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High-performance affinity chromatography for the purification of heparin-binding proteins from detergent-solubilized smooth muscle cell membranes

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Abstract

Heparin and heparan sulfates are regulators of cellular events including adhesion, proliferation and migration. In particular, the antiproliferative effect of heparin on smooth muscle cell (SMC) growth is well described. However, its mechanism of action remains unclear. Numerous results suggest an endocytosis mediated by a still unknown heparin receptor on vascular SMCs. In order to identify a putative heparin receptor on SMCs that could be involved in heparin signalling, affinity chromatography supports were developed. In this paper, we describe high-performance liquid affinity chromatography (HPLAC) supports obtained from silica beads coated with dextran polymer substituted by a calculated amount of diethylaminoethyl functions. With a polysaccharide dextran layer, this type of support can be grafted with specific ligands, such as heparin, using conventional coupling methods. In a previous work, we demonstrated, using butanedioldiglycidyl ether, that silica stationary phases coupled to heparin could be used for the fast elution and good peak resolution of heparin-binding proteins. In the present work, an affinity chromatographic fraction of SMC membrane extracts was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and six heparin-binding proteins from dodecyloctaethyleneglycol monoether-solubilized SMCs were observed. Their M_r values were between 40 and 70 kDa, with three major protein bands at 66, 45 and 41 kDa. These results indicate the usefulness of the chromatographic method for purifying heparin binding proteins from SMC membrane.

Keywords: SMC membranes; Proteins; Heparin; $C_{12}E_8$

1. Introduction

Vascular smooth muscle cells (VSMCs) play a major role in the pathophysiology of vascular walls [1]. Smooth muscle cell (SMC) proliferation is one of the key events in atherosclerosis. Nowadays, it is known that migration and growth of VSMCs are inhibited by heparin and endothelial heparan sulfate proteoglycans [2,3]. Although it is described that heparin is an antiproliferative agent for VSMCs in vitro [2,4,5] and after vascular injury [6,7], the molecular mechanism involved in this effect has not yet been elucidated. Heparin binding sites have been reported with different affinities for a variety of cell types in the vascular system, such as, monocytes [8], macrophages [9,10], VSMCs [11–13] and vascular endothelial cells [14,15], but also in epithelial cells [16], hepatocytes [17,18], Chinese hamster ovary cells [19] and granulosa cells [20]. However, specific cell surface heparin-binding sites were only found in

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VSMCs [11,12] with high affinity ($K_d = 10^{-9}$ *M*) and 10^5 specific heparin-binding sites/cell. The effects of heparin on VSMCs are postulated to be receptor-mediated [11,12]. It is thus interesting to characterize the cell-surface heparin-binding proteins from SMCs. Lankes et al. [21] described a 78 kDa heparin-binding protein from bovine uteri, which was thought to be involved in the inhibition of VSMC proliferation. A possible role of this protein as a receptor for heparin has been suggested. Putative heparin receptors have also been described in other cells. For instance, analysis of surface-iodinated monocytoid cell line (U937) lysates by heparin affinity chromatography revealed a major, 120 kDa, cell surface heparin-binding protein that could be the receptor [8]. Binding sites recognized by heparin have been characterized on the cell surface of mouse uterine epithelial cells by Wilson et al. [16]. Heparin-binding proteins of 14, 18.5 and 31 kDa have been identified in human uterine epithelial cells after binding and elution from heparin–agarose and separation by SDS–PAGE [22]. More recently, Patton et al. [23] isolated a 45-kDa heparin binding polypeptide in porcine aortic endothelial cells. These proteins bind to heparin–Sepharose affinity columns. The importance of the interaction between heparin in solution and membrane proteins of different cellular types is clearly evidenced. However, the specificity of these interactions for the immobilized polysaccharide has still to be demonstrated. In a previous work [24], our results indicated that proteins extracted from cell membranes have affinity for heparin immobilized on a high-performance liquid affinity chromatography (HPLAC) support. We demonstrated that heparin silica-based stationary phases exhibit good separation properties for cellular proteins. Cell extracts were solubilized by different detergents (Triton X-100, octylglucoside and $C_{12}E_8$). We found that $C_{12}E_8$, a dodecyloctaethyleneglycol monoether solubilizing agent for membrane proteins [25], was of interest for both the SMC membrane solubilization and chromatographic elution steps.

