c-Jun N-Terminal Kinase Primes Endothelial Cells at Atheroprone Sites for Apoptosis

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Objective—Atherosclerosis is a focal disease that occurs predominantly at branches and bends of the arterial tree. Endothelial cells (EC) at atherosusceptible sites are prone to injury, which can contribute to lesion formation, whereas EC at atheroprotected sites are resistant. The c-Jun N-terminal kinase (JNK) is activated constitutively in EC at atherosusceptible sites but is inactivated at atheroprotected sites by mitogen-activated protein kinase phosphatase-1 (MKP-1). Here, we examined the effects of JNK activation on EC physiology at atherosusceptible sites.

Methods and Results—We identified transcriptional programs regulated by JNK by applying a specific pharmacological inhibitor to cultured EC and assessing the transcriptome using microarrays. This approach and subsequent validation by gene silencing revealed that JNK positively regulates the expression of numerous proapoptotic molecules. Analysis of aortae of wild-type, JNK1−/−, and MKP-1−/− mice revealed that EC at an atherosusceptible site express proapoptotic proteins and are primed for apoptosis and proliferation in response to lipopolysaccharide through a JNK1-dependent mechanism, whereas EC at a protected site expressed lower levels of proapoptotic molecules and were protected from injury by MKP-1.

Conclusion—Spatial variation of JNK1 activity delineates the spatial distribution of apoptosis and turnover of EC in arteries. (Arterioscler Thromb Vasc Biol. 2010;30:546-553.)

Key Words: apoptosis ■ arterial endothelium ■ atherosusceptibility ■ c-Jun N-terminal kinase ■ mitogen-activated protein kinase phosphatase-1

Atherosclerosis is characterized by the accumulation of cells, lipids, and extracellular matrix in the wall of an artery, which can result in occlusion of the vessel lumen. It develops predominantly at branches and bends that are exposed to disturbed patterns of blood flow, whereas regions exposed to uniform flow are protected.1–4 The molecular mechanism underlying the distinct spatial distribution of lesions is likely to involve apoptosis. Regions that are predisposed to atherosclerosis are characterized by relatively high rates of endothelial cell (EC) injury and turnover,5–7 and apoptosis can be induced in cultured EC by the application of atheroprone flow patterns.8,9 A causal relationship between apoptosis and atherosclerosis was established by enforcing expression of a proapoptotic molecule in arterial EC, which enhanced the accessibility of lipoproteins and leukocytes to arteries and initiated lesion formation in hypercholesterolemic mice.10 In addition, focal endothelial apoptosis/injury in atherosclerotic lesions can, in turn, lead to endothelial denudation and exposure of a procoagulant vascular wall, a major cause of coronary thrombosis.11,12 Several proatherogenic agents are known to induce EC apoptosis, including oxidized low-density lipoproteins,13 reactive oxygen intermediaries (eg, H2O214) and proinflammatory mediators (eg, lipopolysaccharide [LPS],15 tumor necrosis factor [TNF]-α16) by activating distinct signaling pathways that converge to cleave procaspase-3 into the active form of caspase-3. Cleaved caspase-3, in turn, executes apoptosis by activating numerous downstream effectors that trigger DNA fragmentation and other hallmarks of apoptotic cell death.17

We recently studied EC in the murine aorta and demonstrated that c-Jun N-terminal kinase (JNK), a mitogen-activated protein kinase, can be activated by proinflammatory stimuli at atherosusceptible sites but not at atheroprotected sites.18 The spatial distribution of JNK activity in the arterial tree is controlled by mitogen-activated protein kinase phosphatase-1 (MKP-1), a negative regulator of JNK, which is induced by blood flow at atheroprotected sites.18 Here, we demonstrate that JNK induces numerous proapoptotic molecules in EC and that the spatial variation of JNK activation in the arterial tree delineates the spatial distribution of apoptosis and turnover of EC.

