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Glycosaminoglycans exposed on the endothelial cell surface

Binding of heparin-like molecules derived from serum

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We have analyzed the glycosaminoglycans exposed on the surface of endothelial cells cultured in vitro. The cells were labelled with [^{35}S]sodium sulfate. Chondroitin sulfate, heparan sulfate and dermatan sulfate are the main sulfated glycosaminoglycans present on the cell surface. They are synthesized by endothelial cells. However, the electrophoretic analysis and corresponding autoradiography of the glycosaminoglycans removed from the endothelial cell surface shows the presence of heparin-like compound which is not synthesized by the cells as it is unlabelled by [^{35}S]sodium sulfate. We show here that a proteolytic treatment of the commonly used serum for cell culturing, reveals the presence of an heparin-like anticoagulant activity. These results suggest that the endothelial cells bind endogenous heparin-like molecules present in serum.

Heparin; Glycosaminoglycans; Endothelial cell

1. INTRODUCTION

One of the remarkable features of the coagulation system is its ability to respond rapidly to seal a wound site, limiting the blood clotting to the site of the wound. During platelet activation and fibrin formation there are endogenous mechanisms that tend to limit thrombus formation. The known mechanisms are the generation of prostacyclin [1], the activation of protein C [2], fibrinolysis [3], and the presence of the naturally occurring inhibitor antithrombin III, which is potentiated by the presence of heparin [4]. Endothelial cells synthesize heparan sulfate with high affinity for antithrombin III, which can function to accelerate the inactivation of coagulation factors by antithrombin III on the endothelial cell surface [5]. We have previously shown that endothelial cells are able to bind and uptake [^{3}H]heparin added to culture medium, and that they also release its low molecular weight degradation products, thus indicating a metabolism of heparin [6,7]. We have also shown that BACE cells contain heparin; however, as heparin extracted from these cells is consistently unlabelled with [^{35}S]sodium sulfate, we hypothesized that heparin contained in BACE cells comes from serum [6]. In this paper we have analyzed the glycosaminoglycans (GAGs) removed by trypsin from endothelial cell surface and those present in the medium of BACE cells after labelling with [^{35}S]sodium sulfate. The results indicate that endothelial cells bind on their surface heparin-like molecules which are not synthesized by the cells. In addition, we show that serum contains an heparin-like anticoagulant activity, which can be measured by thrombin time assay after an extensive proteolysis. We discuss the exposure of heparin related compounds derived from serum on the endothelial cell surface as it relates to the control of clotting extension and the repair of the injured vessel wall.

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2. MATERIALS AND METHODS

Endothelial cells isolated from bovine adrenal capillary endothelium (BACE) kindly provided by Dr. M. Ziche, Dept. of Pharmacology, University of Florence, Italy, have been described in our previous report [6]. BACE cells were grown in DMEM plus 10% foetal calf serum (Grand Island Biological Co., Gibco, Grand Island, NY, USA).

Cells were labelled with [^{35}S]sodium sulfate (New England Nuclear, Boston, MA, USA) as described in [6]. Cells were washed 5 times in phosphate-buffered saline, treated with 0.25% trypsin (Gibco, USA) for 5 min at 37°C and centrifuged at $200 \times g \times 10$ min. Medium and trypsinate were lyophilized before isolating GAGs following the modified Folch-extraction procedure as previously described [6]. The GAGs extracted in aqueous phases were fractionated stepwise on DEAE-Sephacel (Pharmacia, Uppsala, Sweden). Fractions eluted with 0.6 M and 2.0 M NaCl were dialyzed against distilled water, concentrated and analyzed on cellulose acetate electrophoresis at pH 1.0 [8] and at pH 5.0 [9]. Standard chondroitin sulfate A (CSA), chondroitin sulfate C (CCS) and dermatan sulfate (DS) were obtained from Seikagaku Kogyo Co., Tokyo; hyaluronic acid (HA) was from Sigma Chem. Co., St. Louis, MO and heparan sulfate (HS) from Upjohn International Inc., Kalamazoo, MI. Standard heparin (HP 756, 150 IU/mg) was a kind gift of Prof. Pietro Bianchini, Opocrin Research Laboratories, Modena, Italy. [^{3}H]-labelled heparin was obtained from New England Nuclear, Boston, MA, USA. Nitrous acid treatment was performed directly on cellulose acetate sheet [9]. Cellulose acetate electrophoreses were subjected to direct autoradiography. In addition, aliquots of the ^{35}S-labelled GAGs extracted both from BACE cells and trypsinate were combined, mixed with standard

