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Fibrin-VLDL receptor-dependent pathway promotes leukocyte transmigration by inhibiting Src kinase Fyn and is a target for fibrin β 15–42 peptide

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Abstract

According to the current view, binding of fibrin degradation product E₁ fragment to endothelial VE-cadherin promotes transendothelial migration of leukocytes and thereby inflammation, and fibrin-derived β 15–42 peptide reduces leukocyte transmigration by competing with E₁ for binding to VE-cadherin and, in addition, by signaling through Src kinase Fyn. However, the very low affinity of β 15–42 to VE-cadherin raised a question about its ability to inhibit E₁-VE-cadherin interaction. Further, our previous study revealed that fibrin promotes leukocyte transmigration through the VLDL receptor (VLDLR)-dependent pathway and suggested a possible link between the inhibitory properties of β 15–42 and this pathway. To test such a link and the proposed inhibitory mechanisms for β 15–42, we performed *in vitro* experiments using SPR, ELISA, and leukocyte transendothelial migration assay, and *in vivo* studies with wild-type and VLDLR-deficient mice using mouse model of peritonitis. The experiments revealed that β 15–42 cannot inhibit E₁-VE-cadherin interaction at the concentrations used in the previous *in vivo* studies leaving the proposed Fyn-dependent signaling mechanism as a viable explanation for the inhibitory effect of β 15–42. While testing this mechanism, we confirmed that Fyn plays a critical role in controlling fibrin-induced transendothelial migration of leukocytes and found that signaling through the VLDLR-dependent pathway results in inhibition of Fyn thereby increasing leukocyte transmigration. Furthermore, our *in vivo* experiments revealed that β 15–42 inhibits this pathway thereby preventing inhibition of Fyn and reducing leukocyte transmigration. Thus, this study clarifies the molecular mechanism underlying the VLDLR-dependent pathway of leukocyte transmigration and reveals that this pathway is a target for β 15–42.

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Conflict of interest

None.

Keywords

Fibrinogen; fibrin; VLDL receptor; leukocyte transmigration; inflammation

Introduction

Besides its prominent role in hemostasis, in which conversion of fibrinogen into fibrin results in its polymerization and sealing damaged vasculature to prevent blood loss, fibrin(ogen) is involved in various physiological and pathological processes including inflammation. For example, it was shown that fibrinogen is required for efficient inflammatory responses *in vivo*¹ and fibrin deposition contributes to the pathogenesis of intraabdominal abscess formation.² It was also reported that fibrinogen promotes transendothelial migration of leukocytes,³ which is a key step in the inflammatory response, and it was proposed that fibrinogen promotes this process by bridging leukocytes to the endothelium.^{3–5} In addition, various fibrin degradation products were shown to also contribute to inflammation. Among them, the D dimer and E fragment derived from the terminal D and central E regions of fibrin, respectively, were found to induce the synthesis and secretion of pro-inflammatory interleukin-1.^{6,7} Another fibrin(ogen) degradation product, the β 15–42 fragment, which derives from the N-terminal portions of fibrin β chains,^{8,9} was shown to be an effective neutrophil chemoattractant¹⁰ and a potent inhibitor of platelet adhesion and spreading on polymerized fibrin.¹¹

More than a decade ago, Petzelbauer and collaborators¹² discovered a remarkable cardioprotective property of the β 15–42 fragment. Specifically, they found that a synthetic β 15–42 peptide corresponding to this fragment significantly reduces infarct size in rat models of myocardial ischemia-reperfusion injury.¹² The cardioprotective effect of β 15–42 was subsequently confirmed in rodent and pig models of myocardial ischemia-reperfusion injury.^{13,14} The results of Phase II clinical trials conducted on human patients with early acute myocardial infarction revealed that administration of β 15–42, which was under development as an anti-inflammatory drug called FX06, as an adjunctive agent along with reperfusion resulted in a significant reduction of the necrotic core zone and infarct size.^{15,16} In addition, it was shown that β 15–42 reduces pulmonary, myocardial, liver, and small intestine damage in a pig model of hemorrhagic shock and reperfusion,¹⁷ and exhibits organ protective effect in a pig model of hemorrhagic shock and resuscitation.¹⁸ It was also demonstrated that β 15–42 significantly attenuates ischemia-reperfusion injury in cardiac and renal transplantation models,^{19,20} protects against kidney and liver ischemia-reperfusion injury,^{21–23} and exerts protective effect during acute lung injury.²⁴ Furthermore, it was shown that β 15–42 preserves endothelial barrier function in animal models for Dengue shock syndrome and LPS-induced shock,²⁵ and improves survival and neurocognitive recovery and ameliorates vascular leakage after cardiopulmonary resuscitation.²⁶ The β 15–42 peptide was also used for successful treatment of vascular leak syndrome in Ebola virus disease patient.²⁷

Taking into account a significant effect of the β 15–42 fragment on various inflammation-related processes mentioned above, it is important to understand the molecular mechanism

underlying its biological activity for a better design of potent modulators of such processes. To explain the cardioprotective effect of this fragment, Petzelbauer and collaborators¹² performed various experiments with the NDSK-II fragment, which represents the central region of fibrin including the β 15–42 sequences, and a synthetic β 15–42 peptide (Fig. 1A). Based on their finding that NDSK-II can induce leukocyte transmigration and β 15–42 blocks this process, they proposed that naturally occurring fibrin degradation product E₁ fragment, which is an analog of NDSK-II, promotes leukocyte transmigration *in vivo* by bridging leukocytes to the endothelium through the interaction with CD11c integrin on leukocytes and with VE-cadherin on endothelial cells.^{12,28} In this bridging model, the β 15–42 peptide was proposed to compete with E₁ for binding to VE-cadherin thereby blocking this binding and inhibiting leukocyte transmigration (Fig. 1B). In addition, Gröger and collaborators^{25,29} proposed a signaling function for β 15–42, which signals through the Src kinase Fyn resulting in inhibition of GTPase protein RhoA and maintaining the integrity of the endothelial barrier (Fig. 1C).

