Vol. 141, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 26, 1986 Pages 158-164

THE RELEVANCE OF GLYCOSAMINOGLYCAN SULFATES TO APO E INDUCED LIPID UPTAKE BY HEPATOCYTE MONOLAYERS

B Oswald, F Shelburne, B Landis, A Linker, and S Quarfordt

Department of Medicine, Duke University Medical Center, and Department of Biochemistry, University of Utah, Veterans Administration Medical Centers, Durham, North Carolina, and Salt Lake City, Utah

Received September 15, 1986

The binding of Apolipoprotein E supplemented triglyceride emulsions to sulfated glycosaminoglycans demonstrated specificity for the carbohydarate polymers. Glucosamine containing glycosaminoglycans with relatively less sulfate had little affinity for the Apo E emulsion whereas those with more sulfate (i.e. heparin and sulfated heparans) effectively bound the emulsion. Galactosamine containing glycosaminoglycans (chondroitin 4 sulfate and dermatan sulfate) demonstrated no binding. The Apo E induced uptake of triglyceride emulsions by hepatocytes was inhibited by highly sulfated polysaccharides (i.e. heparin, dextran sulfate) but other glycosaminoglycans which did not bind the emulsion were ineffective in this inhibition. The same sulfated compounds which inhibited the hepatocyte Apo E emulsion interaction effectively released hepatic lipase from isolated hepatic perfusions. Glycosaminoglycan sulfates which did not bind the Apo E supplemented emulsions and did not inhibit hepatocyte association were ineffective in releasing lipase. A heparan mixture isolated from human liver was much less effective in inhibiting Apo E induced association of emulsions with hepatocytes, than heparin. A highly sulfated octasaccharide fraction isolated from bovine liver heparin inhibited more effectively than the human heparans but less than the heparin. Inhibition of Apo E mediated hepatocyte emulsion association was produced by a one hour exposure of the cells to either heparinase or heparanase. The heparanase was more active than the heparinase and both were effective in the presence of protease inhibitors. Enzymes hydrolyzing chondroitin sulfates and hyaluronic acid were inneffective in inhibiting the Apo E induced association. The specific binding of human low density lipoprotein to the hepatocyte was much less effected by the heparanase exposure than the Apo E mediated binding.

The sinusoidal surface of hepatocytes contain an appreciable amount of heparan sulfate (1,2). Some of this surface heparan sulfate may play a role in the binding of hepatic lipase and have other functions in the hepatic metabolism of lipoproteins.

Apolipoprotein E binds to heparin and confers heparin binding on emulsions or lipoproteins with which it associates (3). The addition of apolipoprotein E

0006-291X/86 $1.50

to a triglyceride emulsion produces rapid hepatic uptake and metabolism of the triglyceride by perfused (4) and cultured (5) rat liver suggesting a function for glycosaminoglycans sulfates in this process. The inhibition of Apo E induced heparin binding of emulsions by Apo C proteins (3) and the similar inhibition of hepatic Apo E rich lipoprotein uptake by Apo C protein (4) also suggests a role for glycosaminoglycan sulfates.

MATERIALS AND METHODS

Human apolipoprotein E was prepared from the delipidated very low density lipoprotein of hypertriglyceridemic humans and rat apo E from delipidated rat lipoproteins by molecular sieve, ion exchange and heparin affinity chromatography (6,4). The apolipoprotein was evaluated for purity by both Sodium dodecyl sulphate polyacrylamide gel electrophoresis (7) and urea polyacrylamide gel electrophoresis (9). Human low density lipoprotein was isolated by standard ultracentrifugation (9) and chromatographied on a heparin sepharose column. The human low density lipoprotein was radioiodinated with [^{125}I] by a standard method (10).

The glycosaminoglycans chondroitin-4 sulfate, dermatan sulfate, heparin and hyaluronic acid were purchased from Sigma (St. Louis MO). Glycerol tri [1^{14}C] oleate was obtained from Amersham (Arlington, Illinois). Soy bean trypsin inhibitor was bought from Cooper Biomedical (Pa). Lung heparans with various sulfate contents and hepatic heparans were prepared as previously described (11). Alpha-2-macroglobulin was a gift of Dr. S. Pizzo, (Duke University Medical Center, Durham, N.C.) Heparinase and heparanase were purchased from Seikagahu Kogyo Co., (Japan). The various glycosaminoglycans were immobilized on Sepharose 4B by a previously described (4) variation of the method of Iverius (12). Glycosaminoglycan affinity interaction was determined as described from this laboratory (3) employing a glycerol [1^{14}C] triolein egg lecithin stabilized emulsion having a ratio of triolein/phospholipid cholesterol of 5/4/1 and prepared as previously described (4). The emulsions were supplemented with either human or rat Apo E in a ratio of 100 µg triglyceride to 7.5 µg apoprotein. Sulfate composition of the glycosaminoglycans were determined by the procedure of Antonoupolous (13).

