Signal Transducer and Activator of Transcription-1 Is Critical for Apoptosis in Macrophages Subjected to Endoplasmic Reticulum Stress In Vitro and in Advanced Atherosclerotic Lesions In Vivo

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Background—Macrophage apoptosis is a critical process in the formation of necrotic cores in vulnerable atherosclerotic plaques. In vitro and in vivo data suggest that macrophage apoptosis in advanced atheroma may be triggered by a combination of endoplasmic reticulum stress and engagement of the type A scavenger receptor, which together induce death through a rise in cytosolic calcium and activation of toll-like receptor-4.

Methods and Results—Using both primary peritoneal macrophages and studies in advanced atheroma in vivo, we introduce signal transducer and activator of transcription-1 (STAT1) as a critical and necessary component of endoplasmic reticulum stress/type A scavenger receptor–induced macrophage apoptosis. We show that STAT1 is serine phosphorylated in macrophages subjected to type A scavenger receptor ligands and endoplasmic reticulum stress in a manner requiring cytosolic calcium, calcium/calmodulin-dependent protein kinase II, and toll-like receptor-4. Remarkably, apoptosis was inhibited by ~80% to 90% (P<0.05) by STAT1 deficiency or calcium/calmodulin-dependent protein kinase II inhibition. In vivo, nuclear Ser-P-STAT1 was found in macrophage-rich regions of advanced murine and human atheroma. Most important, macrophage apoptosis was decreased by 61% (P=0.034) and plaque necrosis by 34% (P=0.02) in the plaques of fat-fed low density lipoprotein receptor null Ldlr<sup>−/−</sup> mice transplanted with Stat1<sup>−/−</sup> bone marrow.

Conclusions—STAT1 is critical for endoplasmic reticulum stress/type A scavenger receptor–induced apoptosis in primary tissue macrophages and in macrophage apoptosis in advanced atheroma. These findings suggest a potentially important role for STAT1-mediated macrophage apoptosis in atherosclerotic plaque progression. (Circulation. 2008;117:940-951.)

Key Words: apoptosis ■ atherosclerosis ■ cholesterol ■ macrophage ■ plaque

In advanced atherosclerosis, death of macrophages in the setting of defective phagocytic clearance of apoptotic cells contributes to the development of plaque necrosis.1,2 Plaque necrosis, in turn, is thought to promote plaque disruption and arterial thrombosis, which are the proximate causes of acute cardiovascular events.1-3 Our laboratory established an important principle of advanced lesional macrophage death, namely involvement of the endoplasmic reticulum (ER) stress pathway known as the unfolded protein response (UPR).4,5 Some laboratories have discovered important evidence that the UPR is activated in intimal cells, including macrophages, in advanced murine and human plaques.6-9 In particular, Myoishi et al<sup>6</sup> recently showed a dramatic rise in UPR markers, including the transcription factor CHOP (GADD153), and intimal cell apoptosis in autopsy specimens from humans with vulnerable and ruptured plaques but not stable lesions and in atherectomy specimens from humans with unstable angina but not stable angina. Although the UPR is primarily an ER repair pathway, a branch of the UPR involving the effector CHOP can trigger apoptosis when the cell senses that repair is no longer possible.4,10 In terms of causation, we have shown that advanced lesional macrophage death and plaque necrosis are decreased in atherosclerotic apolipoprotein E–deficient (Apo<sub>e<sup>−/−</sup></sub>) mice in the setting of ER stress prevention<sup>9</sup> or CHOP deficiency (Edward Thorp, PhD; Gang Li, PhD; George Kuriakose, MSc; David Ron, MD; and I.T., unpublished data, 2007).
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Our work on the UPR began with a model of advanced lesional macrophage death that is present in advanced plaques, namely intracellular accumulation of lipoprotein-derived free cholesterol (FC).\(^1\) FC enrichment of macrophages, like many ER stressors, activates the UPR through depletion of ER luminal calcium.\(^12,13\) Since then, mechanistic studies have led to a broader concept of advanced lesional macrophage death beyond the FC model. These studies have shown that any combination of inducers of ER stress and ligands for the macrophage type A scavenger receptor (SRA), both of which are expressed prominently in advanced lesions, triggers macrophage apoptosis.\(^14,15\) Macrophage SRA recognizes a number of lesional molecules and atherogenic lipoproteins, including those used to enrich macrophages with cholesterol in the FC model.\(^16\) The SRA also is a pattern recognition receptor of the innate immune system, and endotoxin-free SRA ligands activate other pattern recognition receptors, notably toll-like receptor-4 (TLR4).\(^15,17,18\) In this context, our studies have shown that SRA ligands trigger 2 critical proapoptotic events in ER-stressed macrophages: TLR4-mediated activation of a proapoptotic MyD88 pathway\(^15\) and SRA-mediated suppression of a prosurvival TLR4-TRIF-interferon (IFN)-β pathway.\(^14,15\)

In this report, we show that apoptosis of ER-stressed macrophages also requires signal transducer and activator of transcription-1 (STAT1) and calcium/calmodulin-dependent protein kinase II (CaMKII) in a process involving cytosolic calcium and TLR4. Most important, we provide evidence that activated STAT1 is present in atheromata and that lesional macrophage apoptosis is suppressed in the setting of STAT1 deficiency.