GAGs (CSA and HP 756) and applied to a column of DEAE cellulose (DE-52, Whatman) (1 cm × 4 cm). The column was washed extensively with 0.05 M LiCl in 0.05 M acetate buffer, pH 4.0, and was then eluted with a linear gradient extending from 0.05 to 1.5 M LiCl in the same acetate buffer. Fractions of 2.0 ml were collected and analyzed for ^{35}S-radioactivity and uronic acid. Hexuronic acid was measured by the carbazole method of Bitter and Muir [10].

Aliquots of 1 ml of foetal calf serum were submitted to proteolytic digestion as described elsewhere [11]. Briefly, papain (EC 3.4.22.2; Calbiochem) was added for 24 h at 60°C. Samples were then boiled and, at intervals of 24 h, the following enzymes, each at final concentration of 1 mg/ml, were added sequentially: trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4 21.1), collagenase (EC 3 4.24.3) (Sigma Chem. Co.) and pepsin (EC 3.4.23 1) (Boehringer-Mannheim). Samples were boiled at the end of each incubation. Samples were centrifuged at 3000 × g × 10 min and the thrombin time assay was performed on 100 μl of the clear supernatants [11]. For the quantitative evaluation of the anticoagulant activity thrombin time assay was performed in the presence of increasing concentrations of standard heparin

3. RESULTS

Fig. 1 shows the electrophoresis of the complex mixture of GAGs extracted from BACE cells and trypsinate analyzed on cellulose acetate electrophoresis at pH 5.0 and the corresponding autoradiography. GAGS co-migrating with standard chondroitin sulfate A, dermatan sulfate and heparan sulfate are clearly identified. These GAGs are actively synthesized by the cells as shown in the corresponding autoradiography. The presence of *N*-sulfated GAGs is demonstrated by nitrous acid treatment performed on trypsin-removable GAGs (Fig. 2). Chondroitin sulfate A and heparan sulfate are the main GAGs synthesized by BACE cells and are exposed on the cell surface. Dermatan sulfate, which is resistant to treatment with nitrous acid is also present. However, this type of analysis does not allow separation between heparan sulfate and the fractions of heparin which are not precipitated in barium acetate (so-called fast-moving heparin fraction) [12], as demonstrated by the autoradiography of the standard mixture containing ^{3}H-labelled heparin shown in Fig. 1. The presence of heparin in a GAG mixture can be better recognized with an electrophoretic analysis performed at pH 1.0.

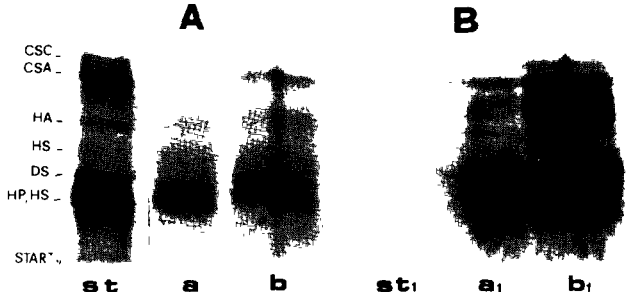

Fig. 1. Cellulose acetate electrophoresis of GAGs from BACE cells and trypsinate. (A) The electrophoretic run at pH 5.0 in 0.1 M barium acetate of GAGs extracted from BACE cells (a) and trypsinate (b). (B) This is the corresponding autoradiography. St, standard mixture; CSC, chondroitin sulfate C; CSA, chondroitin sulfate A; HA, hyaluronic acid, DS, dermatan sulfate; HS, heparan sulfate; HP, fast moving ^{3}H-labelled heparin. Lanes St_1, a_1 and b_1 correspond to lanes St through b on the sheet.

