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Internalization and Metabolism of Endogenous Heparin by Cultured Endothelial Cells

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SUMMARY. We have studied the ability of bovine adrenal capillary cells cultured in vitro to uptake and metabolize heparin. We have previously demonstrated that endogenous heparin can be extracted from human plasma (Vannucchi, S. et al., (1985) Biochem.J. 227, 57-65), and here we show that also endothelial cells contain heparin. However, experiments with (^{35}S)sodium sulfate labeling indicate that these cells do not synthesize de novo heparin, but they uptake it from culture serum. Bovine adrenal capillary endothelial cells are able to bind and uptake (^{3}H)heparin added to culture medium and they also release its low molecular weight degradation products, thus indicating a metabolism of heparin. We discuss about the role of endothelial cell-mediated uptake and metabolism of endogenous heparin in relationship with circulating heparin. We also discuss about these events as related to some of the antithrombogenic properties of the endothelium.

Several evidences suggest that heparin is involved in the pathophysiology of the blood vessel wall. In vivo and in vitro studies indicate that endothelial cells bind heparin (1-3).Other reports demonstrate that heparin is involved in angiogenesis; it plays a role in the mobilization of capillary endothelial cells (4, 5), and it interacts with endothelial cell growth factor (6). Taylor et al. have demonstrated that protamine, which neutralizes heparin, is an inhibitor of angiogenesis (7),

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ABBREVIATIONS: BACE, bovine adrenal capillary endothelium; DMEM, Dulbecco's modified Eagle's basal medium; ATIII, Antithrombin III; GAG_S, glycosaminoglycans; GuHCl, guanidinium hydrochloride.

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whereas other studies claim that heparin, together with steroids, inhibits angiogenesis (8, 9). Heparin also binds to a high affinity receptor on vascular smooth muscle cells and it inhibits their proliferation (10-12).

It has also been shown that heparin can be extracted from plasma and from different blood cells (13-18). In a previous study we have described a procedure to extract endogenous heparin from human plasma, and we have suggested that plasma heparin might be assembled as a "masked" compound, inactive as anticoagulant (13).

In this study we have used a slightly modified procedure to detect endogenous heparin in cultured bovine adrenal capillary endothelial (BACE) cells, and we propose a role for endothelial cells in uptaking and metabolizing heparin; this might represent a way to maintain some of the antithrombogenic properties of the endothelium.

MATERIALS AND METHODS

Cell population. Endothelial cells isolated from bovine adrenal capillary endothelium (BACE) were kindly provided by Dr.M.Ziche, Dept. of Pharmacology, University of Firenze, Italy, and they have been utilized at passages 20 through 30. The endothelial origin of the cell population has been shown (4, 19). Their morphology has also been checked by electron microscopy analysis. BACE cells were grown in DMEM plus 10% foetal calf serum (Grand Island Biological Co. - GIBCO - Grand Island, N.Y., U.S.A.).

Labeling and extraction of glycosaminoglycans (GAG_S). Cells were labeled with 10 μCi/ml of (^{35}S)sodium sulfate (New England Nuclear, Boston, M.A., U.S.A.) for 48 hours in their medium, and then with additional 50 μCi/ml of (^{35}S)sodium sulfate in sulfate-free medium for 24 hours. Cells were washed five times in phosphate-buffered saline and then treated with 0.25% trypsin (GIBCO, U.S.A.) for 5 min at 37°C. Cells were then centrifuged at 200 x g for 10 min, and resuspended in 0.4 M guanidinium HCl (GuHCl), pH 9.5 at the concentration of 10^7 cells/ml. 19 volumes of chloroform/methanol (2:1, v/v) were added to each sample and the mixture was shaken for 1 hour at 4°C. Formation of phases was induced by adding 1/5 of the final volume of GuHCl, pH 9.5. Upper phases were collected and filtered on glass fibre filters (Whatman, GF/A, 2,1 cm); filters were collected, added to lower phases and reextracted with the lower phases. Lower phases,

including interface material, were reextracted with 0.4 M GuHCl/methanol (1:1, v/v), ph 9.5. This procedure was repeated 3 times and then further reextraction of lower phases was performed with 4 M GuHCl/methanol (1:1, v/v), pH 9.5, for 3 other times.

