Extracellular Matrix Metalloproteinase Inducer (CD147) Is a Novel Receptor on Platelets, Activates Platelets, and Augments Nuclear Factor κB–Dependent Inflammation in Monocytes

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Abstract—In atherosclerosis, circulating platelets interact with endothelial cells and monocytes, leading to cell activation and enhanced recruitment of leukocytes into the vascular wall. The invasion of monocytes is accompanied by overexpression of matrix metalloproteinases (MMPs), which are thought to promote atherosclerosis and trigger plaque rupture. Following interaction with itself, the extracellular matrix metalloproteinase inducer (EMMPRIN) induces MMP synthesis via a little-known intracellular pathway. Recently, we showed upregulation of EMMPRIN on monocytes during acute myocardial infarction. EMMPRIN also stimulates secretion of MMP-9 by monocytes and of MMP-2 by smooth muscle cells, indicating that it may be an important regulator of MMP activity. Expression of EMMPRIN on platelets has not been described until now. Here, we demonstrate that resting platelets show low surface expression of EMMPRIN, which is upregulated by various platelet stimulators (flow cytometry). EMMPRIN is located in the open canalicular system and in α granules of platelets (according to electron microscopy and sucrose gradient ultracentrifugation). Platelet stimulation with recombinant EMMPRIN-Fc induced surface expression of CD40L and P-selectin (according to flow cytometry), suggesting that EMMPRIN-EMMPRIN interaction activates platelets. Coincubation of platelets with monocytes induced EMMPRIN-mediated nuclear factor κB activation (according to Western blot) in monocytes with increased MMP-9 (zymography), interleukin-6, and tumor necrosis factor-α secretion (according to ELISA) by monocytes. In conclusion, EMMPRIN may represent a novel target to diminish the burden of protease activity and inflammation in atherosclerosis. (Circ Res. 2008;102:302-309.)

Key Words: platelet receptor ■ inflammation ■ leukocytes ■ metalloproteinases ■ plaque

Atherosclerosis is an inflammatory progressive disease of the vascular wall and is the among the most common underlying causes of morbidity and mortality within the developed world. The rupture of the vulnerable atherosclerotic plaque represents a key process that often leads to acute myocardial infarction or stroke.1 Vulnerable atherosclerotic lesions are characterized by an accumulation of inflammatory cells and matrix metalloproteinases (MMPs), which degrade the protective fibrous cap of the plaque.2–4 Most MMPs derive from immigrated monocytes/macrophages and vascular smooth muscle cells.5 Proteolytic activity is of central importance for plaque progression, during which secreted and membrane-type MMPs (MT-MMPs) enable adherent monocytes to migrate into the vessel wall.6

De novo synthesis of certain metalloproteinases such as MT1-MMP, MMP-2, and MMP-9 is induced by the immunoglobulin-like glycoprotein extracellular matrix metalloproteinase inducer (EMMPRIN) (also known as basigin or CD147).7 The basic mechanisms of this process have been elucidated in fragmentary fashion on tumor cells: EMMPRIN-expressing tumor cells induce the synthesis of MMPs including MMP-1, MMP-2, MMP-3, and MMP-9, as...
well as MT1-MMP and MT2-MMP, in adjacent fibroblasts by means of a transcellular homophilic EMMPRIN-EMMPRIN interaction.\(^8-10\) EMMPRIN expression on various tumor cells has been found to correlate with metastasis development and patient mortality.\(^10,11\)

Recently, we found that certain proatherogenic stimuli upregulate expression of EMMPRIN and MT1-MMP on monocytes in vitro.\(^12,13\) We also showed enhanced surface expression of MMP-9 in monocytes and expression of MMP-2 in smooth muscle cells, indicating that it may display a key regulatory role for MMP activity at the vascular wall.\(^14\)

Platelets are not only responsible for the thromboembolic events occurring in the advanced ruptured plaques that cause acute myocardial infarction and stroke but also play a pathophysiological role in plaque initiation and development.\(^15\) Platelets interact with the vascular endothelium, promoting further bidirectional cell activation.\(^16-19\) In addition, platelets interact with circulating monocytes. At the time of an acute myocardial infarction, increased aggregates of platelets and monocytes have been noted.\(^20\) Monocytes adhere to adherent platelets via P-selectin glycoprotein ligand/P-selectin interaction and via binding of MAC-1 (CD11b/H9251 or to ICAM-2 with fibrinogen as bridging molecules.\(^15\) These adhesive processes induce supplementary inflammatory pathways in platelets and monocytes. Weyrich et al describe nuclear translocation of p65 (RelA), a component of the nuclear factor (NF)-κB family of transcription factors, with subsequent enhancement of the synthesis of monocyte chemotactic protein (MCP)-1 and interleukin (IL)-8 following platelet–monocyte interaction, even though the platelet receptor responsible for this effect is not known.\(^21\) In addition, coinoculation of platelets with monocytes seeded on collagen also induce monocyte MMP-9 activity by means of an unknown receptor or receptors.\(^22\)

