Effective Treatment of Edema and Endothelial Barrier Dysfunction With Imatinib

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Background—Tissue edema and endothelial barrier dysfunction as observed in sepsis and acute lung injury carry high morbidity and mortality, but currently lack specific therapy. In a recent case report, we described fast resolution of pulmonary edema on treatment with the tyrosine kinase inhibitor imatinib through an unknown mechanism. Here, we explored the effect of imatinib on endothelial barrier dysfunction and edema formation.

Methods and Results—We evaluated the effect of imatinib on endothelial barrier function in vitro and in vivo. In human macro- and microvascular endothelial monolayers, imatinib attenuated endothelial barrier dysfunction induced by thrombin and histamine. Small interfering RNA knock-downs of the imatinib-sensitive kinases revealed that imatinib attenuates endothelial barrier dysfunction via inhibition of Abl-related gene kinase (Arg/Abl2), a previously unknown mediator of endothelial barrier dysfunction. Indeed, Arg was activated by endothelial stimulation with thrombin, histamine, and vascular endothelial growth factor. Imatinib limited Arg-mediated endothelial barrier dysfunction by enhancing Rac1 activity and enforcing adhesion of endothelial cells to the extracellular matrix. Using mouse models of vascular leakage as proof-of-concept, we found that pretreatment with imatinib protected against vascular endothelial growth factor–induced vascular leakage in the skin, and effectively prevented edema formation in the lungs. In a murine model of sepsis, imatinib treatment (6 hours and 18 hours after induction of sepsis) attenuated vascular leakage in the kidneys and the lungs (24 hours after induction of sepsis).

Conclusions—Thus, imatinib prevents endothelial barrier dysfunction and edema formation via inhibition of Arg. These findings identify imatinib as a promising approach to permeability edema and indicate Arg as novel target for edema treatment. (Circulation. 2012;126:2728-2738.)

Key Words: Abl-related gene tyrosine kinase ■ edema ■ endothelium ■ imatinib ■ sepsis

The endothelium tightly controls the exchange of fluid from the circulation to the surrounding tissues. Dysfunction of this barrier leads to uncontrolled fluid extravasation and edema,1–3 and characterizes life-threatening conditions like sepsis4 and acute lung injury.4 Despite high mortality rates—up to 50% in sepsis—no treatment is currently available for endothelial barrier dysfunction and edema.1 However, in a recent case report we described fast resolution of pulmonary edema on treatment with imatinib.5

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Imatinib is a small molecule inhibitor, blocking the ATPase activity of the kinases c-Abl, Abl-related gene (Arg/Abl2), platelet-derived growth factor receptor (PDGFR), c-KIT, and discoid domain receptor-1.6 Thus far, imatinib has found its major application in the treatment of Bcr-Abl positive chronic myeloid leukemia and gastro-intestinal stromal tumors,6 whereas nonmalignant proliferative disorders like lung fibrosis7 and pulmonary hypertension8 may form future applications of imatinib. Although designed as a smart drug specifically targeting overactive kinases, imatinib is associated with several side effects. Long-term treatment with imatinib may lead to cardiac failure by inducing cardiomyocyte apoptosis,9 and, of note, long-term treatment with imatinib was associated with subcutaneous edema.10

In the light of these studies the association of imatinib treatment with resolution of edema is surprising. Yet, increas-

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ing evidence indicates that imatinib may protect against edema.11–13 A second case report revealed clinical improvement of acute lung injury on initiation of imatinib,11 whereas two experimental studies demonstrated that imatinib protects against brain edema after stroke.12,13 The mechanism by which imatinib may protect against edema remains largely unclear. The protective effect of imatinib on brain edema was mainly attributed to PDGFR-α inhibition on perivascular astrocytes,12,13 which is unlikely to explain the protective effects of imatinib observed in pulmonary edema. Otherwise, the descriptive character of mentioned case reports5,11 limited mechanistic interpretation, although an effect of imatinib on endothelial barrier function was suggested.5

Little is known about the direct effects of imatinib on the endothelial barrier as main regulator of fluid exchange. In the current study we hypothesized that imatinib reduces edema formation via direct preservation of endothelial barrier integrity. Using in vitro and in vivo models of endothelial barrier dysfunction, we show that imatinib effectively protects against endothelial barrier dysfunction and edema formation.

Methods

Endothelial Barrier Function Assays

Endothelial barrier function was evaluated with horseradish peroxidase (HRP) passage and electric cell-substrate impedance sensing. For measurement of HRP passage, confluent cells were seeded in 1:1 density on 0.33 cm² Costar polycarbonate filters, pore-size 3.0 μm (Corning, Lowell, MA), and grown to confluence in 5 days. For pretreatment, pharmacological inhibitors or vector were dissolved in M199 (Biowhittaker/Lonza, Verviers, Belgium) supplemented with 1% human serum albumin (HSA; Sanquin Blood Supply, Amsterdam, The Netherlands), and added to the upper compartment of the filters during 60 minutes. For stimulation, pretreatment medium was changed for 1%HSA/M199 containing designated inhibitors, HRP 5 μg/mL (Sigma Aldrich, Zwijndrecht, the Netherlands) and thrombin 1 U/mL (Sigma Aldrich). 1% HSA/M199 was added to the lower compartment. At indicated time points, samples were taken from the lower compartment. The HRP concentration was detected by measuring chemoluminescence after addition of TMB/E (Upstate/Milipore, New Brunswick, NJ) and perforated through-and-through with a 21-G needle.17 After extraction of a column of 1 mm feces, the cecum was repositioned in the abdominal cavity. For sham surgery, the cecum was only positioned outside the abdominal cavity and repositioned. The abdominal cavity was closed with a continuous suture. At t = 6 hours and t = 18 hours after surgery, mice were treated with imatinib mesylate (50 mg/kg in PBS) or vector by subcutaneous injection in the neck. At t = 23 hours, 100 μL Evans Blue (1% in PBS) was administered via the tail vein and left circulating for 1 hour. At 24 hours after surgery, mice were anesthetized with fentanyl, midazolam, and acepromazine, and euthanized by withdrawal of 0.5 to 1 mL blood from the heart. Whole blood was collected in heparinized tubes, centrifuged for 10 minutes at 1800g and 4°C. Plasma (75 μL) was added to 150 μL formamide for determination of the Evans Blue concentration in the plasma. The kidneys, liver, and lungs were collected and thoroughly washed in saline. Evans Blue was extracted from organ tissue by incubating organs in 300 μL (kidneys and lungs) or 500 μL (liver lobe) formamide at 55°C. After 48 hours the organs were removed; the remaining formamide was centrifuged (13 500 rpm for 5 minutes) and analyzed spectrophotometrically at 610 nm (Evans Blue) and 740 nm (overlap of hemoglobin in the Evans Blue range). The corrected Evans Blue absorbance was calculated by the following formula: OD610 = [1.426×OD740+0.03].18 After Evans Blue measurement, organs were washed to remove the formamide and air dried at 90°C to determine dry weight. Vascular leakage is presented as the amount of organ Evans Blue absorbance, corrected for organ dry weight and plasma Evans Blue absorbance.

