Macrophage Apoptosis Exerts Divergent Effects on Atherogenesis as a Function of Lesion Stage

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Background—Because apoptotic cell clearance appears to be defective in advanced compared with early atherosclerotic plaques, macrophage apoptosis may differentially affect plaque progression as a function of lesion stage.

Methods and Results—We first evaluated the impact of targeted protection of macrophages against apoptosis at both early and advanced stages of atherosclerosis. Increased resistance of macrophages to apoptosis in early atherosclerotic lesions was associated with increased plaque burden; in contrast, it afforded protection against progression to advanced lesions. Conversely, sustained induction of apoptosis in lesional macrophages of advanced lesions resulted in a significant increase in lesion size. Such enhanced lesion size occurred as a result not only of apoptotic cell accumulation but also of elevated chemokine expression and subsequent intimal recruitment of circulating monocytes.

Conclusions—Considered together, our data suggest that macrophage apoptosis is atheroprotective in fatty streak lesions, but in contrast, defective clearance of apoptotic debris in advanced lesions favors arterial wall inflammation and enhanced recruitment of monocytes, leading to enhanced atherogenesis. (Circulation. 2009;119:1795-1804.)

Key Words: atherosclerosis ■ cholesterol ■ inflammation ■ leukocytes ■ macrophages ■ pathology ■ survival

Atherosclerosis is an inflammatory vascular disease characterized by the intimal accumulation of macrophage foam cells, cell death, and chronic arterial inflammation.1 Macrophage apoptosis has been identified as a prominent feature of atherosclerotic plaques because macrophage cell death is believed to support necrotic core growth. The apoptotic process is controlled by intracellular levels of proapoptotic and antiapoptotic proteins such as those of the Bcl-2 family. Indeed, the relative expression of proapoptotic (eg, Bax and Bak) and antiapoptotic proteins (eg, Bcl-2 and Bcl-xL) of the Bcl-2 family determines the overall sensitivity of the cell to apoptotic stimuli. In macrophages of atherosclerotic lesions, the proapoptotic Bax and Bak proteins predominate, whereas the antiapoptotic Bcl-2 and Bcl-xL are deficient,2,3 thereby arguing for their enhanced susceptibility to apoptosis. However, the impact of macrophage apoptosis on plaque progression remains to be specifically investigated.

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Recent studies have shed light on the potential impact of apoptosis on atherosclerotic lesion progression. Indeed, disruption of either the proapoptotic molecule Bax in bone marrow–derived cells4 or the antiapoptotic factor AIM5 has revealed that apoptosis attenuates early plaque formation. However, because apoptotic cells accumulate preferentially in advanced rather than in early lesions,6,7 macrophage apoptosis may differentially affect plaque progression as a function of lesion stage.8 In addition, apoptotic cell clearance appears to be defective in advanced lesions but efficient in early ones.9 Moreover, apoptotic cells may possess proinflammatory properties, in part as a result of the presence of oxidized phospholipids (oxPLs) at their surface,10,11 which are known triggers of inflammatory responses in arterial tissues.12 In this setting, studies of mice in which components of the apoptotic cell clearance machinery have been deleted13 or of lupus-prone mice characterized by ineffective apoptotic cell clearance14 have revealed that defective apoptotic cell clearance is associated with enhanced atherosclerotic plaque progression.

To understand the impact of macrophage apoptosis on atherosclerosis at both early and advanced stages of lesion progression, we first used a transgenic approach allowing specific protection of macrophages against apoptosis (CD68−hBcl-2 mice). With this approach, we demonstrated that macrophage apoptosis was atheroprotective during the early stages of atherosclerosis, whereas macrophage cell death accelerated plaque progression in more advanced lesions. To mirror this effect, we applied a complementary approach based on sustained induction of lesional macrophage apo-
sis in CD11c-DTR transgenic mice and demonstrated that apoptotic cell accumulation in advanced lesions enhances plaque progression. Finally, short-term induction of macrophage apoptosis provided evidence that apoptotic cell accumulation in advanced atherosclerotic lesions promotes inflammatory gene expression, circulating monocyte recruitment, and accumulation of newly recruited macrophages.

Methods
Transgenic Animals and Atherosclerosis
Studies Design
Transgenic mice overexpressing the antiapoptotic protein hBcl-2 in macrophages (Mo-hBcl-2 mice) were generated using homologous recombination in embryonic stem cells to produce a single-copy transgene insertion at the hypoxanthine phosphoribosyl transferase (Hprt) locus located on the X chromosome. The transgene consists of the human hBcl-2 cDNA from pORF-hBcl-2 (InvivoGen, San Diego, Calif) cloned downstream of the mouse macrophage-specific promoter CD68 from pDRIVE-mCD68 (Invivogen). Mo-hbcl-2 and Apoe<sup>–/–</sup> mice were crossed to obtain Apoe<sup>–/–</sup> and Mo-hBcl-2 Apoe<sup>–/–</sup> mice, and littersmates were used for all experiments. Because the Hprt locus is located on the X chromosome, all experiments were performed with male littersmates to avoid the potential variability of transgene expression related to random inactivation of the X chromosome in females. At 6 weeks of age, mice were fed a Western diet (WD; 0.15% cholesterol and 20% saturated fat) for 5 or 15 weeks. Apoe<sup>–/–</sup> and CD11c-DTR-GFP<sup>15</sup> mice on the C57BL/6J background were obtained from Charles River Laboratories (Wilmington, Mass) and the European Mouse Mutant Archive (Centre National de la Recherche Scientifique, Centre de Distribution, de Typage et d’Archivage animal, Orléans, France), respectively, and crossed to obtain CD11c-DTR Apoe<sup>–/–</sup> mice. To evaluate the impact of sustained induction of macrophage apoptosis in atherosclerotic plaques, Apoe<sup>–/–</sup> mice (8 weeks old) were lethally irradiated, transplanted with 3×10<sup>5</sup> bone marrow cells from CD11c-DTR Apoe<sup>–/–</sup> mice, and fed a WD after a 4-week recovery period. After 5 weeks of WD feeding, mice were injected with either diphtheria toxin (DT; 4 ng/g; Sigma, St Louis, Mo) or PBS every 10 days for 50 days and killed 48 hours after the last injection. To study the impact of short-term induction of lesional macrophage apoptosis, 6-week-old Apoe<sup>–/–</sup> and CD11c-DTR Apoe<sup>–/–</sup> mice were fed a WD for 8 weeks to develop atherosclerotic lesions, injected intravenously with DT (4 ng/g) or vehicle, and killed 48 hours later. All animal procedures were performed with accreditation from the French government and under strict compliance with animal welfare regulations.

