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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.

Keywords

fibrinogen; vascular endothelial cadherin; matrix metalloproteinases; intercellular adhesion molecule-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

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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 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 tested 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.

Materials and methods

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).

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.

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 hours with Fg (2 mg/ml or 4 mg/ml), Fg (4 mg/ml) with anti-ICAM-1 (50 μ g/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 x) 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 2 wells were analyzed. In each well, three to five cell images were analyzed and normalized per cell. Fluorescence intensity in 4 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 μ g/ml), anti-ICAM-1 antibody (50 μ g/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 2x with cold PBS and lysed with cold RIPA buffer (containing 5 mM of EDTA) supplemented with PMSF (1 mM) and PI cocktail (1 μ l/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 μ g) were resolved on 10% SDS-PAGE gel and after electrophoresis at 100V, 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-IIITM 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 sec, 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'-AGAAGCTATGTCCGCAGGAA-3' and (reverse) 5'-GCTCTGCATGTTTGGTCTCA-3' and GAPDH (forward) 5'-AACTTTGGCATTGTGGAAGG-3' (reverse) 5'-ACACATTGGGGGTAGGAACA-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 ChemiDocTM XRS+ System with Image LabTM Software (Life Science Research). To correct the obtained results for possible differences in mRNA load, data are presented as ratio of the IOD of each band of mRNA of interest to IOD of the respective GAPDH band.

Transendothelial electrical resistance

Junctional interaction of MBECs was assessed by measuring transendothelial electrical resistance and the cell adherence to the SEM was assessed by measuring the capacitance as described earlier [12]. MBECs were placed on an electrical cell-substrate impedance system's (Applied Biophysics, Troy, NY) gold microplates and grown to a complete monolayer that covered the 8-wells chamber's microelectrodes. Cells were treated with Fg (4 mg/ml), Fg (4 mg/ml) with 50 μ g/ml anti-ICAM-1 antibody, or with anti-ICAM-1 antibody (50 μ g/ml) alone. In another series of experiments cells were treated with Fg (4 mg/ml), Fg (4 mg/ml) with TIMP-4 (12 ng/ml), or with TIMP-4 (12 ng/ml). In both experiments cells incubated with medium alone were used as a control group. Average values for resistance and capacitance for the last 30 min observation before treatments were taken as baseline for each experimental group. Resistance and capacitance values for each experimental group were collected and plotted as relative (to values of the treatment starting point) resistance or capacitance vs. time.

Statistical analysis

All data are expressed as mean $\pm$ SEM. The experimental groups were compared by one-way ANOVA. If ANOVA indicated a significant difference ($P < 0.05$), Tukey's multiple comparison test was used to compare group means. Differences were considered significant if $P < 0.05$. Differences in cell layer resistance and capacitance between experimental groups were assessed at every 2h.

Results

Immunohistochemical analysis using confocal microscopy showed that Fg caused dose-dependent alterations of VE-cadherin expression in MBECs and enhanced its translocation from cell periphery to cytosol (Fig. 1). These Fg effects were prevented by the presence of function-blocking anti-ICAM-1 antibody (Fig. 1). In another series of experiments treatment

of MBECs with TIMP-4 (inhibits MMP-9 activity [22]) mitigated 4 mg/ml Fg-induced downregulation and translocation of VE-cadherin (Fig. 2).

Treatment of MBECs with high content (4 mg/ml) of Fg caused a significant decrease in the protein content of VE-cadherin compared to that induced by the lower dose (2 mg/ml) of Fg (Fig. 3). This effect was mitigated by the presence of function-blocking antibody against ICAM-1 (Fig. 3). Anti-ICAM-1 antibody alone had no effect on protein expression of VE-Cadherin (Fig. 3).

