Human Blood–Derived Macrophages Induce Apoptosis in Human Plaque–Derived Vascular Smooth Muscle Cells by Fas-Ligand/Fas Interactions

Joseph J. Boyle, David E. Bowyer, Peter L. Weissberg, Martin R. Bennett

Abstract—Human atherosclerotic plaques that rupture are characterized by relatively low vascular smooth muscle cell (VSMC) and high inflammatory cell contents. Ruptured plaques also contain higher numbers of apoptotic VSMCs than do stable lesions, suggesting that VSMC apoptosis may promote plaque rupture. We examined the ability of human monocytes/macrophages to induce apoptosis of VSMCs derived from human carotid plaque, aortic media, and coronary media. Macrophages, but not T lymphocytes, induced a dose-dependent apoptosis of VSMCs, which required monocyte maturation to macrophages and direct cell-cell contact/proximity. VSMC apoptosis was inhibited by neutralizing antibodies to Fas-ligand (Fas-L) or an Fas-Fc fusion protein, indicating the requirement for membrane-bound Fas and Fas-L. Monocyte maturation was associated with increased surface expression of Fas-L, coincident with the onset of cytotoxicity. VSMCs expressed surface Fas, which was increased in plaque VSMCs, and plaque VSMCs also underwent Fas-induced apoptosis. We conclude that human macrophages potently induce human VSMC apoptosis, which requires direct cell-cell interactions and is in part dependent on Fas/Fas-L interactions. Macrophage-induced VSMC apoptosis may therefore directly promote plaque rupture. (Arterioscler Thromb Vasc Biol. 2001;21:1402-1407.)

Key Words: macrophages ■ atherosclerosis ■ apoptosis ■ smooth muscle cells, vascular ■ Fas

Myocardial infarction is caused by rupture of a coronary atherosclerotic plaque,1 with thrombus formation and arterial occlusion. Ruptured plaques are characterized by increased macrophage and T-lymphocyte and decreased vascular smooth muscle cell (VSMC) and collagen contents.2,3 Because VSMCs are the only plaque cells that synthesize structurally important collagen isoforms types I and III,4,5 their loss may promote plaque rupture.5 Reduced numbers of VSMCs in advanced atherosclerotic plaques may be due to decreased proliferation, increased death, or both.6

Apoptosis (programmed cell death) is a regulated form of cell death defined by specific morphological markers, including nuclear and cytoplasmic condensation, nuclear fragmentation, and membrane blebbing.7,8 VSMC apoptosis has been identified in advanced human atherosclerotic plaques5,6 and is increased in unstable versus stable lesions,11 implicating VSMC apoptosis in plaque rupture. Although increasing evidence suggests that VSMC apoptosis is deleterious in advanced atherosclerosis, the stimuli for VSMC apoptosis in atherosclerotic plaques are unknown. In vitro, human plaque–derived VSMCs show increased apoptosis compared with cells from normal media, implying an intrinsic susceptibility to apoptosis in plaque VSMCs.6 However, the local plaque microenvironment may also regulate VSMC apoptosis. For example, immunocytochemistry shows that plaque VSMCs express cytokine activation markers, indicating exposure in vivo to specific cytokines.3 In vitro, soluble inflammatory cell cytokines synergistically induce VSMC apoptosis,12 suggesting that inflammatory cells may reduce VSMC numbers by inducing apoptosis.

In addition to soluble cytokines, apoptosis may be induced by direct interactions of membrane-bound receptors and ligands. Thus, both CD95 (Fas) and tumor necrosis factor (TNF) receptors are expressed by VSMCs in atherosclerotic plaques, and Fas can induce apoptosis in cytokine-primed cells.13 Fas-ligand (Fas-L) is expressed as a membrane-bound protein on activated T lymphocytes and natural killer (NK) cells, inducing apoptosis by direct cell-cell contact.8 Fas-L is also present intracellularly in monocytes/macrophages and may be released on activation.14

We therefore examined whether human inflammatory cells (macrophages and T lymphocytes) induce human VSMC apoptosis. We found that macrophages, but not T lymphocytes or monocytes, potently induce VSMC apoptosis. This event requires direct cell-cell contact or proximity and is partly due to Fas/Fas-L interactions. Plaque macrophages may therefore directly induce VSMC apoptosis in atherosclerosis, promoting plaque rupture.

