831

A heparin-binding protein involved in inhibition of smooth-muscle cell proliferation

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A heparin-binding protein was isolated from bovine uteri and purified to homogeneity. This protein appears as a double band of approx. 78 kDa in SDS/polyacrylamide-gel electrophoresis and has an isoelectric point of 5.2. The binding of heparin to this protein is saturable. No other glycosaminoglycan from mammalian tissue, such as hyaluronic acid, chondroitin sulphate, dermatan sulphate or keratan sulphate, binds to the 78 kDa protein. Dextran sulphate binds in a non-saturable fashion. Certain heparan sulphate polysaccharide structures are required for binding to the 78 kDa protein. Some proteoheparan sulphates, such as endothelial cell-surface proteoheparan sulphate, show only weak interaction with the 78 kDa protein in contrast with a basement-membrane proteoheparan sulphate from HR-9 cells. Antibodies against the 78 kDa protein inhibit binding of proteoheparan [^{35}S]sulphate from basement membranes to smooth-muscle cells. Conventional antibodies, Fab fragments and some monoclonal antibodies, inhibit smooth-muscle cell proliferation in a similar range as that observed for heparin. The protein was detected in a variety of tissues and cells but not in blood cells. A possible role of this protein as a receptor for heparin or heparan sulphate and its function in the control of the arterial wall structure are discussed.

INTRODUCTION

Mainly owing to the work of the groups of Karnovsky, Castellot and Rosenberg, it is now generally established that heparin inhibits smooth-muscle cell (SMC) proliferation in vitro and in vivo [1–5]. Since SMC proliferation is regarded as one of the key events in the pathogenesis of atherosclerosis [6], this inhibitory heparin effect is considered to be of great physiological and pathological significance. On the basis of these findings a model for the pathogenesis of atherosclerosis has been proposed [7].

The molecular basis of this inhibitory heparin effect is not yet clear. Recently it has been suggested that heparin acts intracellularly after uptake by SMC [8,9]. There are also data indicating that the interaction of heparin with SMC is receptor mediated [9,10]. SMC migration can be specifically inhibited by heparin in a dose-dependent, cell-specific and reversible fashion [11]. The effect of heparin on SMC seems to be selective for certain heparin–ligand structures [12].

To study the anti-proliferative heparin effect on SMC growth at a molecular level, it is necessary to isolate the SMC surface protein which is responsible for the binding of heparin. Recently, the isolation of a heparin-binding 38 kDa protein from tissue-cultured SMC-conditioned medium has been described [13]. This protein is specifically expressed by nodular SMC [14]. It is not clear,

however, whether this protein, which is secreted by SMC and is not cell-associated, is involved in growth inhibition by heparin or whether it is just a marker protein for a nodular SMC phenotype.

The heparin effect is restricted to a certain phenotype of SMC. Cells from later passages or from cultures which were grown at 10 % (or more) fetal-calf serum apparently are not growth inhibited by heparin. It may be that the cellular binding protein which promotes the anti-proliferative effect to the cell is only expressed by a particular SMC phenotype which is not the common cellular form of SMC from mass cultures. For this reason we did not use cultured SMC as a source for the isolation of the heparin-binding protein.

In the work presented here, we describe the isolation and partial characterization of a heparin-binding protein with a molecular mass of approx. 78 kDa from bovine uteri. This protein does not bind to other glycosaminoglycans. From SMC proliferation assays in vitro with affinity-purified antibodies from goat anti-serum or with monoclonal antibodies (mAbs) we suggest that this protein is involved in SMC growth inhibition.

MATERIALS AND METHODS

Materials

Germany); heparin (porcine mucosa, grade II) for coupling to Sepharose CL-2B or CL-6B (Sigma, München, W. Germany); heparin for SMC proliferation assays (Sandoz, Basel, Switzerland); Sepharose CL-2B, CL-6B, Sephacryl S-200, S-300, Sephadex G-25, Protein A–Sepharose (Pharmacia, Heidelberg, W. Germany); fetal-calf serum, papain crystalline suspension (EC 3.4.22.2) (Boehringer, Mannheim, W. Germany); heparinase (EC 4.2.2.7) (Miles, München, W. Germany); polyethylene glycol (Merck, Darmstadt, W. Germany); Dulbecco's Minimal Essential Medium, penicillin/streptomycin, trypsin/EDTA solutions (Flow Laboratories, Meckenheim, W. Germany); goat anti-(mouse IgG) serum, FITC-coupled anti-(rabbit IgG) IgG, FITC-coupled anti-(goat IgG) IgG (Dianova, Hamburg, W. Germany); lactoperoxidase or horseradish peroxidase-coupled anti-(rabbit IgG) IgG, horseradish peroxidase-coupled anti-(goat IgG) IgG, horseradish peroxidase-coupled anti-(mouse IgG) IgG (Sigma and Dianova). Keratan sulphate (porcine cornea), chondroitin sulphate (bovine trachea), dermatan sulphate (calf skin), hyaluronic acid (bovine corpus vitreum) and dextran sulphate were generous gifts by Dr. Helmut Stuhlsatz (Central Laboratory, Aachen Technical University, W. Germany). Rabbit anti-(bovine band 4.1) serum was a generous gift of Dr. Joseph A. Madri (Yale University School of Medicine, Pathology Department, New Haven, CT, U.S.A.). The human T lymphoma cell line Jurkat was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.); the human colon adenocarcinoma cell line HT-29 was kindly provided by Drs. J. Fogh and M. Pfreundschuh, Sloan-Kettering Cancer Center, NY, U.S.A.

Amino acid and amino sugar analysis was performed on a Biotronik LC 6000 analyser after hydrolysis in 6 M-HCl or 3 M-HCl at 115 °C for 15 h.

Antibody production and testing

To produce conventional anti-sera, 500 μg of protein in 500 μl of phosphate-buffered saline (PBS) were mixed with the same volume of complete Freund's adjuvant and injected intracutaneously 3 times into rabbits or goats with 14 days between each injection. Anti-sera were produced by coagulation of blood. For affinity purification of the goat antibodies, 10 ml portions of anti-serum were applied to a 78 kDa protein–Sepharose CL-6B column (1.5 cm × 4 cm; approx. 2 mg of protein/ml of gel). The column was washed with 120 ml of PBS and the bound antibodies were then eluted with 0.1 M-Tris/HCl/4 M-guanidinium chloride (pH 7.2). The antibody fraction in the eluate, as determined by 280 mm absorption, was collected, dialysed against 3 × 10 l of water, and freeze-dried.

Fab fragments were produced by papain digestion following a standard protocol [15]. The fragments were purified by chromatography on Protein A–Sepharose and chromatography on Sephacryl S-200 in PBS.

mAbs were produced according to a standard procedure. Female BALB/c mice were immunized by subcutaneous injection of 50 μg of purified heparin-binding protein, mixed with the same volume of complete Freund's adjuvant. The mice were boostered twice at 3 week intervals, first by subcutaneous injection of the same amount of antigen mixed with incomplete Freund's adjuvant, followed by an intraperitoneal injection of

the pure antigen 3 days before the fusion. Fusions were performed by the method of Köhler & Milstein [16].

