ml in Medium 1/BSA containing 10 mM EGTA or various concentrations of $CaCl_2$ as indicated. The cells were incubated at 37°C for up to 120 min, washed 3 times in ice-cold Medium 1/BSA with EGTA or Ca^{+2} as needed, and solubilized in 1.0% deoxycholate. Radioactivity was determined and then the samples were assayed for DNA content. No correction was made for internalized and degraded ^{125}I-HA, since release of radiolabeled breakdown products into the medium occurs only after ~3 h [19]. We previously showed [19] that endocytosis of ^{125}I-HA by LECs in the presence of 1.8 mM Ca^{+2} is 95% competed by a 125-fold excess of non-derivatized HA (i.e., endocytosis is 95% specific).

General

^{125}I-Radioactivity was determined using a Packard Multiprias 2 γ spectrometer. The experimental points on the graphs represent the average of duplicate values. SEM bars are included for all data points unless the symbols were larger than the SEM, in which case they are not shown. DNA was determined by the method of Labarca and Paigen [27] using calf thymus DNA as a standard.

RESULTS

Effect of Ca^{+2} on Specific ^{125}I-HA Binding to Cultured LECs

To determine whether Ca^{+2} has an effect on ^{125}I-HA binding to cultured cells, we incubated LECs in Medium 1/BSA in either 10 mM EGTA (~ 0 mM free Ca^{+2}) or with increasing concentrations of $CaCl_2$ (Fig. 1). Since 50–75% of the total cellular HA receptor content is intracellular [18], ^{125}I-HA binding to LECs was measured at 4°C in the presence of 0.055% digitonin to assess binding to both internal and cell surface HA receptors. That there was little or no significant change in specific ^{125}I-HA binding in the absence of Ca^{+2} (10 mM EGTA) when compared to physiologic Ca^{+2} (1.8 mM) indicates that ^{125}I-HA binding to LEC HA receptors is not dependent on

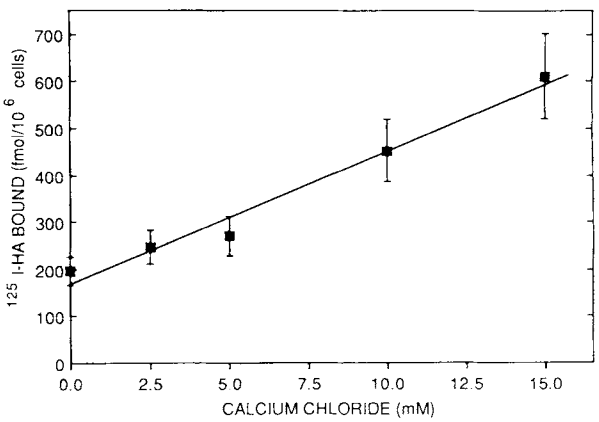

Fig. 1. Effect of Ca^{+2} on specific ^{125}I-HA binding to cultured LECs. LECs were incubated at 4°C with ^{125}I-HA in Medium 1/BSA containing either 10 mM EGTA (no Ca^{+2}) or the indicated concentrations of $CaCl_2$ in the presence of digitonin to assess the number of total cellular HA binding sites. Specific ^{125}I-HA binding was determined as described in Methods. The line determined by least squares linear regression analysis (cc = 0.98) had a slope of 28.3 fmol HA bound/10^6 cells/mM Ca^{+2}.

Ca^{+2}. However, a linear increase in HA binding activity was clearly seen when the Ca^{+2} concentration was increased further. In the experiment in Figure 1 the increase (slope) was 28.3 fmol ^{125}I-HA bound/10^6 cells/mM Ca^{+2} and a three-fold stimulation of specific HA binding was observed at 15 mM Ca^{+2}. In other experiments this stimulation of specific HA binding at 15 mM Ca^{+2} ranged up to thirty-fold compared to the EGTA control. In some experiments the *stimulation* of HA binding by Ca^{+2} appeared to be sigmoidal rather than linear, so that the increase at 10 mM was even greater. This enhanced ^{125}I-HA binding in the presence of 10–15 mM Ca^{+2} is reversed if the cells are subsequently washed in Ca^{+2}-free or EGTA-containing medium (not shown). The ^{125}I-HA binding was 50–80% specific in the absence of Ca^{+2} and at all Ca^{+2} concentrations tested. These levels of specificity are similar to those previously obtained in the presence of physiological concentrations of extracellular Ca^{+2} [18,19]. When the saturation binding curve for cells incubated with ^{125}I-HA in the absence of Ca^{+2} was compared to that for cells incubated in the presence of physiological concentrations of Ca^{+2}, they were also very similar (not shown).