Plasma Lipid Analyses, Quantification of Atherosclerotic Plaques, Immunohistochemistry, Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick-End Label Staining, Analysis of Gene Expression by Quantitative Polymerase Chain Reaction, and Flow Cytometry
All these procedures were performed as previously described<sup>14,16</sup> (see the Methods section in the online-only Data Supplement).

Quantification of Anti-Malondialdehyde-Modified Low-Density Lipoprotein, Anti–Oxidized Low-Density Lipoprotein, E06 Antibodies, and Serum-Oxidized Phospholipids
Antibody titers to oxidation-specific epitopes of malondialdehyde-modified (MDA) low-density lipoprotein (LDL) or copper-oxidized LDL (oxLDL) and T15 clonotypic (E06) natural antibodies were determined as described.<sup>17</sup>

Labeling of Blood Monocytes and Recruitment Assays
Monocyte labeling was performed as previously described.<sup>18</sup> To assess the recruitment of blood monocytes, 6-week-old Apoe<sup>–/–</sup> and CD11c-DTR Apoe<sup>–/–</sup> mice were fed a WD for 8 weeks, injected intravenously with 200 ng DT or vehicle and 250 μL of yellow green beads, and killed 48 hours later.

Statistical Analysis
Statistical calculations were performed with GraphPad Prism, version 4.03. Results were analyzed by Student unpaired t-tests with the Welch correction if variances were unequal or Mann–Whitney U test as indicated in the figure legends. The statistical significance of the differences between 2 groups was compared by ANOVA followed by the Newman–Keuls multiple-comparison test. Values of P<0.05 were considered significant.
Generation and Characterization of Mø-hBcl-2 Mice

To selectively overexpress hBcl-2 cDNA in macrophages, we constructed a vector in which the expression of the human form of the antiapoptotic Bcl-2 gene is driven by the macrophage-specific promoter CD68 (Figure 1A). The CD68-hBcl2 (Mø-hBcl-2) transgene transmitted with the expected frequency, and Mø-hBcl-2 or Mø-hBcl-2 Apoe−/− mice exhibited no obvious developmental or morphological abnormalities on the C57BL/6J genetic background (data not shown). To confirm expression of the transgene, hBcl-2 mRNA and protein were measured in thioglycollate-elicited peritoneal macrophages from Mø-hBcl-2 and control mice by reverse-transcription polymerase chain reaction (Figure 1B) and flow cytometry (Figure 1C). To evaluate the impact of hBcl-2 expression on apoptosis of macrophages, ox-LDL-induced caspase-3 activity was measured. Macrophages derived from Mø-hBcl-2 mice were markedly protected against oxLDL-induced apoptosis compared with nontransgenic controls (P<0.01; Figure 1D).

We next evaluated whether hBcl2 overexpression in macrophages exerted an impact on foam cell formation. Macrophages from Mø-hBcl-2 or control mice displayed similar cholesterol-loading capacity on incubation with acetylated LDL (Figure 1E). In addition, because CD68 also is expressed in circulating monocytes, we assessed the expression of the transgene in this cell type. Cytometric analysis revealed that splenic CD19+ B cells, CD4+ and CD8+ T cells, and CD11chigh dendritic cells from Mø-hBcl-2 mice did not express hBcl2, whereas blood CD115+ monocytes did at a low level (Figure I of the online-only Data Supplement). Consistent with this finding, we found that monocyte count was 40% higher in the Mø-hBcl-2 Apoe−/− mice than in wild-type Apoe−/− mice on both chow and WD (Figure 1F).

Impact of Macrophage Resistance to Apoptosis on Atherogenesis

To assess the impact of macrophage resistance to apoptosis on the progression of early versus advanced atherosclerotic lesions, Mø-hBcl-2 Apoe−/− and Apoe−/− littermates were fed a WD for 5 or 15 weeks. No significant differences in plasma lipid levels or body weight were found between Mø-hBcl-2 Apoe−/− and Apoe−/− mice at either time point (Table 1). After 5 weeks of diet, aortic root lesion area was 50% larger in Mø-hBcl-2 Apoe−/− mice than in Apoe−/− mice (P<0.03; Figure 2A). As expected, 10 additional weeks on diet shifted the early cellular lesions to larger (>10-fold) and more complicated lesions with signs of necrotic core formation. In contrast to the 5-week time point, the mean area of the aortic root lesion in Mø-hBcl-2 Apoe−/− mice was 25% smaller than that in controls after 15 weeks of diet (P<0.04; Figure 2B). At this time point, en face analysis of the descending aorta confirmed this finding; the lesion area was 50% smaller in...
Mφ-hBcl-2 Apoe<sup>−/−</sup> mice compared with controls (P<0.04; Figure 2C).

