Deposition of Platelet RANTES Triggering Monocyte Recruitment Requires P-Selectin and Is Involved in Neointima Formation After Arterial Injury

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Background—Chemokines expressed on atherosclerotic endothelium or deposited by activated platelets have been implicated in monocyte recruitment during atherogenesis and restenosis. Although the involvement of P-selectin in these processes is evident from studies in knockout mice, it has not been elucidated whether delivery of platelet chemokines requires P-selectin, thus serving as a P-selectin–dependent effector function.

Methods and Results—Using immunofluorescence and laminar flow assays, we found that the deposition of the platelet-derived chemokine RANTES and monocyte arrest subsequently triggered by RANTES immobilized on inflamed endothelium are more efficient after preperfusion than after static preincubation of platelets and appear to depend on interactions of platelet but not endothelial P-selectin. This was revealed by the effects of P-selectin antibodies and comparison of P-selectin–deficient and wild-type platelets. Immunohistochemistry detected a substantial luminal expression of RANTES on neointimal lesions in wire-injured carotid arteries of apolipoprotein E (apoE)–deficient mice but not of mice with a combined deficiency in apoE and P-selectin (or platelet P-selectin). As assessed by histomorphometry, treatment of apoE-deficient mice with the RANTES receptor antagonist Met-RANTES markedly reduced neointimal plaque area and macrophage infiltration.

Conclusions—Our data suggest that RANTES deposition and subsequent monocyte arrest are promoted by platelet P-selectin and involved in wire-induced intimal hyperplasia, and that blocking RANTES receptors attenuates neointima formation and macrophage infiltration. This mechanism represents an important component explaining the protection against neointimal growth in P-selectin–deficient mice and may represent a novel approach to the treatment of restenosis or atherosclerosis by the administration of chemokine receptor antagonists. (Circulation. 2002;106:1523-1529.)

Key Words: restenosis • platelets • inflammation • atherosclerosis

Restenosis after arterial injury has been characterized as an accelerated form of atherosclerosis. The neointima formation underlying restenosis represents a complex remodeling process that involves adhesive interactions of platelets and leukocytes with the denuded vessel wall as well as smooth muscle cell proliferation.1-3 After endothelial denudation, exposure of the basement membrane and subendothelial matrix leads to the deposition of fibrin and adherent platelets, which on activation and degranulation express P-selectin and present fibrinogen, thereby mediating leukocyte rolling and arrest, and supporting their inflammatory recruitment.4,5 Because of secretion of cytokines or growth factors, monocyte-derived macrophages may be associated with lesion progression, including the proliferation of migrated smooth muscle cells.2 The increase in neointima is accompanied by a reendothelialization, which is usually completed after 3 weeks.1

The targeted disruption of P-selectin has been shown to protect against spontaneous atherosclerosis in apolipoprotein E–deficient (apoE−/−) mice and against neointima formation and inflammatory cell recruitment after carotid ligation.6,7 Notably, a deficiency in P-selectin abrogated macrophage infiltration and neointima formation after wire-induced endothelial denudation in carotid arteries of lesion-prone apoE−/− mice or femoral arteries.8,9 This may be attributable to diminished monocyte recruitment on adherent platelet monolayers, thrombotic material, or regenerating endothelium, or reduced fibrin deposition and chemokine synthesis by leuko-

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cytes in the absence of P-selectin.8–11 Activated platelets express surface-bound molecules; release inflammatory cyto-
kines, eg, CD40L or interleukin (IL)–1β, resulting in endo-
theelial activation; and secrete chemoattractants, such as
the lipid mediator platelet-activating factor or the chemokines
PF4, ENA-78, or RANTES.12,13 We recently found that
RANTES secreted by stimulated platelets is immobilized on
microvascular or aortic endothelium, triggers monocyte arrest
under flow conditions in vitro and in perfused carotid arteries,
and is detectable on endothelium in carotid arteries of
apoE−/− mice with early atherosclerotic lesions or after
wire-induced injury.13 The efficacy of RANTES deposition by
platelets in flow13 prompted us to test whether interactions
of P-selectin contribute to endothelial immobilization of
RANTES and subsequent monocyte arrest in flow and
whether RANTES may thereby participate in neointima
formation and monocyte infiltration in injured carotid arteries of
apoE−/− mice.