Methods

See the online-only Data Supplement for expanded Methods.

Assay of Macrophage Apoptosis

Midstage and late-stage apoptosis in peritoneal macrophages was assayed by annexin V and propidium iodine staining, respectively, with the Vybrant Apoptosis Assay Kit No. 2 (Molecular Probes, Carlsbad, Calif). At the end of incubation, the macrophages were gently washed once with PBS and incubated for 15 minutes at room temperature with 120 μL annexin-binding buffer (25 mmol/L HEPES, 140 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4, 0.1% BSA) containing 10 μL Alexa Fluor 488-conjugated annexin V and 1 μL of 100-μg/mL propidium iodine. The staining mixture was then removed and replaced with 120 μL annexin-binding buffer. The cells were viewed immediately at room temperature with an Olympus IX-70 inverted fluorescent microscope equipped with filters appropriate for fluorescein and rhodamine, and images were obtained with a Cool Snap charge-coupled device camera mounted with coverslips. TUNEL staining was analyzed with an Olympus IX-70 inverted fluorescent microscope equipped with a Cool Snap charge-coupled device camera and imaging software (Roper Scientific). Fluorescent images were captured and analyzed with image analysis software (Adobe Systems, San Jose, Calif).

Statistical Analysis

Data are presented as mean±SEM. Absent error bars in the bar graphs signify SEM values smaller than the graphic symbols. The significance of paired data was determined by Student t test. Data with >2 groups or ≥2 independent variables were analyzed with ANOVA, followed by the Bonferroni post hoc test. Significance is indicated by an asterisk in the figures with an explanation in the figure legends; nonsignificance is indicated by NS in the figures.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

SRA-Induced Apoptosis in ER-Stressed Macrophages Requires STAT1 and Is Preceded by Serine Phosphorylation of STAT1

During the course of another study investigating an ER stress response mediator called interferon-inducible, double-
stranded RNA-regulated eIF-2α protein kinase (PKR), we conceived the hypothesis that STAT1, the activity of which is modulated by PKR, may play a role in ER stress–induced macrophage apoptosis. To test this idea, we compared SRA/ER stress–induced apoptosis in peritoneal macrophages from wild-type (WT) versus Stat1−/− mice. Confirming our previous work, both intracellular FC enrichment with an SRA-interacting lipoprotein and treatment with the SRA ligand fucoidan plus the UPR activator thapsigargin triggered apoptosis, as indicated by an increase in annexin V staining (Figure 1A and 1B, WT). In contrast, Stat1−/− macrophages were markedly protected from apoptosis by both inducers (80% to 90% inhibition of apoptosis; *P < 0.05), indicating an essential role of STAT1 in this model of macrophage apoptosis (Figure 1A and 1B, Stat1−/−). The decrease in apoptosis in Stat1−/− macrophages could not be explained by a decrease in either SRA (not shown) or CHOP induction (Figure 1C).

STAT1 is activated by phosphorylation of Y701 or S727.21 Y701 phosphorylation is essential for STAT1 dimerization, nuclear translocation, and DNA binding.21 S727 phosphorylation enhances the transcriptional activity of tyrosine-phosphorylated STAT1 or, in some cases, has been reported to participate in signaling in the absence of Y701 phosphorylation.21–23 As shown in Figure 2A, FC loading of macrophages induced serine, whereas tyrosine phosphorylation was not detected, and total STAT1 was not increased. In contrast, very little serine phosphorylation was seen in nonloaded or cholesteryl ester–loaded macrophages, which show no or very little evidence of ER stress.4 As expected, IFNγ induced highly detectable levels of tyrosine phosphorylation and serine phosphorylation of STAT1.21 Previous work has suggested that nuclear Ser-P-STAT1 can occur through serine phosphorylation of a constitutive pool of nuclear STAT1.23 We detected STAT1 in nuclear fractions isolated from untreated macrophages, and Ser-P-STAT1 was increased with FC loading. Although total nuclear STAT1 was modestly increased after FC loading, this increase was much less than that seen with IFNγ, which is known to induce STAT1 nuclear translocation21 (Figure 2B). These data suggest that at least a portion of FC-induced Ser-P-STAT1 occurs through phosphorylation of constitutively nuclear STAT1. It also is possible that at least a portion of the STAT1 was tyrosine phosphorylated but below the limits of detection of our immunoblot assay.
The ability of IFNγ to stimulate both serine and tyrosine phosphorylation of STAT1, the presence of IFNγ in atherosclerotic lesions, and recent evidence that IFNγ promotes advanced plaque progression24,25 led us to explore the effect of the combination of FC loading and IFNγ treatment on Ser-P-STAT1 and apoptosis. FC-loaded macrophages treated with IFNγ showed an increase in Ser-P-STAT1 that was greater than either condition alone (Figure 2).