Methods

Isolation of Peripheral Blood Mononuclear Cells and Subsets

Human peripheral blood mononuclear cells (PBMCs) were prepared from buffy coats by using established methods, and monocyte- and lymphocyte-rich fractions were purified by differential adherence.15,16 Flow cytometry indicated that the adherent fraction was...
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Soluble Fas-L was added at 1.6 mol/L each. To induce apoptosis in VSMC monocultures, agonistic anti-Fas IgM or z-VAD-fmk. (See Methods online at http://atvb.ahajournals.org.) To assess the requirement for inhibitors, neutralizing anti–Fas-L antibody, Fas-Fc fusion protein, and phage-induced apoptosis, cocultures were repeated with apoptosis in- quantified by trypan blue exclusion. To assess mechanisms of macro- 

detection of apoptosis in cocultures of human 

cellular VSMCs and PBMCs 

Monocytes/macrophages and VSMCs were cocultured for 8 days, and the apoptotic index of each cell type was analyzed. (See Methods online at http://atvb.ahajournals.org.) Apoptosis was quantified by fluorescence microscopy with the DNA-binding dyes propidium iodide (PI) and Hoechst 33258, and cell type was determined with the monocyte/macrophage marker CD14-FITC (Figures IIA through IID online at http://atvb.ahajournals.org). To confirm apoptosis, cultures were stained with FITC–annexin-V and PI, examined by immunocytochemistry and flow cytometry, and also subjected to DNA flow cytometry for hypodiploid DNA (Figures IIE through IIH online at http://atvb.ahajournals.org). To assess the requirement for cell-cell proximity or contact, filter-separation coculture was performed with 0.2-μm filter-separation units, and VSMC death was quantified by trypan blue exclusion. To assess mechanisms of macro- 

expression of Fas and Fas-L 

Fas and Fas-L in cultured VSMCs, monocytes, or macrophages were assessed by flow cytometry and Western blotting. Thrombosed coronary arteries from autopsies for acute myocardial infarction were immuno- 

Statistics 

Data were checked for distribution before analysis and analyzed by ANOVA, Dunn’s non-parametric test for multiple simultaneous comparisons, or Pearson’s product-moment correlation coefficient. 

Results 

Quantification of Apoptosis in Coculture 

Apoptosis of VSMCs after 8 days in coculture was assessed with PI/Hoechst 33258 staining by using a modification of established methods.12 CD14-FITC staining was used to identify monocytes, although initial experiments indicated that monocyte apoptosis occurred predominantly in the first 48 hours of coculture. VSMC apoptosis was defined by chromatin condensation and nuclear fragmentation on PI/ Hoechst 33258 staining in CD14-negative cells (Figures IIA through IID online at http://atvb.ahajournals.org). Apoptosis detected by this method was compared with the viability of cells released into suspension, as determined by trypan blue exclusion (Pearson’s correlation coefficient r=0.95). This comparison indicated that PI/Hoechst 33258 staining provided an accurate assessment of cell death in each cell type. 

