The p17 Cleaved Form of Caspase-3 Is Present Within Viable Macrophages In Vitro and in Atherosclerotic Plaque

Thomas Q. Nhan, W. Conrad Liles, Alan Chait, John T. Fallon, Stephen M. Schwartz

Objective—In vitro studies of macrophage death in response to oxidized LDL (oxLDL) were undertaken as a model for the formation of the necrotic core of atherosclerotic plaque.

Methods and Results—Thioglycollate-elicited mouse peritoneal macrophages avidly incorporated both oxLDL and acetylated LDL (acLDL) to become foam cells. oxLDL-treated macrophages, but not acLDL-treated macrophages, showed nearly 100% death, with characteristics consistent with apoptosis, including cell surface phosphatidylserine exposure, intracellular caspase-3 activity, cleavage of caspase-3 substrates, and DNA fragmentation, as shown by TUNEL assay. The activated form of caspase-3 (p17 cleaved form) was present in attached, viable macrophages before exposure to oxLDL. This p17 form was also found in apparently viable as well as in TUNEL-positive cells within atherosclerotic lesions of chow-fed apolipoprotein E–deficient (apoE/−) mice. The amount of p17 caspase-3 was reduced by in vitro blockade of FasL with an FasL-blocking antibody and was absent in macrophages from lpr/lpr mice, which lack functional Fas. Moreover, lpr/lpr macrophages resisted oxLDL cytotoxicity.

Conclusions—The naturally occurring Fas-FasL induction of caspase-3 cleavage after macrophage attachment may represent an important physiologic mechanism that primes for cytotoxicity by oxLDL and possibly, other death-inducing molecules. (Arterioscler Thromb Vasc Biol. 2003;23:1276-1282.)

Key Words: macrophages ■ CD95 ■ LDL ■ caspases ■ apoptosis

The definitive feature of the fully developed atherosclerotic plaque in humans is the encapsulated necrotic core.1,2 Rupture of this core leads to sudden coronary death, embolic injury to the brain, and/or ischemic injury to other organs.3,4 Although the sequence of events leading to plaque rupture has long been described in humans, recent observations from our group and others have also documented necrotic core formation from dying macrophages followed by plaque rupture in murine models of atherosclerosis.5,5

Macrophage death is widely accepted to be caused by the toxic moiety of modified LDL.6–11 The etiologic agent responsible for plaque macrophage death has been suggested variously to be unesterified cholesterol12,13 or oxidized LDL.10,14–20 With the identification of terminal dUTP nick end-labeling (TUNEL)–positive cells in the plaque, Fas, and FasL, the assertion has been made that the agent initiating the apoptotic pathway initiated by Fas.21,22 This interpretation of data, however, is somewhat surprising, because Kiener et al23 showed that although monocytes are readily susceptible to Fas-mediated apoptosis, this susceptibility is lost during macrophage differentiation.

In the present study, we show that Fas activation is a normal event that places macrophages in a viable but “death-primed” state required for death by subsequent exposure to oxidized LDL. It is intriguing to consider the possibility that this priming event is distinct from the death event and that death only occurs when sufficient oxidized LDL has accumulated.

Methods

Reagents

Fetal bovine serum (FBS), obtained from Hyclone Laboratories, was heat-inactivated for 1 hour at 65°C. Staurosporine was obtained from Sigma-Aldrich and was used at a dose of 0.1 μmol/L to induce cell death. Z-DEVD-fmk and Z-VAD-fmk were obtained from Enzyme Systems Products. IDUN1529 was generously provided by Idun Pharmaceuticals, San Diego, Calif. Caspase-3 rabbit polyclonal antibody (No. 9662), anti-cleaved caspase-3 rabbit polyclonal antibody (No. 9661), and anti–poly (ADP-ribose) polymerase (PARP) rabbit polyclonal antibody (No. 9542) were obtained from Cell Signaling Technology. Anti-Mac-3 rat monoclonal antibody (mAb; 01781D), anti-cleaved caspase-3 rabbit polyclonal antibody (553702), inhibitory anti-FasL mAb (MFL3), and agonistic anti-Fas mAb (Jo2) were obtained from PharMingen. Native LDL (naLDL), copper-oxylated LDL (oxLDL), and acetylated human LDL (acLDL) were prepared as described previously; the thiobarbituric acid–reactive (TBARS) substances level of naLDL was 1.5 nmol/mg and for oxLDL, 45 nmol/mg before addition of 25 μmol/L butylated hydroxytoluene in the final preparation to prevent further oxidation.24,25
Mouse Peritoneal Macrophage Culture