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Materials and Methods

Reagents and Antibodies
A pharmacological inhibitor of all 3 JNK isoforms (CT536706) was prepared as described previously and dissolved in DMSO (10 mmol/L stock). LPS (R&D), TNF-α (R&D), and antibodies that recognized phosphorylated JNK Tyr183/Thr185 (Cell Signaling Technology), JNK (Cell Signaling Technology), CD31 (BD Biosciences Pharmingen), Receptor interacting protein 1 (RIP1; BD Bioscience), tubulin (Sigma Aldrich), procaspase-3, actin (Abcam), active cleaved caspase-3, and Ki-67 (Abcam) were obtained commercially. TUNEL staining was performed using the in situ cell death detection kit (Roche). Other reagents were purchased from Sigma Aldrich unless otherwise stated.

Endothelial Cell Culture
Human umbilical vein endothelial cells (HUVEC) were collected using collagenase and cultured as described previously.

Microarray Analysis
Total RNA was prepared from HUVEC using a commercially available kit (Omega Bio-Tek), and the purity and integrity of total RNA samples were assessed using a Bioanalyzer (Agilent). High-quality samples were used to generate labeled cRNA using commercially available kits (Affymetrix). Labeled cRNA was hybridized to Human Gene 1.0 ST Arrays (Affymetrix) following standardized procedures. This array contains probes that identify 764,885 transcripts that represent 28,869 genes. Raw data were analyzed using Resolver software (Rosetta) to quantify changes in transcripts differentially expressed in the experimental samples. Functional annotation of coregulated genes was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software.

Gene Silencing
RNA interference was performed using 2 different JNK1-specific siRNA or c-Jun–specific siRNA (Applied Biosystems). Nontargeting scrambled control sequences were also synthesized (Qiagen). Cell cultures that were 70% to 80% confluent were transfected with siRNA or c-Jun–specific siRNA (Applied Biosystems). Nontargeting scrambled control sequences were also synthesized (Qiagen). Cell cultures that were 70% to 80% confluent were transfected with siRNA (5 μmol/L, final concentration) by electroporation (Amaxa) following the manufacturer’s instructions and then incubated in complete growth medium for 24 to 48 hours before analysis.

Comparative Real-Time Polymerase Chain Reaction
Transcript levels were quantified by comparative real-time polymerase chain reaction using gene-specific primers (Supplementary Table I, available online at http://atvb.ahajournals.org) as described previously. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product for the genes of interest and for the housekeeping gene β-actin.

Western Blotting
Levels of JNK1, RIP1, and procaspase-3 were measured in total cell lysates prepared using radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris, pH 8.0) by Western blotting using specific primary antibodies, horseradish peroxidase-conjugated secondary antibodies, and chemiluminescence detection. Tubulin levels were measured to control for equal loading.

c-Jun Activity Assay
The levels of phosphorylated c-Jun and total c-Jun were determined using enzyme-linked immunosorbent assay kits following the manufacturer’s instructions (ActiveMotif). The ratio of phosphorylated c-Jun-to-total c-Jun, which is a measure of c-Jun activity, was calculated for each sample.

Animals
Male mice between 2 and 3 months of age were used. Wild-type C57BL/6 mice were obtained from Charles River Laboratories, UK. The MKP-1 knockout mouse strain (MKP-1−/− [C57BL/6]) was obtained from Bristol-Myers Squibb and the JNK1−/− strain was obtained from Professor Roger Davis, University of Massachusetts. Experiments were performed using groups of at least 4 animals. All experiments were performed within guidelines set by the Federation of European Laboratory Animal Science Associations.

En Face Immunostaining
The expression levels of specific proteins were assessed in EC at regions of the murine aorta that are known to be susceptible to (inner curvature of the arch) or protected from (outer curvature) atherosclerosis by en face staining, followed by laser-scanning confocal microscopy as described previously. Aortae were tested by immunostaining using specific primary antibodies and Alexafluor568-conjugated secondary antibodies. EC were identified by costaining using anti-CD31 antibodies conjugated to Alexafluor488, and nuclei were costained using Draq5 (Biotest). The expression of particular proteins at each site was assessed by quantification of fluorescence intensity for multiple cells (at least 100 per site) using LSM 510 software (Zeiss).

