TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion

Thibaut Quillard1,2, Haniel Alves Araújo1, Gregory Franck1, Eugenia Shvartz1, Galina Sukhova1, and Peter Libby1*

1Division of Cardiovascular Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, USA; and 2INSERM, UMR957, Université de Nantes, Nantes Atlantique Universités, EA3822, 1 Rue Gaston Veil, Nantes 44035, France

Received 9 December 2014; revised 30 January 2015; accepted 1 February 2015; online publish-ahead-of-print 9 March 2015

Aims Superficial erosion of atheromata causes many acute coronary syndromes, but arises from unknown mechanisms. This study tested the hypothesis that Toll-like receptor-2 (TLR2) activation contributes to endothelial apoptosis and denudation and thus contributes to the pathogenesis of superficial erosion.

Methods and results Toll-like receptor-2 and neutrophils localized at sites of superficially eroded human plaques. In vitro, TLR2 ligands (including hyaluronan, a matrix macromolecule abundant in eroded lesions) induced endothelial stress, characterized by reactive oxygen species production, endoplasmic reticulum (ER) stress, and apoptosis. Co-incubation of neutrophils with endothelial cells (ECs) potentiated these effects and induced EC apoptosis and detachment. We then categorized human atherosclerotic plaques (n = 56) based on morphologic features associated with superficial erosion, ‘stable’ fibrotic, or ‘vulnerable’ lesions. Morphometric analyses of the human atheromata localized neutrophils and neutrophil extracellular traps (NETs) near clusters of apoptotic ECs in smooth muscle cell (SMC)-rich plaques. The number of luminal apoptotic ECs correlated with neutrophil accumulation, amount of NETs, and TLR2 staining in SMC-rich plaques, but not in ‘vulnerable’ atheromata.

Conclusion These in vitro observations and analyses of human plaques indicate that TLR2 stimulation followed by neutrophil participation may render smooth muscle cell-rich plaques susceptible to superficial erosion and thrombotic complications by inducing ER stress, apoptosis, and favouring detachment of EC.

Keywords Superficial erosion • Endothelial cells • TLR2 • Neutrophil • Hyaluronan • NET

Translational Perspective Many fatal myocardial infarctions result from superficial erosion of the intima without evidence of fibrous cap rupture and arise from unknown mechanisms. As statin treatment has increased in patients at risk for atherosclerotic events worldwide, and because these agents combat the causes of plaque rupture, understanding the mechanisms of superficial erosion has assumed greater importance. The new observations presented here support the role of TLR2 activation followed by granulocyte participation in the endothelial cell damage and desquamation that characterize these lesions.

Introduction Many fatal myocardial infarctions result from superficial erosion of the intima without evidence of fibrous cap rupture. Erosions associate with female sex, younger age, smoking, hyper-triglyceridemia, and diabetes. Eroded lesions exhibit particular morphological features that contrast with the well-described characteristics of ‘rupture-prone’ lesions. The intima of eroded plaques typically has...
a discontinuous endothelial layer, contains abundant smooth muscle cells and proteoglycans/glycosaminoglycans—in particular hyaluronan—acid and versican—and few macrophages. Disruption of the endothelial layer likely contributes to such acute thrombotic complications. Indeed, induction of endothelial apoptosis in vivo drives endothelial denudation and thrombus formation. Endothelial cell apoptosis in human plaques associates with oscillatory shear stress downstream of plaques, where plaque erosions tend to occur. Furthermore, after experimental production of stenosis in injured rabbit femoral arteries, mural thrombi form in the post-stenotic region that develops a smooth muscle cell-rich neointima recapitulating aspects of typical eroded lesions in humans. These studies support the contribution of EC apoptosis in atheroma exposed to disturbed blood flow.

Increased endothelial apoptosis in such regions likely results from a combination of multiple factors. In experimental atherosclerosis in mice, endothelial cells exhibit TLR2 in areas of disturbed blood flow, hyperlipidaemia increases TLR2 expression, and inhibition of this receptor reduces EC dysfunction produced by low shear stress. In humans, TLR2 protein increases substantially in the endothelium overlying atherosclerotic lesions. In lesions prone to erosion, lipoproteins and proteoglycans could serve as endogenous activators for TLR2.

Plasma from patients with eroded culprit plaques exhibit elevated myeloperoxidase (MPO), produced primarily by polymorphonuclear leukocytes (PMN), compared with those with ruptured lesions. Co-culture of PMN with endothelial cells induces endothelial injury and hypochlorous acid, a major product of MPO, can trigger endothelial cell apoptosis and generation of tissue factor.