Figure 2. FC loading induces serine but not tyrosine phosphorylation of STAT1. A, Macrophages were incubated for the indicated times with medium alone (Control) or medium containing acetyl-LDL (CE-loaded), acetyl-LDL plus 58035 (FC-loaded), or 100 U/mL IFNγ. Whole-cell lysates were then prepared and subjected to immunoblot analysis to detect phospho–S727 STAT1 (Stat1 pS727), phospho–Y701 STAT1 (Stat1 pY701), and total STAT1. B, Nuclear fractions from control, FC-loaded, and IFNγ-treated macrophages were subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, and the nuclear marker nucleophosmin.

Figure 3. IFNγ enhances FC-induced STAT1 serine phosphorylation and STAT1-dependent FC-induced apoptosis. A, Macrophages were incubated for the indicated times with medium alone (Control) or medium containing 58035 (FC), 100 U/mL IFNγ, or acetyl-LDL, 58035, and IFNγ (FC + IFNγ). Whole-cell lysates were then prepared and subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, CHOP, and β-actin. In the CHOP blot, a nonspecific band is indicated by the asterisk. B, Macrophages from WT or Stat1−/− mice were incubated for 13 hours with medium alone (Control) or medium containing 100 U/mL IFNγ, acetyl-LDL plus 58035 (FC), or acetyl-low-density lipoprotein, 58035, and IFNγ (FC + IFNγ). Apoptosis was assayed and quantified as in Figure 1.

*P=0.01 for FC and P<0.001 for FC + IFNγ by Bonferroni after ANOVA.
ER stress by the cholesterol trafficking inhibitor U18666A4 was necessary for STAT1 serine phosphorylation. We recently showed that buffering cytosolic calcium with 1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis [acetoxymethyl ester] (BAPTA-AM) markedly inhibited both FC-induced and thapsigargin/fucoidan-induced apoptosis.15 To test the role of cytosolic calcium in STAT1 serine phosphorylation, we incubated FC-loaded macrophages with increasing concentrations of BAPTA-AM or equivalent volumes of vehicle control. As shown in Figure 5A, BAPTA-AM suppressed FC-induced serine phosphorylation of STAT1 in a dose-dependent manner.

One mechanism by which cytosolic calcium might participate in STAT1 serine phosphorylation is by activating CaMKII, which may directly phosphorylate STAT112 and/or lead to its phosphorylation by enhancing TLR4 signaling (see Discussion).15,29,30 As shown in Figure 5B, FC loading led to a rapid and marked enhancement of CaMKII threonine phosphorylation, which is a marker of its activation. At the 30- and 60-minute time points, the degree of activation was similar to that of the calcium ionophore A23187, a known potent activator of CaMKII. Similar results were found with fucoidan plus thapsigargin (data not shown). Note that the time course of CaMKII activation by FC loading or by thapsigargin plus fucoidan precedes the onset of STAT1 serine phosphorylation in these cells. To show a functional role for CaMKII activation in both STAT1 serine phosphorylation and apoptosis in FC-loaded macrophages, we used 2 structurally diverse CaMKII inhibitors. The data in Figure 5C and 5D show that the chemical CaMKII inhibitor KN93,31 but not the inactive homologue KN92, and the peptide CaMKII inhibitor AIP32 markedly suppressed FC-induced STAT1 serine phosphorylation. Most important, KN93 but not KN92 suppressed FC-induced apoptosis by 92% (P<0.05) (Figure 5E). Note that neither KN93 nor AIP decreased the uptake or ER trafficking of lipoprotein-derived FC or the induction of CHOP (data not shown and Figure 5D). In summary, these data indicate that cytosolic calcium and CaMKII are essential for STAT1 serine phosphorylation and apoptosis in the SRA-ER stress model. We also conducted experiments on 2 additional macrophage models, namely mouse bone marrow–derived and human peripheral blood–derived macrophages. In both of these cell types, the SRA–ER stress model exclusively induced STAT1 serine phosphorylation via a pathway mediated by cytosolic calcium and CaMKII (see the Figure in the online-only Data Supplement), suggesting the universality of this signaling pathway among macrophages.