En Face TUNEL Staining
DNA fragmentation was measured in EC in murine aortae by en face TUNEL staining, followed by laser-scanning confocal microscopy. Animals were euthanized with CO2 inhalation and aortae were perfused in situ with phosphate-buffered saline and then perfusion-fixed with 2% formalin before harvesting, as described previously. Fixed aortae were tested by TUNEL staining following the manufacturer’s recommendations (Roche). EC were identified by costaining using Griffonia lectin conjugated to Rhodamine (Vectorlabs) or by using anti-CD31 conjugated to phycoerythrin, and nuclei were costained using Draq5 (Biotest). Staining was assessed by confocal microscopy and apoptotic EC, characterized by TUNEL staining colocalized with pyknotic or fragmented nuclei, were quantified in multiple fields of view.

Statistics
Differences between samples were analyzed using an unpaired Student t test or a χ2 test (*P<0.05; **P<0.01; ***P<0.001).

Results

JNK Acts as a Positive Regulator of Proapoptotic Molecules in Cultured EC
We examined the physiological role of JNK in cultured EC using a pharmacological inhibitor (CT536706). Pretreatment of HUVEC with CT536706 suppressed subsequent phosphorylation of c-Jun by TNF-α, confirming that this compound can inhibit JNK activation in cultured EC (Supplementary Figure I, available online at http://atvb.ahajournals.org). We studied the transcriptional programs regulated by JNK using microarrays. Treatment of HUVEC with CT536706 altered the constitutive expression of 1123 transcripts (116 upregulated and 1007 downregulated) that belong to several functional groups, including inflammation, proliferation, apoptosis, and cell signaling (Supplementary Table II, Supplementary Figure IIA), suggesting that JNK regulates the basal expression of numerous molecules that possess diverse physiological functions. Of particular note, proapoptotic transcripts were significantly enriched in the JNK-dependent group. Treatment of HUVEC with TNF-α altered the expression of 241 transcripts (165 upregulated and 76 downregulated), including regulators of apoptosis, leuko-
cyte adhesion, and cell signaling (Supplementary Table II, Supplementary Figure IIB), a finding that is consistent with previous studies.24,25 Pretreatment of HUVEC with the JNK inhibitor suppressed the induction by TNF-α/H9251 of 134 transcripts, and this group was enriched with molecules with proapoptotic or proinflammatory functions (Supplementary Table II, Supplementary Figure IIB). TNF-α/H9251 also induced antiapoptotic molecules, but the majority of them (60%) were not influenced by CT536706 (Table and data not shown). In summary, our findings suggest that JNK regulates proinflammatory, proapoptotic, and other physiological processes in both quiescent and TNF-α–treated EC.

The microarray study was validated by independent quantitative reverse-transcription polymerase chain reaction experiments that confirmed that pharmacological inhibition of JNK leads to reduced basal expression of molecules that positively regulate apoptosis, eg, caspase-3, DKK1 (Table and Figure 1A), proinflammatory activation, eg, vascular cell adhesion molecule-1, E-selectin (Table, Supplementary Figure III), or both of these processes, eg, Toll-like receptor (TLR) 4, RIP1 (Table, Figure 1A). We focused on a subset of proapoptotic molecules and demonstrated by Western blotting that pharmacological inhibition of JNK reduced the expression of RIP1 and procaspase-3 proteins in HUVEC (Figure 1B). Further validation was obtained using JNK1-specific siRNA, which suppressed the expression of JNK1 but did not influence JNK2 (Figure 1C). Silencing of JNK1 significantly reduced the expression of RIP1 and caspase-3, whereas a scrambled, nontargeting control had no effect (Figure 1C). Silencing of the downstream transcription factor

<table>
<thead>
<tr>
<th>Genes</th>
<th>Basal state</th>
<th>TNFα-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular cell adhesion molecule 1 (VCAM-1)</td>
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</tr>
<tr>
<td>E-selectin</td>
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</tr>
<tr>
<td>Interleukin 8 (IL-8)</td>
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<tr>
<td>Bone morphogenetic protein 4 (BMP4)</td>
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<td>Caspase 3</td>
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</tr>
<tr>
<td>Bcl-2 related protein A1</td>
<td></td>
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</tr>
</tbody>
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JNK regulated

- Positively
- Negatively
- Unaltered

NA indicates not applicable; NT, not tested.
c-jun also led to a significant reduction in RIP1 and caspase-3 levels (Figure 1D). Thus, we conclude that JNK-c-Jun signaling positively regulates the expression of proapoptotic molecules in cultured EC.

