Phagocytosis and Macrophage Activation Associated With Hemorrhagic Microvessels in Human Atherosclerosis

Mark M. Kockx, Kristel M. Cromheeke, Michiel W.M. Knaapen, Johan M. Bosmans, Guido R.Y. De Meyer, Arnold G. Herman, Hidde Bult

Objective—Previously, we demonstrated that activated inducible NO synthase (iNOS)-expressing foam cells in human carotid plaques often produce autofluorescent (per)oxidized lipids (ceroid). Here, we investigate whether intraplaque microvessels can provide foam cells with lipids and trigger macrophage activation.

Methods and Results—Microvessels (vWf immunoreactivity), activated macrophages (iNOS immunoreactivity), and ceroid were systematically mapped in longitudinal sections of 15 human carotid endarterectomy specimens. An unbiased hierarchical cluster analysis classified vascular regions into 2 categories. One type with normal vWf expression and without inflammatory cells was seen, and another type with cuboidal endothelial cells, perivascular vWf deposits, and iNOS and ceroid-containing foam cells was seen in 4 (27%) of 15 plaques. The perivascular foam cells frequently contained platelets (glycoprotein Ibα) and erythrocytes (hemoglobinugm, iron), pointing to microhemorrhage/thrombosis and subsequent phagocytosis. Similar lipid-containing cells, expressing both ceroid and iNOS, were generated in atherosclerosis-free settings by incubating murine J774 macrophages with platelets or oxidized erythrocytes and also in vivo in organizing thrombi in normocholesterolemic rabbits.

Conclusions—Focal intraplaque microhemorrhages initiate platelet and erythrocyte phagocytosis, leading to iron deposition, macrophage activation, ceroid production, and foam cell formation. Neovascularization, besides supplying plaques with leukocytes and lipoproteins, can thus promote focal plaque expansion when microvessels become thrombotic or rupture prone. (Arterioscler Thromb Vasc Biol. 2003;23:440-446.)

Key Words: atherosclerosis ■ microvessels ■ inducible NO synthase ■ hemorrhage ■ erythrocytes

Intermittent growth is a characteristic of human atherosclerosis. This could be the consequence of recurrent rupture of the fibrous cap followed by thrombus organization into the plaque. Other studies have suggested a causative role of hemorrhages of intraplaque microvessels in carotid plaque rupture. Paterson et al proposed the vascularization theory of plaque evolution by demonstrating hemosiderin deposition in early atheromatous plaques, and they related this to repeated intraplaque capillary rupture, but it remains unclear how hemorrhages contribute to lipid accumulation. Ceroid is one of these lipid components and consists of insoluble mixtures of oxidized lipids and proteins, which mark sites of previous oxidative events. Macrophages play a central role in the production of this fluorescent pigment, and extracellular ceroid deposits ultimately accumulate in the necrotic core of the plaque. Furthermore, the upregulation of inducible NO synthase (iNOS), a major ancillary pathway of host defense by activated macrophages, is a characteristic feature of foam cell–rich plaque regions. Recently, we showed that iNOS, which is predominantly expressed in macrophages, often colocalizes with ceroid or platelet-derived amyloid β in advanced human plaques. Because the reasons

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for these associations are unclear, we investigated the role of microvessels in plaque progression. To this end, the distribution of microvessels, ceroid and iNOS (as a marker of macrophage activation), was systematically mapped in human carotid artery plaques. The expression of von Willebrand factor (vWf) in the endothelial cells of intraplaque microvessels is highly variable, ranging from undetectable to thick perivascular deposits. The latter are due to increased vWf biosynthesis during atherogenesis. Therefore, vWf was used as a marker of endothelial cell activation. For an unbiased identification of topographical associations, the results were subjected to a cluster analysis. Finally, the formation of iNOS-expressing ceroid-containing foam cells was demonstrated in normocholesterolemic settings on erythropagocytosis by macrophages in experimental thrombi in rabbit carotid arteries and in murine J774 macrophages in culture.