All animal experiments were performed with approval of the Animal Ethical Committees of the VU University Medical Center or the University of Illinois at Chicago.

Statistical Analyses

Data are reported as mean±standard error of the mean (SEM). n refers to the number of independent experiments with cells from different donors, unless stated otherwise. With the hypothesis that imatinib decreases endothelial hyperpermeability via an effect on Arg activation, the effect of interventions (imatinib, siRNAs) on vascular permeability for fluid would be analyzed.
endothelial barrier function and activation of specific signaling molecules was tested for statistical significance. For comparison of 2 conditions a Student t test was used, for comparison of 3 conditions a 1-way ANOVA with Tukey post hoc test or a repeated measures ANOVA with Bonferroni post hoc test was used when appropriate, as indicated in the figure legends. P values <0.05 were considered statistically significant. Additional methods and materials used for this study can be found in the online-only Data Supplement.

**Results**

**Imatinib Attenuates Disruption of the Endothelial Barrier by Thrombin and Histamine**

The direct effect of imatinib on endothelial barrier function was evaluated in isolated human endothelial cell monolayers under basal and stimulated conditions. Short-term treatment of human lung microvascular endothelial cells and human umbilical vein endothelial cells (HUVECs) with imatinib did not affect endothelial barrier function under basal conditions (Figure 1A and 1D). However, imatinib dose-dependently attenuated endothelial barrier disruption by thrombin with an optimal dose at 10 μmol/L in HUVECs (Figure I in the online-only Data Supplement). Imatinib 10 μmol/L effectively protected against endothelial barrier dysfunction, shown by a 46% and 44% reduction in thrombin-induced macromolecule passage (Figure 1A and 1D) and a 9% and 28% attenuation of the thrombin-induced decrease in endothelial electric resistance (Figure 1B and 1E). Immunostaining of the cell–cell junctional protein β-catenin (green) and the nuclei (blue) in human umbilical vein endothelial cells (HUVECs). Arrows indicate presence of intercellular gaps. Scale bars, 10 μm. Representative images of n=3 experiments. D, Effects of imatinib on endothelial barrier function under basal and thrombin stimulated conditions as measured by macromolecule passage over HUVEC monolayers. *P<0.05, ***P<0.001 compared with Vector + thrombin in Bonferroni post hoc test of repeated measures ANOVA (n=4 donors). E, Absolute endothelial resistance of confluent HUVEC monolayers during thrombin stimulation. Inset, The thrombin response (% decrease in resistance) in imatinib- versus vector-pretreated cells. **P<0.01 (n=5 donors). F, Absolute endothelial resistance of confluent HUVECs during stimulation with histamine (10−5 mol/L). Inset, Effects of imatinib on the histamine response (% decrease in resistance). *P<0.05 (n=4 donors).
lial barrier dysfunction, independent of endothelial cell type or barrier-disruptive agent.

**Imatinib Exerts its Protective Effect via Inhibition of the Tyrosine Kinase Abl-Related Gene (Arg)**

To elucidate the kinase through which imatinib exerts its protective effect on endothelial barrier function, we performed siRNA knock-downs of the known imatinib-sensitive tyrosine kinases (c-Abl, Arg, PDGFR, c-KIT, discoid domain receptor-1) and evaluated the effects on thrombin-induced endothelial barrier dysfunction. Knock-down of PDGFR, c-Ab/β, c-Abl, c-KIT, or discoid domain receptor-1 did not affect the thrombin response (Figure 2A) and decrease in endothelial resistance (Figure 2B). Knock-down of Arg and treatment with imatinib similarly attenuated the thrombin response, whereas imatinib had no additive protective effect in Arg-depleted cells (Figure 2C), indicating that imatinib exerts its protective effects predominantly via inhibition of Arg. To establish whether Arg is activated during endothelial barrier dysfunction, we measured CrkL phosphorylation at Tyr207 (an exclusive target for c-Abl and Arg)19 in c-Abl–depleted cells. Thrombin induced robust CrkL phosphorylation in c-Abl–depleted cells, which could be prevented by imatinib or combined c-Abl/Arg knock-down (Figure 2D and 2E). Arg was also activated on stimulation with the barrier-disruptive agents VEGF and histamine (Figure 2F). These findings identify Arg as a novel mediator of endothelial barrier dysfunction. Arg-mediated endothelial barrier dysfunction can be effectively inhibited with imatinib.

**Arg Inhibition Prevents Loss of Cell–Matrix Interaction During Endothelial Stimulation**

Our next step was to analyze the effect of imatinib on processes regulating endothelial barrier function. A functional endothelial barrier is characterized by low actomyosin tension and stable cell–cell junctions. During endothelial barrier dysfunction increased actomyosin contraction and disruption of cell–cell junctions result in gap formation.1,2 Tight adhesion of endothelial cells to the subcellular matrix counteracts cell retraction, and as such limits junction disruption and gap formation.2,20,21 Imatinib did not affect RhoA/Rho kinase activity or calcium-dependent signaling (Figure 3), as main determinants of actomyosin contraction. Moreover, imatinib did not visibly change the morphology of actin fibers (data not shown). Resolving the endothelial resistance measurements into separate components reflecting cell–cell contact and cell–matrix interaction14,15 displayed that Arg inhibition with siRNA or imatinib predominantly attenuated the loss of cell–matrix interaction during thrombin stimulation (Figure 4A and 4B and Figure V in the online-only Data Supplement). Using immunofluorescence and live-cell imaging of the focal adhesion marker paxillin we indeed observed...
that imatinib enhanced the formation of focal adhesions, in particular at the cell periphery (Figure 4C and 4D and Figure VI and Movie I in the online-only Data Supplement). Furthermore, the activity of Rac1—a GTPase known to reinforce both cell–matrix interaction\(^{21}\) and cell–cell junctions\(^{1,2}\)—was enhanced by imatinib during thrombin stimulation (Figure 4E). Together, these data indicate that imatinib limits Arg-mediated endothelial barrier dysfunction by enhancing Rac1 activity and by enforcing adhesion of endothelial margin areas to the extracellular matrix.