**JNK1 Regulates the Expression of Proapoptotic Molecules in the Arterial Tree**

We previously demonstrated that levels of phosphorylated JNK are higher in EC at an atherosusceptible site compared to an atheroprotected site, whereas endothelial expression of total JNK1 is similar in these 2 regions (data not shown). We concluded that JNK is preferentially activated at the atherosusceptible region and therefore predicted that JNK-dependent proapoptotic signaling molecules will be expressed preferentially at this site. Consistent with this hypothesis, en face staining of wild-type mice revealed that EC in the susceptible site express higher levels of RIP1 (Figure 2A) and caspase-3 (Figure 2B) compared to EC in the protected site. Genetic deletion of JNK1 reduced RIP1 (Figure 2A) and procaspase-3 (Figure 2B) levels in the susceptible site, indicating that JNK1 is essential for their expression in this region.

**JNK1 Delineates the Spatial Distribution of Caspase-3 Activation in the Arterial Tree**

We examined whether JNK1 regulates caspase-3 activation at the susceptible site by studying EC in aortae of wild-type, JNK1/−/−. We treated with vehicle alone or remained untreated. A, Transcript levels were quantified by real-time polymerase chain reaction. Mean values (±SD) were calculated from data pooled from 3 independent experiments. B, Procaspase-3, RIP1, and tubulin levels were assessed by Western blotting. Representative of 2 independent experiments. C and D, HUVEC were treated with JNK1-specific siRNA (JNK1a or JNK1b), c-jun-specific siRNA, or with a scrambled nontargeting sequence (Scr) and were incubated for 24 hours. JNK1, JNK2, c-Jun, and tubulin levels were determined by Western blotting. Transcript levels were quantified by real-time polymerase chain reaction. Mean values (±SD) were calculated from data pooled from 3 (C) or 2 (D) independent experiments.
LPS-treated wild-type animals (Figure 3, panel 3). Because MKP-1 suppresses JNK activation at the protected site, we examined whether it is required for the suppression of caspase-3 activation. Genetic deletion of MKP-1 enhanced caspase-3 activation in response to LPS at the protected site (Figure 3, compare panels 3 and 7), indicating a cytoprotective role for MKP-1.

***JNK1 Activity Delineates the Spatial Distribution of EC Turnover in the Arterial Tree***

EC apoptosis was assessed by detecting DNA fragmentation by TUNEL staining. To ensure that apoptotic cells were of endothelial origin, we performed 2 series of experiments in which EC were costained using either Griffonia lectin (Figure 4, Supplementary Figure IV) or anti-CD31 (Supplementary Figure V). TUNEL-positive nuclei were not detected in EC at the susceptible site in untreated wild-type mice but could be induced at this site by LPS (Supplementary Figure IV, Figure 4, Supplementary Figure V, compare panels 2 and 4). Genetic deletion of JNK1 suppressed DNA fragmentation in response to LPS (Figure 4, Supplementary Figure V, compare panels 4 and 6). These data suggest that although JNK1 regulates constitutive expression of proapoptotic proteins at the susceptible site, it is not sufficient to induce EC apoptosis. However, EC in this region are primed for apoptosis in response to LPS through a JNK1-dependent mechanism. In wild-type animals, EC at the protected site were resistant to the induction of apoptosis by LPS (Figure 4, Supplementary Figure V, panel 3). Genetic deletion of MKP-1 enhanced DNA fragmentation in response to LPS in EC at the protected site (Figure 4, compare panels 3 and 7), indicating that MKP-1 is required for the maintenance of EC viability in this region. Genetic deletion of MKP-1 did not influence apoptosis in the susceptible region, which is consistent with our previous observation that MKP1 is not expressed at this site.