Methods

Cell Culture, Cell Isolation, and Reagents

Human microvascular endothelial cells (HMVEC) (Clonetics) and
monocytic Mono Mac 6 cells were cultured as described.13,14
Platelets were isolated by centrifugation of platelet-rich plasma on a
40% human serum albumin cushion and Sepharose-2B (Pharmacia)
gel filtration or using ADIgel separation columns (American
Diagnostica Inc.), and kept in assay buffer (HBSS, 10 mmol/L
HEPES, 1 mmol/L Ca2+/Mg2+, and 0.5% human serum albumin) at
37°C.1,7 The peptide antagonists Met-RANTES15 and 8-73GRO-α16
were kindly provided by Drs A. Proudfoot (Serono Pharmaceutical,
Geneva, Switzerland) and I. Clark-Lewis (University of British
Columbia, Vancouver, BC, Canada), respectively. The monoclonal
antibody (mAb) 2PH1 to murine P-selectin glycoprotein ligand-1
(PSDL-1) was from Pharmingen, the mAbs G1 and S12 to
PSGL-1 mAbs (10 ng/mL) were kindly provided by Dr R. McEver (University of
Oklahoma Health Sciences Center, Oklahoma City), and goat polyclonal
antibody reactive with murine or human RANTES (C-19) was
from Santa Cruz Biotechnologies. Thrombin-receptor activating
peptide (TRAP) was from Bachem, recombinant IL-1β from
PeproTech. Other reagents were from Sigma.

Platelet Perfusion, Monocyte Arrest, and
Immunofluorescence on Endothelium

Laminar flow assays were performed as described.4,14 Confluent
HMVEC in Petri dishes (arrest) or on glass coverslips (immuno-
fluo-rescence) activated with IL-1β (10 ng/mL) for 12 hours were
preperfused in a flow chamber at 1.5 dyn/cm2 or preincubated in
stasis with TRAP (2 μmol/L)—stimulated platelets (105 cells/mL)
for 20 minutes at 37°C. Platelets were pretreated with P-selectin or
PSGL-1 mAbs (10 μg/mL) and filtered to remove excess mAb, or
platelets isolated from P-selectin−/− or wild-type mice were used for
preperfusion. Mono Mac 6 cells (106/mL) were resuspended or
pretreated with Met-RANTES (1 μg/mL) for 15 minutes in assay
buffer, kept at 37°C, and perfused at 1.5 dyn/cm2. The number of
monocytes firmly adherent by primary interaction with HMVEC
after 5 minutes was quantified in multiple fields recorded by video
microscopy. Detection of surface-bound RANTES was performed as
described.13 HMVEC fixed in 3.7% formaldehyde and blocked
against unspecific binding were reacted with C-19 overnight at 4°C
and with fluorescein isothiocyanate–conjugated IgG for 30 minutes
at 25°C. Images were recorded with a Leica DMRBE microscope.
Staining of adherent platelets followed this protocol using a CD41
mAb (Diavac.com) as the primary antibody. Serum levels for peptide
antagonists were measured by ELISA (R&D Systems). Lipid
variables determined by routine procedures did not significantly differ
between groups (data not shown). Expression of P-selectin and
RANTES receptors on HMVEC was analyzed by flow cytometry as
described.14