In a separate experiment, the binding of ^{125}I-HA to intact or permeable LECs was determined in the presence of 10 mM EGTA or 1.8 mM Ca^{+2}. As expected, a small and not statistically significant difference was seen in the binding of ^{125}I-HA to the cell surface with or without Ca^{+2}. Intact LECs bound 31.8 ± 6 and 23.4 ± 5 fmol HA/10^6 cells in the presence or absence of 1.8 mM Ca^{+2}, respectively. LECs, permeabilized with digitonin, bound 360 ± 32 and 365 ± 78 fmol HA/10^6 cells in the presence or absence of 1.8 mM Ca^{+2}, respectively. These above data demonstrate that neither the surface nor the intracellular HA receptors require Ca^{+2} in order to bind HA and that the Ca^{+2}-stimulation of HA binding is not apparent at 1.8 mM Ca^{+2}; higher concentrations are needed to distinguish the Ca^{+2}-stimulated HA binding activity.

Effect of Ca^{+2} and Hyperosmolarity on Binding and Endocytosis of ^{125}I-HA by LECs

Although Ca^{+2} is not required for HA binding to cultured LECs, Ca^{+2} can nonetheless cause a large enhancement of specific HA binding. To determine if Ca^{+2} is required for HA receptor function (i.e., endocytosis), LECs were incubated at 37°C with ^{125}I-HA in the presence of either 10 mM EGTA or 1.8 mM Ca^{+2} (Fig. 2). Again, at zero time (4°C) there was little difference in ^{125}I-HA binding. Surprisingly, neither the extent nor the rate of ^{125}I-HA uptake was affected by EGTA. After 2 h in the absence of Ca^{+2}, the LECs had internalized 249 ± 19 fmol ^{125}I-HA/10^6 cells compared to 236 ± 10 fmol/10^6 cells in the presence of 1.8 mM Ca^{+2}. We conclude that, therefore, Ca^{+2} is not required for the LEC HA receptor to function normally and to endocytose ligand efficiently in cultured LECs.

Hyperosmolarity inhibits the endocytosis of a variety of receptors that use coated pits, including those for asialoglycoproteins [28] and chemotactic peptide [29]. McGary et al. [19] determined that internalization of ^{125}I-HA in the presence of 1.8 mM Ca^{+2} is mediated by a coated pit pathway in LECs by demonstrating that sucrose-induced hyperosmolarity inhibited ^{125}I-HA endocytosis by > 90%. Sucrose does not affect the binding of ^{125}I-HA to LECs and does not affect cell viability [19]. To determine if the ^{125}I-HA is internalized via coated pits in the absence of Ca^{+2}, LECs were incubated in either 10 mM EGTA or 1.8 mM Ca^{+2} in the presence or absence of 0.4 M sucrose (Fig. 2). Endocytosis under both conditions was inhibited ~90% by sucrose. These data indicate that the coated pit mediated endocytic function of this HA receptor is not dependent on extracellular Ca^{+2}.

Since greater than physiologic concentrations of Ca^{+2} increase the specific binding of ^{125}I-HA to