Methods

The ethics committees of Middelheim Hospital and Antwerp University approved the studies.
Human Atherosclerosis
Carotid artery endarterectomy specimens were obtained from 15 patients (stenosis >70% [digital subtraction angiography and duplex ultrasound], mean age 66 years), cut along their longitudinal axis, and fixed in 4% formaldehyde within 5 minutes after collection. Decalcification was performed in Bouin’s fixative (15 vol saturated picric acid, 5 vol of 40% formaldehyde, and 1 vol acetic acid) for 48 hours. Whole-mount longitudinal sections of paraffin-embedded specimens were mounted on 3-aminopropyltriethoxysilane-coated slides.11

Experimental Thrombosis in Rabbit Carotid Arteries
Male New Zealand White rabbits (n=38, weight 2.5 to 3.5 kg, normal diet) were anesthetized (sodium pentobarbital, 30 mg/kg IV). The right carotid artery was selected to induce thrombus by repeated inflation of an oversized (2.5-mm) angioplasty balloon.15 The uninjured contralateral artery served as the control. One animal was investigated immediately. The others were anesthetized again at 7 (n=6), 14 (n=13), or 21 (n=18) days after angioplasty, and both carotid arteries were removed and fixed in 60% methanol, 30% 1,1,1-trichloroethane, and 10% glacial acetic acid (Methacarn). Transversal sections of paraffin-embedded specimens were mounted on aminopropyltriethoxysilane-coated slides.

Immunohistochemistry
The following primary antibodies were used: rabbit polyclonal anti-human vWF (1:500, Dako), iNOS (1:1000, Biomol Research Laboratories), heme oxygenase-1 (1:100, Transduction Laboratories), hemoglobin (1:100, Lipshaw Immunon), and monoclonal anti-human glycoprotein Ib (GPIb; clone G28E5; 1:3000, gift from Dr M.F. Hoylaerts, Center for Molecular and Vascular Biology, KUL, Leuven, Belgium) for human tissue and polyclonal sheep anti-human vWF (1:200, The Binding Site), monoclonal anti-human iNOS (1:25, Transduction Laboratories), and anti-rabbit macrophage RAM-11 (1:100, Dako) for rabbit tissue.

After inactivation of endogenous peroxidase (3% H2O2), monoclonal antibodies were detected with goat anti-mouse peroxidase antibody (Jackson); sheep polyclonal antibodies, with rabbit anti-sheep peroxidase (Jackson); and rabbit polyclonal antibodies, with the polyclonal Envision System (Dako). For demonstration of the antibody (Jackson); sheep polyclonal antibodies, with rabbit anti-human iNOS (1:100, Lipshaw Immunon), and monoclonal anti-human glycophorin Ibo (GPIbs, clone G28E5; 1:3000, gift from Dr M.F. Hoylaerts, Center for Molecular and Vascular Biology, KUL, Leuven, Belgium) for human tissue and polyclonal sheep anti-human vWF (1:200, The Binding Site), monoclonal anti-human iNOS (1:25, Transduction Laboratories), and anti-rabbit macrophage RAM-11 (1:100, Dako) for rabbit tissue.

Iron Deposition
To detect iron deposits, Perls’ stain was used. To study colocalization, this was performed on the iNOS-immunostained slide by using an unbiased counting frame with a set of regularly spaced points.16 The points hitting immunoreactive structures were counted and expressed as percentage of the points in the intima.

Cell Culture
Murine macrophages (5×10^6/800 μL, cell line J774A.1, American Type Culture Collection) were grown in RPMI and allowed to adhere in culture slides (Becton Dickinson Labware) at 37°C in 5% CO2,95% air as described.13 Thereafter, macrophages were incubated for 41 hours in DMEM with or without 10^5 washed human platelets13 or 4×10^10 oxidized (CuSO4+H2O2) erythrocytes (ox-RBCs).12 The cell-free supernatant was stored at −20°C for nitrite measurements (Griess reaction) as an index of iNOS activity.12 The cells were fixed with paraformaldehyde (1%, 2 minutes) followed by methanol (−20°C, 6 minutes), air-dried, and stained with oil red O.

Statistical Analysis
Results are given as mean±SEM. For an unbiased classification, all ROIs were subjected to a hierarchical cluster analysis (procedure K-means cluster, SPSS release 10, SPSS Inc). Vessel density, the presence of subendothelial vWF deposits, and INOS density were used as input variables after z transformations to obtain equal scaling (mean 0, SD 1). A minimum size of 10 ROIs was used as the criterion to select the number of categories. Differences among means were evaluated with an ANOVA (Bonferroni post hoc test); correlations were determined by the Spearman test. A value of P<0.05 was considered significant.

