C1-Inhibitor Decreases the Release of Vasculitis-Like Chemotactic Endothelial Microvesicles

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ABSTRACT
The kinin system is activated during vasculitis and may contribute to chronic inflammation. C1-inhibitor is the main inhibitor of the kinin system. In this study, we investigated the presence of the kinin B1 receptor on endothelial microvesicles and its contribution to the inflammatory process. Compared with controls (n=15), patients with acute vasculitis (n=12) had markedly higher levels of circulating endothelial microvesicles, identified by flow cytometry analysis, and significantly more microvesicles that were positive for the kinin B1 receptor (P<0.001). Compared with microvesicles from wild-type cells, B1 receptor-positive microvesicles derived from transfected human embryonic kidney cells induced a significant neutrophil chemotactic effect, and a B1 receptor antagonist blocked this effect. Likewise, patient plasma induced neutrophil chemotaxis, an effect decreased by reduction of microvesicle levels and by blocking the B1 receptor. We used a perfusion system to study the effect of patient plasma (n=6) and control plasma (n=6) on the release of microvesicles from glomerular endothelial cells. Patient samples induced the release of significantly more B1 receptor-positive endothelial microvesicles than control samples, an effect abrogated by reduction of the microvesicles in the perfused samples. Perfusion of C1-inhibitor–depleted plasma over glomerular endothelial cells promoted excessive release of B1 receptor-positive endothelial microvesicles compared with normal plasma, an effect significantly decreased by addition of C1-inhibitor or B1 receptor-antagonist. Thus, B1 receptor-positive endothelial microvesicles may contribute to chronic inflammation by inducing neutrophil chemotaxis, and the reduction of these microvesicles by C1-inhibitor should be explored as a potential treatment for neutrophil-induced inflammation.


Activation of the kinin/contact system is proinflammatory, inducing pain and capillary leakage symptomatic of severe vascular inflammation.1 The kinin system consists of a cascade of proteins ultimately leading to the release of bradykinin from high molecular weight kininogen.1 In addition to bradykinin, other kinins are released during inflammation, including a 13-amino acid peptide termed proteinase 3-kinin (PR3-kinin) cleaved by neutrophil-derived proteinase 3 (PR3) and thus presumably active during conditions associated with neutrophil influx, such as the chronic vascular inflammatory condition vasculitis.2 Bradykinin binds to the constitutively expressed B2-receptor, whereas des-arg9-bradykinin and PR3-kinin bind to the B1-receptor (B1R), expressed primarily during chronic inflammation.2,3 The major inhibitor of the kinin system is C1-inhibitor.1

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The kinin system may be assembled and activated on endothelial cells and neutrophils, as well as on blood cell-derived microvesicles (MVs) with a proinflammatory effect. MVs are vesicular membrane blebs (0.1–1.0 μm in diameter) shed by cells during activation, injury and/or apoptosis that contain proteins, RNA, histones, and other cellular components from the parent cell. MVs can be found in the plasma of healthy individuals but are increased during inflammatory conditions. MVs can promote inflammation and thrombosis and thus prolong the inflammatory process. This may be achieved by membrane exposure of, or release of, proinflammatory cytokines, chemokines, selectins, adhesion molecules, and complement factors as well as phosphatidylserine, which may activate coagulation factors, and tissue factor (predominantly on monocytic MVs), thus promoting thrombosis. MVs may also transfer the kinin B1R. In the circulation, MVs may interact with cells by receptor binding, membrane fusion or endocytosis/engulfment of the entire MV, as reviewed by Meckes and Raab-Traub. Thus, MVs may facilitate intercellular communication.

Endothelial cells also react to stress by shedding MVs (termed endothelial cell-derived microvesicles; EMVs). EMVs are increased in various conditions associated with vascular damage, such as diabetes and hypertension. Circulating EMVs as well as platelets and leukocytes have been detected systemically in patients with vasculitis. The kinin system may be assembled and activated on endothelial cells and neutrophils, 4 as well as on blood cell-derived microvesicles (MVs)5 with a proinflammatory effect. MVs may interact with cells by receptor binding, membrane fusion or endocytosis/engulfment of the entire MV, as reviewed by Meckes and Raab-Traub.15 Thus, MVs may facilitate intercellular communication.

EMVs in Vasculitis Plasma Are B1R-Positive

Plasma from patients with vasculitis (n=12) and healthy controls (n=15) were analyzed by flow cytometry for the presence of EMVs, defined as positive for CD105 and/or CD144. Patient plasma exhibited significantly more EMVs than controls. Furthermore, a significantly higher amount of EMVs were B1R-positive compared with controls (Figure 1). Of the total number of patient EMVs, a median of 41% (range, 8%–69%) were B1R-positive compared with 13% (range, 0%–49%) in controls. No correlation was found between B1R-positive EMV levels and variables such as specific diagnosis, presence/absence of immunotherapy, creatinine levels, or BVAS (Table 1). Patient EMVs were also found to be IL-8-positive, with a median of 86% (range, 51%–97%; n=5). Most patient EMVs that were B1R-positive were also IL-8-positive, with a median of 92% (range, 86%–96%). Fewer control EMVs were IL-8-positive, with a median of 46% (range, 6%–65%; n=5, data not shown).

MV with B1R Induce Neutrophil Migration

Experiments were designed to investigate if the presence of B1R on MVs induced neutrophil chemotaxis. Neutrophils migrated toward MVs derived from human embryonic kidney (HEK) cells transfected with B1R (HEK<sup>B1R</sup>) cells or MVs derived from wild-type cells (HEK<sup>WT</sup> cells; 70 μg/ml MVs). Enhanced migration was demonstrated when MVs derived from HEK<sup>B1R</sup> cells were placed in the lower compartment compared with MVs derived from HEK<sup>WT</sup> cells (Figure 2A). Neutrophil migration toward MVs derived from HEK<sup>B1R</sup> cells was significantly reduced by preincubation with the B1R-antagonist R715. Neutrophil migration toward MVs derived from HEK<sup>WT</sup> cells was comparable with MVs derived from HEK<sup>B1R</sup> cells preincubated with the R715 B1R-antagonist.