To probe the mechanisms that underlie superficial erosion, this study tested the hypothesis that TLR2 activation triggers endothelial apoptosis and denudation and thus contributes to the acute thrombotic complications of atherosclerosis. The results demonstrate that TLR2 ligation alone suffices to promote EC production of reactive oxygen species, ER stress, and apoptosis, processes that increase markedly in presence of neutrophils. Co-incubation of EC with TLR2 agonists and with neutrophils also augmented EC desquamation in vitro. Analysis of human endarterectomy specimens showed a correlation between apoptosis of ECs with TLR2 and neutrophil presence in lesions complicated by superficial erosion, but not in plaques with characteristics considered ‘rupture-prone’. These observations support the involvement of TLR2 in superficial erosion of human atheroma, providing novel insight into the mechanism of this increasingly common cause of acute coronary syndromes.

**Methods**

For a full description of the methods used, see Supplementary material online.

**Human atheromata selection**

Our laboratory has systematically collected specimens of human carotid plaques obtained at endarterectomy (n = 295) by protocols approved by the Human Investigation Review Committee at the Brigham and Women’s Hospital. We used morphologic criteria to categorize plaques as ‘erosion prone’, ‘stable’, or ‘rupture-prone’ lesions based on plaque morphology, smooth muscle cell content, macrophage content, fibrotic/necrotic core area, and endothelial apoptosis. We used well-established criteria to select plaques with thin fibrous cap and high macrophage and low smooth muscle cell content, which we classified as ‘Rupture-prone’. We also selected proteoglycan-rich plaques with many smooth muscle cells and few macrophages, that we denoted ‘smooth muscle cell (SMC)-rich plaques’.

Since regions of plaques that have undergone superficial erosion lack endothelium, we assessed apoptotic endothelial cells by double fluorescent staining (CD31 and TUNEL), and classified plaques as either low-EC apoptosis SMC-rich plaques (‘stable’) or high EC-apoptosis SMC-rich plaques (‘erosion prone’). Two investigators independently performed the analysis and classification of the tissue specimens, with 85% concordance. The selection of samples for further analysis (immunofluorescent staining for TLR2 and PMN, see below) required concordance of both observers.

**Results**

**TLR2 stimulation induces endothelial stress and apoptosis**

To test the hypothesis that TLR2 mediates endothelial damage associated with superficial erosion, we assessed the EC response to TLR2 activation in vitro. Toll-like receptor-2 stimulation of cultured HSiVEC by lipoteichoic acid (LTA) or by Pam3 for 4 h induced EC activation, monitored by augmented expression of the adhesion molecules E-selectin (22.6- and 56.8-fold increase, respectively) and intercellular adhesion molecule-1 (ICAM-1; 3.4- and 2.3-fold increase), and of IL-8 (9.3- and 14.0-fold increase), a chemokine particularly implicated in recruitment of PMN (Figure 1A and B). Toll-like receptor-2 agonists provoked less endothelial activation than the prototypical pro-inflammatory cytokine TNFα, consistent with the model we propose that TLR2 ligation serves as an early stimulus, priming the ECs for further activation and damage.

We further tested whether TLR2 ligation promoted EC ER stress and release of ROS. Pam3 or LTA stimulation induced a time-dependent augmentation of a biomarker of ER stress, the chaperone GRP78, and phosphorylated transcription factor eukaryotic initiation factor 2-α (eIF2α) (Figure 1C). We evaluated the production of ROS using H2DCFDA, a fluorescent ROS sensor. Exposure to TLR2 activators increased release of ROS by ECs (Figure 1D).

We also tested the hypothesis that TLR2 agonists increase EC apoptosis. The hallmark of apoptotic cells, cleaved caspase-3 protein, rose after TLR2 stimulation of EC for 24 h (Figure 1E). Caspase-3 and -7 activity also increased significantly in ECs in response to TLR2 agonists (68 and 60% increases following LTA or Pam3) (Figure 1F). We further tested the hypothesis that TLR2 ligation impairs the ability of EC to repair desquamative injury. Toll-like receptor-2 activation impaired the capacity of cultured EC to cover an injured area compared to non-treated controls (Figure 1G). Hyaluronan, an endogenous ligand for TLR2, prominent in eroded lesions, produced similar effects on ECs, supporting its involvement in this process (Figure 2). Together, these data demonstrate that TLR2 ligation triggers EC activation, stress, and apoptosis, and impairs injury responses, potentially facilitating superficial erosion and limiting its repair in plaques rich in endogenous agonists for TLR2 (e.g. hyaluronan).
The presence of neutrophils markedly aggravates Toll-like receptor-2-mediated endothelial cell stress