Results
Human Atherosclerosis
Each section showed American Heart Association (AHA) stages I to V.1 Complicated plaques (AHA VI) were present in 14 specimens, often at the dorsal wall of the carotid sinus.
Thrombi were noted in 4 specimens (Table). All specimens contained iNOS-expressing macrophages (positive ROIs 7% to 50%, mean density 1.2% to 16.0%); ceroid was detected in 13 specimens (positive ROIs 2% to 52%, mean density 0.4% to 18.2%); and microvessels were detected in 14 specimens (positive ROIs 14% to 76%, mean density 2.1 to 25.8 vessels/mm²). Mean densities of iNOS and ceroid were not affected by sex, the maximum AHA score, or thrombus (P > 0.05). However, vascular density was higher in specimens with thrombus (16±5 microvessels/mm², n=4) than in specimens without thrombus (6±1 microvessels/mm², n=11, P = 0.027 by ANOVA). There was a strong positive correlation between the mean vascular density and iNOS expression in plaques (R_{Spearman} = 0.64, P = 0.01).

The distribution of microvessels, intracellular ceroid, and iNOS-expressing macrophages was mapped for each plaque; Figure 1 shows an example. Microvessels were present in fibrous regions, often close to the media, or in foam cell–rich areas in the proximal shoulder. In the former vessels, vWF was either undetectable or present in flat endothelial cells; macrophages expressing iNOS or ceroid were rare. In contrast, microvessels in foam cell–rich regions often contained thick perivascular vWF deposits and were frequently associated with iNOS-expressing ceroid-containing macrophages. Finally, avascular clusters of iNOS-expressing foam cells frequently surrounded the necrotic core, commonly in association with ceroid.

These qualitative observations were substantiated by a cluster analysis of all ROIs (n=592). This unbiased assessment identified 4 relatively homogeneous groups (Figure 2). Category A was found in all sections and contained 413 ROIs in which microvessels and iNOS were rare or absent (Figures 2 and 3A). Category B consisted of 69 ROIs in 14 sections (93%) with a high microvascular density but few iNOS-expressing cells or perivascular vWF deposits (Figures 2 and 3B). Category C contained 49 ROIs in 4 sections (27%) with high vascular densities, cuboidal endothelial cells, subendothelial vWF deposits, and macrophages containing iNOS, ceroid, and lipid droplets (Figures 2 and 3C). Finally, category D consisted of 61 ROIs in all plaques with many iNOS-containing macrophages but few microvessels. Ceroid was more abundant in categories C and D compared with categories A and B (Figure 2). Adding ceroid to the cluster analysis produced a very similar classification (results not shown).

The third indication of the association between activated macrophages and microvessels was obtained by selecting ROIs containing microvessels, irrespective of their density. Macrophages expressing iNOS were more abundant in ROIs with perivascular vWF deposits (mean density 0.21±0.04, high...
Figure 3. Most fibrous regions of human plaques were devoid of vessels (A); others contained microvessels (mv) with intracellular vWF in flat endothelial cells (B). Microvessels in foam cell–rich regions often showed increased endothelial vWF expression and perivascular vWF deposition (C). Other foam cell–rich regions with ceroid-containing macrophages (*) were devoid of microvessels (D). Immunostaining for vWF is shown (A through D). In perivascular foam cells (category C), phagocytosis of platelets (immunostaining for GPlbα, E) and erythrocytes (immunostaining for hemoglobin, F) was demonstrated. Several perivascular macrophages showed expression of heme oxygenase-1 (G) and iNOS (H, red-brown; pink nuclei, neutral red, arrowhead), which often colocalized (arrow) with iron (blue-green, double staining). Bar=20 μm.

Figure 4. Progressive macrophage infiltration (RAM-11), iNOS expression, iron deposition, and neovascularization during thrombus organization. Results are expressed as positive area within tissue circumcised by the internal elastic lamina. Values are mean±SEM (n=6 to 18).

Colocalization Study
Because we were interested in associations of microvessels, activated macrophages, and ceroid, the regions (classified in category C) were studied in greater detail. These microvessels were found not only between the plaque shoulder and necrotic core (Figure 1) but also in the fibrous cap at sites of plaque rupture. Macrophages around microvessels with cuboidal endothelial cells and perivascular vWF deposits often showed signs of platelet and erythrophagocytosis. The platelet marker GPIbα was detected as a cytoplasmic granular stain, and hemoglobin, heme oxygenase-1, and iron deposits were noted in the macrophages (Figure 3E through 3H). In these regions, iNOS and heme oxygenase-1 were demonstrated as granular staining patterns in the cytoplasm. Finally, a strong colocalization of iron deposits and iNOS expression in the macrophages was found (Figure 3H). The presence of erythrocytes, iron deposits, and hemoglobin in plaque regions with microvessels points to microhemorrhages of these microvessels.

