**Mer Receptor Tyrosine Kinase Signaling Participates in Platelet Function**

Cailin Chen, Quan Li, Andrew L. Darrow, Yuanping Wang, Claudia K. Derian, Jing Yang, Lawrence de Garavilla, Patricia Andrade-Gordon, Bruce P. Damiano

**Objective**—Recently, mice made deficient in growth arrest–specific gene 6 product (Gas6) or in which Gas6 gene expression was inhibited were shown to have platelet dysfunction and to be less susceptible to thrombosis. The aim of this study was to define and characterize the relevant Gas6 receptor or receptors involved in platelet function.

**Methods and Results**—Using RT-PCR and Western blot analysis we found that mer was the predominantly expressed subtype in mouse and human platelets, whereas axl and rse were not detected. We generated mer-deficient mice by targeted disruption of the mer receptor gene. Platelets derived from mer-deficient mice had decreased platelet aggregation in responses to low concentrations of collagen, U46619, and PAR4 thrombin receptor agonist peptide in vitro. However, the response to ADP was not different from wild-type platelets. Knockout of the mer gene protected mice from collagen/epinephrine–induced pulmonary thromboembolism and inhibited ferric chloride–induced thrombosis in vivo. Tail bleeding times, coagulation parameters, and peripheral blood cell counts in mer-deficient mice were similar to wild-type mice.

**Conclusion**—Our data provide the first evidence that mer, presumably through activation by its ligand Gas6, participates in regulation of platelet function in vitro and platelet-dependent thrombosis in vivo. (Arterioscler Thromb Vasc Biol. 2004;24:1118-1123.)

**Key Words:** receptor tyrosine kinase ■ Gas6 ■ mer ■ platelet ■ thrombosis

Mer (c-mer, Nyk, Eyk)1-2 tyrosine kinase belongs to a family of transmembrane receptors that also includes axl (Ark, Ufo, Tyro7)3 and rse (Tyr03, Dtk, Etk, Bt, Tif).4,5 This family of proteins has been implicated in reversible cell growth arrest,6 survival,7 proliferation,7-9 and cell adhesion.10-12 Recently, the product of the growth arrest–specific gene 6 (Gas6) was identified as a heterophilic ligand for axl, rse, and mer, whereas protein S was identified as the ligand for the rse receptor.13-16 Both Gas6 and protein S belong to the vitamin K–dependent protein family. Proteins in this family, which also includes prothrombin; coagulation factors VII, IX, and X; protein C; and protein Z, are characterized by posttranslational γ-carboxylation of certain glutamic acid residues by carboxylase, using vitamin K as a cofactor.6,17 Gas6 is structurally similar to protein S with 48% amino acid identity. Protein S is a cofactor for activated protein C that inactivates the coagulation factors Va and VIIIa18 and is thus involved in the anticoagulation cascade. Genetic deficiency of protein S in humans is one of the most severe inherited risk factors for thrombosis.19 Gas6, originally isolated as a growth arrest–specific gene from quiescent fibroblasts,6 has recently been shown to participate in the regulation of platelet activation and aggregation.20 Gas6 amplified platelet aggregation and secretion in response to known platelet agonists including ADP, collagen, and the thromboxane A2 analogue U46619.20 Inactivation of the Gas6 gene prevented venous and arterial thrombosis in mice, and protected against fatal collagen/epinephrine–induced thromboembolism.20 In addition, Gas6 antibodies inhibited platelet aggregation in vitro and protected mice against fatal thromboembolism in vivo.20

The aim of the present study was to define and characterize the relevant Gas6 receptor or receptors involved in platelet function. We first investigated the receptor expression pattern. We found that mer receptor was the predominantly expressed subtype in human and murine platelets, whereas axl and rse were not detected. To investigate whether mer receptor contributes to the regulation of platelet function, we generated mer-deficient mice by targeted disruption of the mer receptor gene. Elimination of mer protein resulted in impaired platelet aggregation induced by collagen, U46619, and PAR4 thrombin receptor agonist peptide at low concentrations in vitro, and inhibition of acute arterial thrombosis in vivo. Our data provides the first evidence that mer, presumably through activation by its ligand Gas6, participates in...
regulation of platelet function in vitro and platelet-dependent thrombosis in vivo.

Methods

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from washed platelets, CHRF-288-11 cells (kindly provided by M. Lieberman, University of Cincinnati), liver or brain tissue with Trizol Reagent (Invitrogen, Calif). First-strand cDNA was prepared with an oligo (dT) primer and Superscript Preamplification System kit (Life Technologies). After reverse transcription, the cDNA product was amplified by PCR following a standard protocol.