Figure 3. The protective effect of imatinib does not involve the RhoA/Rho kinase pathway and calcium-dependent signaling. A, RhoA activity on thrombin (1 U/mL) stimulation in human umbilical vein endothelial cells (HUVECs). Imatinib did not affect thrombin-induced RhoA activation. \(\ ^{*}P<0.05, \ ^{**}P<0.01\) compared with 0 minutes, NS=non significant in Bonferroni post hoc test of repeated measures ANOVA (n=3–4). B, The thrombin response (\% decrease in resistance) in HUVECs treated with imatinib, the Rac kinase inhibitor Y27632, or the combination. Imatinib had an additive effect to Y27632, indicating that imatinib exerts its protective effect independent of Rac kinase activity. \(\ ^{*}P<0.05, \ ^{**}P<0.01\) in Tukey post hoc test of 1-way ANOVA (n=3). C, The thrombin response (\% decrease in resistance) in HUVECs treated with imatinib, the intracellular calcium chelator BAPTA-AM, or the combination. Imatinib had an additive effect to BAPTA-AM, indicating that imatinib exerts its protective effect independent of calcium-dependent pathways. \(\ ^{*}P<0.05, \ ^{**}P<0.01\) in Tukey post hoc test of 1-way ANOVA (n=3). RhoA indicates Ras homolog family member A; and BAPTA-AM, 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid, tetraacetoxymethyl ester.

Figure 4. Abl-related gene (Arg) inhibition improves cell–matrix interaction during thrombin stimulation. A, Absolute endothelial electric resistance attributable to cell–matrix interaction (Alpha) of wild-type vs Arg-depleted human umbilical vein endothelial cells (HUVECs) during thrombin (1 U/mL) stimulation (n=4). B, The effects of thrombin on cell–matrix interaction (\% of wild-type vs Arg-depleted HUVECs). \(\ ^{*}P<0.05\) (n=4). C, Immunofluorescence staining of pY118 paxillin (green) and VE-cadherin (red) for visualization of focal adhesion (FA) formation. Scale bars, 10 \(\mu\)m. Representative images of n=3 to 4 experiments. D, Quantification of the number of FAs during thrombin stimulation as observed during pY118 paxillin staining. \(\ ^{*}P<0.05, \ ^{**}P<0.01\) in Bonferroni post hoc test of repeated measures ANOVA (n=3–4). E, Normalized Rac1 activity in HUVECs during thrombin stimulation. \(\ ^{**}P<0.01\) in Bonferroni post hoc test of repeated measures ANOVA (n=4). scRNA indicates scrambled RNA; siRNA, small interfering RNA.
Effect of Imatinib-Sensitive Tyrosine Kinases on Basal Barrier Function

Although imatinib had no effect on basal barrier function (Figure 1A and 1D), we observed that inhibition of individual imatinib-sensitive kinases did change basal endothelial barrier function. Under nonstimulated conditions PDGFR- and c-KIT–depleted endothelial monolayers displayed improved barrier function, whereas c-Abl depletion reduced barrier function (Figure 5A). The finding that simultaneous inhibition of all these kinases (cq, by imatinib) has no effect on basal barrier function, suggests that the barrier-imparing effect of c-Abl inhibition is balanced by the beneficial effect of PDGFR and c-KIT inhibition, rendering a net zero effect on basal endothelial barrier function. To test this, we treated endothelial cell monolayers with Tyrphostin AG1296, a selective inhibitor of PDGFR and c-KIT. Tyrphostin AG1296 enhanced endothelial resistance up to 35% (Figure 5B). These data indicate that under basal conditions c-Abl inhibition opposes the barrier enforcing effects of Arg/PDGFR/c-KIT inhibition, whereas thrombin-induced endothelial barrier dysfunction was only influenced by Arg.

Imatinib Protects Against Vascular Leakage and Pulmonary Edema Formation In Vivo

To establish the protective effect of imatinib on endothelial barrier function in vivo, we tested imatinib in mouse models of vascular leakage and pulmonary edema. VEGF-induced vascular leakage of albumin was measured by intravenous injection of Evans Blue, followed by injection of VEGF in the skin.3 Vascular leakage was compared between mice pretreated with imatinib and mice pretreated with vector. Imatinib treatment (20 mg/kg) attenuated VEGF-induced extravasation of Evans Blue in the skin by 39% to 55% (Figure 6A and 6B). Next, we measured the effect of imatinib on pulmonary edema formation. Acute pulmonary edema was induced in vivo by intravenous injection of thrombin-receptor activating peptide in mice pretreated with imatinib or vector. Ex vivo, the weight gain of isolated perfused lungs was measured, reflecting the pulmonary vascular permeability for fluid (Kfc).16 Imatinib treatment (50 mg/kg) reduced pulmonary edema formation, shown by 66% reduction of Kfc (Figure 6C). Thus, imatinib effectively prevents vascular leakage and edema formation in vivo.

