RANTES Deposition by Platelets Triggers Monocyte Arrest on Inflamed and Atherosclerotic Endothelium

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- **Background**—Circulating platelets and chemoattractant proteins, such as the CC chemokine RANTES, contribute to the activation and interaction of monocytes and endothelium and may thereby play a pivotal role in the pathogenesis of inflammatory and atherosclerotic disease.
- *Methods and Results*—The binding of RANTES to human endothelial cells was detected by ELISA or immunofluorescence after perfusion with platelets or exposure to their supernatants. Monocyte arrest on endothelial monolayers or surface-adherent platelets was studied with a parallel-wall flow chamber and video microscopy. We show that RANTES secreted by thrombin-stimulated platelets is immobilized on the surface of inflamed microvascular or aortic endothelium and triggers shear-resistant monocyte arrest under flow conditions, as shown by inhibition with the RANTES receptor antagonist Met-RANTES or a blocking RANTES antibody. Deposition of RANTES and its effects requires endothelial activation, eg, by interleukin-1 β , and is not supported by venous endothelium or adherent platelets. Immunohistochemistry revealed that RANTES is present on the luminal surface of carotid arteries of apolipoprotein E–deficient mice with early atherosclerotic lesions after wire-induced injury or cytokine exposure. In a mechanistic model of atherogenesis, monocyte adherence on endothelium covering such lesions was studied in murine carotid arteries perfused ex vivo, showing that the accumulation of monocytic cells in these carotid arteries involved RANTES receptors.
- *Conclusions*—The deposition of RANTES by platelets triggers shear-resistant monocyte arrest on inflamed or atherosclerotic endothelium. Delivery of RANTES by platelets may epitomize a novel principle relevant to inflammatory or atherogenic monocyte recruitment from the circulation. (*Circulation.* 2001;103:1772-1777.)

Key Words: inflammation ■ atherosclerosis ■ platelets ■ peptides ■ monocytes ■ blood flow

ctivation of vascular endothelium during the pathogenesis A of inflammatory conditions, such as atherosclerosis or transplant rejection, leads to a subintimal infiltration with monocytes that is thought to be orchestrated by the sequential involvement of multiple signal molecules, eg, chemokines and their monocyte receptors.^{1,2} Induction of the chemokine monocyte chemotactic protein-1 (MCP-1) is evident in macrophagerich atherosclerotic lesions.^{3,4} MCP-1, its receptor CCR2, and the growth-related activity (GRO)- α receptor CXCR2 contribute to macrophage infiltration and lesion formation in atherosclerosis-prone mouse models.5-7 CXC chemokines, such as interleukin (IL)-8 or GRO- α immobilized to heparan proteoglycans on inflamed endothelium, and MCP-1 can mediate the shear-resistant arrest of monocytes via their receptors and may also be involved in subsequent spreading and emigration.⁸⁻¹⁰ The CC chemokine RANTES, which has been found in arteries with transplant atherosclerosis and has been implicated in allograft rejection,^{11,12} can bind to microvascular endothelium and trigger monocyte arrest under flow conditions.

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Circulating platelets may affect vascular and inflammatory syndromes by bridging between endothelium and monocytes or via their secretory products.¹ Their cooperative cellular interactions are mediated by adhesion molecules, ie, P-selectin or β_2 - and β_3 -integrins binding to fibrinogen, and contribute to thrombus formation and fibrin deposition.^{13–16} On activation, platelets express surface-bound molecules and release of proinflammatory cytokines, eg, IL-1 β , resulting in endothelial activation, and secrete chemoattractants, eg, platelet-activating factor (PAF) and RANTES.^{17–20} Given the crucial role of chemokines in coordinating leukocyte traffic, we tested whether platelets may be involved in monocyte recruitment due to delivery and deposition of chemokines to inflamed endothelium.

