Sensitivity to Fas-Mediated Apoptosis Is Determined Below Receptor Level in Human Vascular Smooth Muscle Cells

Shiu-Wan Chan, Laszlo Hegyi, Stephen Scott, Nathaniel R.B. Cary, Peter L. Weissberg, Martin R. Bennett

Abstract—Despite Fas expression, many cells resist Fas-induced apoptosis. Although differences in surface Fas expression can explain Fas resistance, multiple proteins below receptor level also inhibit Fas-induced apoptosis. To examine the mechanism of Fas resistance, we studied Fas-induced apoptosis in human medial vascular smooth muscle cells (VSMCs) from healthy coronary arteries. VSMCs showed marked heterogeneity to Fas-induced apoptosis, exhibiting both Fas-resistant (98.1 ± 2.3% viable, n = 4, P = NS) and Fas-sensitive (31.3 ± 2.6% viable, n = 3, P < 0.01) cells. Fas-resistant VSMCs expressed surface Fas and could recruit RIP, indicating that functional receptor complexes were formed. However, Fas-resistant cells showed reduced expression of FADD, Fas ligand, and caspases 3, 7, and 8 and increased expression of FLIP and c-IAP-1. Fas-induced apoptosis was associated with cleavage of caspase 3 and blocked by inhibitors of caspase 3 or 8 but not caspase 1, 6, or 7. Selective inhibition of caspase 3 or 8 by antisense transfection inhibited Fas-induced apoptosis, but their reexpression could not rescue the Fas-resistant phenotype. In vivo, medial VSMCs showed marked heterogeneity of expression of caspase 3. We conclude that Fas sensitivity is determined not only by expression of surface Fas but by differential expression of Fas-signaling proteins below receptor level. Subpopulations of cells within the same tissue have different sensitivities to apoptosis, determined by expression of specific death-signaling proteins. (Circ Res. 2000;86:1038-1046.)

Key Words: vascular smooth muscle cell ▪ apoptosis ▪ remodeling

Apoptosis is an evolutionarily conserved mechanism of cell death that regulates tissue architecture and homeostasis in both embryogenesis and adult organisms. In virus infection and neoplasia, apoptosis also protects the organism from potentially dangerous cells. Indeed, viruses and neoplastic cells have evolved mechanisms of downregulating apoptosis, and progression of disease is associated with escape or resistance to apoptosis. Although selective apoptosis of cells within a tissue coordinates remodeling of that tissue, it is not known why some cells undergo apoptosis in one spatial location and not in another. Clearly, cell-cell differences in susceptibility to apoptosis may govern whether apoptosis occurs in cells of the same lineage in a complex tissue.

Apoptosis of vascular smooth muscle cells (VSMCs) occurs in both physiological and pathological remodeling of arteries and in disease states such as atherosclerosis and angioplasty restenosis. Arterial remodeling and injury provokes profound medial VSMC apoptosis, which determine arterial caliber, mass, and architecture, irrespective of concomitant VSMC replication. However, despite presumably adjacent medial VSMCs receiving the same apoptotic stimulus, VSMC apoptosis in arterial remodeling or injury is very localized and incomplete, depending on the location of the VSMC in question and reflecting heterogeneity of response. The mechanisms underlying differential sensitivity of VSMCs to the same apoptotic stimulus are unknown.

Apoptosis is regulated through a variety of different pathways, including those regulated by death receptors of the tumor necrosis factor (TNF) family (Fas/CD95, TNF-R1, DR-3/TRAMP, TRAIL-R1, and TRAIL-R2). Binding of Fas ligand (Fas-L) to Fas induces receptor trimerization, recruitment of adapter molecules FADD and RIP to the receptor complex, and recruitment of cysteine proteases (caspases) such as caspase 8 (FLICE) and caspase 2. Caspase 8 becomes proteolytically activated by oligomerization, with subsequent activation of effector caspases (caspases 3, 6, and 7) responsible for cleavage of intracellular substrates required for cellular survival, architecture, and metabolic function. The major active caspases in Fas-mediated apoptosis are caspases 3, 8, 6, and 7, with stepwise appearance of active caspases suggesting a caspase cascade.

Fas is constitutively expressed in cells of many tissues, including epithelial and hematopoietic cells. Importantly, subpopulations of endothelial cells and some mesenchymal
cells express Fas, indicating that Fas-sensitive and -resistant cells of the same lineage may coexist within the same tissue. However, despite cells expressing Fas, many cells may be resistant to Fas-induced apoptosis because of the intracellular sequestration of Fas, downregulation of surface Fas expression, or expression of Fas-L decoy receptors. Fas-induced apoptosis can also be blocked by expression of several intracellular proteins, including Bcl-2 family members binding proteins with inactive caspase domains such as c-FLIP, and inhibitors of caspases such as the inhibitor of apoptosis (IAP) family of proteins. IAPs directly inhibit the enzymatic activity of caspases 3 and 7 (but not caspase 1, 6, 8, or 10) and also indirectly block caspases 3, 6, and 7 processing by inhibiting cytochrome c-induced activation of caspase 9.

