Human endothelial cells produce orosomucoid, an important component of the capillary barrier

Sörensson, Jenny, Göran L. Matejka, Maria Ohlson, and Börje Haraldsson. Human endothelial cells produce orosomucoid, an important component of the capillary barrier. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H530–H534, 1999.—The serum protein orosomucoid (α1-acid glycoprotein) is needed to maintain the high capillary permselectivity required for normal homeostasis. It is not known how the protein executes its action, but it seems to contribute to the charge barrier. Moreover, recent studies suggest that the endothelial glycocalyx is essential for the charge barrier. The main site of orosomucoid synthesis is the liver, but we wanted to explore the possibility that orosomucoid was synthesized in endothelial cells. Primary cultures of human microvascular endothelial cells (HMVEC) from dermal tissue were established. Human liver cells were used as positive controls, and total RNA was prepared from both cell types. Reverse transcription-polymerase chain reaction (RT-PCR) was performed and demonstrated orosomucoid expression. After RT-PCR, the identities of the PCR products were confirmed by sequencing. RNase protection assay performed on total RNA from the HMVEC confirmed the results from the RT-PCR, i.e., orosomucoid mRNA is expressed by endothelial cells. Synthesis of orosomucoid in both liver and endothelial cells was demonstrated by immunoprecipitation. In conclusion, endothelial cells normally produce orosomucoid, which is essential for capillary charge selectivity. We suggest that orosomucoid exerts its effect by interacting with other components of the endothelial glycocalyx.

glycocalyx; α1-acid glycoprotein; capillary permeability

THE MICROVASCULAR ENDOTHELIUM covers a huge surface area in the human body. It constitutes a fine line between the rather static cellular system of various organs and the constantly flowing blood compartment. The endothelial cells have important functions required in the blood-tissue interface. The endothelium produces, for example, thrombomodulin and heparan sulfate to prevent clogging and nitric oxide and prostacyclin to maintain flow patency (33). The cells have also been shown to express vascular permeability factors that have a potent capacity to increase endothelial permeability (10).

This report deals with orosomucoid, a polyanionic protein with a molecular mass of ~41 kDa and a high carbohydrate content (45%). The protein is sialylated, resulting in a pI as low as 2.7 (31). Orosomucoid is a member of the acute-phase protein family, and elevated concentrations are found in inflammation and cancer, after surgery, and during pregnancy (2, 29, 30). The protein is mainly synthesized in the liver, but there is evidence of orosomucoid synthesis in both human breast epithelial cells (12) and leukocytes (3). Orosomucoid is needed to maintain capillary permeability (15), probably by increasing the polyanionic charge selectivity of the endothelial barrier (8, 15, 17). Such an interaction may be the result of binding to endothelial cell receptors. For example, Schnitzer and Pinney (32) found that bovine serum orosomucoid (BSO) binds to the surface of bovine pulmonary microvascular endothelial cells (BLMVEC). The results of Schnitzer and Pinney indicate that BSO binds specifically to BLMVEC in a manner that fulfills many of the requirements of a ligand-receptor interaction. There was no binding to other cells such as fibroblasts and smooth muscle cells. Orosomucoid could also exert its action by affecting the organization of the molecules on the surface of the endothelial cells. Such an effect has not been studied for the components in the glycocalyx, but orosomucoid in vitro affects the three-dimensional arrangement of collagen (11).

These hypotheses suggest an interaction between the capillary barrier (i.e., the endothelial cell) and the serum proteins. An alternative hypothesis can be proposed, however, with the assumption that orosomucoid is locally produced by the endothelial cells. In that case, the impaired capillary permselectivity observed when perfusates devoid of orosomucoid are used may reflect washout of the protein in combination with a slow turnover. In a first step to test this hypothesis, we investigated whether the endothelial cells do produce orosomucoid.