Splenocytes from immune spleens and Ag8 myeloma cells were mixed at a cell ratio of 2:1 and fused in 1.5 ml of 70% (w/v) polyethylene glycol 4000 in Dulbecco's P_i with 15% dimethyl sulphoxide. After fusion, cells were resuspended in hypoxanthine, aminopterine and thymidine (HAT) medium containing 10% heat-inactivated fetal-calf serum. Cells were distributed in 24-well microtitre plates (Costar, MA, U.S.A.) at 2×10^5 cells/well after pre-incubation for 48 h with 2×10^4 non-immune BALB/c peritoneal macrophages. Hybrids were kept in HAT selection medium for 2 weeks. Supernatants from wells with visible colonies were tested by e.l.i.s.a. on purified heparin-binding protein. Positive hybridomas were subcloned by limited dilution on a feeder layer of BALB/c spleen cells (5×10^5 cells/ml). Hybridomas of interest were propagated as intraperitoneal tumours in mice. Ascites fluids were cleared by two consecutive $(NH_4)_2SO_4$ precipitations (50% (w/v) saturation) and extensive dialysis against PBS. The mAbs were further purified by application to Protein A–Sepharose CL-4B. The column was equilibrated in 0.025 M-Tris buffer, pH 8.0, and then eluted with a gradient of 0.025 M-sodium citrate buffer (or glycine/HCl), varying in pH from 6.0 to 3.5. The immunoglobulin eluted at pH 3.5. All mAbs obtained were of IgG isotype as determined by an e.l.i.s.a. using goat anti-(mouse IgG) serum.

E.l.i.s.a. assays were performed following a standard procedure [17]. Plates were coated with 1 μg of protein/ml of PBS. All further washing and incubation procedures, with the exception of the final colour reaction, were carried out by using 1% bovine serum albumin (BSA) in PBS. In those cases when whole cells were used in the e.l.i.s.a. procedure, 1000 SMC were seeded in each well, cultured for 24 h, washed with PBS 3 times, fixed with methanol at room temperature for 10 min and further processed according to the routine standard e.l.i.s.a. procedure.

For Western blot analysis, proteins were separated on SDS/PAGE and subsequently transferred to nitrocellulose as described by Towbin et al. [18]. Specific staining with antibodies was performed as previously described [19]. Positive control experiments for the anti-serum against the band 4.1 were carried out with erythrocyte ghosts as protein source.

mAb W6/32 was from a preparation described previously [20]. Indirect immunofluorescence was performed as described previously [17,21]. Stained cells were examined by a cytofluorograph (Ortho Diagnostic Systems, Westwood, MA, U.S.A.).

Cell-surface iodination and immunoprecipitation

Membrane proteins of the established human colon carcinoma cell line HT-29 were radiolabelled by the lactoperoxidase method described by Goding [22]. In brief, 1×10^7 cells (viability over 95%), suspended in 0.5 ml of PBS, were labelled with 0.5 mCi of ^{125}I in the presence of 10 μg of lactoperoxidase. To this mixture 10 μl each of 0.003% H_2O_2 in PBS, 0.01% H_2O_2 in PBS and 0.03% H_2O_2 in PBS were added (1 min between each step). The reaction was stopped by adding 500 μl of 5 mM-NaI in PBS at 4 °C and mixing. Cells were washed three times by centrifugation at 1000 g and resuspension in PBS, and then were solubilized in 1% Nonidet P-40/

10 mM-Tris/HCl/150 mM-NaCl/10 μM-PMSF/10 μM-aprotinin (pH 7.2) at 4 °C. Nuclei and cell debris were removed by centrifugation at 10000 g and the proteins in the supernatant were non-specifically precipitated by incubation with 100 μl of a 1:1 (v/v) suspension of Protein A–Sepharose in the above-mentioned lysis buffer for 1 h at 4 °C. The mixture was centrifuged at 1000 g and 4 °C and the supernatant was combined with 10 μg of antibody and 20 μg of rabbit anti-(mouse IgG) IgG in 100 μl of PBS. To this solution 20 μl of Protein A–Sepharose (50 % suspension in PBS) was added. The suspension was shaken gently overnight at 4 °C and the gel was washed three times with 10 mM-Tris/HCl/150 mM-NaCl/0.25 % Nonidet P-40, pH 7.4 (washing buffer), by centrifugation at 10000 g and resuspension in 100 μl of washing buffer. The Protein A–Sepharose pellet was then boiled in 50 μl of 0.125 M-Tris/HCl/2 % SDS/20 % glycerol/1 % dithioerythritol/1 mM-EDTA, pH 7.2 (SDS sample buffer), for 5 min and the 1000 g supernatant was then applied to SDS/PAGE.

Cytofluorographic analysis

Cells (1 × 10^6) were incubated with 5 μg of purified mAbs in 50 μl of PBS at 4 °C for 30 min and washed twice. The cells were then incubated with 30 μl of 0.03 % FITC-coupled rabbit anti-(mouse IgG) IgG in PBS at 4 °C for 30 min, washed three times and examined with a fluorescence microscope (Zeiss, Frankfurt, Germany) or a cytofluorograph (Ortho Diagnostic Systems, Westwood, MA, U.S.A.) equipped with an argon laser which was operated at 488 nm. For each run 1.5 × 10^4 cells were analysed with settings used to exclude non-viable cells according to scatter characteristics. The gains were kept at identical settings for all mAbs used in this study. Background controls were done by incubating cells with the second antibody only. The percentage of cells within a region (channel 30–1000, indicating the increase in the intensity of fluorescent staining) minus the percentage of background controls (5–7 %) is given for each antibody.

CNBr coupling to Sepharose

Sepharose gels were exhaustively washed with water, suspended in twice the volume of water, and adjusted to pH 10–11 with 2 M-NaOH. CNBr (50 mg/ml) was added to the gel and the pH maintained between 10 and 11 by adding 2 M-NaOH dropwise. After 3 min of reaction, the gel was washed extensively with 0.2 M-sodium acetate. Ligand (2–5 mg of heparin or protein/ml of gel) in 0.1 M-sodium acetate was added and the mixture was shaken at 4 °C for 24 h, after which period, 50 μl of ethanolamine/ml of gel was added. Shaking was continued for 5 h and the gel was then washed with 4 M-NaCl and PBS.

Isolation of the heparin-binding protein

All isolation procedures were carried out at 0–4 °C. Ten bovine uteri from the slaughter house were rapidly cooled to 0 °C. Blood vessels, ligamentae, mucus, fasciae, etc., were removed and the uteri were then minced, suspended in approx. the same volume of 2 mM-PMSF/2 mM-EDTA in PBS and homogenized with a spinning knife (Star-Mix) at 0 °C. The homogenate was frozen, thawed, and centrifuged at 10000 g and 0 °C for 15 min. To show enrichment of the heparin-binding protein in the microsomes, the supernatant was recentrifuged at 100000 g (60 TI rotor, Beckman L5 ultra-centrifuge) and
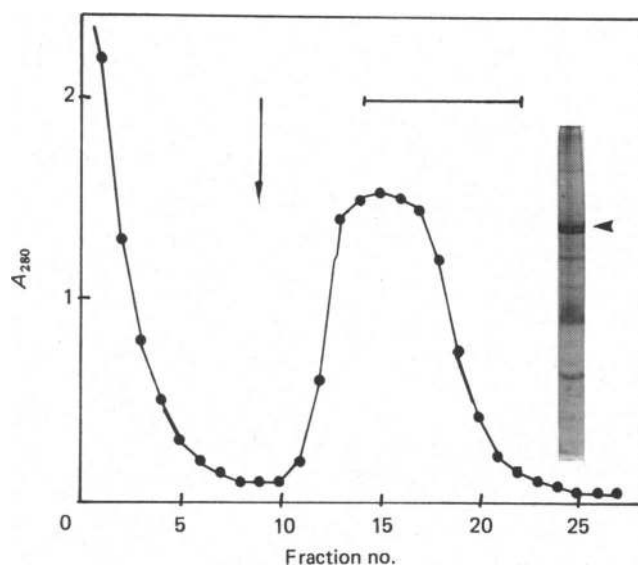
Fig. 1. Chromatography on heparin–Sepharose