We have examined the sensitivity of human arterial medial VSMCs to Fas-induced apoptosis. We found that Fas resistance is due to differential expression of both proapoptotic and antiapoptotic proteins below receptor level. In particular, caspases 3 and 8 are necessary but not sufficient for Fas-induced apoptosis. Heterogeneity of expression of apoptosis-signaling proteins is also demonstrable in vivo, indicating the coexistence of Fas-sensitive and -resistant cells within the same tissue. This heterogeneity may profoundly affect how a tissue remodels after an apoptotic stimulus and identifies a subpopulation of cells that undergoes apoptosis after a defined stimulus.

Materials and Methods

Generation of VSMC Cultures

Healthy medial VSMCs were derived from coronary arteries of patients undergoing cardiac transplantation for nonspecific cardiomyopathy, as previously described, and human atherosclerotic plaque VSMCs from carotid endarterectomy specimens of patients with symptomatic carotid disease. VSMC cultures with extended life span were generated, as previously described, and used for experiments examining cleavage of caspases (see below). The present study was conducted in accordance with guidelines of the local ethical committee, and informed consent was obtained for the use of normally discarded tissue.

Induction of Apoptosis

Apoptosis was induced by the addition of 10 μg/mL cycloheximide (CHX) (Sigma)+1 μg/mL anti-Fas antibody IgM clone CH-11 (α-Fas) (05-201, Upstate) for 24 hours or CHX+recombinant Fas-L (50 ng/mL) (a generous gift from Dr Peter Kiener, Bristol-Myers Squibb, Princeton, NJ). Alternatively, apoptosis was induced by transferring cells to medium containing 0% FCS for 24 hours.

Time-Lapse Videomicroscopy

Time-lapse videomicroscopy was performed as previously described.

XTT Assay Plus Peptide Inhibition

Cells were seeded at a suitable density (from 3000 cells to 2×10⁴ cells per well in a 96-well plate (Falcon) overnight. After induction of apoptosis for 24 hours, 50 μL of XTT (Boehringer Mannheim) was added to each well, and readings were taken 24 hours later at 492 nm, corrected against 690 nm. The protective effect of synthetic peptide inhibitors was tested by adding serial dilutions of zVAD-fmk, DEVD-CHO, IETD-CHO (Bachem), or YVAD-CHO (Star) to cells for 24 hours before and during induction of apoptosis.

Flow Cytometry

Flow cytometry for Fas or Fas-L, was performed, as previously described, using anti-Fas antibody IgM (05-201, Upstate) or anti-Fas-L IgG1 antibodies (F37720, Transduction Labs or NOK-1 (65320C Pharmingen))

Western Blotting

Western blotting was performed, as previously described, using primary antibodies directed against Fas, Fas-L, FADD, caspases 8, 1, 3, and 7, Bcl-2, Bcl-X, RIP, c-IAP-1, FLIP, β-tubulin, and α-smooth muscle actin.

Immunoprecipitation

VSMCs were preincubated in CHX for 24 hours and protein lysates isolated at 0 to 2 hours after addition of IgM Fas. Fas-protein G columns were made by binding 10 μg/mL mouse anti-Fas IgG to protein G Sepharose (16 hours, 4°C). VSMC lysates were incubated with anti–Fas-protein G complexes (3 hours, 4°C), precipitated, separated by 10% SDS-PAGE in nonreducing conditions, and blotted with 0.25 μg/mL anti-RIP antibody.

Immunocytochemistry

Cells were incubated with 1:100 goat anti-p20 caspase 3 antibody (N-19) or control goat polyclonal IgG in blocking buffer for 1 hour and then biotinylated rabbit anti-goat secondary antibody (Santa Cruz) for 30 minutes at room temperature. This was followed by incubation with avidin–biotinylated horseradish peroxidase complex (Santa Cruz) for 30 minutes at room temperature followed by development with 3,3′-diaminobenzidine (Sigma).

Expression/Inhibition of Caspase 3

Full-length human caspase 3 or 8 expression vectors in either the sense or antisense orientation were cotransfected with pCMV β-galactosidase into VSMCs. After 2 hours, apoptosis was induced by anti-Fas IgM and CHX, and after 48 hours, cultures were stained for β-galactosidase activity.

Histochemistry of Tissues

Human healthy coronary arteries were stained with antibodies to caspase 3, Fas, FADD, or caspase 8 or isotype-matched control antibodies at room temperature overnight. Positive staining was detected using HRP or alkaline-phosphatase–conjugated secondary antibodies.

Results

Different VSMC Isolates Are Sensitive or Resistant to Fas-Induced Apoptosis

To examine any heterogeneity in ability to undergo apoptosis in VSMCs from healthy human arteries, we isolated medial VSMCs from healthy coronary arteries. Uncloned, pooled populations of cells from individual patients were used. We examined apoptosis of VSMCs after stimulation of Fas or after growth factor withdrawal. All VSMCs were initially resistant to Fas-induced apoptosis (not shown), as previously demonstrated, but after CHX priming, 3 of 7 separate VSMC cultures showed significant reduction in viability to 15%, 71%, and 8%, respectively, compared with CHX alone. Of the 7 cultures, 4 remained resistant to Fas (Figure 1A). Time-lapse videomicroscopy in response to recombinant Fas-L indicated rates of apoptosis of 8% and 89% at 24 hours in resistant and sensitive cells, respectively (Figure 1B). CHX treatment alone did not differentially induce apoptosis in the absence of Fas activation in either Fas-resistant or -sensitive
cells (Figure 1B). Experiments that were continued to 72 hours revealed that Fas resistance was not due to a time delay but to a persistent and sustained viability (not shown). Fas resistance or sensitivity between VSMCs was not correlated with patient age, gender, drug treatment, or the presence of hypertension. Thus, individual medial human VSMC cultures were either sensitive or resistant to Fas-induced apoptosis, but this was only revealed after CHX priming.