Human VSMCs 

Human aortic VSMCs were obtained from donors from Addenbrooke’s Hospital Transplant Program. Human plaque VSMCs were cultured from carotid plaques that had been removed at carotid endarterectomy. Cells were cultured and characterized by immuno- 

Human Macrophages Induce Apoptosis More Potently Than Do T Lymphocytes 

To examine whether human PBMCs induce VSMC apoptosis, we incubated PBMCs, purified monocytes, or purified T lymphocytes with human VSMCs over a period of 8 days. To determine the role of individual PBMC components in inducing apoptosis, we first used a human VSMC cell line, HCMD-1 E6, which has reproducible levels of apoptosis over many passages in culture.17,18 Increasing ratios of PBMCs to VSMCs increased VSMC apoptosis, with 50% toxicity at ~8:1 (Figure 1). The mono- 

Macrophage-Induced Toxicity Is Due to Apoptosis 

Macrophage-induced cytotoxicity is a well-recognized phenom- 

Human Macrophages Induce Apoptosis in Human Plaque-Derived VSMCs 

The HCMD-1 E6 VSMC cell line has been generated by inserting the human papilloma virus E6 gene into medial VSMCs derived from a normal coronary artery specimen. This manipulation results in stable proliferation and apoptosis over many months in culture but produces slightly increased basal rates of apoptosis compared with untransduced cells. To confirm that monocyte-induced apoptosis was not related to the semitransformed nature of this cell line and to examine
the potential in vivo relevance of monocyte-induced apoptosis in advanced atherosclerosis, we examined macrophage-induced apoptosis in VSMCs from human atherosclerotic plaques (n=4 monocyte donors, n=4 plaque VSMC donors). The control VSMC apoptotic index was 5.2±0.9% (mean±SEM); 8-day coculture with human monocytes induced 81±2.9% apoptosis in plaque VSMCs. Thus, plaque VSMCs were as susceptible to macrophage-induced apoptosis as were HCMED-1 E6 VSMCs or aortic VSMCs (see below) at the same ratio of monocytes to VSMCs.

**Macrophages Induce Dose-Dependent Apoptosis in Aortic Medial VSMCs**

Although the arterial media usually has low levels of VSMC apoptosis, medial VSMC apoptosis is associated with infiltration of inflammatory cells in atherosclerotic aneurysms. We therefore examined macrophage-induced apoptosis of primary human aortic medial VSMCs in direct coculture over 8 days (Figure III online at http://atvb.ahajournals.org). Macrophages induced a dose-dependent apoptosis of human aortic medial VSMCs, with 50% toxicity at a 4:1 ratio of macrophages to aortic VSMCs.

**Macrophage-Induced Apoptosis Requires Cell-Cell Contact or Proximity**

VSMC apoptosis may be induced by a combination of soluble inflammatory cytokines. Macrophages also express proapoptotic cell surface ligands such as Fas-L and TNF-α. To examine whether macrophage-induced VSMC apoptosis was mediated by soluble cytokines or by direct cell-cell contact or proximity, macrophage-induced apoptosis of aortic VSMCs or plaque VSMCs was analyzed by separating the VSMCs and macrophages through a 0.2-μm filter, which permits free passage of soluble mediators (n=4 macrophage donors, n=4 plaque VSMC donors). In monocolours, there was 5.2±0.9% (mean±SEM, percentage of trypan blue–positive cells) plaque VSMC death and 4.5±2.4% HCMED1-E6 VSMC death after 8 days in culture. In direct coculture with macrophages, there was 90±2.2% plaque VSMC death and 98±0.17% HCMED1-E6 VSMC death. Filter-separation coculture reduced VSMC apoptosis to 2±1.2% plaque VSMC death and 2±0.9% HCMED1-E6 VSMC death (Figure IV online at http://atvb.ahajournals.org). Thus, prevention of cell-cell contact completely abrogated the macrophage-induced apoptosis of either HCMED-1 E6 VSMCs or plaque VSMCs, indicating that direct cell-cell contact or proximity is required for macrophage-induced VSMC apoptosis.