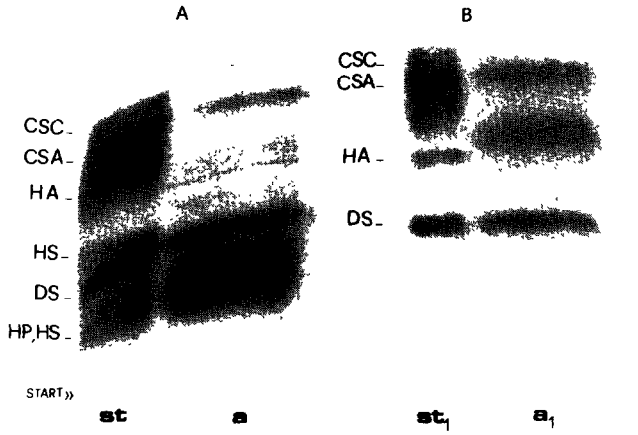

Fig. 2. Nitrous acid treatment of trypsin-removable GAGs from BACE cells. Cellulose acetate electrophoresis of trypsin-removable GAGs before (A) and after treatment with nitrous acid (B). Run at pH 5.0 in 0.1 M barium acetate. St, St1, standard mixture; a, a_1, trypsin-removable GAGs The abbreviations for standard GAGs are as in Fig. 1.

Fig. 3 shows the electrophoretic pattern and the corresponding autoradiography of GAGs extracted from [^{35}S]sodium sulfate-labelled BACE cells, medium, and trypsinate at pH 1.0. The autoradiography clearly shows that GAGs which co-migrate with standard heparin are the only unlabelled compounds. We have further analyzed the ^{35}S-labelled GAGs extracted both from cells and trypsinate by an ion-exchange chromatography on DE-52 eluted with a linear gradient of LiCl. As shown in Fig. 4, only a small portion of the ^{35}S-labelled GAGs is eluted later than a chondroitin sulfate standard, but ahead of heparin, whereas the main fraction of the ^{35}S-labelled GAG is co-eluting with standard chondroitin sulfate. These results demonstrate that BACE cells do not synthesize heparin. However, the unlabelled highly-sulfated compound co-migrating with standard heparin in cellulose acetate electrophoresis shown in Fig. 3 was also extracted from the culture medium containing 10% foetal calf serum. We show in Fig. 5 that the supernatant of the proteolytically digested serum, produces a delay in thrombin time which corresponds to 1.2 μg/ml of standard heparin. Taken together, these results suggest that BACE cells bind the endogenous heparin present in the serum on their surface.

4. DISCUSSION

In vivo and in vitro studies indicated that endothelial cells bind, internalize and degrade exogenous heparin [13,14]. Binding has been implicated in heparin's pharmacokinetics [15].

