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Specific intracellular hyaluronic acid binding to isolated rat hepatocytes is membrane-associated

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Intact isolated rat hepatocytes show a small amount of specific ^{125}I-labeled hyaluronic acid (HA) binding. However, in the presence of digitonin, a very large increase in the specific binding of ^{125}I-HA is observed. Chondroitin sulfate, heparin and dextran sulfate were as effective as unlabeled HA in competing for ^{125}I-HA binding to permeabilized hepatocytes, indicating that the binding sites may have a general specificity for glycosaminoglycans. After rat hepatocytes had been homogenized in a hypotonic buffer, more than 98% of the ^{125}I-HA binding activity could be pelleted by centrifugation at 100000 × *g* for 1 h. Mild alkaline treatment of hepatocyte membranes did not release ^{125}I-HA binding activity, suggesting that the HA binding site is an integral membrane molecule. Furthermore, trypsin treatment of deoxycholate-extracted membranes destroyed the binding activity, as assessed by a dot-blot assay. This suggests that a protein component in the membrane is necessary for ^{125}I-HA binding activity. Rat fibrinogen could be a possible candidate for the HA binding activity because HA binds specifically to human fibrinogen (LeBoeuf et al. (1986) J. Biol. Chem. 261, 12 586). Also, fibrinogen can be found in a quasi-crystalline form in rat hepatocytes and could be pelleted with the membranes. Rat fibrinogen was not responsible for the ^{125}I-HA binding activity, since (1) purified rat fibrinogen did not bind to ^{125}I-HA, and (2) immunoprecipitation of rat fibrinogen from hepatocyte extracts did not decrease the ^{125}I-HA binding of these extracts. We conclude that the internal HA binding sites are membrane- or cytoskeleton-associated proteins and are neither cytosolic proteins nor fibrinogen.

Introduction

Hyaluronic acid (HA), a non-sulfated glycosaminoglycan, has been used in eye surgery, tendon repair and in the treatment of arthritic joints [1–3]. In these and other clinical procedures, large amounts of HA are injected into the body. Fraser, Laurent and co-workers [4,5] have shown that in mammals, liver is the major site of clearance of HA from the blood. These investigators also showed that liver endothelial cells are responsible for the uptake and degradation of circulating HA [6,7]. Our laboratory has also studied HA binding to and metabolism by different cell types in the liver and has confirmed and extended these observations in cultured endothelial cells [8,9]. However, we [8,10] and others [11] have observed that hepatocytes, the parenchymal cells of the liver, are

Abbreviations: HA, hyaluronic acid; BSA, bovine serum albumin; DOC, deoxycholate; EGTA, ethyleneglycolbis(betaaminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid; TBS, Tris-buffered saline; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

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also able to specifically bind HA. Surprisingly, when isolated rat hepatocytes were permeabilized with digitonin to expose intracellular binding sites, there was a remarkably large number of HA binding sites per cell. There are approx. $1.3 \cdot 10^6$ sites per cell based on equilibrium binding studies performed with ^{125}I-HA of $M_r \approx 30000$ [10]. These binding sites are specific for other glycosaminoglycans.

Since there are a large number of HA binding sites per hepatocyte, we believe that they must have an important cellular function. To further characterize the intracellular HA binding site(s) in hepatocytes, we wanted to determine their carbohydrate specificity and whether they are associated with any cellular membranes. If the HA binding sites are membrane-bound, then one can localize the binding site to specific organelle(s). This information would then aid in purifying the molecule and in determining its function.

We recently showed that human fibrinogen can bind specifically to HA [12]. Fibrinogen is a possible candidate for the HA binding activity in hepatocytes, since these cells are the major site of its production. Fibrinogen can also be present in large amounts and is probably too large at 370 kDa to be released from hepatocytes by digitonin treatment [13]. Furthermore, at least a subpopulation of hepatocytes contains such a high concentration of fibrinogen that the protein is stored in dense inclusions within the cell [14]. The goal of the present study was to determine whether the specific ^{125}I-HA binding activity of rat hepatocytes is due to fibrinogen, other cytosolic proteins, or a membrane-associated molecule.