Hepatocyte monolayer cultures were prepared from 250 gram Sprague-Dawley rats by a collagenase perfusion technique modified from Seglen (14). The hepatocytes were maintained in minimal essential media containing 10 mg/ml of bovine albumin for a 36 h interval prior to assaying for Apo E mediated lipid uptake and metabolism. The synthetic triglyceride emulsions used for the assay of hepatocyte uptake and metabolism were the same as those employed in the assay of glycosaminoglycan binding. Incubations of 25 µg of triglyceride containing 7 µg of Apo E were done with hepatocytes in the albumin minimum essential media at 37° for 1 hour. After the incubations the media was harvested, the cells washed twice with 2 ml of 7.5 mM phosphate buffered (pH 7.4), 150 mM NaCl (PBS), and the cells harvested by scraping with a plastic policeman. The cells were centrifuged at 3000 rpm for 5 minutes, re-washed with PBS twice, and then extracted by the method of Dole (15) assaying both organic and aqueous fractions. Additions of glycosaminoglycans to the incubation were made at the same time the emulsion was added. Exposure of the hepatocytes to enzymes degrading glycosaminoglycans was done by adding the various enzymes at concentrations from 3 mIU to 12 mIU of enzyme per ml. These were added to minimum essential medium containing 10 mg/ml of bovine albumin alone or in the presence of 50 µg of either the protease inhibitors α-2- macroglobulin or soybean trypsin inhibitor. After the one hour enzyme exposure, the media was removed and the cells were washed three times with phosphate buffered saline prior to assaying with the Apo E enriched

radiolabeled emulsion. Enzyme exposed hepatocytes were assayed for the appearance of Lactate dehydrogenase by a standard method (16).

Isolated liver perfusions were performed by an established method (17) employing apoprotein free bovine serum albumin (30 mg/ml) in Krebs ringer bicarbonate buffer as perfusate. The glycosaminoglycan (50 µg) was added to the perfusion and the perfusate (about 50 ml) recovered after 10 minutes and aliquots assayed for hepatic lipase by the method of Schotz (18).

RESULTS AND DISCUSSION

Chromatography of an Apo E supplemented glycerol [1^{14}C] triolein emulsion on Sepharose immobilized heparan with relatively low (9% by weight) sulfate content (eluting from Dowex at 0.9 M NaCl) revealed no affinity of the emulsion for the glycosaminoglycan (Fig. 1, Top Panel). Heparan containing relatively more (17%) sulfate (eluting from Dowex at 1.4 M NaCl) exhibited binding of the Apo E enriched emulsion comparable to that of a heparin column (Fig. 1, Top Panel). The Apo E supplemented triglyceride emulsion demonstrated no affinity for either chondroitin or dermatan sulfate (Fig. 1, Bottom Panel).

The addition of Apo E to a triolein emulsion resulted in a 10 fold enhancement in the recovery of the radiolabeled triolein in the hepatocytes (Table 1). Adding heparin to the incubations at a concentration of 10 µg/ml