In the present study, we show, for the first time, that platelets express EMMPRIN in an activation-dependent fashion. Platelet interactions involving EMMPRIN stimulate platelet degranulation. We show that this novel platelet receptor augments monocyte-derived MMP-9 activity and the secretion of various cytokines via NF-κB activation following monocyte–platelet interaction.

**Materials and Methods**

**Reagents**

Aprotinin was from Lixo. Coomassie brilliant blue and Protein Assay Reagent were from Bio-Rad. Medium 199, anti-human \(\beta\)-actin (clone: AC 15), poly-L-lysine, and lipopolysaccharide were from Sigma. Very-low endotoxin (VLE)–RPMI medium 1640 was from Biochrom, and low-toxin fetal calf serum (Clonetics). The IKK inhibitor BMS-345541 was from Calbiochem. The primary mouse anti-human antibodies anti-EMMPRIN, which was purified and fluorescein isothiocyanate (FITC)-conjugated (clone: HIM6), and goat anti-human Fc (IgG, Clone AP113F) were from Becton Dickinson. Activity blocking anti-human antibody anti-EMMPRIN (UMB6) was from Ancell, anti-GPIIIb/IIIa, which was purified and phycoerythrin (PE)-conjugated (clone: S2Z2), anti-P-selectin (clone: CLB-Thromb/6), and anti-CD40L (clone: TRAP-1) were from Beckman-Coulter, and secondary goat monoclonal antibody (mAb) recombinant PE–conjugated anti-mouse from Immunotech. Gold-labeled secondary anti-mouse antibody (5 nm) were from Amer sham. SDS gels containing 10% gelatin were from Invitrogen. Horseradish peroxidase–conjugated secondary anti-mouse antibody and Western blotting Luminol reagent were from Santa Cruz. Precast 6% Tris–borate/EDTA (TBE) gel was from Invitrogen. Epon was from Serva. IL-6 ELISA was from R&D, and IL-10 and tumor necrosis factor (TNF)\(6\) ELISA were from IBL.

**Cells**

Human monocytes were isolated as described.\(^23\) Briefly, mononuclear cells were isolated by centrifugation of citrate phosphate dextrose adenine–anticoagulated blood on a Ficoll gradient (20 minutes, 800g, 4°C). Mononuclear cells were cultured on plastic dishes (0.5 × 10^6 cells/mL) in VLE–RPMI medium 1640 (Biochrom) supplemented with 10% low-tox fetal calf serum (Clonetics). After 24 hours, nonadherent cells were removed by gentle washing. The remaining cells (85% to 90% CD14-positive monocytes) were resuspended using EDTA (0.05% PBS) and washed in VLE–RPMI medium 1640. Washed platelets were prepared as described.\(^19\)

**Flow Cytometry and Gelatin Zymography**

Flow cytometry and gelatin zymography were performed as previously described.\(^14,19,24\) Gelatin zymography was quantified (optical density) by ImageJ software.

**Disruption of Platelets and Preparation of Subcellular Fractions**

Disruption of platelets and preparation of subcellular fractions were performed as previously described.\(^25\) Briefly, isolated platelets were resuspended at a concentration of 5 × 10^9 platelets/mL. The suspension was transferred to a nitrogen cavitation cell disruption bomb, and the nitrogen pressure was raised to 84 kg/cm\(^2\). After 15 minutes, platelets were disrupted as the suspension flowed rapidly out of the disruption bomb and returned to atmospheric pressure. Platelet homogenates were separated by a linear sucrose gradient (30% to 60%) in 5 mmol/L EDTA. Six gradients of 1.5 mL each were run simultaneously at 1.34 × 10^6 g for 2 hours. Gradient fractions were isolated by a siliconized pipette and dialyzed against phosphate buffered KCl. Finally isolated fractions were subjected to Western blot analysis.

**Enzyme-Linked Immunosorbent Assay**

All ELISAs were performed according to the instructions of the manufacturer.

