Acute and Chronic Smooth Muscle Cell Apoptosis After Mechanical Vascular Injury Can Occur Independently of the Fas-Death Pathway

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Abstract—Vascular smooth muscle cell (VSMC) apoptosis has been demonstrated in vascular lesions, such as atherosclerotic and postangioplasty restenotic lesions. Balloon injury also induces VSMC apoptosis. Fas is a death factor that mediates apoptosis when it is activated by its ligand, FasL. Fas-mediated apoptosis was found to be implicated in the pathogenesis of vascular diseases in which Fas/FasL expression was detected. We investigated whether the Fas/FasL interaction mediated acute and chronic VSMC apoptosis and lesion formation in a vascular injury model that may resemble balloon angioplasty. A large spring wire was inserted into the femoral artery of C3H/HeJ (wild-type), C3H-gld (Fas ligand−/−), and C3H-lpr (Fas−/−) mice. The wire was left in place for 1 minute to denude and expand the artery. Massive apoptosis was observed in medial VSMCs from 1 to 7 hours later. There was no difference in the number of apoptotic cells among the 3 groups of mice 4 hours after injury. At 4 weeks, the injured arteries presented signs of concentric neointimal hyperplasia composed exclusively of VSMCs. There was no difference in the degree of neointima hyperplasia (intima/media ratios were as follows: wild type 1.4±0.3, gld 1.0±0.2, and lpr 1.3±0.2) or in the number of apoptotic nuclei among the 3 groups. These findings suggest the existence of other signaling pathways for acute and chronic VSMC apoptosis, at least that induced by mechanical vascular injury. (Arterioscler Thromb Vasc Biol. 2001; 21:1733-1737.)

Key Words: Fas ■ apoptosis ■ balloon injury ■ smooth muscle cells ■ intima

Vascular smooth muscle cell (VSMC) apoptosis has been demonstrated in atherosclerosis and in restenotic lesions after angioplasty.1-2 In animal models of balloon vascular injury, medial VSMC apoptosis and subsequent cell loss were observed early after the injury.3,4 However, the molecular mechanisms of vascular cell apoptosis remain to be elucidated,5,6 and the role of smooth muscle cell apoptosis in vascular remodeling is still a matter of controversy.7-10 It has been postulated that vascular cell apoptosis plays a role in the development of vascular lesions,7-10 because exuberant balloon-induced apoptosis results in enhanced neointimal formation.11 In contrast, it has also been proposed that VSMC apoptosis prevents proliferative vascular disease, because forced induction of VSMC apoptosis by gene modification results in a reduction of vascular lesions.12-15

Fas is a death receptor that transmits apoptosis-inducing signals when activated by its ligand (FasL).16 The Fas/FasL system was first identified in the immune system.17,18 However, at present, there is a large body of evidence indicating that the Fas/FasL interaction controls cell death in a wide variety of cell types under physiological and pathological conditions.17,19-24 Cultured VSMCs express abundant Fas on their surface and undergo apoptosis when stimulated by membrane-bound FasL.12,25 Fas was found to be expressed on apoptotic cells in atherosclerotic lesions,7 and it has been proposed that Fas-mediated apoptosis might be involved in the pathogenesis of atherosclerosis.7,26,27

For a better understanding of the pathogenesis of vascular diseases, genetically modified mice have been used.28 Significant neointima-like hyperplasia was noted in several models of vascular injury prepared according to the perivascular approach.29-31 However, in no mouse model was medial cell apoptosis (which plays a pivotal role in vascular remodeling after balloon angioplasty) induced rapidly after the injury.3,4,11 We recently developed a new mouse model of vascular injury, which may resemble the balloon angioplasty model.32 This vascular injury results in medial smooth muscle cell apoptosis after 1 to 7 hours and marked enlargement of the lumen, followed by robust VSMC proliferation and an intimal lesion.32 The neointima continues to grow for 3 weeks; thereafter, no significant change in the size of the neointima is observed. Neointimal hyperplasia is exclusively composed of α-smooth muscle actin-positive cells.32

In the present study, we took advantage of mouse genetics to study the potential involvement of the Fas/FasL system in acute and chronic VSMC apoptosis and in the development of
vascular lesions. We induced endovascular injury in wild-type, gld (Fas ligand−/−), and lpr (Fas−/−) mice. There was no significant difference in the number of acute and chronic apoptotic cells or in the size of neointimal hyperplasia among the 3 groups. These results suggest the existence of a Fas-independent pathway that mediates acute and chronic smooth muscle cell apoptosis, at least in response to mechanical overexpansion of the artery.