Mouse Carotid Artery Injury Model

ApoE−/− and P-selectin−/− mice (C57BL/6) were from The Jackson
Laboratory. C57BL/6 mice were purchased from MkB (Ry, Den-
mark). P-selectin−/−/apoE−/− mice8,14 were provided by Dr A.L.
Beaudet (Baylor College of Medicine, Houston, Tex) and bone
marrow–transplanted platelet P-selectin−/−/apoE−/− mice will be
described (D. Manka, PhD, et al, submitted for publication,
2002). ApoE−/− mice fed an atherogenic diet containing 21% fat for
1 week before and for 4 weeks after injury were injected intraperitone-
ally with Met-RANTES (n=6; 10 μg), 8-73GRO-α (n=8; 10 μg), or
PBS vehicle (n=6) once daily for 28 days starting before injury.
Mice were anesthetized with intraperitoneal ketamine and xylazine.
After midline neck incision, the left external carotid artery was tied
off distally, and via transverse arteriotomy, a 0.014-in flexible
angioplasty guidewire was advanced by 1 cm. Complete and uniform
endothelial denudation was achieved by 3 passes along the common
carotid artery with a rotating motion.3 At day 28, mice were injected
with pentobarbital for euthanasia and in situ perfusion fixation was
performed with 4% paraformaldehyde at 100 mm Hg. Injured left
and uninjured right arteries were excised and embedded in paraffin.

Quantitative Histopathology and
Immunohistochemistry

Serial sections (5 μm thick) were stained with Movat pentachrome.8
Histomorphometric analysis was performed by individuals blinded
to treatments. For quantitative comparisons, at least 6 sections per
animal within a standardized distance from the bifurcation (1000 to
2000 μm) were analyzed. The areas within lumen and internal and
external elastic lamina were determined by planimetry using Image
Pro Plus 3.0 (Media Cybernetics). Plaque, medial, overall vessel
area, and intima/media ratio were calculated. Immunohistochemistry
was performed as described.3,11 Sections were stained for macro-
phages with mAb F4/80 (Accurate Chemical). For RANTES staining,
slides from apoE−/−/platelet P-selectin−/− and apoE−/− mice were
reacted with antibody C-19. Antibodies were visualized by an
avidin/biotin peroxidase–linked detection system (Vector Laborato-
ries). As a cumulative measure of monocyte infiltration during
neointima formation, foam cells and lipid deposits were identified in
Movat-stained sections within a standardized distance from the
bifurcation (1000 to 2000 μm), and the area covered by both was
determined by blinded observers in digitized images using NIH
Image Software. Data are expressed as percentage of total wall area.

Statistical Analysis

Statistical analysis was performed using Instat software (GraphPad
Software). In vitro data represent mean ± SD from 3 to 6 independent
experiments. Data from animal studies represent mean ± SEM from 6
to 8 mice. Data were compared by 1-way ANOVA and Student-
Newman-Keuls t test to evaluate 2-tailed levels of significance.
Differences with P<0.05 were considered as statistically significant.

Results

Because the endothelial deposition and arrest function of
RANTES secreted by stimulated platelets appear to be more
pronounced after preperfusion than after preincubation in
stasis,13 we were prompted to refine this observation using
submaximally stimulated platelets. As assessed by immuno-
fluorescence and flow assays, preperfusion of platelets stim-
ulated with thrombin (not shown) or TRAP at concentrations
close to the EC50 for degranulation18 resulted in a more
substantial immobilization of RANTES (Figure 1A and 1B) and
subsequent monocyte arrest (Figure 2A) on IL-1β–acti-
vated HMVEC in flow than preincubation with such platelets or
superenatants in stasis. The adhesion of platelets to HM-
VEC was negligible, and CD41 staining was clearly discernible from RANTES staining (not shown). Treatment of monocytes with the receptor antagonist Met-RANTES abolished the enhancement of arrest on HMVEC preexposed to platelets (not shown), clearly indicating that it was triggered by immobilized RANTES.