In the present study, SDS–PAGE analysis of affinity-purified $C_{12}E_8$-solubilized SMC membrane proteins revealed approximately six heparin-binding proteins with molecular masses ranging from 40 to 70 kDa.

2. Experimental

2.1. Materials

The HPLC apparatus is a Merck-Hitachi 655A-12 gradient system from Merck-Clevenot (Nogent sur Marne, France) with a Rheodyne 7161 injection valve, connected to a UV absorbance detector (Model 111D, Gilson) and an integrator (D-2000 GPC Integrator, Merck).

Silica beads were kindly provided by Biosepra (Villeneuve la Garenne, France) and had a diameter of 25–60 μm and a porosity of approximately 3000 Å (specific surface of the beads 15 m^2/g). Dextran T40 (37200 g/mol) and standard proteins were purchased from Pharmacia Biotech (Orsay, France). 2-Chloro-*N*,*N*-diethylaminoethane (DEAE) was from Janssen Chemica (Pantin, France). Phosphate buffered saline (PBS) without calcium and magnesium was from Gibco (Cergy Pontoise, France). Heparin H410 was provided by the Institut Sanofi Recherche (Gentilly, France). Formaldehyde was from Carlo Erba (Nanterre, France), 1,4-butanedioldiglycidyl ether (BDGE), dodecyloctaethyleneglycol monoether ($C_{12}E_8$), benzamidine, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), EDTA, Coomassie brilliant blue R250 and silver nitrate were purchased from Sigma (St. Quentin Fallavier, France). Acrylamide, bisacrylamide, bromophenol blue, Tris base, glycine and phenylmethanesulfonyl fluoride (PMSF) were obtained from Fluka (St. Quentin Fallavier, France). Ammonium peroxodisulfate and SDS were obtained from Interchim (Montluçon, France).

2.2. Cell culture

The isolation of smooth muscle cells from Sprague-Dawley rat aorta explants has been described previously [26,27]. Cells were grown at 37°C with 5% CO_2 in Modified Eagle Medium (MEM, Gibco, Cergy Pontoise, France) supplemented with 2% L-glutamine and 10% fetal bovine serum (Gibco) in 75 cm^2 culture flasks.

2.3. Detergent-solubilized smooth muscle cells

SMC membranes were extracted according to the

method of Clairbois et al. [24]. Briefly, SMCs (10×10^6 cells/75 cm^2 flask) were scraped from the tissue culture flask in ice-cold PBS, pH 7.4, containing 3 m*M* EDTA, 4 m*M* benzamidine and proteases inhibitors (leupeptin, pepstatin A, aprotinin) and transferred to a conical tube. After centrifugation (500 *g*, 5 min, 4°C), cell pellets were resuspended in ice-cold bidistilled water containing the protease inhibitors and stored at −80°C until required. After one cycle of freezing–thawing and centrifugation (3000 *g*, 10 min, 4°C), the pellet was resuspended in 0.01 ml/10^6 cells of solubilization buffer [PBS–0.5%(w/v) $C_{12}E_8$] for 30 min at 4°C with gentle stirring. Then, the membrane suspension was centrifuged (800 *g*, 15 min, 4°C) and stored at −80°C.

2.4. Synthesis of a SiD–BDGE–heparin chromatographic support

Preparation of the SiD–BDGE–heparin support was carried out in two steps. Firstly, porous silica beads were coated with a hydrophilic and cationic DEAE–dextran layer (SiD). Secondly, the biospecific ligand was immobilized by a conventional coupling agent (BDGE). The reaction is performed in two successive steps.