Upper phases were dialyzed, liophylized, resuspended in 1 ml of 0.05 M Tris/HCl, pH 9.0, and loaded onto a column (1 x 3 cm) of DEAE-Sephacel (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. In order to separate heparin, stepwise elution was performed with 10 ml of 0.5 M Tris/HCl, pH 8.0, followed by 10 ml of the same buffer, pH 7.0, then by 10 ml of 0.05 M sodium acetate, pH 5.0 and finally by 10 ml of 0.05 M sodium acetate, pH 4.0.

GAG_s were collected in the last two fractions, eluted respectively with 10 ml of 0.6 M NaCl in 0.05 M sodium acetate, pH 4.0, and with 10 ml of 2 M NaCl in the same buffer. These two last fractions were dialyzed, liophylized and analyzed on cellulose acetate electrophoresis at pH 1.0 (20). Cellulose acetate sheets were subjected to direct autoradiography. GAG_s were also analyzed by Antithrombin III (ATIII) affinity chromatography (21).

Internalization and metabolism of (^{3}H)heparin. BACE cells grown at confluency (1 x 10^6 cells/dish) were incubated with 0.05 μCi/ml of (^{3}H)heparin (New England Nuclear) for 2 hours at 4°C. Cells were washed 3 times in cold buffer and then temperature was shifted to 37°C. Cells were treated with 0.25% trypsin for 1 min at various times as indicated (Fig. 3). Medium and trypsin-removable material were collected; cells were dissolved in 0.2 N NaOH and (^{3}H)heparin was counted by liquid scintillation.

To study the metabolism of (^{3}H)heparin by BACE cells, (^{3}H)heparin was gel-filtered on a G-75 Sephadex column (1 x 90 cm), eluted with 1 M NaCl, flux 4 ml/hour: 2 ml fractions were collected. Fractions 14 through 24 were pooled dyalized and used to label cells as described above. 24 hours after shifting temperature to 37°C, medium was collected and analyzed on a G-75 Sephadex column as described. The entire experiment was performed in the absence of serum to avoid (^{3}H)heparin degradation by serum heparitinase (22).

RESULTS AND DISCUSSION

Fig.1 (A, B and C) shows the electrophoretic pattern and the corresponding autoradiography, of (^{35}S)sodium sulfate labeled GAG_s extracted from BACE cells;the autoradiography clearly shows that GAG_s which comigrate with standard heparin are the only unlabeled compounds.

Fig.2 (A and B) shows the electrophoretic pattern and the corresponding autoradiography of (^{35}S)sodium sulfate labeled GAG_s after affinity chromatography on ATIII-Sepharose column.

