

Immaturity of microvessels in hemorrhagic plaques is associated with proteolytic degradation of angiogenic factors

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Abstract

Aims: We investigated the causes of microvessel immaturity and destabilization in human atherosclerotic lesions.

Methods: Human atherosclerotic carotid plaques (n=24) were classified as non-hemorrhagic (NH) or hemorrhagic (HEM), according to their macroscopic aspect and hemoglobin content. Plaque microvessel density and maturity were quantified by immunohistochemistry. Expression of angiogenic factors was studied by immunohistochemistry, *in situ* hybridization and ELISA. Plaque-conditioned media were tested for plasmin and elastase activities, and for their ability to degrade angiogenic factors and to induce smooth muscle cell migration.

Results: Microvessel density and leukocyte infiltration were increased in HEM compared to NH plaques. Plaque vasculature appeared vulnerable as indicated by the absence of α -actin-positive mural cells in most plaque vessels. Despite increased numbers of angiogenic factor-expressing microvessels and leukocytes in HEM plaques, lower levels of vascular endothelial growth factor, placental growth factor and angiopoietin-1 were found in conditioned media from HEM plaques. However, NH and HEM plaques released similar levels of the vascular destabilizing factor, angiopoietin-2. Addition of recombinant angiogenic factors to plaque extracts showed that all factors but angiopoietin-2 were selectively degraded by plasmin and/or elastase released from HEM plaques. Furthermore, conditioned media from HEM plaques showed a reduced ability to induce smooth muscle cell migration.

Conclusions: Our results provide evidence that immaturity of plaque vessels is associated with degradation of angiogenic factors by hemorrhage-conveyed leukocytes and proteases.

Introduction

Earlier¹ and more recent biochemical² and functional imaging³ data have shown that intraplaque hemorrhages are the main link between plaque progression and the clinical expression of human atherothrombotic disease. Microvessel density increases with plaque complexity⁴ and both unstable and ruptured plaques display higher microvessel density than stable and non-ruptured plaques⁵⁻⁷. Neo-vessels in plaques express leukocyte adhesion molecules⁸, appear leaky^{4,9,10} and microhemorrhages can be observed in their vicinity^{11,12}. When localized in inflammatory and hemorrhagic areas, microvessels are dysmorphic and lack surrounding α -actin-positive mural cells^{12,13}. All these studies suggest that microvessel development and immaturity is involved in intraplaque hemorrhage and leukocyte infiltration, two critical determinants for plaque progression.

We proposed that intramural hematoma/thrombus could be the main source of proteases, including serine proteases, in vulnerable plaques^{14,15} and we demonstrated that the more complicated the plaque, the richer it is in serine proteases¹⁶. In earlier studies we demonstrated *in vitro*¹⁷ and *in vivo*¹⁸ that serine proteases are capable of limiting cell colonization of the proteolytic-rich hematoma/thrombus and thus, of inhibiting lesion repair.

Neo-vessel development and maturation require angiogenic factors, among which the components of the vascular endothelial growth factor (VEGF) and angiopoietin systems have been extensively studied. The VEGF system is involved at different steps of angiogenesis (vessel permeabilization, endothelial cell proliferation, migration and survival, and formation of neo-capillaries), whereas angiopoietins appear to be involved in vessel stabilization/destabilization. Atherosclerotic vascular walls show increased levels of angiogenic factors and receptors, whose expression is associated with macrophages, neo-vessels and smooth muscle cells^{19,20}. Similarly, angiopoietin-1 (ang-1) / -2 (ang-2) and their

receptor Tie-2 are expressed by the atherosclerotic wall, with a reported overexpression of angiopoietin-2 and Tie-2²¹⁻²³.

The relationship between hemorrhage and immaturity of plaque microvessels in human atherothrombosis remains unclear. Sluimer *et al.* recently showed by electron microscopy that endothelial junctions were incomplete in intraplaque neocapillaries¹⁰. Here, we show that immaturity of plaque microvessels in hemorrhagic plaques is associated with degradation of angiogenic factors by plaque proteases. Proteolytic degradation of angiogenic factors within hemorrhagic plaques is likely to create an environment hostile to maturation of neovessels that impairs mural cell recruitment and potentially participates to leukocyte infiltration and further plaque destabilization.

Materials and Methods

Tissue samples

Human carotid endarterectomy samples (n = 24) and healthy mammary arteries (n = 10) were obtained as surgical waste in the absence of patient opposition, in accordance with the French ethical laws (L. 1211-2 to L. 1211-7, L. 1235-2 and L. 1245.2) and following ethics committee advice (CPP Paris Ile de France XI no 07043, Centre Hospitalier de Poissy/Saint-Germain-en-Laye). The investigation conforms with the principles outlined in the Declaration of Helsinki.

Human carotid endarterectomy samples were dissected into culprit (CP, the stenosing segment causing the symptoms which led to surgery) and adjacent non-complicated areas (NP), as previously described^{14,16}.

Healthy mammary arteries were dissected to separate the adventitia from the remaining arterial segment. Each sample from mammary and carotid arteries was cut into small pieces (5 mm³) and separately incubated in RPMI-1640 medium (Gibco) for 24 h at 37°C (6 mL/g of wet tissue)²⁴. The tissue-culture media containing released material were then collected and stored at -80°C until use.

Hemoglobin/Heme determination

The presence of hemorrhage in CP and NP was determined by macroscopical observation and measurement of hemoglobin/heme concentration by addition of formic acid to the tissue-culture media^{14,25}. Heme content was then determined by reading the absorbance at 405 nm²⁵.