**Fas-Resistant VSMCs Show Normal Apoptosis on Serum Withdrawal**

To examine whether Fas resistance represented a generalized failure to undergo apoptosis, we examined the effects of serum withdrawal. All 7 cell lines underwent apoptosis on serum withdrawal (Figure 1C). CHX potentiated serum-withdrawal–induced apoptosis, but both Fas-resistant and -sensitive cells were equally sensitive (not shown). Thus, Fas resistance did not represent a generalized defect in apoptosis, and a partial dichotomy exists between the mechanisms of Fas and serum-withdrawal–induced apoptosis.

**Both Fas-Sensitive and -Resistant Cells Express Fas**

Fas in VSMCs exists both intracellularly and on the cell surface, with only the latter being functional to bind Fas-L and induce apoptosis. Fas-L also exists as a membrane and cytoplasmic protein and in a soluble form, but only the membrane form is a potent inducer of apoptosis. To examine whether Fas resistance represented reduced Fas/Fas-L surface expression, flow cytometric analysis was performed. This showed that both Fas-sensitive and -resistant VSMCs expressed surface Fas to a similar extent (Figure 2). Analysis of permeabilized cells demonstrated that the majority of Fas existed in VSMCs as a cytoplasmic protein, and Fas-L was not detected on the cell surface (Figure 2). Two separate anti–Fas-L antibodies were used, both of which indicated similar expression by flow cytometry and Western blot (see below).

**Differential Expression of Fas-Signaling Proteins in VSMCs**

Binding of Fas-L to Fas recruits the adapter molecule FADD to the receptor complex, first activating caspase 8 and then activating a caspase cascade. Therefore, Fas resistance could...
be due to differences in expression or activation of a number of different molecules. Western blots indicated that Fas-resistant and -sensitive cell lines showed expression of signaling proteins involved in Fas-induced apoptosis (Figure 3). Fas migrated as 2 bands of different molecular masses of ~45 kDa, possibly representing different glycosylation. In contrast, Fas-resistant cells demonstrated lower expression of both FADD and Fas-L (Figure 3) and no detectable expression of caspases 3, 7, and 8. Fas-resistant and -sensitive cells expressed caspase 1 to a similar extent, although caspase 9 expression was variable. Immunocytochemistry of Fas-sensitive and -resistant cells for caspase 3 showed that differences in expression of caspase 3 occurred at a single cell level and cells were either positive or negative for caspase 3 (not shown). Northern blot analysis showed that the differences in caspase 3 expression were due to differences in caspase 3 mRNA levels between Fas-resistant and -sensitive cells (not shown).

Fas-induced apoptosis is regulated also by expression of antiapoptotic proteins, including antiapoptotic members of the Bcl-2 family (Bcl-2/Bcl-XL), FLIP, and IAPs. We found consistently increased expression of FLIP and, to a lesser extent, c-IAP-1, but not Bcl-2 or Bcl-XL in Fas-resistant cells (Figure 3). This indicates a coordinated downregulation of proapoptotic-signaling molecules and increased expression of antiapoptotic molecules in Fas-resistant cells. To analyze whether the different responses to Fas represented differences in expression of antiapoptotic proteins in response to CHX priming, we examined expression of Bcl-2, Bcl-X, c-IAP-1, and FLIP in response to CHX alone, CHX + α-Fas, or serum withdrawal (Figure 3B). Although CHX had a variable effect on expression of all proteins, there was no relative increase in expression of antiapoptotic proteins in the Fas-resistant versus -sensitive cells after CHX priming.

Fas-Resistant and -Sensitive VSMCs Transmit Death Signals From Fas

To examine whether Fas in Fas-resistant cells can efficiently transmit a death signal on ligand binding, we analyzed death signals transmitted from Fas in both cell types. Fas-L binding to Fas recruits adapter proteins FADD and RIP to Fas.26,27 As both FADD and caspase 8 expression (but not RIP) were markedly reduced in resistant cells, we examined the ability of Fas-L to recruit RIP to Fas. In both resistant and sensitive cells, Fas-L caused a time-dependent recruitment of RIP to Fas, with some association being evident at time 0, an increase at 30 minutes, and a return to baseline by 2 hours (Figure 4). Thus, Fas is both present and functional on Fas-resistant and -sensitive cells, and Fas-L engagement and activation of Fas can be achieved under the conditions studied.