Thus, according to the current view, the β 15–42 fragment modulates leukocyte transmigration through two mechanisms, direct inhibition of leukocyte binding and Fyn-dependent signaling. As an inhibitory molecule, β 15–42 is thought to reduce leukocyte transmigration and thereby inflammation by directly inhibiting E₁ fragment-mediated bridging of leukocytes to the endothelium. As a signaling molecule, β 15–42 is thought to contribute to endothelial barrier integrity by inhibiting RhoA through a Fyn-dependent signaling pathway. However, our previous data³⁰ indicating that the affinity of β 15–42 to VE-cadherin is very low raised a question of whether this fragment can indeed compete with the E₁ fragment for its binding to VE-cadherin. Further, our previous study revealed that interaction of fibrin with the VLDL receptor (VLDLR) triggers the VLDLR-dependent pathway that promotes leukocyte transmigration.³¹ We found that this interaction occurs through a pair of fibrin β N-domains,³¹ each of which encompasses β 15–64 sequence.⁸ We also found that the VE-cadherin-binding site is located in the N-terminal half of the β N-domain including β 15–42 sequence⁸ while the VLDLR-binding site is located mainly in its C-terminal half.³² This finding suggested that the inhibitory function of β 15–42 should be independent of the VLDLR-dependent pathway. Surprisingly, our previous experiments performed using a mouse model of peritonitis revealed that the dimeric form of β 15–42, the (β 15–44)₂ fragment, inhibited leukocyte transmigration in wild-type mice but failed to inhibit this process in VLDLR deficient mice.³¹ These findings raised a question of whether the signaling function of β 15–42 may be connected with the VLDLR-dependent pathway. The present study was performed to address these two questions and to further clarify the molecular mechanism underlying the VLDLR-dependent pathway of leukocyte transmigration.

Materials and Methods

Proteins and reagents

Human fibrinogen (plasminogen-, VWF-, and fibronectin-depleted), plasmin, and thrombin were from Enzyme Research Laboratories (South Bend, IN, USA). The fibrin-derived E₁ fragment and its analog, NDSK-II fragment, were prepared as described earlier.^{30,33–35} E₁

and NDSK-II were biotinylated with EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific, Rockford, IL, USA) as recommended by the manufacturer. The β 15–42 and β 15–44 peptides were synthesized by Bachem (Torrance, CA, USA) and β 15–44 was dimerized as previously described³⁰ to produce the dimeric (β 15–44)₂ fragment. Human recombinant VE-cad(1–5) fragment including all five extracellular domains of VE-cadherin was expressed in *Escherichia coli* and purified as described earlier.³⁶ Anti-VLDLR monoclonal antibody (mAb) 1H10^{37,38} was purified from hybridoma supernatants on Protein A-Sepharose (Sigma-Aldrich, St. Louis, MO, USA). Anti-NDSK-II mAb T2G1 against the fibrin β 15–21 region^{39,40} was a gift from Dr. B. Kudryk (New York Blood Center). Anti-Fyn mAb FYN-59 and anti-actin mAb AC-40 were obtained from BioLegend (San Diego, CA, USA) and Sigma-Aldrich, respectively. Goat secondary anti-mouse polyclonal antibodies (H +L) conjugated with HRP and HRP substrate SureBlue Reserve were from KPL (Gaithersburg, MD, USA). Pierce NeutrAvidin conjugated with HRP was from Thermo Scientific. Saracatinib (AZD0530), an inhibitor of the Src kinase family members including Fyn, was from BioVision (Milpitas, CA, USA). Calcein AM fluorescent dye and phorbol 12-myristate 13-acetate (PMA) were from Corning (Bedford, MA, USA) and Promega (Madison, WI, USA), respectively, N-formyl-Met-Leu-Phe (fMLP) and FITC-labeled 40 kDa dextran were from Sigma-Aldrich.

Cell cultures and treatments

Human umbilical vein endothelial cells (HUVECs) obtained from Lonza (Walkersville, MD, USA) were cultured in EBM-2 basal medium supplemented with EGM-2 SingleQuot Kit (Lonza), which contained 10% FBS,³⁸ according to the manufacturer's instruction, and used for experiments at passage 4–6. The HL-60 human promyelocytic leukemia cell line (ATCC, Manassas, VA, USA) was cultured and differentiated into a neutrophil-like lineage as described earlier.^{30,41} All cell cultures were maintained at 37°C in 5% CO₂.

Mice

Wild-type C57BL/6J mice aged 8–12 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). VLDLR deficient (VLDLR^{-/-}) mice on a mixed background were also obtained from the Jackson Laboratory and bred 10 generations into a C57BL/6 background. Littermate mice were used as controls. Since in our previous studies we noted no difference in leukocyte infiltration into the peritoneum between male and female mice, both were used in these experiments. All mice were housed in a pathogen-free facility, and all procedures were performed with approval of the University of Maryland Institutional Animal Care and Use Committee.

Knockdown of Fyn expression in HUVECs

HUVECs were transfected with MISSION lentiviral transduction particles containing shRNA (Sigma-Aldrich) as recommended by the manufacturer. Among five different shRNAs tested, the most potent construct (TRCN0000003099) was selected for our experiments. Transfection was performed at ~ 80 % HUVEC confluency with MOI (multiplicity of infection) of 75 in the presence of polybrene (AmericanBio, Natick, MA, USA) at 8 µg/mL. Infected cells were then treated with 2 µg/mL puromycin (Sigma-Aldrich) for 24 hours. Puromycin-resistant cells were maintained in complete medium under

puromycin selection (0.5 µg/mL puromycin) and were used for no more than 1–2 additional passages. The shRNA-mediated suppression of Fyn expression in HUVECs was tested by immunoblotting.