Histology and immunohistochemistry

Tissues were fixed in 3.7% paraformaldehyde, embedded in paraffin and sectioned at 5 µm. Sections were stained with Perl's Prussian blue for hemosiderin and with Masson's trichrome

for fibrous tissue and general morphology. Immunohistochemistry was performed using mouse monoclonal antibodies to CD31 (clone JC70A, Dako), α -smooth muscle actin (clone 1A4, Dako), CD68 (clone PG-M1, Dako) and CD66b (clone 80H3, Immunotech), and rabbit polyclonal IgG to PIGF (Relia Tech), and goat polyclonal IgG to VEGF (R&D systems), angiopoietin-1 (R&D systems), and angiopoietin-2 (R&D systems). A peroxidase Dako LSAB2+ kit was used for detection. Control irrelevant antibodies (Dako) were applied to assess non-specific staining. 3,3'-diaminobenzidine (Dako) or histogreen (Histoprime) were used as peroxidase substrates. Sections were counterstained with Mayer's hematoxylin or with nuclear fast red.

Microvessel density in the shoulder and cap regions of plaques was quantified by counting CD31-positive vessels on plaque sections (3 fields per plaque from 8 carotid samples per group at x 40 magnification). The percentage of pericyte-covered vessels in the vascular hot spots was calculated from the number of alpha-smooth muscle actin-positive vessels over the total number of CD31-vessels in adjacent serial sections.

Quantification of macrophage and neutrophil infiltration in culprit atherosclerotic plaques was estimated by counting the number of CD68- and CD66b-positive cells in plaque sections (3-5 fields per section from 5 carotid samples per group at x 100 magnification).

***In situ* hybridization**

cDNAs were used for the generation of ^{35}S -RNA probes for VEGF, VEGFR-1, VEGFR-2, angiopoietin-1, angiopoietin-2 and Tie-2²⁶. Sections received 50 μl of hybridization mixture containing 5×10^5 cpm of ^{35}S -UTP-labelled riboprobe and were hybridized at 50°C overnight. Slides were exposed for 4 days on X-ray film (BioMax MR, Kodak) to evaluate the signal intensity and then dipped in NTB2 liquid emulsion (Kodak). The slides were exposed for 4 weeks, developed, fixed, stained with nuclear fast red, and mounted in Eukitt.

ELISA tests

Concentrations of VEGF, PlGF, angiopoietin-1, angiopoietin-2 and soluble Tie-2 in plaque-conditioned media were determined by ELISA kits (R&D Systems), following the manufacturer's instructions.

Plasmin and leukocyte elastase activities

Tissues (~20 mg) were incubated in 0.05 M HEPES buffer pH 7.4, 0.75 M NaCl, 0.05% NP₄₀ with 40 μM of the synthetic plasmin substrate, MeOSuc-Ala-Phe-Lys-AMC (Bachem) or the leukocyte elastase substrate, MeOSuc-Ala-Ala-Pro-Val-AMC (Calbiochem). Substrate hydrolysis was monitored for 2 h by spectrofluorometry (Hitachi F-2000; $\lambda_{\text{Exc}} = 380 \text{ nm}$; $\lambda_{\text{Em}} = 460 \text{ nm}$).

Proteolysis of angiogenic mediators

Recombinant PlGF (100 ng, Relia Tech), VEGF (100 ng, R&D Systems), angiopoietin-1 (200 ng, Relia Tech) and angiopoietin-2 (200 ng, Relia Tech) were incubated for 3 hours at 37°C in 0.05 M HEPES buffer pH 7.4, 0.75 M NaCl, 0.05% NP₄₀ with plasmin (33 nM, American Diagnostica) or leukocyte elastase (33 nM, Calbiochem). *Ex vivo* proteolytic degradation was performed by incubating recombinant angiogenic mediators (VEGF and PlGF : 250 ng; Ang-1/-2 : 500 ng) with CP (n = 8) or NP (n = 8) samples for 3 days at 37°C with or without 66 μM of plasmin inhibitor (H-D-Val-Phe-Lys-CMK, Calbiochem) or leukocyte elastase inhibitor (MeOSuc-Ala-Ala-Pro-Val-CMK, Calbiochem).

Immunoblot

Samples were transferred to nitrocellulose membranes (Pierce) after resolution by 12% SDS–PAGE. Membranes were probed with anti-PIGF (Relia Tech), anti-VEGF (R&D systems), anti-angiopoietin-1 (Zymed Laboratories) and anti-angiopoietin-2 (Zymed Laboratories) and with peroxidase-conjugated secondary antibody (Jackson ImmunoResearch).

MTT assay

Adhesion and survival of vascular smooth muscle cells in response to plaque-conditioned media was assessed by the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Confluent human aortic smooth muscle cells seeded in 48-well plates were incubated for 22 hours at 37°C with 400 μ L of serum-free SM2 medium (Promocell) supplemented with 200 μ L of plaque-conditioned media or RPMI-1640 medium. Non-adherent cells were then eliminated by two washes in PBS and residual viable adherent cells were incubated for 3 hours with 0.5 mg/mL of MTT (Sigma). MTT was then removed, the formazan crystals produced by the reaction dissolved in DMSO, and the absorbance was read at 540 nm.

Cell migration assay

Cell migration was assessed using a modified cell dispersion assay²⁷. Human aortic smooth muscle cells (5×10^5) (Promocell) were seeded inside a 3-mm glass ring placed in the middle of a collagen-coated coverslip in 24-well plates. Four hours after plating, the glass ring was removed and the cells were covered with serum-free SM2 medium (Promocell), containing or not tissue-culture medium from atheromatous carotid samples, for 22 hours at 37°C in a humidified atmosphere. After staining cells with hematoxylin, cell-covered areas were quantified by morphometric analysis using Histolab software (Microvision Instruments, Evry,

France). In this model, cells migrate as an outgrowth from the confluent area initially delimited by the ring (13.25 mm²). Cell migration is expressed as the difference between the surfaces covered by cells in the stimulated versus unstimulated control conditions.

Statistical analysis

Data represent means \pm SEM and were analyzed by the Wilcoxon paired non-parametric test or the Mann-Whitney non-parametric test, when appropriate. Statistical significance was accepted for $p < 0.05$.

