Expression of Platelet-Activating Factor Receptor in Human Carotid Atherosclerotic Plaques
Relevance to Progression of Atherosclerosis

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Background—Human monocyte-derived macrophages synthesize numerous proinflammatory and prothrombotic substances, including lipid mediators, such as platelet-activating factor (PAF), which may play a major role in the onset and perpetuation of atherosclerotic lesions. In addition, both monocytes and macrophages express PAF receptors (PAF-R). The expression of PAF-R is transcriptionally downregulated by oxidized LDL in vitro in primary cultures of monocyte/macrophages. In this study, we evaluated the expression of PAF-R in human carotid plaque tissue, in foam cells isolated from human carotid plaques, and in primary cultures of umbilical smooth muscle cells (SMCs).

Methods and Results—We show that PAF-R was expressed at low levels in foam cells compared with monocyte/macrophages in plaques, as assessed by immunohistochemical staining and in situ hybridization. In addition, low levels of mRNA were also detected by RT-PCR in isolated human carotid foam cells. A prominent finding of our study was the demonstration that contractile SMCs were positive for PAF-R, and its mRNA was extracted from primary cultures of umbilical SMCs.

Conclusions—As macrophages lose their inflammatory phenotype on transformation into foam cells, they may equally lose their capacity of defense against aggression. We postulate that the diminished expression of PAF-R may be deleterious in the context of plaque formation and progression. The observation that arterial SMCs of contractile phenotype express PAF-R opens new avenues concerning the migration of these cells from media to intima and atherosclerotic plaque formation. (Circulation. 2000;102:2569-2575.)

Key Words: atherosclerosis • macrophages • muscle, smooth • cells • lipoproteins
are involved in the development of atherosclerosis (see review14). The treatment of macrophages with oxLDL in vitro is accompanied by a marked decrease in both mRNA expression and PAF-R binding.13 The question thus arose as to whether PAF-R was expressed and eventually modulated in human arteries and in atherosclerotic lesions. For this purpose, we determined, by use of immunohistochemical staining and in situ hybridization, the expression of PAF-R in macrophages and macrophage-derived foam cells in human carotid atherosclerotic plaques in regions containing necrotic and fibrous areas. In addition, we report here for the first time that PAF-R is expressed in arterial SMCs in the media.

Methods
Sample Collection and Tissue Sections
Full-thickness resection of common carotid artery were collected from patients undergoing surgery in the Department of Vascular Surgery at Pitie-Salpetriere Hospital, Paris, France. After resection, the fresh specimens were macroscopically assessed for atherosclerotic lesions and carefully dissected; only specimens without calcifications were cross-sectioned into 2-mm segments, fixed in 10% buffered neutral formalin, processed to paraffin blocks, and stored at room temperature. Serial sections 3 μm thick fixed on slides were stained with hematoxylin, eosin, and saffron.

Antibodies
For immunohistochemistry, the following monoclonal antibodies were used: anti-CD68 (KP-1 clone, Dako) at 1:500 dilution for macrophage identification; anti-α-smooth muscle actin (1A4 clone, Sigma), anti-desmin (D33 clone, Dako), and anti-myosin (smooth, HSM-V clone, Sigma) at 1:1000, 1:100, and 1:300 dilution, respectively, for SMC identification; anti-PAF-R15 at 1:200 to 1:500 dilution and mouse IgG1 (Dako) at 1:10 to 1:500 dilution as negative control; and polyclonal antibody anti-CD3 (Dako) at 1:50 dilution for lymphocyte identification.

Immunohistochemistry
Deparaffinized and rehydrated sections after microwave treatment (2×5 minutes at 900 W in citrate buffer) were incubated for 30 minutes at room temperature with primary antibodies, washed, and incubated for 30 minutes with a Multilink kit (Biosys) for polyclonal antibodies and ABC Vector kit (Biosys) for monoclonal antibodies. After washing, the alkaline phosphatase/anti–alkaline phosphatase complexes (Dako) were added. Fast Red TR substrate system (Dako), gave a red precipitate on positive cells. Slides were counterstained with aqueous hematoxylin and mounted with Immumount (Shandon). Negative controls were obtained by replacing primary antibodies by either mouse IgG1 or an irrelevant antibody.

Isolation and Culture of Human Monocyte-Derived Macrophages
Mononuclear cells were isolated from the blood of healthy, normolipidemic volunteers as described.2 Cells were plated at a density of...
3×10⁶ per well into 6-well Primaria culture dishes (Becton Dickin-
son) in RPMI medium containing gentamicin (40 mg/mL), glutamine
(0.05%), and human serum (10%). At day 12 of culture, monocytes
were differentiated into macrophages and were free of lymphocytes
as assessed by negative anti-CD3 staining and scanning electron
microscopy (data not shown). Total cellular RNA was isolated with
RNA Plus (Bioprobe).

