Free Cholesterol Loading of Macrophages Induces Apoptosis Involving the Fas Pathway*

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Macrophage death is an important feature of atherosclerosis, but the cellular mechanism for this process is largely unknown. There is increasing interest in cellular free cholesterol (FC) excess as an inducer of lesional macrophage death because macrophages accumulate large amounts of FC in vivo, and FC loading of macrophages in culture causes cell death. In this study, a cell culture model was used to explore the cellular mechanisms involved in the initial stages of FC-induced macrophage death. After 9 h of FC loading, some of the macrophages exhibited externalization of phosphatidylserine and DNA fragmentation, indicative of an apoptotic mechanism. Incubation of the cells with Z-DEVD-fluoromethylketone blocked these events, indicating dependence upon effector caspases. Macrophages from mice with mutations in either Fas or Fas ligand (FasL) demonstrated substantial resistance to FC-induced apoptosis, and FC-induced death in wild-type macrophages was blocked by an anti-FasL antibody. FC loading had no effect on the expression of cell-surface Fas but caused a small yet reproducible increase in cell-surface FasL. To determine the physiological significance of this finding, unloaded and FC-loaded Fas-deficient macrophages, which can only present FasL, were compared for their ability to induce apoptosis in secondarily added Fas-bearing macrophages. The FC-loaded macrophages were much more potent inducers of apoptosis than the unloaded macrophages, and this effect was almost completely blocked by an inhibitory anti-FasL antibody. In summary, during the early stages of FC loading of macrophages, a fraction of cells exhibited biochemical changes that are indicative of apoptosis. An important part of this event is FC-induced activation of FasL that leads to Fas-mediated apoptosis. In light of recent in vivo findings that show that apoptotic macrophages in atherosclerotic lesions express both Fas and FasL, we present a cellular model of Fas-mediated death in lesional foam cells.

Cholesterol-loaded macrophages are critical components of atherosclerotic lesions (1), and recent in vivo studies have demonstrated the importance of these cells in lesion progression (2–5). In addition, lesional macrophages may also contribute to the complications of advanced atherosclerosis, such as plaque rupture and acute thrombosis (6). Observational studies have noted that macrophage death is a prominent feature of atherosclerotic lesions (7–10), and it is likely that dying macrophages influence lesion progression. On the one hand, macrophage death, particularly apoptotic death, may actually limit the cellularity of lesions. However, atherogenic and thrombogenic molecules as well as degradative enzymes released from dying macrophages may contribute to atherogenesis, plaque rupture, and acute thrombosis. In this light, plaque rupture and acute thrombosis are strongly associated with the presence of lipid cores in lesions (11), areas that have been shown to contain the debris of dead macrophages (8, 9). Indeed, Bauriedel et al. (10) have reported that atherectomy specimens from patients with unstable angina have approximately twice the number of dead intimal cells when compared with specimens from patients with stable angina.

Possible causes of macrophage death in atherosclerotic lesions include cholesterol-induced cytotoxicity (12, 13), growth factor deprivation (14), and exposure to oxidized lipoproteins and other arterial wall factors such as inflammatory cytokines and nitric oxide (7, 15). This laboratory and others have been particularly interested in death caused by excess cellular free cholesterol (FC), because macrophages in advanced lesions have been shown to accumulate large amounts of FC (16–19), and FC loading of cultured macrophages is a potent inducer of cell death (12, 13). Importantly, a recent study in an induced mutant mouse model has implicated FC loading of macrophages in the acceleration of atherosclerosis (20). FC loading probably kills cells through the inhibition of certain critical plasma membrane enzymes (21–24). In fact, Papahadjopoulos (21) demonstrated 25 years ago that FC-mediated inhibition of two critical plasma membrane enzymes, (Na"K")-ATPase and adenylate cyclase, leads to cellular death and proposed that these events may play an important role in the development of the necrotic core in advanced atheromata.