Results

Hemorrhagic plaques are characterized by a high density of alpha-smooth muscle actin-negative microvessels

Human carotid endarterectomy samples were dissected into culprit plaques (CP) and adjacent non-complicated areas (NP). All CP used in this study displayed a non-ruptured fibrous cap upon macroscopic observation (Fig. 1A). Among 24 endarterectomy samples, 11 CP were non-hemorrhagic and 13 CP presented macroscopically visible intraplaque hemorrhage and significantly higher levels of hemoglobin as compared to other samples, while all NP were non-hemorrhagic (Fig. 1B).

Recent and old intraplaque hemorrhages were identified by the presence of extravasated red blood cells and by positive Prussian blue staining, which reveals hemosiderin, a degradation product of phagocytosed hemoglobin²⁸ (Fig. 1C). In accordance with the classification established by Stary *et al.*²⁹, histologic analysis revealed that non-hemorrhagic samples corresponded to early atherosclerotic lesions (type II and III) and fibrous or calcified lesions of type IV and V. Hemorrhagic plaques included non-ruptured type V lesions with old and/or recent microhemorrhages, and non-ruptured type VIb lesions with large intraplaque hematoma (Fig. 1C).

Microvessel density was increased in hemorrhagic plaques as compared to non-hemorrhagic plaques (Fig. 2A and B). Plaque microvessels predominated in the shoulder region of the plaque, at the interface between the core, the cap and the media (Fig 2A). It is noteworthy that no difference in microvessel density was found between hemorrhagic plaques with and without hematoma (11.82 ± 6.84 versus 12.57 ± 5.99 microvessels/mm², $p = 0.971$, $n=6$). The majority of microvessels in hemorrhagic plaques lacked alpha-smooth muscle actin-positive cells in their wall (Fig. 2A and C). This reduced mural cell coverage of hemorrhagic plaque microvessels indicated that these vessels were structurally immature and fragile. These results

suggest a link between increased neo-vascularization combined with defective blood vessel maturation and intraplaque hemorrhage.

The intraplaque angiogenic balance is altered in hemorrhagic plaques

Microvessel growth and maturation are regulated by angiogenic factors. Differences in microvessel density and maturity between hemorrhagic and non-hemorrhagic plaques may depend on different intraplaque angiogenic balances. Positive immunostaining for VEGF, PlGF, angiopoietin-1 and angiopoietin-2 was detected in both hemorrhagic and non-hemorrhagic plaques (Fig. 3A). Staining for angiogenic factors within plaques was mostly associated with microvessels, their surrounding smooth muscle cells, and was also present in their vicinity, where CD68-macrophages and CD66b-neutrophils were observed (Fig. 3A and C). The expression of angiogenic factors and of their receptors by vessels and inflammatory cells within non-hemorrhagic and hemorrhagic plaques was further confirmed by *in situ* hybridization (Fig. 3B). Taken together, these results show that angiogenic factors and their receptors are expressed in non-hemorrhagic and hemorrhagic culprit plaques, and that endothelial cells and leukocytes are the main cell source of angiogenic factors in plaques. Interestingly, in parallel to their increased microvessel density (Fig. 2A and C), hemorrhagic plaques showed several-fold higher numbers of infiltrating macrophages and neutrophils than non-hemorrhagic plaques (Fig. 3C and D).

Since microvessels and leukocytes are sources of angiogenic factors (Fig. 3A), their content may be increased in hemorrhagic plaques. Quantification of angiogenic factors released into tissue-culture media from healthy mammary arteries and atheromatous carotids revealed that levels of VEGF, PlGF and angiopoietin-1 were significantly increased in atheromatous carotid samples (Fig. 4A-C). Furthermore, levels of PlGF, VEGF and soluble Tie-2 increased with plaque complexity in non-hemorrhagic lesions, as revealed by the higher levels measured

in culprit plaques compared to non-complicated ones (Fig. 4A-C, E). Surprisingly, levels of PlGF, VEGF and angiopoietin-1 were significantly decreased in culprit hemorrhagic plaques when compared to non-hemorrhagic culprit plaques (Fig. 4A-C). In contrast, levels of soluble Tie-2 were increased in hemorrhagic culprit plaques while angiopoietin-2 levels were similar in culprit and non-complicated parts of both hemorrhagic and non-hemorrhagic lesions (Fig. 4D). Thus, pro-angiogenic factor levels are decreased in hemorrhagic culprit plaques while their angiopoietin-2 levels remain stable. This shows that intraplaque hemorrhage is associated with angiogenic imbalance in favor of the vascular destabilizing factor, angiopoietin-2.

Angiogenic factors are degraded by plasmin and leukocyte elastase in hemorrhagic plaques

The reduced levels of angiogenic factors in hemorrhagic plaques may be due to decreased release and/or to degradation of these factors by hemorrhage-associated factors. VEGF has heparin-binding properties and can be retained in the ECM, which may result in apparently lower levels in plaque-conditioned media. However, the addition of heparinase-III (1U/mL) during the preparation of plaque-conditioned media did not affect VEGF levels in non-hemorrhagic or in hemorrhagic plaques (not shown). This suggests that the decreased level of VEGF released by hemorrhagic plaques was not due to increased trapping of VEGF within the ECM.

Consistent with our previous report^{14,16}, a significant increase in plasmin activity was detected in hemorrhagic plaques as compared to non-hemorrhagic and non-complicated plaques (Fig 5A). Leukocyte elastase activity was also significantly elevated in hemorrhagic culprit lesions¹⁴ (Fig 5B). Both proteases (33 nM) degraded *in vitro* VEGF, PlGF and angiopoietin-1 and 2, as evidenced by the presence of proteolytic products with lower molecular weights compared

to the native proteins or by the disappearance of immunoreactive species in western immunoblot after treatment of recombinant growth factor by plasmin and leukocyte elastase (Fig 5C).