Fas-Induced Apoptosis Is Dependent on Activity of a Caspase 3 or 8 Family Member

To examine which caspases are involved in Fas-mediated apoptosis of VSMCs, we studied the effect of peptide inhibitors of the caspase P’ Asp cleavage site. The Y-VAD sequence coupled to aldehyde or fluoromethyl ketone inhibits caspase 1–like caspases with a $K_I$ of <1 nmol/L, whereas the DEVD or IETD sequence is more specific for caspase 3–like or caspase 8–like enzymes, respectively.28–30 Y-VAD is a nonspecific inhibitor of all caspase family members. In 2 Fas-sensitive cell lines, z-VAD, DEVD, and IETD inhibited Fas-induced apoptosis in a dose-dependent manner (Figure 5). Inhibition by z-VAD started at 10 μmol/L and increased to 100% at 600 μmol/L. Inhibition by DEVD or IETD started at 10 μmol/L and increased to 100% at 100 μmol/L. In contrast, Y-VAD did not show any inhibition even at high concentrations (Figure 5), although 100 μmol/L or higher YVAD could inhibit Fas-induced apoptosis in Jurkat cells (not shown). These results imply that caspase 3–like caspases and caspase 8–like caspases are critical to Fas-induced apoptosis in VSMCs. Time-lapse videomicroscopy confirmed that the protective effect of caspase inhibitors was due to a reduction in apoptotic death and inhibitors did not affect cell proliferation (not shown). z-VAD, but not DEVD or IETD, also inhibited serum withdrawal–induced apoptosis of both
Cleavage of Caspases in Fas-Induced Apoptosis in VSMCs

To become enzymatically active, caspases need to be cleaved from a larger inactive zymogen proform into smaller species, which can heterodimerize. To examine which of the downstream effector caspases were activated and cleaved in Fas-mediated VSMC apoptosis, we generated semi-immortalized Fas-sensitive VSMCs. Extensive characterization of these cells showed identical expression of Fas, Fas-L, FADD, individual caspases, or antiapoptotic molecules and sensitivity to Fas-induced apoptosis compared with primary cells (not shown). Cleavage of caspase 3 into the p20 active form was observed at 10 hours after addition of IgM Fas and persisted to 24 hours (Figure 6A). In contrast, there was no cleavage of caspase 7 (Figure 6B) or caspase 1 or 6 (not shown) over the same time course. Cells in 0% FCS for 24 hours did not show any cleavage of caspase 3 (not shown), consistent with the observation that DEVD-CHO does not inhibit apoptosis in 0% FCS (see Figure 1 online at http://www.circresaha.org).

Caspases 3 and 8 Expression Is Required for Fas-Induced Apoptosis

To analyze whether caspase 3 or 8 expression is required, and is sufficient, for Fas-induced apoptosis, we expressed full-length antisense or sense caspase 3 or 8 in Fas-sensitive cells with an expression plasmid encoding β-galactosidase and confirmed increased or reduced caspase protein expression by Western blots (not shown). Fas-induced apoptosis was examined by morphological appearance of live versus dead transfected cells (see Figure 2 online at http://www.circresaha.org and the Table). Transfection of VSMCs induced some apoptosis, which could be increased by treatment with α-Fas and CHX in Fas-sensitive cells and inhibited by suppression of caspase 3 or 8 (89.1% versus 42.7% or 34.1%, respectively). Expression of caspase 3 or 8 in Fas-resistant cells did not induce spontaneous apoptosis. In addition, expression of caspase 3 or 8 did not restore Fas sensitivity in Fas-resistant cells. Thus, Fas-induced apoptosis of VSMCs requires caspases 3 and 8, but caspase 3 or 8 alone is not sufficient to rescue the Fas-resistant phenotype, supporting the theory that

Figure 5. Caspase involvement in Fas-induced apoptosis. Inhibition profiles (absorbance at 495 nm as a measure of cell viability) of anti–Fas-induced apoptosis (CHX+α-Fas) in a Fas-sensitive cell line by increasing concentrations of peptide inhibitors zVAD-fmk (A), DEVD-CHO (B), IETD-CHO (C), and YVAD-CHO (D) compared with cells in medium containing 10% FCS alone (Un) or with CHX+α-Fas alone. Data are mean±SEM; n=3. *P<0.05, **P<0.01 vs (CHX+Fas).

Figure 6. Caspase 3 cleavage in Fas-induced apoptosis. Cleavage of caspase 3 (A) and caspase 7 (B) over a time course of 24 hours after treatment with anti-Fas antibody (1 μg/mL)+CHX. Arrow indicates the cleaved product of caspase 3.
multiple defects in Fas signaling may be responsible for this phenotype.

Differential Expression of Fas-Related Proteins In Vivo

To examine whether the differential expression of signaling proteins that we observed in cultures was also seen in vivo, we analyzed expression of caspase 3 (Figure 7), caspase 8, Fas, and FADD in fixed sections of human coronary arteries. Fas and FADD were expressed in the majority of medial VSMCs in healthy arteries (not shown). In contrast, medial VSMCs showed marked heterogeneity of expression of caspase 3. The majority of medial VSMCs were caspase 3–negative, but 1% to 2% were caspase 3–positive (Figure 7). Caspase 8 showed a similar distribution to caspase 3 (not shown).