To exclude the possibility that the attenuation of vascular leakage resulted from a smaller hydrostatic pressure difference or a decrease in microvascular perfusion, we measured the effect of imatinib on these parameters in an experimental set-up similar to the Miles assay and the Kfc measurements. First, systemic blood pressure was measured using radio telemetry. Blood pressure was monitored before and after administration of imatinib (Figure VIIA in the online-only Data Supplement). Comparing mean arterial pressure 5 minutes before and 30 minutes after administration of imatinib, no effect of imatinib on mean arterial pressure was observed (Figure 6D; Figure VIIA in the online-only Data Supplement). Subsequently, the effect of imatinib on microvascular perfusion was evaluated in skin and muscle by contrast-enhanced ultrasonography. Comparing microvascular blood volume (as measure of microvascular perfusion) before and after administration of imatinib, we found that imatinib did not decrease microvascular perfusion in skin (Figure 6E and 6F; Figure VIIIB in the online-only Data Supplement) or muscle (Figure VIIC–VIIE in the online-only Data Supplement), but rather caused a nonsignificant increase.

Together, these experiments indicate that the protective effect of imatinib on vascular leakage cannot be explained by smaller hydrostatic pressure differences or decreased microvascular perfusion. As proof-of-concept these data therefore support the hypothesis that imatinib prevents vascular leakage through a direct protective effect on the endothelial barrier.

Imatinib Treatment Attenuates Vascular Leakage During Sepsis

To evaluate the effect of imatinib in a clinically relevant disease model, sepsis was induced by CLP,17 and mice were treated with imatinib or vector 6 hours and 18 hours after induction of sepsis (Figure 7A). For evaluation of vascular leakage, Evans Blue was administered intravenously 23 hours after induction of sepsis, and organs were harvested 1 hour after Evans Blue administration. Septic mice treated with vector showed a 2- to 3-fold increase in Evans Blue in the kidneys, which was attenuated by 50% in septic mice treated with imatinib 50 mg/kg (Figure 7B). A similar trend was
observed for the liver, although post hoc analyses did not show a statistical difference between vector- and imatinib-treated mice (Figure 7C). In the lungs, a slight increase of Evans Blue was observed in septic mice treated with vector. This increase was not significant, mainly because not all mice developed vascular leakage in the lungs (Figure 7D). Yet, the number of mice that developed pulmonary vascular leakage on sepsis was significantly higher in vector-treated mice than in imatinib-treated mice (0/5 in the sham group versus 3/6 in the CLP/H11001 vector group versus 0/5 in the CLP/H11001 imatinib group; P<0.05 in a t test). This animal study demonstrates that imatinib attenuates vascular leakage in a clinically relevant disease model and indicates that imatinib is also effective when imatinib treatment is initiated after induction of disease.

Discussion

Here we show that treatment with imatinib is an effective therapeutic approach to endothelial barrier dysfunction and vascular leakage. Imatinib attenuated endothelial barrier dysfunction in human endothelial cells isolated from multiple origins and stimulated with a variety of barrier-disruptive agents. Specifically, we found that imatinib exerts its protective effects via inhibition of Arg, a thus far unknown mediator of endothelial barrier dysfunction. Imatinib limited Arg-mediated endothelial barrier dysfunction by enhancing Rac1 activity and enforcing adhesion of endothelial cells to the extracellular matrix. The barrier-protective effect of imatinib was established in in vivo models of vascular leakage and pulmonary edema.

Effect of Imatinib on Endothelial Barrier Function

Our finding that short-term treatment with imatinib protects against endothelial barrier dysfunction and edema formation provides first mechanistic insight regarding previous case reports on patients in whom initiation of imatinib treatment was followed by fast resolution of pulmonary edema.5,11
Combining in vitro and in vivo measurements of endothelial barrier dysfunction and vascular leakage, we found that imatinib protects against edema formation by enforcing the endothelial barrier. Although edema formation and vascular leakage may also be affected by changes in blood pressure, microvascular perfusion, or vascular remodeling, these factors are less likely to underlie the protective effect of imatinib. Alteration of blood pressure and microvascular perfusion as explanation for edema resolution was excluded in this study, because (1) imatinib did not affect systemic blood pressure in an experimental set-up similar to the Miles assay or Kfc measurements, (2) the pressure and the flow in the pulmonary circulation was kept constant in the Kfc measurements, and (3) no effects of imatinib on microvascular perfusion were observed. The acute character of the in vivo experiments further excludes chronic vascular remodeling as explanation for the protective effects of imatinib on edema formation and vascular leakage. Therefore, we conclude that imatinib protects against edema formation by preservation of endothelial barrier integrity.

Whereas the Miles assay and the Kfc measurements serve as proof-of-concept experiments in which imatinib was given as pretreatment and possible confounders were excluded, the clinical relevance of the protective effect of imatinib on endothelial barrier function was evaluated in a murine model of sepsis (CLP). This experiment mimics the clinical setting, because CLP is considered the most reliable disease model available for sepsis, and because the treatment sequence in this experiment mimicked the clinical sequence of development of disease and subsequent initiation of treatment. In septic mice we found that imatinib reduced vascular leakage of Evans Blue in the kidneys by 50%, resembling the attenuating effect found in the Miles assay. In addition, the number of septic mice developing vascular leakage in the lungs was significantly lower in the imatinib-treated group than in the vector-treated group. As reported previously, a high interindividual variation was observed for vascular leakage in liver and the lungs, which may account for the lack of significance in post hoc analyses.

The optimal protective effect of imatinib on endothelial barrier function was already achieved at concentrations be-
between 5 and 10 μmol/L in vitro. These concentrations correlate with plasma levels in patients treated with imatinib for chronic myeloid leukemia or gastrointestinal stromal tumors. Also, the dosage used in our in vivo experiments resembles imatinib dosages used in the clinical setting. The slight difference in treatment concentration between our in vivo experiments (20–50 mg/kg) and clinical treatment dosage (5–10 mg/kg) is compensated by the higher metabolism and the lower half-life of imatinib in mice (T1/2 2–4 hours in mice) compared with human (T1/2 18 hours). Therefore, this study not only explains how imatinib may protect against edema, but also proposes imatinib administration as promising approach to edema resulting from endothelial barrier dysfunction.

Role of Arg in Endothelial Barrier Dysfunction

The protective effects of imatinib on endothelial barrier function resulted predominantly from inhibition of the non-receptor tyrosine kinase Arg. Knock-down of Arg mimicked the effect of imatinib on endothelial barrier function, and imatinib did not have an additive effect in Arg-depleted cells. To the best of our knowledge, this is the first report showing that Arg is involved in endothelial barrier dysfunction. The importance of Arg as mediator of endothelial barrier dysfunction was illustrated by the fact that Arg inhibition with imatinib reduced the thrombin response up to 44%, whereas the finding that Arg is activated on endothelial stimulation with the barrier-disruptive agents thrombin, VEGF, and histamine stresses its relevance.