First step: to minimize the nonspecific interactions between the silica support and proteins, silanol groups on native silica beads were neutralized by a positively charged modified dextran. Synthesis of DEAE–dextran was achieved by a controlled reaction of the hydrochloride, 2-chloro-*N*,*N*-diethylaminoethane hydrochloride, with the native polymer in alkaline medium at 55°C for 30 min. The percentage of DEAE groups on dextran was calculated by elemental analysis of nitrogen. The coating of silica beads was performed by impregnation and crosslinking of DEAE–dextran [24,28,29]. Briefly, a 10% aqueous solution of DEAE–dextran polymer (under alkaline conditions) was adsorbed onto silica beads by gentle impregnation; then, the adsorbed polymer was crosslinked by a 0.3% (v/v) diethyl ether solution of BDGE. The amount of crosslinked polymer (DEAE–dextran) on the silica phase was determined by elemental analysis of carbon.

Second step: the immobilization of heparin on the coated support (or SiD) was performed as previously described [24] using BDGE as a coupling agent (Fig. 1). After washing, the amount of immobilized heparin (5±1 mg per gram of SiD support) was determined by elemental analysis.

2.5. Chromatographic elution

All eluents were prepared from bidistilled water that had been degassed and filtered through 0.22 μm Millipore filters prior to chromatography.

The stainless steel column (12.5×0.4 cm I.D.) was packed with 1 g of SiD–BDGE–heparin support using the slurry method. The stationary phase was equilibrated with $C_{12}E_8$ buffer (PBS–0.1% (w/v) $C_{12}E_8$, pH 7.4).

All extracts were subjected to HPLAC separation using detergent concentrations that were approximately ten times above the critical micellar con-

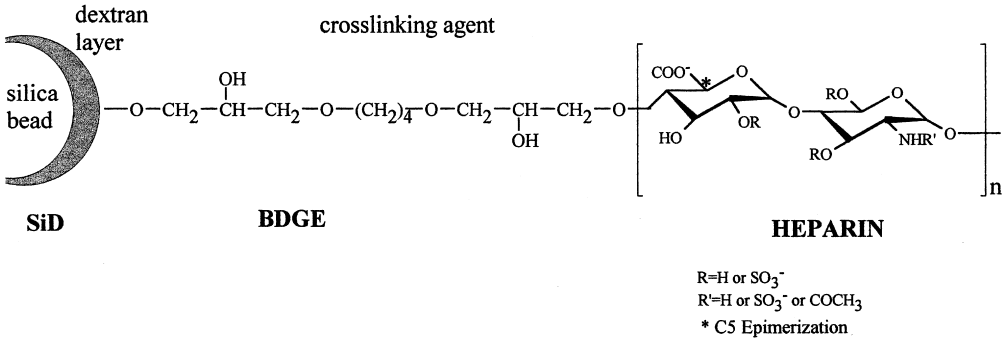

Fig. 1. Structure of the SiD–BDGE–heparin support.

centration (CMC), to avoid the aggregation of membrane proteins. Cell membrane extracts were loaded on the column at a flow-rate of 0.5 ml/min. The elution was performed either by using a linear sodium chloride gradient from 0.15 to 2 *M* in $C_{12}E_8$ aqueous buffer or by using a direct step of 2 *M* NaCl. Chromatographic fractions were collected every minute (fraction collector Model 203, Gilson). The effluent was analyzed by an on-line UV absorbance detector (Model 111B, Gilson) at 280 nm.