**Small Interfering RNA–Mediated Gene Silencing of EMMPRIN**

Small interfering (si)RNA for EMMPRIN exon sequence and nonsilencing control siRNA were obtained from Qiagen (2-For-Silencing). The following EMMPRIN duplex was used: sense, 5'-(GGG CCA UGC UGG UCU GCA A)dTdT-3'; antisense, 5'-(UUU CAG ACC AGC AUG GCC G)dTdC-3'. Nonsilencing control siRNA (Qiagen) was used as a negative control. Transfections were performed in 24-well plates with a complex of 1 μg of siRNA (20 μmol/L) and 6 μL of Effectene transfection reagent (Qiagen) 36 hours before the subsequent experiments. Successful suppression of EMMPRIN surface expression was routinely confirmed by fluorescence-activated cell-sorting analysis.

**Generation of Chimeric EMMPRIN-Fc Fusion Protein and of Human Fc Fragments**

EMMPRIN-Fc fusion protein and human Fc fragments were cloned and manufactured in an eukaryotic Flp-In CHO cell–based expression system (Invitrogen), as previously published.\(^14\)

**Protein Purification**

The culture supernatant of stable recombinant EMMPRIN-Fc– or recombinant Fc–expressing CHO cells was collected, centrifuged
(3800g, 30 minutes, 4°C), and filtered (0.2 μm). The proteins were precipitated by addition of 1.2-volume ammonium sulfate (761 g/L) and stirring overnight at 4°C. The proteins were pelleted by centrifugation (3000g, 30 minutes, 4°C), dissolved in 0.1-volume PBS, and dialyzed against PBS overnight at 4°C. The protein solution was clarified by centrifugation (14000g, 30 minutes, 4°C), filtrated (0.2 μm), and loaded on a Protein A column that had been equilibrated with binding buffer (HiTrap Protein A HP, Amersham Pharmacia Biotech AB, Uppsala, Sweden). The column was washed with binding buffer (20 mmol/L sodium phosphate buffer, pH 7.0, 0.02% NaN₃) until absorbance at 280 nm was <0.01 and was eluted with elution buffer (100 mmol/L glycine, pH 2.7). The eluted fractions (900 μL) were immediately neutralized with 100 μL of neutralization buffer (1 mol/L Tris–HCl, pH 9.0, 0.02% NaN₃), pooled, dialyzed against PBS overnight at 4°C, aliquoted, and frozen at −20°C. The column was neutralized with binding buffer, washed with 20% (vol/vol) ethanol, and stored in a refrigerator. The purity of the EMMPRIN-Fc was controlled by Coomassie blue–stained SDS-PAGE gel electrophoresis.

Western Blots and Real-Time RT-PCR

Western blots and real-time PCR were performed as previously described. PCR products were measured and visualized on 6% TBE gel.

Transmission Electron Microscopy

Cover slips precoated with poly-L-lysine (10 μg/mL) were incubated with platelets and saturating concentrations of a primary mouse mAb anti-human GPIb/IIa (Beckman-Coulter) or anti-human EMMPRIN (Becton Dickinson). Platelets were incubated with primary antibody, and unbound superfluous antibody was removed by gentle washing, followed by centrifugation and coincubation with saturating concentrations of a 5 mmol/L gold-labeled secondary anti-mouse antibody (Amersham), followed by gentle washing to remove superfluous mAbs. Afterward, platelets were fixed with glutaric aldehyde and polymerized with Epon for transmission electron microscopy.

Statistical Analysis

Results with normally distributed continuous variables were reported as means±SD and were analyzed by unpaired t test or ANOVA, followed by the Scheffe test, as appropriate. In general, P<0.05 was regarded as significant.

Results

Platelets Contain EMMPRIN and Express It on the Cell Surface

Various methods were used to identify EMMPRIN as a protein and receptor on platelets. Flow cytometry showed a low expression of EMMPRIN on the surface of resting platelets (Figure 1A). However, in response to various stimuli (thrombin, ADP, collagen), EMMPRIN surface expression increased within minutes (Figure 1A and 1B). The kinetic shows a similar time course to P-selectin. Transmission electron microscopy revealed that EMMPRIN is mainly localized within the open canalicular system (OCS) of platelets and is transferred to the cell surface during the shape changes on platelet stimulation (Figure 2A). Control staining with the gold-labeled secondary antibody alone showed no detectable signal in resting and stimulated platelets (data not shown). The comparable kinetic of EMMPRIN and P-selectin upregulation prompted us to study whether EMMPRIN is expressed in α granules. Therefore Western blot analysis of isolated subcellular platelet fractions was performed. In fact, α granules of resting platelets contain EMMPRIN (Figure 2B). After thrombin stimulation of platelets, EMMPRIN is no longer detectable in α granules but appears to be concentrated in the plasma membrane, which is in concordance with the transmission electron microscopic and flow cytometric analysis and suggests that a combination of shape changes and platelet degranulation contributes to EMMPRIN upregulation after platelet stimulation.