Immunoblotting

To assess Fyn levels in HUVECs, the cells were lysed in Pierce RIPA buffer (Thermo Scientific) containing 1 mM EDTA and 0.2 mM PMSF, the lysates were subjected to SDS-PAGE in 4–12% Bis-Tris gel, and the proteins were transferred to nitrocellulose membranes. Fyn and actin were detected by immunoblotting with anti-Fyn mAb FYN-59 and anti-actin mAb AC-40, respectively, followed by secondary anti-mouse HRP-conjugated IgG. The blots were developed with SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific). ImageJ software (NIH) was used to quantify band densities on the immunoblots.

Surface plasmon resonance analysis

The inhibitory effect of the β 15–42 fragment on the interaction of NDSK-II with VE-cadherin was tested by surface plasmon resonance (SPR) using the BIAcore 3000 biosensor (BIAcore AB, Uppsala, Sweden), essentially as we described earlier.^{30,36} Briefly, immobilization of the VE-cad(1–5) fragment to the surface of CM5 sensor chip was performed using the amine coupling kit as previously described.⁸ Binding experiments were performed at 10 µL/min flow rate in the binding buffer HBS-P (GE Healthcare-Biosciences AB, Uppsala, Sweden) containing 1 mM CaCl₂. NDSK-II at 1.5 µM without or with increasing concentrations of β 15–42, or increasing concentrations of β 15–42 were injected, and association/dissociation between them and immobilized VE-cad(1–5) was monitored in real time by the change in the SPR signal (response). To regenerate the chip surface, complete dissociation of the bound NDSK-II and/or β 15–42 fragments was performed as described earlier.⁸ To evaluate the affinity of NDSK-II to VE-cadherin, increasing concentration of NDSK-II in the same buffer as above were injected and the association/dissociation between it and immobilized VE-cad(1–5) was monitored as described above. Experimental data were analyzed using BIAevaluation 4.1 software supplied with the instrument. The dissociation equilibrium constant, K_d , was calculated with the equation $K_d = k_{\text{diss}}/k_{\text{ass}}$, where k_{ass} and k_{diss} represent kinetic constants that were estimated by global fitting analysis of the association and dissociation data, respectively, using the 1:1 Langmurian interaction model (kinetic analysis). To confirm the kinetic analysis, K_d was also estimated by analysis of the association data using the steady-state affinity model (equilibrium analysis).

Solid-phase binding assay (ELISA)

To test the inhibitory effect of β 15–42 on binding of biotinylated NDSK-II or E₁ to the VE-cad(1–5) fragment, wells of Immulon 2HB microtiter plates were coated overnight at 4°C with 2 µg/mL VE-cad(1–5) in coating buffer (0.1M Na₂CO₃, pH 9.5). The wells were then blocked with Blocker BSA in TBS (Tris-buffered saline) (Thermo Scientific) for 1 hour at room temperature. Following washing with the binding buffer (TBS containing 0.05% Tween 20 and 1mM CaCl₂), biotinylated NDSK-II or E₁, each at 1.5 µM in the binding buffer, was pre-incubated with β 15–42 at 12 or 120 µM, and with (β 15–44)₂ at 12 µM or

with mAb T2G1 at 0.5 μ M, for 30 min at 37°C, and 100 μ L aliquots of the mixtures were added to the wells and incubated overnight at 4°C. Bound NDSK-II or E₁ fragment was detected by the reaction with NeutrAvidin conjugated with HRP (1 hour at 37°C). TMB microwell peroxidase substrate, SureBlue Reserve (KPL), was added to the wells, and the amount of bound E₁ or NDSK-II was measured spectrophotometrically at 450 nm after termination of the reaction with KPL TMB stop solution.

Endothelial permeability assay

Permeability of cultured HUVEC monolayers was measured using FITC-labeled 40 kDa dextran flux. The experiments were performed in 24-well plates containing 8- μ m pore size PET membrane inserts (Corning, Durham, NC, USA). HUVECs were seeded onto the insert membrane precoated with 0.1% gelatin (Millipore, Temecula, CA, USA) and grown to confluence for 3 days without medium in the lower chamber. FITC-labeled dextran was added to the monolayers at 1 mg/mL in 200 μ L of complete endothelium cell medium. The inserts were placed into the wells, each of which contained 700 μ L of the medium. The samples were removed from the lower chambers after 1 hour of incubation and FITC-dextran was quantified with fluorescence plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA) at 480 nm/530 nm.

Leukocyte transendothelial migration assay

Transmigration experiments were performed as we described earlier.^{30,31,38,42} Briefly, HUVEC monolayers on 8- μ m pore size PET membrane inserts were prepared as described above. The monolayers were then washed with IMDM and serum-starved for 2 hours before experiments. Calcein AM-labeled differentiated HL-60 cells were stimulated with 100 nM PMA, washed with IMDM, and 5×10^5 cells in IMDM containing 1.5 μ M NDSK-II without or with 100 nM Saracatinib or 0.5 μ M mAb 1H10 were added on top of HUVEC monolayers. The inserts were placed into the wells each containing 100 nM chemoattractant fMLP in IMDM. Transmigration proceeded for 4 hours and migrated HL-60 cells were recovered from the bottom of wells and quantified with fluorescence plate reader.