Materials and Methods

Materials. Digitonin was from Kodak Chemical Co., Rochester, NY. Protein A was from Genzyme, Boston, MA. Bovine serum albumin (BSA), collagenase (type I), Percoll, HA from human umbilical cord, rat fibrinogen, calf thymus DNA, chondroitin sulfate (mixed isomers), *N,N'*-diacetylchitobiose, glucuronic acid, *N*-acetylglucosamine, polygalacturonic acid, dextran sulfate and Nonidet P-40 were obtained from Sigma Chemical Co., St. Louis, Mo. Goat anti-rat fibrinogen IgG and human fibrinogen were from US Biochemical Corp., Cleveland, OH. Nonimmune goat IgG was prepared as previously described [15]. Deoxycholate (DOC) and inulin were obtained from Matheson, Coleman and Bell Inc., Cincinnati, OH. Nitrocellulose and dot-blot manifold Model No. SRC-76/0 were from Schleicher and Schuell, Keene, NH. Protein A was coupled to CNBr-activated Sepharose 4B from Pharmacia as described by the manufacturer. Dextran was also obtained from Pharmacia Chemicals. Heparin (highly sulfated) was obtained from V labs Inc., Covington, LA. Desulfated chondroitin sulfate was prepared according to the procedure of Nagasawa and Inoue [16]. The unique alkylamine derivative of HA and its Bolton-Hunter adduct were prepared and iodinated as described [17] with the following modifications. The starting HA had $M_r \approx 60000$ and a 2-fold molar excess of sodium periodate to reduced ends was used during the synthesis. To remove unreacted diaminohexane from the HA-amine, the reaction mixture was adjusted to pH 2.5 with acetic acid and ethanol was added to precipitate HA-amine [17]. The pellet was dissolved in distilled water, the pH was adjusted to 11 with NaOH and then the HA-amine was precipitated with ethanol again. The procedure of an acidic ethanol precipitation followed by a basic ethanol precipitation was repeated two or three times until the HA amine was purified from the free amine

Media and buffers. Medium 1/BSA is a modified Eagle's medium supplemented with 2.4 g/l of Hepes, 0.22 g/l $NaHCO_3$, and 0.1% (w/v) BSA (pH 7.4). Buffer 1 contains 143 mM NaCl/6.8 mM KCl/10 mM Hepes (pH 7.4). TBS contains 154 mM NaCl/10 mM Tris-HCl (pH 7.4). Buffer A contains 0.2% Nonidet P-40 in TBS. Extraction buffer contains 0.5% DOC in 20 mM Tris-HCl (pH 8.0) with 133 μM PMSF.

Hepatocyte preparation. Rat hepatocytes were prepared from male Sprague-Dawley rats (Harlan, Houston, TX) by the collagenase perfusion procedure of Seglen [18] with minor modifications [19]. Before use, the cells were first incubated at 37°C for 1 h in medium 1/BSA to allow recovery from the isolation procedure. After the incubation, the cells were chilled, washed, and then loaded onto a discontinuous Percoll gradient to separate nonparenchymal cells and dead hepatocytes from via-

ble hepatocytes [20]. The viable hepatocytes are denser and pellet to the bottom of the gradient. These cells (98% viability, 99% purity) were washed with buffer 1 and stored for up to 1 h on ice before use.

Immunoprecipitation. The protein A-Sepharose was washed by centrifugation three times with 20 mM sodium phosphate (pH 7.4) in a Beckman Model B microfuge and incubated for 1 h with 5% BSA in the same buffer. If cell extracts were used, then extract protein (3 mg/ml) replaced the BSA as a blocking agent. The protein A-Sepharose was washed three times and resuspended with the same buffer. Different concentrations of anti-rat fibrinogen or non-immune goat antibodies were added to 150 μl of a 20% (v/v) suspension of protein A-Sepharose, and incubated for 1 h at 4°C. The Sepharose was then pelleted, washed twice and incubated with cell extract as described in Fig. 2.

Dot-blot assay. The assay is based on the 'Western' blot procedure of Burnette [21]. The dot-blot assay was developed to allow for easy separation of ^{125}I-HA bound to proteins from the free ^{125}I-HA by first adsorbing the soluble proteins to nitrocellulose. Nitrocellulose and Whatman 3 MM chromatography paper were soaked in TBS and placed in a dot-blot manifold with the nitrocellulose above the chromatography paper, and this was subjected to vacuum. Variable amounts of protein were adsorbed to the nitrocellulose by adding the protein solution to the individual wells. No more than 40 μg of protein was added to any one spot of nitrocellulose. Then 500 μl of TBS was added to each well. The TBS was allowed to filter through the nitrocellulose and the vacuum was turned off. The nitrocellulose was removed from the manifold, incubated in buffer A with 5% BSA for 1 h, and then incubated with 6 μg/ml of ^{125}I-HA in buffer A plus 5% BSA in a sealed plastic bag. An identically treated piece of nitrocellulose was incubated with a 150-fold excess of nonradiolabelled HA. After a 2 h incubation, the plastic bags were opened and the nitrocellulose was transferred to a 7 × 22 cm plastic tray, rinsed quickly with TBS followed by two 10 min washes by agitation with buffer A, then quickly rinsed with TBS and air-dried. The blotted areas were cut out with a cork borer, placed into gamma tubes and ^{125}I radioactivity was determined. The specific binding was determined by subtracting the radioactivity associated with the nonspecific binding sample from the total binding.