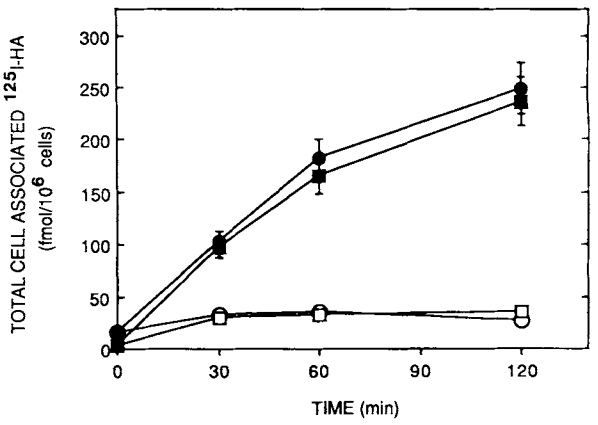

Fig. 2. Effect of Ca^{+2} depletion and hyperosmolarity on the endocytosis of ^{125}I-HA by LECs. Intact cultured LECs were incubated at 37°C with 2.0 μg ^{125}I-HA/ml in Medium 1/BSA containing either 10 mM EGTA (■,□) or 1.8 mM $CaCl_2$ (●,○), with (□,○) or without (■,●) 0.4 M sucrose for the indicated times. The cells were washed and cell-associated radioactivity was determined as described in Methods. The zero time point represents specific cell surface ^{125}I-HA binding at 4°C.

digitonin permeabilized LECs, we also determined the effect of elevated Ca^{+2} levels on endocytosis in a separate experiment (Fig. 3). Whether cells were in the presence of EGTA or 1.8 mM Ca^{+2}, there was no difference in their ability to bind ^{125}I-HA at time zero (4°C) or to endocytose ^{125}I-HA at 37°C; these results are identical to those shown in Figure 2. However, in the presence of 10 mM Ca^{+2}, a dramatic increase was seen in the amount of specific cell surface binding (note the time zero value) as well as the amount of total cell-associated ^{125}I-HA. No significant difference was seen for 30 min, indicating an initial lag in the apparent Ca^{+2}-stimulated uptake of HA. After 30 min, a linear increase in the amount of cell-associated radioactivity occurred and did not plateau by 120 min. The apparent rate of uptake was 3.5×10^4 ^{125}I-HA molecules/cell/min, which is about twice the maximum rate of specific HA endocytosis this receptor can mediate at saturation in the presence of 1.8 mM Ca^{+2} [19]. In this experiment, the HA concentration was well below that needed for saturation. If the stimulated HA uptake at 10 mM Ca^{+2} was really due to internalization via a coated pit mechanism, then hyperosmolar conditions should inhibit uptake by ~90%. When LECs were incubated in the presence of 10 mM Ca^{+2} and 0.4 M sucrose, however, there was very little decrease in the amount of cell-associated ^{125}I-HA (Fig. 3). This suggests that, if in fact the ^{125}I-HA is being internalized, a coated pit mechanism is not involved.

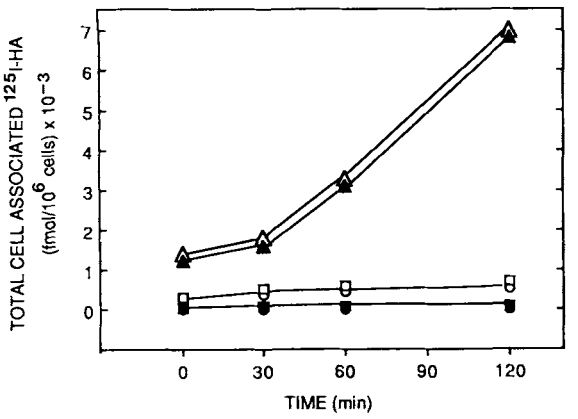

Fig. 3. Effect of hyperosmolarity on the endocytosis of ^{125}I-HA by LECs in the presence of 10 mM Ca^{+2}. Cultured LECs were incubated at 37°C with 2.0 μg ^{125}I-HA/ml in Medium 1/BSA containing either 10 mM EGTA (■,□), 1.8 mM $CaCl_2$ (●,○), or 10 mM $CaCl_2$ (▲,△), with (▲,■,●) or without (△,□,○) 0.4 M sucrose. At the indicated times, the cells were washed and radioactivity was determined as described in Methods. The zero time point represents specific cell surface ^{125}I-HA binding at 4°C.