Differential Expression of Fas-Related Proteins in Atherosclerosis

Differential expression of apoptosis-signaling proteins in vivo would be predicted to alter sensitivity of VSMCs to apoptosis in diseases in which apoptosis induced via those pathways is activated. Human atherosclerosis is characterized by an accumulation of VSMCs and inflammatory cells, both T lymphocytes and macrophages, which are a source of both soluble and membrane-bound death ligands. Apoptosis has been demonstrated in VSMCs in human plaques, and both Fas and caspase 3 activation have been implicated. To examine whether human plaque intimal VSMCs were of the Fas-sensitive or -resistant phenotype, we analyzed protein expression of Fas, FADD, caspase 3, and caspase 7 in 4 separate isolates of human plaque intimal VSMCs isolated from carotid endarterectomy specimens. Plaque VSMCs expressed variable amounts of caspases 3 and 7, but no isolate was negative for these caspases. FADD and Fas were also uniformly expressed by cultured plaque VSMCs. Examination of these plaque-derived VSMCs isolated all lines to be Fas-sensitive (see Figure 3 online at http://www.circresaha.org).

Discussion

Although Fas is widely expressed, and surface Fas is detectable on many cells, cells vary considerably in their sensitivity

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<th>Fas-sensitive cells</th>
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<tr>
<td>α-Fas</td>
<td>89.1</td>
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<td>Vector caspase 3 (AS) + α-Fas</td>
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<tr>
<td>Vector caspase 3 (AS)</td>
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<tr>
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<td>34.1</td>
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<tr>
<td>Vector caspase 8 (AS)</td>
<td>24.2</td>
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<tr>
<th>Treatment</th>
<th>Fas-resistant cells</th>
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<td>CHX alone</td>
<td>22.1</td>
</tr>
<tr>
<td>α-Fas</td>
<td>24.7</td>
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<tr>
<td>Vector caspase 3 (S) + α-Fas</td>
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<td>Vector caspase 3 (S)</td>
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<tr>
<td>Vector caspase 8 (S) + α-Fas</td>
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<td>Vector caspase 8 (S)</td>
<td>24.1</td>
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<th>Treatment</th>
<th>Apoptotic Transfected Cells, %</th>
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<tr>
<td>CHX alone</td>
<td>23.4</td>
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<tr>
<td>CHX alone</td>
<td>22.1</td>
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Mean percentage of apoptotic cells for VSMCs was treated with either CHX alone or anti-Fas (1 μg/mL) (α-Fas) after transfection with pCMVβ-galactosidase and either sense (S) or antisense (AS) vector caspase 3 or caspase 8. Apoptotic transfected cells were identified by expression of β-galactosidase in a cell with an apoptotic morphology.
to Fas-induced apoptosis. Thus, delivery of a cross-linking anti-Fas antibody in vivo induces fulminant hepatocellular apoptosis and failure without obvious effects on many other cells or organs that express Fas.\(^5\)\(^6\) Fas-induced apoptosis absolutely requires FADD because FADD-null cells are resistant to Fas.\(^7\) However, Fas-induced apoptosis can also be inhibited by IAPs, FLIP, Bcl-2, and Bcl-X\(_L\), although the relative contributions of these proteins to Fas resistance in vivo are unknown.

Different isolates of human medial VSMCs show markedly different responses to Fas-induced apoptosis, although all isolates undergo apoptosis after growth factor withdrawal. Fas-resistant VSMCs show reduced expression of proapoptotic proteins involved in Fas signaling, including Fas-L, FADD, and caspases 3, 7, and 8, and increased expression of FLIP and c-IAP-1. Fas in both resistant and sensitive cells is expressed at similar levels both in total and on the cell surface and is functional to transmit a death signal through RIP, indicating that Fas resistance is determined below receptor level. We found that Fas-mediated apoptosis depends on the activity of a DEVD-inhibitable caspase, which is identified as caspase 3, because Fas-mediated apoptosis of Fas-sensitive cells is associated with cleavage of caspase 3 but not caspase 6 or 7. Caspase 8 is also required for Fas-induced apoptosis because selective inhibition of caspase 3 or 8 inhibits apoptosis in Fas-sensitive cells. However, caspase 3 or 8 is not sufficient alone for Fas-induced apoptosis, because expression alone cannot rescue Fas sensitivity in Fas-resistant cells, as would be predicted by the multiple differences in expression of Fas-signaling proteins. Finally, we found that VSMCs in the media of human coronary arteries in vivo also show heterogeneous expression of caspase 3, with caspase 3–positive and caspase 3–negative cells being normal components of the vessel wall. In contrast, VSMCs isolated from atherosclerotic plaques are caspase 3–positive and Fas-sensitive.