A fundamental issue related to FC-induced macrophage death is whether the cellular and biochemical processes associated with apoptosis are involved. This is an important question because death by apoptosis may have different physiological and pathophysiological consequences compared with death by necrosis (see “Discussion”). Indeed, macrophages in atherosclerotic lesions have been shown to have cellular characteristics of both apoptosis and necrosis (25). In this report we have used a series of biochemical assays, inhibitory antibodies, and genetic mutations to show that a portion of macrophages ex-
posed to excess FC undergoes apoptosis and remarkably that the Fas pathway is involved in this process. Given recent in vivo data showing that apoptotic macrophages in atherosclerotic lesions express both Fas and Fas ligand (FasL) (26, 27), our data suggest a cellular model of Fas-mediated death in lesional foam cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Falcon tissue culture plastic ware were used in these studies was purchased from Fisher Scientific. Tissue culture media and other tissue culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum (FBS), obtained from Hyclone Laboratories (Logan, UT), was heat-inactivated for 1 h at 65 °C. T-Dependently-labeled propidium iodide (PI) was added to the cells for 15 min at room temperature according to the manufacturer’s instructions (Molecular Probes). Cells were immediately viewed with a 40× objective using an Olympus IX-70 inverted fluorescence microscope equipped with filters for staining. PI counterstain is also added that stains all of the cells because it is restricted to the inner leaflet of the plasma membrane. Moreover, during early apoptosis before the loss of membrane integrity, phosphatidylserine appears on the outer leaflet of the plasma membrane (32). Thus, early apoptotic cells interact with the phosphatidylserine-binding protein, annexin V, which in this assay is conjugated to the green stain Alexa 488 (31). The membrane-impermeable nucleic acid stain PI (red-orange) is excluded by early apoptotic cells but stains necrotic cells. As illustrated in Fig. 1, A–C, macrophages incubated in control medium or medium containing either acetyl-LDL or compound 58035 showed very little evidence of cell death. Macrophages incubated with acetyl-LDL and compound 58035, however, showed a substantial number of dead cells, and most stained with annexin V but not PI (Fig. 1D). Quantification of data from a large number of cells verified that FC loading, but not cholesterol ester loading or compound 58035 by itself, induced phosphatidylserine externalization in macrophages (Fig. 1E).

Another characteristic of apoptotic death is fragmentation of DNA, and this event can be detected in situ by the TUNEL assay in which terminal deoxynucleotidyltransferase is used to transfer dNTP-digoxigenin to the free 3′-OH ends of fragmented nuclear DNA. The attached dNTP-digoxigenin is then visualized by fluorescein-labeled anti-digoxigenin (33). PI is also included in this assay, but because TUNEL staining is performed on fixed cells, all of the cells stain red. As illustrated in Fig. 2, A and B, FC loading of macrophages caused a marked increase in the number of TUNEL-positive cells; quantitative data from 700–1000 cells are shown in Fig. 2C.

To directly show the involvement of caspases in FC-induced apoptosis, peritoneal macrophages were loaded with FC in the absence or presence of the irreversible caspase inhibitor Z-DEVD-fmk (34) (Fig. 3). This inhibitor was originally designed to inhibit effector caspases 3 and 7, but it may inhibit other caspases when used in cell culture experiments at high doses (34). Partial inhibition was noted at 20 μM, and almost complete inhibition was seen at 100 μM. These data, together with the annexin and TUNEL data shown in Figs. 1 and 2, demonstrated that FC loading of macrophages for 9 h leads to a series of biochemical events that are characteristic of apoptosis.