Methods

Cell Culture, Cell Isolation, and Reagents

Human microvascular endothelial cells (HMVECs), human aortic endothelial cells (HAECs) (both Clonetics), human umbilical vein

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endothelial cells (HUVECs), or Mono Mac 6 cells were cultured as described.8,12 Monocytes were isolated from leukocyte-rich plasma by hyperosmotic NycoPrep 1.068 density gradient centrifugation (Nycomed), yielding a purity of 80% to 85% unactivated cells.²¹ Platelets were isolated by centrifugation of platelet-rich plasma¹³ on a 40% human serum albumin (HSA) cushion at 1200g and Sepharose-2B (Pharmacia) gel filtration of the interface and kept in HHMC (HBSS, 10 mmol/L HEPES, 1 mmol/L Ca2+ and Mg2+, 0.5% HSA) at 37°C. Flow cytometry of surface P-selectin revealed that platelets were not activated (not shown). The RANTES receptor antagonist Met-RANTES generated by retention of the initiation methionine,22 PAF antagonist L-659,989 (MS&D),23 and monoclonal antibody (mAb) VL-1 to RANTES24 have been described. P-selectin mAb AK-4 was from Pharmingen, and goat polyclonal Abs to murine RANTES (C-19) or MCP-1 (R-17) were from Santa Cruz. Recombinant RANTES, IL-1B, and murine tumor necrosis factor (TNF)- α were from PeproTech. Other reagents were from Sigma Chemical Co.

Monocyte Recruitment on Endothelium or Adherent Platelets in Shear Flow

Laminar flow assays were performed as described.8,13,25 Confluent ECs activated with IL-1 β (10 ng/mL) for 12 hours or surfaceadherent platelet layers formed by binding to silane-treated glass slides¹³ and activated with thrombin were assembled as the lower wall of a flow chamber on the stage of an Olympus IMT-2 microscope. Endothelium was preperfused at a shear rate of 1.5 dyne/cm² or preincubated in stasis with platelets (10⁸ cells/mL) or supernatants for 20 minutes at 37°C after platelet stimulation with thrombin (0.5 U/mL) for 5 minutes. Preexposure to platelets or supernatants was also performed in the presence of the blocking RANTES mAb VL-1 (10 µg/mL). An isotype control mAb had no effect (not shown). Mono Mac 6 cells or monocytes (10⁶ cells/mL) were resuspended and pretreated with Met-RANTES (1 μ g/mL) for 15 minutes in HHMC (Mg^{2+}/Ca^{2+} added shortly before assays), kept at 37°C during assays, and perfused at 1.5 dyne/cm². The number of monocytes firmly adherent by primary interaction with endothelium after 5 minutes of accumulation was quantified in multiple fields by analysis of images recorded with a JVC 3CCD video camera and recorder. Data were analyzed by ANOVA.

ELISA, Flow Cytometry, and Immunofluorescence

Detection of soluble or surface-adherent RANTES was performed by a modified ELISA as described.¹² For flow cytometry,¹² platelets were reacted with P-selectin mAb AK-4, RANTES mAb VL-1, or isotype controls (10 μ g/mL) in HHMC with 0.5% BSA for 30 minutes, stained with FITC-conjugated goat anti-mouse IgG mAb for 30 minutes on ice, and analyzed in a FACScan (Becton Dickinson). Immunofluorescence was performed as described.⁸ Briefly, HMVECs grown on glass coverslips were activated with IL-1 β and treated as above, fixed in 3.7% formaldehyde, and incubated for 2 hours at room temperature with 10% heat-inactivated HSA in PBS to block nonspecific binding. Cells were reacted with VL-1 mAb overnight at 4°C and incubated with FITC-conjugated IgG for 30 minutes at 25°C. Images were recorded with a Leica DMRBE fluorescence microscope with an ×100 oil immersion objective.

Immunohistochemistry and Ex Vivo Perfusion of Murine Carotid Arteries

Carotid arteries from apo $E^{-/-}$ mice (Jackson Laboratories, Bar Harbor, Me) fed a Western-type diet (21% fat) for 5 weeks or from C57BL/6 mice (Hilltop, Scottdale, Pa) were paraffin-embedded and cut into 5-µm sections (3 to 4 mice per treatment). Some apo $E^{-/-}$ mice were wire-injured as reported,²⁶ and some mice were treated with TNF- α (1 µg IP) 4 hours before the artery was harvested. Endogenous peroxidase was blocked with 0.45% H₂O₂ in methanol. Antigens were retrieved by boiling slides in unmasking solution. Slides were allowed to cool and were blocked in buffer containing fish skin oil gelatin, normal horse serum (5%), and an avidin-