2.6. Identification of proteins by SDS–PAGE

Proteins were desorbed from the heparinized support with NaCl as described above and separated according to their molecular masses by reducing SDS–PAGE. Electrophoresis was performed according to the method of Laemmli [30]. Bromophenol blue was used as the tracking dye. Protein samples (from 4 to 20 μg in 100–150 μl), prepared under reducing conditions, were electrophoresed on a 7.5% SDS–polyacrylamide gel. All fractions in the chromatographic peak were pooled, dialyzed against PBS, mixed with SDS sample buffer and loaded on the gel, without heating. We did not observe any change in the migration of proteins when samples were not boiled before loading on the gel (data not show). A constant current of 35 mA was applied until the bromophenol blue tracking dye front exited from the bottom of the gel. After electrophoresis, the gel was first stained with Coomassie brilliant blue and then using the silver staining procedure: Gels were soaked immediately after electrophoresis in an aqueous solution containing 50% (v/v) methanol, 10% (v/v) acetic acid and 0.25% (w/v) Coomassie brilliant blue R250 for 20 min, and destained in 5% ethanol, 7.5% acetic acid overnight. Gels were stained using silver nitrate according to the method of Wray et al. [31], which can detect proteins at the nanogram level. This staining method is reported to be 50–100 times more sensitive than the Coomassie brilliant blue method.

The molecular masses of the different protein bands were estimated using several standard proteins with molecular masses ranging from 53 to 212 kDa [myosin (212 kDa), α2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), glutamate dehydrogenase (53 kDa)].

2.7. Protein content assay

Protein content was determined by the bicinchoninic acid (BCA) method [32] using bovine serum albumin as the protein standard (Micro BCA protein assay reagent kit, Interchim, France). The protein concentration for each sample was determined using a calibration curve obtained at 570 nm.

3. Results and discussion

We have prepared porous silica supports coated with a hydrophilic dextran layer. This coated polymer was crosslinked, activated and derivatized by heparin (Fig. 1). The affinity support was used to characterize membrane proteins of rat aorta SMCs. Cells were washed and solubilized using 0.5% (w/v) $C_{12}E_8$. After centrifugation to remove any insoluble materials, cell lysates were applied to the heparin affinity column.

3.1. Identification of heparin-binding proteins

The preliminary experiments in high-performance affinity chromatography on the heparin coated support (SiD–BDGE–heparin) were performed with a $C_{12}E_8$-based chromatographic buffer (PBS, 0.1% $C_{12}E_8$, pH 7.4) to prevent aggregation and precipitation of membrane proteins. A 100-μl volume of $C_{12}E_8$-solubilized extract (approximately 3.6 mg/ml) was loaded onto the column and the bound proteins were eluted with a linear NaCl gradient (0.15–2 *M*). As an example, we report in Fig. 2A a typical chromatogram with desorbed proteins obtained using a linear 10 min NaCl gradient. The proteins eluted as a single peak, F1 (Peak F1; ~2 ml) at 2 *M* NaCl. The F1 fraction desorbed at 2 *M* NaCl was pooled, dialyzed, concentrated and analyzed by SDS–PAGE followed by silver staining [31]. The electrophoretic pattern of fraction F1 is presented in Fig. 2B. Proteins present in the starting extract of detergent-solubilized SMC plasma membranes are shown in Fig. 2B, lane 1. After purification by HPLAC, the proteins can be resolved into approximately six discrete bands (lane 2). The molecular masses of the different protein bands were estimated by SDS electrophoresis. There were three major bands, with