To determine whether lesional macrophage number was equally influenced in Mφ-hBcl-2 Apoe<sup>−/−</sup> mice, CD68-positive area was quantified by immunohistochemistry on aortic sinus sections. After 5 weeks of diet, macrophage area was 50% greater in Mφ-hBcl-2 Apoe<sup>−/−</sup> mice than in littermate controls (P<0.01; Figure 3A), whereas after 15 weeks of this diet, the macrophage area was 35% lower in Mφ-hBcl-2 Apoe<sup>−/−</sup> mice (P<0.01; Figure 3B). We next quantified apoptotic cell accumulation in aortic sinus lesions. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end label (TUNEL)–positive cells were detectable only in advanced lesions (15 weeks) but not in early ones (5 weeks), consistent with earlier reports. At 15 weeks of diet, apoptotic cells in plaques from Mφ-hBcl-2 Apoe<sup>−/−</sup> mice were 3-fold less abundant than in controls (P<0.003; Figure 3C), corresponding to a 3-fold reduction in apoptotic cell content per 1 mm<sup>2</sup> lesion (P<0.003; Figure 3D). Because macrophages undergoing apoptosis in advanced plaque express tissue factor, we investigated its pattern of expression in early and advanced lesions of Mφ-hBcl-2 Apoe<sup>−/−</sup> mice and controls. Tissue factor staining localized at the site of lipid accumulation mainly in acellular areas of advanced lesions (online-only Data Supplement Figures II and III). However, no difference in staining was revealed between Mφ-hBcl-2 Apoe<sup>−/−</sup> mice lesions and controls at this advanced stage of plaque growth.

**Impact of Macrophage Resistance to Apoptosis on Circulating Levels of Autoantibodies Against Oxidation-Specific Epitopes and Levels of Oxidized Phospholipids**

Apoptotic cells contain bioactive oxPL and other oxidized lipids on their cell surface and are highly immunogenic, leading primarily to an enhanced IgM response to oxidation-specific epitopes in immunized mice. In this context, we evaluated serum levels of autoantibodies to oxidation-specific epitopes and the quantity of circulating oxPL on apoB-100 lipoproteins. After 5 weeks of WD, levels of IgG1, IgG2c, IgG3, and IgM anti–MDA-LDL and anti-oxLDL antibodies were comparable in Apoe<sup>−/−</sup> and Mφ-hBcl-2 Apoe<sup>−/−</sup> mice (Figure 4A and 4B). Plasma levels of these IgG subspecies were not different between the 2 groups at 15 weeks of diet (Figure 4C and 4D); however, a significant increase in the titer of IgM anti–MDA-LDL antibodies (P<0.05; Figure 4C) and a trend toward higher IgM anti-oxLDL titers (Figure 4D) were observed in Mφ-hBcl-2 Apoe<sup>−/−</sup> mice compared with Apoe<sup>−/−</sup> mice. Moreover, titers of the T15/E06 natural antibody, which binds to oxPL, were more elevated in Mφ-hBcl-2 Apoe<sup>−/−</sup> mice after 15 weeks (Figure 4E). Without reaching statistical significance, a similar trend could be observed at the 5-week time point (Figure 4E). Next, because atherosclerotic lesions, including the apoptotic cells within them, are a likely source of oxPLs with proinflammatory and immunogenic properties, we measured circulating levels of oxPLs bound to apoB-100 lipoproteins in both groups of mice. After 5 and 15 weeks of the diet, the relative amounts of oxPL/apoB-100 were significantly lower in Mφ-hBcl-2 Apoe<sup>−/−</sup> mice compared with controls at each time point (Figure 4F).

**Effect of Sustained Induction of Apoptosis of Lesional Macrophages on Atherosclerotic Plaque Progression**

Because increased resistance to apoptosis of macrophages was associated with a reduced progression of advanced plaques, we designed an experimental strategy to evaluate whether sustained induction of apoptosis in established lesions would mirror this effect. To address this point, we used
CD11c-DTR-GFP transgenic mice that were developed to allow conditional depletion of CD11c-positive cells in vivo through administration of DT. The expression of the DT receptor (DTR)–green fluorescent protein (GFP) fusion protein driven by the CD11c promoter was evaluated on aortic root lesions of CD11c-DTR Apoe−/− mice by immunostaining and revealed that GFP-expressing cells represent a subpopulation of CD68+ lesional cells (Figure 5A). In this context, administration of DT should lead to the induction of apoptosis in a subpopulation of lesional phagocytes, including macrophages in CD11c-DTR Apoe−/− mice. Indeed, a marked accumulation of TUNEL+ cells was observed in lesions of CD11c-DTR Apoe−/− mice compared with controls 48 hours after DT injection (Figure 5B).

Because ongoing apoptosis of macrophages is thought to occur continuously during atherogenesis, we evaluated the impact of sustained induction of apoptosis of lesional cells on plaque progression. However, long-term repeated injection of DT in CD11c-DTR Apoe−/− mice was previously shown to be lethal. To circumvent this problem, the hematopoietic system of mice was selectively reconstituted with CD11c-DTR cells by lethally irradiating Apoe−/− mice, followed by reconstitution with CD11c-DTR Apoe−/− bone marrow cells. Plasma total cholesterol and triglyceride levels were similar in DT- and PBS-treated animals fed a WD for 12 weeks. However, we observed a 25% increase in plasma free cholesterol concentration 48 hours after the final DT injection (Table 2). We further examined this phenomenon in another set of animals and found that this effect was transient, lasting 48 to 72 hours, and correlated with DT-induced depletion of CD11c-positive cells (data not shown). Importantly, although the proportion of splenic dendritic cells was markedly decreased in Apoe−/− mice reconstituted with bone marrow cells from CD11c-DTR Apoe−/− mice 48 hours after DT injection, the percentage of blood monocytes remained unchanged (online-only Data Supplement Figure IV). Apoptotic cell accumulation was markedly increased in lesions of chimeric mice treated with DT compared with PBS-injected controls (P<0.0001; Figure 5C). Moreover, aortic root lesion area was 20% larger in transplanted mice treated with DT compared with animals treated with PBS (P=0.01, Figure 5D), whereas a larger difference was observed in the descending thoracic and abdominal aorta (5-fold) in the DT group compared with controls (P<0.0001; Figure 5E). Surprisingly, the degree of CD68+ macrophage immunoreactivity was similar in both groups (data not shown) 48 hours after the final DT injection and may reflect an increased recruitment of circulating monocytes (see below), an effect that could mask the loss of lesional macrophages in the DT-treated group.