**Figure 4.** STAT1 serine phosphorylation in SRA-engaged, ER-stressed macrophages is amplified by ER stress and requires TLR4 activation. Whole-cell lysates were subjected to immunoblot analysis to detect STAT1 pS727 and total STAT1 under the following conditions. A, Macrophages were incubated for the times indicated with acetyl-LDL and 58035 STAT1 under the following conditions. A, Macrophages were incubated for the times indicated with acetyl-LDL or acetyl-LDL, 58035 (FC-loaded) or acetyl-LDL, 58035, and 1 μmol/L U18666A (FC-loaded+U18666A). B, Macrophages were incubated for the times indicated with 0.5 μmol/L thapsigargin, 50 μg/mL fucoidan plus thapsigargin, or fucoidan alone. C, Macrophages from WT or Tlr4−/− mice were incubated for the times indicated under FC-loading conditions.
assess expression of Ser-P-STAT1 in murine and human atheromata (Figures 6 and 7). In mouse lesions, Ser-P-STAT1 was present in numerous macrophage foam cells, as assessed by staining adjacent sections with anti–Mac-3 antibody (Figure 6A and 6B) and Oil Red O (Figure 6D). As illustrated by these images, Ser-P-STAT1 staining also was observed in the endothelial cells lining the lumen, which was PECAM-1 positive (not shown), and in smooth muscle cells in the media, which were β-actin positive (Figure 6F). In human lesions, staining of Ser-P-STAT1 was found in the advanced stages called pathological intimal thickening and fibroatheroma (Figure 7B and 7C).
but not in the early stage of diffuse intimal thickening (Figure 7A). In the advanced lesions, most of the Ser-P-STAT1 colocalized with macrophages (Figure 7B and 7C). Note that Ser-P-STAT1 was found in the nuclei of these cells (Figure 7C, bottom middle) and in areas that were TUNEL positive, a marker of apoptosis (Figure 7C, bottom right). Of interest, some of the Ser-P-STAT1 in the most advanced fibroatheroma was found in macrophages surrounding necrotic areas (Figure 7C, top middle, asterisk).

To further investigate a causal link between STAT1 and lesional macrophage apoptosis, we compared advanced plaques of Western diet–fed Ldlr−/− mice reconstituted with either WT or Stat1−/− bone marrow. The mice were fed the Western diet for 10 or 12 weeks. Plasma lipoprotein cholesterol and body weight were similar between the 2 groups of mice (Figure 8A for 10-week protocol; data not shown for 12-week protocol). In the 10-week study, overall lesion areas were similar (Figure 8B and 8C). However, the number of TUNEL-positive cells in macrophage-rich regions was decreased by 61% (P=0.034) in the Stat1−/−→Ldlr−/− lesions, and a trend toward decreased plaque necrosis existed that did not quite reach statistical significance (P=0.078) (Figure 8C). Note that total macrophage area was not affected by STAT1 deficiency (120.0±11.8×103 and 111.5±21.3×103 μm² in WT and Stat1−/− bone marrow recipients, respectively; P=0.72; see Discussion).

Plaque necrosis likely results from the eventual cellular necrosis of macrophages that become apoptotic but are not subsequently cleared by phagocytes.1–2 Therefore, we predicted that as the lesions in the 2 groups of mice progressed, the difference in necrotic core areas would become statistically significant, whereas apoptotic macrophages per se would become less numerous and less different between the 2 groups of mice. As shown by the data in Figure 8D, the necrotic cores were larger in the 12-week-diet mice, and a statistically significant difference was present in the necrotic core area (34% decrease in the Stat1−/−→Ldlr−/− lesions; P=0.02) but not the number of TUNEL-positive cells. In summary, STAT1 deficiency in bone marrow–derived cells in Ldlr−/− mice has a substantial protective effect on apoptosis in the macrophage-rich lesions of advanced plaques and on plaque necrosis.

**Discussion**

Increasing evidence from a number of laboratories suggests that an ER stress–based model of macrophage apoptosis plays an important role in advanced lesional macrophage death and plaque necrosis.4–9 The work reported here adds critical new components to this model by demonstrating essential roles for STAT1 and CaMKII in macrophage apoptosis in vitro and for STAT1 in advanced lesional macrophage apoptosis and plaque necrosis in vivo.

Figure 6. STAT1 is serine phosphorylation in atherosclerotic lesions from Ldlr−/− mice. Adjacent frozen sections of an aortic root lesion from an Ldlr−/− mice fed a Western-type diet for 12 weeks were immunostained with anti–Ser-P-STAT1 or anti-Mac3 (macrophages) (A and B) or anti-Ser-P-STAT1, oil red O, nonimmune immunoglobulin G (IgG), and α-actin (C through F). Note examples of brown stain in the nuclei of the intimal cells (red arrows), endothelial cells (green arrows), and smooth muscle cells (SMC) in the media (blue arrows). The dark streaks at the intima-media interface in E represent nonspecific staining.
Further studies are required to define at a precise molecular level how the proapoptotic components elucidated in this study fit into the overall scheme of the multihit model of macrophage apoptosis. Our working hypothesis is depicted in Figure 9. We suggest that ER stress triggers 2 key proapoptotic processes: UPR/CHOP and another pathway in which ER stress–induced cytosolic calcium activates CaMKII, which in turn leads to serine phosphorylation of proapoptotic STAT1. Activation of the TLR4-MyD88 pathway by SRA ligands, which is critical for apoptosis, also contributes to STAT1 serine phosphorylation. SRA ligands additionally promote apoptosis through SRA-dependent suppression of prosurvival IFNβ.