Ca^{+2}-Stimulated Bound ^{125}I-HA Is Not Internalized

The increase in cell-associated ^{125}I-HA over time at 37°C in the presence of 10 mM Ca^{+2} may represent either internalization via a non-coated pit pathway or the extracellular accumulation of radiolabel without internalization. If the ^{125}I-HA accumulates extracellularly in the presence of 10 mM Ca^{+2}, then washing with an EGTA-containing buffer should remove the majority of this radiolabel. If, however, the ^{125}I-HA was actually internalized, then the EGTA wash should not significantly decrease the amount of cell-associated ^{125}I-HA. When LECs were incubated at 37°C with ^{125}I-HA in the presence of 10 mM Ca^{+2} and then washed at 4°C in an EGTA-containing buffer, there was a 74% reduction in the amount of cell-associated ^{125}I-HA (Table I). The amount of ^{125}I-HA resistant to the EGTA wash was equal to the amount of ^{125}I-HA endocytosis mediated by the HA receptor (i.e., the amount of ^{125}I-HA uptake, when the incubation and washes were both performed in the presence of EGTA). The rate of endocytosis calculated after removing the extracellular ^{125}I-HA with EGTA was identical to the rate calculated for endocytosis under physiological conditions of Ca^{+2}. We conclude from the above experiments that at 37°C the increased ^{125}I-HA bound in the presence of 10 mM Ca^{+2} is not internalized by LECs but rather accumulates extracellularly.

TABLE I. ^{125}I-HA Accumulation by LECs in the Presence of 10 mM Ca^{+2} Is Extracellular, Not Internal*

Incubation condition	^{125}I-HA Bound (fmol/10^6 cells)		
	Cell surface (4°C)	Cell-associated (37°C) wash buffer	
		EGTA	Ca^{+2}
10 mM EGTA	11 ± 9	287 ± 2	336 ± 36
10 mM $CaCl_2$	62 ± 32	361 ± 27	1,452 ± 64

*Cultured LECs were incubated at 37°C or 4°C for 2 h with 4 μg ^{125}I-HA/ml in Medium 1/BSA with either 10 mM EGTA or 10 mM $CaCl_2$. At the end of the incubation, the samples incubated at 4°C were washed in the same media. The samples at 37°C were chilled to 4°C and divided into two sets. One set was washed with ice-cold Medium 1/BSA containing 10 mM EGTA and the other set was washed with Medium 1/BSA containing 10 mM $CaCl_2$.

Ca^{+2}-Dependent HA Binding Sites Can Only Be Detected on the Cell Surface

To confirm the above conclusion, we compared the amount of specific ^{125}I-HA binding in intact or permeable LECs in the presence of 10 mM EGTA or 10 mM Ca^{+2} (Fig. 4). Binding of HA in the presence of EGTA increased ~743% after permeabilizing the cells with digitonin, demonstrating that digitonin exposes additional intracellular HA receptors as reported previously [18,19]. However, in the presence of 10 mM Ca^{+2}, permeabilization with digitonin increased HA binding by only ~46%. This relatively minor increase in the Ca^{+2}-dependent HA binding detected after digitonin permeabilization could be due to the presence of the Ca^{+2}-independent HA receptors, rather than intracellular Ca^{+2}-dependent sites that are exposed after permeabilization. According to this hypothesis, if the total cellular ^{125}I-HA binding to the Ca^{+2}-independent sites is subtracted from the HA binding in the presence of 10 mM Ca^{+2}, then the cell surface and total cellular Ca^{+2}-dependent HA binding should be the same. In fact, this is the result obtained (see open bars in Fig. 4). The value for the calculated total cellular Ca^{+2}-dependent HA binding activity was 199.4 fmol/10^6 cells and the value obtained for the cell surface only was 196.5 fmol/10^6 cells. These data suggest that the Ca^{+2}-dependent HA binding sites are expressed predominately, if not exclusively, on the surface of the cells and not intracellularly.