Mouse model of peritonitis

Experiments with mice were performed as we described earlier.^{30,31,38} Briefly, wild-type or VLDLR^{-/-} mice (7 per group, aged 8–12 weeks) were injected intraperitoneally (i.p.) with 3.85% Bacto Fluid thioglycollate (1 mL per mouse) to induce leukocyte infiltration into the peritoneum. To test the effect of β 15–42, (β 15–44)₂, and Saracatinib on leukocyte infiltration, mice received an intravenous (i.v.) injection (via the tail vein) of the peptides at 80 μ M or Saracatinib at 0.8 μ M, all in 200 μ L PBS (Phosphate Buffered Saline, Lonza) prior to i.p. injections of thioglycollate. Mice in control groups received an i.v. injection of the same volume of PBS. Four hours after the injections, each group of mice was euthanized, injected i.p. with 3 mL ice-cold PBS, total lavage fluid was withdrawn, and total cell number in lavage fluid was determined using a hemocytometer.

Statistical analysis

Statistical analysis was performed using Student's *t*-test (two sided) with a P value of less than 0.05 being considered significant. All statistical analyses were performed in SigmaPlot 13.0 software (Systat Software, San Jose, CA, USA).

Results

Effect of the $\beta 15$ –42 fragment on interaction of the NDSK-II and E₁ fragments with VE-cadherin

Although the proposed direct inhibition mechanism implies inhibition of the E₁ fragment-VE-cadherin interaction by the $\beta 15$ –42 fragment (Fig. 1B), comparison of affinities of $\beta 15$ –42 and E₁ to VE-cadherin suggests that this mechanism may not be entirely correct. Indeed, our previous study revealed that while the affinity of E₁ to VE-cadherin is high ($K_d = 69$ –79 nM)³⁶, that of $\beta 15$ –42 is very low ($K_d = 267$ μ M).³⁰ Further, the determined K_d value of 267 μ M for $\beta 15$ –42 is more than 20-fold higher than the concentrations of this fragment (~5–12 μ M) that inhibited leukocyte transmigration in the previous *in vivo* experiments.^{12,30} These facts strongly suggest that $\beta 15$ –42 may inhibit leukocyte transmigration via a different mechanism. To test this suggestion, the following experiments were performed.

Since NDSK-II is an analog of the E₁ fragment, has practically the same affinity to VE-cadherin as that of E₁, as we determined here by SPR ($K_d = 97 \pm 14$ nM, Fig. 2, inset), and is usually used to stimulate transendothelial migration of leukocytes instead of the E₁ fragment,^{12,30,31} we first tested the inhibitory effect of the $\beta 15$ –42 fragment on interaction of NDSK-II with the VE-cad(1–5) fragment, which represents the extracellular portion of VE-cadherin,³⁶ using SPR. Since $\beta 15$ –42 has much lower mass than NDSK-II (3 kDa vs 58 kDa) and in control experiments exhibited a very low binding to VE-cad(1–5) (Fig. 2), one could expect a significant drop in SPR signal if the inhibition occurs. However, when NDSK-II at 1.5 μ M with increasing concentrations of $\beta 15$ –42 (0–12 μ M) was added to immobilized VE-cadherin, the association/dissociation curves were practically the same (Fig. 2) indicating practically no inhibitory effect of $\beta 15$ –42 on the interaction of NDSK-II with VE-cad(1–5). These results indicate that the $\beta 15$ –42 fragment at 12 μ M, the inhibitory concentration used in the previous *in vivo* experiments,^{12,30} does not inhibit interaction of NDSK-II with VE-cadherin.

Next, we performed similar experiments by ELISA; however, in these experiments we compared the inhibitory effect of $\beta 15$ –42 on the interaction of NDSK-II and the E₁ fragment with the immobilized VE-cad(1–5) fragment. When of NDSK-II at 1.5 μ M was added to VE-cad(1–5), a significant binding was observed. This binding was not inhibited by the addition of $\beta 15$ –42 either at 12 μ M or at 120 μ M, a 10-fold higher concentration (Fig. 3A). In control experiment, the T2G1 monoclonal antibody, which was shown to inhibit fibrin-VE-cadherin interaction,³⁶ almost completely inhibited interaction of NDSK-II with VE-cad(1–5). Similar results were obtained with the E₁ fragment (Fig. 3B), whose interaction with VE-cad(1–5) was also not inhibited by $\beta 15$ –42 either at 12 μ M or at 120 μ M (Fig. 3B). At the same time, the dimeric ($\beta 15$ –44)₂ fragment, which has much higher affinity to VE-cadherin than $\beta 15$ –42 ($K_d = 715$ nM vs 267 μ M)³⁰ and was used as a control, exhibited

significant inhibition even at 12 μM . Altogether, these experiments clearly show that the $\beta 15\text{--}42$ fragment cannot inhibit the interaction of either NDSK-II or the E_1 fragment with VE-cadherin at the concentrations used in the previous *in vivo* experiments.^{12,30} This, in turn, indicates that the direct inhibition mechanism proposed to explain the inhibitory effect of $\beta 15\text{--}42$ ^{12,28} (Fig. 1B) is incorrect. This also leaves the proposed Fyn-dependent signaling mechanism for the inhibitory action of the $\beta 15\text{--}42$ fragment^{25,29} (Fig. 1C) as a viable explanation for the inhibitory effect of $\beta 15\text{--}42$ on leukocyte transmigration.

Effect of Fyn inhibitor on transendothelial migration of leukocytes *in vitro*

The proposed signaling mechanism induced by the $\beta 15\text{--}42$ fragment suggests that this fragment mediates its activity through Src kinase Fyn, which is proposed to associate with p190RhoGAP ultimately resulting in inhibition of the GTPase protein RhoA thereby preventing cell contraction and maintaining endothelial barrier²⁵ (Fig. 1C). To test this mechanism, we studied the effect of Fyn inhibitor Saracatinib on leukocyte transmigration using *in vitro* leukocyte transendothelial migration assay. The experiments revealed that this inhibitor significantly increases leukocyte transmigration (Fig. 4), in agreement with the proposed Fyn-dependent signaling mechanism.²⁵ Interestingly, this inhibitor increased leukocyte transmigration practically to the same level as the fibrin-derived NDSK-II fragment, which is the simplest mimetic of fibrin⁴² and is an efficient stimulator of leukocyte transmigration,^{12,30} and addition of Saracatinib to NDSK-II had no additive effect on this process (Fig. 4). These results suggest that NDSK-II and Saracatinib function in the same pathway revealing that NDSK-II may also act as Fyn inhibitor. Together, these results suggest that the previously described fibrin-induced VLDLR-dependent pathway of transendothelial migration of leukocytes³¹ may lead to inhibition of Fyn thereby preventing inhibition of RhoA and increasing leukocyte transmigration.

