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Expression of blood clotting Factor VIII:C gene in capillary endothelial cells

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The essential role of Factor VIII:C (FVIII:C, anti-hemophilia factor A) as a cofactor for Factor IX_a-dependent activation of Factor X has been established. In this paper, we describe that capillary endothelial cells from bovine adrenal medulla express active FVIII:C gene. Accumulation of FVIII:C in conditioned media from an 8-day-old culture is approximately twice as high as that stored in the cell when immunoprecipitated FVIII:C was analyzed for its ability to convert Factor X to Factor X_a. Analysis of [^{35}S]methionine-labeled and immunoprecipitated FVIII:C from cells or conditioned media on SDS-PAGE under fully denatured conditions indicated that the newly synthesized FVIII:C consists of heavy chain of M_r 200,000 and light chain of M_r 46,000. The secreted FVIII:C in the non-reduced condition however, has a molecular weight of 270,000 which suggests that in native protein, the heavy and light chains are held together by S–S bonds. Furthermore, susceptibility of the immunoprecipitated FVIII:C to *N*-glycanase digestion establishes that the endothelial cells derived FVIII:C contains asparagine-linked carbohydrate side chains.

Blood coagulation factor VIII:C; Capilliary endothelial cell; Gene expression; *N*-linked glycoprotein; Disulphide bridge; Adrenal medulla

1. INTRODUCTION

Factor VIII:C, (FVIII:C) is an essential cofactor for Factor IX_a dependent activation of Factor X and itself can be activated proteolytically by a variety of coagulation enzymes including thrombin [1]. Its plasma concentration is extremely low (50–150 ng/ml) and deficiency of FVIII:C leads to classic hemophilia (hemophilia A), a bleeding disorder that affects 10–20 per 100,000 males. The disease is transmitted by X-chromosomal inheritance [2] in which blood clots slowly, prothrombin is converted to thrombin at an abnormally slow rate and a rebleeding phenomena is frequently seen. The cloning of cDNA and the gene for human plasma Factor VIII:C has provided some information about its gene structure [3–6]. Various sites for the biosynthesis of FVIII:C have been proposed. Reports of liver transplantation in patients with hemophilia and end-stage liver disease [7,8] have supported the earlier animal studies [9] about the role of liver in FVIII:C synthesis. Northern blot analysis with Factor VIII:C cDNA probes has demonstrated that liver, placenta, kidney [3,10], spleen, lymph node, pancreas, muscle and fetal heart [11] contain Factor VIII:C mRNA. Several cell types or tissues on the other hand appear to lack Factor VIII:C mRNA when examined by immunochemical methods using light or electron microscopy [12,13]. FVIII:C, however, has been localized in hepatic sinusoidal endothelium by immunoperoxidase staining using a monoclonal antibody to FVIII:C [14].

In the present paper we describe that the capillary endothelial cells from bovine adrenal medulla synthesize FVIII:C. It is a constitutive asparagine-linked glycoprotein which converts Factor X to Factor X_a in the presence of Factor IX_a, phospholipid and Ca^{2+} and is distinct from the von Willebrand Factor (vWF).

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Monoclonal antibody (IgG1k) to human FVIII:C (lack cross-reactivity with vWF present in hemophilic plasma) was obtained from Boehringer-Mannheim. COATEST Factor VIII:C assay kit was a product of Helena Laboratories. [^{35}S]Methionine and [^{14}C]methylated protein standards were purchased from Amersham. Immobilon was a gift from Millipore Corporation. vWF Immunoblot was supplied by Ramco Laboratories, Inc. *p*-Nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolylphosphate-toluidine salt (BCIP), SDS, acrylamide, bis-acrylamide, TEMED, Tween 20 and β-mercaptoethanol were purchased from Bio-Rad Laboratories. Peroxidase conjugated anti-rabbit mouse IgG was a product of Miles Laboratory, 3,3'-diaminobenzidine, PMSF, TPCK, soybean trypsin inhibitor, aprotinin, leupeptin, cycloheximide and DTT were obtained from Sigma Chemical Co. Actinomycin D was a product of Calbiochem. Minimal essential medium with Earle's salt (EMEM), glutamine, trypsin-versene and penicillin-streptomycin were supplied by Biofluides Inc. Fetal bovine serum (FCS) was a product of HyClone Laboratories. Protein A-Sepharose CL-4B was obtained from Pharmacia. *N*- and *O*-glycanases were purchased from Genzyme. All other chemicals were of highest purity grade.