**Macrophages but Not Monocytes Induce VSMC Apoptosis**

In atherosclerosis, monocytes migrate into the vessel wall, differentiate into macrophages, and come into proximity with VSMCs. To analyze whether monocytes or macrophages induce VSMC apoptosis, we examined VSMC apoptosis induced by monocytes/macrophages of increasing age in culture. (See Results online at http://atvb.ahajournals.org.) This experiment indicated that the onset of VSMC cytotoxicity occurred at culture days 6 to 8, coincident with monocyte differentiation to macrophages, but not before.

**Macrophages Reduce Viable VSMC Numbers**

Macrophages in plaques also express and secrete mitogens for VSMCs. To examine whether macrophage-induced VSMC apoptosis effectively reduced VSMC numbers and the contribution of macrophage apoptosis, we counted live VSMCs, monocytes/macrophages, and lymphocytes in cocultures. In control plaque and HCMED1-E6 VSMC monocultures, live VSMC numbers did not vary significantly over time: cell numbers at days 0, 2, 4, 6, and 8 were 1000, 1051±230, 1178±216, 1210±166, and 1688±185 plaque VSMCs (mean±SEM cells per well, n=4 experiments), respectively, and 20 000, 20 470±1840, 19 760±1026, 21 440±2270, and 20 250±2000 HCMED1-E6 VSMCs, respectively (P>0.05, ANOVA). In coculture with macrophages, live VSMC numbers fell only at and after culture days 6 to 8: cell numbers at days 0, 2, 4, 6, and 8 were 1000, 1338±128, 1264±197, 624±176, and 38±11, respectively, for plaque VSMCs and 20 000, 22 700±2170, 25 380±1650, 1420±440, and 93±50 for HCMED1-E6 VSMCs, respectively (mean±SEM), corresponding to increased apoptosis. In coculture with VSMCs, live monocyte numbers fell slightly at the outset but were maintained thereafter: cell numbers at days 0, 2, 4, 6, and 8 were 8000, 6100±470, 4850±580, 5730±690, and 4060±490, respectively, for plaque VSMC coculture and 100 000, 82 500±8770, 90 800±10 900, 92 000±15 270, and 86 800±11 760, respectively, for HCMED1-E6 coculture. By comparison with macrophage/VSMC dose-response curves (Figure 1), this reduction in monocyte number (and thus, the monocyte to VSMC ratio) would not have appreciably altered macrophage-induced VSMC apoptosis. The number of live lymphocytes at culture day 8 was not significantly different from that at the start of culture (start ratio 8:1; after 8 days, VSMCs were 20 880±5610 and lymphocytes were 189 240±41 770, yielding a final ratio of 9:1). Thus, leukocytes reduced the number of viable VSMCs from culture days 6 to 8.

**Macrophages Induce VSMC Apoptosis Through Fas/Fas-L**

We have previously shown that human VSMCs can be induced to undergo apoptosis through Fas/Fas-L interactions on their cell surface. Fas/Fas-L–induced apoptosis has also been implicated in VSMC apoptosis in atherosclerosis and may promote apoptosis of cytokine-stimulated VSMCs. To demonstrate whether macrophage-induced VSMC apoptosis was also mediated through Fas/Fas-L, we assessed apoptosis in macrophage/VSMC cocultures in the presence of the neutralizing anti–Fas-L antibody NOK-1 (Figure 2A). Western blotting confirmed the specificity of NOK-1 for Fas-L (not shown) or with increasing concentrations of Fas-Fc fusion protein (Figure 2B). In both cases, there was significant inhibition of macrophage-induced VSMC apoptosis. Control IgG and inhibitor reagents had no effect on macrophage apoptosis or VSMC apoptosis in monocultures and no effect on macrophage-induced VSMC apoptosis in cocultures. At the same time point, there was no effect of Fas-Fc or anti–Fas-L on macrophage apoptosis. To confirm that the inhibition of apoptosis was due to inhibition of Fas on VSMCs and not Fas on macrophages, we preincubated macrophages with a neutralizing anti-Fas antibody before incubation with VSMCs. Prior blockade of macrophage Fas did not reduce macrophage-induced VSMC apoptosis (not
Several detection methods indicated that macrophage-induced VSMC death was apoptotic. However, to confirm that VSMC death required caspase activity, the broad-spectrum caspase inhibitor z-VAD-fmk was added to cocultures. z-VAD-fmk (10 μmol/L) significantly inhibited macrophage-induced VSMC apoptosis (Figure 2A, *P* < 0.05), also confirming that death was apoptotic.