To determine whether the decreased levels of angiogenic factors in conditioned media from hemorrhagic plaques originate from the enrichment of these lesions in proteases, recombinant angiogenic factors were incubated with tissue samples and then analyzed by immunoblotting (Fig 5D). No degradation was observed when recombinant factors were incubated with the non-complicated adjacent segments (NP) of culprit plaques. When incubated with non-hemorrhagic culprit plaques (CP/NH), only a slight degradation was observed for VEGF and angiopoietin-1. In contrast, almost complete disappearance of immunoreactive species was obtained when VEGF, PlGF and angiopoietin-1 were incubated with hemorrhagic culprit plaques (CP/Hem), whereas angiopoietin-2 appeared to be degraded to a lesser extent (Fig. 5D and E). Proteolysis of VEGF, PlGF and angiopoietin-1 and -2 was partially prevented by the addition of specific plasmin and elastase inhibitors (Fig 5E). These results suggest that degradation of angiogenic factors by plasmin and leukocyte elastase may account for the angiogenic imbalance of hemorrhagic culprit plaques. However, the partial character of this inhibition suggests that proteases others than elastase and plasmin are also involved in the degradation of growth factors in hemorrhagic plaques.

Hemorrhagic plaques have a reduced capacity to induce smooth muscle cell migration

Decreased levels of growth factors may affect mural cell recruitment and survival leading to immaturity of neovessels and subsequent bleeding. For this reason, we compared the effect of tissue-culture media, conditioned by non-hemorrhagic and hemorrhagic plaques, on vascular smooth muscle cell spreading, migration and survival.

Measurement of the area covered by the migrated smooth muscle cells in response to plaque extracts showed that both non-complicated and culprit plaques were able to induce migration of smooth muscle cells (Fig. 6A and B). Interestingly, while non-complicated and non-hemorrhagic culprit plaques displayed a similar ability to induce smooth muscle cell migration, the presence of hemorrhage in culprit plaques decreased this ability (Fig. 6A and B). Differences in the area covered by smooth muscle cells in response to plaque-conditioned media may also result from detachment and cell death of smooth muscle cells. We thus investigated the effect of plaque-conditioned media on the adhesion and survival of smooth muscle cells. Incubation of confluent vascular smooth muscle cell cultures with conditioned media from non-hemorrhagic or hemorrhagic plaques did not affect their adhesion and induced a similar increase in smooth muscle viability when compared to unstimulated control cells, as assessed by MTT assay (Fig. 6C). Thus, the reduced area covered by smooth muscle cells in response to hemorrhagic plaque-conditioned media was not due to decreased cell death or adhesion in these conditions. Taken together, these results show that hemorrhagic plaques have a reduced capacity to induce spreading and migration of plaque-stabilizing smooth muscle cells.

Discussion

Immaturity of plaque microvessels is a source of intraplaque hemorrhage, a critical factor in atherosclerotic plaque progression and destabilization^{13,30}. In the present study, we investigated the mechanisms interfering with plaque microvessel maturation. In agreement with recent studies^{10,13}, we show a link between hemorrhage and the growth of immature microvessels within human carotid atherosclerotic plaques. In fact, we found that hemorrhage occurred in plaques characterized by a high density of immature microvessels that lack α -actin-positive mural cells.

The extent and quality of angiogenesis depend on several factors including the expression of angiogenic factors. We show that VEGF, PlGF, ang-1, ang-2, VEGFR-2 and Tie-2 are expressed in microvessels from both hemorrhagic and non-hemorrhagic plaques. Consistent with previous reports^{19-21,23}, positive immunostaining for VEGF and PlGF was also associated with inflammatory cells. Higher levels of VEGF, PlGF and angiopoietin-1 were released by carotid plaques than by healthy mammary arteries and, among plaques, the highest concentrations were released by culprit non-hemorrhagic plaques. Altogether, these data are consistent with the reported angiogenic potential of plaques^{31,32}. It is noteworthy that, while hemorrhagic plaques were highly vascularized, non-hemorrhagic culprit plaques were less invaded by neovessels. This paradoxical result suggests that the density of microvessels in the plaque is not only defined by the levels of released angiogenic factors, but most likely also involves additional factors. For instance, it was recently shown that CD40 ligand-positive microparticles from human atherosclerotic plaques stimulate endothelial proliferation and angiogenesis³³. Moreover, angiogenesis is a complex process that requires destruction of the basement membrane and local degradation of the extracellular matrix (ECM) to allow migration and proliferation of endothelial cells and the release of ECM-bound growth factors. Thus, proteolytic activities may promote the growth of plaque microvessels and the associated

risk of hemorrhage. Supporting this hypothesis and confirming our previous observations^{14,16,34,35}, we show that hemorrhagic plaques are enriched in leukocytes, and in elastase and plasmin activities.

However, a highly proteolytic environment may also limit the scarring process and vessel maturation. Hemo-thrombus-associated protease activities were indeed shown to impede mesenchymatous cell spreading, proliferation and survival^{17,18,36}. Therefore, proteolysis of growth factors by intraplaque proteases may prevent the maturation of plaque microvessels. Our results indicate that degradation of angiogenic factors by plasmin and leukocyte elastase accounts for the decreased levels of VEGF, PlGF and ang-1, and for the increased solubilization of Tie-2 in hemorrhagic plaques compared to non-hemorrhagic plaques. It is noteworthy that plasmin and elastase inhibitors produced only a partial inhibition of growth factor degradation, suggesting that other intraplaque proteases may also be involved in this phenomenon. Indeed, it has been shown previously that hemorrhagic plaques are also enriched in MMPs and mast cell tryptase^{10,14}.

Interestingly, the levels of the vascular destabilizing protein ang-2 were similar in non-hemorrhagic and hemorrhagic plaques. Therefore, it appears that proteases alter the angiogenic balance in hemorrhagic plaques, in particular degrading ang-1 and its receptor Tie-2 while ang-2 levels remained unchanged. These modifications in the angiogenic balance of hemorrhagic plaques corroborate recent findings showing that the balance between ang-1 and ang-2 is in favor of ang-2 in atherosclerotic plaques with high microvessel density²³.

