Published in final edited form as:

Adv Exp Med Biol. 2011 ; 705: 453–464. doi:10.1007/978-1-4419-7877-6_24.

Importance of a Factor VIIIc-Like Glycoprotein Expressed in Capillary Endothelial Cells (eFactor VIIIc) in Angiogenesis

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Keywords

Angiogenesis; Mannosylphospho dolichol synthase; Unfolded protein response; ER stress; Apoptosis; Cell cycle; N-linked glycoproteins; Tunicamycin; Lipid-linked oligosaccharide

24.1 Introduction

Factor VIII is a large, 2,332-residue plasma glycoprotein that acts as a regulatory cofactor in the process of blood coagulation [1–3]. It binds to activated factor IX (factor IXa) in the presence of calcium and negatively charged phospholipids at the surface of activated platelets to form a membrane-associated, proteolytically active complex. Upon complex formation, the $V_{\max}$ of factor IXa is increased by approximately 200,000-fold, promoting the rapid activation of its substrate, the serine protease factor X. The proteolytic conversion of factor X to its active form, factor Xa, is a central control point in the coagulation cascade, leading to activation of thrombin, formation of a fibrin mesh, and establishment of a stable blood clot. The binding of factor VIIIc and other activated proteins to these membrane surfaces allows for localization of the procoagulation process to sites of vascular damage.

The factor VIII sequence contains six sequential domains arranged in the order of A1–A2–B–A3–C1–C2 (Fig. 24.1) [4–6]. The A domains are homologous to one another and display sequence similarity to the copper-binding protein ceruloplasmin. They are flanked by short spacer sequences that are highly acidic. The C domains are also homologous to each other and have a weak homology to the discoidin protein-fold family (e.g. the lipid-binding domain of galactose oxidase) [7, 8]. The circulating form of factor VIII protein is a metal-bridged heterodimer consisting of a heavy chain (A1–A2–B) and a light chain (A3–C1–C2). This form of factor VIII is bound tightly to von Willebrand factor (vWF). Factor VIII is processed further by specific thrombin cleavages into a heterotrimeric form. This active form, factor VIIIc, dissociates from vWF and binds to negatively charged phospholipids on activated platelet surfaces. The carboxyl terminal C2 domain of factor VIII contains binding sites for vWF and for negatively charged phospholipids. The binding of factor VIIIc to membranes involves stereoselection for *O*-phospho-L-serine, the negatively charged head group of phosphatidylserine (PS) [9]. The binding of factor VIII or VIIIc to vWF or PS is

mutually exclusive, even though the activation of factor VIII involves cleavages outside the C2 domain [10].

Factor VIIIc deficiency has been documented in a congenital bleeding disorder, hemophilia A. The current estimate suggests one case of hemophilia A occurs in every 5,000 live births in the USA. We have seen expression of a factor VIIIc-like molecule in capillary endothelial cells, i.e. eFactor VIIIc, but its role in the capillary endothelial cell function, e.g. angiogenesis, has not yet been established. In this article, we present evidence that (a) an extracellular signal stimulates eFactor VIIIc N-glycosylation, which consequently enhances angiogenesis, and (b) eFactor VIIIc supports capillary endothelial cell invasion in Matrigel™.

24.2 Establishment of a Capillary Endothelial Cell Line to Study Angiogenesis In Vitro

A nontransformed capillary endothelial cell line has been established from the microvasculature of bovine adrenal medulla [11]. Dissociated cells, when cultured in Eagle's minimal essential medium (EMEM) with Earl's salt containing fetal bovine serum and antibiotics, attached to one another in an end-to-end and side-to-side fashion. On successive days, they became more flattened, elongated, and established more contact, developing extensive and ordered networks of cells that generated macroscopic capillary-like structures.

Ultrastructural studies indicated extension of thin processes; cytoplasm filled with rough endoplasmic reticulum; and numerous surface cisterns of smooth, coated, and small vesicles exhibiting both exo and endocytosis. Dispersed mitochondria of the long tubular type parallel to the cellular axis were frequently seen. Both tight and gap junctions, as well as intercellular filaments and intercellular spaces reminiscent of capillary lumen, were also present [11].