Patient-Derived B1R-Positive MVs Are Chemotactic for Neutrophils

Plasma from patients with vasculitis induced neutrophil migration (Figure 2B). The chemotactic effect was lowered significantly by reduction of the amount of MVs (Figure 2B).
Table 1. Description of patients included in this study

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age at Sampling</th>
<th>Diagnosis</th>
<th>ANCA</th>
<th>Clinical Findings</th>
<th>Creatinine, a (\mu)mol/L</th>
<th>BP at Admission, mmHg</th>
<th>BVAS Score</th>
<th>Blood Sample Taken Before/After Start of Immunosuppressive Therapy</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>43</td>
<td>HSP</td>
<td>—</td>
<td>Purpura, nephritis, arthritis</td>
<td>89</td>
<td>170/100</td>
<td>15</td>
<td>B</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>16</td>
<td>HSP</td>
<td>—</td>
<td>Purpura, arthritis</td>
<td>69</td>
<td>122/70</td>
<td>3</td>
<td>B</td>
</tr>
<tr>
<td>3(^b)</td>
<td>M</td>
<td>80</td>
<td>MPA MPO</td>
<td>—</td>
<td>Rash, nephritis</td>
<td>259</td>
<td>168/72</td>
<td>23</td>
<td>B</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>62</td>
<td>GPA PR3</td>
<td>—</td>
<td>URT symptoms, pulmonary granuloma, temporal arteritis</td>
<td>61</td>
<td>130/90</td>
<td>6</td>
<td>B</td>
</tr>
<tr>
<td>5(^b)</td>
<td>F</td>
<td>19</td>
<td>SLE</td>
<td>—</td>
<td>Rash, myalgia, scleritis, nephritis</td>
<td>62</td>
<td>110/60</td>
<td>17</td>
<td>A</td>
</tr>
<tr>
<td>6(^b)</td>
<td>F</td>
<td>76</td>
<td>GPA PR3</td>
<td>—</td>
<td>Gingivitis, rhinitis, hematuria</td>
<td>74</td>
<td>130/70</td>
<td>14</td>
<td>B</td>
</tr>
<tr>
<td>7(^b)</td>
<td>F</td>
<td>12</td>
<td>GPA PR3 MPO</td>
<td>—</td>
<td>Fever, arthralgia, nephritis</td>
<td>312</td>
<td>120/70</td>
<td>15</td>
<td>A</td>
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<tr>
<td>8(^b)</td>
<td>F</td>
<td>61</td>
<td>GPA MPO</td>
<td>—</td>
<td>Polychondritis, cough; rhinitis, nephritis</td>
<td>54</td>
<td>150/80</td>
<td>13</td>
<td>B</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>10</td>
<td>HSP</td>
<td>—</td>
<td>Purpura, arthritis, nephritis</td>
<td>47</td>
<td>102/60</td>
<td>7</td>
<td>B</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>51</td>
<td>GPA PR3</td>
<td>—</td>
<td>Hemoptyis, cough, nephritis</td>
<td>132</td>
<td>164/96</td>
<td>8</td>
<td>A</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>73</td>
<td>MPA MPO</td>
<td>—</td>
<td>Hearing loss, nephritis</td>
<td>173</td>
<td>155/93</td>
<td>15</td>
<td>A</td>
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<tr>
<td>12</td>
<td>F</td>
<td>68</td>
<td>GPA PR3</td>
<td>—</td>
<td>Myalgia, arthralgia, nephritis</td>
<td>86</td>
<td>136/79</td>
<td>13</td>
<td>A</td>
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<tr>
<td>13</td>
<td>F</td>
<td>67</td>
<td>GPA MPO</td>
<td>—</td>
<td>Myalgia, confusion, nephritis</td>
<td>148</td>
<td>130/60</td>
<td>22</td>
<td>B</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>9</td>
<td>HSP</td>
<td>—</td>
<td>Purpura, myalgia, nephritis, arthritis</td>
<td>51</td>
<td>110/62</td>
<td>18</td>
<td>B</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>13</td>
<td>GPA PR3</td>
<td>—</td>
<td>Dyspnea, pulmonary infiltrates, arthralgia, nephritis</td>
<td>84</td>
<td>120/70</td>
<td>24</td>
<td>B</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>17</td>
<td>GPA MPO</td>
<td>—</td>
<td>Dyspnea, myalgia, arthralgia, gastrointestinal symptoms, nephritis</td>
<td>86</td>
<td>115/75</td>
<td>26</td>
<td>B</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>13</td>
<td>GPA PR3</td>
<td>—</td>
<td>Myalgia, pansinusitis, exophthalmos, pulmonary infiltrates</td>
<td>48</td>
<td>110/75</td>
<td>17</td>
<td>B</td>
</tr>
</tbody>
</table>

M, male; —, no ANCA detected; B, before; MPA, microscopic polyangiitis; MPO, myeloperoxidase; GPA, granulomatosis with polyangiitis; URT, upper respiratory tract; F, female; A, after.

*Normal creatinine values of 45–90 \(\mu\)mol/L for women and 60–105 \(\mu\)mol/L for men.