One of the most striking findings in the present study is the profound resistance of human VSMCs to Fas, despite Fas expression on the cell surface, and transmission of a death signal from Fas. The observation that Fas-induced apoptosis requires CHX priming suggests that intracellular regulation of Fas-induced apoptosis is responsible for this resistance. In recent studies,\(^1\) we identified that the sensitivity of human VSMCs to Fas-induced apoptosis is governed in part by the surface expression of Fas. Fas in VSMCs is mostly expressed intracellularly in the Golgi complex, where it is questerated from Fas-L binding. However, transport of intracellular Fas by p53 priming of the cells increased surface expression of Fas and increased Fas-induced apoptosis. In the present study, we have shown that a further level of complexity in Fas-induced apoptosis of VSMCs exists. Both Fas-sensitive and -resistant VSMCs expressed similar levels of surface and total Fas, but there were marked differences in expression of Fas-L and FADD in addition to differences in expression of caspases 3, 7, and 8. The occurrence of multiple defects in proteins responsible for signaling apoptosis from Fas suggests that there may be a coordinated up or downregulation of proteins on the same pathway signaling death. This does not appear to be an artifact of culture, because primary cells isolated from tissue specimens showed the same differences in expression of these proteins as cells cultured over multiple passages. In addition, caspase 3 heterogeneity is present in arteries in vivo. Although the mechanism of such coordinated regulation of proapoptotic and antiapoptotic molecules is unknown, coordinated inhibition of Fas, TNF-R1, FADD, and caspase 8 with increased bcl-2 expression has been found in other studies.\(^8\) Our results suggest that sensitivity to Fas-induced apoptosis is determined below cell surface death receptor expression and may involve the complex and coordinated interplay between expression of different proteins.

Although multiple caspases are activated in Fas-induced apoptosis, including caspases 8, 3, 6, and 7,\(^6\) our results indicate that caspases 3 and 8 are critical regulators of Fas-induced apoptosis. Thus, caspase 3 showed both differences in expression in Fas-sensitive versus -resistant cells and was cleaved when Fas-sensitive VSMCs underwent Fas-mediated apoptosis. Moreover, caspase 3 cleavage was seen in Fas-induced apoptosis but not in apoptosis due to serum withdrawal, which was not blocked by DEVD-CHO. In contrast, a number of other caspases, including caspases 1, 6, 7, and 9, were not differentially expressed in Fas-sensitive versus resistant cells, were not cleaved when cells underwent Fas-induced apoptosis, or both. Finally, inhibition of caspases 3 and 8 in Fas-sensitive cells by transfection of antisense cDNA or inhibition of caspase 3 or 8 family enzymes by peptide substrate mimetics inhibited Fas-mediated apoptosis. However, caspase 3 or 8 expression alone was not sufficient to restore Fas sensitivity in Fas-resistant cells, implying that multiple defects are responsible for the resistant phenotype.

The role of caspase 3 in Fas-induced apoptosis is presently controversial. Some studies have identified that Fas-induced apoptosis is inhibited by DEVD peptides,\(^39\)\(^40\) and caspase 3 cleavage and activation have been observed.\(^39\)\(^40\) In contrast, other studies have identified that inhibition of caspase 3 does not block DNA fragmentation, and caspase 3 is not cleaved in all cases of Fas-induced apoptosis.\(^43\) Much of this controversy has been explained by a recent study showing that the requirement for caspase 3 is critically dependent on cell type and inducer of apoptosis.\(^44\) Furthermore, the morphological features of apoptosis in caspase 3–deficient cells were highly variable according to cell type. In VSMCs, we found that Fas-mediated apoptosis of human VSMCs requires caspase 3. Although caspase 3 cleavage is seen in Fas-induced apoptosis associated with characteristic apoptotic morphology, there was no difference in nuclear morphology, cell shrinkage, or cell membrane blebbing and no evidence of PARP cleavage in apoptosis induced by low serum conditions in caspase 3–positive or caspase 3–negative cells (not shown). This indicates that distinct DNases may be activated in apoptosis by caspase 3–dependent or caspase 3–independent pathways.

Although the role of Fas-L in immune-privileged sites is well established, with expression being a mechanism of inducing apoptosis in cytotoxic T lymphocytes directed against “self” proteins, the role of Fas-L in the vessel wall is uncertain. Clearly, the resistance to Fas-induced apoptosis between sensitive and resistant cells is relative, because overexpression of membrane Fas-L can induce apoptosis of VSMCs both in vitro and in vivo.\(^45\) However, this does not mean that selective killing of VSMCs by Fas-L does not
occur in vivo. In endothelial cells, Fas-L expression can protect the vessel wall from leukocyte migration. Fas-L and Fas may represent a rapid mechanism of autodeletion of VSMCs, where increased membrane expression of either Fas-L or Fas is induced by other stimuli, such as DNA damage. Indeed, the relatively high level of cytoplasmic Fas-L expression in Fas-sensitive VSMCs, which also express downstream Fas-signaling proteins, may ensure that VSMCs undergo autocrine destruction by relocating Fas-L to the cell surface, analogous to Fas trafficking in VSMCs.

There is increasing evidence that the adult vessel media consists of VSMCs from distinct cell lineages, characterized by retention of different phenotypes and growth characteristics when placed in culture. Thus, clonal cell lines can be derived from mammalian arteries, with different rates of cell proliferation and apoptosis being retained in culture and different phenotypes seen in medial VSMCs isolated from human arteries. After injury in vivo, most cells do not undergo cell proliferation, but a small number proliferate many times, indicating that after the same initial stimulus, subpopulations of VSMCs within the healthy vessel wall undergo a different response. Human medial VSMCs also exhibit phenotypic heterogeneity, and intimal VSMCs show very different phenotypes to that of medial VSMCs.