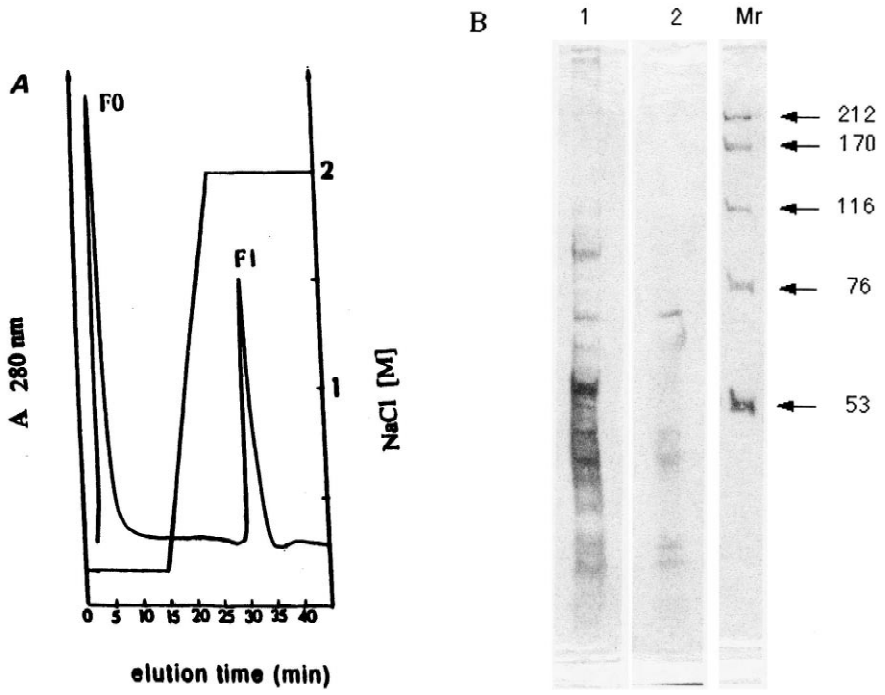

Fig. 2. (A) Typical elution using a linear salt gradient of 100 μl of $C_{12}E_8$ extract on SiD–BDGE–heparin. Elution conditions: Flow-rate, 0.5 ml/min; column, 12.5×0.4 cm I.D. Adsorption buffer: PBS, 0.1% (w/v) $C_{12}E_8$, 0.15 *M* NaCl, pH 7.4. Desorption buffer: PBS, 0.1% (w/v) $C_{12}E_8$, 2 *M* NaCl, pH 7.4. (B) SDS–PAGE profile of proteins. Lane 1, VSMC plasma membranes extracted with 0.5% (w/v) $C_{12}E_8$. Lane 2, proteins purified from a SiD–BDGE–heparin column. Separation of proteins by 7.5% SDS–PAGE under reducing conditions. Proteins were visualized by the silver staining procedure according to Wray et al. [31]. Molecular masses of the proteins were determined using known molecular mass marker proteins. Arrows indicated the position of the molecular mass standards, myosin (212 kDa), α2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamate dehydrogenase (53 kDa).

estimated molecular masses of 66, 45 and 41 kDa. It is interesting to note that Patton et al. [23] recently described the presence of a 45-kDa protein on endothelial cell membranes that binds to a heparin sepharose support. Similarly, epithelial membrane heparin binding proteins were also reported [22] with a M_r values of 31 and 18.5 kDa for the two major protein bands, and 45 kDa for a less abundant protein. However, the analogy for the different cell types remains to be studied.

3.2. Chromatographic parameters

The elution temperature, flow-rate, desorption conditions (competing agent, salinity), amount of protein injected and the nature of the elution buffer are important parameters in chromatography. The separation conditions between the heparin ligand and the binding proteins were studied. The first step was to study the effect of ionic strength on protein desorption using elution buffers with NaCl concentrations varying from 0.5 to 2 *M*. Corresponding elution profiles are reported in Fig. 3A–D. The resolution of desorbed proteins increased with ionic strength. At 1 *M* NaCl, the retained proteins are desorbed in a single peak (Fig. 3B). At 1.5 and 2 *M* NaCl, the retained proteins were desorbed in two peaks with good chromatographic resolution (Fig. 3C,D).

Variation of the kinetics of the NaCl gradient was then studied. Using a rapid 10 min linear gradient (Fig. 2A), proteins were desorbed in a single peak at the end of the process, i.e. at 2 *M* NaCl. Longer salt gradients were used (Fig. 4A–D) to improve the quality of chromatographic resolution. When the slope of the gradient decreased, proteins were de-