Expression of VE-cadherin mRNA was significantly less after treatment of MBECs with high (4 mg/ml) concentration of Fg in comparison to VE-cadherin mRNA expression in cells treated with 2 mg/ml Fg (Fig. S1). However, expression of VE-cadherin mRNA in cells treated with normal (2 mg/ml) and high (4 mg/ml) content of Fg was less than in the control group (Fig. S1). Presence of function-blocking anti-ICAM-1 antibody ameliorated the Fg-induced decreased VE-cadherin mRNA expression, but was still less compared to that in the control group (Fig. S1).

At the higher content (4 mg/ml) Fg decreased electrical resistance between MBECs and increased capacitance of the cell monolayer system (Figs. S2 and 4). These effects of Fg were mitigated by the presence of anti-ICAM-1 antibody (Fig. S2) and TIMP-4 (Fig. 4).

Discussion

Our previous study showed that an increase in blood content Fg induces disruption of EC layer integrity so that it allows macromolecular leakage through it [11]. We have also shown that Fg dose-dependently decreases EC junctional integrity [12]. In the present study, we found that Fg dose-dependently decreases expression of adherence junction protein VE-cadherin and causes its disordered distribution along the cells surface. Since VE-cadherin was still present after Fg-treatment, it is possible that it was partially translocated from membrane to cytosol. These data suggest that Fg binding to its endothelial receptor ICAM-1 decreases VE-cadherin expression and this may occur through activation of extracellular-signal-regulated kinase-1/2 (ERK1/2) [11,12,14]. Earlier we showed that at higher content, Fg binding to ECs causes formation of F-actin [11]. It was suggested that formation of F-actin may increase gaps between the ECs and enhance vascular permeability [6]. Since VE-cadherin is anchored to actin filaments [13,24], Fg-induced formation of F-actin can be a mechanism for pulling VE-cadherin into the cellular cytosol and disrupting its cell membrane lining. This coincides with recent findings indicating that EC focal adhesion is highly dynamic [25] and that an increase in blood-brain barrier permeability involves cellular translocation of gap-junction protein occludin [26], which is known to be also anchored to actin [13].

Binding of Fg to ICAM-1 activates ERK1/2 signaling pathway in ECs [14], which has shown to be involved in activation of MMP-9 [27,28]. It has been demonstrated that activation of MMPs leads to degradation of endothelial junction proteins [29] and increases microvascular permeability [6]. Our data indicate that at an enhanced concentration, Fg activates endothelial MMP-9, which is known to cause vascular remodeling and an increase in its permeability [30,31]. Thus, the present study demonstrates that Fg-induced downregulation of VE-cadherin can occur through activation of MMP-9, which causes mainly degradation of membrane-associated VE-cadherin and has lesser effect on its translocation. Thus, an enhanced binding of Fg to endothelial ICAM-1 and the resultant activation of ERK-1/2 signaling [14] may lead to two independent effects, one is activation of MMP-9 and another formation of F-actin. The first would cause degradation of VE-cadherin and the other its translocation to cytosol.

In the present study we demonstrated that increase in Fg content correlates with increased dysfunction of ECs. Decrease in relative electrical resistance between MBECs indicated opening of cell junctional gaps, while increase in capacitance of the cell monolayer showed extent of cell detachment from the SEM. These effects of Fg were ameliorated in the presence of MMP-9 activity inhibitor, TIMP-4 suggesting that at higher concentration Fg affects both endothelial cell-cell and cell-SEM interactions. Since VE-cadherin is an adherence junction protein and located in a close vicinity of the cell basal side [13,18], changes in its expression or location may affect cell-to-cell [19,32] as well as cell to SEM interactions [32]. Changes in these interactions can positively alter EC layer junctional integrity as well as its attachment the SEM.

Simpson-Haidaris et al [33] suggested that binding of intact Fg to integrins on EC surface induces conformational change exposing the fibrinogen β^{15-42} domain that directly binds to VE-cadherin [34]. This can be a mechanism for a movement of Fg through EC layer seen in our study [11]. Since it took longer time for Fg to leak through EC layer than to albumin [11], this can be explained by the difference in Stokes radii of these proteins. As Fg induces changes in cell layer junctions it can leak through the cell layer and bind to VE-cadherin [34]. Accumulation of immobilized Fg on SEM makes it easily accessible for thrombin, which converts Fg to fibrin. Since digestion of Fg by plasmin *in vivo* is quite rare [35,36] increased deposition of fibrin during various inflammatory diseases such as hypertension, stroke, or diabetes is a result of an increase in concentration of undegraded Fg, its enhanced binding to vascular endothelium, and subsequent transmigration to SEM where it is converted to fibrin.