Macrophages were harvested from the peritoneum of 30 g C57BL6/J or lpr/B6 (approximately 8 to 12 weeks old) male mice (The Jackson Laboratories, Bar Harbor, Me) 4 days after intraperitoneal injection of 2 mL (4% wt/vol) thioglycollate broth and were then cultured on plastic chambered slides, 100-mm tissue-culture plates, or 6-well tissue-culture plastic plates in 5% (vol/vol) FBS in RPMI 1640 for 1 day. After 1 day of attachment, the cells were washed 3 times with phosphate-buffered saline (PBS) and incubated for the indicated times with 30 µg/mL naLDL, oxLDL, or acLDL, or an equivalent volume of PBS in 5% FBS in RPMI. For long-term culture, the medium was changed every 4 days.

ApoE−/− Mouse Atherosclerotic Lesions

Apolipoprotein E–deficient (ApoE−/−) mice on a C57BL6/J background (Jackson Laboratories) were fed a chow diet (Harlan-Teklad) and water ad libitum and were humanely killed at 39.5 weeks using CO2. Tissue sections were obtained and prepared as described previously.26 Adjacent sections were stained with anti-cleaved caspase-3 rabbit polyclonal antibody (PharMingen and Cell Signal-Ing Technology), in situ TUNEL (Roche), and DAPI (please see online Methods available at http://atvb.ahajournals.org).

Caspase-3 Activity Assays

Caspase-3 activity was measured by two different commercially available methods. PhiPhiLux (OncoImmunin) was used to assess caspase-3–like activity in whole cells. EnzChek (DEVD-AMC, Molecular Probes) was used to measure caspase-3–like activity in macrophage cell lysates. Please see online Methods available at http://atvb.ahajournals.org.

Western Blots

For each sample, ~3×10^6 cells were lysed with CHAPS with addition of zVAD.27 Each lane of a 12% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gradient gel was loaded with 20 µg protein. Electrophoresed samples were transferred onto polyvinylidine difluoride membranes. They were probed with polyclonal antibodies for cleaved caspase-3, uncleaved caspase-3, or PARP. Please see online Methods available at http://atvb.ahajournals.org.

Data Analysis

Results are shown as mean±SEM, calculated from at least 3 separate experimental samples. Statistical significance of differences in mean values was determined by ANOVA with the use of InStat software (GraphPad Software), and data were plotted with Prism software (GraphPad). Statistically significant differences were defined at P<0.05.

Results

Differential Effects of naLDL and Modified LDL on Macrophage Viability In Vitro

After 1 day of attachment in vitro, macrophages developed long projections extending 2 to 4 times the cell body diameter. The cells aggregated to form clusters, and their perikaryon showed a granular appearance. Studies were initiated 1 day after isolation, and macrophages were incubated with naLDL, acLDL, and oxLDL for 4 days. LDL-induced effects on macrophages depended on the nature of modifications to LDL. There was no change in appearance of the macrophages exposed to 30 µg/mL naLDL for 4 days compared with macrophages incubated with an equivalent volume of PBS (Figure 1). No lipid accumulation was seen in response to PBS or naLDL (Figure 1a and b). In contrast, the cytoplasm of cells incubated with modified LDL (oxLDL or acLDL) for 4 days showed prominent Nile red–positive spots (Figure 1c and 1d).28

After incubation with 30 µg/mL oxLDL, all macrophages died or showed signs of apoptosis within the 4-day period (eg, overall rounding, nuclear condensation, and detachment from the substratum). These effects were absent when macrophages were incubated with 10 µg/mL oxLDL (data not shown). In contrast, macrophages incubated with naLDL or acLDL for 4 days showed minimal signs of cell death, even though macrophages treated with acLDL had the morphological appearance of foam cells after being stained with Nile red (Figure 1b and 1c). This death occurred, even though oxLDL...
contained 20 μmol/L butylated hydroxytoluene (an antioxidant), which was intended to reduce direct oxidant activity.

Evidence of oxLDL-Induced Apoptosis of Macrophages

Macrophage death was first quantitatively determined by annexin V/propidium iodide (PI) double staining with the use of fluorescence-activated cell sorting system analysis. Nearly 100% intact (PI-negative) macrophages treated with oxLDL for 4 days were annexin V–positive (Figure 2). In contrast, 10% or less of cells were annexin V–positive after 4 days incubation with PBS, naLDL, or acLDL (Figure 2). Evidence of massive cell death was not limited to annexin V staining data. Caspase-3 is commonly believed to be the “final executioner” in programmed cell death. As shown in Figure 3a and Figure II (online supplement available at http://atvb.ahajournals.org), we found that oxLDL induced caspase-3 activity in 100% of wild-type C57BL6/J macrophages. Finally, as shown in Figure 4, 100% of cells were TUNEL-positive by 4 days. At 2 hours, the frequency of macrophages incubated with PBS or naLDL was negligible, but with oxLDL, TUNEL frequency rose to ~20% (data not shown). As observed by others, acLDL also induced a modest increase in TUNEL frequency to ~15% (Figure 4). Thus, by all of the usual criteria, oxLDL at least at 30 μg/mL but not at 10 μg/mL is able to induce massive, apoptotic cell death in macrophages.