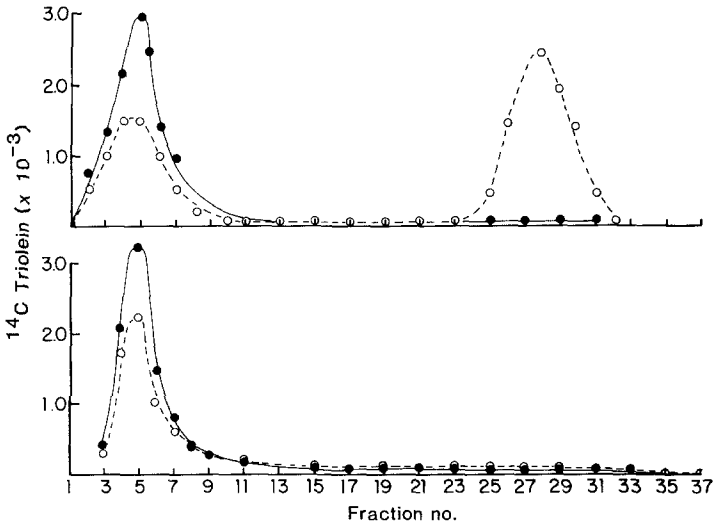

Figure 1. The binding of Apo E Supplemented Triolein emulsion for Affinity columns containing (Top panel ●——● 0.9 Heparan and O-------O 1.4 Heparan). Bottom panel O-------O Dermatan Sulfate ●——● Chondroitin-4Sulfate). The gradient was 0.05 m NaCl to 1.5 M NaCl in 2 mM PO_4 pH 7.4.

TABLE 1. GLYCOSAMINOGLYCAN INHIBITION OF APO E MEDIATED TRIOLEIN HEPATOCYTE INTERACTION ([a])

SUBSTRATE	GLYCOSAMINOGLYCAN	HEPATOCYTE TRIOLEIN RECOVERY
		nmol/h/10^6 Cells
Triolein without Apo E		0.33 (0.023)[b]
Triolein with Apo E		3.76 (0.52)[b]
	Heparin (10 µg)	0.44 (0.08)[b]
	Chondroitin Sulfate (200 µg)	4.23 (0.58)
	Dermatan Sulfate (200 µg)	3.50 (0.35)
	Hyaluronate (200 µg)	3.98 (0.24)
	Heparan Sulfate 0.9 (200 µg)	3.60 (0.42)
	Heparan Sulfate 1.4 (200 µg)	2.08 (0.30)[c]

(a) Data represent triplicate determinations for each assay using lecithin stabilized triolein (31.8 nmol/ml) in the absence and presence of Apo E (0.2 nmol/ml). The recoveries were significantly different from the Apo E enriched emulsion at (b) $p < 0.01$ and (c) < 0.05 by t test.

inhibited the stimulation (Table 1). Chondroitin and dermatan sulfate as well as hyaluronate at higher concentrations (200 µg/ml) were ineffective. The Dowex isolated heparans demonstrated no inhibition for the heparan with 9% sulfate and significant inhibition for the heparan with 17% sulfate.

The ability of a glycosaminoglycan or sulfated polysaccharide to inhibit Apo E supplemented emulsion interaction with hepatocytes was correlated with the ability of this compound to release hepatic lipase from perfusions of isolated livers (Table 2). Heparin and sulfated dextran effectively inhibited the Apo E mediated emulsion hepatocyte association and both released a significant amount of hepatic lipase into the perfusate. Chondroitin sulfate and dermatan sulfate both of which had little inhibitory potential for the Apo

TABLE 2. RELATION BETWEEN HEPATIC LIPASE RELEASE AND INHIBITION OF APO E MEDIATED HEPATOCYTE LIPID ASSOCIATION (a)

SUBSTRATE	POLYMER	HEPATOCYTE TRIOLEIN RECOVERY	HEPATIC PERFUSATE LIPASE ACTIVITY
		nmol/h/10^6 Cells	nmol FFA/ml
Triolein		0.33 (0.23)	
Triolein Apo E		3.76 (0.52)[b]	
	Heparin	0.44 (0.08)[b]	3,696
	Chondroitin Sulfate	4.23 (0.58)[b]	347
	Dermatan Sulfate	3.50 (0.35)[b]	761
	Dextran Sulfate	0.54 (0.16)	3,678

(a) Hepatocyte recovery data are means of triplicate determinations using substrate concentrations described in Table 1 and the lipase recoveries are means of duplicates. Each of the sulfated carbohydrates was added to the liver perfusate at 0.5 mg/ml and to the culture concentrations described in Table 1.
(b) Significantly different than Apo E free emulsion at $p < 0.01$.