\(^b\)These patients were previously described, including their kinin levels.\(^2,23\)
Likewise, addition of the B1R-antagonist decreased neutrophil migration (Figure 2C). Addition of anti-human CXCL8/IL-8 further reduced chemotaxis (35.7 μm; range, 31–42.5, data not shown). This implies that plasma from patients with vasculitis as well as toward the same samples with reduced MV content (median 7.5% of the original EMVs). Neutrophil migration was decreased in MV-reduced vasculitis plasma. (C) Purified neutrophils migrated toward plasma from patients with vasculitis preincubated with or without the B1R-antagonist R715. Neutrophil migration was decreased in samples preincubated with the B1R-antagonist. Experiments in (B and C) were carried out at separate occasions. DMEM and PBS were used as the negative controls. N-formyl-met-leu-phe (fMLP) was used as the positive control. ***P<0.001; **P<0.01; *P<0.05.

**Figure 2.** Neutrophils migrate toward B1R-expressing MVs. Isolated neutrophils from healthy donors migrated through a membrane filter in a Boyden chamber assay. The y-axis represents the distance of neutrophil migration. (A) HEKwt and HEKB1R cell-derived MVs (70 μg/ml) and DMEM (negative control) were placed in the lower compartment. More neutrophil migration was induced by MVs from HEKB1R compared with HEKwt and HEKB1R preincubated with the B1R-antagonist R715. (B) Purified neutrophils migrated toward plasma from patients with vasculitis as well as toward the same samples with reduced MV content (median 7.5% of the original EMVs). Neutrophil migration was decreased in MV-reduced vasculitis plasma. (C) Purified neutrophils migrated toward plasma from patients with vasculitis preincubated with or without the B1R-antagonist R715. Neutrophil migration was decreased in samples preincubated with the B1R-antagonist. Experiments in (B and C) were carried out at separate occasions. DMEM and PBS were used as the negative controls. N-formyl-met-leu-phe (fMLP) was used as the positive control. ***P<0.001; **P<0.01; *P<0.05.

Likewise, addition of the B1R-antagonist decreased neutrophil migration (Figure 2C). Addition of anti-human CXCL8/IL-8 further reduced chemotaxis (35.7 μm; range, 31–42.5, data not shown). This implies that plasma from patients with vasculitis contains MVs with chemotactic properties, indicated by the presence of B1Rs, as well as other chemotactic substances, such as IL-8.

**Release of B1R-Positive EMVs from Primary Glomerular Endothelial Cells**

The release of B1R-positive EMVs was analyzed after perfusion of plasma from patients with vasculitis (n=6; ANCA-positive vasculitis n=4, patients 3, 4, 7, and 15; HSP n=2, patients 2 and 14; Table 1) and controls (n=6) over primary glomerular endothelial cells (PGECS) using a microfluidic perfusion system. Patient preperfusion samples exhibited higher amounts of circulating EMVs positive for B1R compared with controls. Perfusion of plasma over PGECS led to release of B1R-positive EMVs. Significantly more EMVs were released from PGECS when perfused with patient plasma compared with controls, and these EMVs were B1R-positive (Figure 3). No marked difference in PGECS response was noted when using samples from patients with ANCA-positive vasculitis or patients with HSP. Samples from two patients with ANCA-positive vasculitis (patients 7 and 15) were compared with the same samples after IgG depletion by protein-G to remove ANCA. Removal of IgG did affect the release of EMVs in the perfusion system (data not shown), suggesting that ANCA did not contribute to EMV release.

Plasma from patients with vasculitis in which MV levels were reduced (7.5% of the original quantity of EMVs) did not exhibit an increase in the number of EMVs after perfusion over PGECS (Supplemental Figure 1A). Likewise, an increase of B1R-positive EMVs was not registered when the vasculitis plasma with reduced MVs was perfused over the cells (Supplemental Figure 1B).

**C1-Inhibitor and B1R-Antagonist Reduced Release of B1R-Positive EMVs from PGECS**

C1-inhibitor–depleted plasma exhibited lower total EMVs (CD144-positive EMVs) and B1R-positive EMVs before perfusion (median EMVs, 90; range, 38–250×10^6/ml; B1R-positive EMVs median, 8; range, 1–24×10^6/ml) in comparison to normal plasma (median EMVs, 330; range, 80–536×10^6/ml; B1R-positive EMVs median, 53; range, 8–76×10^6/ml). After perfusion, EMV release was higher in the C1-inhibitor–depleted
plasma compared with normal plasma (Figure 4A). C1-inhibitor–depleted plasma with added C1-inhibitor (Berinert) exhibited lower EMV release that did not reach statistical significance when compared with C1-inhibitor–depleted plasma and control plasma (Figure 4A). Perfused C1-inhibitor–depleted plasma also exhibited significantly elevated B1R-positive EMVs that were reduced in the presence of C1-inhibitor (Figure 4B), suggesting that the lack of C1-inhibitor triggered kinin system activation on PGECs and EMVs. Kinin system activation was confirmed by measuring kinin levels in C1-inhibitor–depleted plasma perfused over PGECs showing a higher level in the absence of C1-inhibitor (227.2 pg/ml) compared with a sample with added C1-inhibitor (20.6 pg/ml). Likewise, addition of the B1R-antagonist (R715) to C1-inhibitor–depleted plasma reduced total EMV release and B1R-positive EMVs (Figure 4). Taken together, these results suggest that C1-inhibitor and B1R-antagonist reduced the total number of EMVs as well as levels of B1R-positive EMVs.

B1R-Positive EMVs Are Chemotactic for Neutrophils

C1-inhibitor–depleted plasma perfused over PGECs and containing B1R-positive EMVs (Figure 4) exhibited chemotactic activity toward neutrophils. This activity was specific for B1R because it was significantly reduced in the presence of the B1R-antagonist R715 (Figure 5).