General. Protein was measured using the method of Bradford [22] with BSA as a standard. ^{125}I radioactivity was determined using a Packard Multiprias 2 gamma spectrometer.

Results and Discussion

Specific ^{125}I-HA binding to intact and digitonin-treated hepatocytes

Isolated rat hepatocytes were found to have at least 9000 specific surface ^{125}I-HA binding sites/cell (Table I), as others have also reported [11]. These binding sites could represent a cell surface component involved in anchoring cells in the tissue to HA in the extracellular matrix. Unfor-

TABLE I

SPECIFIC ^{125}I-HA BINDING TO INTACT AND DIGITONIN-PERMEABILIZED HEPATOCYTES

Isolated rat hepatocytes at $4 \cdot 10^6$ cells/ml in medium 1/BSA with 133 μM phenylmethylsulfonyl fluoride were treated with or without 0.055% digitonin [13] for 20 min at 4°C, washed three times by centrifugation, and resuspended at 10^7 cells/ml. Surface ^{125}I-HA binding was determined on intact hepatocytes while the total cellular ^{125}I-HA binding activity (surface and intracellular) was determined in the digitonin-treated cells. Intact and digitonin-treated cells (10^6) were incubated at 4°C for 1 h in medium 1/BSA (0.2 ml) with 6 μg/ml ^{125}I-HA in the presence or absence of 600 μg/ml of nonradioactive HA in medium 1/BSA. The intact cells were centrifuged for 5 min at 300 × *g* over 1 ml of 40% Percoll in PBS and the cell pellets were washed once with medium 1/BSA. The digitonin-treated cells were washed three times by centrifugation. Radioactivity and specific binding were determined as described in Materials and Methods. Cell number was determined by DNA content [23] using a measured value of 18 μg DNA/10^6 hepatocytes. Numbers in parentheses indicate the percent of the total cellular ^{125}I-HA binding found on the cell surface. The nonspecific binding of ^{125}I-HA to permeabilized hepatocytes was 35–55% of the total binding.

Expt.	^{125}I-HA binding (fmol ^{125}I-HA bound/10^6 cells)	
	surface only	surface and intracellular
1	4.6 (3%)	154
2	30.4 (16%)	190
3	10.7 (8.4%)	128
4	11.7 (6.8%)	171

tunately, equilibrium binding studies performed on intact cells did not consistently demonstrate a saturation binding curve for HA. A high level of nonspecific binding also added variability to the data. Therefore, we could not estimate the total number of binding sites by Scatchard analysis.

An unexpected finding, however, was that in the presence of the permeabilizing detergent digitonin, a larger number of specific ^{125}I-HA binding sites were detected inside these cells (Table I). About 92% of the total cellular sites are internal at a subsaturating concentration of ^{125}I-HA. Equilibrium binding studies were successfully performed on permeabilized hepatocytes using ^{125}I-HA of $M_r \approx 30000$ [10]. The K_d was approx. $3 \cdot 10^{-7}$ M and the average number of binding sites per cell was $1.3 \cdot 10^6$. The larger ^{125}I-HA used in the present experiments ($M_r \approx 60000$) had a K_d of approx. $1 \cdot 10^{-7}$ M and the number of binding sites was about 400000/cell (not shown). If the same ratio of surface to total HA binding is observed at saturation, and if the K_d of the surface and intracellular binding sites are the same, then the maximum number of surface HA ($M_r \approx 60000$) binding sites per cell would be approx. 32000.

In many receptor systems, especially those involved in ligand clearance and degradation, the majority of cellular receptors are internal [24]. These receptors can mediate endocytosis of their respective ligands and in most cases undergo receptor recycling. However, in other studies, we have found that hepatocytes do not endocytose ^{125}I-HA in the medium [8,10]. Therefore, these intracellular HA binding sites cannot be involved in receptor recycling and their function is unknown.