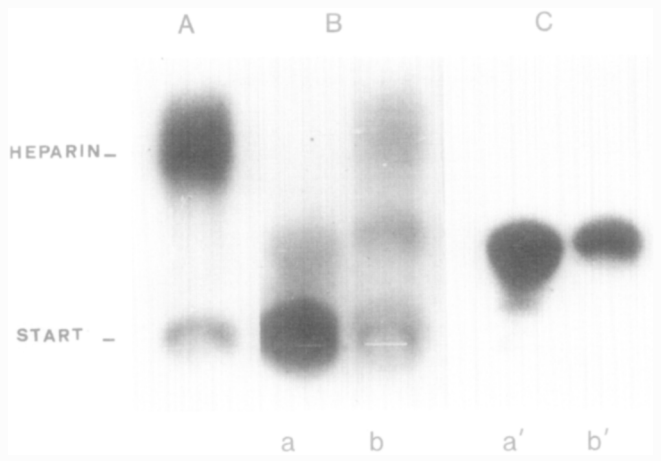

Figure 1. Analysis of GAG_s extracted from BACE cells. BACE cells were labeled with (^{35}S)sodium sulfate and treated with trypsin as described. Cellular GAG_s were separated on DEAE-Sephacel column. A: shows cellulose acetate electrophoresis at pH 1.0 (20) of standard heparin. B: shows cellulose acetate electrophoresis at pH 1.0 of GAG_s eluted in 0.6 M NaCl fraction (a), and of GAG_s eluted in 2 M NaCl fraction (b). C: shows the corresponding autoradiography, where lanes a' and b' correspond to lanes a and b on the cellulose acetate sheet.

The electrophoretic pattern shown in Fig.2 (A) demonstrates that material eluted from ATIII-Sepharose column at 0.4 M NaCl is in fact comigrating with standard heparin. The corresponding autoradiography (Fig.2, B) shows that this material is unlabeled. Fig.2 (B) also shows other GAG_s with low and high affinity for ATIII - eluted respectively at 0.4 and 2 M NaCl - which are labeled with (^{35}S)sodium sulfate; however, none of these compounds actually comigrates with standard heparin (Fig.2, A).

Data shown in Figs.1 and 2 clearly demonstrate that BACE cells do contain heparin; however, because heparin extracted from these cells is consistently unlabeled with (^{35}S)sodium sulfate, we hypothesize that BACE cells are unable to biosynthesize *de novo* heparin. Therefore we assume that heparin contained in BACE cells is coming from serum. We have previously demonstrated that human plasma contains heparin (13) and we have also detected endogenous heparin in foetal calf serum (data not shown). In order to verify this hypotesis, we have tested the ability of BACE cells to uptake and metabolize (^{3}H)heparin added to culture