Macrophages and VSMCs Express Fas-L and Fas

Our results suggest that Fas-L on the surface of macrophages induces VSMC apoptosis by binding to Fas on the surface of VSMCs. To confirm that Fas-L/Fas are expressed by cultured monocytes, macrophages, or VSMCs, we examined protein expression of Fas-L and Fas by Western blots and flow cytometry of cultured plaque VSMCs, monocytes, and macrophages. Figure 3A demonstrates that all 3 cell types express both Fas-L and Fas. We previously found that Fas in normal human VSMCs is mostly intracellular, where it is nonfunctional, with little expression on the cell surface. Similarly, Fas-L is expressed on the surface of inflammatory cells but may be cleaved by a metalloproteinase, resulting in soluble Fas-L, which is far less potent. We therefore examined surface expression of Fas and Fas-L in monocytes/macrophages and VSMCs. Both plaque and normal aortic VSMCs expressed surface Fas, with plaque VSMCs showing higher surface expression (Figure 3B). Monocytes and macrophages also expressed surface Fas. In contrast, surface Fas-L was detected only after monocyte differentiation into macrophages (Figure 3B), although permeabilized monocytes and macrophages expressed abundant intracellular Fas-L (not shown).

Activation of Fas Induces Apoptosis in Plaque VSMCs

The demonstration that macrophage-induced VSMC apoptosis is partly inhibited by neutralizing antibodies to Fas-L or Fas-Fc fusion proteins suggests that plaque VSMCs may undergo apoptosis after activation of Fas. However, it has been shown previously that normal human VSMCs do not undergo Fas-induced apoptosis unless primed by inflammatory cytokines. To examine whether plaque VSMCs are sensitive to Fas-induced apoptosis, we incubated plaque VSMCs with agonistic anti-Fas antibody or soluble Fas-L. The control plaque VSMC apoptotic index was 7.8 ± 1.8% (mean ± SEM, *n* = 4 plaque VSMC donors). This value increased to 59 ± 6.8% with soluble Fas-L and to 40.5 ± 3% with agonistic anti-Fas (Figure V online at http://atvb.ahajournals.org). Thus, cross-linking anti-Fas antibody and soluble Fas-L induced plaque VSMC apoptosis in the absence of any other priming of the VSMCs.

In Ruptured Plaques, Macrophages Express Fas-L and VSMCs Express Fas

For macrophages to induce VSMC apoptosis by Fas-L in vivo, plaque macrophages should express Fas-L and plaque VSMCs express Fas.
should express Fas. This hypothesis was tested immunocytochemically (Figure VI online at http://atvb.ahajournals.org). Indeed, ruptured coronary plaques (n=5 subjects) contained macrophages doubly immunoreactive for Fas-L and CD68 (Figure VI online at http://atvb.ahajournals.org). VSMCs within ruptured plaques expressed Fas and α-actin (Figure VI online at http://atvb.ahajournals.org). Thus, in human ruptured coronary plaques, macrophages expressed Fas-L and VSMCs expressed Fas.

Discussion

Ruptured atherosclerotic plaques histologically have a relatively thin fibrous cap, contain few VSMCs, and have a larger lipid pool and higher inflammatory cell content than do stable lesions.2 Recently, increased levels of VSMC apoptosis have been found in unstable versus stable plaques.11 The inverse correlation between VSMC and inflammatory cell numbers in ruptured plaques and the increased levels of VSMC apoptosis suggest that inflammatory cells may induce VSMC apoptosis, thus directly contributing to plaque rupture. We have shown in the current study that human blood–derived macrophages induce apoptosis of VSMCs derived from human plaques and normal media. VSMC apoptosis induced by macrophages requires direct cell-cell contact or proximity and is mediated in part by Fas–Fas-L interactions. In contrast, human T lymphocytes were ineffective at inducing VSMC apoptosis.