**FC Loading of Mouse Peritoneal Macrophages Induces Apoptotic Changes**—Peritoneal macrophages from C57 mice were incubated for 9 h with medium alone, medium containing acetyl-LDL to induce cholesteryl ester loading, medium containing compound 58035 to inhibit acyl-CoA:cholesterol O-acyltransferase, or medium containing both acetyl-LDL and the acyl-CoA:cholesterol O-acyltransferase inhibitor to effect FC loading (30). Apoptosis of live macrophages was first analyzed by annexin V staining. In intact cells, phosphatidylserine is restricted to the inner leaflet of the plasma membrane. However, during early apoptosis before the loss of membrane integrity, phosphatidylserine appears on the outer leaflet of the plasma membrane (32). Thus, early apoptotic cells interact with the phosphatidylserine-binding protein, annexin V, which in this assay is conjugated to the green stain Alexa 488 (31). The membrane-impermeable nucleic acid stain PI (red-orange) is excluded by early apoptotic cells but stains necrotic cells. As illustrated in Fig. 1, A–C, macrophages incubated in control medium or medium containing either acetyl-LDL or compound 58035 showed very little evidence of cell death. Macrophages incubated with acetyl-LDL and compound 58035, however, showed a substantial number of dead cells, and most stained with annexin V but not PI (Fig. 1D). Quantification of data from a large number of cells verified that FC loading, but not cholesterol ester loading or compound 58035 by itself, induced phosphatidylserine externalization in macrophages (Fig. 1E).

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**FC-induced Apoptosis in Macrophages Involves the Fas Pathway**—The cell-surface receptor Fas, when engaged by membrane-bound FasL, is a well documented initiator of apoptosis (35, 36). To determine the role of the Fas pathway in FC-induced apoptosis in macrophages, we took advantage of naturally occurring mutations in FasL in gld mice and in Fas in lpr mice (37). As shown in Fig. 4A, peritoneal macrophages
from wild-type, gld, and lpr mice were incubated under control or FC-loading conditions for 9 h and then assayed for apoptosis by the annexin assay. Remarkably, there was a 70% decrease in apoptosis in both the gld and lpr mice despite the fact that the macrophages from each of the three types of mice accumulated similar amounts of FC (Fig. 4A, inset). To test the role of the Fas pathway in wild-type macrophages, we determined the effect of a rabbit anti-FasL antibody on FC-induced apoptosis. As shown in Fig. 4B, FC-induced apoptosis was blocked by the anti-FasL antibody but not by a non-immune rabbit IgG, and the percent inhibition was very similar to that observed with gld and lpr macrophages. Thus, triggering of the Fas pathway by FC loading can account for a substantial portion of FC-induced apoptosis in macrophages.

We next sought to explore how FC loading might activate the Fas pathway. To test the possibility that FC loading increases the expression of cell-surface Fas, FACS analysis using an anti-Fas antibody was conducted. Unloaded macrophages showed clear evidence of cell-surface Fas (Fig. 5A), but Fas expression did not change after FC loading for 2, 4 (Fig. 5B), or 8 h. Next, the expression of cell-surface FasL was assayed by FACS analysis. As expected, FasL expression was very low in control macrophages (Fig. 5C). Upon FC loading, however, there was a small but reproducible increase in the expression of this molecule.

To determine whether this small increase in cell-surface FasL expression or perhaps FC-induced conformational activation of FasL might have biological significance, we conducted an experiment in which control versus FC-loaded lpr macrophages were compared for their ability to induce apoptosis in...
secondarily added macrophages. First, ~200,000 lpr macrophages ("primary" macrophages) were plated on the culture dish and incubated in control medium or in medium containing acetyl-LDL and compound 58035 for 2–4 h. lpr macrophages were used for this phase of the experiment. These macrophages are able to present FasL to other cells but display only minimal apoptosis themselves because of the absence of functional Fas (above). The medium containing acetyl-LDL and compound 58035 was removed, and ~50,000 new macrophages ("second-

ary" macrophages) in medium without lipoproteins were added to these cultures for an additional 4 h, after which the cells were observed for annexin staining. In control experiments, the primary lpr macrophages were incubated for the full 6–8 h in control or FC-loading medium and then observed directly (i.e., no secondary macrophages).