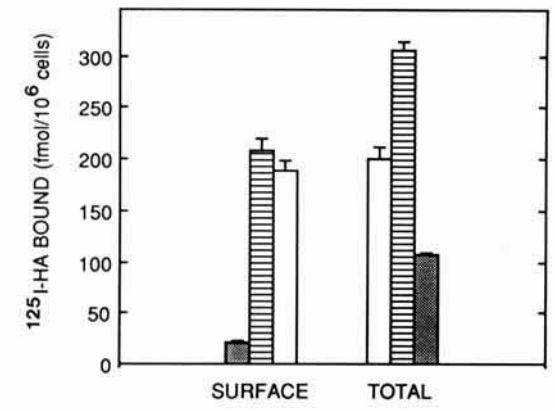

Fig. 4. The Ca^{+2}-dependent ^{125}I-HA binding sites are located on the LEC surface. Specific ^{125}I-HA binding to cultured LECs was determined at 4°C in either 10 mM EGTA (solid gray bars) or 10 mM $CaCl_2$ (striped bars) and, as indicated on the abscissa, in the presence or absence of 0.055% digitonin to assess, respectively, total (surface plus intracellular) cellular or only surface HA binding sites. The open bars show the calculated differences between the amount of ^{125}I-HA bound in the presence of Ca^{+2} minus the amount bound in the absence of Ca^{+2} (i.e., plus EGTA). The open bars are shown next to one another to emphasize that they are essentially identical. The calculated Ca^{+2}-dependent increases in HA binding were 196.5 and 199.4 fmol/10^6 cells for the surface and total, respectively.

DISCUSSION

To our knowledge, all the published studies by us and others on the HA receptor in LECs have been performed in the presence of physiological concentrations of Ca^{+2}. Inclusion of Ca^{+2} in these previous studies was very reasonable, since the LEC HA receptor functions in an environment with ~1.8 mM Ca^{+2}. While characterizing the ^{125}I-HA binding activity in detergent soluble extracts from LEC membranes, we noted that although Ca^{+2} was not required for the specific binding of ^{125}I-HA, increasing Ca^{+2} concentrations stimulated specific HA binding to these extracts in a dot blot assay [22]. This Ca^{+2}-stimulated ^{125}I-HA binding is not an artifact induced by the detergent solubilization, since the effect of Ca^{+2} on the binding of ^{125}I-HA is also seen with cultured LECs. Since a Ca^{+2}-dependent ^{125}I-HA binding activity is associated with cultured LECs, it was, therefore, critical to determine if this Ca^{+2}-stimulated activity was mediated through the endocytic HA receptor of interest or another unrelated molecule. We conclude that the LEC Class II endocytic HA receptor is Ca^{+2}-independent and does not mediate the Ca^{+2}-dependent HA binding activity for the following reasons: 1) Ca^{+2} is not required by the LEC receptor for the efficient binding or subsequent endocytosis of HA. Studies performed on intact and permeabilized cells in the presence of EGTA demonstrate that binding to the bona fide HA receptor is not dependent on any divalent cations. Likewise, endocytosis of HA occurs in the absence of any extracellular divalent cations. 2) The Ca^{+2}-dependent binding of ^{125}I-HA to LECs can be detected only on the external surface of the cells and not intracellularly. 3) The increased ^{125}I-HA bound in the presence of 10 mM Ca^{+2} at 37°C is not internalized after binding to the cell surface but only accumulates excellularly. 4) In studies using detergent extracts of LEC membranes, we find that the Ca^{+2}-dependent and independent HA binding activities have different biochemical properties [22].

Several studies have examined the structure of HA under varying conditions of ionic strength, pH, temperature, solvents, and divalent cations.

Equilibrium dialysis experiments have shown HA to have a high capacity to bind Ca^{+2} (one Ca^{+2} ion/2 disaccarides) with a low affinity for the cation; K_a = 710 M^{-1} [30]. The presence of Ca^{+2} can have a dramatic effect on HA structure [31–33]. Light scattering data suggest that HA molecules become more compact in the presence of 5 mM Ca^{+2} [31]. Although Ca^{+2} causes the HA molecules to assume a more compact configuration, the molecules are non-aggregating [31]. Therefore, the increase in binding we observe is probably not due to an increase in HA self-association. At concentrations >5 mM, the free energy of HA increases, presumably due to a more unstable secondary structure(s) that may make the HA molecule more flexible. This explains why Ca^{+2} can transform a highly viscous HA solution into one of low viscosity [31]. It is interesting to note that 5 mM Ca^{+2} is a concentration at which we see a clearly significant stimulation of ^{125}I-HA binding to LECs.

