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Fibrinogen alters mouse brain endothelial cell layer integrity affecting vascular endothelial cadherin

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ABSTRACT

Many inflammatory diseases are associated with elevated blood concentration of fibrinogen (Fg) leading to vascular dysfunction. We showed that pathologically high (4 mg/ml) content of Fg disrupts integrity of endothelial cell (EC) layer and causes macromolecular leakage affecting tight junction proteins. However, role of adherence junction proteins, particularly vascular endothelial cadherin (VE-cadherin) and matrix metalloproteinase-9 (MMP-9) in this process is not clear. We tested the hypothesis that at high levels Fg affects integrity of mouse brain endothelial cell (MBEC) monolayer through activation of MMP-9 and downregulation of VE-cadherin expression and in part its translocation to the cytosol.

The effect of Fg on cultured MBEC layer integrity was assessed by measuring transendothelial electrical resistance. Cellular expression and translocation of VE-cadherin were assessed by Western blot and immunohistochemical analyses, (respectively). Our results suggest that high content of Fg decreased VE-cadherin expression at protein and mRNA levels. Fg induced translocation of VE-cadherin to cytosol, which led to disruption of cell-to-cell interaction and cell to subendothelial matrix attachment. Fg-induced alterations in cell layer integrity and their attachment were diminished during inhibition of MMP-9 activity.

Thus Fg compromises EC layer integrity causing downregulation and translocation of VE-cadherin and through MMP-9 activation. These results suggest that increased level of Fg could play a significant role in vascular dysfunction and remodeling.

1. Introduction

Fibrinogen (Fg) is a high molecular weight plasma adhesion glycoprotein. It is a biomarker and a cause of inflammation as well as a high risk factor for many cerebrovascular and cardiovascular disorders [1]. Increased blood content of Fg accompanies inflammatory diseases such as hypertension [2,3], diabetes [4], and stroke [5]. Normal level of Fg in blood is around 2 mg/ml [3,6], while during different cardiovascular diseases its blood level ranges 3.6–4 mg/ml. Active participation of Fg in many blood flow related abnormalities such as increase in erythrocyte aggregation [7], platelet thrombogenesis [8], blood coagulation [9], and cell-cell interaction [10] are well known. However, finding that an increased content of undegraded Fg compromises vascular endothelial cell (EC) layer integrity is relatively new [6,11,12].

Increase in vascular permeability is one of the indications of inflammation [13]. Macromolecular leakage through EC layer may occur via two transcellular and paracellular pathways [13]. While transcellular pathway involves movement of solutes and plasma components through the EC, movement of plasma components via paracellular pathway involves changes in tight, gap, and adherence junction proteins [13]. Binding of Fg to its endothelial surface receptor intercellular adhesion molecule-1 (ICAM-1) and the resultant activation of ERK1/2 signaling [14] may be a possible mechanism for disruption of EC layer integrity and increased permeability to albumin through paracellular pathway [11,12]. We also showed that enhanced content of Fg causes an increase in EC layer permeability to albumin by widening gaps between the ECs through formation of filamentous actin (F-actin) [11], downregulation and translocation to cytosol of tight junction proteins (TJP) occludin and tight junction-associated proteins such as zona occludin-1 (ZO-1) and zona occludin-2 (ZO-2) [12].

Matrix metalloproteinases (MMPs) are zinc-dependent endoproteinases. They are expressed in different cell types including

Abbreviations: EC, endothelial cells; Fg, fibrinogen; F-actin, filamentous actin; ICAM-1, intercellular adhesion molecule-1; MMP-9, matrix metalloproteinase-9; MBECs, mouse brain endothelial cells; TJP, tight junction proteins; VE-cadherin, vascular endothelial cadherin; ZO-1, zona occludin-1; ZO-2, zona occludin-2; FIU, fluorescence intensity units; IOD, integrated optical density; TIMP-4, tissue inhibitor of metalloproteinases-4.

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ECs [15] and involved in various physiological and pathological processes, especially in sub endothelial matrix (SEM) degradation and vascular remodeling. Activation of MMP-9, plays an important role in decreasing of brain vascular endothelial layer integrity, and causing macromolecular leakage [16]. Activated MMP-9 degrades EC junction proteins [17] and thus causes disruption of EC layer integrity.

Endothelial specific adhesion molecule Cadherin-5 or vascular endothelial cadherin (VE-cadherin) located at the basal side of ECs [13,18] is present in of all types of vessels [19]. It mediates Ca^{2+} dependent interactions through extracellular domain [20]. The small intracellular domain binds various cytoplasmic proteins (p-120, plakoglobin, β-catenin) [20]. Thus the cytoplasmic structural components of ECs are linked to adhesion junctions [20]. Presence of VE-cadherin at cell contacts essentially indicates the extent of permeability of blood vessels [18].

In the present study we tasted the hypothesis that at pathologically high level Fg alters VE-cadherin expression and its localization and thus affects mouse brain endothelial cell (MBEC) layer integrity.

2. Materials and methods

2.1. Reagents and antibodies

Human Fg (FIB-3, depleted of plasminogen, von-Willebrand factor, and fibronectin) was purchased from Enzyme Research Laboratories (South Bend, IN). Purified function-blocking anti-mouse ICAM-1 (CD54) antibody was obtained from BioLegend (San Diego, CA). Anti-mouse VE-cadherin antibody and 4,6-diamidino-2-phenyl-indole HCl (DAPI) were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody conjugated with Alexa-fluor 594, anti-VE-cadherin-antibody, VE-cadherin primers, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Invitrogen (Carlsbad, CA). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum were obtained from ATCC (Manassas, VA). Protease inhibitor (PI) cocktail and antibody against β-actin were from Sigma–Aldrich Chemicals Co. (St. Louis, MO). Radio-immunoprecipitation assay (RIPA) buffer with ethylenediaminetetraacetic acid (EDTA) was from Boston BioProducts (Ashland, MA). Tissue inhibitor of metalloproteinases-4 (TIMP-4) was purchased from Abcam (San Francisco, CA) and 37% formaldehyde was from Fisher Scientific (Pittsburgh, PA).