In search for signaling pathways underlying the barrier-disruptive actions of Arg, we found that inhibition of Arg by genetic knock-down or imatinib treatment prevented the loss of cell–matrix interaction during endothelial stimulation. This was accompanied by enhanced formation of focal adhesions (FAs), particularly at the periphery of the cell. As proposed by Ingber, adhesion of cells to the subcellular matrix is one of the ways for a cell to remain cell shape and counteract contractile forces during cell retraction. Cell–matrix interaction is mainly achieved through FAs, multifaceted protein complexes that connect extracellular matrix proteins to the intracellular cytoskeleton. The spatial distribution of FAs is an important determinant of endothelial barrier function, as redistribution of FAs to the cell periphery has previously been associated with improved endothelial barrier integrity. Fibroblast studies have demonstrated that Arg inhibits this redistribution by reducing formation and increasing turnover of peripheral FAs. Compared with wild-type, Arg-deficient fibroblasts show larger and denser FAs, mainly located at the cell periphery. These studies support our finding that Arg inhibition with imatinib increases the number of FAs at the cell periphery.

In addition, we found that imatinib enhanced the activity of Rac1, a GTPase known to enforce both cell–cell interaction and cell–matrix interaction. Rac1 activity may enforce endothelial cell–cell junctions via mediators like angiopoietin-1. A direct interrelation between Arg, Rac1, and integrin-mediated adhesion was recently suggested in a fibroblast study that demonstrated that Arg inhibits Rac1 activity and integrin function.

Figure 8 shows an overview of the protective effect of imatinib during endothelial barrier dysfunction. Imatinib inhibits Abl-related gene (Arg), which is activated upon binding of barrier-disruptive agents to their receptor. Arg activation leads to disassembly of peripheral focal adhesions (FAs) and can be inhibited with imatinib. Peripheral FAs improve cell–matrix interaction and contribute to endothelial barrier integrity by counteracting contractile forces and supporting cell–cell contacts. In addition, Arg inhibition with imatinib enhances Rac1 activity, which supports both cell–cell contacts and cell–matrix interaction.
addition, imatinib enhances Rac1 activation, which in turn improves cell–cell contact and cell–matrix interaction. The enhanced cell–matrix interaction, by supporting cell–cell contacts and counteracting contractile forces, limits cell retraction and gap formation.20

**Potential Further Improvement of Endothelial Barrier Function by Imatinib Derivatives**

Considering previous reports on subcutaneous edema as side effect of imatinib, it is also important to note that in the concentrations used in this study, imatinib did not affect basal endothelial barrier integrity. This difference might first of all be explained by treatment duration. Subcutaneous edema as side effect may result from chronic PDGFR inhibition (several months to years) in pericytes and consequent disturbed vascular support.32 Second, because c-Abl inhibition impairs endothelial barrier function33 and imatinib inhibits both Arg and c-Abl, the protective effect of imatinib may depend on the balance of Arg and c-Abl expression in a specific vascular bed. In none of the various macro- and microvascular endothelial cell types that we tested, c-Abl inhibition with imatinib impaired barrier function. This suggests that c-Abl inhibition by imatinib has a limited effect on barrier function. However, the opposing effects of Arg/PDGFR/c-KIT versus c-Abl on endothelial barrier function suggest that imatinib-derivatives lacking c-Abl as target may further improve treatment of endothelial barrier dysfunction.

**Clinical Implications**

For several reasons, this study may have direct clinical value. Imatinib had an optimal protective effect at 10 μmol/L, which correlates with plasma concentrations in patients on imatinib treatment.23,24 The barrier protective effect observed in our study was independent of anatomic location, species, endothelial phenotype, and barrier-disruptive agent, indicating a broad applicability of imatinib. Endothelial barrier protection was already achieved after short-term treatment (30 minutes pretreatment in vivo), whereas initiation of imatinib treatment after induction of sepsis was also shown to be effective. This may facilitate edema treatment in acute conditions like sepsis, but also limit side-effects. As noted before, this study elucidates previous clinical observations favoring imatinib treatment in edema.5,11 Combining our study with these clinical observations provides bench-to-bedside evidence for a protective effect of imatinib on endothelial barrier dysfunction and supports clinical development of imatinib as therapeutic approach to edema.

**Conclusion**

Thus, imatinib prevents endothelial barrier dysfunction and edema formation via inhibition of Arg. These findings identify imatinib treatment as a promising approach to permeability edema and indicate Arg as novel target for edema treatment.

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**Disclosures**

None.

**References**


Endothelial barrier dysfunction is a major contributor to morbidity and mortality in the critically ill. Loss of the endothelial barrier follows exposure of the endothelium to inflammatory mediators and drives vascular leakage and edema formation. To date endothelial barrier function and vascular leakage still lack appropriate therapy. This study shows that imatinib—an US Food and Drug Administration–approved tyrosine kinase inhibitor—directly protects the endothelial barrier under inflammatory conditions. With the use of endothelial cells isolated from various vascular beds, it was shown that imatinib attenuates the loss of endothelial barrier on stimulation with inflammatory mediators. Imatinib protects against endothelial barrier dysfunction predominantly by inhibition of the tyrosine kinase Abl-related gene (Arg), a novel mediator of endothelial barrier disruption. The effect of imatinib on endothelial barrier was established in various mouse models of vascular leakage. Notably, imatinib attenuated vascular leakage in a murine model of sepsis, even when imatinib treatment was initiated considerable time after induction of sepsis. This study carries important clinical implications. First, imatinib may form a suitable therapy for treatment of diseases characterized by vascular leakage. The longstanding experience with imatinib, together with the fact that imatinib concentrations used in this study parallel plasma values in cancer patients, are apparent advantages in this case. Logical first steps in further development of imatinib involve Phase I and II trials to evaluate safety and efficacy of imatinib in patients with profound vascular leakage. Second, the identification of Arg as a novel and drugable target opens perspectives for more specific pharmaceutical interventions.
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Effective treatment of edema and endothelial barrier dysfunction with imatinib