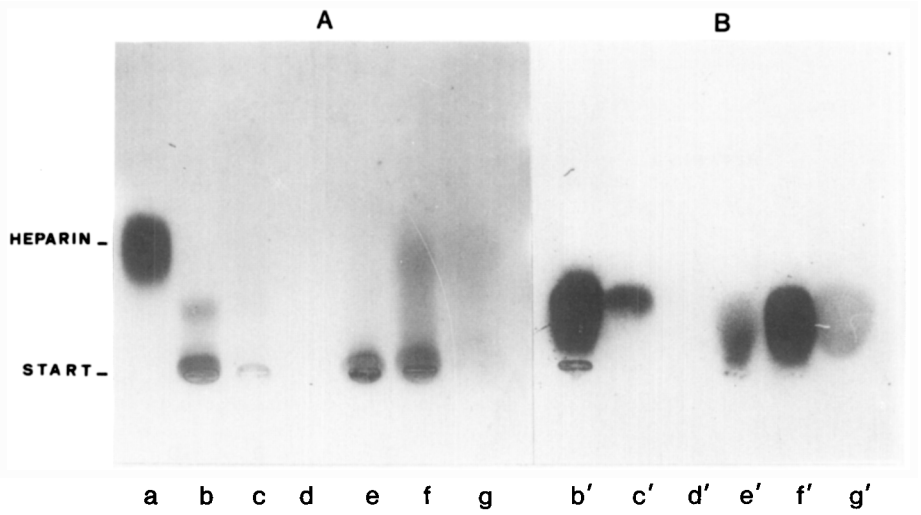

Figure 2. Analysis of GAG_S separated by affinity chromatography on ATIII-Sepharose column. BACE cells were labeled as in Fig. 1, and GAG_S extracted as described. GAG_S eluted in 0.6 and 2 M NaCl from DEAE-Sephacel column were loaded onto a 1 x 3 cm ATIII-Sepharose column (13). Fractions containing GAG_S with low and high affinity for ATIII were then analyzed by cellulose acetate electrophoresis at pH 1.0 (A). Lane a shows separation of standard heparin. Lanes b, c and d show separation of GAG_S eluted in 0.6 M NaCl fraction from DEAE-Sephacel and then chromatographed on ATIII-Sepharose column. b: GAG_S eluted in the washing buffer. c: GAG_S with low affinity for ATIII. d: GAG_S with high affinity for ATIII. Lanes e, f and g show separation of GAG_S eluted in 2 M NaCl fraction from DEAE-Sephacel and chromatographed on ATIII-Sepharose. e: GAG_S eluted in the washing buffer. f: GAG_S with low affinity for ATIII. g: GAG_S with high affinity for ATIII. B: is the autoradiography of the cellulose acetate sheet in A; lanes b', c', d', e', f' and g' correspond to lanes b through g on the sheet.

medium. Fig.3 shows that BACE cells uptake (^{3}H)heparin - bound at 4°C - after shifting temperature to 37°C. (^{3}H)heparin increases inside BACE cells in about 15 min and in the same time course, surface-associated heparin - that is, heparin in trypsin-removable material - decreases (Fig.3). However, after 30 min we observe that heparin in the medium increases, whereas heparin associated with cells or with the cell surface, concomitantly decreases (Fig.3). This indicates that BACE cells bind and internalize (^{3}H)heparin and thereafter they release it (or its degradation products) into culture medium.

DISCUSSION

The binding data obtained in this study is consistent with there being a single class of high affinity receptors for atriopeptin III in rabbit lung. The value obtained for the apparent dissociation constant of the receptor is similar to those reported for other tissues (6-8), and is consistent with atrial peptide levels measured in plasma (12,13). The relative binding affinities of a number of atrial peptide analogs also correlated well with their relaxant activities on contracted vascular tissue strips. The location of atrial peptide receptors in lung is not known at present, although it has been reported that these factors appear to act on the vasculature rather than on airway smooth muscle (10).

It is known that atrial natriuretic factors are released from the atria of the heart, and that the right atrium contains a higher concentration of atrial peptides than the left (14). Atriopeptins can survive passage through the lung (15), as would be required of a circulating hormone (16,17). Thus, when atrial peptides are released from the right atrium, they would first encounter the pulmonary circulation before being transported to other tissues. It is not unreasonable, therefore, to speculate that receptors for atrial peptides exist in the lung vasculature and that atrial peptides, via their vasorelaxant, diuretic and natriuretic activities, could be of use in the therapeutic treatment of pulmonary edema or hypertension. Further studies will be required, however, to determine whether atrial peptides do play a role in the regulation of pulmonary function.

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mined by gel-filtration analysis of (^{3}H)heparin associated with cells 1 hour after endocytosis (data not shown). We have also observed that BACE cells release into culture medium low molecular weight degradation products (Figs.3 and 4). We do not know whether this catabolism takes place in extracellular sites (23) or inside the cells; however, because BACE cells are able to uptake intact heparin, we suggest that heparin catabolism may occur after its binding and internalization.

Our results are in accord with previous reports describing heparin-like compounds associated with the endothelial luminal surface (24). Our data also indicate that endothelial cells in culture biosynthesize GAG_s with low and high affinity for ATIII, but they are unable to synthesize de novo heparin. Subfractions of heparan sulfate with strong anticoagulant activity have been detected in the vascular endothelium and in cultured endothelial cells (25); these may account for the most part of GAG_s with high affinity for ATIII that we are observing in BACE cells.

Therefore, at least two mechanisms might confer antithrombogenic properties on the endothelium; on one side, production of GAG_s with high affinity for ATIII and strong anticoagulant activity: on the other side, uptake and metabolism of endogenous plasma heparin with subsequent release of its degradation products. We have previously demonstrated that plasma heparin is assembled as a "masked" compound, inactive as anticoagulant (13); binding, internalization and metabolism of endogenous plasma heparin by endothelial cells could then be a way to "unmask" circulating heparin and to make it available on the endothelial cell surface.

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