Activation of Caspases in oxLDL-Induced Macrophage Death

The appearance of apoptosis led us to expect an increase in caspase-3 activity when macrophages were incubated with oxLDL. This increase in enzyme activity (Figures 3a and II online) was abrogated when cells were incubated with z-DEVD-fmk or DEVD-CHO (Figures 3c and II online). Pretreatment of macrophages with z-DEVD-fmk before assay (PhiPhiLux) decreased caspase-3 activity in a dose-dependent manner in macrophages incubated with oxLDL (Figure 3c). Additional confirmatory experiments were performed with cell lysates from the same macrophages to measure caspase-3 activity with the DEVD-AMC assay (Figure II online). Competition experiments performed in cell lysates with z-DEVD-fmk and PhiPhiLux showed that oxLDL-induced caspase-3 activity that was present after incubation with oxLDL could be reduced (Figures 3c and II online).
Effects of Pan-Caspase Inhibitors and Fas on oxLDL-Induced Macrophage Death

As reported by others for more modest amount of death in response to accumulation of free cholesterol in macrophages,12 we found that macrophages from lpr mice were resistant to even extreme levels of cytotoxicity induced by oxLDL (Figure 3b). Caspase-3 activity was not detected in lpr macrophages after incubation with oxLDL (Figure 3b). On the basis of these observations and the ability of caspase inhibitors to block caspase-3 activity after oxLDL incubation, we expected that death itself could be blocked or attenuated by caspase inhibition.

Staurosporine treatment of macrophages produced ∼60% cell death, as measured by TUNEL assay. This apoptotic death was blocked by z-VAD-fmk (data not shown). Similar results were observed when caspase inhibitors were used to block Fas-mediated death of Swiss 3T3 cells (data not shown). Unexpectedly, neither z-VAD-fmk (a pan-caspase inhibitor) nor IDUN1529 (another pan-caspase inhibitor with greater specificity for caspase-3)12 was able to block macrophage death in response to oxLDL, as measured by TUNEL assay at 24 hours or 4 days (Figure 4). Similarly, pretreatment with either z-VAD-fmk or IDUN1529 did not affect cell surface phosphatidylserine exposure on macrophages after a 4-day treatment with oxLDL (data not shown). These data suggest that once death has been initiated by oxLDL, caspase activity increases but is not required to initiate cell death.

Presence of p17 Caspase-3 in Viable Macrophages In Vitro

The requirement for Fas in the absence of a requirement for caspases during oxLDL-mediated death might be interpreted as evidence that events caused by Fas ligation had already occurred before exposure to oxLDL. To test this hypothesis, Western blots were used to examine cleavage of caspase-3 in response to macrophage attachment. Positive and negative controls for detection of cleaved caspase-3 were generated in Swiss 3T3 cells with and without Jo-2 Fas–activating mAb treatment (Figures 5 and IV; online supplement available at http://atvb.ahajournals.org). Only the 17-kDa form of cleaved caspase-3 was detected in Jo-2–treated Swiss 3T3 cells. Sublethal stimulation of Fas in Swiss 3T3 cells with Jo-2 also led to the cleavage of procaspase (32 kDa) into other multiple, cleaved, caspase-3 maturation products (21, 19, and 17 kDa; data not shown), but only the p17 form of cleaved caspase-3 was observed in cell lysate of attached C57BL6/J macrophages (Figure 5a). Staurosporine-treated macrophages also showed only the 17-kDa form of cleaved caspase-3 (data not shown). In contrast,as shown in Figures 5a and IVa online, p17 caspase-3 was present in normal, adherent macrophages, whereas freshly obtained peritoneal macrophages showed only the pro- form (32 kDa) in the absence of oxLDL or other death stimuli (Figures 5b and IVb online). Both the 32- and the 17-kDa cleaved forms of caspase-3 were present in both viable, adherent macrophages and in oxLDL-treated, dying macrophages (Figure 5a and 5b). The level of p17 increased slightly after a 4-day incubation with oxLDL (Figure 5a). The level of p32 caspase-3 in oxLDL-treated macrophages remained at the level observed in viable control macrophages (Figure 5b). Data from densitometric measurement of 3 separate Western blots with anti–p17 caspase-3 and anti–p32 caspase-3 are shown in Figures IVa and IVb (online). Perhaps paradoxically, the overall level of p17 caspase-3 in macrophages was increased by incubation with the pan-caspase inhibitors, IDUN1529 or z-VAD-fmk. This might represent evidence for activation of caspase-3 by noncaspase proteolytic pathways.33