This scheme raises a number of critical issues that require further investigation. Among these is whether STAT1 serine phosphorylation per se is required for apoptosis, which is consistent
Figure 8. STAT1 plays a role in advanced lesional macrophage apoptosis and plaque necrosis in female Stat1+/−→Ldlr−/− mice. A, The table shows plasma cholesterol and body weight of Ldlr−/− mice transplanted with Stat1+/+ or Stat1−/− bone marrow and then fed a Western-type diet for 10 weeks starting 6 weeks after transplantation. The graph shows pooled plasma samples from 3 Stat1+/+ and 3 Stat1−/− recipient mice that were fractionated by fast protein liquid gel-filtration chromatography and then assayed for cholesterol. None of the differences in cholesterol, lipoproteins, or body weight were statistically significant. T. Chol indicates total cholesterol; HDL, high-density lipoprotein. B, Hematoxylin and eosin staining of proximal aortas from Ldlr−/− mice fed a Western diet for 10 weeks that were transplanted with bone marrow from Stat1+/+ and Stat1−/− mice. Total lesion area was 493.7±40.5 and 380.6±24.4 μm² in Stat1+/+→Ldlr−/− and Stat1−/−→Ldlr−/− mice, respectively. Bar=20 μm. C, TUNEL (red), DAPI (blue), macrophage (brown), and SMC (brown) staining of lesions similar to those in B. Bar=20 μm. The graph shows quantification of lesion area, TUNEL-positive cells, and necrotic area (Nec) in the lesions of Stat1+/+→Ldlr−/− and Stat1−/−→Ldlr−/− mice. *P=0.034 by Student t test. D, Hematoxylin and eosin staining of proximal aortas from Ldlr−/− mice fed a Western diet for 12 weeks that were transplanted with bone marrow from Stat1+/+ and Stat1−/− mice (n=18 for both groups of mice). Bar=20 μm. The graph shows quantification of TUNEL-positive cells and necrotic area in the lesions of Stat1+/+→Ldlr−/− and Stat1−/−→Ldlr−/− mice. P=0.02 by Student t test.
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with our data and with previous work showing a proapoptotic role of Ser-P-STAT1 in apoptosis in other systems. However, definitive proof requires comparing SRA/ER stress–induced apoptosis in macrophages containing S727- with Y701-mutated STAT1. Until then, we cannot definitively rule out the possibility that apoptosis requires Y701 phosphorylation and that Tyr-P-STAT1 in our SRA–ER stress model is below the limit of immunoblot detection. In pilot studies, we found that apoptosis induced by thapsigargin and fucoidan was markedly suppressed in peritoneal macrophages from S727A-STAT1/− mice, but results with FC-induced apoptosis were not shown). See Discussion for details and for a description of the areas of uncertainty in this model.

Figure 9. Integration of calcium, CaMKII, and STAT1 into the multihit pathway of macrophage apoptosis. According to this working hypothesis, ER stress–induced increase in cytosolic calcium triggers 2 proapoptotic hits: UPR/CHOP and a pathway involving CaMKII and Ser-P-STAT1. TLR4 activation also contributes to STAT1 serine phosphorylation. Ser-P-STAT1 is depicted as a separate pathway from CHOP because studies with Chop−/− and Stat1−/− macrophages showed that CHOP is neither upstream nor downstream of Ser-P-STAT1 (data not shown). See Discussion for details and for a description of the areas of uncertainty in this model.