There are a number of important points to emerge from this study. First, cytotoxicity to VSMCs was mediated by macrophages, not by cytotoxic T lymphocytes or NK cells. Cytotoxic T lymphocytes and NK cells were not detected by flow cytometry in the monocyte subtraction. Macrophage-related cytotoxicity has previously been seen directed against ingested microbes22 or toward tumor cells,23 but there are few reports of macrophage-induced apoptosis of untransformed or uninfected cells. Within the vasculature, macrophages have been shown to induce endothelial cell death within the developing retina, and abnormal macrophage-induced endothelial cell apoptosis results in the persistence of primitive vascular networks,24 suggesting that macrophages may function in embryogenesis. PBMCs preactivated by irradiation or bacterial lipopolysaccharide have also been shown to induce apoptosis of human umbilical vein endothelial cells, although the pathophysiological relevance and the responsible leukocyte subset were not studied.25

Second, we have demonstrated that macrophage-induced killing of VSMCs occurs through induction of apoptosis. Macrophages have previously been reported to induce cytotoxicity of tumor cells. Although the mode of cell death was not analyzed, cell death was due to nitric oxide radicals and oxygen free radicals23 and thus may have occurred by necrosis of target cells. In contrast, apoptosis of VSMCs was clearly shown in the current study by the fluorescence microscopic appearance of condensed nuclei after PI/Hoechst 33258 staining, annexin-V fluorescence, and flow cytometry of hypodiploid peaks of fragmented DNA.

We found that macrophage-induced apoptosis required direct cell-cell contact or proximity and is therefore not likely to have been mediated by soluble cytokines. Previous studies have shown that a combination of inflammatory cell cytokines, including interleukin-1β, TNF-α, and interferon-γ, can induce apoptosis of rat and human VSMCs and can also prime VSMCs to Fas-induced apoptosis.12,13 Although these studies suggested that inflammatory cells induce apoptosis via soluble cytokines, it was not demonstrated whether inflammatory cells could produce sufficient quantities of cytokines to reproduce the effect of exogenous recombinant cytokines. In contrast, cell-free supernatants of activated PBMCs have been shown to induce apoptosis of human umbilical vein endothelial cells, although the mechanism of this effect was not studied and direct cell-cell–mediated apoptosis was a more potent mechanism of killing.25 In contrast, we have demonstrated that direct cell-cell–induced apoptosis by inflammatory cells is a far more potent mechanism for induction of VSMC apoptosis. This does not exclude a role for cytokine-induced priming of VSMCs to undergo apoptosis but suggests that soluble cytokines alone may not be responsible for VSMC apoptosis in atherosclerosis.

We have also shown that macrophage-induced VSMC apoptosis is mediated in part by Fas/Fas-L interactions on the surface of cells. Fas (CD95) is a receptor of the TNF receptor superfamily. On ligation by Fas-L, it induces apoptosis by a cascade of cysteine proteases (caspases), particularly caspase-8 and caspase-3 (reviewed in Ashkenazi and Dixit26). Although Fas-L exists in both membrane-bound and soluble forms, the membrane-bound form is the more potent27 and is partly responsible for cytotoxic T lymphocyte–induced target cell apoptosis.28 Although human monocytes contain high levels of intracellular Fas-L, which can be released on activation,14 Fas-L has not been shown previously to mediate human monocyte/macrophage-induced apoptosis.