**Effect of Short-Term Induction of Lesional Macrophage Apoptosis in Established Atherosclerotic Lesions**

To further examine the molecular and cellular mechanisms that may underlie the enhanced plaque progression observed...
on sustained induction of lesional macrophage apoptosis, we evaluated the impact of short-term DT treatment on vascular inflammation in established lesions. For this purpose, Apoe<sup>−/−</sup> and CD11c-DTR Apoe<sup>−/−</sup> mice with established atherosclerotic lesions were injected with DT and either PBS or DT, respectively, and the cellular response to lesional induction of apoptosis was evaluated 48 hours after injection. We first determined the content of apoptotic cells in the aortic sinus of the 3 groups of mice 2 days after injection. TUNEL staining revealed marked accumulation of apoptotic cells in CD11c-DTR Apoe<sup>−/−</sup> mice treated with DT compared with the control groups, thereby confirming that DT injection had induced apoptosis in lesions (P<0.05; Figure 6A and 6B). The persistence of apoptotic cells 2 days after DT injection in CD11c-DTR Apoe<sup>−/−</sup> mice is consistent with defective clearance of dead cells in advanced plaques as previously suggested<sup>20</sup> (P<0.05; Figure 6A and 6B). By comparison, TUNEL staining was not observed in the spleens of CD11c-DTR Apoe<sup>−/−</sup> mice 48 hours after DT treatment, suggesting efficient removal in this tissue (data not shown).

The accumulation of apoptotic cells in the lesions of the CD11c-DTR Apoe<sup>−/−</sup> mice was associated with dense DAPI staining indicative of accumulation of cells in TUNEL<sup>+</sup> areas (Figure 6A). These cells stained positively for the newly recruited macrophage marker CD11b<sup>+</sup> (Figure 6A), and quantitative analysis revealed that these CD11b<sup>+</sup> macrophages accumulated in the lesions of CD11c-DTR Apoe<sup>−/−</sup> mice treated with DT to a greater degree compared with controls (P<0.05; Figure 6C). Interestingly, we noted that

**Table 2. Lipid Levels and Body Weight of CD11c-DTR Apoe<sup>−/−</sup>→Apoe<sup>−/−</sup> Chimeric Mice Injected With PBS or DT After 12 Weeks of WD**

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<thead>
<tr>
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<th>+PBS</th>
<th>+DT</th>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>1035.2±45.0</td>
<td>1174.1±101.0</td>
<td>NS</td>
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<tr>
<td>Free cholesterol, mg/dL</td>
<td>302.5±9.5</td>
<td>377.8±27.0</td>
<td>&lt;0.05</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>125.4±15.1</td>
<td>124.3±11.1</td>
<td>NS</td>
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<tr>
<td>Free cholesterol/total cholesterol, %</td>
<td>29.3±0.4</td>
<td>33.0±3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.1±0.9</td>
<td>27.2±1.2</td>
<td>NS</td>
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NS indicates not significant.

*Figure 5. Sustained induction of lesional macrophage apoptosis increased atherosclerotic plaque progression. Expression of the CD11c-DTR-GFP transgene by macrophages in CD11c-DTR Apoe<sup>−/−</sup> mice lesions was determined by immunohistochemistry. The DTR-GFP fusion protein–expressing cells were localized with an anti-GFP antibody, and lesional macrophages were detected with CD68 staining on consecutive serial sections of CD11c-DTR Apoe<sup>−/−</sup> mice lesions (A). Apoptotic cells were detected by TUNEL staining on aortic root sections of either CD11c-DTR Apoe<sup>−/−</sup> mice treated with DT or PBS or Apoe<sup>−/−</sup> mice treated with DT 48 hours after injection. Photographs illustrate representative TUNEL staining from both groups of mice (B). Apoptotic cell accumulation was determined in CD11c-DTR Apoe<sup>−/−</sup>→Apoe<sup>−/−</sup> chimeric mice injected with DT and PBS-injected controls (C). The degree of atherosclerosis in Apoe<sup>−/−</sup> mice reconstituted with bone marrow cells from CD11c-DTR Apoe<sup>−/−</sup> that received DT or vehicle was determined by ORO staining of both aortic root sections (D) and the descending aorta (E) after 12 weeks of diet. Photographs illustrate representative ORO staining of aortic root sections or of the descending aortas from both groups of mice. *P<0.05; **P<0.0001.
Atherosclerotic lesions were stained with the monocyte/macrophage marker CD11b for visualization of newly recruited macrophages (green), the TUNEL method for detection of apoptotic cells (red), and DAPI for staining of nuclei (blue) (A). Photomicrographs are representative of 5 to 6 mice analyzed per group (magnification ×200). The ratio of TUNEL-positive areas to ORO-positive staining areas was calculated for each mouse (B). The degree of newly recruited macrophage (CD11b+) accumulation relative to lesion area was determined (C). Values represent the mean±SEM of 5 to 6 mice per group. *Statistically significant difference between CD11c-DTR Apoe−/− and control groups, P<0.05.

were significantly increased (P<0.05 each) in CD11c-DTR Apoe−/− mice treated with DT compared with control groups.

Effect of Short-Term Induction of Lesional Macrophage Apoptosis in Established Atherosclerotic Lesions on Recruitment of Circulating Monocytes

The observation that induction of apoptosis within the lesion was associated with higher levels of chemokines and the presence of CD11b+ cells strongly suggests that apoptotic cell deposition triggered the recruitment of monocytes. Thus, we designed an experiment to dynamically measure the level of monocyte infiltration in response to accumulation of apoptotic cells in the atherosclerotic vascular bed. Systemic injection of fluorescein-labeled microspheres (FITC-M) was used to label monocytes in the circulation. Within 18 hours after intravenous injection of microspheres, ~1% of leukocytes were FITC-M+, of which two thirds were monocytes (data not shown). Overall, ~10% of blood monocytes were phagocytically labeled with this approach (Figure 8A). Only a few beads were detected in atherosclerotic lesions of Apoe−/− mice 48 hours after injection of FITC-M, reflecting the “basal” recruitment of monocytes into the plaque (Figure 8B). The majority of the beads that had not been phagocytosed by peripheral blood leukocytes were found in the spleen (data not shown). In CD11c-DTR Apoe−/− mice, monocytes were labeled in vivo with FITC-M, whereas apoptosis was simultaneously induced in the lesions by DT injection. Forty-eight hours after injection, aortic root sections were prepared and stained both for TUNEL to detect apoptotic cells and with DAPI to visualize nuclei, whereas recruited monocytes were discriminated with green bead fluorescence. As shown in Figure 8C, DT injection induced apoptotic cell accumulation in lesions of CD11c-DTR Apoe−/− mice, which in turn promoted recruitment of circulating monocytes as evidenced by detection of FITC-M+ cells in the lesions of these mice; in contrast, rare beads were detected in control groups.