EMMPRIN Induces Platelet Degranulation

Consequently, we studied the potential functional consequences of EMMPRIN expression on platelets. We hypothesized that EMMPRIN engagement on platelets might stimulate platelet activation. Because homophilic binding of EMMPRIN has been described, we cloned EMMPRIN and manufactured recombinant soluble chimeric EMMPRIN-Fc fusion protein. Incubation of resting platelets for 2 hours with recombinant EMMPRIN-Fc substantially induced platelet degranulation, as evidenced by enhanced surface expression of P-selectin (CD62P) and CD40L (Figure 3A). Fc fragments alone were used as controls and did not show any effect. To strengthen the concept of EMMPRIN binding to platelets, platelets were pretreated with medium, with Fc or with EMMPRIN-Fc for 30 minutes. Platelets were washed and incubated with a FITC-conjugated goat anti-human Fc IgG mAb and analyzed by flow cytometry. In fact, an increased binding of the anti-Fc antibody to the Fc-EMMPRIN–pretreated platelets indicates a binding of EMMPRIN-Fc to the cell membrane (Figure 3B).

EMMPRIN on the Platelet Surface Induces MMP-9 Production by Monocytes

As previously reported by Galt et al, platelets augment the MMP-9 activity of monocytes. Based on the original role of EMMPRIN in stimulating protease activity by means of a process of intercellular dialog, we speculated that EMMPRIN on the platelet surface might contribute to the MMP-9 induction in monocytes. To investigate the capacity of EMMPRIN on the platelet surface to activate proteases, we coinubcated isolated human platelets with isolated monocytes in which surface expression of EMMPRIN had been reduced by pretreatment with EMMPRIN-specific siRNA. Alternatively, monocytes were pretreated with an EMMPRIN activity–blocking mAb. In accordance with Galt et al, we found that interplay between freshly isolated platelets and monocytes amplifies MMP-9 activity (Figure 4). In fact, EMMPRIN-blocking mAb and siRNA-mediated knockdown of EMMPRIN expression on monocytes reduced this effect, suggesting that EMMPRIN-EMMPRIN binding contributes to MMP-9 secretion on platelet–monocyte interactions. Together, these results indicate that EMMPRIN on the platelet surface interacts with EMMPRIN on monocytes to stimulate MMP-9 activity.

EMMPRIN Signaling Activates NF-κB

The EMMPRIN-mediated intracellular activation pathways are incompletely understood. Because protease activation in monocytes is linked to NF-κB signaling, we speculated that cell–cell communication via EMMPRIN engagement might activate NF-κB. NF-κB is a critical regulator
of innate and adaptive immunity and regulates many key inflammatory genes linked to atherosclerosis. NF-κB can be activated by 2 different activation cascades. The classic pathway involves the activation of the IKK complex with subsequent degradation by phosphorylation of IκBα and translocation of untied NF-κB dimer (p65/p50) into the nucleus. The alternative NF-κB activation cascade is mediated through IKK1 and results in the processing of p100 to p52, resulting in the nuclear transfer of the relB-p52 dimer.27

To test the hypothesis that EMMPRIN-EMMPRIN binding evokes protease stimulation by activating NF-κB, we pretreated human isolated monocytes with an IKK inhibitor and then incubated them with recombinant EMMPRIN. As depicted in Figure 5A, stimulation with EMMPRIN led to activation of MMP-9, which was hindered by inhibition of the IKK complex. Western blot analysis showed that EMMPRIN activated NF-κB via the IκBα complex (Figure 5B). Additionally, polymyxin B did not affect MMP-9 secretion on EMMPRIN-Fc treatment (data not shown), which excludes the possibility that a bacterial lipopolysaccharide contamination may account for the stimulatory effects.

EMMPRIN on the Platelet Surface Stimulates NF-κB–Dependent Cytokines in Monocytes

NF-κB is known to activate the synthesis of cytokines in monocytes such as proinflammatory IL-6 and TNFα in addition to antiinflammatory cytokines such as IL-10.27,28 In fact, in an isolated system, adhesion of human monocytes to immobilized recombinant EMMPRIN-Fc strongly induced the secretion of IL-6, TNFα, and IL-10 (Figure 6, right). Pretreatment of monocytes with either an IKK inhibitor or with EMMPRIN-specific siRNA sharply hindered EMMPRIN-mediated cytokine induction. Consistently, platelet–monocyte coincubation induced secretion of IL-6 and TNFα by monocytes (Figure 6, left), which was prevented by monocyte pretreatment with EMMPRIN-specific siRNA. Notably, increased IL-10 secretion could be detected in only the
isolated system of monocyte adhesion to EMMPRIN-Fc but not on cellular interactions. Together, these results indicate that EMMPRIN not only stimulates MMPs but also activates broader inflammatory processes via NF-κB translocation, with prevailing proinflammatory response at cellular interplay.