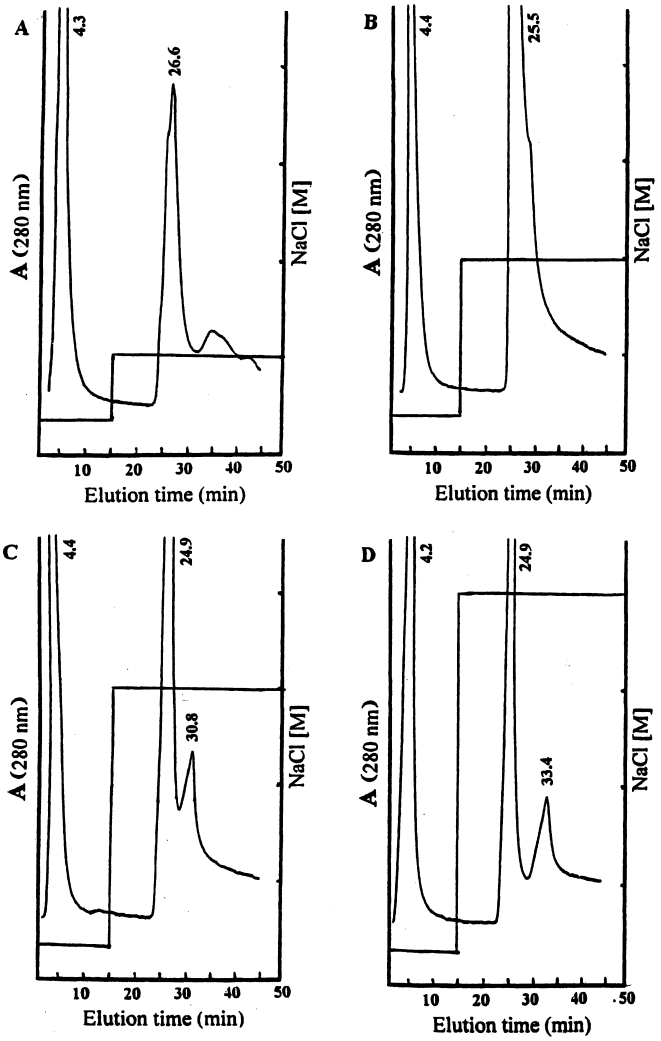

Fig. 3. Elution of 90 μl of $C_{12}E_8$ extract on SiD–BDGE–heparin using different steps of salt elution. (A) 0.5 *M* NaCl, (B) 1 *M* NaCl, (C) 1.5 *M* NaCl and (D) 2 *M* NaCl. Elution conditions: Column, 12.5×0.4 cm I.D.; flow-rate, 0.5 ml/min. Adsorption buffer: PBS, 0.1% (w/v) $C_{12}E_8$, 0.15 *M* NaCl, pH 7.4. Desorption buffer: PBS, 0.1% (w/v) $C_{12}E_8$, 0.5–2 *M* NaCl, pH 7.4.

sorbed in a single peak with a shoulder or in two or even three peaks. In particular, the molarities at which desorption occurred were 0.88 and 1.07 *M* NaCl with a 20-min gradient (Fig. 4A), 0.7 and 0.95 *M* for a 30-min gradient (Fig. 4B), and 0.52 and 0.84 *M* NaCl for a 40-min gradient (Fig. 4C). The third peak, at 1.4 *M* NaCl in Fig. 4D, was obtained using a different desorption protocol. Variation of the slope of the gradient allowed different peak resolution. This indicated that the kinetic parameters play an important role in the elution of these proteins on our HPLAC stationary phase. Future experiments will be devoted to studying the nature of the proteins obtained in the different peaks and to compare them with those obtained with different cell types.

4. Conclusion

Heparin plays an important and complex role in blood vessel biology. It interacts with monocytes and