Discussion

Our data suggest that macrophage survival exerts proatherogenic effects during the early stages of atherosclerotic plaque progression, whereas it reduced plaque burden when lesions were at a more advanced stage. Second, we report that accumulation of apoptotic macrophages in established lesions has a major incidence on the vascular inflammatory response by promoting inflammatory gene expression, circulating monocyte recruitment, subsequent accumulation of newly recruited macrophages, and ultimately plaque progression.

The present experiments demonstrated that protection of macrophage cell death in Mø-hBcl-2 Apoe−/− mice enhanced lesion size compared with Apoe−/− littermate controls at early stages of plaque development (5 weeks of WD). At this early stage, lesions in both Apoe−/− and Mø-hBcl-2 Apoe−/− mice did not contain TUNEL-positive cells, consistent with previous studies that revealed that apoptotic cells are detectable only in advanced lesions.6,7 The absence of apoptotic cells in early lesions might reflect an efficient clearance mechanism at this stage rather than the absence of apoptosis6,9 because notably the free cholesterol–induced apoptosis pathway has...
been shown to be operative (unfolding protein response activation) in the early phases of plaque development.\textsuperscript{7,21} Furthermore, if lesions are larger in \( M\theta-hBcl-2 \) Apoe\(^{-/-}\) mice compared with Apoe\(^{-/-}\) mice, that might suggest that hBcl-2 overexpression could protect the lesion from an apoptotic activity masked by an efficient clearance machinery, thereby increasing the overall cellularity of the plaque and its subsequent growth.

In contrast, at a more advanced stage of atherosclerosis (15 weeks on WD), we observed smaller lesion size in \( M\theta-hBcl-2 \) Apoe\(^{-/-}\) mice compared with Apoe\(^{-/-}\) controls. This finding was associated with a lower abundance of apoptotic cells present in the lesions of \( M\theta-hBcl-2 \) Apoe\(^{-/-}\) mice. Because the opposite situation was noted after 5 weeks on the WD, our data indicate that a marked shift occurred within the following 10-week period. Therefore, we speculate that this period of plaque growth was associated with a growing impairment of apoptotic cell clearance mechanisms. Thus, in this context, enhanced macrophage resistance to apoptosis would confer protection against plaque progression. This hypothesis is consistent with studies in mice in which components of the

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**Figure 7.** Monocyte marker and small inducible chemokine mRNA expression is increased in the lesions of CD11c-DTR Apoe\(^{-/-}\) mice treated with DT. The levels of CD11b (A), CCR2 (B), CX3CR1 (C), VCAM-1 (D), and small inducible chemokines MCP-1 (E), MIP-1\(\alpha\) (F), MIP-1\(\beta\) (G), and MIP-2 (H) mRNA were determined by quantitative polymerase chain reaction in the aortas of each group of mice. Values represent the mean±SEM of 5 to 6 mice per group. *Statistically significant difference between CD11c-DTR Apoe\(^{-/-}\) and control groups, \( P<0.05.\)
apoptotic cell clearance machinery have been deleted as well as with other models of ineffective apoptotic cell clearance (lupus-prone mice) that suggested that lesional apoptotic cell accumulation from early lesion stages was associated with increased atherosclerotic plaque progression. Another potential explanation for such a dual effect was that overexpression of bcl-2 preferentially protected macrophage foam cells from potential inducers of apoptosis present in advanced versus early lesions (such as high levels of oxLDL), thereby reflecting the various potential pathways that can trigger cell death in plaques. Alternatively, the presence of macrophages protected from apoptosis in advanced lesions of Mh-Bcl-2 Apoe−/− mice might favor the phagocytosis of plaque debris and might therefore delay or limit the adverse impact of apoptotic cell accumulation, thereby contributing to the limitation of plaque growth. It is noteworthy that monocytes were constitutively increased in Mh-Bcl-2 Apoe−/− mice, an effect most likely due to leakage of the CD68-hBcl2 transgene in this cell type. As a result, increased resistance to apoptosis of macrophages but equally, to a certain degree, monocytes may have contributed to plaque development in Mh-Bcl-2 Apoe−/− mice at an early stage. Alternatively, the increased monocyte count could also have participated in the observed early plaque progression in those mice. Nevertheless, such a scenario would not hold true in advanced lesions and/or would be markedly counterbalanced by the protected effect afforded by increased resistance of macrophage to apoptosis.

By using the CD11c-DTR system, we provide equal evidence that lesional accumulation of apoptotic cells could induce inflammatory signals and favor monocyte recruitment, thereby further demonstrating that advanced lesions progress when apoptotic cells accumulate. Indeed, we demonstrate that the accumulation of dead cells in plaques induces a proinflammatory milieu as assessed by elevated expression of small inducible chemokines (MCP-1, MIP-1α, MIP-1β, and MIP-2) and monocyte markers. Our results are consistent with recent studies showing that membranes of apoptotic cells contain significant amounts of active oxPLs, which are known inducers of inflammation in murine arteries. It is interesting to note that circulating oxPL levels were decreased in Mh-hBcl-2 Apoe−/− mice compared with controls after both 5 and 15 weeks on WD, potentially as a result of both decreased macrophage apoptosis and higher titers of IgM antibodies (particularly oxPL-specific E06 titers), which might facilitate oxPL clearance.