The impetus for this study was to explore pathways that may be involved in promoting macrophage apoptosis in atherosclerosis. The ultimate significance of lesional macrophage apoptosis likely depends on lesion stage. In early lesions, rapid and efficient phagocytic clearance of apoptotic macrophages appears to limit lesion cellularity and progression. Of interest, STAT1 may have a separate role in these early lesions that is independent of macrophage death because STAT1 deficiency in APOE−/− mice blocks foam cell formation and early lesion development. In advanced lesions, however, evidence exists that clearance of apoptotic cells is defective, leading to postapoptotic macrophage necrosis, inflammation, and eventually overall plaque necrosis. In this context, the multihit model of macrophage apoptosis is likely most relevant to advanced lesions. For example, immunoblots have shown that CHOP is expressed only in advanced lesions and that manipulation of ER stress in vivo is positively associated with advanced atherosclerotic lesions, not negatively associated with early lesion progression. In the case of STAT1 deficiency, a clear trend toward decreased plaque necrosis was present. However, the maximum effect on plaque necrosis may lag behind that of macrophage apoptosis because plaque necrosis likely results from the progressive coalescence of apoptotic macrophages after they become secondarily necrotic. Another prediction from this idea and from the fact that the anti-macrophage antibody used in our study recognizes necrotic apoptotic macrophages is that total macrophage area should be similar in Stat1−/−→Ldlr−/− and Stat1−/−→Ldlr−/− lesions, exactly as we observed experimentally. More fundamentally, we clearly did not observe an increase in lesion area in the Stat1−/− group, which is what is found when early lesional macrophage apoptosis is blocked. In terms of other studies linking STAT1 to advanced plaque progression, in vivo data suggest that interleukin-10, which suppresses STAT1 activity, may protect advanced atheroma from macrophage apoptosis and plaque necrosis. Moreover, Koga et al. reported that blocking the function of the STAT1 activator IFNγ stabilized advanced plaques in APOE−/− mice. Thus, pending further in vivo studies, local inhibition of STAT1 activity may represent a potentially promising therapeutic strategy to prevent the progression of relatively benign lesions to those with increased macrophage apoptosis and plaque necrosis.

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Disclosures

None.
References


CLINICAL PERSPECTIVE

In industrialized societies, virtually all young adults have atherosclerosis. Most of these lesions are asymptomatic and will remain so for the rest of the person’s life. However, a small percentage will progress to a dangerous stage involving plaque breakdown, acute luminal thrombosis, and acute vascular events like myocardial infarction and sudden cardiac death. Thus, a major goal is to elucidate the cellular-molecular events involved in benign-to-vulnerable plaque progression. A key feature of vulnerable plaques is necrotic cores, which likely promote plaque breakdown and acute thrombosis. Necrotic cores are “graveyards of dead macrophages,” a prominent cell type in atherosclerosis. This study used a cell-culture model of macrophage death to explore death-promoting molecules that may be relevant to advanced atherosclerosis. These experiments revealed an important role for a calcium-signaling pathway involving 2 molecules, calcium/calmodulin-dependent protein kinase II and signal transducer and activator of transcription-1 (STAT-1). Both mouse and human advanced atheromata have activated STAT-1. Most important, when macrophages were made deficient in STAT-1 in a mouse model of advanced atherosclerosis, macrophage death and plaque necrosis were diminished. Two important caveats of this study need to be mentioned. First, the processes of macrophage death and plaque necrosis are complex, so the calcium/calmodulin-dependent protein kinase II–STAT1 pathway represents only 1 piece of the puzzle. Second, the mouse is a poor model of plaque disruption and acute thrombosis. Thus, additional studies are needed to explore other pathways involved in advanced lesional macrophage death, and improved mouse models are required to prove the hypothesis that macrophage death and plaque necrosis promote plaque disruption and acute thrombosis. Nonetheless, this study provides important new molecular-cellular information related to the progression of advanced atherosclerotic lesions—information that someday may be translated into therapy designed to block benign-to-vulnerable plaque transformation.
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In the version of the article, “Signal Transducer and Activator of Transcription-1 Is Critical for Apoptosis in Macrophages Subjected to Endoplasmic Reticulum Stress In Vitro and in Advanced Atherosclerotic Lesions In Vivo,” by Lim et al that was posted online on January 28, 2008 (DOI: 10.1161/CIRCULATIONAHA.107.711275), several errors occurred.

In the Figure 6 legend, starting 12 lines from the top, the wrong color arrows are indicated. The text should read “... endothelial cells (green arrows) and smooth muscle cells (SMC) in the media (blue arrows).”

On the same page, left column, ninth line from the top, the callout for Figure 7C should read, “Figure 7C, top middle, asterisk” rather than “Figure 7C, bottom left, asterisk.”

In the Figure 7 legend, 9 lines from the top, the sentence should begin, “The lower middle image shows...” rather than “These lower middle images show...”

In the Figure 8 legend, the wording for panel C was incomplete. It should read, “C, TUNEL (red), DAPI (blue), macrophage (brown), and SMC (brown) staining of lesions similar to those in B.”

Also in the Figure 8 legend, the following sentence, currently located toward the end of the legend, should have appeared with the description of panel B: “Total lesion area was 493.7±40.5 and 380.6±24.4 μm² in Stat1−/−→Ldlr−/− and Stat1−/−→Ldlr−/− mice, respectively.”

These errors have been corrected in the current online version and in the final print version of the article in the February 19, 2008, issue of the journal (Circulation. 2008;117:940–951). The authors regret these errors.

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SUPPLEMENTAL METHODS (Lim et al.)