Generation of mer−/− Mice

A 12.5-kb DNA fragment from a 129SvEvBrd murine genomic clone covering most of the mer gene was used in the targeting construct. A 107-bp sequence of the first coding exon containing transcription and translation initial codons was deleted and replaced by a neomycin selection cassette. The DNA construct was introduced into 129SvEvBrd embryonic stem (ES) cells by electroporation and cultured in the presence of 400 μg/mL of G418 and 0.2 μmol/L ganciclovir. Individual clones were screened for homologous recombination by Southern blot analysis. The targeted ES clones were microinjected into blastocysts from C57BL/6 mice. Male chimeras were bred with C57BL/6 females to obtain heterozygous mice. The mer-deficient mice in order to investigate the involvement of this Gas6 receptor in platelet function.

Blood flow velocity was measured using a Doppler Master (Crystal Biotec Holliston). A strip of parafilm (50×1×1 mm) was inserted under the carotid proximal to the flow probe and a filter paper (Whatman 2, 1×4 mm) was placed between the parafilm and the artery. Another filter paper (1×4 mm) was placed over the carotid and the parafilm was withdrawn. Eight μL of anhydrous ferric chloride (FeCl3, EM Science) at a concentration of 10, 7.5, or 5% in distilled H2O was dropped onto the 2 filter papers. Time from FeCl3 application to flow decline <5% of baseline was recorded during a 30-minute observation period. Results were evaluated statistically using the proportional hazard regression (PHREG) procedure with an analysis of maximum likelihood yielding an overall probability value across the different FeCl3 concentrations. P<0.05 was considered significant.

Measurement of Hematological and Coagulation Parameters

A hematology analyzer (Advia 120 Hematology System, Bayer Diagnostics) was used with 150 μL whole-blood samples collected in 10% sodium citrate to determine blood cell counts, hematocrit, and hemoglobin. An ACL-100 Automated Coagulation Analyzer (Coulter Corp) was used to analyze prothrombin time (PT) and activated partial thromboplastin time (aPTT) from plasma samples. Tail bleeding time was determined in 2- to 3-month-old mice. The tip of the tail (5 to 6 mm from the tip) was snipped. Blood was blotted onto filter paper, and the time to cessation of bleeding was recorded. Statistical analysis consisted of grouped t tests with P<0.05 considered significant.

Results

Expression of mer, axl, and rse in Human and Murine Platelets

We first performed RT-PCR to determine the receptor expression patterns of the 3 Gas6 receptors mer, axl, and rse in washed human platelets. First-strand cDNA synthesis was then used to amplify the 3 receptors. A strong expression of mer in human platelets was also detected (Figure 1A). The cDNA was further analyzed by the amplification of the housekeeping gene GAPDH (data not shown). Gas6 was highly expressed in human platelets (Figure 1A). The cDNA was used to detect and rse mRNA expression in platelets, but 623- and 558-bp bands corresponding to the axl and mer genes, respectively, were detected in brain tissue (Figure 1A). A similar expression pattern was seen in platelet precursor cells, the megakaryocytic cell line CHRF-288 (Figure 1A). The receptor expression pattern was confirmed at the protein level by Western blot analysis (data not shown). We further analyzed the receptor expression pattern in murine tissues. Using RT-PCR, in a similar experimental setting, mer receptor was the only receptor detected in mouse platelets (Figure 1B). No appreciable PCR products for axl and rse were detected, indicating no significant axl or rse mRNA expression in mouse platelets. Interestingly, both axl and mer were expressed in liver tissue, whereas all 3 receptors were expressed in brain tissue (Figure 1B). The similar expression pattern in human and murine platelets, in which mer is the only receptor detected, provided the rationale for generating mer-deficient mice in order to investigate the involvement of this Gas6 receptor in platelet function.

Generation of mer-Deficient Mice

The knockout mice were obtained from Lexicon Genetics Inc (The Woodlands, Tex). The strategy for generating mer-null
The disruption of mer gene transcription was confirmed by Southern blot analysis (Figure 2B). A 3.4-KB band was detected in wild-type (mer+) mice, whereas disruption of the mer gene (mer−) resulted in a 2.0-KB band. As expected, both bands were observed in heterozygous (mer+) mice. A Northern blot analysis was performed to confirm the loss of mer gene transcription. As shown in Figure 2C, the transcript was detected in mer+/− mice, whereas a dramatic reduction of mer message was observed in mer−/− mice, and no signal was detected in mer−/− mice. Western blot analysis from liver cell extracts further verified the absence of mer protein translation in mer−/− mice (Figure 2D).