**Isolation of Macrophages and SMCs From Human Arteries**

Human arteries with atherosclerotic lesions, obtained on carotid
endarterectomy, were immediately placed into Hanks’ balanced salt
solution and were digested with collagenase. Macrophages were
isolated by use of monoclonal antibodies and magnetic
microspheres.16

Primary cultures of human arterial SMCs from the inner media of
human uterine arteries were established by explant technique. 17 Total
cellular RNA was isolated by SNAP isolation kit (Invitrogen).

**cRNA Synthesis**

A 2-kb fragment of human PAF-R cDNA in pBluescript (a gift from
Dr. Shimizu, Tokyo, Japan) was used for antisense or sense cRNA
probes13 labeled with digoxigenin-UTP (Boehringer). Before use, the
dig-cRNA probes were diluted and denatured at 100°C for 5 minutes.

**In Situ Hybridization**

Before hybridization, the sections were dewaxed and treated for 15
minutes with 0.4% pepsin/0.04 mol/L HCl, washed, and treated with
0.1 mol/L EDTA for 45 minutes at room temperature. Some of
the slides were pretreated with DNase 1 U/µL at 37°C for 15 minutes
or with RNase (20 µg/mL RNase A + 1 U RNAase H + 10 U
RNase 1) for 60 minutes at 65°C; pretreatments were blocked with
0.1 mol/L EDTA (5 minutes), and slides were washed and dehy-
drated in 100% ethanol.18,19 Before labeling, sections were rehydrated
and prehybridized in hybridization buffer with formamide (1:1; Amersham). The hybridizations were started by adding digoxigenin-
UTP–labeled riboprobe (30 µL per slide, 1:10 to 1:30) and were
incubated overnight at 65°C in a humidified box. Washes were
performed with 2×SSC with 50% formamide at 65°C for 60 minutes
and with decreasing concentrations of SSC up to 0.1×SSC for 30
minutes at room temperature. After 3 washes in TBS containing
0.1% Tween 20, pH 7.5, sections were treated with 1% anti-
digoxigenin alkaline phosphatase goat serum for 90 minutes at room
temperature. Specific labeling was revealed with 4.5 µL/mL tetra-
zolium nitro blue chloride/3.5 µL/mL 5-bromo-4-chloro-3-indolyl
phosphate in 0.1 mol/L Tris buffer (pH 9.5) containing 0.1 mol/L
NaCl, 0.05 mol/L MgCl₂, and 0.1% Tween 20 for 1 to 24 hours in the
dark. Reaction was stopped by washing in the same buffer; air-dried
slides were mounted with Eukitt.

**Reverse Transcription–Polymerase Chain Reaction**

First-strand cDNA was obtained with total RNA (0.12 to 5 µg), and
PCR was performed in the presence of specific oligonucleotides for
β-actin, PAF-R (L1/C1, H1/C1), as in Reference 13. The RT-PCR
products were analyzed by fractionation of 10-mL aliquots on a 2%
agarose/TAE gel. Control samples analyzed in the absence of reverse
transcriptase were free of genomic DNA.

**Results**

The aim of this study was to evaluate, by in situ hybridization
and immunohistochemical staining, the expression pattern of
PAF-R in vascular wall, including expression in macrophages
of atherosclerotic plaques, and to determine whether changes

[Figure 3. Detection of PAF-R mRNA by RT-PCR in macro-
phages isolated from human carotid plaques. Total RNA (120
ng) was used for RT-PCR procedures. After RT, PAF-R cDNA
fragment was amplified by a 35-cycle PCR with primer set cor-
responding to promoter 1 of PAF-R. PCR products were visual-
ized by ethidium bromide staining after agarose gel electro-
phoresis. M indicates 100-bp Promega size markers; lanes 1
and 3, negative control for PCR; and lanes 2 and 4, 2 different
preparations of mRNA isolated from human plaque macrophages.]

[Figure 4. Expression of PAF-R in SMCs in media of human
artery (magnification ×40). Sections of carotid arteries were
stained with anti-α-actin (A), anti-desmin (B), anti-myosin (C),
anti-PAF-R (D), and mouse IgG1 for negative control (E).]
in this expression in monocyte/macrophages versus foam cells could predict the progression of atherosclerosis.