Specificity of ^{125}I-HA binding to digitonin-treated hepatocytes

Rat hepatocytes in suspension were incubated with ^{125}I-HA in the presence of digitonin and either in the absence or presence of unlabeled HA or other potential saccharide competitors (Fig. 1). Chondroitin sulfate (52% inhibition), heparin (62% inhibition) and dextran sulfate (50% inhibition) were able to compete as effectively as nonradioactive HA (52% inhibition) for binding of ^{125}I-HA. This result suggests that the intracelluiar HA binding sites in hepatocytes, like the HA receptor on

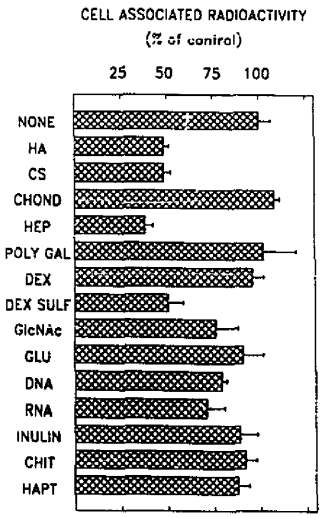

Fig. 1. Specificity of ^{125}I-HA binding to permeabilized hepatocytes. Purified hepatocytes ($2 \cdot 10^6$ cells/ml) were incubated in suspension with 10^{-7} M ^{125}I-HA in the presence of 0.055% digitonin for 1 h at 4°C and in the presence or absence of 600 μg/ml of the indicated saccharides. The cells were then washed by centrifugation and bound ^{125}I-HA was determined as described in Materials and Methods. The amount of cell-associated radioactivity is expressed as a percentage of the control value (no competitor present). Each bar represents the mean and the sample S.D. of two different experiments done in triplicate ($n = 6$) with the exception of DNA, RNA, polygalacturonic acid, glucuronic acid, and *N,N'*-diacetylchitobiose, which represent one experiment ($n = 3$). The abbreviations are as follows: CS, chondroitin sulfate; CHOND, chondroitin (desulfated); HEP, heparin; POLY GAL, polygalacturonic acid; DEX, dextran; DEX SULF, dextran sulfate; GlcNAc, *N*-acetylglucosamine; CHIT, *N,N'*-diacetylchitobiose; HAPT, haptoglobin.

endothelial cells [7,9,10], may have a general specificity for several glycosaminoglycans. Among the other competitors tested, dextran, inulin, *N*-acetylglucosamine, *N,N'*-diacetylchitobiose, glucuronic acid, polygalacturonic acid, chondroitin and haptoglobin were either ineffective or very weak competitors for ^{125}I-HA binding to permeabilized hepatocytes. Strongly anionic polymers like DNA (21% inhibition) and RNA (29% inhibition) were weak competitors for ^{125}I-HA binding to permeabilized cells. These data suggest that charge is essential for HA recognition by the binding sites in hepatocytes. Nonetheless, the fact that desulfated chondroitin, which is still nega-

TABLE II

SPECIFIC ^{125}I-HA BINDING TO CRUDE SUBCELLULAR FRACTIONS OF ISOLATED RAT HEPATOCYTES

Hepatocytes were incubated for 45 min at 4°C in 15 mM Tris-HCl (pH 7.4)/1 mM EGTA/1 mM EDTA/0.1 mM PMSF. The cells were then homogenized using a Tekmar tissuemizer (Model SDT-1810) at setting 50 for 30 s. 99% of the cells were disrupted and more than 80% of the nuclei remained intact. All centrifugations were at 4°C. The homogenate was successively centrifuged at $800\times g$ for 5 min, $10000\times g$ for 10 min and in two experiments, $100000\times g$ for 1 h. The $100000\times g$ pellet was not analyzed, since higher centrifugal force made it difficult to resuspend the membrane pellets and perform the direct, solid phase binding studies. Instead, the supernatants from the $10000\times g$ and $100000\times g$ centrifugations were analyzed for ^{125}I-HA binding using the dot-blot assay as described in Materials and Methods. Membrane fractions from the other pellets were resuspended and incubated with 6 μg/ml of ^{125}I-HA with and without 600 μg/ml of nonradioactive HA for 1 h at 4°C in medium 1/BSA. The membrane fractions were washed three times by centrifugation at $10000\times g$ for 10 min and radioactivity was determined. Numbers in parentheses indicate the percent of the total binding in that fraction.