Effect of the $\beta 15\text{--}42$ and $(\beta 15\text{--}44)_2$ fragments and Fyn inhibitor on leukocyte transmigration in wild-type and VLDL receptor-deficient mice

Our previous studies discovered that fibrin and NDSK-II, which has practically the same affinity to VLDLR ($K_d = 9.6 \pm 1.9$ nM determined by SPR, data not shown) as fibrin ($K_d = 9.2$ nM),³¹ promote transendothelial migration of leukocytes through the VLDLR-dependent pathway^{31,42} and that $\beta 15\text{--}42$ inhibits leukocyte transmigration.³⁰ These findings raised the possibility that the inhibitory properties of $\beta 15\text{--}42$ may also occur via the VLDLR-dependent pathway. This connection is reinforced by our previous experiments, which revealed that the dimeric $(\beta 15\text{--}44)_2$ fragment has little effect on leukocyte transmigration in VLDLR-deficient mice³¹. However, the relatively high affinity of $(\beta 15\text{--}44)_2$ for VE-cadherin ($K_d = 146\text{--}715$ nM)³⁰ makes it difficult to unambiguously define the specific mechanism involved. Therefore, we performed similar experiments in which monomeric $\beta 15\text{--}42$ was used instead of $(\beta 15\text{--}44)_2$. Namely, we tested the effect of the $\beta 15\text{--}42$ fragment on infiltration of leukocytes (neutrophils) into the peritoneum in wild-type and VLDLR-deficient mice using the mouse model of peritonitis. The experiments revealed that in wild-type mice $\beta 15\text{--}42$ inhibited neutrophil infiltration into the peritoneum by about 30% (Fig. 5), which is in a good agreement with the results of our previous *in vivo* experiments.³⁰ At the same time, this fragment had practically no effect on leukocyte infiltration in VLDLR-deficient mice. This finding suggests that the inhibitory activity of $\beta 15\text{--}42$ requires the

presence of the VLDL receptor, i.e. that the inhibitory properties of this fragment are connected with the VLDLR-dependent pathway of leukocyte transmigration.

To further explore this finding, we performed the following experiments using the same mouse model of peritonitis. Namely, we tested the effect of Fyn inhibitor Saracatinib and $\beta 15-42$ -containing fragment on leukocyte transmigration in wild-type mice. Since $\beta 15-42$ and its dimeric version, $(\beta 15-44)_2$, both inhibited leukocyte transmigration in wild-type mice and failed to inhibit it in VLDLR-deficient mice in the present (Fig. 5) and our previous studies³¹, and since dimeric $(\beta 15-44)_2$ exhibits about 2-fold higher inhibitory activity than $\beta 15-42$,³⁰ we used in these experiments $(\beta 15-44)_2$ to increase the inhibitory effect. The results obtained revealed that $(\beta 15-44)_2$ inhibited infiltration of leukocytes (neutrophils) into the peritoneum practically to the same extent as we demonstrated earlier,^{30,31} while the Fyn inhibitor prevented $(\beta 15-44)_2$ from inhibiting this process (Fig. 6). This indicates that $(\beta 15-44)_2$ does not inhibit leukocyte transmigration when Fyn is inhibited, and suggests that the inhibitory action of $(\beta 15-44)_2$ takes place upstream of Fyn action, i.e. that it inhibits the VLDLR-dependent pathway thereby preventing inhibition of Fyn.

Interestingly, the Fyn inhibitor had no effect on infiltration of leukocytes when added in the absence of $(\beta 15-44)_2$ (Fig. 6). To explain these results, one should keep in mind that, in contrast to the transmigration assay, in which leukocyte transmigration was stimulated by fibrin mimetic NDSK-II (see Fig. 4), in the peritonitis model leukocyte transmigration is mediated by the VLDLR-dependent pathway and their infiltration into the peritoneum is stimulated by the interaction of fibrin deposited on inflamed endothelium with the VLDL receptor, as we previously discussed.⁴² This in turn inhibits Fyn increasing RhoA-mediated barrier opening. Since Fyn is inhibited by fibrin binding to the VLDL receptor, the Fyn inhibitor has no effect. These results are in agreement with the proposed Fyn-dependent signaling mechanism²⁵ which suggests that inhibition of Fyn should prevent inhibition of RhoA whose activity causes loss of function of the endothelial barrier resulting in increased leukocyte transmigration (Fig. 1C).

Effect of fibrin-VLDLR interaction on leukocyte transmigration in Fyn-knockdown HUVECs

To further test the above suggestion that the VLDLR-dependent pathway may promote leukocyte transmigration through the inhibition of Fyn, we studied the effects of NDSK-II, which stimulates leukocyte transmigration, and monoclonal antibody mAb 1H10, which was shown to specifically inhibit fibrin-VLDLR interaction and leukocyte transmigration,³⁸ on transendothelial migration of leukocytes in wild-type and Fyn-knockdown HUVECs. To prepare Fyn-knockdown HUVECs, wild-type HUVECs were treated with specific shRNA to suppress Fyn production as described in Materials and Methods. Immunoblot analysis revealed that such shRNA suppressed Fyn expression in Fyn-knockdown HUVECs by more than 80% (Fig. 7, inset A).