DISCUSSION

MV s have been shown to be important mediators of cell-to-cell communication and they also reflect the state of activation of the parent cell from which they were shed. Here, we show that EMVs carry kinin receptors during chronic vascular inflammation, and that the presence of B1R on MVs induced neutrophil chemotaxis. Thus, MVs actively promote and sustain the inflammatory state. B1R-positive EMVs were observed in patients with vasculitis and reproduced in an in vitro perfusion system using patient plasma as well as C1-inhibitor–depleted plasma. Importantly, both C1-inhibitor and a B1R-antagonist inhibited release of B1R-positive EMVs by blocking activation of the kinin system on the endothelium. These results suggest that C1-inhibitor can block the release of chemotactic EMVs during vascular inflammation.

C1-inhibitor inhibits plasma kallikrein, thereby preventing release of kinins. Patients with vasculitis have elevated levels of kinins, both systemically and locally in the kidneys. High levels of bradykinin are also found in patients with hereditary angioedema because of mutations in C1-inhibitor or neutralizing autoantibodies. We have previously shown that patients with vasculitis have normal C1-inhibitor levels. Moreover, genetic sequencing of the C1-inhibitor reactive site did not show alterations. Thus patients with vasculitis most probably have circulating C1-inhibitor capable of reducing the release of B1R-positive EMVs. Kinin system activation may, however, overwhelm the inhibitor and thus levels of C1-inhibitor, although normal, may not suffice to inhibit excessive release of chemotactic B1R-positive EMVs. The results presented here suggest that treatment with C1-inhibitor may, to a certain extent, prevent neutrophil influx at inflammatory sites.

The kinin system is activated in both children and adults with vasculitis. The components of the kinin system play a role in the intrinsic coagulation pathway as well as in the induction of inflammation. During this process there is a
local reduction of BP, promotion of inflammation and pain, inhibition of platelet aggregation, induction of fibrinolysis, and capillary leakage. B1R, expressed during chronic inflammation, binds des-arg⁴-bradykinin and des-arg¹⁰-kallidin, as well as PR3-kinin²⁻³ and the receptor remains on the plasma membrane after ligand binding. Thus, the presence of the B1R on the surface of cells suggests that the ligand is bound, as the receptor is internalized under resting conditions, in the absence of signaling. Ligand binding activates endothelial formation of nitric oxide³⁰ and participates in the induction of pain. EMVs most probably do not possess the cellular machinery required for internalization of the B1R, and would thus be expected to continuously express the receptor.

B1R stimulation leads to neutrophil migration, as shown in both in vivo and in vitro models.³²⁻³⁴ Our results strengthen these findings, showing that even circulating B1R-positive MVs induce chemotaxis, and reduction of MV levels in plasma of patients with vasculitis or blockade of the B1R in the plasma reduced chemotaxis. Importantly, the B1R was demonstrated in kidneys from patients with GN, including vasculitis, and a B1R-antagonist ameliorated the inflammatory response in a mouse model of GN.³⁵ Thus, the presence of B1R on EMVs suggests that it is expressed on these cells during vasculitis. Perfusion experiments demonstrated that patient plasma induced release of B1R-positive EMVs from PGECS, which would be expected to be a major source of EMVs in vasculitis as glomerular injury is predominant. The results suggest that B1R-positive EMVs could induce neutrophil chemotaxis at sites of inflammation.

Endothelial cell injury is a major manifestation of vasculitis in general, and circulating endothelial cells have been detected, indicating vascular wall injury.EMVs are elevated during vasculitis¹⁸⁻¹⁹ and decrease upon treatment, suggesting that

Figure 4. C1-inhibitor and B1R-antagonist reduce release of B1R-positive EMVs after perfusion of PGECS. (A) Perfusion of normal plasma, C1-inhibitor–depleted plasma, and C1-inhibitor–depleted plasma complemented with C1-inhibitor, over PGECS at a shear stress of 5 dynes/cm² for 5 minutes induced the release of EMVs (CD144-positive). C1-inhibitor–depleted plasma showed higher levels of total EMVs compared with control plasma. Complementing the C1-inhibitor–depleted plasma with C1-inhibitor reduced the total number of EMVs. (B) Cells perfused with C1-inhibitor–depleted plasma exhibited significantly higher levels of B1R-positive EMVs than control plasma. Addition of C1-inhibitor significantly reduced the number of B1R-positive EMVs. (C) The amounts of total EMVs released from PGECS perfused with C1-inhibitor–depleted plasma to which the B1R-antagonist R715 was added were significantly reduced compared with cells perfused with C1-inhibitor–depleted plasma. (D) PGECS exposed to C1-inhibitor–depleted plasma in the presence of the B1R-antagonist exhibited significantly lower levels of B1R-positive EMVs.

Samples were run using a CyFlow Cube 8 flow cytometer. **P<0.01; *P<0.05. C1INH, C1-inhibitor; C1INH-dpl plasma, C1-inhibitor–depleted plasma.
Neutrophils migrate toward B1R-positive endothelial MVs. Purified neutrophils, from healthy donors, migrated through a filter in a Boyden chamber toward a chemoattractant. The y-axis represents the distance of neutrophil migration. PBS was used as the negative control. C1-inhibitor–depleted plasma (pre-incubated with or without the B1R-antagonist R715) derived from perfusion experiments over PGECs was used as the chemotactant. Migration of neutrophils was decreased in plasma incubated with the B1R-antagonist R715. \( P < 0.05 \). C1INH-dpl plasma, C1-inhibitor–depleted plasma.

Figure 5.