Expt.	^{125}I-HA binding (fmol ^{125}I-HA bound/10^6 cells)			
	$800\times g$ pellet	$10000\times g$ pellet	$10000\times g$ supernatant	$100000\times g$ supernatant
1	49.6 (25%)	141 (72%)	5.6 (3%)	–
2	71.4 (48%)	58 (39%)	19.8 (13%)	–
3	83.3 (28%)	126 (43%)	83.9 (29%)	< 4 (< 2%)
4	165.0 (48%)	158 (46%)	21.0 (6%)	< 4 (< 2%)

tively charged, does not compete for the binding of ^{125}I-HA supports the idea that both charge and secondary structure are very important for ^{125}I-HA binding to hepatocytes.

Specific ^{125}I-HA binding to crude subcellular fractions of isolated rat hepatocytes

Since the treatment of hepatocytes with 0.055% digitonin may not release very large cytoplasmic proteins (e.g., $M_r > 200000$; [13]), the HA binding sites may either be membrane-bound or a cytosolic protein(s). To address this question, viable isolated rat hepatocytes were homogenized in a hypotonic buffer and the distribution of the ^{125}I-HA binding activity was determined between the cell membranes versus the supernatant (Table II). Each crude membrane fraction showed ^{125}I-HA binding activity. In three experiments, the average ^{125}I-HA binding in membrane fractions pelleting at $\leqslant 10000\times g$ for 10 min was greater than 86% of the total HA binding activity recovered. The remaining ^{125}I-HA binding activity in the supernatant (14%) could be entirely pelleted by centrifugation at $100000\times g$ for 60 min, at 4°C. Thus, the HA binding activity is associated with membranes and insoluble cytoplasmic protein(s).

Carbonate treatment of rat hepatocyte membranes

Since the HA binding activity is membrane-associated, we wanted to determine whether the binding site is part of an integral membrane molecule. One criterion for an integral membrane molecule is that it is retained in membranes after mild alkaline treatment [25]. Extrinsic, peripheral proteins are removed by such treatment. Table III shows the effect of sodium carbonate treatment on total cellular membranes after the removal of nuclei and unbroken cells. The treated membranes

TABLE III

EFFECT OF SODIUM CARBONATE TREATMENT ON ^{125}I-HA BINDING ACTIVITY OF MEMBRANES

Cells were homogenized as in Table II, except 5 mM $MgCl_2$ was included in the homogenization buffer at 4°C, and all the following procedures were done at 4°C. After homogenization, the cells were diluted 1:1 with homogenization buffer plus 0.5 M sucrose. The homogenates were centrifuged for 10 min at $800\times g$ to remove nuclei and the postnuclear supernatants were adjusted to 0.1 M sodium carbonate (pH 11.0). The control received the same volume of PBS. The membrane suspensions were incubated for 30 min, and then centrifuged for 1 h at $100000\times g$. The pelleted membranes were solubilized for 1 h ($(5–7)\cdot 10^6$ cell equivalents/ml) in 20 mM Tris with 0.5% DOC (pH 8.0) at 4°C, filtered through a 0.45 μm filter and assayed for protein. HA binding activity was assayed by the dot-blot procedure. Since mild alkaline treatment of membranes releases protein, the same number of cell equivalents were assayed for both the treated and nontreated membranes. The ^{125}I-HA specific binding was determined from the slope of the line derived from plotting ^{125}I-HA specific binding versus cell equivalents. Each line had at least 12 points for the least-squares regression analysis, and the correlation coefficients ranged from 0.760 to 0.900. n.d., not determined.

Expt.	Treatment	Specific ^{125}I-HA binding (fmol/10^6 cells)	Specific ^{125}I-HA binding activity (pmol/mg protein)
1	PBS	315	1.35
	carbonate	280	n.d.
2	PBS	104	0.75
	carbonate	186	3.62

TABLE IV

EFFECT OF TRYPSIN TREATMENT ON ^{125}I-HA BINDING ACTIVITY OF SOLUBILIZED MEMBRANES

Postnuclear supernatants were prepared as described in Table III and then pelleted at 100000×*g* for 1 h at 4°C. The pelleted membranes were extracted with 0.5% DOC in 20 mM Tris (pH 8.0) for 1 h at 4°C. The extract (450 μg protein in 0.8 ml) was treated with the indicated amount of trypsin or 300 μg/ml of soybean trypsin inhibitor for 30 min at 37°C in the presence of 2 mM $CaCl_2$. The trypsin digestion was stopped by the addition of 300 μg/ml of soybean trypsin inhibitor. The specific ^{125}I-HA binding to 30 μg of membrane extract protein was determined by the dot-blot assay as described in Materials and Methods. The average of triplicates and the sample S.D. is shown.