Ang-1 promotes endothelial cell barrier integrity and it is conceivable that the reduction in ang-1 levels in hemorrhagic plaques favors microvessel permeability. Furthermore, imbalance between angiopoietin-1 and angiopoietin-2 is thought to play a role in brain arteriovenous malformations³⁷, which lead to recurrent cerebral hemorrhages, and in the impairment of

pericyte recruitment and vessel maturation after myocardial ischemia in diabetic patients³⁸. We show that hemorrhagic plaques had a reduced potential to induce smooth muscle cell migration when compared to non-hemorrhagic plaques. Pericytes and periendothelial intimal smooth muscle cells share common markers such as smooth muscle actin, suggesting a common mesenchymatous origin³⁹. Proteolytic degradation of growth factors in hemorrhagic plaques may impair the spreading and recruitment of stabilizing smooth muscle cells and pericytes, and contribute to the compromised structural integrity of microvascular endothelium in atherosclerotic arteries¹⁰. Since VEGF, PlGF and angiopoietins are angiogenic factors that mainly relate to endothelial cell function, it is likely that the reduced capacity of hemorrhagic plaques to induce smooth muscle cell migration also involves degradation of other growth factors. In addition, plaque proteases may also destabilize plaque vasculature by directly damaging microvessels. Indeed, extravasation of red blood cells occurs by passage not only across the endothelium but also through the basement membrane, which implies degradation or rupture of this structure surrounding the vessels.

Of note, in this study, plaques that displayed signs of acute rupture were excluded. Thus, the presence of hemorrhage did not result from recent acute plaque rupture, fissure, or erosion but most likely from angiogenesis as it is now well established³⁰. However, a role of old plaque ruptures and thrombi in the mechanisms leading to the degradation of intraplaque growth factors and to immaturity of plaque microvessels cannot be excluded.

In conclusion, while immaturity of plaque vessels has been proposed to cause intraplaque hemorrhage^{10,30}, our results suggest that on the other hand, intraplaque hemorrhage may further destabilizes the plaque vasculature by accelerating the deposition within the lesion of leukocytes and proteases, which alter the angiogenic balance. Our findings designate proteases as potential targets for the normalization of plaque vasculature, a clinical strategy that has been proposed for plaque stabilization⁴⁰. The present study also provides new insight

into the role of intraplaque hemorrhage in creating a self-perpetuating mechanism of lesion progression and may explain the reported recurrence of intraplaque hemorrhages after a first hemorrhagic event³.

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Conflict of interest

The authors state that there are no conflicts of interest.

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Figure legends

Figure 1. Characterization of non-hemorrhagic and hemorrhagic plaques. A. Representative human atheromatous culprit plaques classified as non-hemorrhagic or hemorrhagic, according to their macroscopical aspect. **B.** Verification of the hemoglobin content in conditioned media from non-hemorrhagic (NH) and hemorrhagic (H) culprit plaques (CP), and from their adjacent non-complicated segments (NP) by a chromogenic assay. **C.** Masson's trichrome (upper panels) and Prussian blue (lower panels) staining of non-hemorrhagic and hemorrhagic culprit plaques. The presence of extravasated red blood cells and of hemosiderin, a degradation product of phagocytosed hemoglobin, allowed the localization of intraplaque hemorrhage (white arrows). Non-hemorrhagic plaques corresponded to early atherosclerotic lesions (type II and III) and fibrous lesions of type IV and V. Hemorrhagic plaques included type V lesions with old and/or recent microhemorrhages, and VIb lesions with large intraplaque hematoma. **Inset:** extravasated red blood cells (black arrows) present in the vicinity of microvessels in hemorrhagic areas. Bars = 500 μm .

Figure 2. Hemorrhagic plaques have increased microvessel density and decreased microvessel pericyte coverage. **A.** Representative photomicrographs of CD-31 and alpha-smooth muscle actin immunostaining of non-hemorrhagic and hemorrhagic plaques. Microvessels were mainly located in the shoulder and cap regions. Bar = 500 μm . Arrows indicate the position of the high magnification view of CD31-positive vessels and corresponding alpha-smooth muscle actin staining (insets). **B.** Immunohistochemical quantification of microvessel density in non-hemorrhagic and hemorrhagic culprit plaques (n = 8-12 plaques per group). **C.** Comparison of microvessel pericyte coverage between non-hemorrhagic and hemorrhagic culprit plaques. Pericyte coverage of plaque microvessels was estimated by calculating the percentage of CD31-positive vessels which were α -smooth muscle actin-positive in adjacent serial sections (n = 8-12 plaques per group).

Figure 3. Immunohistochemical localization and *in situ* hybridization analysis of components of the VEGF and angiopoietin systems. **A.** Immunohistochemical localization of angiogenic factors in non-hemorrhagic and hemorrhagic plaques. VEGF, PlGF, ang-1 and ang-2 staining was associated with microvessels and with infiltrating inflammatory cells. Arrows indicate positive immunostaining in vessels and surrounding inflammatory cells. Bar = 100 μm . **B.** *In situ* hybridization analysis of angiogenic factor expression in non-hemorrhagic and hemorrhagic plaques. mRNA of VEGF, VEGFR-2, ang-1, ang-2 and Tie-2 were found to be associated with vessels and inflammatory cells of non-hemorrhagic and hemorrhagic plaques. Bar = 50 μm . **C.** Immunostaining of CD68- and CD66b-positive cells in non-hemorrhagic and hemorrhagic culprit plaques. Bar = 100 μm . **D.** Immunohistochemical quantification of CD68- and CD66b-positive cells in sections from non-hemorrhagic and hemorrhagic plaques (n = 5 plaques per group).

Figure 4. The intraplaque content in angiogenic factors varies with plaque complexity.

Tissue-culture media from the medial layer of healthy mammary arteries (Mam), non-complicated plaques (NP) and non-hemorrhagic (NH) and hemorrhagic (Hem) culprit plaques (CP) were analyzed by ELISA for PIGF (A), VEGF (B), ang-1 (C), ang-2 (D) and soluble Tie-2 (E).