For this purpose, full-thickness resections of common carotid arteries were obtained from surgical intervention and were characterized as intact vessels composed of well-defined intima, media, and adventitia layers. Atherosclerotic plaques were localized in both intima and media and were composed of a necrotic core adjacent to cholesterol cleft, covered by a fibrous cap. Selected specimens showed neither plaque rupture nor thrombus formation (Figure 1A).

Macrophages and foam cells were dispersed or grouped within the fibrous cap, the base and the shoulder region of the plaque, and the necrotic core. The specimens showed different stages of plaque progression. Some of them were fibrous, others were rich in foam cells in areas adjacent to necrotic core. Monocytes were observed in the lumen and adherent to endothelial cells. The presence of monocyte/macrophages and macrophage-derived foam cells was confirmed by immunohistochemical staining with monoclonal antibodies against CD68 (Figure 1A). Microscopic and transmission electron microscopic observations showed that monocytes and macrophages did not contain lipid droplets in their cytoplasm, in contrast to foam cells, which were much bigger and contained such droplets (data not shown).

Monocytes located in the lumen and adjacent to endothelial cells were strongly stained with the monoclonal antibodies directed against PAF-R (Figure 1B). Small macrophages in the subendothelium and within the atherosclerotic plaques expressed more diffuse and heterogeneous cytoplasmic staining. In contrast, the macrophage-derived foam cells found in plaque and necrotic areas were only weakly stained or even negative (Figure 1B). These results confirm our former in vitro observation that the expression of PAF-R is downregulated in monocyte/macrophage primary cultures after treatment with oxLDL. Similar results were observed in specimens from 5 different patients.

The expression of PAF-R mRNA was visualized by in situ hybridization using cRNA labeled with digoxigenin coupled to UTP. The optimal conditions for in situ hybridization have been established for both PAF-R and scavenger receptors, the latter serving as a positive control. Scavenger receptor labeling showed a strong nuclear staining in macrophages and foam cells (data not shown).

The specific labeling of nuclei of macrophages and foam cells was obtained with antisense riboprobe for PAF-R in the presence of DNase (Figure 2A). The negative controls performed with the sense RNA probe or the antisense RNA probe with RNase treatment resulted in no labeling (Figure 2B). Similar results were obtained with monocyte-derived macrophages in culture (data not shown).

Finally, the presence of PAF-R expression in CD14-positive macrophages isolated from human plaques was confirmed by RT-PCR. Strong signals corresponding to PAF-R mRNA expression are shown in specimens obtained from 2 different patients (Figure 3). The primers used for amplification of cDNA corresponded to the promoter 1 of PAF-R, which is characteristic for macrophages.

SMCs located in the media were strongly stained with the specific myosin, desmin, and α-actin SMC antibodies (Figure 4A, 4B, and 4C) and were equally positively stained with monoclonal antibodies against PAF-R (Figure 4D). The signal was located in the cytoplasm of SMCs, and the extracellular matrix was negative. In contrast, the cells of SMC origin present in the plaque were solely positive for α-actin and did not express PAF-R (Figure 5). Such cells corresponded to dedifferentiated SMCs, because they contained neither myosin nor desmin.

These results were further confirmed by in situ hybridization with the antisense cRNA probe of PAF-R (Figure 6A). Several SMCs in the media were stained, and the pattern of positive cells was comparable to the immunostaining performed with anti–PAF-R antibodies (Figure 4C); again, the dedifferentiated SMCs in plaque areas were negative (Figure 6B).

To confirm the presence of PAF-R mRNA in SMCs, we have extracted total mRNA from primary cultures of human arterial SMCs isolated from the inner media of human uterine arteries. We show in Figure 7 the presence of PAF-R mRNA both in human SMCs and in monocyte-derived macrophages, as visualized by RT-PCR. Because SMCs contained more β-actin mRNA than did macrophages, we deduced that they expressed less PAF-R mRNA.

**Discussion**

Because atherosclerosis is an inflammatory disease,1 we evaluated the cellular expression of PAF-R, which is the target not only for one of the most potent proinflammatory mediators, PAF, but also for its analogues generated on nonenzymatic oxidation of phosphatidylcholine.2 Thus, we show for the first time that PAF-R is expressed not only in monocyte/macrophages and foam cells but also in SMCs of contractile phenotype of the human carotid artery. We have
shown that PAF-R is expressed in primary cultures of human monocyte/macrophages and is diminished, at the transcriptional level, by incubation of macrophages with oxLDL. In the present study, we extended our in vitro observation into an in vivo pathological situation of atherosclerotic lesion formation in human carotid arteries. We show that the macrophage/foam cells expressed less PAF-R antigen than did monocyte/macrophages. As pointed out earlier, the decrease in PAF-R expression in macrophages induced by oxLDL may be relevant to the motility of macrophages and foam cells in the lesions. The reduction of PAF-R numbers at the surface of foam cells may impair motility and the capacity to respond to extracellular signals. In addition, the ability of oxLDL to diminish gene expression has been documented and led to the hypothesis that the suppression of an acute inflammatory response may be implicated in a state of chronic, low-level inflammation.