The Ca^{+2}-stimulated binding of HA to LECs could be due to an effect of Ca^{+2} either on the HA or the LECs or both. For example, it is possible that the Ca^{+2} effect is due to the more compact structure of HA induced by Ca^{+2}. The compact HA molecules would take up less space and reduce the steric hindrance between molecules. Therefore, more HA molecules would have access to the cell surface HA receptors and an increase in binding could result. Laurent et al. [20] noted that the smaller the size of the HA polymer, the greater the number of binding sites. However, this probably does not explain the Ca^{+2}-stimulated phenomenon we observe. For the following reasons, the Ca^{+2} effect is more likely to be on the LECs than on the HA. First, the Ca^{+2}-stimulation of binding is detected only on the cell surface. If the increased binding was due to smaller more compact HA molecules having increased accessibility to receptors, we should have also detected a profound increase in HA binding to intracellular receptors in the presence of digitonin. This was not the case. Second, the increase in HA binding in the presence of 10 mM Ca^{+2} varied from three- to thirty-fold. Yet when ^{125}I-HA binding to the Ca^{+2}-independent HA receptors was measured in the same experiments, more consistent results were obtained. One would not expect to see such variability in HA binding activity if the increase was due purely to the conformation of the HA. Third, preliminary studies indicate that the extent of Ca^{+2}-stimulated HA binding is related to the length of time the LECs are in culture and on the cell density. In addition, there is a 30 min lag before one sees the Ca^{+2} -stimulation of ^{125}I-HA uptake by LECs (Fig. 3). This lag period was not eliminated by incubating the HA at room temperature for 30 min in the presence of 10 mM $CaCl_2$ before exposure to LECs. These results suggest that the effect of supraphysiological Ca^{+2} on HA binding is on the LECs rather than HA.

Since the Ca^{+2}-dependent HA binding to LECs is not to the HA receptor, what molecule(s) is responsible? Extracellular HA binding proteins have been isolated from sera [34,35], the liver [36], the brain [37], and the dermis [38]. It is possible that the extracellular matrix also normally contains Ca^{+2}-dependent HA binding proteins secreted by resident cells. The function of these molecules may be to organize the HA or other GAGs in the matrix in response to a Ca^{+2} signal. Gabriel and Carr [31] postulated that local changes in pH and ion concentrations induced by cells can cause local structural changes in an HA-rich extracellular matrix. In some tissues, it is possible that the local concentration of Ca^{+2} may be high enough in vivo to achieve these stimulatory effects on HA binding. For example, the GAGs and proteoglycans in cartilage, which interact with HA and play a role in its organization [1,39,40], are known to sequester Ca^{+2} [1]. This interaction is of very low affinity and, therefore, the local concentration of free Ca^{+2} might remain very high. Ca^{+2}-induced conformational changes in HA could change the density of the matrix and affect the ability of cells to migrate through the matrix. The extracellular HA binding molecule detected on LECs may also be involved in cell adhesion and/or migration. For example, the integrins [41] and CAMS (cell adhesion molecules [42]) are families of cell surface proteins that are involved with cell-cell adhesion and cell-matrix interactions. The interaction between some members of these families of proteins and their respective ligands is Ca^{+2}-dependent.

While there is extensive literature on the effects of Ca^{+2} on HA conformation, there are few reports that address the effects of Ca^{+2} on the interactions between HA and proteins. In one case, HA binding to C1 complement protein is actually inhibited by Ca^{+2} as well as Mg^{+2} [43]. Another Ca^{+2}-dependent HA binding activity has been identified in permeabilized hepatocytes [44]. HA binding to this molecule also increases in a linear fashion with increasing Ca^{+2} concentra-

tion. The authors concluded that this Ca^{+2}-stimulated HA binding activity was different from the Ca^{+2}-independent intracellular HA binding protein. This Ca^{+2}-dependent HA binding activity is associated with crude membranes from isolated hepatocytes [45] but the precise cellular localization is unknown. Further study is needed to determine the relationship between the LEC and the hepatocyte Ca^{+2}-dependent HA binding activities and if Ca^{+2}-dependent HA binding activities are also present in other cell types.

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