2.2. *Culturing of endothelial cells*

The stock culture of capillary endothelial cells was maintained in EMEM containing 10% FCS (heat-inactivated), glutamine (2 mM),

penicillin (50 units/ml) and streptomycin (50 μg/ml) and subcultured once a week as described before [15,16]. Cells with 80–99% confluency were used in this study.

2.3. *Measurement of FVIII:C activity in endothelial cell lysate*

The generation of activated Factor X by Factor IX_a is greatly stimulated by FVIII:C which is considered as a cofactor in this reaction [17–19]. The assay is designed to give a linear correlation between the amount of generated Factor X_a and the FVIII:C content [20]. Factor X_a is then determined by measuring the release of *p*-nitroaniline from a chromogenic substrate. In a typical bioassay (COATEST Assay), immunoprecipitated FVIII:C from different samples were used for acid-stopped method as described in the supplier's manual.

2.4. *Biosynthesis of Factor VIII:C*

The cells were cultured for 8 days in complete EMEM, washed with methionine-free and serum-free media and labelled with [^{35}S]methionine (30–40 μCi/ml) in methionine-free and serum-free medium containing 1 μg/ml of aprotinin at 37°C. At an appropriate time the medium was removed, and the cells were lysed. Aliquots of conditioned media and cell extracts were immunoprecipitated with a mouse monoclonal antibody to FVIII:C (1:40 dilution) for 3 h at 4°C followed by 12 h with 50 μg/ml of Protein A-Sepharose CL-4B (30 mg/ml) in the presence of PMSF (1 mM), TPCK (200 μM), soybean trypsin inhibitor (1 μg/ml) and leupeptin (1 μM). The Protein A-Sepharose:antigen–antibody complexes were washed twice with NET buffer, twice with washing buffer, and once with PBS, pH 7.4. The immunecomplexes were then analyzed by SDS-PAGE (7.5 or 10%) followed by autoradiography. For non-reducing gels, β-mercaptoethanol was omitted from the sample buffer as well as from the washing buffer. To study the effect of actinomycin D or cycloheximide, the cells were preincubated with actinomycin D (2 μg/ml) or cycloheximide (1 μg/ml) for 1 h at 37°C. The rest of the procedure was the same as described above.

2.5. *N- and O-glycanase digestion of Factor VIII:C*

The immunoprecipitated samples were suspended in 50 μl of 20 mM sodium citrate, boiled for 5 min and then incubated with *N*-glycanase (5 U/ml) for 2 h [21]. For *O*-glycanase digestion, the immunoprecipitated FVIII:C samples were solubilized in Tris-Buffer (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 0.025% SDS, 0.25% DTT, 5 mM EDTA) and then incubated with 100 mU/ml of *O*-glycanase at 37°C for 24 h in a total volume of 55 μl [21]. The samples were then prepared for SDS-PAGE as described above.

2.6. *Immunoblotting of von Willebrand Factor*

The proteins in cell lysate and conditioned media were separated on 10% SDS-PAGE according to the procedure of Laemmli [22] and then transferred to Immobilon at 70 V for 18 h in Tris-glycine buffer system [23,24]. After electrotransfer and blocking, the membrane was incubated with alkaline phosphatase-conjugated anti-von Willebrand Factor antibody (1:500 dilution) for 2 h at room temperature. The blot was then washed and the color was developed with NBT and BCIP.

3. RESULTS AND DISCUSSION

3.1. *Biological activity of endothelial cell derived FVIII:C*

To support the observation that these endothelial cells are synthesizing FVIII:C in active form, immunoprecipitated FVIII:C from the cell lysate as well as from the conditioned media were subjected to a chromogenic assay where the conversion of Factor X to Factor X_a has been quantitated by measuring the proteolytic release of *p*-nitroaniline bound to a synthetic peptide (S-2222). The release of *p*-nitroaniline was quantitative and dependent upon the amount of the cell lysate

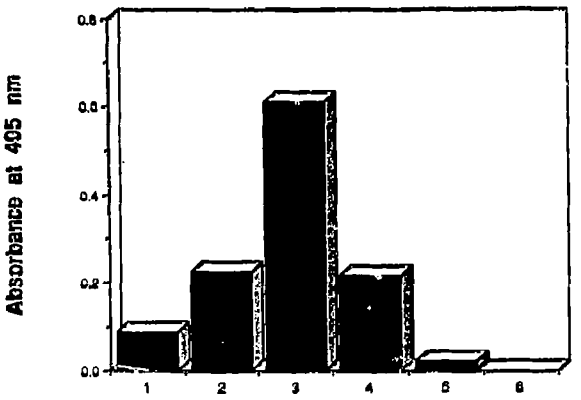

Fig. 1. Measurement of FVIII:C activity. Samples from cell lysate, conditioned media, bovine plasma and human plasma were immunoprecipitated with anti-FVIII:C antibody as described in section 2 and assayed for FVIII:C-dependent Factor X_a generation by measuring *p*-nitroaniline spectrophotometrically at 405 nm. 1, cell lysate (200 μl); 2, conditioned media (200 μl); 3, fresh bovine plasma (20 μl); 4, stored human plasma (20 μl); 5, cell culture media containing 10% fetal bovine serum (200 μl); 6, cell lysate containing 1 μg/ml of aprotinin (200 μl).