The 10000 g supernatant from uterus homogenate was adjusted to 0.1 M-Tris/HCl/0.1 % Chaps/1 mM-PMSF/1 mM-EDTA, pH 7.85 (elution buffer), and applied to the top of a heparin–Sepharose Cl-6B column (50 ml). The column was washed with 500 ml of the elution buffer and further eluted with a linear gradient (500 ml + 500 ml) of elution buffer and elution buffer + 1 M-NaCl. Fractions were assayed for 280 nm absorption, pooled as indicated, and investigated on SDS/PAGE. Electrophoresis of the pooled fraction is shown.

the microsomal pellet, suspended in 0.1 M-Tris/HCl/0.1 % Chaps/1 mM-PMSF/1 mM-EDTA, pH 7.85, then used as a source for further processing. In most cases, however, the 10000 g supernatant was directly used.

The supernatant was adjusted to pH 7.85 with 2 M-Tris, and applied to a heparin–Sepharose CL-6B column (50 ml, 2.5 cm × 10 cm). The column was then washed with ice-cold 0.1 M-Tris/HCl/0.1 % Chaps/1 mM-PMSF/1 mM-EDTA, pH 7.85 (elution buffer), until the 280 nm absorption baseline was reached. Elution was continued with a gradient (500 + 500 ml) of elution buffer and 1 M-NaCl + elution buffer. Fractions of 50 ml were collected at 200 ml/h. Portions (200 μl) were adjusted to 80 % ethanol and incubated at 4 °C for 5 h. After centrifugation at 10000 g the precipitates were solubilized in 50 μl of SDS sample buffer and subjected to SDS/PAGE. Fractions were pooled as indicated in Fig. 1, concentrated 5:1 by distillation in vacuo and chromatographed in portions on Sepharose CL-6B (5 cm × 100 cm) with 0.5 M-NaCl/0.1 M-Tris/HCl/0.1 % Chaps/1 mM-PMSF/1 mM-EDTA, pH 7.4, as eluant. Fractions of 20 ml were collected at 200 ml/h, analysed for 280 nm absorption, pooled as indicated in Fig. 2(a), diluted 1:5 with water and adsorbed onto 20 ml of heparin–Sepharose (1.6 cm × 10 cm). The column was washed with 100 ml of 0.1 M-Tris/HCl/0.1 % Chaps/1 mM-PMSF/1 mM-EDTA, pH 7.85, and the protein was eluted with 50 ml of the same buffer + 1 M-NaCl. The protein peak, determined by 280 nm absorption, was collected, concentrated 3:1 by distillation in vacuo and chromatographed on Sephacryl S-300 (2 cm × 100 cm) with 0.5 M-NaCl/0.1 M-Tris/HCl/0.1 % Chaps/1 mM-PMSF/1 mM-EDTA, pH 7.4, as eluant. Fractions of
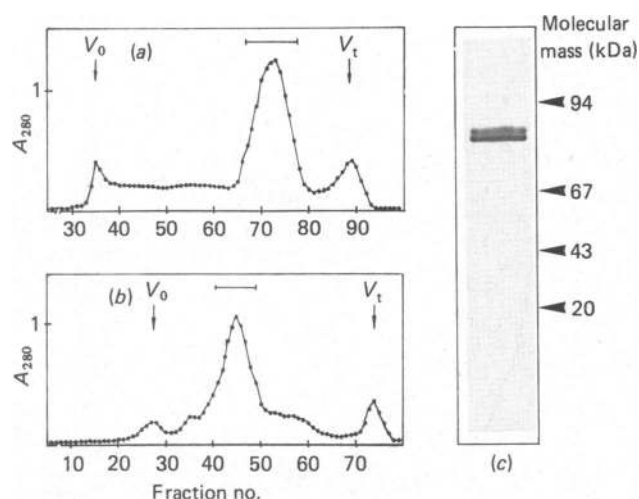
Fig. 2. Chromatography on Sepharose CL-6B and Sephacryl S-300

(a) The pooled fractions from the heparin–Sepharose chromatography shown in Fig. 1 were chromatographed on Sepharose CL-6B (5 cm × 100 cm) in 0.5 M-NaCl/ 0.1 M-Tris/HCl/0.1 % Chaps/1 mM-PMSF/1 mM-EDTA, pH 7.4. Fractions of 20 ml were collected at 200 ml/h and assayed for 280 nm absorption. Arrows indicate V_0 and V_t. Fractions were pooled as indicated. (b) Fractions from a second heparin–Sepharose CL-6B chromatography after chromatography on Sepharose CL-6B (see above) were chromatographed on Sephacryl S-300 (2 cm × 100 cm) with the same eluant as described under (a). Fractions of 4 ml were collected at 50 ml/h and assayed for 280 nm absorption. Arrows indicate V_0 and V_t. Fractions were pooled as indicated and investigated on SDS/PAGE. (c) SDS/PAGE of the purifed 78 kDa protein. Molecular mass standards are given in kDa. Gel staining was carried out with Coomassie Blue.

4 ml were collected at 50 ml/h, analysed for 280 nm absorption and pooled as indicated in Fig. 2(b). Since the purified protein was easily degraded by freeze-thawing and standing at room temperature, etc., long-time storage was usually performed in 80 % ethanol at 4 °C, or in 0.01 % mercaptoethanol/50 % glycerol/ 1 mM-PMSF/1 mM-EDTA at −20 °C. Determination of the isoelectric point was carried out as described previously [24].

Digestion of the heparin-binding protein with endoglycosidase F (EC 3.2.1.96)

Treatment of the purified heparin-binding protein with endoglycosidase F (EndoF; Boehringer Mannheim, Germany) which cleaves N-linked oligosaccharide chains from proteins, was performed as previously described [23]. The protein was suspended in 20 µl of 100 mM-Tris/ HCl, pH 8. SDS [0.5 % (w/v)] 2-mercaptoethanol [1 % (v/v)], 10 µM-EDTA and 2 µl of EndoF (0.048 unit/µl) were added for 1–2 h at 37 °C. SDS-sample buffer (20 µl, 2-fold concentrated) was added and the incubation mixture was then boiled and applied to SDS/PAGE.

SMC proliferation assays

All SMC tissue cultures were isolated from bovine aortic media. For proliferation assays, tissue cultures of passages 1 or 2 were utilized. SMC proliferation assays

were performed by the method of Castellot et al. [4,5]. For testing antibodies or Fab fragments, 5 µg of antibody or Fab fragments/ml were used in 20 % serum SMC tissue cultures. All inhibition assays were done in triplicate and in at least three independent series of experiments. In all series of inhibition assays positive control experiments were performed with various concentrations of heparin (0.1 µg–1 mg/ml). Maximal growth inhibition by heparin was usually achieved at concentrations of 100 µg of heparin/ml and higher. This inhibition value was then compared with the data obtained with the antibodies and Fab fragments. Negative control experiments were performed with 5 µg of standard goat or standard mouse IgG/ml.