These results suggest that caspase-3 is constitutively cleaved to its active form in cultured macrophages. If true, one would also expect to see evidence of proteolysis of caspase-3 substrates. As shown in Figure 5c, the short form of PARP (87 kDa), a classic caspase-3 substrate,34 was present in these cells. Presence of the short form of caspase-3, along with evidence of PARP cleavage, supports the hypothesis that caspase-3 is activated in these cells.

Thus, whereas p17 is present and may be enzymatically active with some substrates, p17 proteolytic activity is low or restricted to a certain subset of substrates (Figures 5a and 5c). p17 was absent in lpr macrophages, even when these cells were incubated with oxLDL. Finally, when wild-type macrophages were treated with an FasL-blocking antibody (MFL3), the amount of p17 was markedly decreased in a dosage-dependent manner (Figures 5d and IVd online).
Presence of p17 Caspase-3 In Vitro and In Vivo

An obvious question raised by these data is whether the p17 observed on Western blots might reflect a small subpopulation of apoptotic cells, even in the absence of oxLDL. To eliminate this possibility, immunohistochemistry was used to identify the presence of p17 caspase-3 in vitro in C57BL6/J peritoneal macrophages to determine whether all or subsets of macrophages have this cleaved form of caspase-3. As shown in Figure 6a, freshly obtained mouse peritoneal macrophages failed to stain. When cells were attached for 24 hours, p17 was detected in all macrophages with the Pharmingen anti-cleaved caspase-3 rabbit polyclonal antibody (Figure 6a).

To extend these data to an in vivo model, we examined macrophages in frozen sections of ApoE−/− mouse atherosclerotic lesions. DAPI was used to show the cellularity within the lesion of these sections (Figure 6b, blue). The identity of these cells as macrophages was established with use of an anti–Mac-3 mAb (Figure 6b, brown). The frequency of p17 caspase-3–positive, foamy macrophages (Figure 6b, green) was much greater than that of TUNEL–positive cells (green) within the lesion. On the basis of studies of adjacent sections, a minority of cells showed neither TUNEL nor p17 caspase-3. Most of the plaque macrophages, perhaps 60%, were positive for p17 caspase-3. Of these cells, a smaller number were also positive for TUNEL (Figure 6b).

Discussion

oxLDL is able to induce massive death of macrophages after 4 days in culture via an Fas-dependent mechanism. Despite the requirement for Fas and evidence that the cell death observed had classic features of apoptosis, caspase inhibitors did not block this form of death.

Our data resolve an apparent contradiction in the literature. Macrophages, like monocytes, have high levels of Fas and FasL, but unlike monocytes, macrophages do not die when Fas is ligated.23 Instead, macrophages become activated, releasing cytokines.23 Yao and Tabas,12 however, reported that death was due to an accumulation of unesterified cholesterol as a result of Fas-FasL activation. This conclusion was based on evidence that accumulation of cholesterol derived from acLDL in the presence of an acyl coenzyme A:cholesterol acyltransferase inhibitor killed wild-type but not lpr macrophages. Similarly, we found that oxLDL was unable to mediate cell death in macrophages from lpr mice. Additionally, an FasL-blocking mAb was able to attenuate the level of p17 caspase-3. A simple explanation of these data is that Fas is preactivated in macrophages when they attach, and the events downstream of that activation are necessary for death in response to either free cholesterol or oxLDL. The nature of the “execution” is unclear.

In support of this hypothesis, as shown in Figure 5, the canonical 17-kDa proteolytic form of caspase-3, the “executor” caspase,29 is absent in lpr macrophages after attachment but present in attached, viable, wild-type macrophages, independent of exposure to oxLDL or any other form of LDL. Although levels of caspase activity were too low for detection by fluorometric assays, activity of this endogenous p17 caspase-3, or some other caspase downstream unable to respond to PhiPhiLux, was suggested by the presence of PARP cleavage (Figure 5c).