Discussion
The present study indicates that EMMPRIN may play an important role in mediating the interplay of cardiovascular cells. Specifically, our study shows (1) that EMMPRIN is expressed by platelets and is upregulated on platelet activation; (2) that binding of EMMPRIN to platelets induces platelet activation and degranulation; and (3) that EMMPRIN-mediated binding of platelets to monocytes induces the secretion of MMP-9 and various cytokines in an NF-κB–dependent fashion. Thus overall, our findings indicate that EMMPRIN-mediated cellular interactions may act in a proatherogenic manner at the vascular wall.

EMMPRIN on Platelets
Electron microscopy revealed that EMMPRIN is localized in the OCS of platelets and is transferred to the external surface following stimulation of the platelets with activators such as thrombin, ADP, or collagen (Figure 2A). Surface-connected OCS contain major glycoproteins (GP) such as GPIIb and GPIIb-IIIa. Immunocytochemical techniques such as flow cytometry are limited in their ability to display antigens within the OCS because mAbs do not completely gain access to OCS targets such as GPIIb-IIIa. The change in shape that activated platelets undergo with externalization of the OCS may therefore at least in part account for the observed upregulation of EMMPRIN measured by flow cytometry (Figure 1). Moreover, the kinetic of the EMMPRIN surface expression response was similar to P-selectin, a protein that is stored in α and dense granules. Because channels of the surface-connected OCS of human platelets serve as conduits for the discharge of α granule products secreted during the platelet release reaction, Western blot analysis of isolated protein fractions of platelet lysates was performed. These experiments revealed that EMMPRIN is additionally stored within α granules of resting platelets. In stimulated and degranulated platelets, EMMPRIN was no longer detectable in the subcellular fraction of α granules and was concentrated in the membrane fraction (Figure 2B). In summary, our data indicate that externalization via OCS conjointly with a degranulation process contribute to EMMPRIN activation–dependent upregulation.

Binding of EMMPRIN to its homophilic ligand EMMPRIN further activates platelets, as shown by increased surface expression of P-selectin (CD62P) and CD40L, indicating that EMMPRIN may play a role in platelet recruitment.
Using an anti-Fc antibody, we could indirectly demonstrate a binding of EMMPRIN-Fc to the platelet membrane, suggesting a homophilic binding. Thus, homophilic EMMPRIN–EMMPRIN interactions between platelets may support a vicious cycle whereby increased EMMPRIN expression on the platelet surface leads to increased EMMPRIN-mediated platelet activation, in turn leading to increased EMMPRIN expression and so on. However, it should be noted that ADP-induced platelet aggregation as an expression of late platelet response was not affected by platelet prestimulation with EMMPRIN (data not shown).

Platelet–Monocyte Interactions

Galt et al have shown previously that on coincubation with platelets, monocytes increase their production of MMP-9, and the authors speculate that binding of platelet P-selectin to P-selectin glycoprotein ligand-1 on monocytes mainly accounts for this effect.22 We have now identified EMMPRIN as a receptor on platelets that may at least contribute to MMP-9 activation in monocytes. Monocyte-derived MMP-9 activity was induced by coincubation with platelets, an effect which was abrogated by EMMPRIN knockdown using siRNA technology or by an EMMPRIN activity blocking mAb.

The pathways by which EMMPRIN mediates intracellular activation are not well understood. We showed that EMMPRIN activates NF-κB in monocytes. Isolated human monocytes were pretreated with medium or with an IKK inhibitor (10 μmol/L, BMS-345541; Calbiochem). After 1 hour, monocytes were stimulated with recombinant EMMPRIN-Fc fusion protein (5 μg/mL) or with Fc fragment of human IgG (2 μg/mL, negative control) or with lipopolysaccharide (LPS) (1 μg/mL, positive control). After 24 hours, cell supernatants were subjected to gelatin zymography to determine MMP-9 activity. One representative zymograph and the quantitative analysis of 3 is shown. *P<0.05.
In summary, EMMPRIN is a novel receptor on platelets. EMMPRIN-mediated cellular interaction between EMMPRIN on the cell surface induces platelet activation and NF-κB-driven monocyte activation. EMMPRIN may play an important role at sites of vascular inflammation and thus presents a potential future therapeutic antiinflammatory target.

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Disclosures
None.

References


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