Binding-inhibition assays with SMC

A confluent monolayer of SMC (approx. 1.3×10^6 cells) was washed 5 times with 5 % BSA/0.5 % goat standard IgG in PBS. The cells were incubated with 5 units of heparinase in 1 ml of 5 mM-CaCl$_2$/PBS at 20 °C for 90 min and then washed 5 times with 5 % BSA/0.5 % standard goat IgG in PBS. HR9-medium (8000 c.p.m.) proteoheparan [35S]sulphate (1.5×10^5 c.p.m./µg), from a preparation described earlier [25], was added in 1 ml of 5 % BSA/0.5 % standard goat IgG in PBS in the absence or presence of 5 µg of conventional goat antibody or mAb 38/87/ml. Incubation was continued for 45 min at room temperature and the cells were then washed 10 times with 5 % BSA/0.5 % standard goat IgG in PBS at 0 °C. The cell layer was dissolved in 0.1 M-Tris/HCl/7 M-urea/2 % SDS (pH 7.2) and measured in a scintillation counter.

Binding assays with the heparin-binding protein

Heparin, dextran sulphate, chondroitin sulphate, dermatan sulphate and keratan sulphate were adjusted to concentrations of between 0.5 µg/ml and 5 mg/ml in 10 ml of 1 mM-EDTA/0.1 % BSA/0.1 % Chaps in PBS (basic buffer) and applied to a heparin-binding protein–Sepharose CL-6B column (1 ml; 0.5 cm × 5 cm, approx. 2 mg of protein). The column was washed with 10 ml of the basic buffer and the bound substances were desorbed with 1 M-NaCl in PBS. The polysaccharide content in the unbound and in the bound material was determined by the orcinol reaction [26] with the same polysaccharide, at various concentrations, as standard.

In another experiment, 100 ng of basement-membrane heparan [35S]sulphate [25] or 100 ng of endothelial-cell surface heparan [35S]sulphate [27] (based on the amount of GlcN in the hydrolysate) was applied to the same heparin-binding protein column as described above. The column was first washed with 5 ml of basic buffer and then eluted with a linear gradient (10 ml + 10 ml) of basic buffer and basic buffer +0.5 M-NaCl. Fractions of 500 µl were collected and assayed for 35S radioactivity.

RESULTS

Isolation and partial characterization of the heparin-binding protein from bovine uteri

All isolation procedures were carried out at 0–4 °C in the presence of 0.1 % Chaps and protease inhibitors 1 mM-PMSF and 1 mM-EDTA.

Microsomes or the microsomal supernatant (10000 g supernatant) from bovine uteri were chromatographed
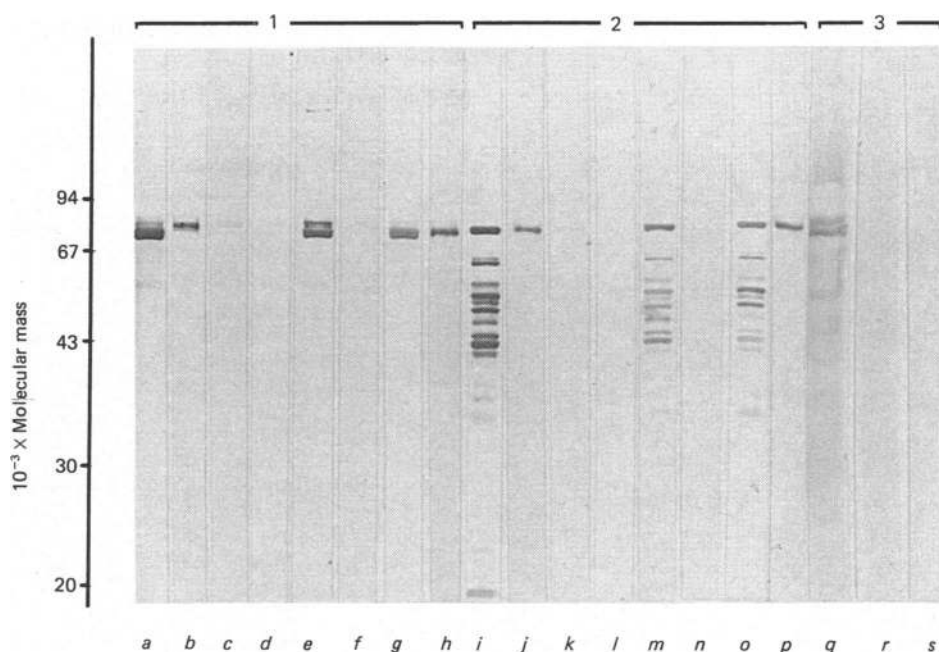
Fig. 3. Western blot analysis of anti-78 kDa protein antibodies

Western blot analysis was performed with purified intact heparin-binding proteins (section 1), proteolytical cleavage products of heparin-binding proteins, obtained after freeze-thawing or standing at room temperature (section 2), and of homogenates of human renal cortex (microsomal fraction, kindly provided by Dr. F. Momburg, Institute of Immunology and Genetics, German Research Cancer Center, Heidelberg, Germany) (section 3). Specific staining was done with mAb 38/87 (lanes *a*, *i* and *q*), mAb 13/83 (lanes *b* and *j*); mAb 45/11 (lanes *h*, *p* and *r*), rabbit anti-(bovine band 4.1) serum (lanes *c* and *k*; generous gift by Dr. J. Madri, Yale University School of Medicine, New Haven, CT, U.S.A.), mAb W6/32 directed against HLA class I antigen (lanes *d* and *l*), mouse fusion serum (lanes *e* and *m*), mouse pre-immune serum (lanes *f*, *n* and *s*) and affinity-purified goat anti-78 kDa protein antibodies (lanes *g* and *o*). As reference proteins, molecular mass standards (Pharmacia) were used.

on heparin–Sepharose CL-6B. Elution of the bound material with a linear NaCl gradient resulted in a broad protein elution peak as indicated by 280 nm absorption. Every fraction was analysed by SDS/PAGE. The material eluting at the beginning of the gradient consisted of many different proteins. These substances were not investigated any further.

Between 0.3 and 0.45 M-NaCl, a dominant protein eluted as a band of approx. 78 kDa in SDS/PAGE with another faint band of slightly higher molecular mass (Fig. 1). Both proteins were co-enriched in all further purification procedures, which involved chromatography on Sepharose CL-6B (Fig. 2*a*) and Sephacryl S-300 (Fig. 2*b*).

As shown in Fig. 3, section 1, for the intact heparin-binding molecule and in Fig. 3, section 2, for proteolytic lysates obtained by freeze-thawing, both proteins are recognized by affinity-purified antibodies from goat anti-serum and also by mAb 38/87. mAb 13/83 specifically detects the upper band. These results suggest that there is structural similarity between the two substances and that the upper band contains an additional domain which represents the epitope for mAb 13/83. mAb 38/87 detected most of the proteolytic fragments of the heparin-binding protein whereas mAb 13/83 reacted only with the intact upper band. mAb 38/87 and mAb 45/11 were applied in Western blots of proteins derived from human kidney lysates (Fig. 3, section 3). Again, both protein bands were stained with mAb 38/87. No band of higher molecular mass was detected in the Western blot analysis

indicating that the 78 kDa protein is not a breakdown product of higher molecular mass compounds.