24.3 Influence of Microenvironment and Extracellular Signaling on Angiogenesis

The capillary endothelial cell population doubled in 56 h when cultured in the presence of 10% fetal bovine serum (G1 = 24 h, S = 8 h, G2 + M = 24 h [12]) in an atmosphere of 95% air and 5% CO₂. A dramatic shift in cell morphology and a considerable amount of cell death occurred when they were cultured in the absence of CO₂. In the absence of CO₂, the cells failed to attach and died within 24 h. Supplementation with 10 mM Hepes–NaHCO₃, pH 7.4, improved cell attachment, but proliferation and cell-to-cell contact remained low [13].

Cells cultured in a media containing 2% fetal bovine serum increased the cell doubling to 68 h due to an extension of the G1 phase to 36 h. On the other hand, culturing the cells in the presence of 2 mM 8Br-cAMP in a 2% fetal bovine serum-containing media increased cell proliferation by ~70% and exhibited cell cycle shortening. During this treatment, almost one half of the cell population entered in the S phase, and the cellular morphology indicated

increased mitosis. Bcl-2 expression and caspase-3, -8, and -9 activity remained unchanged. Western blot results indicated that the expression of the cytosolic chaperones HSP-70 and HSP-90 was enhanced by 1.4- to 1.6-fold in 8Br-cAMP-treated cells with a significant reduction in the expression of endoplasmic reticulum (ER) chaperones GRP-78/Bip and GRP-94 [14]. Metabolic labeling with ^{35}S -methionine as a function of time followed by immunoprecipitation and autoradiography indicated no change in the GRP-78/ Bip level between 3 and 32 h in control cells (Fig. 24.2).

24.4 Expression of Factor VIIIc-Like Protein in Capillary Endothelial Cells

Upon culturing, the cells were fixed and treated with a mouse monoclonal antibody to human factor VIIIc, followed by a fluorescently labeled secondary antibody and analyzed microscopically. The result indicated perinuclear localization of factor VIIIc (Fig. 24.3a). To provide further support for factor VIIIc expression, capillary endothelial cells were labeled with ^{35}S -methionine (40 $\mu\text{Ci/mL}$; Sp. Act. 1,400 Ci/mmol) for 2 h at 37°C. Factor VIIIc from the cell lysate and the conditioned media was immunoprecipitated with an antifactor VIIIc monoclonal antibody and analyzed by 7.5% SDS-PAGE followed by autoradiography. The newly synthesized factor VIIIc consisted of a heavy chain (M_r 200,000 Da) and a light chain (M_r 46,000 Da) in the cell lysate. The values for the heavy and light chains for the secretory factor VIIIc were M_r 210,000 and M_r 40,000 Da, respectively (Fig. 24.3b). Secretory factor VIIIc, on the other hand, has a molecular mass of M_r 270,000 under nonreducing conditions, suggesting that the heavy and light chains are held together by S-S bonds [15]. To analyze the functional status of factor VIIIc, the immune complexes were subjected to a chromogenic assay (COATEST, Kabivitrum, Stockholm, Sweden), where the conversion of Factor X to Factor Xa was quantified by spectrophotometrically measuring the proteolytic release of *p*-nitroaniline bound to a synthetic substrate (S-2222). Both cell lysate and the conditioned media contained factor VIIIc-like activity (Fig. 24.3c).

24.5 Endothelial Factor VIIIc Is an Asparagine-Linked Glycoprotein

Cells were metabolically labeled with [^{35}S]methionine, and factor VIIIc in the conditioned media was immunoprecipitated. The glycosylation status of factor VIIIc was analyzed by SDS-PAGE followed by autoradiography after digesting the immunoprecipitate with *N*-glycanase (PNGase F). Reduction in the molecular mass of the light chain by ~8,000 Da (i.e. from M_r 46,000 to M_r 38,000 Da) supported that the light chain contains ~17% asparagine-linked (*N*-linked) glycans. Digestion with *O*-glycanase did not exhibit any appreciable change in the molecular mass, indicating that factor VIIIc may not contain covalently attached *O*-linked glycans (Fig. 24.4).

24.6 Regulation of Factor VIIIc N-Glycosylation by Transmembrane Signaling

Anatomically, endothelial cells interface blood and the interstitium and are in constant contact with various growth factors, cytokines, chemokines, and other bioactive molecules present in the circulation. Therefore, we have tested the effect of the two circulatory

hormones insulin and isoproterenol (a β -agonist and a synthetic catecholamine) on factor VIIIc N-glycosylation.