To examine whether apoptosis at the susceptible site is associated with EC proliferation, we measured the expression of Ki-67 protein (a marker of proliferation) by en face staining. Ki-67–positive EC nuclei were detected at a low rate...
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atheroprotected sites are resistant. This principle was proven

apoptosis in response to a noxious stimulus, whereas EC at

knowledge that EC at atherosusceptible sites are primed for

apoptosis.10 Here, we demonstrated for the first time to our

endothelium can arise through several processes, including

which are key drivers of atherogenesis. Injury of the vascular

accessibility of the vessel wall to leukocytes and lipoproteins,

atherosusceptible sites.5–7 Thus, their phenotype enhances the

higher rates of injury and turnover compared to EC at

characterized by enhanced proinflammatory activation and

location in the arterial tree. EC at atherosusceptible sites are

The physiology of EC varies considerably according to their

expression of numerous proapoptotic molecules in EC is consistent with previous

observations that JNK can activate proapoptotic signaling

pathways35,36 and induce proapoptotic transcripts by activat-

Our finding that JNK regulates the expression of numerous

proapoptotic molecules in EC is enhanced in atherosclerotic lesions,31 a poly-

morphism that attenuates TLR4 activity is associated with a
decreased risk of atherosclerosis in humans,32 and TLR4

signaling in EC can be activated by oxidized low-density lipoprotein,33 which is known to accumulate in plaques.

BMP4 may also contribute to EC activation and apoptosis
during atherogenesis because its expression in EC is greatly

increased at atherosusceptible sites compared to protected

sites.28 In addition, RIP1 may influence numerous physiolog-

ical activities in EC, including proliferation, activation, and

viability by signaling downstream from TNF receptors.29,34

Our finding that JNK regulates the expression of numerous

proapoptotic molecules in EC is consistent with previous

observations that JNK can activate proapoptotic signaling

pathways35,36 and induce proapoptotic transcripts by activat-

ing the transcription factor c-Jun.37,38

En face staining of the murine aorta revealed that JNK

regulates the basal expression of procaspase-3 and RIP1 at an

atherosusceptible site. Although proapoptotic proteins were

expressed at enhanced levels at susceptible sites, EC apopto-

sis was not detected in untreated animals. However, EC

apoptosis was induced at the susceptible site in wild-type

animals by LPS through a JNK1-dependent mechanism.

Under these conditions, EC apoptosis occurred in ñ3% EC at

the susceptible site, a rate that is consistent with previous

studies of EC viability in LPS-treated rodents.8 We suggest

that JNK1 primes EC in the susceptible region for apoptosis

using a model noxious agent, LPS,15 and it will be important

in future studies to examine whether EC at atheroprone sites

are primed for apoptosis in response to other proatherogenic

molecules such as oxidized low-density lipoproteins13 and
during early atherogenesis.

We discovered that the molecular mechanism for priming

of EC for apoptosis involves JNK1, which regulates the basal

expression of proapoptotic molecules in EC and governs the

spatial distribution of EC apoptosis in the arterial tree. A

previous study by our group18 demonstrated that JNK is

activated constitutively in EC at atherosusceptible sites but is

expressed in an inactive form at atheroprotected sites. Here,

we used cell culture and in vivo models to examine whether

JNK can influence proatherogenic processes in EC and

potentially contribute to local atherosusceptibility. The func-

tion of JNK was investigated in cultured EC in the presence

or absence of a specific pharmacological inhibitor using a

genomics platform followed by functional annotation. This

approach and subsequent validation by gene silencing con-

firmed previous reports that JNK positively regulates the

induction of proinflammatory transcripts,26,27 which influence

atherosclerosis by regulating the recruitment of leukocytes to

the vessel wall. Moreover, analysis of wild-type and

JNK1−/− mice revealed that JNK1 plays an essential role in

vascular cell adhesion molecule-1 expression in EC in vivo (data not shown). We also demonstrated for the first time to

our knowledge that JNK positively regulates endothelial

expression of TLR4, bone morphogenetic protein (BMP) 4,

and RIP1, which are molecules known to trigger dual proin-

flammatory and proapoptotic/necrotic signaling path-

ways.28–30 TLR4, a component of the innate immune system,

has been implicated in atherosclerosis because its expression

in EC is enhanced in atherosclerotic lesions,31 a poly-

morphism that attenuates TLR4 activity is associated with a
decreased risk of atherosclerosis in humans,32 and TLR4

signaling in EC can be activated by oxidized low-density

lipoprotein,33 which is known to accumulate in plaques.