We provide dynamic evidence that the accumulation of CD11b+ newly recruited macrophages in areas of apoptotic cell deposition most probably arose from recruitment of circulating monocytes. Our data are consistent with in vitro experiments that revealed that apoptotic cells, through their oxPLs, can induce endothelial cell activation and subsequent monocyte adhesion and provide the first evidence in vivo that apoptotic cells can stimulate the migration of phagocytes, as previously demonstrated in vitro. Some discrepancies may exist between our results and those of Stoneman and colleagues, who reported that short-term DT treatment of CD11b-DTR-GFP mice triggered macrophage apoptosis in lesions without inducing plaque inflammation, whereas long-term DT treatment of atherosclerotic CD11b-DTR-GFP mice had no impact on plaque progression. The fact that monocytes are CD11b+ cells that were massively depleted in DT-treated CD11b-DTR-GFP mice, thus significantly impairing recruitment to the lesion and consequently reducing the potential impact on vascular inflammation and plaque development, may partly explain the differences with our data. One limitation for the use of the CD11c-DTR model in the present study, however, was the elevation in plasma cholesterol observed after long-term DT treatment of these mice. We cannot exclude that such an effect may have accelerated plaque progression in DT-treated CD11c-DTR Apoe−/− mice. Nevertheless, it is doubtful that a periodic, temporary, and moderate elevation in plasma cholesterol levels might explain the 4-fold increase in lesion size we observed in the aortas of these mice.

Conclusions

The present study provides strong support for a divergent impact of apoptosis on plaque progression as a function of the stage of lesion development, thereby providing a rationale for the association between apoptotic cell accumulation and plaque progression. Considered together, our data suggest that reducing apoptotic cell accumulation in advanced atherosclerotic...
plaque that may be beneficial in attenuating either recruitment of monocytes, the resulting local inflammatory response, or both.

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Disclosures
None.

References

Acute ischemic syndromes are related primarily to rupture of unstable plaques, leading to thrombus formation and occlusive complications. Unstable plaques are typically constituted of the most prominent cell types in atherosclerosis: macrophages and macrophage-derived foam cells. Most of these macrophages are postapoptotic and form a graveyard of dead collapsed cells that might contribute to constitution of the necrotic core. Genetic manipulations of apoptotic genes have been shown to differentially alter atherosclerotic lesion size in murine models of atherosclerosis; however, the potential beneficial or detrimental role of apoptotic macrophage death in plaque development remains controversial. To address this question, we created transgenic mice in which both the lifespan of macrophages was increased in response to elevated resistance to apoptosis (CD68-hBcl2) and targeted induction of lesional macrophage apoptosis (CD11c-DTR) could be achieved. These data provide the first in vivo evidence that macrophage apoptosis is atheroprotective in fatty streak lesions; in contrast, however, defective clearance of apoptotic debris in advanced lesions favors arterial wall inflammation and enhanced recruitment of monocytes, thereby leading to enhanced atherogenesis. Considered together, these findings suggest that attenuating macrophage apoptosis in advanced plaques represents a promising therapeutic strategy.
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Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage.

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MATERIALS AND METHODS

Animals.

Transgenic mice overexpressing the anti-apoptotic protein hBcl-2 under the macrophage-specific promoter CD68 (Mφ-hBcl-2 mice) were generated using homologous recombination in embryonic stem (ES) cells to produce a single-copy transgene insertion at a defined site in the mouse genome as described previously. Site-specific integration of the transgene was achieved at the hypoxanthine phosphoribosyl transferase (Hprt) locus on the X chromosome as previously described. Briefly, in the Hprt vector (derived from pMP8SKB), the 5' homology region consists of 4 kb of DNA derived from the 5' upstream region flanking the Hprt locus. The 3' homology region contains proximal portions of the Hprt gene including sequences capable of restoring Hprt gene function in the BK4s ES cell line. This ES cell line is characterized by an inactivating deletion in the proximal portion of the Hprt gene. The Mφ-hBcl-2 transgene consists of the human hBcl-2 cDNA from pORF-hBcl-2 (Invivogen) cloned downstream of the mouse macrophage-specific promoter CD68 from pDRIVE-mCD68 (Invivogen) and the polyadenylation signal from the mouse EF1 gene inserted 3' relative to the hBcl-2. The Mφ-hBcl-2 cassette was ligated into the polylinker of the targeting vector between the two Hprt homology
domains. The fidelity of the construct was determined by restriction mapping and sequencing. The transgene was introduced by electroporation into BK4s ES cells on a mixed genetic background (50% 129Sv/50 % C57BL/6). Targeted ES cells were injected into blastocysts, implanted into pseudopregnant females and chimeric mice were generated. These chimeras transmitted the transgene through their germ line, and a number of mice bearing the transgene were obtained. Mice overexpressing hBcl-2 under the control of the CD68 promoter (Mϕ-hBcl-2 mice) were genotyped using the following primers: forward, 5’-CAGGAACCTATTATGCTGGC-3’ and reverse, 5’-AGGAAGAGCTACAGAACAACCA-3’.

The PCR amplification cycle was as follows: 30 s at 94°C, 30 s at 56°C and 60 s at 72°C for 35 cycles; and the amplicon size was 800 bp. Apoe−/− and CD11c-DTR-GFP mice on the C57BL/6J background were obtained from Charles River and The European Mouse Mutant Archive (EMMA) respectively and crossed to obtain CD11c-DTR Apoe−/− mice.