24.6.1 Regulation by Insulin

Insulin, a vital hormone of metabolism and a growth factor, stimulated growth of the liver, spleen, and heart during mammalian embryogenesis, as well as the growth of Reuber H35 hepatoma cells [16–18]. On the other hand, insulin downregulated the proliferation of capillary endothelial cells [19, 21]. This raised a question about the insulin receptors on the capillary endothelial cell surface. Examination of insulin receptors on the capillary endothelial cell membrane indicated that (1) ^{125}I -insulin binding plus internalization reached to a steady state in 20 min; (2) acid-washable fraction accounted for nearly half of the total specifically bound insulin; and (3) the dissociation constants (K_d) for insulin receptor in acid-washable fraction were 4.0×10^{-11} M (high affinity) and 4.7×10^{-9} M (low affinity), with a total number of 210,000 receptors per cell [20].

To evaluate the effect of insulin on factor VIIIc biosynthesis and glycosylation, cells were metabolically labeled with [^{35}S]methionine in the presence or absence of different concentrations of insulin (0.01, 1.0, and 10.0 $\mu\text{g/mL}$) and as a function of time (30 min to 24 h). Factor VIIIc in the cell lysate as well as in the conditioned media was immunoprecipitated with a mouse monoclonal antihuman factor VIIIc antibody and analyzed by SDS-PAGE followed by autoradiography. [^{35}S]-Methionine incorporation in the heavy chain (M_r 210,000 Da) of cellular factor VIIIc was maximum at 1.0 $\mu\text{g/mL}$ insulin, followed by 0.01 and 10.0 $\mu\text{g/mL}$ in 2 h compared to the untreated controls. This was different from the light chain (M_r 46,000 Da). Maximum incorporation of radioactivity was observed at 10.00 $\mu\text{g/mL}$ insulin, followed by 0.01 and 1.0 $\mu\text{g/mL}$. Secreted heavy and light chains, however, behaved differently. Maximum radioactivity was incorporated in the heavy and light chains at 1.0 $\mu\text{g/mL}$ insulin concentration, followed by 0.01 and 10.0 $\mu\text{g/mL}$. When the cellular and the secretory components were combined for analysis for each insulin concentration, the incorporation of radioactivity into the heavy and light chains was always higher in each insulin concentration. To emphasize further, a higher level of radioactivity was present in secretory factor VIIIc when the cells were labeled for 2 h in the presence of 1 $\mu\text{g/mL}$ insulin [22].

To address whether insulin increases the glycosylation of factor VIIIc, cells were double labeled with [^3H]mannose and [^{35}S]methionine in the presence or absence of insulin (1.0 $\mu\text{g/mL}$). Factor VIIIc in the cell lysate and in the media was immunoprecipitated with antifactor VIIIc antibody and subjected to SDS-PAGE. Bands corresponding to the heavy chain (M_r 210,000 Da) and the light chain (M_r 46,000 Da) were excised from the gel, and the radioactivity was quantified in a liquid scintillation spectrometer. The ratio of [^3H]mannose to [^{35}S]methionine in factor VIIIc was increased by 83% after insulin treatment (Table 24.1). The rate of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$, lipid-linked oligosaccharide (LLO) biosynthesis and its turnover in cells were also increased. Mannosylphospho dolichol synthase (DPMS) activity was increased by 1.5- to 2-fold. Inability of actinomycin D to inhibit the DPMS activity suggested against increased transcription. Intracellular transport of 2-deoxyglucose was increased by ~30% under this condition [23].

Epinephrine is a “fright, flight, or fight” hormone and prepares the cell for either combat or escape upon binding with β -adrenoreceptors. Therefore, the presence of 24 Factor VIIIc-Like Glycoprotein Expressed in Capillary Endothelial Cells 459 β -adrenoreceptors on capillary endothelial cells was evaluated. Examination of [^3H]dihydroalprenolol ([^3H]DHA) binding indicated the presence of β -adrenoreceptors with two different affinities on the capillary endothelial cell plasma membrane. The dissociation constants (K_d) were $2.7 \pm 0.9 \times 10^{-10}$ M and $2.96 \pm 0.31 \times 10^{-9}$ M with the corresponding B_{max} of 5.1 ± 0.05 and 70.0 ± 0.2 pmol/mg protein, respectively. Inhibition of [^3H]DHA binding with atenolol (a β_1 -antagonist) and ICI 118,551 (a β_2 -antagonist) has suggested that the $\text{IC}_{50\text{cor}}$ ($=K_i$) for atenolol and ICI 118,551 for high-affinity site were $8.0 \pm 3.0 \times 10^{-14}$ M and $25.0 \pm 8.0 \times 10^{-14}$ M, respectively. This indicated that both atenolol and ICI 118,551 were able to displace the bound ligand effectively, but the β_1 -selective antagonist atenolol was three times more potent than its β_2 counterpart, ICI 118,551 [23].