As shown in Fig. 6A (first two bars), 8 h of FC loading had very little effect on the lpr macrophages themselves, confirming the data shown in Fig. 4A. When wild-type (C57) macrophages were added to unloaded lpr macrophages, there was also very little apoptosis (Fig. 6A, bar 3). However, when these same macrophages were added to FC-loaded lpr macrophages, apoptosis was increased substantially. 2 Similar results were obtained in two duplicate experiments. In addition, a similar experiment was conducted where the secondary macrophages were isolated from gld mice, because these macrophages have a blunted apoptosis response to FC loading (Fig. 4A). Thus, this experiment further tests the importance of the primary cells in inducing apoptosis. Similar results to those shown in Fig. 6A were obtained; FC loading of lpr macrophages led to only minimal apoptosis, and almost no apoptosis was observed when gld macrophages were added to unloaded lpr macrophages (Fig. 6B). When gld macrophages were added to FC-loaded macrophages, however, apoptosis was increased. 2 Finally, to show direct involvement of FasL in this process, gld macrophages were added to underlying FC-loaded macrophages in the presence of anti-FasL IgG or control non-immune IgG (Fig. 6C). As before, FC-loaded underlying macrophages were much more potent inducers of apoptosis than unloaded macrophages, and most importantly, the induction of apoptosis was substantially blocked by the inclusion of the anti-FasL antibody. In summary, FC loading of macrophages triggers FasL-dependent apoptosis upon addition of secondarily added macrophages, suggesting that FC loading leads to FasL activation.

2 Although inspection of the dishes appeared to show that most of the apoptotic cells were lying on top of the underlying cells, this was difficult to quantify, and so the data are expressed as percent of total cells in the dish (i.e., ~250,000). If, however, we had expressed the percent apoptosis as per the 50,000 secondary macrophages, the actual percent apoptosis would have been much higher.
DISCUSSION

Our study of FC-loaded macrophages has revealed four key findings. (a) A fraction of macrophages exposed to FC loading shows biochemical features of apoptosis, including caspase-dependent externalization of phosphatidylserine and fragmentation of DNA. (b) Inhibition of the Fas pathway by three independent means (Fas mutation, FasL mutation, and inhibitory anti-FasL antibody) partially blocks FC-induced apoptosis. (c) FC loading increases expression of cell-surface FasL. (d) FC loading of macrophages bearing FasL (but not Fas) is able to induce apoptosis when exposed to macrophages bearing Fas (but not FasL) in a FasL-dependent manner. A working model to emerge from these findings is that FC loading through the induction and possibly activation of cell-surface FasL triggers Fas-mediated apoptosis.

The events studied in this report represent those occurring relatively early during the process of FC loading. The biochemical changes observed during this time period are consistent with the morphological studies of Kellner-Weibel et al. (24), which demonstrate cytosolic and nuclear condensation in FC-loaded macrophages. With more prolonged FC loading, however, the macrophages demonstrate signs more characteristic of necrosis, such as swelling of organelles and disruption of the plasma membrane. A key question to emerge from these findings therefore is how early apoptosis might affect later necrosis. Future experiments in which Fas- or caspase-inhibited macrophages are studied after prolonged FC loading will address this point.

The mechanism of FC-mediated induction of the Fas pathway is one of the more novel findings to emerge from this study. FC-loaded macrophages showed no increase in cell-surface Fas but did show an approximately 2–3-fold increase in cell-surface FasL. Because neither FasL mRNA nor total cellular FasL protein changed, these data most likely indicate that FasL was translocated from internal stores to the cell surface as has been reported previously with the activation of human monocytes (38). In this regard, Hannan and Edidin (39) have shown that cholesterol enrichment of cells induces the translocation of a glycoposphatidylinositol-anchored protein from internal stores to cholesterol-rich domains of the plasma membrane. Although other studies implicating up-regulation of FasL in triggering apoptosis have noted a similar modest increase in cell-surface FasL (40), we cannot be certain that the increase in cell-surface FasL in our system is enough to account for the observed induction of apoptosis. It is possible that a change in cell-surface FasL conformation, perhaps induced by an increase in the FC/phospholipid ratio of the plasma membrane, may have led to activation of this molecule. Another important mechanistic issue is related to the observation that inhibition of the Fas pathway did not totally inhibit apoptosis in FC-loaded macrophages. Future studies will attempt to identify other pathways such as those involving other death receptors and other death pathways.