**Design of Atherosclerosis studies.**

Mϕ-hBcl-2 and Apoe−/− mice were crossed to obtain Apoe−/− and Mϕ-hBcl-2 Apoe−/− mice. 4 to 5 independents breeding pairs of mice were crossed to generate littermate controls for each experiment and correspond to 93.75 to 98.44% of the genetic background of C57BL/6J mice. Since the Hprt locus is located on the X chromosome, all experiments were performed using male littersmates to avoid the potential variability of transgene expression related to random inactivation of the X chromosome in females. Animals were housed in a conventional animal facility with a 6 AM to 6 PM dark/light cycle. Mice were weaned at 21 days and fed a normal mouse chow diet ad libitum (RM1, SAFE). At 6 weeks of age, they were fed a Western diet consisting of 0.15% cholesterol and 20% saturated fat (SAFE, France) for 5 or 15 weeks. To
evaluate the impact of induction of macrophage apoptosis in atherosclerotic plaques, \textit{Apoe} \(^{-/-}\) mice (8-weeks-old) were lethally irradiated and transplanted with 3 \(\times\) \(10^6\) bone marrow cells from \textit{CD11c-DTR Apoe} \(^{-/-}\) mice. After a 4 week recovery period, mice were changed to a Western diet (0.15% cholesterol and 20% saturated fat). After 5 weeks of Western diet feeding, mice were injected with either Diphteria toxin (DT, 4ng/g, Sigma) or PBS every 10 days for 50 days and sacrificed 48 hours after the last injection. All animal procedures were performed with accreditation from the French government and under strict compliance with Animal Welfare Regulations.

\textbf{Acute induction of apoptosis study}

\textit{Apoe} \(^{-/-}\) and \textit{CD11c-DTR Apoe} \(^{-/-}\) mice were weaned at 21 days and fed a normal mouse chow diet ad libitum (RM1, SAFE). At 6 weeks of age, they were fed a Western diet consisting of 0.15% cholesterol and 20% saturated fat (SAFE, France) for 8 weeks to develop atherosclerotic lesions. After 8 weeks of this diet, mice were injected \textit{i.v.} with DT (4ng/g) or vehicle and sacrificed 48 hours later.

\textbf{Plasma lipid analyses and lipoprotein profiles.}

Blood samples were collected at sacrifice following an overnight fast in Microvette tubes (Sarstedt). Plasma total cholesterol (Roche Diagnostics), free cholesterol (Wako) and triglyceride (Biomérieux) concentrations were measured by enzymatic colorimetric assays using an automatic system (Konelab) as previously described \(^4\).
Analysis of atherosclerotic plaques and immunohistochemistry.

Mice were sacrificed under isoflurane anesthesia and perfused with sterile ice-cold PBS. Hearts were collected, fixed in 10% formalin solution for 30 minutes followed by overnight incubation in phosphate-buffered 20% sucrose solution at 4°C. Hearts were then embedded in Tissue-Tek OCT compound (Sakura Finetek). Atherosclerotic lesions were quantified through the aortic root using Oil red O (ORO) staining as previously described. Briefly, approximately 60 sections, 10 µm thick, were cut through the proximal aorta. Every tenth section was stained with oil red O (0.5% in propylene glycol) for 4 hours and then counterstained with Mayer haematoxylin for 3 minutes. Images were captured using a Zeiss Axiovision microscope and a Canon camera. The extent of atherosclerosis was measured with color thresholding to delimit areas of oil red O staining. For immunohistochemistry, aortic root cryosections (10 µm) were air-dried and fixed in 10% formalin for 30 minutes. Sections were then blocked for 60 minutes with 3% BSA in PBS and then incubated with anti-CD68 antibody (Serotec, clone FA-11, 1:300), anti-CD11b antibody (Serotec, clone 5C6 1:100) or anti-GFP antibody (Invitrogen, 1:100) or control antibody overnight at 4°C. After washing, a biotinylated goat anti–rat or anti-rabbit Ig secondary antibody (PharMingen, 1:300) was added, followed by streptavidin–horseradish peroxidase (PharMingen). The signal was enhanced using the tyramide signal amplification (TSA) kit (Du Pont NEN Research Products) according to the manufacturer's protocol and the sections were counterstained for nuclei with DAPI (Vector Laboratories).

TUNEL staining.

TUNEL staining was performed as previously described. Briefly, cryosections (10 µm thickness) were air-dried and fixed in 10% formalin for 30 minutes. Sections were then placed in
0.1M Citrate buffer (pH 6.0) and submitted to 750W microwave irradiation for 1 min. Slides were washed in PBS and blocked by immersion in Tris-HCl 0.1M pH 7.5 containing 3% BSA and 20% normal bovine serum for 30 min. After washing, TUNEL reaction mixture (TMR Red In situ Cell Death Detection Kit, Roche Applied Science) was added and slides were incubated for 60 min at 37°C. Sections were washed in PBS and counterstained for nuclei with DAPI (Vector Laboratories).

**Analysis of gene expression by Q-PCR.**

Real-time quantitative RT-PCR was performed as previously described. Briefly, RNAs were prepared using TRIzol reagent (Invitrogen) from tissue or cells isolated from mice at sacrifice. Each RNA preparation was hybridized with random hexamer (Promega) and reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). All reactions were performed in duplicate or triplicate and HPRT (hypoxanthine guanine phosphoribosyl transferase) was used as a housekeeping gene to account for variability in the initial quantities of cDNA. In all PCR assays and for each primer set, expression of a control cDNA (pool of reverse transcribed-RNA prepared from different mouse tissues) was included and used as an inter-run calibrator. Expression data were based on the crossing points calculated from LightCycler data analysis software and corrected for PCR efficiencies of both the target and the reference genes.

**Flow cytometry**

To assess hBcl-2 protein expression, thioglycolate-elicited peritoneal macrophages recovered from both *Mφ-hBcl-2* and *wt* mice were permeabilized (permeabilization buffer, eBiosciences) and pre-incubated for 5 min with Fc blocker (BD Pharmingen) and stained for 30 min at 4°C
with a phycoerythrin-labelled anti-human Bcl-2 antibody (clone 6C8, BD Pharmingen) as previously described. To determine monocyte count, blood was harvested, red blood cells were lysed in ACK buffer (ammonium chloride solution) and cells were resuspended in PBS containing 1% BSA and 0.01% sodium azide. Cells were subsequently pre-incubated for 5 min with Fc blocker and incubated for 30 min at 4°C with antibodies directed against CD45 (clone 30F11, Miltenyi), F4/80 (clone BM8, eBiosciences) and CD115 (clone AFS98, eBiosciences). Cells were then analysed on a FC 500 flow cytometer (Beckman Coulter) using CXP software.