Trypsin (μg/ml)	Preincubated with trypsin inhibitor	Specific ^{125}I-HA binding (fmol/10^6 cells)	Binding activity remaining (% of control)
0	–	265±31.3	93
10	–	185± 2.4	65
30	–	48±11.2	17
30	+	284± 8.5	100

were then solubilized with DOC and ^{125}I-HA binding activity was determined using the dot-blot assay. The results from one experiment showed an increase in HA binding activity in membranes that had been treated with carbonate (pH 11.0). This is reasonable, since we have found that high pH removes prebound ^{125}I-HA from hepatocytes [10], and therefore treatment at high pH may expose binding sites previously occupied by HA. Since HA binding activity was not lost in either experiment, we conclude that the HA binding activity is tightly associated with membranes and is very likely an integral membrane molecule.

Trypsin treatment of DOC-extracted hepatocyte membranes

Next, we wanted to determine whether a protein component is necessary for the binding of ^{125}I-HA. DOC extracts of crude hepatocyte membranes, that first had nuclei removed, were then treated with trypsin and subsequently assessed for ^{125}I-HA binding activity using the dot-blot assay (Table IV). There was reduced HA binding activity in the trypsin-treated samples. The control, 30 μg/ml of trypsin pretreated with 300 μg/ml of soybean trypsin inhibitor, demonstrated that the loss of HA binding activity was due only to this proteinase and not to other contaminating enzymatic activities. This strongly suggests that the binding site is a protein or has a protein component necessary for ^{125}I-HA binding activity.

Western blots of carbonate-treated membranes

Since HA binding activity can be determined by immobilizing extracts on nitrocellulose, and carbonate treatment enriches the HA binding activity, a 'Western' blot [21] of extracts from carbonate-treated membranes should identify HA binding protein(s). Extracts of membranes treated with carbonate were electrophoresed on a 7.5% SDS polyacrylamide gel [26]. The proteins were transferred to nitrocellulose by electrophoresis and allowed to bind ^{125}I-HA as described in Materials and Methods. When the nitrocellulose was subsequently autoradiographed, unfortunately, no bands were seen. We believe that the binding activity is destroyed by the SDS treatment.

Specific ^{125}I-HA binding to purified rat and human fibrinogen

The recent finding that human fibrinogen specifically binds to HA ($M_r \approx 30000$) with a K_d of about $2 \cdot 10^{-7}$ M [12] raised the possibility that this protein could be the intracellular HA binding site. Furthermore, fibrinogen can be found in a quasi-crystalline form [14] in rat hepatocytes, and therefore could cocentrifuge with membranes from homogenized cells. Thus, rat fibrinogen could still be responsible for the observed ^{125}I-HA binding in permeabilized rat hepatocytes or crude membranes. Two approaches were taken to address this question. The first approach was to determine directly the ability of ^{125}I-HA to bind to purified rat fibrinogen using the dot-blot assay. Fig. 2 shows the results of a dot-blot assay with purified rat and human fibrinogens, and with DOC extracts of hepatocytes. The HA binding activity in permeabilized hepatocytes can be detergent-solubilized and subsequently detected by the dot-blot assay. Human fibrinogen and hepatocyte extracts demonstrate a linear increase in specifically bound ^{125}I-HA with increasing protein concentration. Rat fibrinogen, however, did not show an increase in ^{125}I-HA binding. Several different lots of rat

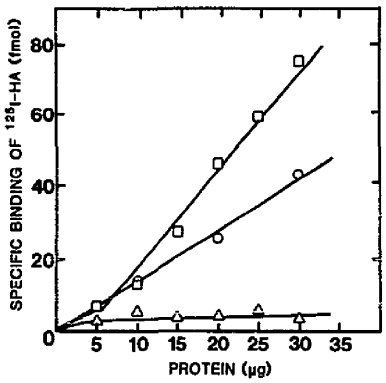

Fig. 2. Detection of ^{125}I-HA binding to soluble proteins. Increasing amounts of human fibrinogen (○), rat fibrinogen (△) or protein from whole cell extracts (□) were adsorbed to nitrocellulose and specific ^{125}I-HA binding was determined as described in Materials and Methods. Extracts were prepared by suspending cells in extraction buffer to $5 \cdot 10^6$ cells/ml. The sample was rotated at 4 °C for 30 min, centrifuged at 10000 × g for 30 min, and the supernatant was removed and used immediately or stored at −70 °C. Each point represents the average of triplicates.

fibrinogen were tested, and, although none showed HA binding activity, they could all be clotted by the addition of thrombin, indicating they did contain functional fibrinogen. This result suggests that, unlike the human protein, purified rat fibrinogen cannot bind HA. Therefore, rat fibrinogen is probably not responsible for the intracellular HA binding seen in the hepatocyte extracts.