Previous studies have shown that macrophages express cell-surface Fas and can undergo apoptosis when exposed to cells bearing FasL such as T cells (45). Interestingly, agonistic anti-Fas antibody, which can induce apoptosis in certain cell types, was found not to induce apoptosis in Fas-bearing macrophages (45). Consistent with this finding, we were unable to induce...
apoptosis using agonistic anti-Fas antibody or soluble FasL in either control macrophages or FC-loaded gld macrophages (data not shown). Thus, macrophages, like T cells (46) and smooth muscle cells (47), require membrane-bound FasL to engage the Fas receptor. In fact, soluble FasL was inhibitory in our system (data not shown), which is similar to the situation with T cells as reported by Suda et al. (46). Because release of soluble FasL from the surface of cells (including monocytes) occurs physiologically (38, 48), it is possible that this process can inhibit Fas-induced apoptosis in macrophages in vivo. Previous work examining cell-surface FasL expression on macrophages has revealed either undetectable (49) or low (45) levels under normal conditions, consistent with the data reported herein. It is likely that this constitutively low level of cell-surface FasL expression protects macrophages from death under normal conditions.

The most important issue to arise from these studies is the potential physiologic relevance of Fas-mediated apoptosis in FC-loaded macrophages. As mentioned in the Introduction, both FC-loaded macrophages and macrophage death are notable features of atherosclerotic lesions. Moreover, Cai et al. (26) have shown that apoptotic macrophages in atherosclerotic lesions, but not non-apoptotic cells, express Fas. In another study, Geng and colleagues (27) showed the presence of numerous TUNEL-positive cells in regions of carotid atherosclerotic plaques that contained FasL-positive macrophages. Whereas these observations do not prove causal relationships, they are consistent with the possibility that some of the macrophage death known to occur in lesions is triggered by FC loading and mediated by the Fas pathway.

If these events do occur, what effect might they have on lesion progression? In one possible scenario, apoptosis of FC-loaded macrophages might be a protective response that allows the “safe” destruction of these potentially harmful lesional foam cells (33). In this scenario, the cells would condense into “apoptotic bodies,” followed by phagocytosis by neighboring macrophages and perhaps exit from lesions without release of cellular contents (50, 51). In another scenario, apoptosis of FC-loaded macrophages may contribute to lesion pathology, particularly lipid core development, by contributing to the release of harmful molecules. As mentioned above, apoptosis can precede what has often been referred to as “necrosis” and does not always prevent release of cellular contents from dying cells (52–57). In addition, the phagocytosis of apoptotic bodies may be inhibited by the presence of oxidized lipoproteins and lipids in atherosclerotic lesions or by the cholesterol-loaded state of the phagocyte, as has been demonstrated in cell culture studies (58, 59). Moreover, even if the initial response were the engulfment of foam cell apoptotic bodies by neighboring macrophages, these phagocytes would now be engorged with apoptotic foam cell “remnants,” including abundant lipids. Eventually, the capacity of lesional macrophages to safely carry out this process might be exceeded. Finally, the induction of FasL by FC loading of macrophages may have atherogenic effects other than those related to apoptosis, as shown by the recent study of Schneider et al. (60) that demonstrated that FasL-transduced arteries in hypercholesterolemic rabbits had increased numbers of intimal smooth muscle cells. Future in vivo models in which the macrophage Fas pathway is genetically altered will be required to definitively address these hypotheses.

In summary, the data reported herein demonstrate that FC loading of macrophages leads to an apoptotic response that is partially dependent on induction of the Fas pathway. In vivo, lesional macrophages accumulate excess FC, have been shown to bear Fas and FasL, and undergo apoptosis. Therefore, the model described herein provides the basis for future studies directed toward the role of macrophage death in general and Fas-mediated apoptosis in particular in the pathophysiology of atherosclerosis.

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