blocking agent (all Vector Laboratories) to reduce unspecific background staining. For staining, slides were incubated with goat polyclonal Ab C-19 (1.5 µg/mL) or R-17 (1 µg/mL), 5% normal horse serum, and biotin at 4°C overnight, reacted with biotinylated secondary horse anti-goat Ab, avidin-biotin peroxidase complex, and 3,3'-diaminobenzidine substrate. Slides were counterstained with hematoxylin, dehydrated with xylene, and mounted, and images were recorded. Perfusion of carotid arteries from $apoE^{-/-}$ mice ex vivo was performed as described.27 Arteries were infused with or without RANTES (150 ng/mL) for 30 minutes and washed. Mono Mac 6 cells treated with or without pertussis toxin (PTX) (200 ng/mL) were labeled with calcein (0.5 µg/mL, Molecular Probes), resuspended at 3×10^6 cells/mL, and pretreated with Met-RANTES (1 μ g/mL) for 15 minutes. Suspensions were perfused in isolated carotid arteries at 10 µL/min. Perfusion and accumulation of labeled monocytic cells were observed by stroboscopic epifluorescence illumination (Strobex; Chadwick-Helmuth) by intravital microscopy (Axioskop FS; Carl Zeiss) with an SW20 immersion objective.

Results and Discussion

RANTES Triggers Monocyte Arrest on Endothelium Preconditioned by Platelets

To investigate whether platelets or their secretory products "prime" endothelium to support initial monocyte arrest, endothelial monolayers activated with IL-1 β were exposed to resting or thrombin-stimulated platelets in stasis or at low shear or were preincubated with their supernatants. Subsequently, human monocytic Mono Mac 6 cells were perfused at a shear rate of 1.5 dyne/cm², and cells adherent primarily to endothelium were counted after 5 minutes of accumulation. Exposure of activated HMVECs to thrombin-stimulated platelets by preperfusion or in stasis triggered a 2-fold increase in shear-resistant monocytic cell arrest (Figure 1A). Preincubation of activated HMVECs with supernatants from thrombin-stimulated platelets, but not thrombin alone or resting platelets, was sufficient to increase arrest (Figure 1A). This implicates soluble platelet products and excludes direct effects of thrombin.

Because platelets release RANTES on stimulation and degranulation,²⁰ we studied its role in monocyte arrest on HMVECs primed by platelets. Pretreatment of monocytic cells with a peptide RANTES receptor antagonist, Met-RANTES,²² abolished firm arrest induced by exposure to stimulated platelets or supernatants (Figure 1A). Similar inhibition was achieved by preincubation of HMVECs in the presence of a blocking RANTES mAb (Figure 1A) but not with an MCP-1 peptide antagonist. This reveals the involvement of monocytic RANTES receptors and indicates that the secreted platelet product mediating arrest is RANTES. The increase in monocyte adhesion after pretreatment with stimulated platelets was observed only on IL-1*β*-activated HM-VECs but not on resting HMVECs, which support only minimal adhesion (data not shown). In contrast, exposure of IL-1 β -activated HUVECs to platelets or their supernatants did not affect monocytic cell arrest (Figure 1B). The fact that binding of RANTES is clearly detectable on activated HMVECs but not on resting HMVECs or activated HUVECs (Reference 12; P.J.N., unpublished data) suggests that cytokine activation is necessary for binding of RANTES to HMVECs and that cell type-specific immobilization of RANTES is required for its function. Experiments performed with isolated human blood monocytes confirmed that Met-



Figure 1. RANTES receptor antagonist Met-RANTES blocks monocyte recruitment on activated endothelium triggered by secretory products of stimulated platelets. Confluent HMVECs (A, C), HUVECs (B), or HAECs (D) activated with IL-1 β (10 ng/mL) were stimulated with thrombin (0.5 U/mL), preperfused at 1.5 dyne/cm², or preincubated with platelets stimulated with thrombin (0.5 U/mL) or their supernatants for 20 minutes with or without RANTES mAb VL-1 (C). Mono Mac 6 cells (A, B) or monocytes (C, D), untreated or pretreated with Met-RANTES (1 μ g/mL), were perfused at 1.5 dyne/cm² on activated endothelial cells. Number of firmly adherent cells was determined after accumulation for 5 minutes (mean±SD, n=6). **P*<0.05 vs untreated monocytes.

RANTES inhibited arrest on HMVECs when primed by exposure to stimulated platelets or supernatants (Figure 1C). Moreover, preperfusion of IL-1 β -activated HAECs with stimulated platelets or exposure to their supernatants triggered monocyte arrest that was blocked by Met-RANTES (Figure 1D), extending the relevance of our results to the arterial macrovasculature. Thus, RANTES released by platelets and bound to the surface of inflamed endothelium supports monocyte arrest in flow.