Our findings suggest that apoptosis in advanced atherosclerosis may occur in a subpopulation of VSMCs. Despite most medial VSMCs and intimal VSMCs in healthy coronary arteries being caspase 3–negative, we found that all plaque intimal VSMCs isolated from carotid endarterectomy specimens showed expression of Fas-signaling proteins, including caspase 3, and no isolate was caspase 3–negative. As inflammatory cells within plaques such as macrophages and T lymphocytes express surface Fas-L, the competence of plaque intimal VSMCs to undergo Fas-induced apoptosis strongly suggests that Fas and Fas-L induce VSMC apoptosis in atherosclerosis. Indeed, Fas has been shown to colocalize with apoptotic VSMCs in atherosclerotic plaques.

In summary, we have demonstrated that human medial VSMCs show marked heterogeneity of expression of proteins regulating apoptosis both in vitro and in vivo. In particular, the sensitivity of VSMCs to apoptosis from defined stimuli may be determined below receptor level. Different sensitivity to apoptosis of subpopulations of medial VSMCs may regulate vessel wall architecture and profoundly influence vessel remodeling and the response to injury.

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Materials and Methods

Generation of vascular smooth muscle cell cultures

Normal medial VSMCs were derived from coronary arteries of patients undergoing cardiac transplantation for non-ischaemic cardiomyopathy as described previously \(^1\), and human atherosclerotic plaque VSMCs from carotid endarterectomy specimens of patients with symptomatic carotid disease. Briefly, cultures were obtained from specimens from individual patients of both sexes including a range of ages between 34 and 62 years (mean age of patients was 47.8 years). Characterisation of VSMCs as medial from normal vessels or intimal from endarterectomy tissues were identified by histological examination of the arteries or endarterectomies removed (not shown). Cells were characterised as VSMCs by culture morphology and immunocytochemical staining pattern at passage 3 (\(\alpha\)-sm-actin positive, vimentin positive, VWF negative, desmin negative and smooth muscle myosin positive)(not shown). Cells from individual patients were maintained as separate cultures. The generation of VSMC cultures with extended life span was performed by stable infection with virus oncogenes including Human Papilloma Virus Type 16 E6 or Simian Virus SV40 Large T antigen as previously described \(^2\).

Primary VSMCs or VSMC cultures were maintained in tissue culture in 75cm\(^2\) flasks in medium 199 (Sigma, St Louis, Mo) supplemented with 20% foetal calf serum (Sigma), Penicillin/Streptomycin/2mM Glutamate (Sigma) and 0.01M HEPES (Fisher, UK) in a
37°C incubator with 5% CO₂. Subconfluent cells were passaged by trypsinisation in 0.05% trypsin in phosphate-buffered saline (PBS).

**Time-lapse videomicroscopy**

Cells grown in a 25cm² tissue culture flask were gassed, and filmed over a period of 24-48 hrs using an Olympus OM-70 microscope enclosed in a plastic environment chamber and maintained at 37°C by an external heater. The time-lapse equipment consisted of a Sony 92D CCD camera with a Panasonic 6730 time-lapse video recorder. Films were analysed for morphology of apoptosis and cell death rates as previously described² using an observer blind to cell type and treatment conditions. Apoptotic cell death events were scored midway between the last appearance of normality and the point at which the cell became fully detached and fragmented, an interval of typically 60-90 minutes. Apoptotic cells were counted out of a total of approximately 300 cells in three different fields from three repeated experiments.

**Flow cytometry**

10⁵-10⁶ cells were incubated with 5μg/ml anti-Fas antibody IgM (#05-201, Upstate) or anti-Fas-Ligand antibody IgG1 (F37720, Transduction Labs, Lexington, Ky or NOK-1 (65320C Pharmingen)) for 30min on ice with or without a 30min permeabilisation pre-incubation with ice-cold 70% ethanol on ice. Cells were then washed once in 1ml 2% FCS/PBS before addition of 1:100 of either FITC-conjugated anti-mouse IgM (Sigma) or anti-mouse IgG1 (Sigma) antibody for 30min on ice. Cells were washed in 1ml 2%
FCS/PBS, resuspended in 0.5ml 2% FCS/PBS and counted for 10,000 events with an excitation wavelength of 488nm and an emission wavelength of 535nm.

**Western blotting**

Protein lysates were isolated from cultured cells into 2x loading buffer (0.125M Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue). Equal amounts of proteins were loaded onto a SDS-PAGE gel and then transferred onto an Immobilon-P membrane (Millipore, Bedford, Ma). After blocking in 5% non-fat skimmed milk (Marvel, UK) in TBS (0.9% NaCl, 10mM Tris pH 7.4) for 1hr at room temperature with shaking, the membrane was incubated with primary antibody in 0.1% Tween-20 (Sigma), 5% Marvel, TBS overnight at 4°C with shaking. The primary antibodies used were directed against Fas (sc-715, Santa Cruz, Ca), Fas-L (F22120 and F37720, Transduction Labs Lexington, Ky and NOK-1 (65320C Pharmingen), FADD (F36620, Transduction), FLICE (caspase 8) (05-477, Upstate, Lake Placid, NY), caspase 1 (06-503, Upstate), caspase 3 (C31720, Transduction), caspase 7 (M64620, Transduction), Bcl-2 (14371E, Pharmingen), Bcl-X (66461A, Pharmingen), RIP (R41220, Transduction), c-IAP-1 (AF818, R&D Systems), FLIP (210-736-C100, Alexis), β–tubulin (T4026, Sigma), and α-smooth muscle actin (A2547, Sigma). The antibodies chosen bind to all of the uncleaved and some cleaved forms of individual caspases, but do not appear to cross-react between caspases. The membrane was then incubated with secondary antibody in 0.1% Tween-20, 5% Marvel, TBS for 2hrs at room temperature with shaking. Signals were detected using ECL (Amersham, UK).
Immunocytochemistry