Macrophage Activation and Ceroid Formation in Experimental Thrombus
To prove that erythrophagocytosis evokes macrophage activation and foam cell formation in an atherosclerosis-free setting, mural thrombi were induced in the rabbit carotid artery.15 Neither macrophages, iNOS, neovessels, nor iron was found immediately after thrombus formation or in uninjured vessels (not shown). Seven days after injury, scattered RAM-11 immunoreactive macrophages were present between the erythrocytes, and they increased until week 3 (Figure 4). A significant fraction of these macrophages expressed iNOS. From day 14, microvessels appeared, and the macrophages formed multinucleated giant cell foam cells and showed iron deposits, which increased until week 3. At that time, autofluorescent ceroid was clearly visible in the macrophages (please see online Figure I, available at http://www.atvb.ahajournals.org). Strong positive correlations (P<0.001) existed between RAM-11 and iron deposition (R_{Spearman}=0.68), iNOS and iron deposition (R_{Spearman}=0.73), microvessels and RAM-11 (R_{Spearman}=0.70), and microvessels and iNOS (R_{Spearman}=0.62).

Macrophage Activation and Ceroid Formation In Vitro
J774 macrophages transformed into foam cells containing cytoplasmic oil red O–positive lipid droplets on incubation with ox-RBCs or platelets for 41 hours (Figure 5). Fluorescent ceroid pigment was seen in macrophages exposed to ox-RBCs; this became more evident 2 days later (not shown). Increased nitrite concentrations were produced by macrophages incubated with ox-RBCs (2.0±0.3 μmol/L, control 0.2±0.1 μmol/L, n=4, P=0.027) or platelets (0.8±0.3 μmol/L, control 0.4±0.2 μmol/L, n=4, P=0.029 by paired t tests).

Discussion
This quantitative study of human carotid artery plaques demonstrated (1) a highly significant association between the overall densities of microvessels and activated iNOS-expressing macrophages; (2) that macrophages were particularly abundant around a subset of microvessels with activated endothelial cells, as indicated by perivascular vWF deposits; (3) that in those regions, phagocytosis of erythrocytes and
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of endarterectomy specimens, presumably because a much
more cases (93%) than in triple-stained cross sections (60%) were
omitted. This could predispose to underestimation of vWf-
classification is also debated, an unbiased hierarchical clus-
ter analysis was used to categorize the data. This algorithm
takes not only the presence or absence but also the abundance
developments in each variable into account and classifies the microvessels
into 2 categories. One category (B, present in all but 1 plaque) was
lined by flat endothelial cells without vWf deposits and contained few perivascular macrophages. Those microvessels
were present in fibrous regions and at the base of the plaque. The other category (C) was lined by cuboidal endothelial
cells showing increased expression and perivascular deposits of vWf. Ceroid-containing foam cells of macrophage origin,
which were activated as demonstrated by their iNOS expres-
sion, surrounded the microvessels of the second category. Those microvessels were detected in 27% of the specimens,
but their incidence may increase when more sections per specimen are analyzed.

By use of different approaches, a higher microvessel
density has been demonstrated in lipid-rich plaques compared
with fibrous plaques, and microvessels in shoulder regions
are often associated with mast cells and T cells as well. Furthermore, endothelial cells in lipid-rich plaques express
increased levels of intercellular adhesion molecule-1, vascular
adhesion molecule-1, and E-selectin. Those findings indicate that microvessels in lipid-rich plaques are important
in the continuous recruitment of macrophages in atheroscle-
rotic plaques. In this concept, the recruited macrophages
transform into foam cells as a consequence of uptake of plasma-derived LDL.

The present study suggests an additional mechanism of
foam cell formation. First, we demonstrated immunoreactiv-
ity of the platelet marker GPIbα, as well as hemoglobin,
heme oxygenase-1, and iron, in the cytoplasm of macro-
phages around microvessels in certain lipid-rich plaque re-
gions, pointing to microhemorrhages and subsequent phago-
cytosis of platelets and erythrocytes. Furthermore, microvessels, located around GPIbα-immunoreactive macro-
phages, often contained platelet-rich microthrombi. Eryth-
rophagocytosis and processing of hemoglobin lead to the
accumulation of heme and iron in the macrophage. This could
explain the expression of heme oxygenase-1 in these macro-
phages, inasmuch as heme is a potent inducer of heme
oxygenase-1. Heme oxygenase-1 protects the macrophages
against oxidative stress and cell death by regulating cellular
iron. Interestingly, iron and ceroid colocalization has been
demonstrated in human plaques. Moreover, erythrophagocy-
tosis increases the capacity of the macrophage to oxidize
LDL, and iron promotes ceroid formation. Collectively,
these data suggest that hemorrhages occur in certain foam
cell–rich areas and that macrophages surrounding those
microvessels transform into activated foam cells producing
ceroid pigment and iron as a result of repeated phagocytosis of platelets and erythrocytes.