MATERIALS AND METHODS

Cell culture. Primary human microvascular endothelial cells (HMVEC; d-Neo 2505) were obtained from Clonetics Bio Whittaker (Walkersville, MD). The cells were cultured in gelatin-coated 75- or 162-cm² culture flasks (Costar, Cambridge, MA) in a humidified, 5% CO₂ atmosphere at 37°C in medium 131, a special medium for microvascular endothelium, containing microvascular growth supplement, penicillin, streptomycin, and amphotericin B (Cascade Biologics, Portland, OR). The cells tested negatively for mycoplasma as shown by both the manufacturer and our laboratory. The cells were also tested for occurrence of von Willebrand factor (factor VIII) and showed expression of this endothelial cell marker. The manufacturer also showed that the cells stained positively for acetylated low-density lipoprotein incorporation and negatively for smooth muscle α-actin. Before RNA
preparation, the HMVEC were starved in medium 131 containing 0.5% FCS for 24 h.

Preparation of nucleic acid and proteins. Total RNA was prepared from HMVEC in passage 7 and from human liver tissue according to the method described by Chomczynski and Sacchi (6), followed by preparation of proteins with the use of Tri-reagent according to the enclosed kit protocol (Sigma, St. Louis, MO).

Polymerase chain reaction primers. Sense and antisense primers located from 206 to 227 bp and 585 to 605 bp in the human orosomucoid cDNA sequence (4) were used to detect the orosomucoid transcript. See Table 1 for sequences.

Reverse transcription-polymerase chain reaction. Synthesis of cDNA was carried out with 1 µg of RNA from HMVEC or liver cells. The reverse transcription (RT) reaction was carried out for 50 min at 42°C and 5 min at 70°C in RT buffer (Promega, Madison, WI) in the presence of 1 µg of random primer (Promega), 15 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega), 20 U RNasin (Promega), and dNTP mix (deoxy-CTP, -GTP, -TTP, and -ATP), with 10 mM of each base in a total volume of 20 µl. For the polymerase chain reaction (PCR) amplification, PCR buffer [50 mmol/l KCl, 10 mmol/l Tris·HCl (pH 8.3), 1.5 mmol/l MgCl2, and 0.001% (wt/vol) gelatin (Perkin-Elmer Cetus, Norwalk, CT)], 0.3 µmol/l of each sense and antisense primer, and 2.5 µl Taq DNA polymerase (Promega) were used. This mixture was added to 5 µl from each RT reaction, and the samples were amplified with orosomucoid primer for 30 cycles (15 s of denaturation at 94°C, 15 s of annealing at 56°C, and 30 s of elongation at 72°C; Perkin-Elmer, GeneAmp PCR system 9600). After the amplification reaction was completed, the PCR products were separated and visualized by ultraviolet detection. Sequencing was performed to confirm the identity of the PCR products (ABI Prism 377, Perkin-Elmer). The homology of the PCR product compared with the complete sequence of human orosomucoid mRNA was elucidated using Seqman software.

Probe. The PCR products (from HMVEC and human liver) were purified from the agarose gel using ion-exchange transfer membranes (DEAE membranes, NA 45; K&b Lab, Spånga, Sweden), and the DNA was then eluted and precipitated. The pBluescript SK plasmid (Stratagene, AH Diagnostics, Skarholmen, Sweden) was cleaved using the restriction enzyme Sma I. The ends of the sample DNA were blunted with mung bean nuclease (New England Biolabs, Beverly, MA). The sample DNA was then inserted into the plasmid overnight at 16°C using T4 ligase. Competent bacteria (DH5α) were transformed and seeded on agar plates with carbenicillin, isopropylthiogalactoside, and X-Gal and cultured overnight at 37°C. The transformed colonies were harvested and cultured in large flasks overnight in a 37°C shaking incubator. A plasmid minipreparation (Plasmid minipreparation kit, Quiagen, Hilden, Germany) was made to confirm that the sample DNA from the PCR reaction was inserted into the plasmid DNA. A large preparation was then made to collect the insert from the total amount of bacteria (Plasmid maxipreparation kit, Quiagen). The collected inserts were sequenced (ABI Prism 377, Perkin-Elmer), and the homologies of the inserts from the plasmids to the orosomucoid cDNA sequence were elucidated. RNase protection assay followed by gel separation. RNA samples (20 µg) were hybridized overnight at 45°C with 32P-labeled orosomucoid probe and then digested with RNase (RPA II kit, Ambion). The RNA/RNA hybrids were precipitated, dissolved, and separated on a mini 6% polyacrylamide, 8 mol/l urea gel (Novex, San Diego, CA). The signal from the protected fragments was visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation. Proteins obtained from HMVEC and liver were precipitated together with rabbit anti-human orosomucoid (Dako, Glostrup, Denmark) at a concentration of 1:100. The mixture was allowed to precipitate overnight at 4°C. Protein G-Sepharose beads (Santa Cruz Biotechnology, Heidelberg, Germany) were then added, and the samples were allowed to blend for an additional 3 h. After washing and denaturation were completed, precipitated samples were run on a 10–12% bis-Tris polyacrylamide gel (Nupage bis-Tris 10–12%, Novex) according to the protocol by Ohlsson et al. (22). Blotting of the proteins to a polyvinylidene difluoride membrane was performed in a standard manner, and then the membrane was blocked in 5% nonfat dry milk plus 0.25% gelatin for 1 h at room temperature. The membrane was then incubated with anti-α1-acid glycoprotein monoclonal antibody (mouse; Sigma) at a dilution of 1:10,000, washed, and finally incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse-HRP; Amersham Life Science, Amersham, UK) at a dilution of 1:7,000. The protein-antibody complexes were visualized by enhanced chemiluminescence (ECL + and Hyperfilm ECL, Amersham) according to the kit instructions.