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Supplemental Methods

Reagents

Imatinib (as used for \textit{in vitro} experiments) was purchased from ChemieTek (Indianapolis, IN) and dissolved in dimethylsulphoxide (DMSO) to a stock concentration of 10mM. Imatinib mesylate (used for \textit{in vivo} experiments) was purchased from SelleckChem (Houston, TX) and dissolved in phosphate-buffered saline (PBS) to a stock concentration of 5mg/mL. Tyrphostin AG1296 was obtained from Sigma Aldrich (Steinheim, Germany), Y27632 from Tocris Cookson Ltd (London, United Kingdom), BAPTA-AM from Calbiochem (EMD/Merck, Darmstadt, Germany). Thrombin was purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). VEGF was from Invitrogen (Camarillo, CA), histamine from Tocris Bioscience (Ellisville, MO). Thrombin Receptor Activating Peptide (TRAP, TFLLRN) was from dr. S.M. Vogel. Small interference RNAs (siRNAs) against c-Abl, PDGFR-\(\alpha\) and -\(\beta\), Arg, c-KIT, DDR-1 and scrambled RNA (scRNA) were from Santa Cruz Biotechnology (Santa Cruz, CA). The following antibodies were used: anti-c-Abl (#2862), anti-PDGFR-\(\alpha\) (#3174) and -\(\beta\) (#3169), anti-pTyr207 CrkL (#3181), anti-CrkL (#3182) and anti-DDR-1 (#5583), all from Cell Signaling Technologies (Danvers, MA), anti-\(\beta\)-actin (Sigma Aldrich), anti-VE-cadherin (SC-6458, Santa Cruz), anti-\(\beta\)-catenin (clone 8E7, Upstate/Millipore, Temecula, CA), anti-pTyr118 Paxillin (44722G, Invitrogen) and anti-Arg (NBP1-18875, Novus Biologicals, Littleton, CO).

Endothelial cell culture

\textit{Human pulmonary microvascular cells} were isolated from human lung tissue, as previously described.\(^1\) Cells were cultured in EGM2-MV culture medium (EBM2 medium supplemented with 5% foetal bovine serum, human epidermal growth factor, fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, hydrocortisone, ascorbic acid, gentamicin and amphotericin according to the manufacturers protocol [BioWhittaker/Lonza, Verviers, Belgium], and with penicillin [100U/mL] and streptomycin [100\(\mu\)g/mL]) and seeded on gelatine-coated 25cm\(^2\) culture flasks. Cells were grown to confluence at 37\(^\circ\)C and 5% CO\(_2\), with a change of culture medium every other day. They were extensively characterized as endothelial cells by the presence of endothelial markers and the absence of epithelial, lymphatic and smooth muscle cell markers. Cells were cultured up to passage 7, for experiments passage 4-7 cells were used.
Human skin microvascular endothelial cells were isolated from human foreskin, as previously described, and cultured with EGM2-MV culture medium. Cells were grown to confluence at 37°C and 5% CO₂, with a change of culture medium every other day, and extensively characterized as endothelial cells.

For human umbilical vein endothelial cells, umbilical cords from healthy donors were obtained from the Amstelland Ziekenhuis, Amstelveen. Cells were isolated, and extensively characterized as previously described. After isolation, cells were resuspended in M199 medium (Biowhittaker/Lonza), supplemented with penicillin 100U/mL and streptomycin 100µg/mL (Biowhittaker/Lonza), heat-inactivated human serum 10% (Sanquin Blood Supply, Amsterdam, The Netherlands), heat-inactivated new-born calf serum 10% (Gibco, Grand Island, NY), crude endothelial cell growth factor 150µg/mL (prepared from bovine brains), L-glutamine 2mmol/L (Biowhittaker/Lonza), and heparin 5U/mL (Leo Pharmaceutical Products, Weesp, The Netherlands). Cells were cultured at 37°C and 5% CO₂, with a change of culture medium every other day. Cells were cultured up to passage 2, for experiments passage 1-2 cells were used. Unless indicated otherwise, experiments were performed with human umbilical vein endothelial cells.

The use of human tissue for isolation of endothelial cells was approved by the Medical Ethical Committee of the VU University Medical Center. Patients gave informed consent for the use of tissue for research purposes.

Transfections
Endothelial cells were transfected with Amaxa Technology (Amaxa Biosystems, Lonza), according to the manufacturers protocol. 80-90% confluent cells were trypsinized and transfected with indicated siRNAs. Transfected cells were seeded on gelatine-coated ECIS arrays or 5cm² culture wells. For control of transfection efficiency, confluent transfected cells were lysed 48h after transfection. 20µg of total protein/condition was electrophoresed, transferred to nitrocellulose membranes and immunoblotted for c-Abl, Arg, PDGFR-α and -β, c-KIT and DDR-1 to analyze protein expression (Supplemental Figure S4I,J).
**Plasmids & Viral transduction**

Lentiviral particles were isolated from the supernatant of HEK293 cells transiently transfected with third-generation packaging constructs and lentiviral expression vectors encoding Paxillin-mCherry (gift of Dr. Olivier Pertz, University of Basel, Switzerland) or VE-cadherin-GFP. HUVECs were infected with supernatant containing lentiviral particles in the presence of 8 µg/ml polybrene overnight.

**Immunofluorescence imaging**

Cells were seeded on glass coverslips coated with glutaraldehyde 0.5% (Fluka, St. Gallen, Switzerland) -crosslinked gelatine. Cells were seeded in 1:1 density and grown to confluence in 48-72 hours. For pretreatment, culture medium was changed to 1% human serum albumin (HSA, Sanquin Blood Supply) in M199, containing pharmacological inhibitors or vector. After 60min of pretreatment, thrombin was added to the wells for a final concentration of 1U/mL. At indicated time points, medium was replaced by 2% paraformaldehyde (37°C, Sigma Aldrich), followed by 15min incubation on ice. Cells were permeabilized with Triton X-100 0.05% (Sigma Aldrich) in PBS, and incubated overnight with primary antibodies against β-catenin, VE-cadherin or pY118-Paxillin. Subsequently, cells were washed and incubated with FITC- or Cy3-labeled secondary antibodies (Invitrogen, Paisly, United Kingdom) and rhodamine/phalloidin (Invitrogen) for 1h at room temperature. Cells were washed, and sealed with Vectashield mounting medium containing DAPI (Vector Laboratories Inc, Burlingham, CA) for nuclear staining. Imaging was performed with an Axiovert 200 Marianas™ inverted wide-field fluorescence microscope, using a 63x Zeiss oil objective (NA 1.4). Images were analyzed with Slidebook software (Intelligent Imaging Innovation, Denver, CO) and ImageJ (National Institutes of Health). For quantification of focal adhesions, pY118-Paxillin images were equally adjusted for contrast. After conversion to black/white images, the number of focal adhesions was counted with the Analyze Particle module in ImageJ.