Figure 5. The spatial distribution of endothelial proliferation is

regulated by JNK1. The expression of a marker of cell prolifera-

tion (Ki-67) was assessed by en face staining of susceptible or

protected regions of the aorta in wild-type or JNK1−/− mice that

were either treated with LPS (intraperitoneal injection; 4 mg/kg

for 6 hours) or remained untreated (red; n=3 per group). Endo-

thelial marker is CD31 (green). Nuclear were counterstained

(DNA, purple). Representative images and the proportion of

Ki-67–positive EC (mean±SD) are shown.

at the susceptible site in untreated mice but could be induced

at this region by LPS (Figure 5, compare panels 2 and 4). By

contrast, EC at the protected site did not express Ki-67

constitutively and were relatively resistant to Ki-67 induction

by LPS (compare panels 3 and 4). Genetic deletion of JNK1

suppressed Ki-67 expression at the susceptible site (compare

panels 4 and 6), indicating that JNK1 is required for EC

proliferation at this region. Thus, we conclude that JNK1

activation enhances EC turnover at the susceptible site by

positively regulating apoptosis and proliferation.

Discussion

The physiology of EC varies considerably according to their

location in the arterial tree. EC at atherosusceptible sites are

characterized by enhanced proinflammatory activation and

higher rates of injury and turnover compared to EC at

atheroprotected sites.5–7 Thus, their phenotype enhances the

accessibility of the vessel wall to leukocytes and lipoproteins,

which are key drivers of atherogenesis. Injury of the vascular

endothelium can arise through several processes, including

apoptosis.10 Here, we demonstrated for the first time to our

knowledge that EC at atherosusceptible sites are primed for

apoptosis in response to a noxious stimulus, whereas EC at

atheroprotected sites are resistant. This principle was proven

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by elevating expression of proapoptotic signaling molecules such as procaspase-3 and RIP1, which can be subsequently activated by noxious stimuli. Although this is the first evidence to our knowledge that EC can be primed for apoptosis by elevated expression of proapoptotic proteins, an analogous mechanism has been described for the induction of apoptosis in T lymphocytes in response to T-cell receptor signaling, which upregulates procaspase-3 at a transcriptional level. In addition to the induction of proapoptotic transcripts, JNK1 can regulate apoptosis by alternative mechanisms, including cleavage of Bid (a member of the Bcl2 family) into tBid, which alters mitochondrial permeability to release the proapoptotic factor Smac/Diablo, which activates the initiator caspase-8, and by activation of the E3 ubiquitin ligase Itch, which ubiquitinates the cytoprotective protein c-FLIP, leading to its degradation. Therefore, it is plausible that JNK1 regulates EC apoptosis at susceptible sites through mechanisms that operate at both transcriptional and nontranscriptional levels.

Our study also revealed that EC at atherosusceptible sites were primed for proliferation in response to LPS via a JNK1-dependent mechanism. Although it is plausible that JNK1 influences EC proliferation directly, a previous study suggested that JNK is a negative regulator of EC division. Instead, we favor the possibility that JNK1 influences proliferation indirectly by driving EC apoptosis, which in turn causes neighboring EC to proliferate because of loss of EC–EC contact inhibition. Thus, increased EC proliferation may compensate for EC loss attributable to apoptosis and provide an important repair mechanism at atherosusceptible sites. Nevertheless, EC turnover is likely to influence atherosclerosis by causing transient, local loss of EC, which may increase the accessibility of the artery wall to lipoproteins and inflammatory cells.