Cells grown on 8-well chamber slides were fixed in −20°C methanol for 10 min on ice. Endogenous peroxidase activity was blocked in 1% H₂O₂ in PBS for 10 min at room temperature. Cells were blocked in 1% normal rabbit serum/2% BSA (Sigma) in PBS for 1 hr at room temperature.

Expression of caspases 3 and 8

Full-length human caspases 3 or 8 were cloned as EcoR1/Mun1 fragments into the retrovirus expression vector LXPOP, or BamH1/Kpn1 fragments into pcDNA3 respectively, in either the sense or antisense orientation. Fas-sensitive VSMCs were cotransfected with 5 μg of antisense caspase vector or vector control and 5 μg pCMV β-Gal, or Fas-resistant cells transfected with 5 μg sense caspase vector or vector control and 5 μg pCMV β-Gal using Superfect™ (Qiagen) following the manufacturer’s recommendations. After 2 hours, apoptosis was induced by anti-Fas IgM and cycloheximide, and cells filmed by time-lapse videomicroscopy for 48 hours. After this period, cell cultures were fixed and stained for β-Galactosidase activity. Alternatively, cells transfected with either sense or antisense caspase vector then placed into antibiotic selection (5 μg/ml) until a pooled population of resistant cells could be obtained (approx. 2 weeks), and analysed for caspase expression by Western blots.

Histochemistry of tissues

Paraffin-embedded sections were obtained from regions of macroscopically normal coronary arteries derived from recipient hearts from cardiac transplantation patients. 10
separate specimens were studied, which included approximately 25 normal vessels. Sections were deparaffinized, rehydrated, decalcified in 3% citric acid overnight and antigens unmasked by either microwave treatment, 1% SDS or 0.01% pronase (Sigma). Endogenous peroxidase activity was blocked in 3% H₂O₂ for 5min at room temperature. Sections were blocked in 20% normal goat or rabbit serum/TBS (0.05M Tris pH 7.6, 0.15M NaCl) for single staining or 20% normal rabbit serum/TBS for double staining for 20min at room temperature.

Sections were incubated with 1:100 monoclonal anti-caspase 3 antibody (C31720, Transduction Labs), 1:20 anti-Fas antibody (F22120, Transduction Labs), 1:20 anti-FADD antibody (SC L-18, Santa Cruz) for single staining or 1:100 polyclonal goat anti-caspase 3 p20 antibody (N-19, SC1226, Santa Cruz) for double staining at room temperature overnight. After incubation with primary antibody, sections were incubated with biotinylated goat anti-mouse antibody or rabbit anti-goat antibody for 25min, followed by incubation with StreptABComplex/horseradish peroxidase (DAKO, UK) for 25min at room temperature. The signals were detected with 1mg/ml diaminobenzidine (DAKO) and 0.02% H₂O₂. For double staining, the sections were blocked in 20% normal goat serum/TBS for 20min and were further processed by incubation with 1:400 monoclonal anti-α smooth muscle actin (Sigma) for 30min. Sections were then incubated with alkaline phosphatase-conjugated goat anti-mouse antibody (DAKO) for 30min, developed with Vector Red (Vector, Burlingame, Ca), counterstained and mounted in Permount (Fisher Scientific, UK).
References - online


Figure Legends to online Figures

Figure 1 online

Apoptosis in the presence of caspase inhibitors

Time-lapse videomicroscopic analysis of apoptotic deaths following treatment with (A) α-Fas+CHX or (B) 0% FCS in the absence (Un) or presence of peptide inhibitors (300µg/ml).

Figure 2 - online

Requirement for caspase 3 in Fas-induced apoptosis

Fas-resistant VSMCs transfected with pCMV β-Gal + LXPOP caspase 3 (sense) stained for β-Galactosidase activity, showing transfected cells with either normal or apoptotic (arrow) morphology.

Figure 3- online

Expression of apoptosis signalling molecules in human atherosclerotic plaque VSMCs

(A) Western blot of 4 separate isolates of human plaque intimal VSMCs derived from carotid endarterectomy specimens for caspases 3 and 7, FADD, Fas or α-smooth muscle actin. (B) Mean percentage viability ±SEM of 4 separate isolates of human intimal plaque VSMCs following treatment with an agonistic anti-Fas antibody (1µg/ml) + 10µg/ml cycloheximide (CHX)(clear bar) or CHX alone (solid bar). n=3* p<0.01 vs. control.
Figure 1
Online