Figure 5. Plasmin and elastase enzymatic activities and proteolysis of angiogenic factors in hemorrhagic plaques

In situ plasmin (A) and elastase (B) enzymatic activities were determined using selective fluorogenic substrates in non-complicated (NP) and non-hemorrhagic (NH) and hemorrhagic (Hem) culprit plaques (CP). C-D. Western blot analysis of angiogenic factor degradation by plasmin, elastase and atherosclerotic plaques. Recombinant VEGF, PIGF, ang-1 and ang-2 were incubated with either (C) purified plasmin and leukocyte elastase or (D) tissue samples of non-complicated (NP) or culprit (CP) areas of hemorrhagic (Hem) and non-hemorrhagic plaques (NH). E. Recombinant angiogenic factors were incubated with tissue samples of culprit areas of hemorrhagic plaques in the presence or absence of a plasmin inhibitor (H-D-Val-Phe-Lys-CMK), or a leukocyte elastase inhibitor (MeOSuc-Ala-Ala-Pro-Val-CMK). Plasmin and elastase inhibitors partially prevented proteolysis by hemorrhagic culprit tissue sample. Arrows indicate the molecular weights of the recombinant growth factors.

Figure 6. The ability of atherosclerotic plaques to induce smooth muscle cell migration is reduced by intraplaque hemorrhage. A. Representative photographs of migrating smooth muscle cells stained with H&E after a 22-hour incubation with tissue culture medium from non-hemorrhagic culprit plaque (CP/NH, left panel) or hemorrhagic complicated plaque (CP/Hem, right panel). Bar, 1 mm. B. Comparison of smooth muscle cell migration among

cells stimulated with supernatant from non-complicated plaques (NP) and non-hemorrhagic (NH) and hemorrhagic (Hem) culprit plaques (CP). Results are expressed as the difference between cell-covered areas in stimulated and unstimulated control conditions. The increase in surface in the control group was 6.65 ± 0.39 mm². **C.** Effect of plaque-conditioned tissue-culture media on smooth muscle cell adhesion and survival assessed by the MTT assay.