**Live-Cell Imaging**

For live-cell microscopy, cells were plated on Lab-Tek chambered 1.0 borosilicated coverglass slides coated with 3 mg/ml fibronectin. Cells were imaged using a widefield microscope (NIKON Eclipse TI) with a 60x Apo TIRF oil objective (NA 1.49) and an EMCCD camera (Andor). Images were enhanced for display with an unsharp mask filter and by adjusting brightness and contrast settings in ImageJ.
**RhoA and Rac1 activity assay**

For analysis of RhoA and Rac1 activity, 5 cm² confluent cells/condition were pretreated with imatinib (10µM) or vector (DMSO 0.1%) and stimulated with thrombin (1U/mL) during indicated intervals. After stimulation, cells were washed with ice-cold PBS and lysed with lysis buffer provided with the G-LISA Activity Assay kit (Cytoskeleton Inc, Denver, CO). After centrifugation of the cell lysates, the supernatant was snap-frozen and stored at -80°C. RhoA and Rac1 activity was determined with the G-LISA RhoA and Rac1 Activity Assay, according to the manufacturers protocol.

**Telemetry measurement of systemic arterial blood pressure**

To assess whether imatinib affects transendothelial passage through effects on systemic blood pressure, male Balb/c mice (strain ByJ, Charles River, 25-30gr) were anesthetized with isoflurane (2-2.5% v/v). After induction of anesthesia, a telemetric device (Type: TA11PA-C10, Data Science International, St. Paul, MN) was inserted in a subcutaneous pocket with a pressure-sensing probe positioned in the aortic arch (via the internal carotid artery). After two weeks of recovery blood pressure measurements were initiated, followed by induction of anesthesia according to the protocol of the Miles assay. Upon induction of anesthesia with fentanyl, midazolam and acepromazine, the blood pressure was measured for 45 minutes to allow normalization of the blood pressure changes induced by anesthesia. After 45 minutes, imatinib mesylate was administered intraperitoneally (20mg/kg, 5mg/mL in PBS), and blood pressure was monitored for 60 minutes to evaluate effects of imatinib on systemic arterial blood pressure.

**Measurement of microvascular perfusion**

To assess whether imatinib affects transendothelial passage through effects on microvascular perfusion, we quantified skin perfusion in C57/BL6J mice (20-30 gr) by contrast-enhanced ultrasonography. Mice were anesthetized with fentanyl, midazolam and acepromazine, followed by cannulation of the right jugular vein (t = 0min) and shaving of the right hind limb. An echo probe was positioned perpendicular to the right hind limb, for cross sectional ultrasound analysis of the proximal adductor muscle group and the overlying skin. After surgery a baseline ultrasound recording (t = 45min) was performed (Sequoia 512, Siemens Medical Systems, Germany); ultrasound recording was maintained throughout the rest of the experiment. The baseline ultrasound recordings were followed by continuous infusion of phosphatidylcholine/polyethylene glycol stearate-coated decafluorobutane-
filled microbubbles, which were manufactured at the Erasmus University Rotterdam as described. Microbubbles were infused via the right jugular vein at an infusion rate of 5 µl/min. Two to three minutes after initiation of microbubble infusion a steady state was reached (t = 60min). To quantify perfusion of skin and muscle in detail, contrast replenishment curves were generated by low-frequency (5 MHz, 1sec) microbubble destruction followed by continuous high-frequency (14 MHz) imaging of microbubble replenishment. Replenishment curves were fitted using the one phase exponential association equation Y=A*(1-exp(-β*t)), in which Y is the microbubble signal and t is time. The value A is the maximal Y value, representing blood volume, whereas the β is a rate constant, representing the red blood cell velocity. Contrast replenishment curves were generated in triplo. To specifically quantify perfusion of the microcirculation, the contrast signal from large arteries was eliminated. This was achieved by subtraction of the contrast signal recovered during the first 0.5 seconds after microbubble destruction. After measuring perfusion under control conditions, imatinib mesylate (50mg/kg in PBS) was administered intraperitoneally (t = 90min) in each mouse. Thirty minutes after imatinib administration (t = 120min) ultrasound measurements described above were repeated. The perfusion parameters were calculated for both skin and muscle, by quantifying contrast signal in different regions of interest during image analysis. Ultrasound data were analyzed off-line using MatLab and GraphPad Software; the ultrasound signal was corrected for differences in blood microbubble density by dividing the skin or muscle microbubble signal by the ultrasound signal in the femoral artery. Triplo measurements were averaged for each mouse.