TABLE 3. EFFECT OF HEPATIC GLYCOSAMINOGLYCANS ON APO E INDUCTION OF LIPID RECOVERY IN HEPATOCYTE MONOLAYERS

GLYCOSAMINOGLYCAN (% SULFATE)	CONCENTRATION μg/ml	INCREASE PRODUCED BY APO E OVER CONTROL
None		6.6(0.5)
Heparin (39%)	10	0.7
Human Heparan (15%)	200	6.3(0.4)
Human Heparan (7%)	200	5.1(0.3)[b]
Bovine Heparin Octasaccharide (29%)	200	2.4(0.4)[c]

(a) Values are means of either duplicate or quadruplicate (with standard errors) determinations at each glycosaminoglycan concentration. The Apo E increment was determined by dividing the hepatocyte recovery in the absence of Apo E into that in its presence. The values in ( ) under glycosaminoglycan are the % sulfate by weight. Significantly different from the incubation without glycosaminoglycan at (b) $p < 0.05$. (c) $p < 0.01$.

E mediated increment in hepatocyte lipid recovery released little enzyme activity.

Human hepatic heparans were much less active in inhibiting the hepatocyte triolein recovery than heparin (Table 3). Significant inhibition was observed for the heparan containing 7% sulfate but the more sulfated heparan was without effect. An octasaccharide fragment of beef hepatic heparin significantly inhibited but was not as effective as intact mucosal heparin possibly because of its smaller size or lower sulfation.

TABLE 4. THE EFFECT OF ENZYMES WHICH DEGRADE GLYCOSAMINOGLYCANS ON THE RECOVERY OF APO E EMULSIONS IN HEPATOCYTE MONOLAYERS

ENZYME	mIU([b])	RATIO OF APO E INCREMENT WITH AND WITHOUT ENZYME
Chondroitinase ABC	12	1.27(0.31)
Hyaluronidase	12	1.18(0.24)
Heparinase	3	.38(0.18)[c]
	6	.37(0.21)
	12	.52(0.20)
Heparanase	3	.32(0.11)[c]
	6	.25(0.09)[d]
	12	.20(0.07)[d]

(a) Values are means of quadruplicate determinations at each concentration. The increment in hepatocyte lipid recovery produced by Apo E observed in the absence of enzyme was divided into the recovery noted in the assay after one hour of enzyme exposure.

$$\text{Ratio} = \frac{\frac{\text{nmol cell TG Apo E}}{\text{nmol cell TG}}\ \text{Enzyme exposed}}{\frac{\text{nmol cell TG Apo E}}{\text{nmol cell TG}}\ \text{No Enzyme}}$$

(b) mIU milli international unit corresponds to the liberation of 1 nmol of Δ 4,5 hex uranic acid per minute.

(c) Significantly different than the value for hyaluronidase at $p < 0.05$.

(d) $p < 0.02$.

Incubations of hepatocyte monolayers for one hour with enzymes degrading glycosaminoglycans produced no morphologic evidence of cell toxicity or increments in media lactic dehydrogenase activity. Chondroitinase and hyaluronidase (Table 4) had no effect on the Apo E mediated hepatocyte lipid recovery. Heparinase treatment blunted this effect of Apo E but was not dose dependent and the effect was not consistent as reflected in the large standard errors. Heparanase produced a consistent dose dependent decrease in Apo E mediated hepatocyte triolein recovery. The decrease of Apo E induced hepatocyte recovery produced by 6 mIU/ml of heparanase to 25% of the untreated cells was further decreased to 19% and 16% in the presence of soybean trypsin inhibitor and α-2-macrogobulin respectively. Neither of the protease inhibitors produced any inhibition in the absence of the heparanase.

The specific binding of [I^{125}] low density lipoprotein to the rat hepatocyte monolayer was only modestly reduced by the preincubation of the cells with 6 mIU of heparanase (Figure 2).

The presence of appreciable amounts of heparan sulfate at the sinusoidal surface of the hepatocyte (2) possibly has a functional role in hepatic lipoprotein transfer. However, both apoproteins B and E which could have