There is evidence to suggest that the protein is located on the cell surface from immunoprecipitation of surface-radioiodinated HT-29 (human carcinoma cell line) cell lysates (Fig. 4). A band of 78 kDa was precipitated by mAb 38/87. As negative controls, we used mAb 45/11, which did not cross-react to human species and an mAb against HLA class I antigen. Some lower molecular mass proteins were also unspecifically absorbed to the Protein A–Sepharose immunocomplex when using mAb 38/87 and control antibodies.

The molecule has some striking similarities to erythrocyte protein 4.1 as indicated by molecular mass, staining pattern on endothelial cells and SMC and susceptibility to proteolytic degradation. Analogues of this erythrocyte submembraneous skeleton protein have recently been described for lymphocytes [28], fibroblasts [29] and endothelial cells [30,31]. In Western blot analysis with an anti-serum against bovine band 4.1 from a preparation described previously ([30] a generous gift of Dr. Joseph A. Madri) we did not observe an affinity of this anti-serum to the 78 kDa protein stronger than the background signal obtained with the negative control antibodies.

The protein turned out to be very sensitive to proteolytic attacks. Freeze-thawing or standing at room temperature for several days resulted in breakdown of the molecule. The protein aggregated and precipitated rapidly in the absence of detergents and in low-salt
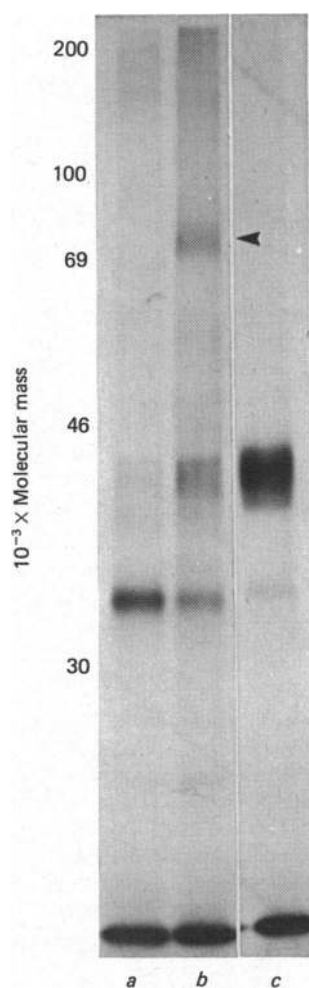
Fig. 4. Immunoprecipitates of surface-labelled human carcinoma cells HT-29

SDS/PAGE analysis under reducing conditions was performed with immunoprecipitates of antibodies against the heparin-binding protein (mAb 45/11, lane *a*; mAb 38/87, lane *b*) and HLA class I antigen (mAb W632, lane *c*) with lactoperoxidase ^{125}I-labelled cell lysates derived from cell line HT-29. The arrow indicates the precipitated band of the 78 kDa protein. As reference proteins, ^{14}C-labelled molecular mass standards (Amersham & Buchler, Braunschweig, Germany) were used.

Table 1. Amino acid and amino sugar analysis of the heparin-binding protein

All numbers are related to 1000 amino acid residues.

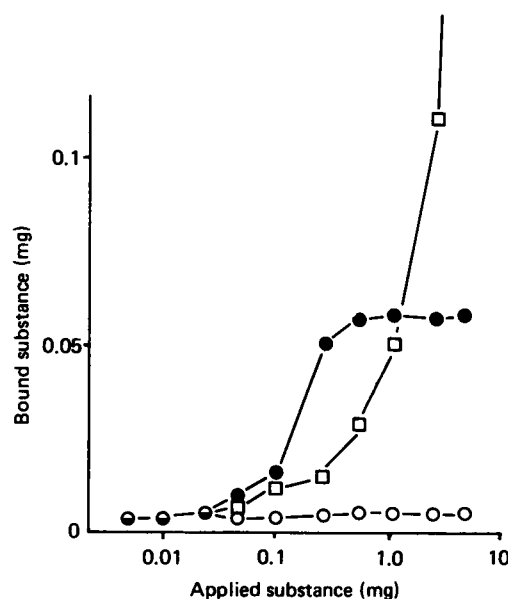| Amino acid	
Asp	98
Thr	53
Ser	67
Glu	167
Pro	57
Gly	77
Ala	78
Val	51
Met	7
Cys	9
Ile	38
Leu	82
Tyr	24
Phe	35
His	19
Lys	83
Arg	56
GalN	< 0.1
GlcN	5

Fig. 5. Binding of various polyanions to the 78 kDa protein

Polyanions such as heparin (●), dextran sulphate (□), hyaluronic acid, chondroitin sulphate, dermatan sulphate and keratan sulphate (○) were adjusted to concentrations of 0.5 μg/ml–5 mg/ml in 10 ml of 1 mM-EDTA/0.1% BSA/0.1% Chaps in PBS and applied to a 78 kDa protein–Sepharose CL-6B column (1 ml, approx. 2 mg of protein). The column was washed with 10 ml of the same buffer and the bound substances were desorbed with 1 M-NaCl in PBS. The eluate was investigated for carbohydrate content with the orcinol reaction, compared with quantitative standards of the same polyanion.

buffers. The amino acid composition is given in Table 1. Glucosamine, but not galactosamine, was detected. Since *O*-linked glycoprotein oligosaccharides contain galactosamine, we conclude that the oligosaccharide moiety is Asn-*N*-linked. Glucosamine was present in amounts of four GlcN or as one *N*-linked complex-type oligosaccharide per molecule. The isoelectric point was found to be 5.2.

Binding specificity of heparin to the 78 kDa protein

Binding of glycosaminoglycans or other polyanions to a protein as the heparin-binding protein from bovine uteri may simply be due to an ion-exchange effect or cross-linking interactions by divalent cations such as calcium (for review see [32]). A lot of proteins will bind to heparin at low pH or in the presence of Ca and this may not be related to any physiological process.

We defined specificity of heparin binding to the 78 kDa protein in the following way: binding of heparin is not only due to ion-exchange and cross-linking effects (taking place at high pH and in the presence of EDTA),
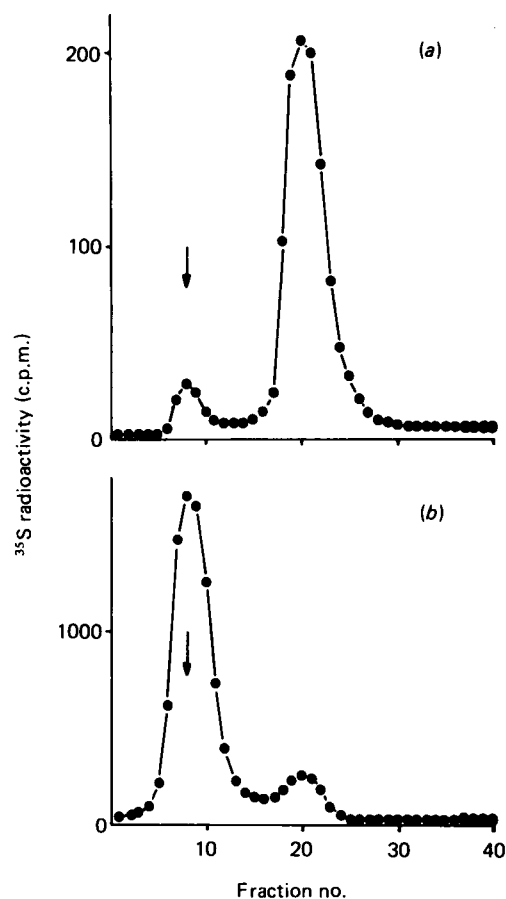
Fig. 6. Binding assay with endothelial cell-surface proteoheparan sulphate (a) and a basement-membrane proteoheparan sulphate from HR-9 cells (b)

Proteoheparan sulphate 100 ng in 1 mM-EDTA/0.12% BSA/0.1% Chaps in PBS (basic buffer) was absorbed to the same column as described under Fig. 5 and eluted with a linear gradient of basic buffer and basic buffer + 0.5 M-NaCl (10 ml + 10 ml). Fractions of 500 μl were collected and analysed for radioactivity. The arrow indicates the start of the gradient.

but also heparin binds to the protein with a higher affinity than other related polyanions.