They are adequate biomarkers of endothelial injury during vasculitis. Circulating EMVs in patients with vasculitis were shown to be E-selectin positive. Furthermore, levels of EMVs were higher in pediatric patients with vasculitis that developed thromboembolic events. In this study, high levels of EMVs could not be correlated to patient diagnosis, BVAS, renal function, or immunosuppressive treatment, probably because of the limited number of patients and their heterogeneity. Nevertheless, reducing MV levels in vasculitis plasma decreased EMV release from PGECs, and a novel function of EMVs contributing to the local inflammation was demonstrated. Both B1R and IL-8, demonstrated on EMVs, were found to be chemotactic. Blockade of B1R reduced the chemotactic potential of EMVs, an effect further enhanced, albeit minimally, by additional blockade of IL-8 on the EMVs, showing that EMVs have potent inflammatory properties. The chemotactic potential of B1R-positive MVs was not specific for EMVs, as even MVs derived from HEK\(^{B1R}\) cells induced neutrophil migration. Although not addressed here, we speculate that even other MVs bearing B1R, such as neutrophil-derived MVs, could induce a similar chemotactic and proinflammatory effect.

The factor/s inducing kinin system activation and EMV release during vasculitis are most probably numerous. EMVs have been associated with vascular injury and metabolic derangements and may in themselves be injurious to endothelial cells. MV release may be beneficial for the parent cell as a means to rid the cell of unwanted components, and general abrogation of MV release may thus not be favorable. C1-inhibitor reduced the total release of EMVs and, more specifically, those that were B1R-positive. As B1R-positive MVs induced neutrophil chemotaxis, a major feature of inflammation, the effect of C1-inhibitor on B1R-positive EMV release could be beneficial. We therefore suggest that C1-inhibitor should be explored as a treatment for neutrophil-associated vascular inflammation.

**CONCISE METHODS**

**Patients and Controls**

Blood samples were available from 17 patients (ten females and seven males, from children and adults; median age, 43 years) with vasculitis treated at the Department of Nephrology and the Department of Pediatrics, Section of Pediatric Nephrology, Skåne University Hospital, Lund and Malmö, Sweden. Details regarding patients’ age, sex, clinical and laboratory findings, diagnosis, and BP are given in Table 1. None of the patients were on hemodialysis at, or before, the time of sampling. Samples were obtained at onset or during the acute phase of disease and used for flow cytometry analysis and perfusion assays. Disease activity was assessed using the BVAS (Table 1). Blood was also available from 27 healthy adult controls (15 women, 12 men; median age, 42 years) not using any medications at the time of sampling. These samples were used for flow cytometry analysis, perfusion and neutrophil chemotaxis assays. Samples from the patients and controls were obtained after written consent of the subjects or their parents. The study was carried out with the approval of the Regional Ethics Review Board of Lund University.

**Blood Samples**

Whole blood was drawn into citrated tubes and centrifuged to obtain platelet-poor plasma and platelet-free plasma. These samples were analyzed for the presence of EMVs and used in perfusion experiments. In certain plasma samples the EMV content was reduced by centrifugation to 7.5% (range, 5.7%–11.3%) of the original EMV content. For details see Supplemental Material.

**Culture of HEK Cells and Isolation of MVs**

HEK\(^{wt}\) and HEK cells stably expressing human B1R (HEK\(^{B1R}\)) were cultured as detailed in the Supplemental Material. Medium from cells was collected and centrifuged at 2500 \( \times \)g for 5 minutes and further at 100,000 \( \times \)g for 3 hours to collect the pellets containing MVs. The concentration of MVs derived from HEK\(^{wt}\) and HEK\(^{B1R}\) was 70 \( \mu \)g/ml (corresponding to 36.7 \( \times \)10\(^6\) MVs/ml). Kinin levels were
measured in the supernatants of HEK<sup>wt</sup> and HEK<sup>B1R</sup> cells using an ELISA kit described below, and were found to be below the detection limit.

**Culture of PGECs**
PGECs (Cell Systems, Kirkland, WA) were cultured as described in the Supplemental Material and used in perfusion experiments.

**Perfusion Experiments over PGECs**
A semiautomated microfluidic perfusion system (VenaFlux; Cellix, Dublin, Ireland) was used to mimic shear in the microvasculature. Microcapillary channels (Vena8 Endothelial+ biochips; Cellix) were coated with fibronectin (Sigma-Aldrich, St. Louis, MO) and the PGEC suspension as described. PGECs were prestimulated with 200 μM histamine (Sigma-Aldrich) in Dulbecco phosphate buffered saline (DPBS; PAA Laboratories) for 10 minutes at a shear stress of 5 dynes/cm<sup>2</sup> obtained with a Mirus Evo nanopump (Cellix). Histamine prestimulated samples mimic the activated endothelium present during inflammation. Histamine-stimulated endothelial cells were found to release more EMVs, confirming previous results, and were therefore used in all described experiments.

Plasma samples from patients and controls were perfused over the PGECs at 2–5 dynes/cm<sup>2</sup> for 5–15 minutes, as described in the Supplemental Material. In certain experiments, IgG was depleted from plasma by adsorption to a Protein G Sepharose column (Amersham Biosciences, Uppsala, Sweden). After perfusion, the presample (plasma before perfusion over PGECs) and the perfused sample were collected and centrifuged for 5 minutes at 10,000×g. Dilution of the perfused samples was adjusted for by protein concentrations. These samples were later assayed by flow cytometry for detection of EMVs.

C1-inhibitor–depleted plasma (Milan Analytica AG, Rheinfelden, Switzerland) as well as vasculitis plasma were diluted 1:1 in filtered DPBS and similarly perfused over the cells at a shear stress of 5 dynes/cm<sup>2</sup> for 5 minutes. In certain experiments C1-inhibitor (Berinert, Marburg Germany) or B1R-antagonist R715 (1 μM; a kind gift from D. Regoli, Universite de Sherbrooke, Quebec, Canada) was added to the plasma just before perfusion.