In view of clinical evidence that PMN activation accompanies superficial erosion,\textsuperscript{11} and of the ability of TLR2 agonists to increase IL-8 expression shown here, we hypothesized that PMN recruitment to erosion prone lesions follows and amplifies TLR2-mediated EC stress. We co-cultured freshly isolated human PMN with human EC pretreated with TLR2 for 4 h. Polymorphonuclear leucocytes induce inflammatory gene expression in unstimulated EC. The presence of both TLR2 ligands and PMN potentiated the increased expression of E-selectin (31.4-fold compared with LTA-treated ECs only), ICAM-1 (12.4-fold), VCAM-1 (23.1-fold), and IL-8 (18.5-fold) (Figure 3A). Exposure to both TLR2 agonists and PMN also augmented EC expression of the ER stress markers DDIT-3 and GADD34 (Figure 3B). Moreover, the addition of PMN promoted the production of ROS by
H₂DCFDA-loaded ECs after TLR2 stimulation. Whereas increasing the number of PMN added to the non-treated EC did not alter the oxidant generation, EC treated with TLR2 agonists increased ROS generation in proportion to the number of PMN (Figure 3C). 

Assessment of the induction of cell death used flow cytometry of CD146+ to quantify apoptosis of ECs exposed to TLR2 agonists, PMN, or both. Consistent with the above findings, TLR2 ligation with either Pam3 or LTA increases the number of apoptotic ECs in the presence of PMN (Figure 3D). The increase in apoptotic ECs during co-incubation with PMN achieved significance only in the presence of TLR2 agonists.

To affirm the participation of TLR2 in these effects of LTA or Pam3, we inhibited TLR2 with a blocking antibody and/or with specific siRNAs (Figure 4). Toll-like receptor-2 blockade significantly reduced the overexpression of the activation markers E-selectin, VCAM-1, and IL-8 following LTA or Pam3 stimulation (Figure 4B).

**Neutrophils and Toll-like receptor-2 stimulation impair endothelial adherence**

Impaired EC attachment to its substrate could also contribute to superficial erosion. Neutrophils contain abundant MMP2 and
MMP9, non-fibrillar collagenases that might modulate cell adherence by degrading basement membrane proteins.\textsuperscript{14} Gelatin zymographic analysis of supernatants of ECs showed significantly higher MMP2 activity in EC treated with disease-relevant endogenous ligands for TLR2 abundant in plaques that develop superficial erosions (Figure 5A).\textsuperscript{9,10} Hyaluronan induced activity of MMP2 and MMP9 to levels similar to those seen with LTA. In addition, PMA, a known strong inducer of NETs, also increased EC MMP2 and MMP9 activity. Moreover, TLR2 stimulation augments the elaboration of the active form of MMP9 and its activity in EC, and to an even greater extent when co-cultured with PMN (Figure 5B). Pre-treatment of EC with an antibody which blocks TLR2 before PMN addition largely prevented the increased MMP9 activity in co-culture supernatants (Figure 5C).

Endothelial cell adherence and endothelial integrity rely on tight junctions. Vascular endothelial (VE)-cadherin is one of the major proteins that maintain and regulate endothelial permeability by allowing adherens junctions between ECs.\textsuperscript{15} Upon TLR2 stimulation, EC contained less VE-cadherin, while maintaining its expression on the cell membrane surface. Co-culture with PMN strongly diminished VE-cadherin expression in EC. Toll-like receptor-2 activation together with exposure to PMN accentuated the reduction and internalization of VE-cadherin (Figure 5D and E).

An in vitro detachment assay furnished further insight into how TLR2 and PMN might influence EC adherence. After 24 h of incubation, TLR2 ligand treatment augmented cell detachment (a 64% increase compared with untreated cells). Co-culture of EC with
PMN aggravated EC detachment (Figure 5F), implicating both TLR2 activation and PMN in endothelial desquamation.