It is unclear whether quiescent microvessels in fibrous
regions (category B) will eventually become fragile and prone to rupture because of endothelial cell activation
and recruitment of inflammatory cells. Ongoing inflammation,
matrix-degrading metalloproteinases released from macro-
phages, or proteases secreted by accumulating mast cells
could invoke proteolytic damage to the microvessels and
facilitate intraplaque microhemorrhages. Previously, we
reported that foam cell accumulation is associated with
smooth muscle cell death. Therefore, it would be interesting
to investigate whether apoptosis of vascular cells leads to the
transition of the foam cell–rich vascular regions (category C)
to avascular areas (category D) around the necrotic core.
Furthermore, erythrophagocytosis could also promote expan-
sion of the necrotic core, inasmuch as hemoglobin-derived iron and phagocyte-generated oxidants collaborate to cause macrophage dysfunction.

Because human plaques already contain plasma-derived lipoproteins, further studies were performed in normcholesterolemic rabbits and with macrophages in culture to prove that the lipids in perivascular foam cells can originate from phagocytosed membranes. The in vivo experiment confirmed the formation of macrophage-derived foam cells in organizing thrombi after platelet or erythrocyte phagocytosis in the absence of elevated LDL levels.

To exclude the possibility that increased oxidation of LDL after erythropagocytosis might explain the accumulation of lipids and ceroid during thrombus organization, murine J774 macrophages were incubated with human blood platelets or ox-RBCs. Platelet phagocytosis evoked foam cell formation, confirming older reports, and macrophage activation, as indicated by the iNOS metabolite nitrite. Recently, we reported that platelet phagocytosis also stimulates tumor necrosis factor-α and cyclooxygenase-2 expression, that priming with interferon-γ drastically raises J774 activation, and that the activation occurs in human macrophages as well. In the present study, we show that macrophages after uptake of ox-RBCs, presumably via scavenger receptors, (1) transform to foam cells as well, (2) become activated, as indicated by the iNOS activity, and (3) produce autofluorescent pigments with characteristics of ceroid in foam cells around microvessels in human plaques in spite of the serum-free environment. Thus, both experiments directly support the hypothesis that lipids and ceroid in foam cells around microvessels could be derived from platelets and erythrocytes. Previously, we demonstrated that ceroid and iNOS expression are frequently associated in macrophages in human plaques. The present study could explain this phenomenon because we now show that erythropagocytosis, similar to platelet phagocytosis, stimulates iNOS expression and because iron is known to promote lipid peroxidation and ceroid formation. The function of iNOS expression is not clear, but possible roles in atherogenesis and thrombus vascularization have to be considered because plaque progression and angiogenesis during wound healing are reduced in iNOS-deficient mice.

In summary, the cluster analysis provided an unbiased confirmation of the abundance of activated microvessels in certain lipid-rich atherosclerotic plaques reported by others, and their findings were extended by demonstrating platelet and erythrocyte phagocytosis by the perivascular foam cells. Moreover, these foam cells were activated (as indicated by their iNOS expression) and produced autofluorescent pigments that were compatible with ceroid. During thrombus organization and also in vitro, the same foam cells, ceroid formation, and iNOS upregulation could be induced in macrophages by platelet or erythropagocytosis in atherosclerosis-free settings. Therefore, we conclude that in some plaques, microhemorrhages or thrombi initiate the phagocytosis of platelets and erythrocytes. This not only explains iron deposition, but it also invokes nonimmune macrophage activation, foam cell formation, and ceroid production. These focal processes may contribute to plaque expansion and may eventually promote plaque vulnerability.

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Kockx: iNOS expressing macrophages and microhemorrhages

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**Figure Legends:**

**Figure I.** Transversal sections of an organizing thrombus (3 weeks) in the carotid artery of a normocholesterolemic rabbit showing microvessels (vWF stain A,C) and a dense macrophage infiltration (RAM-11 stain, B). The macrophages contain ceroid pigment (C*) and express iNOS (D). Bar=20 µm