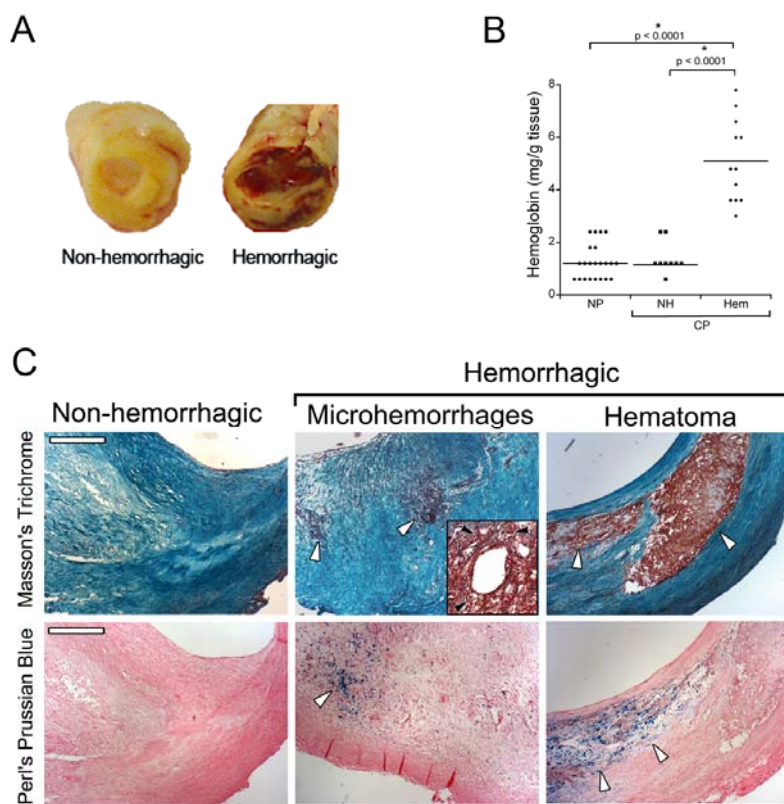


Figure 1

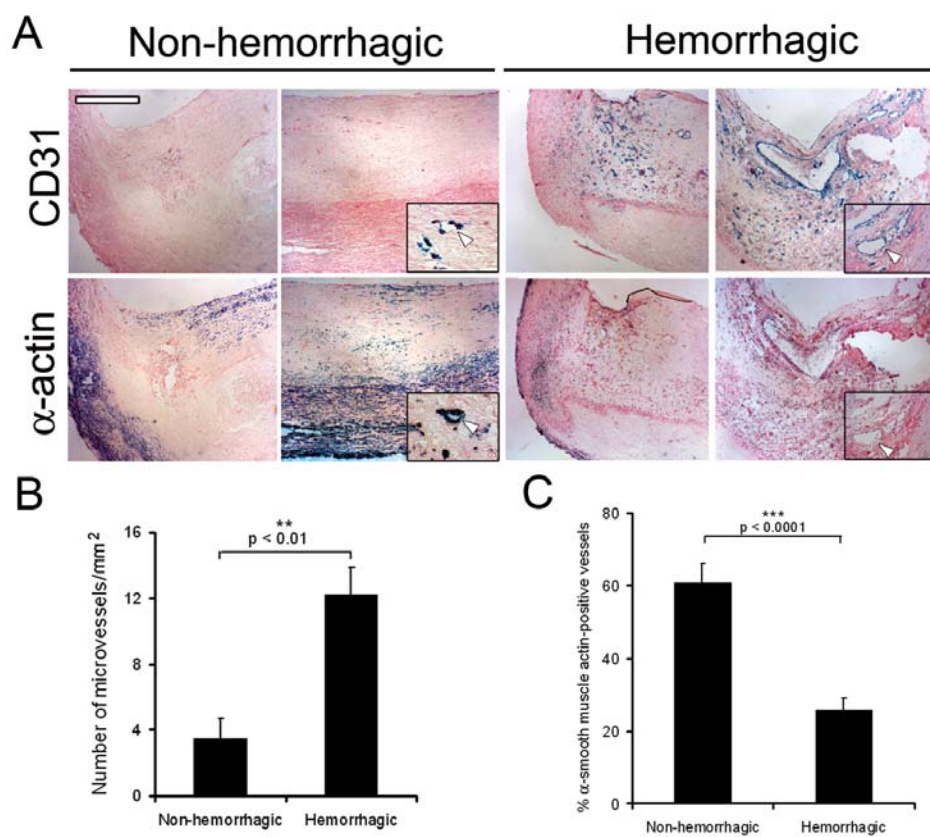


Figure 2

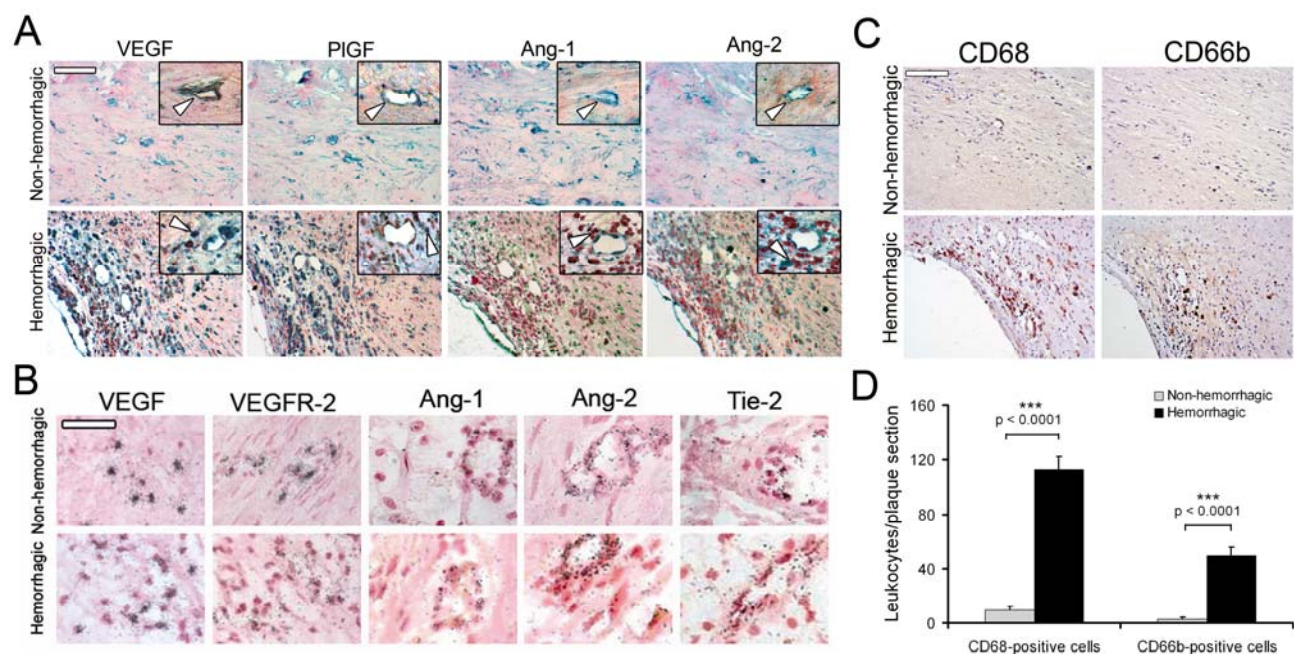


Figure 3