As expected, in control experiments with wild-type HUVECs using leukocyte transmigration assay, NDSK-II stimulated transendothelial migration of leukocytes and mAb 1H10 inhibited NDSK-II-induced leukocyte transmigration (Fig. 7). At the same time, no stimulating effect of NDSK-II or inhibitory effect of this mAb on NDSK-II-induced leukocyte transmigration was observed with Fyn-knockdown HUVECs (Fig. 7). This finding

further confirms that the fibrin-VLDLR-dependent pathway requires Fyn to promote transendothelial migration of leukocytes. It should be noted that leukocyte transmigration was increased in Fyn-knockdown HUVECs compared to that in wild-type HUVECs. Our additional experiments using endothelial permeability assay revealed a significant increase in permeability of Fyn-knockout HUVECs for FITC-labeled dextran (Fig. 7, inset B). These data are in agreement with the proposed key role of Fyn in the maintenance of endothelial integrity.²⁵

Discussion

Numerous studies demonstrated that fibrin-derived $\beta 15-42$ fragment efficiently inhibits transendothelial migration of leukocytes and thereby inflammation in various inflammation-related animal models¹²⁻²⁷ (see Introduction). Two molecular mechanisms have been proposed to explain its anti-inflammatory action, the direct inhibition mechanism and Fyn-dependent signaling one (Fig. 1). We tested both mechanisms in the present study. The results obtained allowed us to establish a link between the inhibitory properties of the $\beta 15-42$ fragment and the previously discovered fibrin-induced VLDLR-dependent pathway of leukocyte transmigration,³¹ and to clarify the molecular mechanism underlying this pathway.

The first mechanism proposed to explain the inhibitory properties of the $\beta 15-42$ fragment suggests that fibrin degradation product E_1 fragment bridges leukocytes to the endothelium through the interaction with leukocyte CD11c integrin and endothelial VE-cadherin, thereby promoting transendothelial migration of leukocytes, and $\beta 15-42$ inhibits leukocyte transmigration by competing for E_1 -VE-cadherin interaction^{12,28} (Fig. 1B). In the present study, we provide direct experimental evidence that at the concentrations inhibiting leukocyte transmigration in the previous *in vivo* experiments^{12,30} $\beta 15-42$ cannot compete with the E_1 fragment for E_1 -VE-cadherin interaction due to its very low affinity to VE-cadherin. Moreover, the E_1 fragment may exist in the circulation only in a complex with the D dimer in which it cannot interact with CD11c, as we previously discussed.⁴² Altogether, these facts indicate that the proposed direct inhibition mechanism^{12,28} is incorrect.

The second proposed mechanism suggests that, in addition of being an inhibitory molecule, $\beta 15-42$ is also a signaling molecule which preserves endothelial barrier function thereby reducing leukocyte transmigration by inhibiting stress-induced opening of endothelial cell junctions.^{25,29} This Fyn-dependent mechanism includes dissociation of the Src kinase Fyn from VE-cadherin-containing junctions upon exposure of endothelial cells to $\beta 15-42$ followed by association of Fyn with p190RhoGAP that inhibits GTPase protein RhoA²⁵ (Fig. 1C). Since RhoA is known to regulate actin dynamics and junction stability,⁴³ its inactivation prevents cell contraction and maintains endothelial barrier function. While testing this mechanism, we found the molecular target for the VLDLR-dependent pathway of leukocyte transmigration and revealed a link between the inhibitory properties of the $\beta 15-42$ fragment and this pathway.

One of the major findings of the present study is that the inhibitory properties of the $\beta 15-42$ fragment are connected with its inhibition of the fibrin-induced VLDLR-dependent pathway which promotes leukocyte transmigration. This is evident from our present (Fig. 5) and

previous³¹ *in vivo* experiments using mouse model of peritonitis which clearly indicate that both $\beta 15-42$ and $(\beta 15-44)_2$ did not inhibit infiltration of leukocytes into the peritoneum in VLDLR-deficient mice. Another major finding is that the VLDLR-dependent pathway results in inhibition of Fyn. There are two lines of evidence for this finding. First, *in vitro* experiments revealed that Fyn inhibitor Saracatinib and NDSK-II both exhibited similar stimulating effect on leukocyte transmigration and their combined effect was not additive (Fig. 4) suggesting that they both inhibit Fyn. Second, our experiments with Fyn-knockdown HUVECs revealed that NDSK-II does not stimulate leukocyte transmigration in such cells and mAb 1H10, which inhibits fibrin-VLDLR interaction and reduces leukocyte transmigration in wild-type HUVECs and wild-type mice³⁸, does not inhibit this process in Fyn-knockdown HUVECs (Fig. 7).

In summary, the present study clarifies the molecular mechanism underlying fibrin-induced VLDL receptor-dependent pathway of transendothelial migration of leukocyte and identifies it as a target for the fibrin-derived $\beta 15-42$ fragment. The data obtained are consistent with the model presented in Fig. 8, which combines our major findings with the previously proposed role of Fyn in inactivation of RhoA,²⁵ whose activity causes loss of function of the endothelial barrier resulting in increased leukocyte transmigration. This model suggests that interaction of fibrin or its mimetic NDSK-II with the VLDL receptor triggers the VLDLR-dependent pathway which leads to inhibition of Fyn (Fig. 8A). When Fyn is inhibited by this pathway, RhoA is active and fibrin-induced leukocyte transmigration is maximal. When this pathway is inhibited by the $\beta 15-42$ or $(\beta 15-44)_2$ fragments (Fig. 8B), as demonstrated in the present study, active Fyn associates with p190RhoGAP and inhibits RhoA, as was suggested by Gröger and collaborators²⁵. Such inhibition prevents stress-induced opening of endothelial junctions and fibrin-induced leukocyte transmigration. The only discrepancy between this model and our experimental data is that infiltration of leukocytes into the peritoneum in VLDLR-deficient mice (Fig. 5), in which the VLDLR-dependent pathway is absent, was higher than one would expect when Fyn is not inhibited by this pathway. This was also observed in our previous study³¹. A possible explanation is that in such mice the absence of this pathway is compensated by other pathway(s) controlling the activity of Fyn.