**Kinin ELISA**
Kinin levels in HEK supernatants and perfused C1-inhibitor–depleted plasma samples were measured using the MARKIT-M bradykinin-ELISA kit (Dainippon Pharmaceutical, Osaka, Japan) as per the manufacturer’s instructions.

**Detection of MVs Derived from Endothelial Cells and B1R on the EMVs**
Plasma samples and samples collected from perfusion experiments were incubated with mouse anti-human CD144 (conjugated with phycoerythrin, 1:200; peridinin chlorophyll protein-cyanin5.5, 1:600; or FITC, 1:200) and mouse anti-human CD105:peridinin chlorophyll protein-cyanin5.5 at 1:800. MVs labeled with either or both antibodies were considered to derive from endothelial cells.

To detect B1R, rabbit anti-human B1R from two sources (in-house developed antibody<sup>42</sup> at 10 μg/ml final concentration or BDKRB1 at 1:50; Abgent, San Diego, CA) was incubated with the plasma or perfusion samples. To detect IL-8, mouse anti-human IL-8:phycoerythrin at 1:800 was used. Flow cytometry was performed using a BD FACSCan<sup>®</sup> Cytometer and FACSDiva Software version 6.0 (Becton Dickinson Immunocytometry Systems, San Jose, CA) or a CyFlow Cube 8 flow cytometer (Sysmex Partec, Norderstedt, Germany) with FCS Express 4 Flow Research Edition software version 4.07.0003 (De Novo Software, Glendale, CA). The latter flow cytometer detects vesicles of submicron size and therefore identifies more events corresponding to MVs. Details are given in the Supplemental Material.

**Neutrophil Migration Assay**
Neutrophils were purified from citrated whole blood using Lymphoprep (Fresenius Kabi, Oslo, Norway), as previously described<sup>43</sup>, and adjusted to a concentration of 2×10<sup>6</sup> cells/ml. A neutrophil migration assay was performed with a Boyden chamber microslide filter assay (Neuro Probe, Gaithersburg, MD). Purified neutrophils from healthy donors (<i>n</i>=10) were placed in the upper compartment of the chamber. MVs derived from HEK<sup>wt</sup> cells, HEK<sup>B1R</sup> cells, and PGECs in perfusion experiments were placed in the lower compartment with or without preincubation with 1 μM B1R-antagonist R715, for 30 minutes at room temperature. Alternatively, plasma (diluted at 1:4) was placed in the lower compartment, in the presence or absence of the B1R-antagonist R715. In certain experiments, anti–hCXCL8/IL-8 (1 μg/ml; R&D Systems, Minneapolis, MN) was added to the plasma 30 minutes before running the assay. Migration in the membrane (measured in micrometers) was assayed by light microscopy (Zeiss Axiostar Plus; Zeiss, Göttingen, Germany). For details, see the Supplemental Material.

**Statistical Analyses**
To compare EMV levels between patient and control samples, in plasma as well as in the perfusion experiments, the Mann–Whitney U test was used. Correlations between B1R-positive EMVs and variables such as diagnosis, treatment, creatinine levels, and the BVAS were carried out using Spearman correlation coefficient. Neutrophil migration and perfusion experiments were compared using Kruskal–Wallis and Dunn multiple comparisons test. The Wilcoxon test was used to compare neutrophil migration in paired samples. A <i>P</i> value ≤0.05 was considered significant. Statistical analysis was performed using GraphPad prism software (version 7; GraphPad Software, La Jolla, CA).

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DISCLOSURES
None.

REFERENCES


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CORRECTION


Firstly, the opening passage of the sixth paragraph is corrected to read as follows: “There are a few limitations to the current study. For example, the authors defined EMVs by the expression of CD105$^+$ and/or CD144$^+$. Importantly, CD105 (endoglin) may also be expressed by monocytes. Nevertheless, CD144 is exclusive to endothelial cells. As described in previous work by the same researchers, the microvesicle size described ranged from 8-451 nm, suggesting that the microvesicles analyzed were most likely derived from the plasma membrane.”

Secondly, the final two sentences of the seventh paragraph are corrected to read as follows: “Interestingly, using flow cytometric analysis of plasma from patients with ANCA vasculitis, the authors recently found high levels of leukocyte-derived microvesicles bearing B1R and showed that microvesicles shuttle B1R between leukocytes and endothelial cells.”

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Supplement

C1-inhibitor decreases release of vasculitis-like chemotactic endothelial microvesicles

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Supplementary Methods

Patients and controls

Blood samples were available from 17 patients (10 females, 7 males, children and adults, median age 43 years) with vasculitis treated at the Department of Nephrology and the Department of Pediatrics, section of Pediatric Nephrology, Skåne University Hospital, Lund and Malmö, Sweden. Details regarding patients’ age, sex, clinical findings, diagnosis, renal function and blood pressure are given in Table 1. None of the patients were on hemodialysis at, or before, the time of sampling. Vasculitis was defined according to the Chapel Hill nomenclature. \(^1\) Samples were obtained at onset or during the acute phase of disease and used for flow cytometry analysis and perfusion assays. Disease activity was assessed using the Birmingham Vasculitis Activity Score (BVAS, Table 1). \(^2\) Blood was also available from 27 healthy adult controls (15 women, 12 men, median age 42 years) not using any medications at the time of sampling. These samples were used for flow cytometry analysis, perfusion and neutrophil chemotaxis assays. Samples from the patients and controls were obtained after written consent of the subjects or their parents. The study was carried out with the approval of the Regional Ethics review board of Lund University.
Blood samples

Whole blood was drawn by venipuncture into vacutainer tubes containing 0.5 mL of 0.129 M sodium citrate (Becton Dickinson, Franklin Lakes, NJ). These samples were analyzed for the presence of endothelial microvesicles (EMVs), perfusion experiments and neutrophil chemotaxis assays. The first tube after venipuncture was discarded. The samples were centrifuged within 30 min from sampling for 15 min at 2600 x g to produce platelet-poor plasma. The platelet-poor plasma was further centrifuged for 5 min at 9900 x g at room temperature to obtain platelet-free plasma. In certain plasma samples the EMV content was reduced by centrifugation at 20000 x g for 40 min resulting in 7.5 % (range 5.7-11.3 %) of the original EMV content. The supernatant was divided into aliquots and stored at -80ºC.