**Differential accumulation of neutrophils and neutrophil extracellular traps in smooth muscle cell-rich vs. ‘rupture-prone’ human atheromata**

Human atheromata that undergo superficial erosion typically have many smooth muscle cells, but notably fewer macrophages than ruptured plaques. We probed the mechanisms that might participate in endothelial loss in a collection of human lesions with morphologic characteristics ascribed to superficially eroded vs. ‘rupture-prone’ plaques (i.e., those with few SMCs but many inflammatory cells, and thin fibrous caps). In this regard, we scrutinized our human endarterectomy tissue collection (n = 295) and classified plaques by morphologic criteria as either rich in smooth muscle cells, or ‘rupture-prone’ as described in the Methods section (see Supplementary material online, Figure S1). Two investigators performed the classification independently. We performed further histological analyses on samples (n = 56) independently and concordantly categorized by both investigators according to their morphology, SMC, and macrophage content.

We quantified luminal EC apoptosis after EC and TUNEL co-staining. Some of the ‘SMC-rich plaques’ had very few luminal apoptotic ECs whereas others contained some clusters of apoptotic ECs on the luminal surface (Figure 6A). We characterized the SMC-rich lesions into two groups: ‘low-EC apoptosis SMC-rich plaques’ (n = 10) and ‘high EC apoptosis SMC-rich plaques’ (n = 16) according to the criteria described in the Methods section.

To assess the association TLR2 and of PMN with endothelial apoptosis in human lesions, we stained the sections for the PMN marker neutrophil elastase (NE). Clusters of apoptotic ECs coincided with nearby regions of high neutrophil accumulation in ‘SMC-rich plaques’. In contrast, ‘rupture-prone’ plaques seldom showed this association (Figure 6B). Co-localization of an independent neutrophil marker, MPO, with NE in human lesions affirmed recognition of PMN (see Supplementary material online, Figure S2).

Neutrophil extracellular trap formation can trigger endothelial apoptosis, and NETs associate with apoptotic EC in human atheroma. Some NETs localize at the luminal surface of plaques, as disclosed by the presence of neutrophils identified by NE.
Counterstaining with DAPI revealed both intracellular and extracellular DNA. Smooth muscle cell (SMC)-rich plaques with low-EC apoptosis exhibited few if any NETs (Figure 6B and D). In contrast, in the ‘SMC-rich plaques with high apoptosis’, most of the PMN adjacent to clusters of apoptotic ECs co-localized with NETs. Double staining of plaques with NE and extracellular citrullinated histone-4 corroborated the presence of NETs in these areas (Figure 6B and D).

**Toll-like receptor-2 expression and neutrophils correlate with endothelial cell apoptosis primarily in smooth muscle cell-rich plaques**

Quantification of the total number of NE-positive cells showed more PMN in ‘SMC-rich plaques with high apoptosis’ and ‘rupture-prone’ lesions compared with atheromata with low apoptosis. Neutrophil extracellular traps (DAPI) and citrullinated-histone-positive PMN consistently colocalized. SMC-rich plaques with high apoptosis exhibited more TLR2 staining than ‘rupture-prone’ lesions, but we observed no significant difference between SMC-rich plaques with high or low endothelial apoptosis (Figure 7A).

To assess further correlations between TLR2 and the number of apoptotic EC, PMN accumulation, and NETs, we combined all the ‘SMC-rich plaques’ and compared them with ‘rupture-prone’ plaques, using the number of apoptotic ECs as a dependent variable. The total number of apoptotic ECs at the plaque lumen correlated with PMN infiltration in ‘SMC-rich plaques’ (Figure 7B). Moreover, more abundant apoptotic ECs also correlated with more of NET-rich regions (Figure 7C and D) and TLR2 staining (Figure 7E). In contrast, the rupture-prone plaques showed no correlation between the number of apoptotic ECs and PMN, NETs, or TLR2 staining.

**Discussion**

Knowledge regarding the mechanisms of superficial erosion has lagged considerably behind the understanding of plaque rupture, despite the growing clinical importance of this mechanism of acute thrombotic complication of atherosclerosis. Post-mortem studies show that superficially eroded plaques typically contain abundant smooth muscle cells and proteoglycans, but may have little lipid...
core and few macrophages in stark contrast to ruptured plaques. Although impaired endothelial integrity characterizes eroded lesions, the underlying mechanisms remain obscure. This study probed the novel hypothesis that TLR2 mediates aspects of altered endothelial functions that may contribute to superficial erosion.