Surface Binding of Platelet-Derived RANTES to Activated Endothelium

We next studied whether RANTES secreted by stimulated platelets is deposited on HMVECs. ELISA confirmed that after stimulation, platelets released substantial amounts of RANTES into supernatants (Figure 2A). Flow cytometry detected a marked expression of P-selectin but not RAN-TES on the surface of thrombin-stimulated platelets (Figure 2B). Cell surface ELISA, however, demonstrated that incubation of IL-1 β -activated HMVECs or HAECs with thrombin-stimulated but not resting platelets or supernatants under rotation resulted in a solid immobilization of RANTES but not MCP-1 to the surface of HMVECs or HAECs but not of resting HMVECs or resting or activated HUVECs (Figure 2C). These observations support the conclusion that cytokine activation is necessary to induce specific endothelial binding sites for RANTES. Immuno-fluorescence analysis of IL-1 β -activated HMVECs confirmed that preperfusion with resting platelets did not result in specific staining for RANTES (Figure 2D). After exposure of HMVECs to thrombin-stimulated platelets in shear flow, an intense staining was observed (Figure 2D), indicating substantial immobilization of RANTES on the endothelial surface. Deposition after platelet preperfusion appeared to be more pronounced than after incubation with platelets or supernatants in stasis (Figure 2D). This may imply a potential role for signals promoting degranulation of platelets in flow.

RANTES Is Not Involved in Monocyte Arrest on Adherent Platelets

Platelets may also be important for leukocyte recruitment when adherent to surfaces exposed by endothelial injury or denudation. Accumulation of neutrophils on stimulated platelets in shear flow involved activation of the β_2 integrin Mac-1 by the lipid mediator PAF but not chemokines acting via CXCR2.¹³ Similarly, the PAF antagonist L-659,989²³ but not Met-RANTES inhibited monocyte arrest on thrombin-stimulated platelet layers in shear flow (Figure 2E). This is most likely due to an inability of platelets to bind RANTES, whereas PAF is retained in lipid membranes.¹³ Thus, monocyte-platelet interactions in shear flow are triggered by PAF, whereas RANTES secreted by platelets is insufficient to support arrest unless immobilized by activated endothelium.

Luminal Deposition of RANTES in Atherosclerotic and Injured Arteries

RANTES expression has been detected on endothelium of coronary arteries undergoing transplant-associated accelerated atherosclerosis.11 To assess the relevance of RANTES deposition in the context of atherogenesis in vivo, immunohistochemistry was performed after wire-induced injury on carotid arteries from lesion-prone $apoE^{-/-}$ mice fed a western diet and from wild-type mice treated with TNF- α . RANTES was detectable in the intima and media (eg, in mononuclear cell infiltrates) of early atherosclerotic lesions in apoEmice; most accentuated staining, however, was seen on the luminal surface of the arterial wall, within thrombotic material juxtaposed to the lesions and possibly in association with the endothelium (Figure 3A). A similar pattern of staining for RANTES was found in human carotid atherectomy specimens with advanced lesions (not shown). The finding that in situ hybridization did not reveal RANTES mRNA in inflamed endothelium²⁸ suggests paracrine delivery of RAN-TES to such sites. By contrast, no staining for RANTES was observed in carotid arteries of apoE^{+/+} mice (Figure 3B) or with isotype control (Figure 3C). Four weeks after a wireinduced injury to the carotid artery of apoE^{-/-} mice,²⁹ selective RANTES staining was found on the surface lining of the neointimal lesions (Figure 3D). Stimulation with TNF- α resulted in marked RANTES staining throughout the



Figure 2. RANTES secreted by thrombinstimulated platelets is deposited on activated HMVECs or HAECs but is not involved in monocyte arrest on platelets. RANTES in supernatants of thrombin-activated (0.5 U/mL) or resting platelets was quantified by ELISA (mean±SD, n=7) (A). Surface expression of P-selectin and RANTES (dotted line) or isotype control (bold line) on activated platelets was analyzed by flow cytometry (B). After rotating preincubation with resting or stimulated platelets or their supernatants, levels of surface-bound RANTES or MCP-1 on HMVECs or HAECs were determined by ELISA. Incubation of activated HMVECs with recombinant RANTES (0.5 µg/mL) resulted in a specific OD of 0.19 ± 0.02 (n=4), whereas after incubation of resting HMVECs or resting or activated HUVECs with stimulated platelets or supernatants, all specific OD readings obtained were <0.02. One representative of 4 comparable experiments is shown (C). RANTES immobilization on HMVECs after preperfusion of platelets or preincubation with their supernatants was detected by immunofluorescence (D). Monocytes pretreated with Met-RANTES (1 µg/mL) or PAF antagonist L-659,989 (1 µmol/L) were perfused at 1.5 dyne/cm² on thrombinstimulated, surface-adherent platelets. At 5 minutes, firmly adherent monocytes were counted (mean±SD, n=6) (E).