Materials
Tissue culture media, cell culture reagents, and heat-inactivated FBS (GIBCO BRL) were purchased from Invitrogen. The acyl-coenzyme A-cholesterol acyltransferase (ACAT) inhibitor 58035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide (1) was from J. Heider, formally of Sandoz (East Hanover, NJ). A 10-mg/ml stock was made in DMSO and used at a concentration of 10 µg/ml in all experiments. Recombinant mouse IFNγ was from the PBL Biomedical Laboratories. The Vybrant Annexin V/Propidium Iodide Apoptosis Assay kit #2 was from Molecular Probes. LDL (d, 1.020-1.063 g/ml) from fresh human plasma was isolated by ultracentrifugation (2). Acetyl-LDL was prepared from LDL by reaction with acetic anhydride, as described previously (3), and used at a concentration of 50 µg/ml in all experiments. All other chemical reagents, including tunicamycin, thapsigargin, concanavalin A, and fucoidan, were purchased from Sigma-Aldrich.

Endotoxin Testing
Reagents used in this study were tested for endotoxin contamination by using the Limulus Amebocyte Lysate (LAL) kit (Cambrex, Walkersville, MD) and found to have <0.06 EU/ml endotoxin at working dilutions.

Eliciting and Culturing Mouse Peritoneal Macrophages
Macrophages were obtained from 8–10-wk-old female C57BL/6J mice (Jackson Laboratory); Stat1−/− mice on a C57BL/6J background (4); or Tlr4del mice on a C57BL/10ScNJ background and wild-type C57BL/10ScSnJ background (Jackson Laboratory). The macrophages were harvested either three days after intraperitoneal (i.p.) injection of concanavalin A (5) or after immunization with methyl-BSA (6). For the latter method, 2 µg/ml methyl-BSA in 0.9% saline was emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco). Mice were immunized intradermally with 100 µl of the emulsion. After 14 days, the immunization protocol was repeated, except incomplete Freund’s adjuvant was used instead of CFA. Seven days later, the mice were injected i.p. with 0.5 ml PBS containing 100 µg methyl-BSA and then the macrophages were harvested 4 days after that by peritoneal lavage. All macrophages were
cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 20% L-cell conditioned medium for 48-72 h, at which point they were typically at ~90% confluency.

**Immunoblot Analysis**

Cell lysates were prepared by homogenization in 1X sample buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue, and boiled at 100°C for 5 min. Cytosolic and nuclear extracts were isolated using the Nuclear Extraction Kit (Panomics) according to the manufacturer's protocol. Cell extracts were electrophoresed on 4–20% gradient SDS-PAGE gels (Invitrogen) and transferred to 0.45-µm nitrocellulose membranes. The membranes were blocked in Tris-buffered saline, 0.1% Tween 20 (TBST), containing 5% (w/v) nonfat milk at room temperature for 1 h and then incubated with primary antibodies diluted in TBST containing 5% (w/v) nonfat milk or 5% BSA (w/v) at 4 °C overnight. This incubation was followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 h. Proteins were detected by SuperSignal West Pico-enhanced chemiluminescent solution (Pierce Chemical Co.). When required, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce Chemical Co.) for 15 min at room temperature and reprobed for β-actin (loading control) or other proteins. Antibodies against total STAT1, GADD 153 (CHOP), and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. Antibodies against phospho-Thr286/287 CaMKII, CaMKII, phospho-Ser727 STAT1, and phospho-Tyr701 STAT1 were purchased from Cell Signaling Technology. Anti-nucleophosmin antibody was purchased from Zymed, and anti-β-actin mouse monoclonal antibody was from Chemicon International. The HRP-conjugated donkey anti–mouse and donkey anti–rabbit IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

**Plasma Cholesterol Assays**

Total plasma cholesterol was determined using an enzymatic kits from Wako Chemicals GmbH. Plasma high-density lipoprotein (HDL) cholesterol was determined after dextran sulfate-Mg²⁺ precipitation of apoB-containing lipoproteins. Lipoprotein-cholesterol profiles were determined by FPLC gel-filtration fractionation consisting of two Superose 6 columns connected in series (Amersharm Pharmacia).
Immunohistochemistry of Murine and Human Atherosclerotic Lesions

Ldlr−/− mice on the C57BL/6 background were fed a "Western-type" diet (21% anhydrous milk fat and 0.15% cholesterol from Harlan-Teklad; TD88137) for 10 weeks. After anesthetization, the hearts of the mice were perfused with PBS. The hearts and proximal aortae were harvested, perfused ex vivo with PBS, embedded in OCT compound, snap frozen in an ethanol-dry ice bath, and stored at −70°C. Sections of proximal aortae (6-μm thick) were prepared at −20°C by using a Microm Microtome Cryostat HM 505 E and placed on poly-L-lysine-coated slides (Fisher Scientific). The sections were then fixed in ice-cold acetone for 10 min, air dried for 10 min, and stored at −70°C until use. All of the following procedures were conducted at room temperature.