References


**Supplemental Figures**

**Supplemental Figure S1**

Dose-dependent inhibition of thrombin-induced macromolecule passage by imatinib. Horse radish peroxidase (HRP) passage over human umbilical vein endothelial cell monolayers under basal and stimulated (thrombin 1U/mL) conditions. Monolayers were pretreated with increasing concentrations of imatinib. Average values of $n = 4$ experiments, which all showed the same effect.
Supplemental Figure S2

Immunofluorescence staining of the adherens junction protein VE-cadherin (green) and nuclei (blue) in human umbilical vein endothelial cells after 30 minutes of thrombin (1U/mL) stimulation. Arrows indicate loss of junctions and presence of intercellular gaps. Scale bars represent 10µm. Representative images of n = 3 experiments.
Supplemental Figure S3  Barrier protective effects of imatinib in microvascular endothelial cells isolated from human foreskin. Absolute endothelial electrical resistance of confluent human foreskin microvascular endothelial cell monolayers during thrombin 1U/mL stimulation (n = 3 donors).
Supplemental Figure S4 Knock-down of the imatinib sensitive kinases in human umbilical vein endothelial cells (HUVECs). (A) Absolute endothelial electrical resistance of HUVECs transfected with scRNA or siRNA against PDGFR-α, PDGFR-β or PDGFR-α/β. The endothelial resistance was measured under basal conditions and during thrombin (1U/mL) stimulation. Average values of n = 4 experiments. (B) The thrombin response (% decrease in resistance) in wild type versus PDGFR-α, PDGFR-β or PDGFR-α/β depleted endothelial cells. No statistical difference was observed (n = 4). (C) Absolute endothelial resistance of HUVECs transfected with scRNA or siRNA against c-Abl during thrombin stimulation (n = 4). (D) The thrombin response (% decrease in resistance) in wild type versus c-Abl-depleted endothelial cells. No statistical difference was observed (n = 4). (E) Absolute endothelial resistance of HUVECs transfected with scrambled RNA or siRNA against c-KIT during thrombin stimulation (n = 3). (F) The thrombin response (% decrease in resistance) in wild type versus c-KIT-depleted endothelial cells. No statistical difference was observed (n = 3). (G) Absolute endothelial resistance of HUVECs transfected with scrambled RNA or siRNA against DDR-1 during thrombin stimulation (n = 4). (H) The thrombin response (% decrease in resistance) in wild type versus DDR-1-depleted endothelial cells. No statistical difference was observed (n = 4). (I) Expression of PDGFR-α and -β. HUVECs were transfected with scrambled RNA or siRNA against PDGFR-α and -β and grown to confluence (24h). As no PDGFR protein could be detected, confluent cells were serum starved for 24h with medium containing 1% (1%s) or 2% (2%s) new-born calf serum, or cultured on normal growth medium (cM199). After 24 hours cells were lysed and cell lysates were analyzed for expression of PDGFR-α or -β by Western Blot. Lysates of human lung fibroblasts (FB) served as
positive control for PDGFR expression. M = marker. Representative blots of n = 3 experiments. (J) Expression of Abl-related gene (Arg), c-Kit, c-Abl and DDR-1 in HUVECs treated with scrambled RNA versus siRNA against Arg, c-Kit, c-Abl and DDR-1, respectively. Cells were transfected with scrambled RNA or the indicated siRNA and grown to confluence (48h). Lysates of confluent cells were analyzed for protein expression by Western Blot. c-Abl served as loading control during Arg knock-down to exclude aspecificity of the siRNA.
Supplemental Figure S5

Computational modeling of endothelial electrical resistance, yielding resistance attributable to cell-matrix interaction (Alpha) and the resistance attributable to cell-cell contact (Rb) in human umbilical vein endothelial cells (HUVECs). (A) Absolute endothelial electrical resistance attributable to cell-cell contact (Rb) of wild-type versus Arg-depleted endothelial cells during thrombin stimulation (n = 4). (B) The effects of thrombin on cell-cell contact (%) of wild-type versus Arg-depleted cells. * P < 0.05 in paired t-test (n = 4). (C) The effects of thrombin on cell-matrix interaction (%) of HUVECs treated with imatinib or vector. * P < 0.05 in paired t-test (n = 4). (D) The effects of thrombin on cell-cell contact (%) of HUVECs treated with imatinib or vector. * P < 0.05 in paired t-test (n = 4).
Supplemental Figure S6 Immunofluorescence staining of pY118 Paxillin (green) and VE-cadherin (red) in human umbilical vein endothelial cells for visualization of focal adhesion formation during thrombin stimulation (1U/mL). This figure represents the time course of the images shown in Figure 4C. Scale bars represent 10µm. Representative images of n = 4-6 experiments.
Supplemental Figure S7

Effect of imatinib administration on blood pressure and microvascular perfusion. (A) Systemic arterial blood pressure of male Balb/cJ mice as measured by radio telemetry. Induction of anesthesia and administration of imatinib 20mg/kg (intraperitoneally) were performed according to the protocol used in the Miles assay. n = 3 mice. (B) Representative picture of contrast-enhanced ultrasonography of the right hind limb. This picture shows a cross section of the hind limb at the level of the adductor muscle group. Gas-filled microbubbles were used for contrast enhancement, which show up as green/white (contrast rich) against a dark (contrast poor) background. The red dots and connecting lines show the region of interest used for determination of the microvascular perfusion of the skin. The data analysis of this ultrasonography is presented in Figure 6 of the main manuscript. (C) Cross section of the hind limb at the level of the adductor muscle group, in which red dots and connecting lines show the region of interest used for determination of the microvascular perfusion of muscle. (D) Analysis of microvascular perfusion of the muscle by contrast-enhanced ultrasonography. The graph shows representative contrast replenishment curves after microbubble destruction in a single mouse before and after treatment with imatinib 50mg/kg. The plateau of the replenishment curve represents the microvascular blood volume [A] of the muscle. An extensive explanation of the ultrasonography analysis is provided in the Methods section of the main manuscript. A.U. = Arbitrary Units. (E) The effect of imatinib 50mg/kg on microvascular tissue perfusion of muscle, shown by the microvascular blood volume [A] before (5min) and after (30min) treatment with imatinib. NS = non significant in paired t-test (n = 3 mice).
Legends to Supplemental Video

**Supplemental Video 1** Imatinib attenuates thrombin-induced opening of endothelial junctions and promotes formation of focal attachments during thrombin stimulation. Time lapse recordings of human umbilical vein endothelial cells expressing VE-cadherin-GFP and Paxillin-mCherry that are stimulated with thrombin (1U/mL) at t=119 minutes. The right movie shows cells that were pretreated with imatinib at t=60 minutes, and the left movie shows control cells without imatinib pretreatment. This is a representative recording of 6 movies per condition using endothelial cells from two donors.

**Movie information**

- **Time resolution:** 1 minute per frame
- **Addition of imatinib/mock:** t = 60 minutes
- **Addition of thrombin:** t = 119 minutes
- **Green channel:** VE-cadherin-GFP
- **Red channel:** Paxillin-mCherry