We next studied the role of P-selectin interactions in RANTES deposition on HMVEC and its functional consequences in flow. Pretreatment of platelets with a blocking but not a nonblocking P-selectin mAb inhibited RANTES immobilization (Figure 1C and 1D) and RANTES-mediated monocyte arrest (Figure 2B) on IL-1β-activated HMVEC in flow. This was confirmed using murine platelets, as murine RANTES has sufficient cross-species homology to activate human receptors. Preperfusion with platelets from wild-type but not P-selectin−/− mice led to substantial deposition of RANTES (Figure 1E and 1F) and subsequent monocyte arrest on activated HMVEC.

**Figure 1.** Deposition of RANTES by stimulated platelets on endothelium is more efficient under flow than in stasis and requires platelet P-selectin. HMVEC activated with IL-1β were preperfused at 1.5 dyne/cm² (A and C through H), or preincubated in stasis (B) with TRAP-stimulated human platelets pretreated without (A, B, G, and H) or with nonblocking mAb S12 (C) or blocking mAb G1 (D) to P-selectin, or were preperfused with stimulated murine wild-type (E) or P-selectin−/− platelets (F). HMVEC were treated with S12 (G) or G1 (H) mAb. RANTES immobilized on HMVEC was detected by immunofluorescence. Shown are representative images. Scale bar, 10 μm.

**Figure 2.** Monocyte recruitment on activated endothelium in flow after preperfusion of platelets depends on platelet P-selectin. IL-1β-activated HMVEC were untreated (control), pretreated in stasis, or preperfused with stimulated human platelets or their supernatants (A). Platelets were pretreated with blocking (G1) or nonblocking (S12) mAb to P-selectin and filtrated (B). Murine wild-type platelets pretreated with or without PSGL-1 mAb or P-selectin−/− platelets were preperfused (C). Monocytic Mono Mac 6 cells were perfused at 1.5 dyne/cm², and the number of firmly adherent cells was determined at 5 minutes. Data are mean±SD from 3 to 6 independent experiments. *P<0.05 vs control, **P<0.05 vs platelet, ***P<0.05 vs wild type.
RANTES has been detected in arteries with transplant vasculopathy but also juxtaposed to microthrombotic material on the surface of endothelium covering early atherosclerotic or neointimal lesions in carotid arteries of apoE−/− mice after wire-induced injury. To test the relevance of P-selectin for RANTES deposition in vivo, we performed immunohistochemistry for RANTES in carotid arteries from apoE−/− mice as compared with P-selectin+/−/apoE−/− mice 4 weeks after arterial injury. Whereas no staining was seen using isotype control (Figure 3A), robust transmural staining was found in tumor necrosis factor-α–treated mice. RANTES was preferentially detectable on the luminal surface of endothelium covering neointimal lesions in apoE−/− mice (Figure 3B) but not in either P-selectin+/−/apoE−/− mice (Figure 3C) with minimal intimal hyperplasia8,9 or in pPS+/−/apoE−/− mice (Figure 3D). In apoE−/− mice, discrete expression of RANTES was also evident in neointimal cells with a smooth muscle cell phenotype (Figure 3B).

Because the targeted disruption of P-selectin prevents neointima formation in apoE−/− mice, we studied whether effects of RANTES could be a component involved in intimal hyperplasia after arterial injury, given the crucial role of P-selectin in RANTES deposition and monocyte arrest on endothelium in vitro. We used the RANTES receptor antagonist Met-RANTES12,13 and 8-73GRO-α, an antagonist for CXCR2 and murine KC. The intraperitoneal injection of these peptide antagonists for RANTES or GRO-α resulted in substantial serum levels at 1 hour (eg, 9.63±5.69 ng/mL in controls for Met-RANTES), which remained elevated at 48 hours (data not shown). As apparent by representative histopathologic sections (Figure 4A through 4C) and confirmed by histomorphometry (Figure 4D), the treatment of mice for 28 days after wire-induced injury by daily intraperitoneal injections of Met-RANTES but not 8-73GRO-α reduced neointimal plaque area in carotid arteries of apoE−/− mice by almost 40% (P<0.05). Accordingly, the intima/media ratio was decreased by 30% (P<0.05) with Met-RANTES but not with 8-73GRO-α (Figure 4E).