During the last decade, the actions of TLRs have garnered interest in atherosclerosis pathophysiology, since mice lacking TLRs and TLR polymorphisms in humans associate with altered disease indices. While TLR4 appears to potentiate primarily the macrophage responses to lipids and inflammation, TLR2 affects macrophages and vascular cells more broadly, and correlates with increased inflammation and matrix degradation in human lesions. Toll-like receptor-2 expression associates with dysfunctional endothelium in vivo and recent work recently reported that TLR2 ligation induced overexpression of adhesion molecules in endothelial cells. Our study substantially expands these findings and provides evidence in support of TLR2’s contribution to superficial erosion, a late complication of already established atherosclerotic lesions. Building on the observation by Hansson’s group that the endothelium overlying atherosclerotic lesions exhibit heightened expression of TLR2, our data indicate that SMC-rich lesions have accentuated expression of TLR2, and the presence of this receptor correlates directly with augmented apoptosis of luminal EC in human plaques. That superficial erosion complicates plaques rich in glycosaminoglycans, and hyaluronan can activate TLR2 heightened our interest in this particular TLR in this context.

Our results further support the involvement of hyaluronan as a disease-relevant endogenous TLR2 agonist, as human plaques that have caused fatal thrombosis due to superficial erosion typically harbour particularly abundant hyaluronan and proteoglycans.
Figure 7  Correlations between endothelial cell apoptosis, toll-like receptor-2, and polymorphonuclear leucocyte in smooth muscle cell-rich and rupture-prone lesions. Quantification of neutrophils, cit-H4-positive polymorphonuclear leucocyte and neutrophil extracellular trap numbers at lumen and percentage of toll-like receptor-2 staining per plaque (A). Correlation analysis comparing total number of luminal neutrophils and number of apoptotic endothelial cell in smooth muscle cell-rich plaques and rupture-prone plaques (B). Correlation between cit-H4-positive polymorphonuclear leucocyte and apoptotic endothelial cell (C). Correlation between neutrophil extracellular trap numbers (cells showing co-localization of neutrophil elastase and cit-H4 in 4′,6-diamidino-2-phenylindole extracellular projections in lumen), and apoptotic endothelial cell (D). Correlation between percentage of toll-like receptor-2 staining per plaque and number of apoptotic endothelial cell (E). Bars represent mean ± SD. (*P < 0.05.)
Figure 8 A schema of a potential pathophysiologic pathway to superficial erosion as a cause of thrombotic complications of atherosclerotic plaques. The bottom of the diagram depicts a longitudinal section of an artery affected by a proteoglycan-rich atherosclerotic plaque. The darker brown indicates accumulation of the proteoglycans such as hyaluronan, versican, and biglycan. The left side of this diagram (1) shows some of the putative triggers for causing endothelial damage as an underlying cause of superficial erosion. Such triggers include pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), and other ligands for innate immune receptors including TLR2, the subject of this report. These ligands bind to pattern recognition receptors on the surface of the endothelial cell. Hyaluronic acid, a common constituent of plaques that have undergone superficial erosion, may act as a TLR2 ligand. Various apoptotic stimuli derived from the inflammatory cells in plaques as well as modified lipoproteins can promote apoptosis of endothelial cells. Matrix degrading enzymes such as the matrix metalloproteinases can attack constituents of the basement membrane that provides a substrate for endothelial cell adhesion mediated by integrins among other adhesion molecules of the basal surface of the endothelial cell. The non-fibrillar collagenases MMP-2 and MMP-9 and the activator of MMP-2, MMP-14, enzymes often overexpressed in plaques, may sever the tethers of the endothelial cell to the intimal surface. The right side of this diagram (2) shows some of the consequences of erosion. Once an endothelial cell has desquamated (as depicted by the damaged endothelial cell with the pyknotic nucleus) the dying endothelial cell can release microparticles that bear tissue factor that can accelerate the activity of factors VII and X to augment thrombin formation and ultimately the conversion of fibrinogen to fibrin yielding an increase in blood coagulation. Exposure of the sub-endothelial matrix can provide a substrate for granulocyte adhesion, activation, and degranulation. Granulocytes are a source of reactive oxygen species such as hypochlorous acid, HOCl, a product of myeloperoxidase (MPO), and also superoxide anion (O$_2^-$). Granulocytes arrive after the initial disruption in the endothelial monolayer according to this scheme. Granulocytes can also release the calgranulin family member MRP-8/14 implicated in inflammation and other aspects of atherothrombosis. Dying granulocytes release DNA and histones contributing to the formation of neutrophil extracellular traps (NETs). These structures can form a nidus for extension of thrombosis and entrapment of further blood leukocytes amplifying the local inflammatory response. Exposure of the sub-endothelial extracellular matrix macromolecules can activate platelets causing them to degranulate and release pro-inflammatory mediators such as interleukin-6 and RANTES. Activated platelets also release plasminogen activator inhibitor-1 (PAI-1), a major inhibitor of endogenous fibrinolytic enzymes. PAI-1 can thus reduce fibrinolysis and increase clot stability.
Other endogenous molecules that could serve as TLR2 ligands in vivo include fatty acids and oxidized phospholipids (OxPAPC). Our results also show that TLR2 agonism augmented the activities of the type IV collagenases MMP-2 and 9, proteinases particularly apt for degrading components of the basement membrane to which endothelial cells attach. Disruption of this substrate might also promote EC death by anoikis in vivo. Indeed, TLR2 agonism rendered EC monolayers more susceptible to desquamation in an in vitro assay.