Mer-Deficient Mice Showed Normal Phenotype and Hemostasis

Mer−/− mice were born at the expected Mendelian frequency. The mice were viable, fertile, and appeared healthy. There were no genotypic differences in litter size, weight, and size of mice. There were no significant gross pathologic changes in major organs in mer-deficient mice. As shown in the Table, there were no differences in red and white blood cell counts between mer+/+ and mer−/− animals. Platelet counts were similar in mer+/+ (863.3×10^9/L) and mer−/− mice (828.3×10^9/L). Moreover, there was no evidence of spontaneous bleeding in mer−/− mice. Although tail-clip bleeding time was slightly greater in mer-deficient mice (9.33±0.1 minute, n=8, in mer−/− mice versus 7.98±0.5 minutes, n=8, in mer+/+ mice), the difference was not statistically significant. There were no genotypic differences in prothrombin and activated partial thromboplastin times.

**Impaired Platelet Aggregation in Vitro**

We next examined platelet function in mer+/+ and mer−/− mice using in vitro platelet aggregation assays. Platelets from mer+/+ littermates dose-dependently aggregated in response to ADP, collagen, the thromboxane A2 receptor agonist...
U46619, and the PAR4 thrombin receptor agonist peptide AYPGKF (Figure 3). The platelet aggregation response to ADP was similar in mer−/− and mer+/+ mice at a variety of agonist concentrations (data not shown). In contrast, a decreased aggregation response was observed in mer−/− mice in response to 2 μg/mL of collagen (Figure 3A). A reduced response in mer−/− in comparison to mer+/+ mice was also noted at 1 μg/mL and 0.75 μg/mL of collagen (Figure 3B and data not shown). Although platelet aggregation in response to 1 or 5 μmol/L U46619 was similar in mer−/− and mer+/+ mice (Figure 3C), the response to a lower concentration of 0.5 μmol/L U46619 was reduced in mer−/− mice (Figure 3D). Similarly, the response to lower concentrations of AYPGKF was reduced in mer−/− mice, whereas the response to higher concentrations was similar in mer−/− and mer+/+ mice (Figure 3E and 3F).

Impaired Platelet Function in Vivo
Platelets are known to play a central role in both arterial and venous thrombosis. To determine whether mer deficiency affects platelet function in vivo, we used 2 thrombosis models: collagen/epinephrine–induced pulmonary embolism and ferric chloride–induced arterial thrombosis. Male littermate offspring of mer−/− and wild-type mice (mer+/+) from 3 individual experiments using PRP pooled from 4 animals. Large squares on the x axis represent 30-second intervals; y axis is percent aggregation (% light transmission) in 10% interval.

In the ferric chloride–induced thrombosis model, time to cessation of blood flow and the proportion of arteries patent at the end of the experiment were assessed. Three concentrations of anhydrous FeCl3 (10%, 7.5%, and 5%) were evaluated. Eight mice for each ferric chloride group were tested. Overall, increasing concentrations of FeCl3 resulted in a more rapid progression to blood flow cessation. However, time to cessation of blood flow at each of the FeCl3 concentrations was prolonged in mer−/− mice compared with mer+/+ mice (Figure 4B, P=0.037). The differences were not pronounced at the highest FeCl3 concentration (mean time to cessation of blood flow of 10.5 and 8.9 minutes in mer−/− and mer+/+ mice, respectively, and no vessels were patent after 30 minutes). However, the differences were more pronounced in the 7.5% FeCl3 group. A greater proportion of vessels were patent at the end of the experiment (30 minutes) in mer−/− compared with mer+/+ mice (33.3% of mer−/− versus no vessel patent in mer+/+ mice). Moreover, the most pronounced protection was observed with the lowest dose of 5% FeCl3. The proportion of vessels patent at the end of the experiment (30 minutes) was markedly increased in mer−/− mice (87.5% compared with 40% in mer+/+ animals).