Immunoprecipitation of rat fibrinogen from whole cell extracts

The second approach was to test whether rat fibrinogen is involved in the ^{125}I-HA binding activity of the cell extracts. This protein was first removed from hepatocyte extracts by immunoprecipitation. DOC extracts of rat hepatocytes were treated with anti-fibrinogen IgG bound to protein A-Sepharose to remove fibrinogen (Fig. 3). No significant decrease ($P > 0.9$) in ^{125}I-HA binding was observed in hepatocyte extracts treated with either immune (Fig. 3A) or nonimmune (Fig. 3B) antibodies compared to untreated extracts, when analyzed by Student's t-test.

Since there was no effect on ^{125}I-HA binding after immunoprecipitation with anti-rat fibrinogen IgG, controls were performed to verify that these antibodies were active and could immunoprecipitate rat fibrinogen under these conditions. First, a spectrophotometric clotting assay showed that the immune, but not the nonimmune, IgG interfered with thrombin-induced fibrin formation and polymerization. Second, fibrinogen immobilized on nitrocellulose paper was able to bind at least 10-times more ^{125}I-protein A after incubation with the immune IgG compared to the nonimmune IgG. Third, 10 μg of fibrinogen was immunoprecipitated by 100 μg of immune antibodies prebound to protein A-Sepharose with 0.1% DOC present. The highest anti-fibrinogen antibody concentration in Fig. 3A should have therefore immunoprecipitated all the fibrinogen in the extracts *. Since the antibody can bind to and immunoprecipitate rat fibrinogen, and since no loss of ^{125}I-HA binding was observed with the immunoprecipitation of fibrinogen from hepatocyte extracts, we conclude that rat fibrinogen is not responsible for the ^{125}I-HA binding activity of these extracts.

What are the intracellular HA binding sites in hepatocytes if they are not fibrinogen? One possibility in an HA binding protein shown to be present in acid extracts of homogenized whole rat liver [28]. It is not known, however, whether this protein is associated with hepatocytes, the extracellular matrix or another cell type in the liver. Also, the isolation procedure for this HA binding protein is the same procedure used to isolate hyaluronectin from brain [29]. Hyaluronectin is an HA binding glycoprotein localized in the extracellular matrix of many tissues, but is mainly present in adult brain [30]. Another possibility is that the intracellular HA binding activity is related to the presence of heparan sulfate, HA and other glycosaminoglycans in the nucleus and the cytoplasm [31–34]. The significance of finding extracellular matrix components and binding activity for these components inside cells or in nuclei is presently unknown. A third

* Assuming a basal rat of fibrinogen synthesis in hepatocytes of 20 pmol/10^6 cells per 24 h [27] and 1 mg total protein/10^6 cells, we calculate that only 0.6 μg of fibrinogen would be expected in each immunoprecipitated sample in Fig. 3.

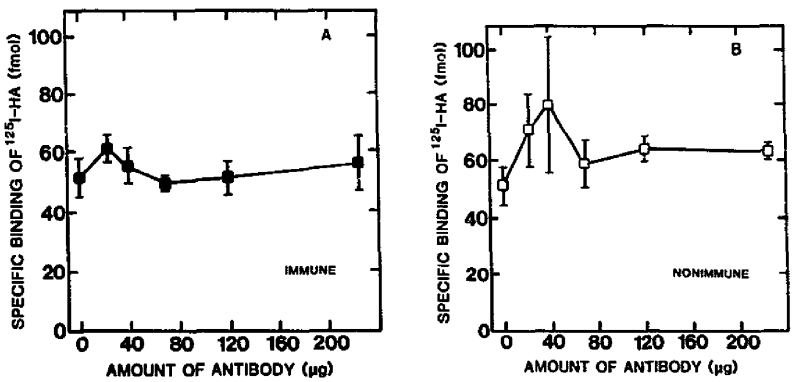

Fig. 3. ^{125}I-HA binding to immunoprecipitated whole cell extracts. DOC cell extracts (100 μl; 1.6 mg protein/ml) were incubated with the indicated amounts of immune (A) or nonimmune (B) antibodies that had been prebound to protein A-Sepharose as described in Materials and Methods. The suspensions were incubated for 2 h at 4°C and then centrifuged. The supernatant (15 μl) was adsorbed onto nitrocellulose and a dot-blot assay was performed in triplicate. Error bars indicate the sample S.D.

possibility is that the intracellular HA binding activity could be due to proteins and enzymes involved in the biosynthesis of HA and its export to the cell surface and the extracellular matrix. Further studies to characterize the intracellular HA binding activity are in progress.