In conclusion, at higher level of Fg, its enhanced binding to endothelial apical side receptors alters EC layer junctional integrity and its attachment to the SEM through activation of MMP-9. High content of Fg causes downregulation VE-cadherin expression and its translocation to cytosol. Activated MMP-9 digests junction proteins and particularly VE-cadherin causing an increase in the EC layer gap openings. In addition, Fg binding to ECs activates ERK1/2 signaling and causes formation of F-actin [11], which can be a mechanism for Fg-induced disarrangement of VE-cadherin lining and its translocation. Combined, these effects of Fg may lead to an enhanced cerebrovascular permeability seen in our study (unpublished data).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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Highlights

- Fibrinogen (Fg) binding to endothelial ICAM-1 downregulates VE-cadherin expression.
- This effect of Fg occurs through activation of MMP-9.
- Fg-induced activation of MMP-9 disrupts endothelial cell layer junctional integrity
- Fg-induced activation of MMP-9 causes detachment of endothelial cells from matrix

Figure 1. Effect of Fg on VE-cadherin expression in MBECs

Examples of confocal microscopy images of VE-cadherin expression in MBECs after treatment with medium alone (Control), 2 or 4 mg/ml of Fg, (Fg2 and Fg4, respectively), 4 mg/ml Fg with anti-ICAM-1 antibody (Fg4 + anti-ICAM-1), or ICAM-1 antibody alone (anti-ICAM-1).

VE-cadherin is indicated by red color and MBEC nuclei are blue. Fg-induced disruption of VE-cadherin lining between the cells are indicated by arrows.

P < 0.05 for all. * - vs. Control, † - vs. Fg2, ‡ - vs. Fg4; n=3 for all groups

Figure 2. Fg-induced VE-cadherin expression in MBECs

Examples of confocal microscopy images of VE-cadherin expression in MBECs after treatment with medium alone (Control), 4 mg/ml Fg (Fg4), 4 mg/ml Fg with TIMP-4 (Fg4+TIMP-4), or with TIMP-4 alone (TIMP-4).

VE-cadherin is indicated by red color and MBEC nuclei are blue. Fg-induced disruption of VE-cadherin lining between the cells are indicated by arrows.

$P < 0.05$ for all. * - vs. Control, ‡ - vs. Fg4; $n=3$ for all groups

Figure 3. Fg-induced changes in VE-cadherin protein expression in MBECs

Examples of changes in VE-cadherin protein content in MBECs treated with medium alone (Control), 2 or 4 mg/ml of Fg (Fg2 and Fg4, respectively), 4 mg/ml Fg in the presence of anti-ICAM-1 antibody (Fg4 + anti-ICAM-1), or anti-ICAM-1 antibody alone (anti-ICAM-1).

Relative protein expression is reported as a ratio of integrated optical density (IOD) of each band to the IOD of the respective β -actin band.

$P < 0.05$ for all. * - vs. Control, † - vs. Fg2, ‡ - vs. Fg4; # - vs. Fg4+anti-ICAM-1. $n=4$ for all groups.

Figure 4. Fg-induced changes in MBEC layer integrity and adhesion

Changes in relative resistance (left) and capacitance (right) of MBECs treated with 4 mg/ml of Fg (Fg4), 4 mg/ml Fg in the presence of TIMP-4 (Fg4+TIMP-4), medium alone (Control), or TIMP-4 alone.

Note: The first vertical dashed line indicates cell treatment with TIMP-4, while the second vertical dashed line indicates the cell treatment with Fg
 $P < 0.05$ for all. * - vs. Control; $n=4$ for all groups.