Our observation of p17 caspase-3 in murine atherosclerotic lesions (Figure 6b) is supported by observations shown in human lesions in a review article.24 It is important to point out that none of these data, in vivo or in vitro, prove that caspase-3 is the critical product of Fas activation required for death. In the case of studies of unesterified cholesterol, the authors were able to inhibit death by using a pan-caspase inhibitor; however, the cells were never pretreated with the inhibitor, so we cannot ascertain whether further induction of caspases is needed for that form of cell death. Our data suggest that subsequent caspase activation is not needed for oxLDL-mediated death. Thus, additional Fas activation after exposure to LDL may or may not be required for the modest
level of cell death induced by free cholesterol or the cataclysmic level of cell death after exposure to oxLDL.

Evidence for such a functional role for Fas activation with caspase-3 activation already exists in other cell types. Caspase-3 activation is required for T-cell proliferation, and its inhibition leads to reduced or absent T-cell proliferation in response to tumor necrosis factor-α. Caspase-3–mediated signaling has also been implicated in the regulation of myogenesis. In addition, Fas ligation during erythropoiesis does not induce cell death but inhibits differentiation of immature erythroblasts by caspase-mediated degradation of the transcription factor GATA-1.

This concept of Fas activation as a critical event in the formation of the atherosclerotic lesion is supported by a recent study of gene therapy. Schneider et al have reported accelerated lesion formation after adenovirus-mediated FasL transfection of foam cell–rich, rabbit atherosclerotic lesions. Presumably, their goal was to accelerate cell death, perhaps decreasing lesions size; however, there was no evidence of increased death in transfected lesions. Thus, an Fas-dependent, activated state of viable macrophages may be an important phase in lesion development, independent of cell death. In this regard, it is interesting to note that in vivo, unlike our in vitro model, only a portion of macrophages within the plaque were positive for p17 caspase-3. It would be of interest to know whether that proportion is increased when FasL is overexpressed.

Given the apoptotic pattern of cell death, we were surprised that caspase inhibitors did not block oxLDL-mediated death of macrophages. This was not a failure of caspase inhibitors to access their targets, because z-VAD-fmk blocked staurosporine-induced death. Furthermore, the more modest level of apoptosis observed in response to unesterified cholesterol was also blocked with z-VAD-fmk. We also showed that a related inhibitor, z-DEVD-fmk, was able to block the oxLDL-induced increase in caspases fluorometric activity but not of the TUNEL signal in macrophages 24 hours after treatment with oxLDL (Figures 3 and 4).

The failure of the caspase inhibitors does not rule out a role for caspases during oxLDL-induced death. The half-life of z-VAD-fmk in aqueous solution has been reported to be as short as 1 hour (Enzyme Systems, personal communication). Thus, the kinetics of inactivation may be very important. For example, free radicals or detergents produced by oxLDL are likely to damage mitochondria. Disruption of the mitochondrial membrane can lead to release of Smac/DIABLO that can antagonize the endogenous inhibitor for caspase-3 and -7, IAP. Perhaps release of inhibition occurs so rapidly that the inhibitors are unable to act. Alternatively, mitochondrial membrane disruption may release additional caspase-independent death, including factors such as apoptosis-inducing factor. This form of mitochondrial death would not be inhibited by caspase inhibitors but might be enhanced by as-yet-undefined effects of Fas-FasL activation.

Although mitochondrial damage presumably does occur during oxLDL-mediated death, in the absence of an inhibitor for this damage, we cannot prove that mitochondria are the only target. Lougheed et al have shown that cholesterol uptake by macrophages leads to an expansion of the endosomal compartment, and Li et al showed that uptake of oxLDL into the acidic vacuolar compartments (late endosomes, prelysosomes, and lysosomes) of macrophages leads to disruption of membrane stability and subsequent leakage of cathepsin D into the cytoplasm. The release of cathepsin D or other lysosomal proteases could themselves be cytotoxic or, again, a massive, continuous activation of caspases could overwhelm the inhibitors.

Finally, we wish to point out how very little is known about the mechanisms leading to macrophage death in the plaque. Does this death occur, as suggested by our data with oxLDL, as a calamitous, sudden event when a critical level of oxLDL has accumulated, or is death more gradual, as might be expected on the basis of the moderate cell death seen with unesterified cholesterol? Is either mechanism critical in vivo? A recent study has demonstrated an accumulation of unesterified cholesterol in the formation of acellular, necrotic cores. A priori arguments about likely intraplaque levels of oxLDL or the likelihood that free cholesterol accumulates in vivo to the level seen in vitro with an acyl coenzyme A:cholesterol acyltransferase inhibitor are unlikely to be resolved, because the relevant quantitative measurements are difficult to make in vivo. However, the common role for Fas in both forms of death and in the physiological activation of macrophages may open an experimental and therapeutic pathway. Certainly, it would appear important to know more about the functional properties of macrophages that have passed this step in their differentiation.

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