In order to select the conditions which allow discrimination between non-specific and specific interactions we chose the following conditions for binding assays with heparin: (1) pH higher than 7.0; (2) presence of other proteins (BSA) and detergents (Chaps); (3) presence of chelating agents (EDTA). For binding assays with heparin and various other polyanions we constructed a 78 kDa protein–Sepharose CL-2B column. The polyanions were applied in 10 ml portions and at various concentrations and were measured in the eluate by the orcinol reaction.

Under these conditions we determined a saturable heparin-binding characteristic (Fig. 5). We did not observe binding in any of the other common glycosaminoglycans from mammalian tissue. Dextran sulphate, a polyanion which does not occur physiologically, binds to the 78 kDa protein in a non-saturable fashion (Fig. 5).

The binding of the 78 kDa protein to polyanions is not

only selective to heparin over other glycosaminoglycans but also has a binding specificity to certain heparan sulphate structures. A basement-membrane proteoheparan sulphate from HR-9 cells and a cell-surface proteoheparan sulphate from bovine aortic endothelial cells were first applied to a 78 kDa protein column and then desorbed with a linear gradient of 0–0.5 M-NaCl (Fig. 6). There is a much stronger affinity of the basement-membrane component compared with the cell-surface proteoheparan sulphate from bovine aortic endothelial cells.

Localization of the 78 kDa protein

Affinity-purified goat antibodies and mAbs against the 78 kDa protein were used to investigate the localization of this protein in tissues and cells by histo-immunofluorescence. This protein seems to be widely distributed on endothelial and epithelial cells as well as on skeleton muscle and SMC (Fig. 7). From the staining pattern we conclude that the 78 kDa protein is either located on the cell surface or approximate to it. As two examples we show immunofluorescent staining with conventional goat antibodies on skeleton muscle and on tissue-cultured SMC. Since the human T leukaemia cell line Jurkat did not react with mAb 38/87 against the heparin-binding molecule in cytofluorographic analysis (Fig. 8), we presume that this antigen is not present on blood cells. This result also underlines the dissimilarity to band 4.1. In contrast, the human colon carcinoma cell line HT-29 [33], gave a strong fluorescence staining with mAb 38/87 (Fig. 8). Cell line HT-29 shows some variety in intensity of fluorescence after labelling with mAb 38/87. This may point either to heterogeneity within the cell population or to cell-cycle dependence of antigen expression. In the same assay mAb 13/83 did not stain HT-29 cells or Jurkat cells.

Reaction of SMC with anti-78 kDa antibodies

SMC were seeded in e.l.i.s.a. plates and the titre of affinity-purified goat antibodies was determined in comparison with 78 kDa protein-coated wells. As shown in Fig. 9, there are parallel titres against the 78 kDa protein and the tissue-cultured SMC. SMC were grown to confluence (approx. 50000 cells/cm²) and digested with heparinase for 90 min to remove bound heparan sulphate. Cells were then incubated with 50 ng of a basement-membrane proteoheparan [35S]sulphate from a preparation described previously [25] in the absence or presence of affinity-purified goat antibodies or mAbs against the 78 kDa protein. As shown in Fig. 10, binding of the proteoheparan [35S]sulphate to SMC is inhibited by 70% in the presence of goat antibodies and by 40% in the presence of mAb 38/87.

We wanted to investigate whether the growth inhibitory effect of heparin on SMC can be influenced by antibodies against the 78 kDa protein. SMC were coated with affinity-purified goat antibodies, their Fab fragments or mAbs before addition of heparin. Surprisingly, treatment with the antibodies or the Fab fragments alone inhibited SMC growth; results are given in Fig. 11. As a heparin standard we applied the lowest heparin concentration which gave maximal SMC growth inhibition, typically 100 μg/ml. With the exception of mAb 13/83, all antibodies as well as Fab fragments from goat antibodies inhibited SMC proliferation. The same proteoheparan sulphates which have been investigated for
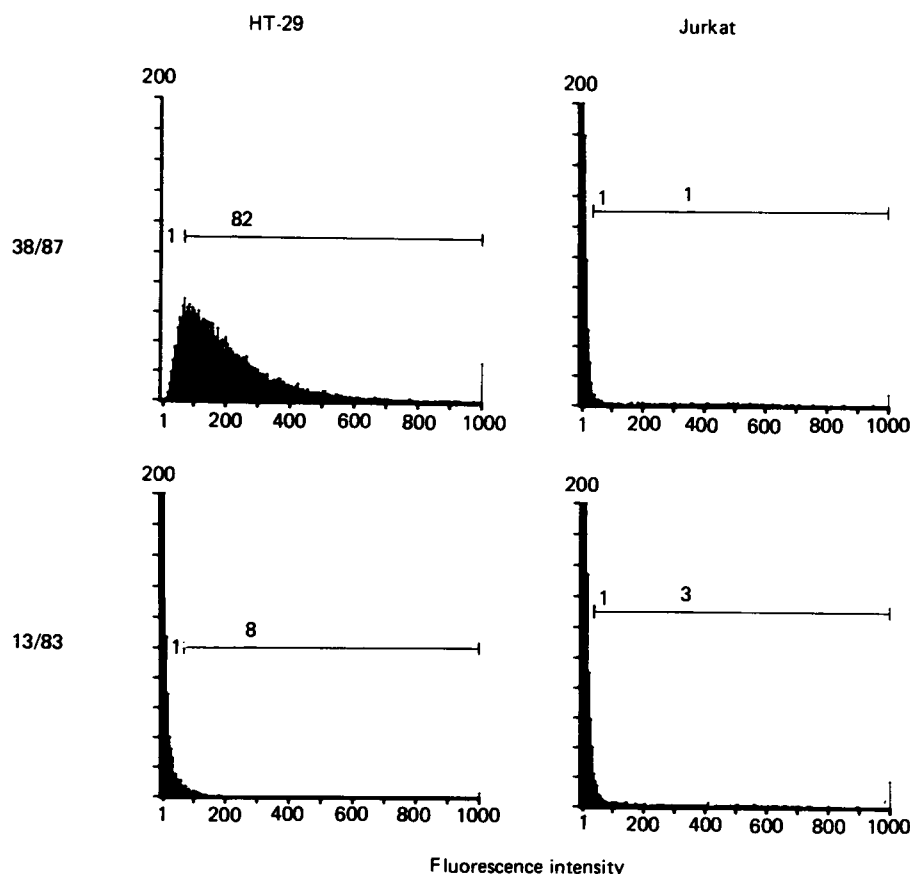
(a)