In this paper we demonstrate that endothelial cells

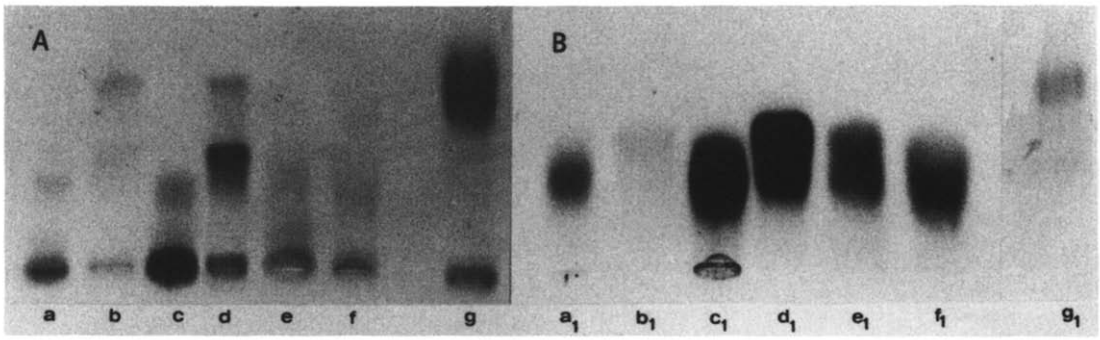

Fig. 3. Analysis of GAGs extracted from BACE cells, medium and trypsinate by cellulose acetate electrophoresis at pH 1.0. BACE cells were labelled with [^{35}S]sodium sulfate and treated with trypsin as described. GAGs from medium, trypsinate and cells were extracted and submitted to ion-exchange chromatography on DEAE-Sephacel. (A) Cellulose acetate electrophoresis at pH 1.0: (a,c,e) GAGs eluted at 0.6 M NaCl fraction extracted from medium (a), trypsinate (c), and cells (e); (b,d,f) GAGs eluted in 2 0 M fraction extracted from medium (b), trypsinate (d) and cells (f); (g) standard heparin (HP 756) mixed with ^{3}H-labelled heparin. (B) This is the corresponding autoradiography of the cellulose acetate sheet in (A); lanes a_1, b_1, c_1, d_1, e_1, f_1 and g_1 correspond to lanes a through g on the sheet.

expose on their surface endogenous heparin-like compounds. It is well known that endothelial cells synthesize heparan sulfate with high affinity for antithrombin III [5]. However, we show here that endothelial cells expose heparin-like molecules which are not synthesized by the cells, but they are instead derived from serum. In a previous paper we described how heparin can be extracted from human plasma or serum using a modified Folch-extraction procedure [16]. Unfortunately this method yielded results difficult to reproduce: further results lead us to suggest that interactions with proteins are responsible for the difficulty in isolating endogenous heparin from plasma or serum [17]. According to this hypothesis, a proteolytic digestion of plasma proteins reveals the presence of an heparin-like anticoagulant activity in plasma [11]. In this paper we describe that very similar results are obtained on foetal calf serum used for cell cultures. These results suggest that heparin bound on the cell surface is derived from serum. We have previously shown how activated platelets release heparin [18]. However, heparin-like molecules are also present in circulating plasma under an undetectable form [16,17], probably due to the interactions between heparin and proteins [19]. Whatever the origin of these compounds, whether platelets or the plasmatic compartment undergoing clotting cascade activation, this binding can represent an important mechanism to limit blood clotting at the areas of endothelial cell damage avoiding clot propagation on uninjured endothelium. Furthermore, heparin stimulates endothelial cell proliferation by interacting, and increasing the effect of fibroblast growth factor [20]. It has recently been reported that endothelial cells synthesize fibroblast growth factor which they sequester in their matrix [21]. Thus, a binding of heparin after clotting on undamaged endothelium could participate in vessel wall repair by stimulating endothelial cell regeneration.

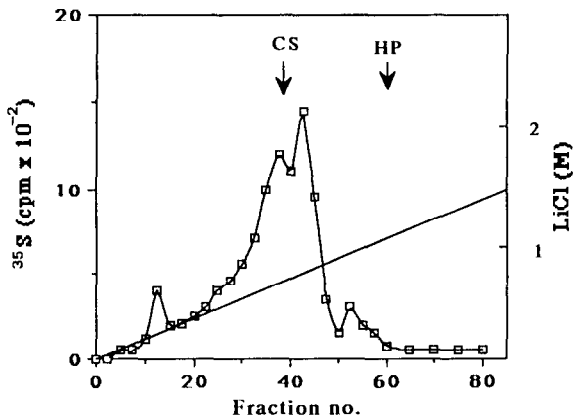

Fig. 4. Ion-exchange chromatography on DEAE-cellulose of ^{35}S-labelled GAGs from BACE cells and trypsinate. Aliquots of the ^{35}S-labelled GAGs extracted both from cells and trypsinate were combined, mixed with standard heparin and chondroitin sulfate A and applied to a column of DEAE-cellulose (DE-52). The elution was performed as described in section 2. The arrows on the top of the figure indicate the peak elution positions of chondroitin sulfate (CSA) and heparin (HP) standards. (—), the LiCl linear gradient.