or conditioned media present in the assay. The results as summarized in Fig. 1 indicated that both cell lysate and the conditioned media contained active FVIII:C (lanes 1 and 2). Factor X_a dependent generation of *p*-nitroaniline, however was twice as high in the conditioned media as in the cell lysate. Both human and bovine plasma controls also showed 10- to 30-fold higher activity (lanes 3 and 4). Since, the release of *p*-nitroaniline from the chromogenic substrate (S-2222) is a proteolytic cleavage, therefore, collection of cell lysate or conditioned media in the presence of aprotinin, a protease inhibitor completely inhibited the FVIII:C dependent Factor X_a activity (lane 6). In addition, since the culture media contained 10% fetal bovine serum, therefore, quantitation of serum borne FVIII:C in the cell culture media indicated the presence of only 1/10th of what has been observed in the conditioned media [5].

The monoclonal antibody used here is specific for FVIII:C and hence, non-interference of FVIII:C dependent Factor X_a production by the immunecomplexes helped in using a cleaner preparation free from contaminating proteases. This is possible only because of antibody recognition site is different from its catalytic site. In addition, use of a complete reaction mixture minus FVIII:C immunoprecipitate as a blank eliminated the possibility of tissue factor/Factor VII as possible contaminants in these preparations. Furthermore, increased level of FVIII:C in the conditioned media ruled out cellular acquisition of Factor VIII:C from the serum present in the culture media.