To evaluate whether the β -adrenergic response of isoproterenol is mediated via intracellular 3',5'-cyclic adenosine monophosphate (cAMP), cells were exposed to isoproterenol (1×10^{-7} M) for 30 min in a serum-free media containing 5×10^{-7} M isobutylmethylxanthine (a phosphodiesterase inhibitor). Extracted cAMP was quantified by radiobinding assay [22]. A nearly 1.5-fold increase in cAMP concentration supported that capillary endothelial cells have functional β -receptors.

Prior to studying the influence of β -adrenoreceptor activation on factor VIIIc glycosylation, cellular glycoproteins were analyzed in the presence or absence of the β -blockers atenolol and ICI 118,551. The cells were labeled with [^3H]mannose (10 $\mu\text{Ci/mL}$) and [^{14}C]leucine (1.25 $\mu\text{Ci/mL}$) in a continuous pulse for 1 h at 37°C in the presence of isoproterenol (1×10^{-9} M) with or without atenolol or ICI 118,551 (5×10^{-8} M). The ratio of [^3H]mannose to [^{14}C]leucine incorporation in proteins was quantified in a liquid scintillation spectrometer after precipitating the proteins with 10% trichloroacetic acid. The results indicated that the ratio of [^3H]mannose to [^{14}C]leucine incorporation into proteins was reduced by ~45% in cells pretreated with either atenolol or ICI 118,551 [24], thus establishing activation of protein N-glycosylation in capillary endothelial cells by isoproterenol. To address whether isoproterenol specifically increases the glycosylation of factor VIIIc, cells were labeled with ^{35}S -methionine in the presence of increasing concentration of isoproterenol (i.e. 1×10^{-9} , 1×10^{-7} , 1×10^{-5} , and 1×10^{-3} M) and analyzed as before. The incorporation of ^{35}S -methionine was highest at 1×10^{-7} M isoproterenol in both cell lysate and in the conditioned media. In the cell lysate, the order was $10^{-7} > 10^{-5} > 10^{-3} > 10^{-9}$ M > control, whereas in the conditioned media, the order was $10^{-7} > 10^{-9} > 10^{-5} > 10^{-3}$ M > control (Fig. 24.5).

To analyze the glycosylation status, cells were dually labeled with ^3H -mannose (10 $\mu\text{Ci/mL}$) and ^{35}S -methionine (40 $\mu\text{Ci/mL}$) for 1 h at 37°C in a low-glucose serum-free/methionine-free DMEM (BioFluids, Inc.) in the presence or absence of isoproterenol (1×10^{-7} M). Factor VIIIc in cell extract and in the conditioned media was immunoprecipitated with the antifactor VIIIc monoclonal antibody and separated on a 10% SDS-PAGE. The protein bands of M_r 200,000 (cellular)/ M_r 210,000 (media) and M_r 46,000 (cellular)/ M_r 40,000 (media) Da were excised, and the radioactivity was quantified in a liquid scintillation

spectrometer. The results indicated that the ratio of ^3H -mannose to ^{35}S -methionine in cellular factor VIIIc was increased by 73% after isoproterenol treatment, whereas that of the secretory factor VIIIc was increased by 45% (Table 24.2).

24.7 Factor VIIIc Expression and Angiogenesis Are Coupled

Angiogenesis, i.e. neovascularization, is key to tumor growth and invasion [24, 25]. Significant components of angiogenesis are endothelial cell migration, capillary budding, establishment of capillary loops, and neovascular remodeling. We have seen above that cAMP-related stimuli increase factor VIIIc N-glycosylation, and we have evaluated further whether the same stimulus would accelerate the capillary endothelial cell proliferation and the lumen formation. To examine the cellular proliferation, synchronized culture of capillary endothelial cells was treated with 2 mM 8Br-cAMP in 2% fetal bovine serum. The cells were collected after every 8 h and counted. There was a time-dependent increase in cellular proliferation (Fig. 24.6a). Photomicrographs indicated a lumen-like structure formation after 4 days of 8Br-cAMP treatment (Fig. 24.6b, c).