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References

1 Gerke, E., Meyer-Schwickerath, G. and Wessing, A. (1985) Surgical Pharmacology of the Eye (Sears, M. and Turakkanen, A., eds.), pp. 411–415, Raven Press, New York.
2 St. Onge, R., Weiss, C., Denlinger, J.L. and Balazs, E.A. (1980) Clin. Orthopaed. 146, 269–275.
3 Weiss, C., Balazs, E.A., St. Onge, R. and Denlinger, J.L. (1981) Semin. Arthritis Rheum. 11, 143–144.
4 Fraser, J.R.E., Laurent, T.C., Pertoft, H. and Baxter, E. (1981) Biochem. J. 200, 415–424.
5 Fraser, J.R.E., Appelgren, L.E. and Laurent, T.C. (1983) Cell Tissue Res. 233, 285–293.
6 Eriksson, S., Fraser, J.R.E., Laurent, T.C., Pertoft, H. and Smedsrod, B. (1983) Exp. Cell Res. 144, 223–228.
7 Laurent, T.C., Fraser, J.R.E., Pertoft, H. and Smedsrod, B. (1986) Biochem. J. 234, 653–658.
8 Raja, R.H. and Weigel, P.H. (1985) J. Cell Biol. 101, 426, abstr.
9 Raja, R.H., McGary, C.T. and Weigel, P.H. (1988) J. Biol. Chem. 263, in press.
10 Raja, R.H. (1986) PhD Dissertation, The University of Texas Medical Branch, Galveston, TX.
11 Truppe, W., Basner, R., Von Figura, K. and Kresse, H. (1977) Biochem. Biophys. Res. Commun. 78, 713–719.
12 LeBoeuf, R.D., Raja, R.H., Fuller, G.M and Weigel, P.H. (1986) J. Biol. Chem. 261, 12, 586-12, 592.
13 Weigel, P.H., Ray, D.A. and Oka, J.A. (1983) Anal. Biochem. 133, 437–449.
14 Callea, F., DeVos, R., Togni, R., Tardanico, R., Vanstapel, M.J. and Desmet, V.J. (1986) Histopathol. 10, 65–73.
15 McAbee, D.D. and Weigel, P.H. (1987) J. Biol. Chem. 262, 1942–1945.
16 Nagasawa, K., Inoue, Y. and Tokuyasu, T. (1979) J. Biochem. 86, 1323–1329.
17 Raja, R.H., LeBoeuf, R.D., Stone, G.W. and Weigel, P.H. (1984) Anal. Biochem. 139, 168–177.
18 Seglen, P.O. (1973) Exp. Cell. Res. 82, 391–398.
19 Clarke, B.L., Oka, J.A. and Weigel, P.H. (1987) J. Biol. Chem. 262, 17384–17392.
20 Dalet, C., Fehlmann, M. and Debey, P. (1982) Anal. Biochem. 122, 119–123.
21 Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
22 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
23 Labarca, C. and Paigen, K. (1980) Anal. Biochem. 102, 344–352.
24 Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W. and Schneider, W.J. (1985) Annu. Rev. Cell Biol. 1, 1–39.
25 Fujiki, Y., Hubbard, A.L., Fowler, S. and Lazarow, P.B. (1982) J. Cell Biol. 93, 97–102.
26 Laemmli, U.K. (1970) Nature 227, 680–685.

27 Qureshi, G.D., Guzelian, P.S., Vennart, R.M. and Evans, H.J. (1985) Biochim. Biophys. Acta 844, 288–295.
28 D'Souza, M. and Datta, K. (1985) Biochem. Int. 10, 43–51.
29 Delpech, B. and Halavent, C. (1981) J. Neurochem. 36, 855–859.
30 Delpech, B. et al. (1986) Front. Matrix Biol. 11, 78–89.
31 Ishihara, M., Fedarko, N.S. and Conrad, H.E. (1986) J. Biol. Chem. 261, 13,575–13,580.
32 Stein, G.S. et al. (1975) Nature 258, 639–641.
33 Furukawa, K. and Terayama, H. (1979) Biochim. Biophys. Acta 585, 575–588.
34 Ripellino, J.A., Bailo, M., Margolis, R.U. and Margolis, R.K. (1988) J. Cell Biol. 106, 845–855.