Breaches in the integrity of arterial intimal endothelium normally undergo rapid repair by coverage by adjacent endothelial cells. We found that TLR2 activation impaired repair of injury to an endothelial monolayer in vitro. Once a small patch of endothelium desquamates, recruitment, and activation of platelets and granulocytes can ensue, favouring formation of NETs. As demonstrated here, PMNs can favour formation of NETs.30 As demonstrated here, PMNs can promote thrombosis associated with superficial erosion. The observations that hyaluronan triggers IL8 release by EC and CD44–hyaluronan interactions promote neutrophil recruitment and adhesion, tighten the links between PMN and superficial erosion. This study documented a particularly strong correlation between apoptotic EC and the presence of NETs in human plaques with a superficial erosion-like morphology. This observation indicates that this mechanism of endothelial apoptosis applies particularly to superficial erosion, but not in ‘rupture-prone’ lesions.

We postulate that superficial erosion of plaques involves two ‘hits’ (Figure 8). The first consists of a phase of initial endothelial injury, mediated by TLR2. Following initial endothelial injury, and death and/or sloughing/desquamation, neutrophils recruited to the scene can mediate a ‘second hit’ by amplifying, sustaining, and propagating the local processes that promote endothelial injury. As thrombosis and coagulation provoked by local tissue factor generation by dying endothelial cells and contact with the subendothelial matrix occurs, granulocytes become trapped in the fibrin strands, and form NETs, as observed here in our human specimens, in accord with observations reported by others.34–36

Our findings point to important distinctions in the fundamental mechanisms that underlie superficial erosion vs. fibrous cap rupture, congruent with the well-recognized morphologic differences between these two dominant causes of clinically important acute thrombotic complications of atherosclerosis. Our data suggest that neutrophils participate prominently in the propagation of superficial erosion, while abundant evidence supports the pathogenic role of monocytes/macrophages in plaque rupture. Apoptosis of SMC may prevail in formation of thin-capped atheroma, while EC apoptosis appears more critical in superficial erosion. Fibrillar collagenases participate in plaque rupture, while the present data implicate the non-fibrillar collagenases in superficial erosion. In contrast, accumulation of proteoglycan rather than paucity of interstitial collagen characterize the lesions complicated by superficial erosion. Indeed, the present study provides mechanistic insight into a likely link between the TLR2 ligand hyaluronan and this path to thrombosis. As statin treatment has increased in patients at risk for atherosclerotic events worldwide, and these agents combat the causes of plaque rupture, understanding the mechanisms of superficial erosion, and their modification has assumed greater importance. The findings presented here provide some novel insight in this regard.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

**Acknowledgements**

Pr. F. William Luscinskas kindly provided the VE-cadherin monoclonal antibody. We thank Drs Michael A. Gimbrone, Jr and Dr Guillermo Garcia-Cardena for helpful conversations. We also thank the Neurobiology Department and the Neurobiology Imaging Facility for consultation and instrument availability that supported this work. This facility is supported in part by the Neural Imaging Center as part of an NINDS P30 Core Center grant #NS072030.

**Funding**

This work was supported by grants from the National Heart, Lung, and Blood Institute (R01 HL080472 to P.L.) and from the Donald W. Reynolds Foundation (to P.L.). H.A. was supported by the Fondation pour la Recherche Médicale, Paris, France. A gift from James Annenbourg La Vea provided support for parts of this work.

**Conflict of interest:** none declared.

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