It should be noted that it is unlikely that the $\beta 15-42$ or $(\beta 15-44)_2$ fragments, which are comparatively bulky and highly positively charged,³² can enter endothelial cells to directly inhibit the VLDLR-dependent pathway. Thus, one can speculate that such fragments may bind to a putative receptor which could transfer a signal to this pathway through some mediators. Similarly, the VLDLR-dependent pathway may also include putative mediators transferring fibrin-induced signal to Fyn. Such putative receptor and mediators remain to be identified. It should also be noted that the $\beta 15-42$ peptide, which was shown to exhibit anti-inflammatory properties in various inflammation related *in vivo* models,¹²⁻²⁷ and the monoclonal antibody mAb 1H10, which we demonstrated earlier to inhibit fibrin-VLDLR interaction³⁸, are both efficient inhibitors of leukocyte transmigration. The present study found that they both inhibit the same VLDLR-dependent pathway of leukocyte transmigration. This finding suggests that this mAb may exhibit similar anti-inflammatory effects as $\beta 15-42$ in the *in vivo* models mentioned above.¹²⁻²⁷ This finding also raises a question of whether mAb 1H10 would be a suitable antagonist of leukocyte transmigration

for the development as anti-inflammatory therapeutics. This question remains to be answered.

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Summary Table

What is known about this topic?

- Fibrin-derived $\beta 15-42$ peptide was shown to inhibit transendothelial migration of leukocytes and thereby inflammation in different animal models of inflammation.
- Two mechanisms for the inhibitory action of $\beta 15-42$ have been proposed: (1) $\beta 15-42$ directly inhibits interaction of fibrin-degradation product fragment E_1 with endothelial VE-cadherin thereby reducing leukocyte transmigration; (2) $\beta 15-42$ also signals through Src kinase Fyn to inhibit GTPase protein RhoA whose activity causes endothelial barrier disruption.
- We have also discovered that interaction of fibrin with the VLDL receptor triggers the VLDL receptor-dependent pathway that promotes leukocyte transmigration and inhibition of this interaction with specific mAbs or some $\beta 15-42$ -containing fragments inhibits this process.

What does this paper add?

- We demonstrate here that $\beta 15-42$ cannot inhibit E_1 fragment-VE-cadherin interaction at the concentrations used in the previous *in vivo* experiments suggesting that the proposed direct inhibitory function for this peptide is incorrect; at the same time our study confirmed signaling function of $\beta 15-42$ through Fyn.
- Our experiments revealed that fibrin-induced VLDL receptor-dependent pathway promotes leukocyte transmigration by inhibiting Fyn. This finding clarifies the molecular mechanism underlying this pathway.
- We also found that $\beta 15-42$ reduces leukocyte transmigration by inhibiting the VLDL receptor-dependent pathway thereby preventing inhibition of Fyn. This finding establishes a link between the inhibitory function of $\beta 15-42$ and this pathway.

Fig. 1. Schematic representation of the proposed mechanisms for the inhibitory actions of the $\beta 15$ –42 fragment. **(A)** Schematic presentation of fibrin and its fragments used in the present study. Fibrin E region and the E₁ and NDSK-II fragments derived from this region are colored blue with their β N-domains colored red; the $\beta 15$ –42 and $(\beta 15$ –44)₂ fragments representing N-terminal halves of these domains are also colored red. **(B)** In the proposed direct inhibition mechanism,^{12,28} fibrin degradation product E₁ fragment bridges leukocytes to the endothelium through the interaction with leukocyte CD11c integrin and endothelial VE-cadherin to promote their transendothelial migration, and the $\beta 15$ –42 fragment directly inhibits E₁-VE-cadherin interaction thereby reducing leukocyte transmigration. **(C)** In the proposed Fyn-dependent signaling mechanism,^{25,29} $\beta 15$ –42 signals to Fyn resulting in

dissociation of the latter from VE-cadherin, association with p190 RhoGAP, and subsequent inhibition of RhoA. Since active RhoA promotes stress-induced opening of endothelial cell adherent junctions and thereby leukocyte transmigration, its inhibition prevents cell contraction and maintains endothelial barrier function.⁴³

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Fig. 2.

Effect of the $\beta 15-42$ fragment on binding of NDSK-II to the immobilized VE-cad(1-5) fragment detected by surface plasmon resonance (SPR). NDSK-II at $1.5 \mu\text{M}$ was added to immobilized VE-cad(1-5) fragment in the absence or presence of increasing concentrations of $\beta 15-42$ (0.6, 1.2, 6.0, $12 \mu\text{M}$). In control experiments, $\beta 15-42$ was added to immobilized VE-cad(1-5) at the same concentrations (0.6, 1.2, 6.0, $12 \mu\text{M}$). The association and dissociation of $\beta 15-42$ and (NDSK-II + $\beta 15-42$) was measured in real time while registering SPR signal (response). The curves are representative of three independent experiments. The inset shows SPR-detected binding of NDSK-II added at 10, 25, 50, 100, 200, 500 nM to immobilized VE-cad(1-5); the dotted curves represent the best fit of the binding data using global fitting analysis (see Material and Methods). The K_d value determined from 3 independent experiments was found to be $97 \pm 14 \text{ nM}$, i.e. very close to those determined earlier for fibrin,⁸ the E_1 fragment,³⁶ and the recombinant $(\beta 15-66)_2$ fragment corresponding to a pair of fibrin βN -domains.^{8,36}

Fig. 3.