To detect phospho-S727 STAT1, the frozen sections were washed 3 times in PBS for 2 min, blocked for 1 h with 10% normal donkey serum (Jackson ImmunoResearch), and incubated for 1 h with 2.7 μg/ml rabbit anti-phospho-S727 STAT1 (Cell Signaling #9177) or, as a negative control, 2.7 μg/ml nonimmune rabbit IgG (Jackson ImmunoResearch). After washing 3 times in PBS, the sections were incubated for 30 min with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch). Finally, the sections were incubated with streptavidin-horseradish peroxidase and the DAB chromogen (both from BD Pharmingen), mounted in Permount, and viewed with an Olympus IX 70 inverted microscope using a ×40 objective. Parallel sections were stained with anti-Mac-3 (BD Pharmingen), anti-α-actin (Abcam), and anti-PECAM-1 (Santa Cruz Biotechnology) antibodies to label macrophages, smooth muscle cells, and endothelial cells, respectively. To stain macrophages in parafinized sections used for in-situ TUNEL analysis, sections were deparaffinized, rehydrated, blocked for 1 h with 10% normal donkey serum (Jackson ImmunoResearch), and incubated overnight with a rabbit anti-mouse macrophage antibody (AIA31240, Accurate Chemical & Scientific Corp.). The subsequent steps were identical to that of staining frozen sections described above. To visualize foam cells, frozen sections were fixed with buffered formalin and stained with Oil Red O for neutral lipid and Harris hematoxylin for nuclei.

For the human coronary artery study, the heart of a patient who had died suddenly of coronary causes was obtained as described previously (7). Coronary segments (3-4 mm in length) were frozen, cryosectioned, and stored at −80°C until use. For immunostaining, the sections were thawed, fixed in cold acetone (−20°C), air-dried, and stained with primary antibodies against phosphorylated STAT1 (Millipore, Lake Placid, NY, Catalog # 07-714,
dilution 1:300) or the macrophage marker CD68 (Dako, Carpinteria, CA, Catalog #M0814, dilution 1:600) for 1 hour. Primary antibodies were labeled using an EnVision+ System, peroxidase kit (Catalog # K4009) for 30 min. Negative control staining for STAT1 was performed with non-immune IgG (Dako, catalog # X0903) at a similar protein concentration. Finally the signal was visualized by a 3-amino-9-ethylcarbozole substrate-chromogen system producing a rose red color; the sections were counterstained with Gill hematoxylin. For co-immunostaining with anti-Ser-P-STAT1 and anti-CD68, anti-Ser-P-STAT1 was first visualized using diaminobenzidine (DAB) tinted with nickel chloride (black color). The sections were then immunostained for CD68 and visualized using an alkaline phosphatase ABC kit (Vector, Burlingame, CA) with Vector red; sections were then counterstained with methyl green.

Apoptosis was identified by TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick-end labeling) using TdT from VasoTACS, Trevigen, Gaithersburg MD. Human tonsil served as a positive control. The reaction produce was visualized with DAB tinted with nickel chloride. The section was then stained with CD68 at 1:600 for 1 hour and developed with alkaline phosphatase red substrate kit (Vector, Burlingame, CA). The sections were then counterstained with methylgreen.

Mouse bone marrow-derived macrophages (mBMDM) were collected as described in Methods and cultured for 7 days to permit differentiation into mature macrophages. (A) mBMDM were incubated with acetyl-LDL plus the ACAT inhibitor 58035 (FC-loaded), 50 μg/ml fucoidan and 0.5 μM thapsigargin (TG+Fuc), or 100 U/ml IFNγ for the indicated times. 

(B) mBMDM were incubated with 50 μg/ml fucoidan and 0.5 μM thapsigargin (TG+Fuc) plus 50 μM KN93 or 50 μM KN92 for 2 h.

Human monocyte-derived macrophages (hMDM) were isolated from normal human buffy coats as previously described (Bottalico et al. [1993] J Biol Chem 268: 8569-8573) and cultured in the presence of 1 ng/ml GM-CSF for 10 days. (C) hMDM were incubated with acetyl-LDL plus the ACAT inhibitor 58035 (FC-loaded), 50 μg/ml fucoidan and 0.5 μM thapsigargin (TG+Fuc), or 100 U/ml IFNγ for the indicated times. 

(D) hMDM were incubated with 50 μg/ml fucoidan and 0.5 μM thapsigargin (TG+Fuc) plus 10 μg/ml BAPTA or vehicle control for the indicated times. Whole-cell lysates were then subjected to immunoblot analysis to detect phospho-S727 STAT1 (Stat1 pS727), phospho-Y701 STAT1 (Stat1 pY701), and total STAT1.