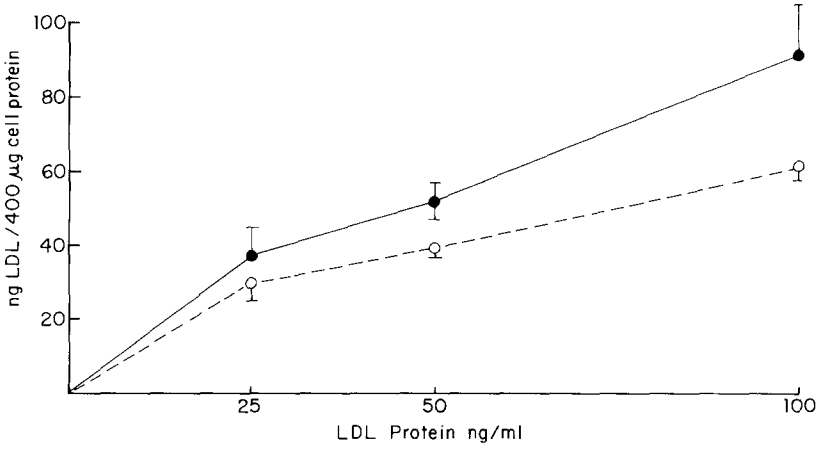

Figure 2. The effect of Heparanase on Low Density Lipoprotein specific Binding to Hepatocytes. Hepatocyte cultures were preincubated without Heparanase ●——● and with Heparanase O-------O as described in text. The cultures were subsequently incubated with the indicated concentrations of [^{125}I] human Low Density Lipoprotein in the presence and absence of 500 µg of luman Low Density Lipoprotein to determine specific binding.

potential affinity for the heparan appear (19,20,21) to have specific receptors on the hepatocyte membrane making the functional relevance of the heparan sulfate in the metabolism of these lipoproteins questionable. Nevertheless, the pronounced heparanase reduction of Apo E mediated hepatocyte triolein recovery and metabolism with little influence on that of low density lipoprotein suggests some role of the hepatocyte surface heparan sulfate in the transport of Apo E rich lipoproteins.

It is possible that the hepatic heparan sulfate which is in the space of Disse (1) and is readily accessible to plasma serves to initially interact with the plasma Apo E rich lipoprotein and subsequently mediate its interaction with the hepatocyte receptor. The glycosaminoglycan sulfate in effect would act to facilitate the interaction of the lipoprotein with the Apo E hepatocyte receptor.

REFERENCES

1. Stow , J.L. Kjellen, L., Unger, E., Hook, M., and Farguhar, M.G. (1985) J. Cell Biol. 100, 975-980.
2. Kjellen, L., Oldberg, A., and Hook, M. (1980) J. Biol. Chem. 255, 10407-10413.
3. Shelburne, F.A., and Quarfordt, S.H. (1981) Chemistry and Biology of Heparin, pp. 207-215, Elsevier, North Holland Inc., Eds: Lumblad, R.L., Brown, W.V., Mann, K.G., and Roberts, H.R.
4. Shelburne, F.A., Hanks, J., Meyers, W., and Quarfordt, S.H. (1980) J. Clin. Invest. 65, 652-658.
5. Quarfordt, S.H., Micholopoulos, G., and Schirmer, B. (1982) J. Biol. Chem. 257, 14642-14647.
6. Brown, W.V., Levy, R.I., and Fredrickson, D.S. (1970) Biochim. Biophys. Acta. 280, 573-595.
7. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
8. Kane, J.P. (1973) Ann. Biochem. 53, 350-364.
9. Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353.
10. Bilheimer, D.W., Eisenberg, S., and Levy, R.I. (1972) Biochim. Biophys. Acta. 260, 212-221.
11. Linker, A., and Hovingh, P. (1973) Carbohyd Res 29, 41-62.
12. Iverius, P-H., and Anfinsen, C.B. (1969) J. Biol. Chem. 244, 5849-5855.
13. Antonopoulis, C.A. (1962) Acta. Chem. Scand. 16, 1521-1522.
14. Seglen, P.O. (1972) Exp. Cell Res. 74, 450-454.
15. Dole, V.P. (1956) J. Clin. Invest. 35, 150-154.
16. Amador, E., Dorfman, L.E., and Wacker, W.E.C. (1963) Clin. Chem. 9, 391.
17. Mortimore, G.E. (1963) Am. J. Physiol. 204, 699-704.
18. Schotz, M.C., Garfinkel, A.S., Huibotter, R.J., Stewart, J.E., (1970) J. Lipid. Res. 11, 68-69.
19. Kinoshita, M., Teramoto, T., Kato, H., et. al. (1985) J. Biochem. 97, 1803-1806.
20. Hui, D.Y., Brecht, Hall, E.A., Friedman, G., Innerarity, T.L., and Mahley, R.W. (1986) J. Biol. Chem. 261, 4256-4267.
21. Schneider, W.J., Goldstein, J.L., and Brown, M.S. (1985) Methods in Enzymology. 109, 405-417.