We observed that EC at the atheroprotected site were resistant to the induction of apoptosis and proliferation by LPS. The underlying mechanism is likely to involve blood flow, which varies in magnitude and direction according to vascular anatomy and alters the physiology of endothelial cells. The application of unidirectional laminar flow (mimicking atheroprotective conditions) can suppress JNK activity, apoptosis, and proliferation in cultured EC, whereas the application of disturbed flow (mimicking atheropromoting conditions) can have the opposite effects. Here, we demonstrate that suppression of EC apoptosis at the atheroprotected site relies on MKP-1, a negative regulator of JNK that is induced by unidirectional laminar flow in cultured EC and is preferentially expressed by EC in the atheroprotected region. Thus, our novel findings suggest that local hemo-dynamics maintain EC viability and reduce EC turnover at atheroprotected sites by inducing persistent expression of MKP-1, which inactivates JNK and suppresses proapoptotic signaling pathways. A recent study revealed that genetic deletion of JNK2, but not JNK1, suppressed uptake of modified low-density lipoprotein by macrophages and reduced lesion development after exposure of apolipoprotein E/– mice to a high-fat diet. This latter study does not, however, preclude a role for JNK1 in endothelial injury during the initiation of atherosclerosis, because this phase of the disease was not studied. In addition, any protective effects of JNK1 genetic deletion may have been overwhelmed by the high atherogenic drive in the apolipoprotein E/– model. Thus, we suggest that further studies are required to examine the potential role of JNK1 in endothelial injury, lipid deposition, and vascular inflammation during early atherogenesis.

Conclusion
In summary, differential activation of JNK1 delineates the spatial variation in proapoptotic gene expression, apoptosis, and turnover in arterial EC, and may govern the spatial distribution of atherosclerotic plaques. Our conclusion has implications for the therapeutic targeting of JNK1 to suppress vascular injury.

Acknowledgments
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In the article, “c-Jun N-terminal Kinase Primes Endothelial Cells at Atheroprone Sites for Apoptosis” by Chaudhury et al, which appeared in the March 2010 issue of the journal (Arterioscler Thromb Vasc Biol. 2010;30:546–553; DOI: 10.1161/ATVBAHA.109.201368), the following Acknowledgment was missing from the article: The work was funded by the British Heart Foundation and the National Heart and Lung Institute Foundation.

The online version has been corrected.

The authors regret the error.

DOI: 10.1161/ATV.0b013e3181fe3c9e
SUPPLEMENT MATERIAL

LEGENDS FOR SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table I
Gene-specific primer sequences for quantitative real-time PCR.

Supplementary Table II
Biological classification of differentially expressed genes regulated by JNK in untreated (basal state) or TNFα-treated EC.

Supplementary Figure I
CT536706 suppresses c-Jun phosphorylation. HUVEC were treated with CT536706 (JNK inhibitor; 1 μM for 1 hour), with vehicle alone or remained untreated, and then stimulated with TNFα (10 ng/ml for 15 minutes) or remained untreated. Levels of phosphorylated c-Jun and total c-Jun were determined by ELISA. Mean ratios (+/- SD) of phosphorylated c-Jun/total c-Jun calculated from two independent experiments are shown.

Supplementary Figure II
Heat maps depicting the effects of CT536706 on the endothelial transcriptome. HUVEC were treated with CT536706 (JNK inhibitor; 1 μM for 5 hours), with vehicle alone or remained untreated. Cultures were co-stimulated with TNFα (10 ng/ml) for the final 4 hours or remained untreated. Each experimental condition was performed in triplicate. Transcript levels in each sample were assessed at a genomic level using Affymetrix® microarrays and analysed using
Rosetta-resolver® and DAVID software. Pro-inflammatory and pro-apoptotic transcripts that were positively regulated by JNK, either in the basal state (A) or in response to TNFα-treatment (B), were identified. Their patterns of expression are superimposed on heat maps where green indicates suppression by the JNK inhibitor and red indicates enhancement.

**Supplementary Figure III**

**JNK positively regulates constitutive and TNFα-inducible expression of pro-inflammatory molecules in EC.** HUVEC were treated with CT536706 (JNK inhibitor; 1 μM for 5 hours), with vehicle alone or remained untreated. Cultures were co-stimulated with TNFα (10 ng/ml) for the final 4 hours or remained untreated. Levels of pro-apoptotic (A), anti-apoptotic (B) or pro-inflammatory (C) transcripts were quantified by real-time PCR. Mean values (+/- SD) calculated from three independent experiments are shown.