3.2. *De-novo synthesis of FVIII:C in the capillary endothelial cells*

In order to demonstrate that FVIII:C is indeed made

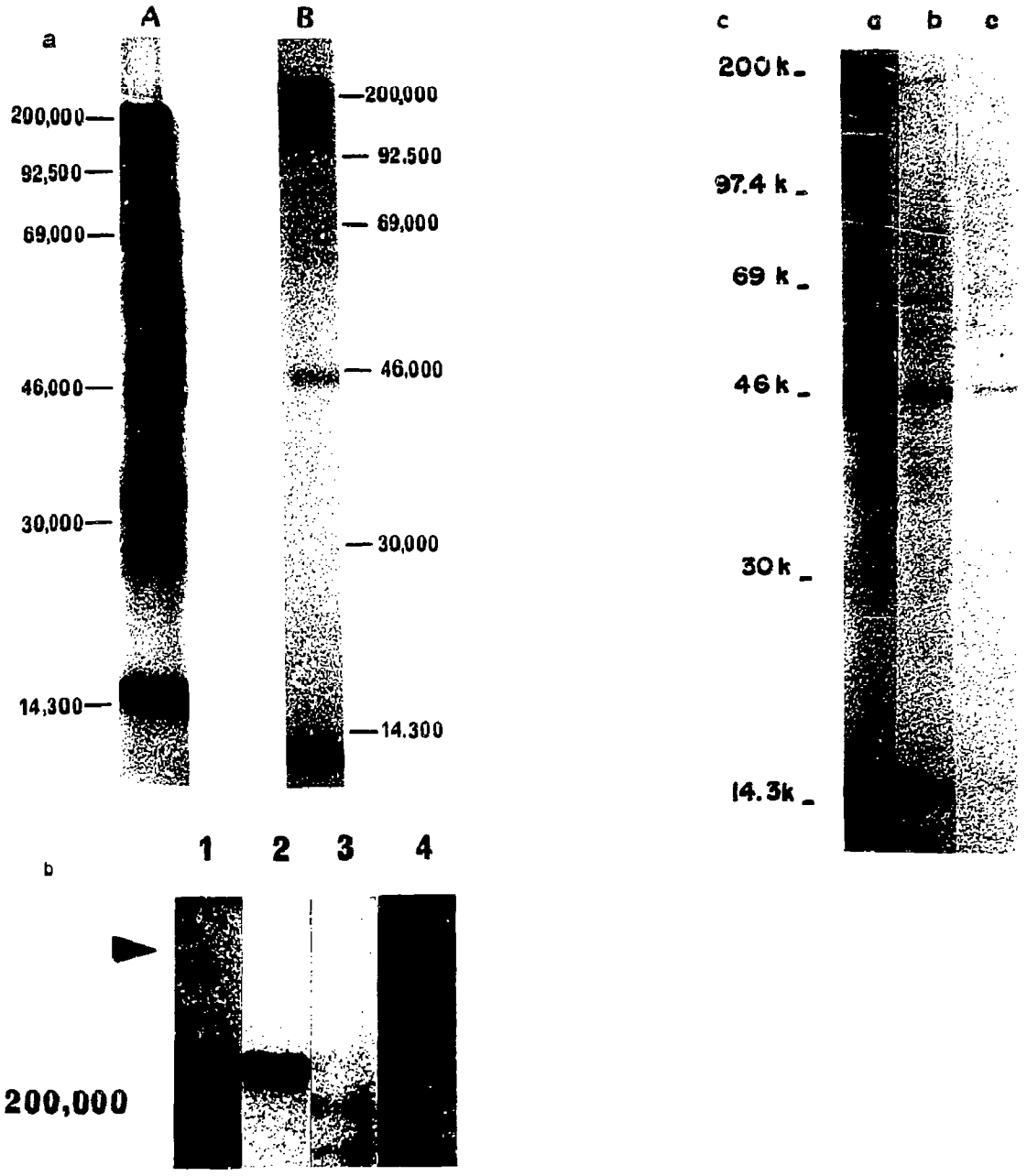

Fig. 2. Synthesis of FVIII:C in capillary endothelial cells. Cells were grown for eight days in 60 mm dish and labelled with [^{35}S]methionine (30 μCi/ml, sp.act. 1400 Ci/mmol) for 2 h at 37°C as described in section 2. Equal aliquots from each sample were analyzed on SDS-PAGE after immunoprecipitation with anti-Factor VIII:C antibody. (a) Detection of Factor VIII:C before and after immunoprecipitation against anti-Factor VIII:C antibody on 7.5% SDS-PAGE under reduced condition; lane A, before immunoprecipitation; lane B, after immunoprecipitation. (b) Migration of FVIII:C on SDS-PAGE under reduced (lanes 1 and 2) and non-reduced (lanes 3 and 4) conditions; lane 1, cell lysate; lane 2, conditioned media; lane 3, cell lysate; lane 4, conditioned media. (c) Effect of actinomycin D and cycloheximide on FVIII:C biosynthesis. The cells were treated with actinomycin D (2 μg/ml) or cycloheximide (1 μg/ml) and then labelled with [^{35}S]methionine (30 μg/ml, sp.act. 1400 Ci/mmol) as described in section 2. The immunoprecipitates were then analyzed on a 10% SDS-PAGE. Lane 1, control; lane 2, actinomycin D; lane 3, cycloheximide.

by these cells, the cells were labelled with [^{35}S]methionine. The labelled proteins when analyzed by SDS-PAGE under reduced condition followed by autoradiography showed the presence of a number of protein species ranging from M_r 200,000 to M_r 15,000. After immunoprecipitation with mouse monoclonal antibody to FVIII:C only M_r 200,000 and M_r 46,000 species were detected (Fig. 2a, lanes A and B). A somewhat diffuse band close to M_r 14,300 in lane B and right at the dye front has been noted whose relationship to M_r 200,000 and M_r 46,000 has not yet been understood except that it is recognized by the antibody. To investigate the size of FVIII:C further, the immunoprecipitates were subjected to SDS-PAGE under both reducing and non-