RESULTS

The expression of orosomucoid mRNA from HMVEC (Fig. 1) and human liver was examined by RT-PCR. Transcripts of the expected size (according to the human orosomucoid cDNA sequence) were detected in both HMVEC and human liver. The 400-bp PCR products were sequenced and compared with orosomucoid (4), and the correct identities of the PCR products were confirmed. Protected fragments of 400 bp were also found when the orosomucoid cDNA was hybridized to RNA from HMVEC (Fig. 2) using RNase protection assay. To elucidate whether the mRNA for orosomucoid is translated into protein, we used immunoprecipitation. The results showed clearly that orosomucoid is present in the cells (see Fig. 3).

DISCUSSION

We demonstrate in the present study that orosomucoid is synthesized by microvascular endothelial cells. This finding should be viewed against the background that orosomucoid plays an important role in transcapillary exchange. Absence of serum proteins in vascular perfusates increases capillary permeability (9, 14, 15). This phenomenon is known as the “protein” or “serum effect” (19). In 1987 Haraldsson and Rippe (15) found that orosomucoid was an important determinant of the capillary permselectivity in rat skeletal muscle. In the absence of orosomucoid there was a normal hydraulic conductance, but the clearance for albumin increased

Table 1. Primers used in PCR amplification

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<tr>
<th>Primer</th>
<th>Primer Sequence</th>
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<tr>
<td>Oroso (F)</td>
<td>5′-GAACTCTTTTTTACCTTCCC-3′</td>
</tr>
<tr>
<td>Oroso (R)</td>
<td>5′-TTCCTGTTTTTTCCTCCT-3′</td>
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Sequences were used as primers to detect orosomucoid transcripts in PCR amplification. Sequences are given as forward (F) and reverse (R) primers.
threefold. Similar data were found for glomerular capillary permselectivity (13) and rat heart capillaries (37). The effect of orosomucoid on permselectivity was verified in frog mesenteric capillaries in a study also demonstrating a charge effect of the protein (8, 17). Recently, a study by Muchitsch et al. (21) showed that orosomucoid antagonized the capillary leakage induced by different inflammatory agonists such as Lys-plasminogen, histamine, platelet-activating factor, and thrombin in vivo in guinea pigs.

These data pinpoint the crucial role of orosomucoid in maintaining the capillary barrier for macromolecules.

Our data thus suggest that the orosomucoid in the glyocalyx capillary barrier is locally produced by the endothelial cells themselves. Such a hypothesis could explain the observations of serum protein hysteresis noted by Huxley and Curry (16). It was found that the permselectivity fell as the serum protein concentration was reduced, but increasing the concentration had less pronounced effects on permeability. Hence, normal capillary permselectivity was never restored, which is difficult to comprehend using the concept of plasma proteins interacting with the capillary barrier. Could this instead be due to a washout of glyocalyx components (such as orosomucoid) produced by the endothelial cells themselves?

Fig. 1. Human dermal microvascular cells (HMVEC-d-Neo 2505) grown to confluence in passage 7 in gelatin-coated 162-cm² culture flasks.