To establish such a coupling more precisely, we used the protein N-glycosylation inhibitor tunicamycin. Tunicamycin is a glucosamine-containing pyrimidine nucleoside and is a competitive inhibitor of *N*-acetylglucosaminyl-1 phosphate transferase of the dolichol pathway in the ER. We have observed that tunicamycin (1 $\mu\text{g/mL}$) treatment reduced the LLO formation in capillary endothelial cells. This resulted in decreased factor VIIIc expression and cellular proliferation (Fig. 24.7).

24.8 Role of Factor VIIIc in Capillary Invasion

Factor VIIIc is a cofactor in the blood coagulation cascade, but its role in capillary endothelial cell physiology needs to be established. One of our hypotheses is that eFactor VIIIc activates matrix metalloproteinases (MMPs) during capillary invasion and supports tumor growth. To test this hypothesis, we have cultured capillary endothelial cells on plates coated with growth-factor-reduced MatrigelTM in the presence and absence of antifactor VIIIc monoclonal antibody as well as tunicamycin. The chemoattractant used in this study was conditioned media from human breast cancer cells MCF7. Capillary endothelial cells were cultured for 24 h, the inserts were processed, and the number of cells passed through the membrane was counted under a microscope after staining with hematoxylin. The control inserts were without MatrigelTM coating. The results in Table 24.3 indicated that antifactor VIIIc monoclonal antibody blocked MatrigelTM invasion of capillary endothelial cells. Tunicamycin at 1 $\mu\text{g/mL}$ showed an increased invasion, but it was reduced to almost one-third at 10 $\mu\text{g/mL}$. One plausible explanation is that tunicamycin needs more time to act and that it needs higher concentrations to neutralize the effect of a high concentration of the growth factor(s) present in the tumor cell-conditioned media.

24.9 Summary

The physiological role of factor VIIIc in the blood coagulation cascade is established. But very little is known about the biosynthetic regulator(s) of this asparagine-linked (*N*-linked) glycoprotein. In addition, precise determination of cell-type specificity of factor VIIIc

biosynthesis has also not been elucidated. Earlier, we demonstrated the expression of active factor VIIIc protein in a capillary endothelial cell line. This endothelial cell factor VIIIc, i.e. eFactor VIIIc, is a 270-kDa N-linked glycoprotein in which the heavy chain (M_r 210,000 Da) and the light chain (M_r 46,000 Da) are joined together by disulfide bridge(s). In addition, we have also demonstrated that factor VIIIc expression precedes the endothelial cell proliferation. In this article, we have presented evidence that factor VIIIc N-glycosylation is upregulated by cAMP signaling. Upregulation of factor VIIIc N-glycosylation consequently accelerates the capillary endothelial cell proliferation, i.e. angiogenesis. This has been supported by the use of a protein N-glycosylation inhibitor, tunicamycin. Capillary endothelial cells treated with tunicamycin exhibit reduced factor VIIIc level and inhibition of cellular proliferation. In addition, we have also provided evidence that factor VIIIc is responsible for tissue invasion during tumor progression. We used Matrigel™ for this study. Cells cultured in the presence of antifactor VIIIc monoclonal antibody failed to invade the Matrigel™ matrix. This suggested that eFactor VIIIc activates MMPs during tumor invasion, a function analogous to what has been observed in factor VIIIc-dependent activation of factor X in blood coagulation. Our study has also suggested that N-glycosylation of factor VIIIc is very much required for its tissue invasion because cells cultured in the presence of tunicamycin have exhibited considerable inhibition of Matrigel™ invasion.

Acknowledgments

The authors greatly appreciate the editorial assistance of Ms. Laura M. Bretaña. The authors also acknowledge the technical help provided by Mr. Jonathan Caldera Colón and Miss Maria Teresa Milán Mello. The authors are also indebted to Dr. Amitava Banerjee for his critical reading of the manuscript and for enhancing the quality of the images. The work has been supported in part by grants from the Department of Defense DAMD17-03-1-0754, the NIH/NCRR/RCMI grant G12-RR03035 (to Krishna Baksi).