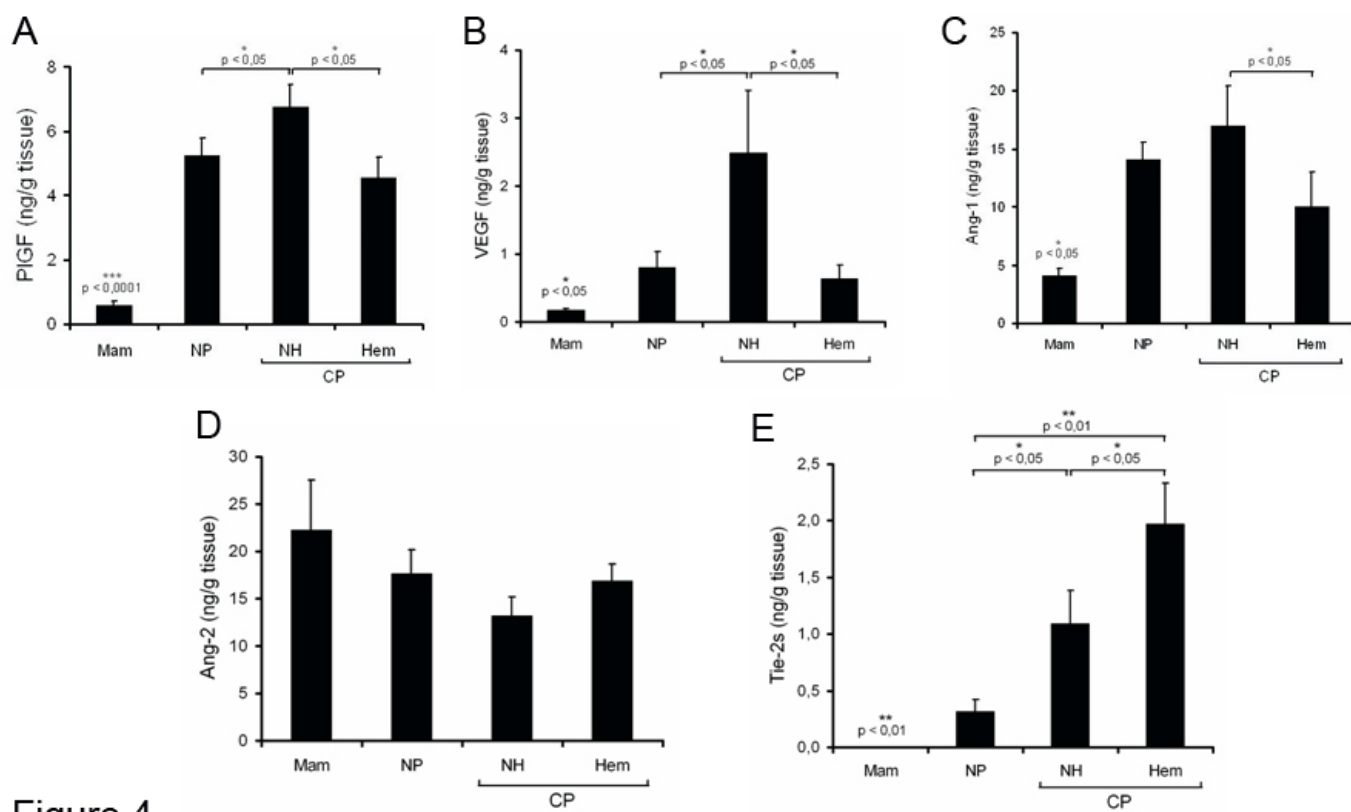


Figure 4

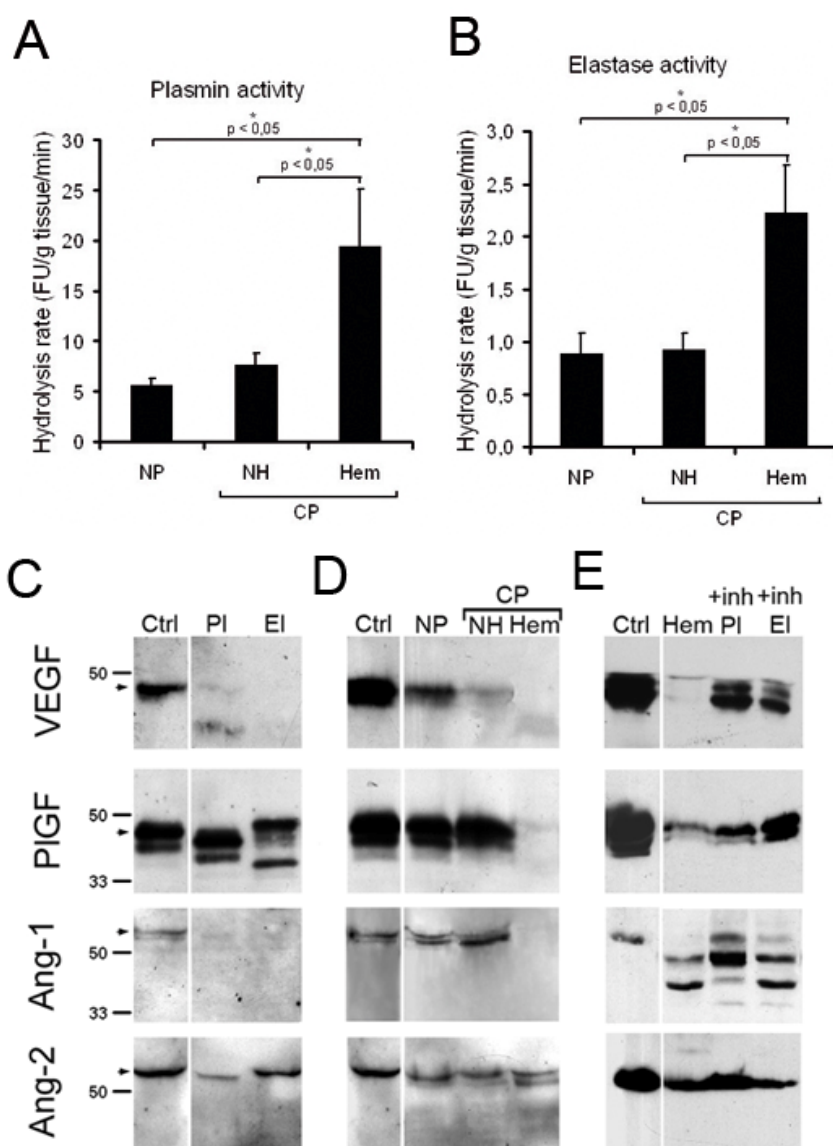


Figure 5

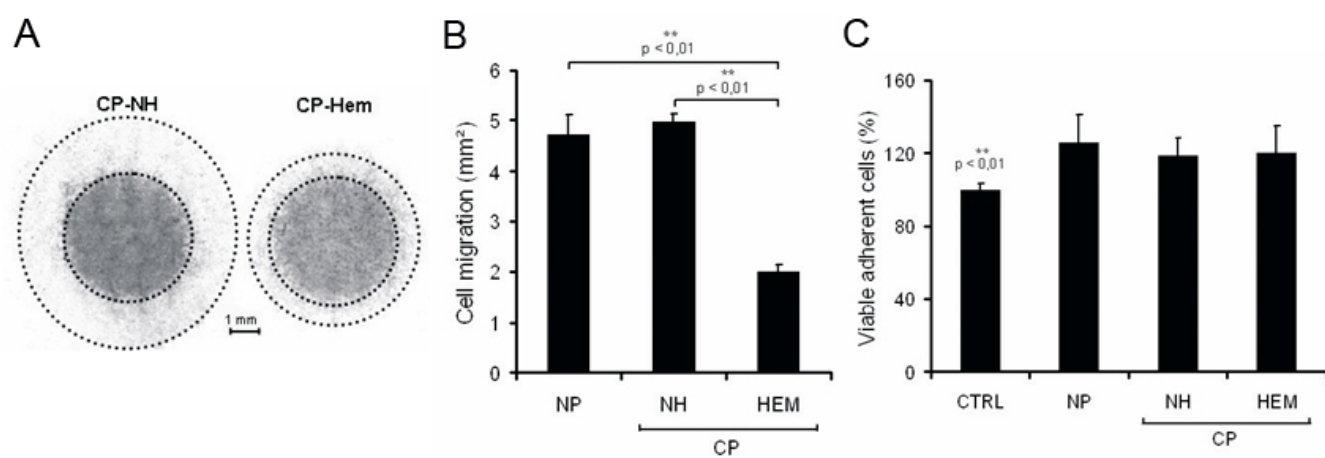


Figure 6