(b)
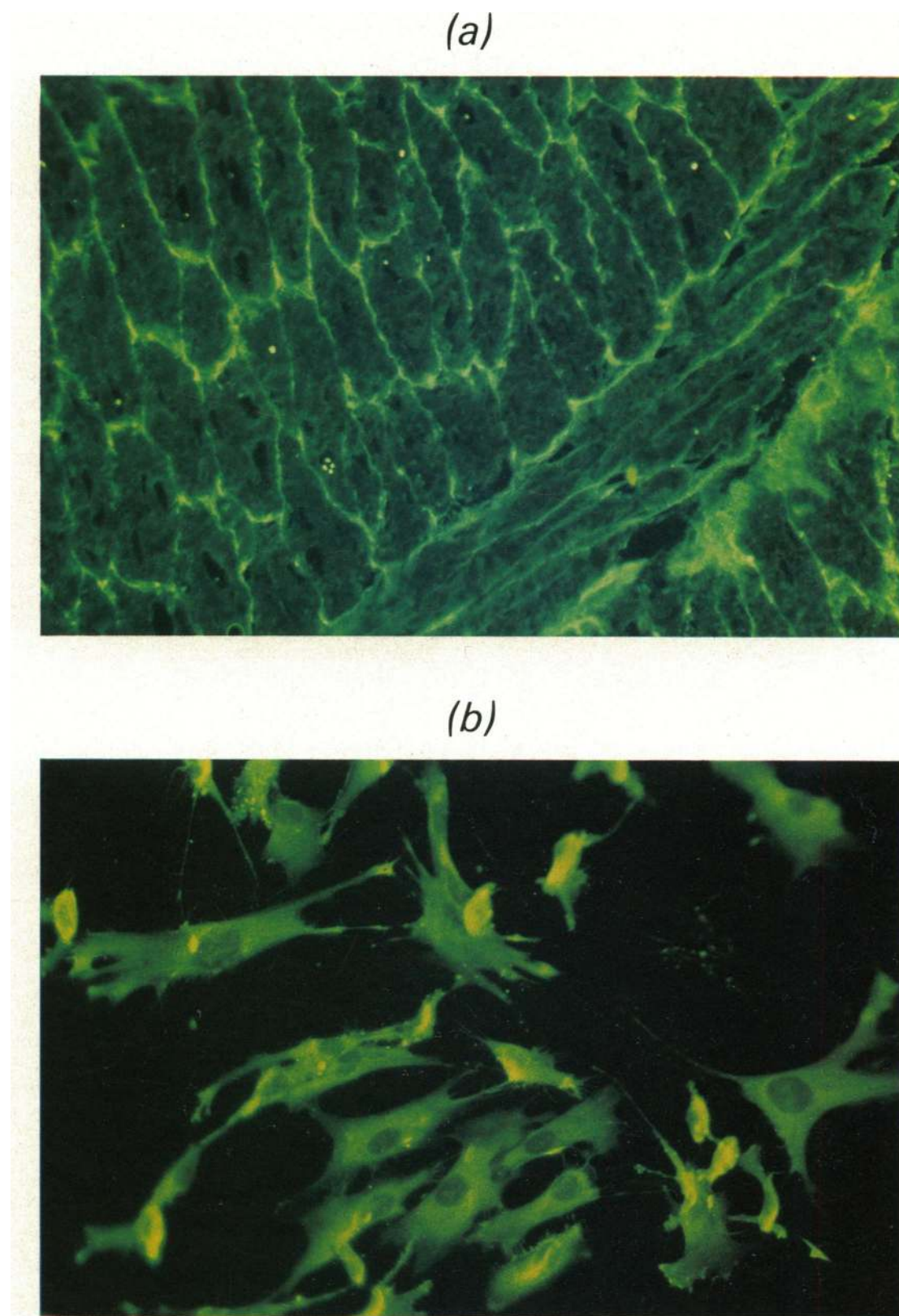
Fig. 7. Indirect immunofluorescence with goat anti-78 kDa protein antibodies

Frozen sections of heart muscle magnified ×154 (*a*) and methanol fixed, tissue-cultured bovine aortic SMC magnified ×620 (*b*) were stained with affinity-purified goat anti-78 kDa protein antibodies. FITC-coupled rabbit anti-(goat IgG) was used as a secondary antibody.

Fig. 8. Cytofluorographic analysis of human cell line HT-29 and human T lymphoma cell line Jurkat

Histograms from cytofluorographic analysis were performed after indirect immunofluorescence reactions with mAbs 38/87 and 13/83. Background controls were done by incubating cells with the second antibody only [FITC-coupled rabbit anti-(mouse IgG) IgG]. For each histogram 1.5×10^4 cells were analysed; numbers in each histogram represent the percentage of positive cells within a region (channel 30–1000) minus the percentage of background controls (5–7%). The channel numbers indicated at the x-axis correlate with fluorescent intensity of cell staining; the y-axis gives the number of cells stained.

their binding to the isolated 78 kDa protein were applied in SMC proliferation assays in concentrations of 100 μg of polysaccharide/ml, based on molar amount of GlcN in the hydrolysate. There is only very little growth inhibition on SMC as determined by the endothelial cell-surface proteoheparan sulphate ·in contrast with the basement-membrane component which inhibits SMC proliferation in the same range as heparin.

DISCUSSION

We have isolated a 78 kDa protein from bovine uteri with specific affinity to heparin. The protein was present in the microsomal pellet of the uterus homogenate. Since affinity, in the case of polyanion–protein interactions, may be a general phenomenon of ion-exchange and cross-linking effects, we have determined the specificity of the heparin–78 kDa protein interaction in terms of selectivity towards heparin over other glycosamino-glycans from mammalian tissue. Only dextran sulphate interacts with the 78 kDa protein in a non-saturable fashion.

For the following reasons this protein may be looked at as a possible heparin or heparan sulphate receptor: (1) the binding of heparin to the protein is specific and

saturable, (2) antibodies against this protein inhibit binding of heparan [^{35}S]sulphate to SMC, (3) antibodies against this protein and their Fab fragments inhibit SMC proliferation.

It has been described in the case of the epithelial growth factor receptor, that antibodies against the receptor protein can mimic the effect of the natural ligand [34,35]. It may be, in the case of the 78 kDa protein, that the antibodies against the 78 kDa protein simulate the effect of heparin as a ligand. The anti-proliferative effect of the antibodies is probably not due to cross-linking of the SMC surface, because Fab fragments show similar activity in SMC proliferation assays compared with intact antibodies.

However, some problems remain to be resolved. The basis for the molecular heterogeneity of the 78 kDa protein is not clear. It is probably not due to different degrees of glycosylation since digestion of the protein with endoglycosidase F, an enzyme which removes N-linked glycoprotein oligosaccharides, did not eliminate the faint band of slightly higher molecular mass (results not shown). By the cross-reactivity of some mAbs to both molecules it is suggested that there is structural similarity between the two proteins. It may be that the main 78 kDa band represents a proteolytic fragment of
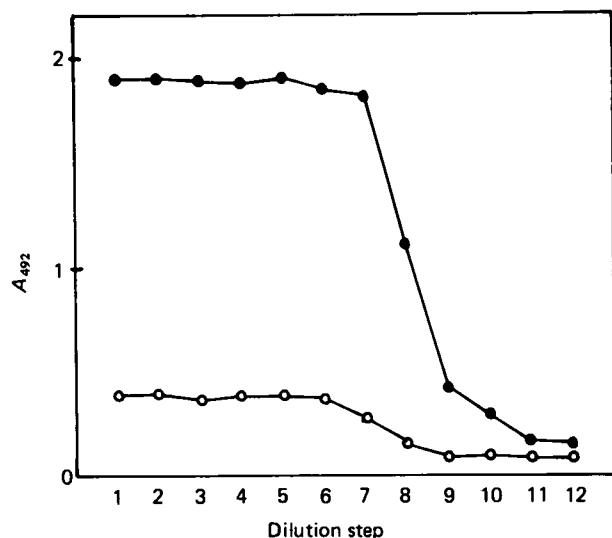
Fig. 9. Titre of goat anti-78 kDa protein antibodies against tissue-cultured SMC and the 78 kDa protein

Microtitre wells were either coated with 1 μg of the 78 kDa protein/ml (●), or seeded with 1000 SMC/well (○). Cells were fixed with methanol and then processed in the routine e.l.i.s.a. procedure. Dilutions were carried out in 12 doubling dilutions. Controls were performed with 78 kDa protein- or SMC-coated wells by omitting the anti-78 kDa protein antibodies. No significant titres were observed in the controls.