Recent studies indicated increased levels of nuclear factors of the peroxisome proliferator–activated receptor (PPAR) family in arterial cells in atherosclerotic plaques. Because they transactivate not only genes bearing the PPRE response elements, they may equally inhibit several inflammatory genes containing nuclear factor (NF)-κB and STAT motifs by protein-protein interactions. Because the PAF-R promoter contains 3 NF-κB–binding elements, it is potentially a good target for the PPAR-mediated suppression of transcription. Our recent unpublished data indicate that PPAR-α is readily involved in such PAF-R gene repression (Hourton et al, under revision in Biochem J). In recent studies, PAF-R gene expression was shown to be transcriptionally modulated by cytokines, and TNF-α was found to activate PAF-R transcription via NF-κB.

Our present observation that the arterial SMCs of contractile phenotype in human carotid arteries are positive for PAF-R suggests that the expression of PAF-R may be important in their proliferation and migration from arterial media into intima; such migration has been described in the development of human atherosclerotic lesions (see review). Thus, neointimal dedifferentiated SMCs promote plaque formation, because they actively participate in extracellular matrix excretion and deposition of calcium. Two recent theories explaining the SMC heterogeneity and different proliferative, migrating, and matrix-producing capabilities are based on (1) the clonal reversion of the adult medial SMCs to an immature phenotype, expressing genes characteristic of a synthetic/proliferative SMC, or (2) the existence of different lineage on embryonic development. In this context, the acquisition and/or expression of PAF-R by SMCs could be a decisive step in their migratory and proliferative potential in the intimal space. Indeed, a recent study showed that mechanical stress induces PAF-R expression in aortic SMCs. We anticipate that in atherosclerosis, the inflammatory reaction in the intima of vessels will favor PAF formation by macrophages and foam cells and equally the generation of PAF and its analogues from oxidized lipoproteins. The diffusion of PAF and/or its active analogues into the media may thus allow the PAF-R–bearing SMCs to be attracted into the intimal space, where they become PAF-R–negative and loose their contractile phenotype.

Figure 6. In situ hybridization staining of PAF-R mRNA in SMCs in media (A) and in plaque (B) (magnification ×40). Sections were hybridized as described in Methods with antisense (A and B) or sense cRNA probe for control (C).

Figure 7. Detection of PAF-R and β-actin mRNA by RT-PCR in cultures of SMCs isolated from human umbilical vein. Total RNA (1 μg) was used for RT-PCR. After reverse transcription, PAF-R cDNA fragment and β-actin were amplified by a 30-cycle PCR with primer set corresponding to promoter 1 of PAF-R (190 bp) and β-actin (838 bp). PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis. A, mRNA isolated from monocyte–derived macrophages; B, mRNA isolated from SMCs.
Only few in situ studies have been reported on PAF-R expression in vessels, and they were mainly related to PAF-induced increases in vascular permeability, showing a widespread PAF-R in microvascular beds and especially its ubiquitous presence on endothelia and in pericytes, fibroblasts, and macrophages associated with microvessels. None of the latter studies have explored the pattern of PAF-R expression in human atherosclerotic lesions. Earlier work using polyclonal anti-PAF-R antibodies showed the presence of PAF-R in human monocytes, neutrophils, B cells, and differentiated myeloid cell lines. Recently, mice lacking PAF-R were generated and showed a marked reduction in systemic anaphylactic symptoms but remained sensitive to endothoxic shock. This model should be helpful to further elucidate the potential role of PAF-R in atherosclerosis.

In conclusion, our data indicate that the in vivo expression of PAF-R in vascular wall is readily diminished in macrophage-derived foam cells of atherosclerotic plaques compared with monocyte/macrophages located in healthy areas. The carotid arteries of all patients studied contained a significant amount of PAF-R–positive SMCs in the media; however, the dedifferentiated SMCs in the plaque areas were negative, as assessed by both immunohistochemical and in situ hybridization detection. The latter finding may be of importance in relation to SMC migration on atherogenesis. We anticipate that the PAF-R expression may be useful as a marker for arterial wall disease, and it may become a valid potential target for intervention.

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