For monocytes to induce apoptosis in VSMCs requires the presence of surface Fas-L on monocytes and Fas on VSMCs. We have demonstrated by Western blotting and flow cytometry that these proteins are both expressed and are present on the cell surface of the requisite cells. VSMC Fas is able to induce apoptosis, as shown by induction of apoptosis by an agonistic anti-Fas antibody or soluble Fas-L in plaque VSMCs. In previous studies, we and others have shown that normal human medial VSMCs are resistant to Fas-induced apoptosis and require priming with p53 activation or cytokine treatment before becoming sensitive.13,19 In addition, the level of surface expression of Fas partly determines sensitivity to Fas-induced apoptosis. However, flow cytometry of plaque VSMCs suggests that these cells have increased surface expression of Fas compared with aortic VSMCs, which may explain why plaque VSMCs undergo Fas-induced apoptosis without prior priming.

The role of Fas/Fas-L in mediating macrophage-induced apoptosis does not exclude other mediators in executing this function. The dose-response curve for Fas-Fc fusion protein shows that partial inhibition of macrophage-induced VSMC apoptosis is due to partial contribution of Fas/Fas-L to apoptosis and is not due to insufficient doses of the inhibitor. This result suggests that macrophage-induced apoptosis can also be due to other membrane-bound ligands/receptors. We have also not totally excluded the possibility that autocrine or paracrine Fas/Fas-L interactions on monocytes/macrophages alter their ability to induce apoptosis. However, prior incubation of macrophages with neutralizing anti–Fas-L antibody did not reduce macrophage-induced VSMC apoptosis, and the induction of apoptosis in plaque VSMCs by agonistic antibodies to Fas or soluble Fas-L suggests that monocyte Fas-L interacts with VSMC Fas to induce apoptosis. In addition, in monocytes and
macrophages, Fas/Fas-L interactions have been implicated in autocrine apoptosis, not activation.\textsuperscript{14} Monocytes in atherosclerotic plaques differentiate into macrophages, possibly related to the phagocytosis of both lipids and apoptotic bodies.\textsuperscript{1} Cultured monocytes also differentiate into macrophages.\textsuperscript{1} The high levels of bystander apoptosis in early monocyte cultures raise the possibility that macrophage maturation in culture may be related to phagocytosis of lipids or apoptotic bodies. Although monocytes cultured to 4 days did not induce significant VSMC apoptosis, by 6 days monocytes/macrophages potently induced VSMC apoptosis. Thus, the time course of macrophage cytotoxicity suggests that differentiation is required for efficient macrophage-induced apoptosis. Consistent with this concept, surface Fas-L was detectable on macrophages only after 5 days in culture, coincident with the onset of cytotoxicity. Importantly, we found no change in the total expression of Fas-L in monocytes compared with macrophages, consistent with previous studies suggesting that Fas-L is mostly intracellular in monocytes\textsuperscript{13} and that both surface expression and release are increased by activation. Consistent with other studies,\textsuperscript{20,26} we found that monocyte differentiation into macrophages is associated with resistance to apoptosis. It is thus conceivable that monocyte differentiation into macrophages in the atherosclerotic plaque is associated with resistance to apoptosis but an increased ability to induce apoptosis in adjacent cells.

For these in vitro findings to be pathophysiologically relevant then, ruptured human plaques should contain macrophages expressing Fas-L and VSMCs expressing Fas. Immunocytochemistry of ruptured plaques confirmed this hypothesis. Although other investigators have shown that VSMCs in stable plaques express Fas,\textsuperscript{13} we can find no prior reports of plaque macrophage Fas-L expression. The finding of Fas-L and Fas in ruptured coronary plaques is consistent with the hypothesis that Fas-mediated macrophage-induced VSMC apoptosis may have in vivo relevance. In conclusion, we have demonstrated that human monocyte-derived macrophages induce human VSMC apoptosis. Apoptosis requires direct cell-cell contact and is mediated in part through membrane-bound Fas/Fas-L. We suggest that macrophage induction of VSMC apoptosis in atherosclerosis may promote plaque rupture.

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