**Supplementary Figure IV**

**Multiple optical slices revealed TUNEL positive EC in the susceptible region of the aorta of LPS-treated mice.**

EC apoptosis was assessed by TUNEL staining (green) at a susceptible region of the aorta in wild-type mice treated with LPS (4 mg/kg for 6 hours; intraperitoneal injection). Endothelial marker (lectin); nuclear counterstain (DNA). Images are shown that were generated from a single site within the susceptible region of an LPS treated wild-type mouse. A single confocal slice is shown (top left). Multiple optical slices were generated at different points along the z-axis from the luminal side (image 1) towards the media (z-stack; lower panels). Z-stacks were compiled and are shown in relation to the x- or y-axes (top right). Apoptosis was characterised by TUNEL-
positive pyknotic or fragmented nuclei (arrows).

**Supplementary Figure V**

**The spatial distribution of EC apoptosis is regulated by JNK1.**

DNA fragmentation in EC was measured by TUNEL staining (green) of susceptible and protected regions of the aorta in wild-type or JNK1^-/-_ mice that were either treated with LPS (intraperitoneal injection; 4 mg/kg for 6 hours) or remained untreated (n=4 per group). EC were detected by counterstaining using anti-CD31 conjugated to PE (red) and nuclei were also counterstained (DNA, purple). Apoptotic EC, characterized by TUNEL staining, were quantified in multiple fields of view. Representative images and the proportion of TUNEL-positive EC (mean +/- SD) are shown.
## Supplementary Table I

<table>
<thead>
<tr>
<th>Gene</th>
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<td>VCAM-1</td>
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<td>5'-CTTGCAATTTCTTTTACACCTGACC-3'</td>
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<tr>
<td>E-selectin</td>
<td>5'-GCTCTGAGCTCGGACAT-3'</td>
<td>5'-GAAGGCTCACTCAACAGGGAATG-3'</td>
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<td>IL-8</td>
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## Supplementary Table II

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Supplementary Figure I

Control DMSO CT536706

c-Jun activation (OD 450)

Vehicle JNK inhibitor TNFα

C-Jun activation (OD450)

0 100 200 300 400 500 600 700 800

**
Supplementary Figure II

A

Vehicle
JNK inhibitor
− − − +

Dickkopf 1
Caspase-3
Receptor Interacting Protein 1
Caspase recruitment domain family, member 6
Bone morphogenesis protein 4
Bone morphogenesis protein 2
Zinc finger protein 346 (JAZ)
MAPK activating death domain
D4, zinc zna double PHD fingers family 2 (DPF2)
Fas-Associated protein with Death Domain (FADD)
Bel-2 antagonist 1 (BAK1)
Cell division cycle and apoptosis regulator (CCAR1)

Rhotekin

B

Vehicle
JNK inhibitor
+ + − −

FOS-like antigen 2
Knuppel-like factor 3 (KLF-3)
Receptor interacting protein 2 (RIP2)
E-selectin
TRAF member associated NF-kappa-B activator
Interferon regulatory factor 1
Inhibitor of apoptosis protein-repeat 3 (IAP-3)
Vascular cell adhesion molecule-1 (VCAM-1)
Intercellular adhesion molecule-1 (ICAM-1)
TNF receptor-associated factor 1 (TRAF1)
Inhibitor of apoptosis protein-repeat 2 (IAP-2)
Serine threonine kinase 4 (Mst-1)
Rel A (p65)
Protein phosphatase 2A

TNFα − + + +

Supplementary Figure III

A

**TLR 4**

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**CARD 6**

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C

**E-selectin**

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**VCAM-1**

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</tbody>
</table>
Supplementary Figure IV
Supplementary Figure V

Protective Susceptible

Untreated

Wild type

JNK1\(^{-/-}\)

DNA merge

TUNEL CD31 DNA merge

TUNEL CD31 DNA merge

TUNEL CD31 DNA merge

TUNEL CD31 DNA merge

% apoptotic cells

Untreated LPS treated

Wild-type JNK1\(^{-/-}\)

Protected

Susceptible

LPS treatment

*