Given the role of platelet RANTES in monocyte recruitment on activated endothelium, we evaluated whether the inhibition of neointima formation by Met-RANTES was associated with reduced macrophage infiltration. Immunohistochemistry revealed a sparse infiltration with F4/80-positive monocytes/macrophages in Met-RANTES–treated mice, as compared with the pronounced infiltration in vehicle-treated or 8-73GRO-α–treated apoE−/− mice (Figure 5A). To obtain an accurate and cumulative measure of macrophage infiltration during the course of neointima formation, we determined the area of foam cells/lipid deposits by morphometry. Consistent with effects on plaque area, treatment with Met-RANTES but not with 8-73GRO-α inhibited macrophage infiltration into the site of arterial injury by 40% (Figure 5B), supporting a role of inflammation in neointima formation and invoking a mechanism underlying protective effects of Met-RANTES.
Discussion

Our data demonstrate that systemic blockade of RANTES receptors inhibits neointima formation and macrophage infiltration in carotid arteries of apoE<sup>−/−</sup> mice after wire-induced injury. The deposition of RANTES by platelets and its subsequent function in triggering monocyte arrest on activated endothelium required platelet P-selectin. The inhibition of RANTES deposition may constitute an important component explaining the profound and well-documented effects of P-selectin deficiency on neointima formation.<sup>8,9</sup>

RANTES secreted by stimulated platelets can be immobilized and presented on activated endothelium, where it enhances monocyte recruitment in flow.<sup>13</sup> Using platelets submaximally stimulated with TRAP, we confirmed that the endothelial deposition of RANTES by platelets was more effective in flow than in stasis or with supernatants. This suggests that flow-dependent interactions of platelets with endothelium may facilitate deposition of RANTES. Both P-selectin and its glycoprotein ligand PSGL-1 are expressed by stimulated platelets and have been implicated in mediating their rolling interactions on endothelium.<sup>22,23</sup> Using blocking mAbs and murine P-selectin–deficient platelets, we were able to dissect that platelet P-selectin but not PSGL-1 was critical for the endothelial deposition of RANTES as a prerequisite for its arrest function. Whereas endothelial but not platelet P-selectin has been involved in platelet rolling in vivo,<sup>22</sup> platelet-endothelial interactions critical for RANTES deposition appear to require platelet rather than endothelial P-selectin. This parallels another in vitro study showing that the adhesive interaction of platelets with endothelium not stimulated to upregulate P-selectin is mediated by platelet P-selectin.<sup>24</sup> Our data suggest that by allowing sufficient contact with HMVEC in flow, P-selectin–dependent interactions of platelets contribute to the deposition of RANTES on endothelium. This may be achieved by its secretion from granules in close proximity to endothelium or by the transfer of platelet microparticles containing RANTES,<sup>25</sup> a hypothesis that is currently under investigation. Consistent with our data, similar mechanisms may explain an involvement of platelet but not endothelial P-selectin in neutrophil-mediated postischemic renal failure in which neutrophil-attracting chemokines may be deposited by platelets.<sup>26</sup>

To confirm the in vivo relevance of P-selectin for RANTES deposition by platelets, we performed immunohistochemistry to detect RANTES immobilized on endothelium covering neointimal lesions in apoE<sup>−/−</sup> carotid arteries after wire-induced injury. Given that RANTES was not detectable on the endothelium of injured arteries in P-selectin<sup>−/−</sup> or P<sup>S</sup>/apoE<sup>−/−</sup> mice, our results infer that efficient RANTES deposition in vivo is dependent on the presence of platelet P-selectin. By providing evidence for a mechanism underlying RANTES deposition, this expands our findings that RANTES is expressed in juxtaposition to microthrombotic material on the endothelial surface covering early atherosclerotic and neointimal lesions in carotid arteries of apoE<sup>−/−</sup> mice.<sup>13</sup> Together with observations that the absence of P-selectin prevents neointima formation,<sup>8,9</sup> these results underscore the concept of using RANTES blockade in an effort to limit neointimal growth after arterial injury.