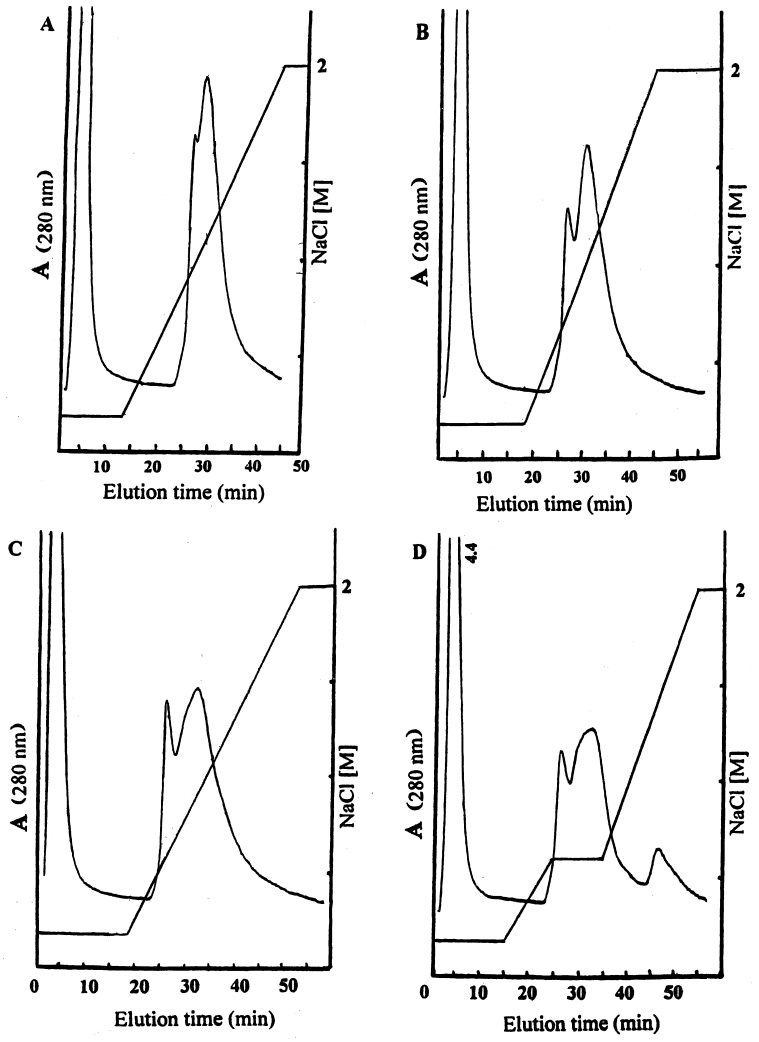

Fig. 4. Elution of 90 μl of $C_{12}E_8$ extract on SiD–BDGE–heparin using different linear salt gradients. (A) 20 min, (B) 30 min, (C) 40 min, (D) 10 min from 0.15 to 0.6 *M* NaCl, a step at 0.6 *M* NaCl followed by a 20-min gradient from 0.6 to 2 *M* NaCl. Elution conditions: Column, 12.5×0.4 cm I.D.; flow-rate, 0.5 ml/min Adsorption buffer: PBS, 0.1% (w/v) $C_{12}E_8$, 0.15 *M* NaCl, pH 7.4. Desorption buffer: PBS, 0.1% (w/v) $C_{12}E_8$, 2 *M* NaCl, pH 7.4.

macrophages as well as endothelial cells and smooth muscle cells. The identification of a putative receptor in smooth muscle cells is essential to the understanding of heparin signalling. In this study, membrane extracts from rat aorta vascular SMCs were solubilized with polyoxyethylenic detergents ($C_{12}E_8$) and subjected to heparin affinity chromatography. SDS–PAGE of high-performance liquid heparin affinity chromatography of SMC lysates revealed proteins with three major bands, at 66, 45 and 41 kDa. Ongoing studies will indicate if one or any of them could represent a specific heparin receptor with antiproliferative activity on SMC growth.