Culture of human embryonic kidney cells and isolation of microvesicles

Wild-type HEK cells (HEK\textsuperscript{wt}) and HEK cells stably expressing the human B1-receptor (HEK\textsuperscript{B1R})\textsuperscript{3} were cultured to confluence in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria) in 10% CO\textsubscript{2} at 37ºC. HEK\textsuperscript{B1R} cells were supplemented with zeocin (400 µg/mL, Invitrogen, Carlsbad, CA) to select for stable transfectants. When confluent, the cells were washed twice in Dulbecco’s phosphate buffered saline (DPBS, PAA Laboratories) and incubated in serum-free medium overnight. Medium from cells was collected and centrifuged at 2500g for 5 min to remove dead cells. The supernatant was centrifuged at 100000 g for 3 hours after which the supernatant was discarded. The pellet containing microvesicles (MVs) was resuspended in DMEM. The concentration of MVs in both suspensions, derived from HEK\textsuperscript{wt} and HEK\textsuperscript{B1R}, was determined by
nanoparticle tracking (NanoSight LM10, Amesbury, UK) and by protein concentration, determined by spectrophotometry, and adjusted to 70 µg/mL (corresponding to 36.7 x10^8 MVs/mL). Kinin levels were measured in the supernatants of HEK^wt and HEK^B1R cells using an ELISA kit described below, and were below the detection limit.

**Culture of primary glomerular endothelial cells**

Primary human glomerular microvascular endothelial cells (PGECs, Cell Systems, Kirkland WA) were cultured to confluence in endothelial growth medium 2-microvascular (EGM-2MV) supplemented with growth factors, 5% fetal bovine serum (all from Lonza, Walkersville, MD) and 1% penicillin/streptomycin and used until passage 10. Cells were detached from the culture flasks using TrypLE (Life Technologies, Grand Island, NY), washed and resuspended in the same medium, without serum, and used in perfusion experiments.

**Perfusion experiments over primary glomerular endothelial cells**

A semi-automated microfluidic perfusion system (VenaFlux, Cellix, Dublin, Ireland) was used to mimic shear in the microvasculature. Microcapillary channels (Vena8 Endothelial+ biochips, Cellix) were coated with fibronectin (100 µg/mL, Sigma-Aldrich, St. Louis, MO) and the PGEC suspension allowed to attach as previously described. PGEC were pre-stimulated with 200 µM histamine (Sigma-Aldrich) in DPBS for 10 min at a shear stress of 5 dynes/cm² obtained with a Mirus Evo nanopump (Cellix). In certain experiments cells were not pre-stimulated with histamine but perfused with DPBS under the same conditions. Histamine pre-
stimulated samples mimic the activated endothelium present during inflammation.\textsuperscript{5} These endothelial cells were found to release more EMVs, confirming previous results\textsuperscript{4} and were therefore used in all described experiments.

Plasma samples from patients and controls were perfused over the PGEC. Gly-Pro-Arg-Pro (10 $\mu$M, Sigma-Aldrich) was added to the plasma before perfusion to prevent fibrin polymerization.\textsuperscript{6} The plasma (100 $\mu$L) was diluted 1:1 in filtered DPBS. In certain experiments IgG was depleted from plasma by adsorption to a Protein G sepharose column (Amersham Biosciences, Uppsala, Sweden).

Plasma samples were perfused at a shear stress of 2-5 dynes/cm$^2$ for 5-15 min. The lower shear rate was chosen in order to spare plasma volume. After perfusion, the pre-sample (plasma before perfusion over PGEC) and the perfused sample were collected and centrifuged for 5 min at 10000 $\times$ g before storage at -80°C. The pre-sample was not filtered in order not to remove pre-existent EMVs as their contribution to release of EMVs from PGEC was of interest. These samples were later assayed by flow cytometry for detection EMVs and the dilution factor adjusted for by protein concentrations (quantitated by spectrophotometry, NanoDrop 1000, NanoDrop Technologies, Wilmington, DE).

C1-inhibitor-depleted plasma (Milan Analytica AG, Rheinfelden, Switzerland) was centrifuged at 10000 g for 5 min and the supernatant diluted 1:1 in filtered DPBS. This plasma was thereafter perfused over the cells at a shear stress of 5 dynes/cm$^2$ for 5 min. In certain experiments C1-inhibitor (final concentration 1 IU in suspension, Berinert, CSL Behring, Marburg Germany) or the B1R antagonist R715 (1 $\mu$M, a kind
gift from D. Regoli, Universite de Sherbrooke, Quebec, Canada) was added to the plasma just before perfusion.

**Kinin ELISA**

Kinin levels in HEK supernatants and perfused C1-inhibitor-depleted plasma samples were measured using the MARKIT-M bradykinin-ELISA kit (Dainippon Pharmaceutical, Osaka, Japan) as per the manufacturer’s instructions as previously described. 7,8

**Detection of microvesicles derived from endothelial cells and B1R on the EMVs**

Plasma samples and samples collected from perfusion experiments were incubated for 20 min in the dark with mouse anti-human CD144 (conjugated with phycoerythrin (PE), 1:200, peridinin chlorophyll protein-cyanin5.5 (PerCP-Cy5.5) 1:600 or fluorescein isothiocyanate (FITC) 1:200) and mouse anti-human CD105:PerCP-Cy5.5, 1:800. MVs labelled with either or both antibodies were considered to derive from endothelial cells. Both single and double stained MVs were considered positive and each microvesicle was registered only once. As isotype controls IgG1:PE, IgG1:FITC and IgG1:PerCP-Cy5.5 were used (all antibodies from BD Biosciences, San Jose, CA except the PE conjugated antibodies which were from eBioscience, San Diego CA).