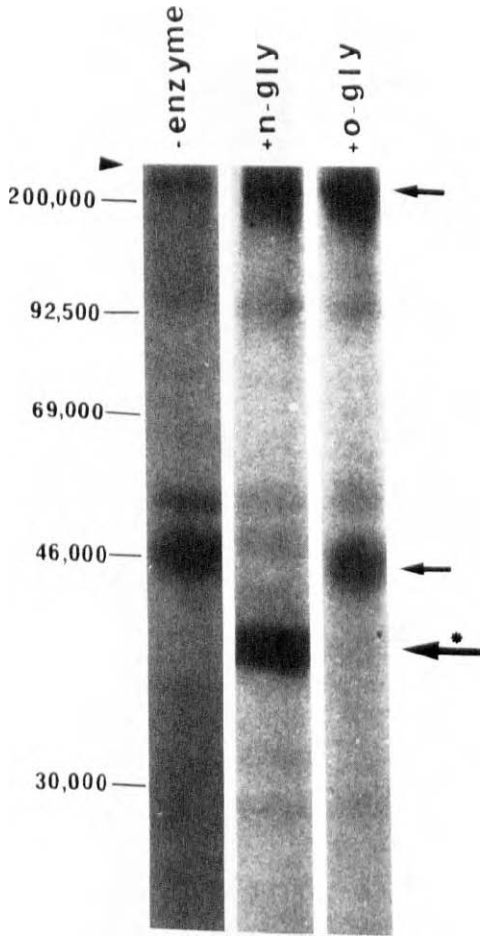

Fig. 3. *N*- and *O*-glycanase digestion of secreted FVIII:C. Cells were cultured for eight days in 60 mm dishes, labelled with [^{35}S]methionine (30 μCi/ml, sp.act. 1400 Ci/mmol) for 2 h at 37°C and the conditioned media was immunoprecipitated with anti-FVIII:C antibody as above. The immunoprecipitates were then digested with *N*- and *O*-glycanases. – enzyme, control; +n-gly, digested with *N*-glycanase; +o-gly, digested with *O*-glycanase.

reducing conditions. Under fully reduced condition, heavy chain of M_r 200,000 and light chain of M_r 46,000 were present in cell lysate (Fig. 2b, lane 1). A minor band just below the M_r 200,000 band has been seen particularly in this preparation which is most likely generated either by a mild proteolysis or by incomplete processing. The molecular weight of the heavy chain was slightly higher (i.e. M_r 215,000) in the conditioned media (Fig. 2b, lane 2). On the other hand, under non-reduced condition though no changes in the molecular weight for cellular components were observed, a protein of M_r 270,000 appeared in the conditioned media in addition of the M_r 215,000 species (Fig. 2b, lane 4). This implied not only the existence of FVIII:C gene in these cells but also the heavy and light chains in the secreted FVIII:C are held together by S–S bond. When the question on the existence of FVIII:C gene was answered by radiolabelling of cells in the presence of actinomycin D (2 μg/ml), it demonstrated reduced ^{35}S-methionine incorporation into FVIII:C whereas cycloheximide (1 μg/ml) completely abolished it (Fig. 2c, lanes a–c).

These observations then provide evidence that capillary endothelial cells are indeed one of the site(s) for Factor VIII:C biosynthesis. Reduction of [^{35}S]methionine labelled FVIII:C in actinomycin D-treated cells as well as its near absence in the presence of cycloheximide further supported the presence of active transcribable FVIII:C gene in these cells. Deduced amino acid sequence from the DNA sequence speculated that the protein core for FVIII:C is 270,000 Da and if fully glycosylated then it could acquire a mol.wt. of M_r 330,000. However, a protein of this size has not yet been documented. In any event, if this is found to be true then endothelial cell derived FVIII:C has a molecular mass between that of human and porcine plasma (M_r 240,000) [25]. Furthermore, the presence of S–S bond in endothelial cells derived FVIII:C put the earlier claim of Ca^{2+} bridge in porcine plasma FVIII:C in a questionable doubt [25].

3.3. *Susceptibility of FVIII:C to* N-*glycanase digestion*

Many plasma proteins are known to carry carbohydrate residues bound to the protein core. After determining that these endothelial cells possess glycosylating activity for the asparagine-linked (*N*-linked) glycoproteins [16], we became interested in studying the protein-carbohydrate linkage in FVIII:C. When the immunoprecipitated FVIII:C from conditioned media was digested with *N*- and *O*-glycanases, we observed that it became susceptible to *N*-glycanase digestion but not to *O*-glycanase (Fig. 3). This indicated that FVIII:C is indeed an asparagine-linked glycoprotein. Reduction in molecular weight of the light chain by ~8,000 Da (i.e. M_r 46,000 to M_r 38,000) indicated that the light chain contains 17% carbohydrate and hence is rich in carbohydrates. This certainly supports the earlier observation that light chain of recombinant FVIII:C is also rich in carbohydrate [27].

3.4. *Detection of von Willebrand Factor*

Immunocytochemical analysis with a polyclonal antibody to human FVIII-associated antigen (FVIII:AR) detected the presence of Factor VIII in these cells [15]. Since, FVIII:C circulates as a complex with von Willebrand Factor (vWF) in the plasma, it was then questioned whether vWF was similarly synthesized in these cells. Consequently, we have analyzed the cell lysate and the conditioned media by Western blotting using alkaline phosphatase-conjugated polyclonal antibody to human vWF raised in rabbit. The results (data not shown) indicated that when the blot was treated with anti-vWF antibody, the human plasma reacted strongly with the antibody and the bovine plasma only weakly, whereas the interactions were completely absent in the endothelial cell lysate and conditioned media. The poor reactivity of the antibody raised against the human pro-

tein prevented definitive demonstration of the absence of vWF in these cells.

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