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References

[1] R. Ross, Annu. Rev. Physiol. 57 (1995) 791–801.
[2] J.J. Castellot, M.L. Addonizio, R.D. Rosenberg, M.J. Karnovsky, J. Cell Biol. 90 (1981) 372–379.
[3] W.E. Benitz, R.T. Kelley, C.M. Anderson, D.E. Lorant, M. Bernfield, Am. J. Respir. Cell. Mol. Biol. 2 (1990) 13–24.
[4] R.L. Hoover, R.D. Rosenberg, W. Haering, M.J. Karnovsky, In vitro Circ. Res. 47 (1980) 578–583.
[5] J.J. Castellot, J. Choay, J.-C. Lormeau, M. Petitou, E. Sache, M.J. Karnovsky, J. Cell Biol. 102 (1986) 1979–1984.
[6] A.W. Clowes, M.J. Karnovsky, Nature 265 (1977) 625–636.
[7] J.R. Guyton, R.D. Rosenberg, A.W. Clowes, Circ. Res. 46 (1980) 625–634.
[8] L. Leung, K. Saigo, D. Grant, Blood 73 (1989) 177–184.
[9] I. Fabian, I. Bleiberg, M. Aronson, Biochim. Biophys. Acta 544 (1978) 69–76.
[10] I. Bleiberg, I. MacGregor, M. Aronson, Thromb. Res. 29 (1983) 53–61.
[11] J.J. Castellot, K. Wong, B. Herman, R.L. Hoover, D.F. Albertini, T.C. Wright, B.L. Caleb, M.J. Karnovsky, J. Cell. Physiol. 124 (1985) 13–20.
[12] D. Letourneur, B.L. Caleb, J.J. Castellot, J. Cell. Physiol. 165 (1995) 676–686.
[13] T. Bârzu, M. Pascal, M. Maman, C. Roque, F. Lafond, A. Rousselet, J. Cell. Physiol. 167 (1996) 8–21.
[14] B. Glimelius, C. Busch, M. Hook, Thromb. Res. 12 (1978) 773–782.
[15] T. Bârzu, P. Molho, M. Tobelen, M. Petitou, J. Caen, Biochim. Biophys. Acta 845 (1985) 196–203.
[16] O. Wilson, A.L. Jacobs, S. Stewart, D.D. Carson, J. Cell. Physiol. 143 (1990) 60–67.
[17] H.J. Ryser, N. Morad, W.C. Shen, Cell Biol. Int. Rep. 7 (1983) 923–930.
[18] M. Ishihara, N.S. Fedarko, H.E. Conrad, J. Biol. Chem. 261 (1986) 13575–13580.
[19] L. Kjellen, A. Oldberg, K. Rubin, M. Hook, Biochem. Biophys. Res. Commun. 74 (1977) 126–133.
[20] M.A. Winner, R.L. Ax, J. Reprod. Fert. 87 (1989) 337–348.
[21] W. Lankes, A. Griesmacher, J. Grunwald, A. Schwartz, R. Albiez, R. Keller, Biochem. J. 251 (1988) 831–842.
[22] N. Raboudi, J. Julian, L.H. Rohde, D.D. Carson, J. Biol. Chem. 267 (1992) 11930–11939.
[23] W. A Patton C, A. Granzow, L.A. Getts, S.C. Thomas, L.M. Zotter, K.A. Gunzel, L.J. Lowe-Krentz, Biochem. J. 311 (1995) 461–469.
[24] A.S. Clairbois, D. Letourneur, D. Muller, J. Jozefonvicz, Int. J. Biochromatogr. (1998), in press.
[25] S. Khamlichi, M.J. Loirat, D. Blanchard, M. le Maire, P. Bailly, J.P. Cartron, O. Bertrand, J. Biochem. Biophys. Methods 29 (1994) 123–134.
[26] M.C. Bourdillon, J.P. Boissel, B. Crouset, A. Perrin, Biomedicine 25 (1976) 263–267.
[27] T. Avramoglou, J. Jozefonvicz, J. Biomater. Sci. Polym. Ed. 3 (1991) 149–154.
[28] X. Santarelli, D. Muller, J. Jozefonvicz, J. Chromatogr. 443 (1988) 55–62.
[29] F.L. Zhou, D. Muller, X. Santarelli, J. Jozefonvicz, J. Chromatogr. 476 (1989) 195–203.
[30] U.K. Laemmli, Nature 227 (1970) 680–685.
[31] W. Wray, T. Boulikas, V.P. Wray, R. Hancock, Anal. Biochem. 118 (1981) 197–203.
[32] K.J. Wiechelman, R.D. Braun, J.D. Fitzpatrick, Anal. Biochem. 175 (1988) 231–237.