2.2. Endothelial cell culture

MBECs were purchased from ATCC (Manassas, VA). The endothelial nature of the cells was verified by uptake of acylated low-density lipoprotein and positive staining for CD-31 [21]. The MBECs were cultured in DMEM Complete medium (ATCC) according to the manufacturer's recommendations at 37 °C with 5% CO_2/air in a humidifier environment and were used at the 5th or 6th passage for the experiments.

2.3. Fg-induced VE-cadherin alterations

Immunohistochemistry and laser-scanning confocal microscopy were used to visualize Fg-induced changes in VE-Cadherin expression and location. MBECs were grown until confluent in 6-well plates. The cells were washed and treated for 20 h with Fg (2 or 4 mg/ml), Fg (4 mg/ml) with anti-ICAM-1 (50 μg/ml) antibody, or anti-ICAM-1 antibody alone. In another group of experiments the cells were treated with Fg (4 mg/ml), Fg (4 mg/ml) with TIMP-4 (12 ng/ml), known as MMP-9 activity inhibitor [22], or TIMP-4 (12 ng/ml) alone. The cells incubated with DMEM alone were used as a control group. After treatment, the cells were washed twice with PBS (phosphate buffered saline) and incubated in 3.7% formaldehyde for 10 min at RT. The cells were washed with PBS and anti-VE-Cadherin antibody (1:250 dilutions) was applied at 4 °C overnight. Appropriate fluorescence-conjugated secondary antibody (1:200 dilutions) was applied for 1 h at RT in dark. Cell nuclei were labeled with DAPI (1:10,000 dilutions) added to the wells for 15 min. The laser-scanning confocal microscope (Olympus FluoView1000, objective 100×) was used for image capture. VE-cadherin (Alexa 594) was visualized using a HeNe-G laser (556 nm) to excite the dye, while emission was observed above 573 nm. Cell nuclei (DAPI-labeled) were visualized using a 405-laser Diod laser (372 nm) to excite the dye, while emission was observed above 456 nm. Off-line image analysis software (Image-Pro Plus) was used to assess VE-cadherin expression. Corresponding AOIs (of the same size in all respective experimental groups) were placed around the cells. For each experimental group two wells were analyzed. In each well, three to five cell images were analyzed and normalized per cell. Fluorescence intensity in four randomly placed AOIs were measured. The results were averaged for each experimental group and presented as fluorescence intensity units (FIU).

Changes in protein content of VE-cadherin induced by Fg in MBECs were assessed by western blot analysis. The cells were grown in 6-well plates (TPP, Trasadingen, Switzerland) until 80% confluent. They were incubated at 37 °C for 24 h with Fg (2 or 4 mg/ml), Fg (4 mg/ml) with anti-ICAM-1 antibody (50 μg/ml), anti-ICAM-1 antibody (50 μg/ml), or serum-free DMEM alone, which was used as a control group. The procedure was done according to the method described earlier [12]. Briefly, after incubation, cells were washed 2× with cold PBS and lysed with cold RIPA buffer (containing 5 mM of EDTA) supplemented with PMSF (1 mM) and PI cocktail (1 μl/ml of lysis buffer). Protein content of the lysate was determined using the Bicinchronic Acid (BCA) protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (30 μg) were resolved on 10% SDS–PAGE gel and after electrophoresis at 100 V, transferred onto a nitrocellulose membrane (Bio-Rad laboratories, Hercules, CA). The blots were incubated with monoclonal anti-VE-Cadherin antibody (1:200 dilutions) overnight at 4 °C with gentle agitation. After incubation, the proteins on blots were detected by secondary antibody (1:3000 dilutions). Then, membranes were stripped and re-probed for content of β-actin as a loading control. The blots were analyzed with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD) as described earlier [23]. The protein expression intensity was assessed by the integrated optical density (IOD) of the area of the band in the lane profile. To correct data for possible differences in the protein load, the measurements presented are the IOD of each band under study (protein of interest) divided by the IOD of the respective β-actin band.

Expression of VE-Cadherin messages was determined using the reverse transcription polymerase chain reaction assay (RT-PCR). The expression of messenger RNAs (mRNAs) for VE-Cadherin was examined using two-step RT-PCR. ImProm-III™ Reverse Transcription System (Promega, Madison, WI) used according to the manufacturer's specifications. cDNA samples were incubated for 2 min at 94 °C, cycled 30 times (at 94 °C for 2 min, 55 °C for 30 s, and 72 °C for 1 min), and extended for 1 min at 72 °C. Products were visualized in a 1% TAE agarose gel, stained with ethidium bromide. Primer was VE-Cadherin (forward) 5′-AGAAGCTATGTCGGCAGGAA-3′ and (reverse) 5′-GCTCTGCATGTTTGGTCTCA-3′ and GAPDH (forward) 5′-AACTTTGGCATTGTGGAAGG-3′ (reverse) 5′-ACACATTGGGGGTAGG AACA-3′. Reactions were carried out in BioRad C1000 Thermal Cycler with Dual 48/48 Fast Reaction Module (Life Science Research, Hercules, CA). Gels were photographed using the Molecular Imager