Fig. 2. RNase-protected human orosomucoid mRNA analyzed by PAGE. See RNase protection assay followed by gel separation in MATERIALS AND METHODS for description of procedure. Lane A, HaeIII ladder; lane B, HMVEC (20 µg RNA); lane C, human liver (20 µg RNA). All samples were run on the same gel, but expression of orosomucoid was lower in cell sample, so we made adjustments for that in analysis.

Fig. 3. Immunoprecipitation of proteins. See Immunoprecipitation in MATERIALS AND METHODS for description of procedure. Lane A, blank (no protein added to reactions); lane B, purified human orosomucoid (0.01 µg protein); lane C, human liver (5 µg protein); lane D, HMVEC (500 µg protein). Bar indicates orosomucoid with a size of ~41 kDa. Orosomucoid can be expressed as several different subpopulations, probably due to charge heterogeneity of carbohydrate side chains (12). In this assay we could detect 2 different subpopulations in human liver and only 1 in HMVEC.
The role of the endothelium in transcapillary exchange has been constantly debated. In the 1940s the work by Pappenheimer and Soto-Rivera (24) suggested that the exchange might take place across passive membranes with functional pores (24). Macromolecules were then suggested to pass through an intracellular route using vesicular transport (18, 23). Physiological studies indicate that such vesicular transport gives a small contribution to the overall transcapillary passage of macromolecules (26). However, most vesicles seem to fuse and form a branching system, enlarging the endothelial surface area, and may occasionally fuse to form large pores (5). Indeed, the passage of solutes across capillary walls can be described by a two-pore model that uses explicit nonlinear, irreversible hemodynamic equations for each functional pore (25). The capillary walls, however, are not static membranes with spherical pores. Instead, the capillary endothelium is a dynamic structure reacting to various stimuli, exposing numerous receptors and enzymes to the blood, and capable of pinocytosis of certain proteins (34).

How can fenestrated endothelial cells be restrictive components of, for instance, the glomerular blood-urine barrier? Luft (20) was one of the first to describe a layer covering the endothelial cells that could be detected by the use of a cationic stain (ruthenium red). This endocapillary layer (the glycoalx) is suggested to be constituted of a fibrous, three-dimensional network of glycoproteins, reinforced by plasma proteins such as albumin and orosomucoid (11). In a study by Adamson and Clough (1) it was shown that the presence of plasma proteins in the perfusate alters the glycoalx configuration and that the thickness of the layer increases, thereby supporting the fiber matrix theory (7). The perfusion (or shear stress) may also affect capillary permeability, conceivably through effects on the glycoalx, as indicated in the study by Ryan and Karnovsky (28). Recently, Rostgaard and Qvortrup (27), using a new fixation technique, described an endocapillary surface coat in the glomerular capillaries that is ~50–100 nm thick. Others have found it to be even thicker (~500 nm) (36). One possibility is that some of the components of the glycoalx may be eluted, among them orosomucoid, when an artificial solution is used to perfuse a capillary bed, thus resulting in a gradual increase in microvascular permeability. Hence, the glycoalx layer may be more important than previously thought in terms of restricting transcapillary exchange of macromolecules (35). Adamson and Clough (1) suggested that the glycoalx may behave as a fiber matrix with size- and charge-restricting properties. In the kidneys, however, there seem to be two separate serial barriers, one charge discriminating and a second size selective. Thus we recently perfused isolated kidneys with solutions of low ionic strength to increase charge interactions (35). We were able to interfere with the charge barrier with no effect on the size barrier, because the clearance of neutral solutes was unaffected (35).

In conclusion, on the basis of our findings, we propose a new hypothesis for how orosomucoid affects the capillary permeability. The endothelial cell produces orosomucoid and other components of its glycoalx. This endothelial cell surface coat seems to be an essential part of the capillary charge barrier, probably acting as an ion exchanger, thereby reducing the concentration of negatively charged solutes in the gel. Orosomucoid contributes to the functional properties of the glycoalx. Thus increased capillary permeability with protein leakage, edema, and/or proteinuria could be due to impaired production of orosomucoid and other essential parts of the glycoalx caused by endothelial dysfunction.

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