The inhibitory effect of Met-RANTES on intimal hyperplasia and macrophage infiltration supports a growing body of evidence that has associated attenuated lesion formation in models of vascular injury with an inhibition of inflammation.<sup>8,9,27</sup> The blockade of RANTES has been described as a powerful tool to suppress monocyte recruitment on different endothelial cell types and in apoE<sup>−/−</sup> carotid arteries.<sup>13</sup> Hence, the reduction of neointima formation reported herein may be due to an inhibition of macrophage infiltration and perpetuating effects on the influx of monocytes or smooth muscle.

Figure 4. The RANTES receptor antagonist Met-RANTES inhibits wire-induced neointimal growth in apoE<sup>−/−</sup> mice. A through C, Representative sections of carotid arteries of apoE<sup>−/−</sup> mice stained with Movat pentachrome 4 weeks after wire-induced injury and daily treatment with vehicle (A), the peptide antagonist Met-RANTES (B), or the K<sub>2</sub>G receptor antagonist 8-73GRO-α (C). Scale bar, 100 μm. D and E, Morphometric analysis of neointima area (D) and intima/media (I/M) ratio (E) were performed using digitized images. Data are mean±SEM from 6 to 8 animals. *<i>P</i>&lt;0.05 vs control.
cells via secretion of growth factors or chemokines. For instance, platelet-derived RANTES can induce the synthesis of chemokines by monocytes/macrophages in cooperation with engagement of PSGL-1 by platelet P-selectin,[10] a function that is likely to be inhibited by Met-RANTES. More pronounced effects of P-selectin deficiency with an absence of intimal hyperplasia and macrophage infiltration have been ascribed to a lack of RANTES. Given that adherent platelets support leukocyte arrest mediated by platelet-activating factor but not that by RANTES, whereas RANTES immobilized on endothelium triggers monocyte arrest,[4,13,14] the involvement of RANTES and its blockade may not be maximally effective during the early response to injury but rather at later stages of monocyte recruitment, eg, during reendothelialization.

Our results emphasize the involvement of RANTES in intimal hyperplasia and monocyte recruitment after arterial injury in mice with nascent atherosclerotic lesions. As the neointimal lesions develop on top of an atherogenic background, this model may be suitable to improve our insights into postinterventional restenosis in humans with obstructive atherosclerosis. The morphology of these accelerated lesions resembles spontaneous atherosclerotic plaques observed after months in apoE−/− mice. Although RANTES, GRO-α, and their receptors have all been implicated in triggering monocyte arrest on atherosclerotic endothelium in uninjured carotid arteries of apoE−/− mice,[13,21] Met-RANTES but not 8-73GRO-α at the same dose reduced injury-induced intimal hyperplasia and concomitant macrophage infiltration. This difference may be due to insufficient concentrations of 8-73GRO-α or to a variable participation of chemokines in native atherosclerosis versus accelerated lesion formation after vascular injury. Because RANTES but not GRO-α can trigger both arrest and transmigration on endothelium in flow,[13,14] this infers a more sensitive or universal involvement of RANTES in completing macrophage recruitment and supports distinct mechanistic scenarios in spontaneous versus postinjury lesion formation.

Conclusions

Our findings may have important implications for an improved understanding of the role of inflammation and potential therapeutic targets in the treatment of atherosclerotic vascular disease. Although a major contribution of adhesion molecules and chemokines to neointima formation after arterial injury has been shown using targeted disruption of the respective genes or blocking mAbs,[6,9,27] our study demonstrates for the first time that the systemic administration of a specific peptide antagonist against a chemokine receptor may be efficient in attenuating neointimal growth. In combination with local stent-based treatment regimens, this may open novel avenues for a molecular strategy to limit neointimal growth.

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