**In vitro foam cell formation and ox-LDL induced apoptosis assays**

Bone marrow–derived macrophages were isolated as previously described and cultured in DMEM supplemented with 20% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM penicillin/streptomycin and 30% L929 cell–conditioned media. The bone marrow–derived macrophages were analyzed after 6-8 days in culture, at which time they had acquired macrophage morphology and were greater than 99% positive for expression of MOMA-2, F4/80, and CD68. BMDM were loaded with 50 µg/ml acLDL for 48 hours and cholesterol content was analysed using Amplex Red Cholesterol Assay Kit (Molecular Probes). In order to determine oxLDL-induced apoptosis, BMDM were treated with copper-oxidized LDL for 24 hours and apoptosis induction was measured using EnzChek Caspase-3 assay (Molecular Probes).

**Quantification of anti-MDA LDL, anti-oxLDL and E06 antibodies.**

Antibody titers to oxidation-specific epitopes of OxLDL were determined as described. Briefly, wells were coated with malondialdehyde-modified LDL (MDA-LDL) or copper-oxidized-LDL (oxLDL) at 5 µg/ml. After blocking, plasma (1:100) was added to wells and binding of IgM,
IgG1, IgG2c and IgG3 was detected by chemiluminescent enzyme immunoassays using alkaline phosphatase-labeled secondary antibodies and the substrate LumiPhos (Lumigen). For the measurement of T15 clonotypic (E06) natural antibodies, a double antibody capture assay was used as described. Briefly, wells were coated with 5µg/ml monoclonal anti-T15-idiotypic antibody AB1-2. After blocking, plasma (1:100) was added to wells and binding of T15 clonotypic IgM antibodies was detected by using 1µg/ml biotinylated AB1-2 followed by alkaline phosphatase-labeled NeutrAvidin and the substrate LumiPhos (Lumigen). For all measurements, luminescence was determined using a Dynex luminometer and antibody binding was measured as relative light units (RLU) measured over 100 ms.

Determination of serum oxidized phospholipids (OxPL).

The OxPL content of mouse ApoB-100-containing lipoprotein particles was assessed by ELISA as described previously. The LF3 and LF5 antibodies (kindly supplied by S. Young) were used to capture and recognize ApoB-100 respectively, while the E06 antibody was used to detect OxPL epitopes on captured ApoB-100 particles. Briefly, wells were coated with the apoB-100-specific monoclonal antibody LF3 at 5 µg/ml. After blocking, the plates were incubated with plasma (1:80) for 1 h at room temperature. Then, biotinylated E06 antibody (2µg/ml) and LF5 antibody (2µg/ml) were added in parallel wells to detect the quantity of oxPL in captured particles and the relative amount of apoB captured in each sample respectively. Then, the plates were incubated with alkaline phosphatase-streptavidin for 1 h followed by the substrate LumiPhos (Lumigen) for 30 min in the dark. Chemiluminescence was read on a Dynex Luminometer, and data were expressed in relative light units (RLU) measured over 100 ms.
Then, the relative quantity of E06 epitopes (OxPL) bound per apoB-100 particle was determined by dividing RLU obtained with the E06 antibody by RLU obtained with the LF5 antibody.

**Labelling of blood monocytes and recruitment assays.**

Monocyte labelling was performed as previously described. Briefly, 1µm yellow green (FITC) beads (250µL, 10^10 beads/ml, Molecular Probes) were injected i.v. for labelling of circulating monocytes. Using this technique, approximately 10% of CD11b^high Side Scatter^low blood monocytes had engulfed at least one bead as described by Tacke and colleagues. To assess the recruitment of blood monocytes, 6-week old Apoe^-/- and CD11c-DTR Apoe^-/- mice were fed a Western diet for 8 weeks and then injected i.v. with 200 ng of DT or vehicle and 250 µL of yellow green beads. After 48h, mice were sacrificed, the aortic root removed, embedded in OCT and 10 µm-cryosections made. The number of monocytes containing fluorescent beads attached to the intimal area or present in atheromatous plaques was quantified for each mouse.

**Statistical analysis.**

Statistical calculations were performed using GraphPad Prism, version 4.03 for Windows. Results were analyzed by Student's unpaired t-tests with Welch's correction if the variances were unequal (aortic root lesion area at 5 weeks) except for “mean macrophage area at 5 weeks”, where the Mann-Whitney U-test was used. The statistical significance of the differences between more than two groups was compared by ANOVA followed by the Newman-Keuls multiple comparison test. P<0.05 was considered significant. Values are expressed as mean ± SEM.
REFERENCES


Supplemental Figure I. hBcl-2 expression in different cell types of the immune system. Intracellular staining of hBcl-2 was performed in splenic CD19+ B cells, CD4+ and CD8+ T cells, CD11c^hi^ dendritic cells and F4/80^+^ macrophages, and in CD115^+^ blood monocytes.
5 weeks

Supplemental Figure II. Tissue factor immunostaining in aortic root lesions of Mφ-hBcl-2 Apoe⁻/⁻ mice and their controls after 5 weeks of Western diet. Each photograph is representative of a single animal.
Supplemental Figure III. Tissue factor immunostaining in aortic root lesions of Mφ-hBcl-2 Apoe−/− mice and their controls after 15 weeks of Western diet. Each photograph is representative of a single animal.
Supplemental Figure IV. Blood monocyte and dendritic cells in CD11c-DTR Apoe<sup>−/−</sup> → Apoe<sup>−/−</sup> chimeric mice injected with DT. Percentage of circulating blood monocyte (CD115<sup>+</sup>)(A) and splenic dendritic cell content(CD11c<sup>hi</sup> MHC-II<sup>+</sup>)(B) were determined by flow cytometry. * P<0.001