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Fig. 24.1.
Domain structure of factor VIII. Arg 372, Arg 740, and Arg 1648 are proteolytic cleavage sites

Fig. 24.2.

Expression of GRP-78 in capillary endothelial cells as a function of time. Cells were labeled with [^{35}S]methionine, and GRP-78 was immunoprecipitated from the cell lysate with anti-GRP-78 monoclonal antibody and analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography

Fig. 24.3.

Expression of factor VIIIc in capillary endothelial cells. **(a)** Cellular localization of factor VIIIc by immunofluorescence microscopy; **(b)** autoradiography of actively synthesized factor VIIIc; and **(c)** bioactivity of immunoprecipitated factor VIIIc: 1 = cell lysate; 2 = conditioned media; 3 = bovine plasma; 4 = human plasma; 5 = EMEM with 10% fetal bovine serum; 6 = cell lysate with a protease inhibitor (aprotinine)

Fig. 24.4.

Factor VIIIc is an asparagine-linked glycoprotein. Cells were labeled with [^{35}S]methionine, and immunoprecipitated factor VIIIc was digested with *N*- and *O*-glycanases and analyzed by SDS-PAGE followed by autoradiography. Lane 1 = control; lane 2 = after digestion with *N*-glycanase; lane 3 = after digestion with *O*-glycanase

Fig. 24.5.

Effect of isoproterenol on factor VIIIc biosynthesis. The cells were labeled with [^{35}S]methionine for 1 h at 37°C in the presence of isoproterenol, and factor VIIIc was analyzed in the cell lysate and in the conditioned media. *Con* control; *a* = 1×10^{-3} M isoproterenol; *b* = 1×10^{-5} M isoproterenol; *c* = 1×10^{-7} M isoproterenol; *d* = 1×10^{-9} M isoproterenol

Fig. 24.6.

Effect of 8Br-cAMP on capillary endothelial cell proliferation and the lumen formation. A synchronized cell population was treated with 2 mM 8Br-cAMP for 0–96 h. The cell numbers were counted in a hemocytometer, and the morphology was monitored under a Nikon PMS microscope. (a) The growth curve; (b) control cells; and (c) cells treated with 2 mM 8Br-cAMP

Fig. 24.7.

Downregulation of factor VIIIc expression and cellular proliferation by tunicamycin. Cells were cultured in the absence or in the presence of tunicamycin (1 $\mu\text{g/mL}$). Factor VIIIc expression was examined after 32 h, whereas the cell number was monitored after every 24 h for 96 h

Table 24.1
Effect of insulin on the ratio of [^3H]mannose to [^{35}S]methionine incorporation in factor VIIIc

Sample	Cellular		Media		Total (cellular + media)	
	Mean $\pm$ SEM	% increase over control	Mean $\pm$ SEM	% increase over control	Mean $\pm$ SEM	% increase over control
Control	1.00 $\pm$ 0.14	–	1.00 $\pm$ 0.27	–	1.00 $\pm$ 0.27	–
Insulin (1.0 mg/mL)	1.82 $\pm$ 0.15	82	1.00 $\pm$ 0.13	0	1.00 $\pm$ 0.13	82

Regulation by a β -agonist isoproterenol

Table 24.2
Effect of isoproterenol on the ratio of [^3H][mannose to [^{35}S][methionine incorporation in factor VIIIc

Sample	Cellular		Media		Total (cellular + media)	
	Mean $\pm$ SEM	% increase over control	Mean $\pm$ SEM	% increase over control	Mean $\pm$ SEM	% increase over control
Control	1.00 $\pm$ 0.14	–	1.00 $\pm$ 0.27	–	1.00 $\pm$ 0.27	–
Isoproterenol ($1 \times 10^{-7}\text{M}$)	1.73 $\pm$ 0.08	73	1.45 $\pm$ 0.08	45	1.45 $\pm$ 0.08	45

Table 24.3

Effect of anti-factor VIIIc monoclonal antibody on Matrigel™ invasion of capillary endothelial cells

Sample	Control insert (cell number)	Growth-factor reduced (cell number)	% invasion
Control	5,668 ± 788	2,078 ± 0	37
Antifactor VIIIc antibody (100 ng)	2,372 ± 612	80 ± 0	2.4
Tunicamycin (1 µg/mL)	3,194 ± 112	1,644 ± 445	51.5
Tunicamycin (10 µg/mL)	3,699 ± 360	673 ± 141	28.2