Fig. 10. Effect of anti-78 kDa protein antibodies on the binding of proteoheparan [^{35}S]sulphate to SMC in culture

Approx. 1.3×10^6 confluent SMC were incubated with 5 units of heparinase for 90 min, washed, and then incubated with 50 ng of basement-membrane proteoheparan [^{35}S]sulphate (1.5×10^5 c.p.m./μg) in 5% BSA/0.5% standard goat IgG in PBS in the absence (a) or presence of mAb 38/87 (b) or affinity-purified goat anti-78 kDa protein antibodies (c) (5 μg/ml each) for 45 min. The cell layer was washed, dissolved, and assayed for radioactivity.

the original protein of slightly higher molecular mass. The protein preparations turned out to be extremely sensitive towards proteolytic cleavage. However, more detailed studies such as peptide mappings are needed in order to elucidate the structural relationship between the two protein bands.

The 78 kDa protein is only soluble in higher salt concentrations and in the presence of detergents, which are properties typical of membrane proteins. We have detected this protein on the surface of human carcinoma cells HT-29 by surface labelling with ^{125}I and by cytofluorographic analysis. This indicates that the 78 kDa protein may be an essential part of the cell surface. Although these results are indicative of a cell-surface-associated membrane protein, we could not yet collect conclusive evidence for a model in which the 78 kDa protein serves as a transmembraneous receptor for heparin or heparan sulphate.

Liposome binding is in some cases regarded as a further indication of the membrane association of a protein. The 78 kDa protein indeed binds to liposomes (results not shown). However, we do not consider this as evidence of a transmembraneous structure for the protein since liposome studies have to be carried out in the absence of detergents. These are conditions under which the protein will precipitate or unspecifically stick to various supramolecular structures including liposomes. For these reasons, we were not able, as yet, to demonstrate a membrane association of the 78 kDa protein. We feel that the only way to elucidate a possible transmembraneous structure, is to investigate the amino acid sequence of the heparin-binding protein and look for the presence or absence of a transmembraneous domain.
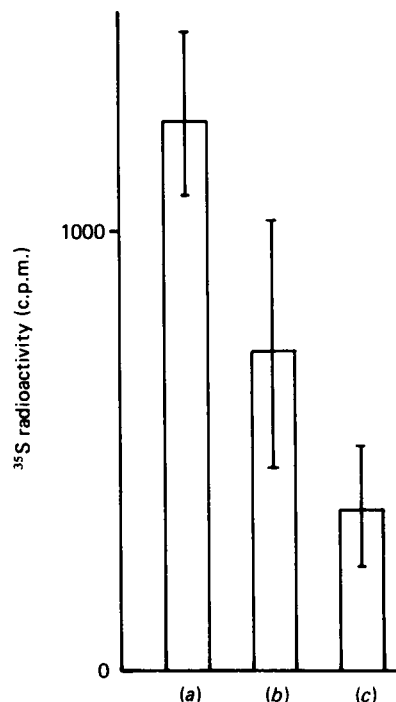
The 78 kDa protein is widely distributed and not only present on SMC. From our histo-immunological investigations its seems to be present in all cells which grow associated to basement-membrane structures. Furthermore, basement-membrane proteoheparan sulphate from HR-9 cells [25] binds to the 78 kDa protein with a much higher affinity than endothelial cell-surface proteoheparan sulphate from another preparation [27]. Both proteoheparan sulphates are eluted from DEAE–Sephacel with the same ionic strength. Inhibition studies with the same proteoheparan sulphates show that only the basement-membrane components have an inhibitory effect on SMC at polysaccharide concentrations of 100 μg/ml. On the basis of these findings, it is possible that the 78 kDa protein belongs to a group of cell-membrane proteins which promote binding of cells to basement-membrane molecules [36,37], and in this particular case, to basement-membrane proteoheparan sulphate. Therefore, the anti-proliferative effect of heparin on the proliferation of SMC may eventually be one phenomenon within the modulating effects of extracellular matrix [38,39] on cell behaviour and cellular phenotype.

One reason for the heparin effect on SMC proliferation gaining so much attention from other scientists, is that it offers the possibility of treating diseases which are
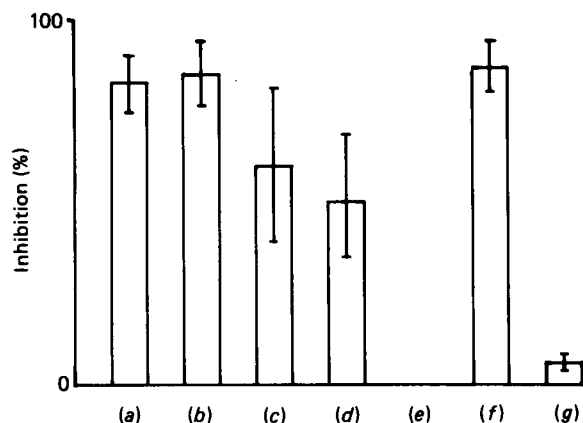
Fig. 11. SMC growth inhibition by heparin, heparan sulphate, anti-78 kDa protein antibodies and their Fab fragments

The proliferation assay was performed according to Castellot et al. [4]. SMC were converted into a growth-arrested state by culturing with 0.5% fetal-calf serum (low serum) for 3 days. Some of the SMC cultures were maintained in low serum (low-serum control). To the other SMC cultures medium with 20% serum (high serum) was added in the absence (high-serum control) or presence of 100 μg heparin/ml (a), affinity-purified goat anti-78 kDa protein antibodies (b), Fab fragments of these antibodies (c), mAb 38/87 (d), mAb 13/83 (e) (5 μg/ml each), 100 μg of basement-membrane heparan sulphate from HR-9 cells/ml (f) or 100 μg of cell-surface heparan sulphate from bovine aortic endothelial cells/ml (g). Cells were cultured for 3 days and counted. High-serum control cell numbers were set as 0% inhibition and low-serum control cell numbers as 100% inhibition. Standard goat IgG or standard mouse IgG in concentrations of 5 μg/ml were added to high-serum cultures as a negative control.

accompanied by SMC growth and change in phenotype with a non-anticoagulant heparin fraction.

The purification of a heparin-binding protein involved in SMC growth inhibition, as described here, allows study of the interaction between heparin and the SMC surface on a molecular level. The isolation procedure of the antithrombin III binding fragment [40] offers the opportunity to construct affinity matrices for the isolation of the heparin oligosaccharide responsible for the inhibition of SMC proliferation.

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