intima and media of wild-type carotid arteries (Figure 3E). MCP-1 was also found in monocyte/macrophage-rich areas of lesions in apo $E^{-/-}$ mice (Figure 3F). All observations were obtained consistently in \geq 3 animals.

Monocyte Arrest in Carotid Arteries of ApoE^{-/-} Mice Involves RANTES Receptors

In a mechanistic investigation of macrophage recruitment in atherogenesis, monocytic cells perfused ex vivo have been shown to accumulate on endothelium covering early atherosclerotic lesions in carotid arteries from apoE^{-/-} mice.27 In this model, attachment of Mono Mac 6 cells was reduced by Met-RANTES (data not shown) but increased by preinfusion of arteries with RANTES (Figure 4). Pretreatment with PTX resulted in a 50% inhibition of monocyte arrest, confirming that it was mediated by G_i protein-dependent and -independent mechanisms (Figure 4). An equivalent inhibition with Met-RANTES indicated that PTX-sensitive arrest was mediated largely by RAN-TES receptors (Figure 4). The PTX-insensitive component was blocked by α_4 mAb (data not shown), reflecting the presence of preactivated α_4 integrins.²⁷ Because efficient cross-species responses of human monocytes in murine vessels are conceivable, given the structural and functional

conservation of RANTES,³⁰ our data suggest that RAN-TES receptors are involved in atherogenic monocyte recruitment.

Implications of Platelet-Derived RANTES for Inflammation and Vascular Disease

Studies on the role of platelets in monocyte recruitment have been based largely on their direct interactions. Our data provide the first evidence that the conveyance of RANTES by platelets and its deposition on the endothelial surface can trigger monocyte arrest to inflamed endothelium of microvascular or arterial origin. Although potential effects of less abundant platelet chemokines or precursors cannot be excluded, our results clearly implicate RANTES and its receptors. This mechanism thus defines a novel principle by which platelets support inflammatory recruitment of monocytes from the circulation in distinct vascular beds, epitomizing a proximal step in an emerging hierarchy.8 The presence of RANTES on the luminal surface of diseased or injured carotid arteries further implies that this concept is relevant for the direct recruitment of monocytes to atherosclerotic or restenotic lesions. In light of the crucial and complex involvement of monocytes in atherogenesis,1 blocking platelet-derived RANTES as a culprit



Figure 3. Immunohistochemistry for RANTES in carotid atherosclerosis after vascular injury or cytokine stimulation. Staining for RANTES (Ab C-19) was seen in paraffin-embedded sections of native early atherosclerotic lesions (eg, in mononuclear cell infiltrates of intima and media and accentuated on luminal surface) of carotid arteries of apoE^{-/-} mice fed a western diet for 5 weeks (A) but not in apo $E^{+/+}$ mice (B) or with isotype control (C). In arteries from apo $E^{-/-}$ mice after wire injury, RANTES staining is concentrated on luminal surface (D), and in arteries of apoE^{+/+} mice treated with TNF- α , RANTES is seen throughout vessel wall (E). Staining for MCP-1 (Ab R-17) is seen in intimal and medial areas of apoE^{-/-} carotid arteries (F). Original magnifications were $\times 100$ or $\times 200$.

for monocyte arrest with peptide analogues, such as Met-RANTES, or selective nonpeptide receptor antagonists may thus serve as a future approach to the prevention and therapy of atherosclerosis and restenosis.



Figure 4. Role of RANTES receptors in monocyte accumulation in carotid arteries of apoE^{-/-} mice. Calcein-labeled Mono Mac 6 cells pretreated with PTX (200 ng/mL) or Met-RANTES (1 μ g/mL) were perfused in explanted carotid segments pretreated with RANTES. Flow was reduced from high shear to 3 dyne/cm² at 0 minutes. Untreated monocytic cells (control) were perfused in same vessel before and after pretreated cells. Accumulation of adherent cells was quantified at indicated time points. Data are representative of 3 independent experiments.

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