Discussion
Intravascular platelet aggregation, although essential for primary homeostasis, also contributes to the underlying pathologies associated with enhanced thrombosis such as myocardial infarction, unstable angina, stroke, and ischemia in other vital organs. Such thrombotic-related episodes remain a major cause of morbidity and mortality globally. Gas6 has been shown to play an important role in platelet function and...
thrombosis. Gas6 is present in α-granules and is secreted after platelet activation, where it presumably activates its receptor or receptors on the surface of platelets. To fully understand how Gas6 and its receptors are involved in the regulation of platelet function, we generated mer knockout mice based on receptor expression patterns in platelets. Our results show that inactivation of the mer gene results in impaired platelet aggregation induced by low concentrations of the platelet agonists collagen, U46619, and the PAR4 thrombin receptor agonist peptide AYPGF. In addition, disruption of the mer gene protects against acute thrombosis triggered by arterial application of ferric chloride and pulmonary thromboembolism induced by collagen/epinephrine.

The 3 receptors for Gas6, mer, axl, and rse proteins, have been shown to be expressed in cells of the mammalian reproductive, immune, vascular, and nervous systems. Although coexpression of more than one of the receptors within the same cell has been reported, some cell types appear to express only a single member of the family. To investigate which of the Gas6 receptors is involved in platelet function, we first performed RT-PCR to determine receptor expression patterns of the 3 receptors in human platelets and the megakaryocytic cell line CHRF-288. Mer was the predominantly expressed receptor in both human platelets and CHRF cells; axl and rse were not detected. This is in contrast to previously reported results by Angelillo-Scherrer and colleagues indicating expression of axl and rse proteins on murine platelets and RNA expression of all 3 subtypes in human platelets. We used 2 sets of oligo nucleotides as PCR primers for each of the human receptors. One set was designed according to the best choices using the PrimerSelect software program (DNASTAR); the other was a primer set published by Angelillo-Scherrer et al. The results from both sets of primers indicated exclusive expression of mer receptor in platelets and megakaryocytes using our methodology. In fact, the experimental conditions were optimized using a brain cDNA library template for positive controls. Moreover, mouse axl transcript was readily detected in both liver and brain tissues, and rse mRNA was detected in brain. In addition, this receptor expression pattern was confirmed at the protein level. However, we cannot rule out the possibility that both axl and rse are expressed at very low levels in platelets and may not have been detected by our analysis method.

Our receptor expression data suggested that the mer receptor might play the major role in Gas6 signaling and potentially in platelet function. Therefore, we generated mer-deficient mice as a tool to address this hypothesis; our in vitro and in vivo studies with these knockout mice support the above hypothesis. Platelets derived from mer−/− mice had impaired platelet aggregation responses in vitro and showed reduced thrombosis in both the collagen/epinephrine- and ferric chloride–induced thrombosis models in vivo. However, the antithrombotic responses were less pronounced than those observed in a study of Gas6-knockout mice. Possible explanations for the differences between mer-deficient and Gas6-deficient mice include possible upregulation of other receptors in mer-deficient mice that compensated for the defect or the existence of other unknown Gas6-responsive receptors on platelets. As with Gas6-deficient mice, mer−/− mice had an apparently normal phenotype, intact hemostasis, and no evidence of spontaneous bleeding.

The decreased platelet response in knockout mice was not apparent for all platelet agonists. Specifically, platelets derived from mer-deficient mice had decreased platelet aggregation response to collagen, U46619, and PAR4 thrombin receptor agonist peptide and, primarily, at low agonist concentrations. The response to ADP was not different from wild-type platelets. The activation of Gas6-receptor appears to play an amplification role in platelets. This is reflected in the differential effect of mer deficiency on low and high agonist concentrations. Moreover, the agonist-specific responses observed with mer−/− platelets, in contrast to Gas6−/− platelets, suggest that mer signaling may contribute to the regulation of specific receptor pathways. Both ADP and thromboxane A2 (TXA2) are released after platelet activation (ADP through release of dense granules, and TXA2 via arachidonic acid metabolism via cyclooxygenase-1) and considered important secondary mediators of platelet aggregation. Lack of effect of mer deficiency on ADP-induced aggregation suggests that the selective impairment of platelet aggregation at low concentrations of the other agonists was not likely a result of diminished dense granule release or ADP receptor function. Our results with ADP are in contrast to those reported for Gas6−/− mice, where platelet aggregation in response to low concentrations of ADP was inhibited. This
Mer Receptor Involvement in Platelet Function

In conclusion, our data provide the first evidence that mer, presumably through activation by its ligand Gas6, participates in regulation of platelet function in vitro and platelet-dependent thrombosis in vivo. Blockade of Gas6-mer receptor signaling may represent a beneficial therapeutic approach for the prevention or treatment of thrombosis. Further studies are needed to determine the downstream elements of Gas6-mer activation involved in platelet function.

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References

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