ELISA-detected effect of the $\beta 15-42$ fragment on binding of NDSK-II (**A**) and the E_1 fragment (**B**) to the immobilized VE-cad(1-5) fragment. NDSK-II or E_1 , each at 1.5 μM , was added without or with 12 and 120 μM $\beta 15-42$ to immobilized VE-cad(1-5) and its binding was measured as described in Materials and Methods. In control experiments, NDSK-II or E_1 , each at 1.5 μM , was added to VE-cad(1-5) in the presence of 0.5 μM mAb T2G1 or 12 μM $(\beta 15-44)_2$, respectively. Each graph shows combined data obtained from two independent experiments performed in triplicate; error bars denote means $\pm$ SD.

Fig. 4.

Effect of Fyn inhibitor Saracatinib on leukocyte (neutrophil) transmigration observed using leukocyte transendothelial migration assay. HUVECs were grown to confluence on gelatin-coated cell culture inserts. Calcein AM-labeled HL-60 cells differentiated into neutrophil-like cells were added to upper chambers on top of the HUVEC monolayers in the presence of PBS (control), 100 nM Saracatinib, 1.5 μ M NDSK-II (fibrin mimetic added to stimulate cell transmigration), or a combination of 1.5 μ M NDSK-II and 100 nM Saracatinib; Saracatinib in both cases was added 30 min prior addition of HL-60 cells to let its diffusion into the cells. The cells were allowed to migrate into the lower chambers containing chemoattractant fMLP for 4 hours at 37 °C, collected, and measured by fluorescence at 530 nm. The results are expressed as percentage of differentiated HL-60 cells (neutrophils) migrated in the presence of PBS (control). The graph shows combined data from two independent experiments performed in triplicate; error bars denote means $\pm$ SD.

Fig. 5.

Inhibitory effect of the β 15–42 fragment on leukocyte (neutrophil) infiltration into the peritoneum in wild-type and VLDL receptor-deficient mice observed using mouse model of peritonitis. The β 15–42 fragment in 200 μ L of PBS at 80 μ M was injected intravenously in wild-type and VLDLR^{-/-} mice; control mice were injected with 200 μ L of PBS. The number of infiltrated leukocytes (neutrophils) was estimated as described in Materials and Methods. Dots represent individual mice, and lines show the mean and standard error of the mean of each group (n = 7). ***P < 0.001.

Fig. 6.

Effect of $(\beta 15-44)_2$ and Fyn inhibitor Saracatinib on leukocyte (neutrophil) infiltration into the peritoneum in wild-type mice observed using mouse model of peritonitis. Fyn inhibitor at 0.8 μM , $(\beta 15-44)_2$ at 80 μM , or a mixture of Fyn inhibitor at 0.8 μM and $(\beta 15-44)_2$ at 80 μM , each in 200 μL of PBS, was injected intravenously in wild-type mice; control mice were injected with 200 μL of PBS. The number of infiltrated leukocytes (neutrophils) was estimated as described in Materials and Methods. Dots represent individual mice, and lines show the mean and standard error of the mean of each group ($n = 7$). *** $P < 0.001$.

Fig. 7.

Effect of NDSK-II and anti-VLDLR mAb 1H10 on leukocyte (neutrophil) transmigration in wild-type and Fyn-knockdown endothelial cells observed using leukocyte transendothelial migration assay. Wild-type and Fyn-knockdown HUVECs were grown to confluence on gelatin-coated cell culture inserts. Calcein AM-labeled HL-60 cells differentiated into neutrophil-like cells were added to upper chambers on top of the HUVEC monolayers in the presence of PBS (control), 1.5 μ M NDSK-II, or a combination of 1.5 μ M NDSK-II and 0.5 μ M mAb 1H10. The cells were allowed to migrate into the lower chambers containing chemoattractant fMLP for 4 hours at 37 °C, collected, and measured by fluorescence at 530 nm. The results are expressed as percentage of differentiated HL-60 cells (neutrophils) migrated in the presence of PBS (control). The graph shows combined data from two independent experiments performed in triplicate; error bars denote means $\pm$ SD; *** P < 0.001. **Inset A** shows the levels of Fyn and actin expression in wild-type (1) and sh-RNA-mediated Fyn-knockdown (2) HUVECs determined by immunoblotting; actin was used as a loading control. Molecular mass standards (Std., MagicMark XP Western standard, Invitrogen, Carlsbad, CA, USA) with indicated molecular masses are shown on the left. **Inset B** shows transendothelial permeability of wild-type and Fyn-knockdown HUVECs determined by measuring the passage of FITC-labeled 40 kDa dextran through HUVEC monolayers as described in Materials and Methods. The graph shows combined data from

two independent experiments performed in triplicate. The data represent permeability of endothelium to FITC-dextran expressed as the mean fold increases $\pm$ SD with respect to wild-type cells.

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Fig. 8.

Schematic representation of the VLDL receptor-dependent pathway of leukocyte transmigration and its inhibition by the β 15–42 or $(\beta$ 15–44)₂ fragments. **(A)** Interaction of fibrin or its mimetic NDSK-II with the VLDL receptor, which occurs through their β N-domains³¹ (shown in red), induces the VLDLR-dependent pathway resulting in inhibition of Fyn, as revealed in the present study. This prevents inhibition of RhoA, which in active state promotes stress-induced opening of endothelial cell adherent junctions and thereby leukocyte transmigration. **(B)** The β 15–42 or $(\beta$ 15–44)₂ fragments inhibit the VLDLR-dependent pathway, as also revealed in the present study, and such inhibition may be triggered by their interaction with a putative receptor. This prevents inhibition of Fyn, which dissociates from VE-cadherin, associates with p190 RhoGAP, and subsequently inhibits

RhoA thereby preventing junction opening and leukocyte transmigration, as was previously proposed by Gröger and collaborators.^{25,29}

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