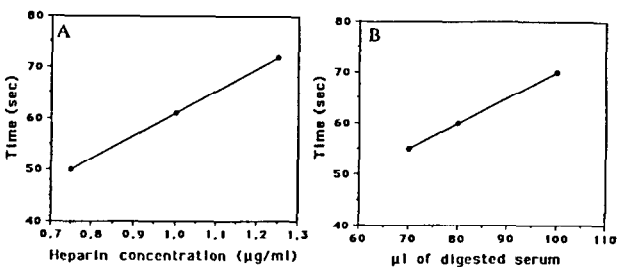

Fig. 5 Thrombin time assay. The thrombin time assay was performed on pooled human plasma in the presence of different concentrations of standard heparin (A) or in the presence of different amounts (70, 80 and 100 μl) of the proteolytically digested serum (B).

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REFERENCES

[1] Moncada, S., Griglewski, R.J., Bunting, S. and Vane, J.R. (1976) Nature 263, 663–665.
[2] Clouse, L.H. and Comp, P.C. (1986) N. Engl. J. Med. 314, 1298–1304.
[3] Sakata, Y., Curriden, S , Lawrence, D., Griffin, J.H. and Loskutoff, D.J. (1985) Proc. Natl. Acad. Sci. USA 82, 1121–1125.
[4] Rosenberg, R.D. and Rosenberg, J.S. (1984) J. Clin. Invest. 74, 1–6
[5] Marcum, J.A. and Rosenberg, R.D. (1984) Biochemistry 23, 1730–1737.
[6] Vannucchi, S., Pasquali, F., Chiarugi, V. and Ruggiero, M. (1986) Biochem. Biophys. Res. Commun. 140, 294–391.
[7] Vannucchi, S., Pasquali, F., Porciatti, F., Chiarugi, V., Magnelli, L. and Bianchini, P. (1988) Thromb. Res. 49, 373–383.
[8] Wessler, E. (1971) Anal. Biochem. 41, 67–72.
[9] Cappelletti, R., Del Rosso, M. and Chiarugi, V (1980) Anal. Biochem. 105, 430–435.
[10] Bitter, J. and Muir, H.M. (1962) Anal. Biochem. 4, 330–334.
[11] Cavari, S., Stramaccia, L. and Vannucchi, S. (1992) Thromb. Res. 67, 157–165.
[12] Oreste, P. and Torri, G. (1980) J Chromatogr. 195, 39–401.
[13] Mahadoo, J., Hiebert, L.M. and Jacques, L.B. (1977) Thromb. Res. 12, 79–80
[14] Glimelius, B., Bush, C. and Hook, M. (1987) Thromb. Res. 12, 773–782.
[15] Van Rijn, J.L.M.L., Trillou, M., Mardiguian, J., Tobelem, G. and Caen, J. (1987) Thromb. Res. 45, 211–222.
[16] Vannucchi, S , Ruggiero, M. and Chiarugi, V. (1985) Biochem. J. 227, 57–65.
[17] Pasquali, F., Oldani, C., Ruggiero, M , Magnelli, L., Chiarugi, V. and Vannucchi, S. (1990) Clin. Chim. Acta 192, 19–28.
[18] Vannucchi, S., Fibbi, G., Pasquali, F., Del Rosso, M., Cappelletti, R. and Chiarugi, V. (1982) Nature 296, 352–353.
[19] Lee, M.K and Lander, A.D. (1991) Proc. Natl. Acad. Sci. USA 88, 2768–2772.
[20] Gospodarowicz, D. and Cheng, J. (1986) J. Cell. Physiol 128, 475–484.
[21] Vlodalsky, J., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J. and Klagsburn, M. (1987) Proc Natl. Acad. Sci. USA 84, 2292–2296.