To detect B1 receptor (B1R), rabbit anti-human B1R from two sources (in-house developed antibody9 10 μg/mL final concentration or BDKRB1 1:50, Abgent, San Diego, CA) was incubated with the plasma or perfusion samples for 20 min at rt. As
a control antibody, polyclonal rabbit IgG (AbD Serotec, Düsseldorf, Germany) was used. After washing in DPBS samples were incubated with porcine anti rabbit:FITC (1:300 Dako, Glostrup, Denmark) for a further 20 min. To detect IL-8, mouse anti human IL-8:PE (1:800) was incubated with the plasma samples for 20 min at rt. As the isotype control IgG2:PE was used (both antibodies from BD Biosciences). Before analysis, suspensions were washed in DPBS to remove unbound antibodies.

**Acquisition and interpretation of flow cytometry data**

Flow cytometry was performed using a BD FACSCanto Cytometer and FACSDiva Software version 6.0 (Becton Dickinson Immunocytometry Systems, San Jose, CA) or a CyFlow® Cube 8 flow cytometer in which samples were run at a flow rate of 0.2 μL/sec (Sysmex, Norderstedt, Partec, Germany) with FCS Express 4 Flow Research Edition software version 4.07.0003 (De Novo Software, Glendale, CA). The latter flow cytometer detects vesicles of submicron size and therefore identifies more events. Forward and side scatter measurements were obtained with gain settings in logarithmic mode and correct color compensation was determined for the analysis of MVs as previously described.\textsuperscript{10} Results are expressed as the number of positive MVs per mL plasma. Fluorescence minus one was used to define events with fluorescence above background levels to set up positive regions.

**Neutrophil migration assay**

Whole blood from healthy donors was drawn as described above into citrate tubes. The first tube was discarded. Whole blood was combined 1:1 with Dextran 2% (Sigma-Aldrich) in NaCl 0.9% and allowed to sediment for 30 min at room temperature. Neutrophils were purified from the supernatant using Lymphoprep
(Fresenius Kabi, Oslo Norway) adjusted to a concentration of 2 x 10^6 cells/mL as previously described. Cells were identified as neutrophils (98%) by flow cytometry using a specific neutrophil marker (CD66b:PE, BD Pharmingen, San Jose, CA). Purified neutrophils were used in neutrophil migration assays performed with a Boyden chamber micropore filter assay (Neuro Probe, Gaithersburg, MD) as previously described. Purified neutrophils from healthy donors (n=10) were placed in the upper compartment of the chamber. MVs from HEK^wt or HEK^B1R (at 36.7 x10^8 MVs/mL because lower concentrations, 3.67 and 1.84 x 10^8 MVs/mL, did not induce chemotaxis) and PGEC (from perfused plasma samples) were placed in the lower compartment. Alternatively, vasculitis plasma or C1-inhibitor depleted plasma (diluted 1:4) were used as chemoattractants in the lower compartment. Chemoattractants were added with or without preincubation with 1 μM B1R antagonist R715, for 30 min at room temperature. In certain experiments anti-hCXCL8/IL-8 (1 μg/mL, R&D systems, Minneapolis, USA) was added to the lower compartment and preincubated with the MVs for 30 min. DMEM or PBS was used as the negative control and formyl-methionyl-leucyl-phenylalanine (fMLP, 1:20000, Sigma-Aldrich) as the positive control. In experiments in which vasculitis plasma was used neutrophils were obtained from donors with blood group O. After 30 min incubation, at 37°C and 5% CO₂, the membrane filters (3 μm pores, Merck Millipore Ltd, Tullagreen, Ireland) between the upper and lower compartments, were fixed, stained with Hematoxylin Carazzi solution (Labservice, Sundsvall, Sweden), and mounted on slides with cedar oil (Sigma-Aldrich) as previously described.13 Migration in the membrane (measured in μm) was assayed by light microscopy (Zeiss Axiostar Plus, Göttingen, Germany).
Statistics

To compare EMV levels between patient and control samples, in plasma as well as in the perfusion experiments, the Mann-Whitney U test was used. Correlations between levels of B1R-positive EMVs and variables such as diagnosis, immunosuppressive treatment, creatinine levels and the BVAS were carried out using Spearman’s correlation coefficient. Neutrophil migration and perfusion experiments carried out with and without C1-inhibitor were compared using Kruskal-Wallis and Dunn’s multiple comparisons test. In certain experiments the Wilcoxon test was used to compare neutrophil migration in paired samples with or without preincubation with the B1R antagonist. A $P$ value of $\leq 0.05$ was considered significant. Statistical analysis was performed using GraphPad prism software (GraphPad Software, Version 7, La Jolla, Ca).
References


Supplementary figure 1

Figure S1. Effect of microvesicle reduction in vasculitis plasma on endothelial microvesicle release from PGECs under perfusion

MV-reduced vasculitis plasma was perfused over PGECs at a shear stress of 5 dynes/cm² for 5 min (in similarity to Figure 3). Endothelial microvesicle release, and B1R-positive endothelial microvesicle release, did not increase compared to pre-perfusion samples. PGEC: primary glomerular endothelial cells. MV: microvesicle. B1R: B1-receptor.