Methods

Animals

Eight-week-old male C3H/HeJ (wild-type), C3H-gld (FasL−/−), and C3H-lpr (Fas−/−) mice were purchased from Japan SLC, Inc (Shizuoka, Japan), kept in microisolator cages under a 12-hour day-night cycle, and fed regular chow. For all surgical procedures, the mice were anesthetized by intraperitoneal injection of 50 mg/kg Nembutal (Abbott Laboratories) diluted in 0.9% sodium chloride solution. All procedures involving experimental animals were performed in accordance with protocols approved by institutional guidelines for animal care of The University of Tokyo and compiled with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised in 1985).

Mouse Femoral Injury Model

Transluminal mechanical injury of the femoral artery was induced by inserting a large wire, as previously described. A copy of the tutorial video of the surgical procedure can be obtained by request to M.S., or the video can be viewed online at http://plaza.umin.ac.jp/~msata/. Briefly, either the left or right femoral artery was exposed through the tail vein at 0.5 g/g. The liver tissues were removed at 17 hours, indicating that the Fas/FasL system was not involved in apoptotic cells among the 3 groups of mice (Figure 1B), which was found where the large wire had been placed in all mice.

Injection of Anti-Fas Antibody

Agonistic anti-Fas antibody (clone Jo2) was purchased from PharMingen. The antibody was diluted in PBS and administered intravenously to 8-week-old male C3H/HeJ and C3H-lpr (Fas−/−) mice through the tail vein at 0.5 μg/g. The liver tissues were removed at death, fixed with 4% paraformaldehyde in PBS (pH 7.4), and embedded in paraffin. The femoral artery was carefully excised, further fixed in 4% paraformaldehyde overnight at 4°C, and embedded in paraffin. The femoral artery and vein were looped together proximally and distally with 6-0 silk suture (Natsume Co) by blunt dissection. The femoral artery and vein were looped together proximally and distally with 6-0 silk suture (Natsume Co) for temporary vascular control during the procedure. A straight spring wire (0.38-mm diameter, No. C-SF-15-15, Cook) was carefully inserted into the femoral artery toward the iliac artery via a small branch between the rectus femoris and vastus medialis muscles. The wire was left in place for 1 minute to denude and dilate the artery. Then, the wire was removed, and the silk suture at the proximal portion of the muscular branch artery was secured. Blood flow in the femoral artery was restored by releasing the sutures placed in the proximal and distal femoral portions. The skin incision was closed with a 5-0 silk suture (Natsume Co) on a Provis AX80 microscope (Olympus) equipped with an epifluorescence optical lens.

Immunohistochemistry

Paraffin-embedded sections (5 μm thick) were deparaffinized and blocked with 1% rabbit serum. Endothelial cells, T lymphocytes, polymorphonuclear cells, and macrophages were revealed by immunostaining with anti-CD31 antibody (clone MEC13.3, PharMingen), anti-CD3ε hamster monoclonal antibody (Santa Cruz Biotechnology), anti-CD11b monoclonal antibody (clone M1/70, Serotec), and anti-F4/80 monoclonal antibody (clone A3-1, Serotec), respectively, followed by the avidin-biotin complex technique and Vector Red substrate (Vector Laboratories, Inc). Smooth muscle cells were identified by immunostaining with an alkaline phosphatase–conjugated monoclonal antibody to α-smooth muscle actin (clone 1A4, Sigma). Sections were counterstained with hematoxylin.

Statistical Analysis

All results are expressed as the mean±SEM. The means were statistically compared by ANOVA, followed by the Student t test. A value of P<0.05 was considered statistically significant.

Results

Rapid Onset of Medial Cell Apoptosis After Mechanical Endovascular Injury in FasL- or Fas-Deficient Mice

A large spring wire was inserted into the femoral artery for 1 minute to denude and expand the artery (wire/lumen ratio 1.9±0.2). As reported for balloon injury models in rat and rabbit arteries,3 acute expansion of the mouse femoral artery induced apoptosis in a large number of medial smooth muscle cells, as determined by the TdT-mediated dUTP nick-end labeling (TUNEL) technique (Figure 1A).32 Massive apoptosis was detected from 1 to 6 hours after injury. At 17 hours, the cellularity of the media declined dramatically, and a few TUNEL-positive cells were detected in the media.32 At 4 hours after injury, a similar frequency of apoptosis was detected in wild-type, gld (FasL−/−), and lpr (Fas−/−) mice. There was no significant difference in the number of apoptotic cells among the 3 groups of mice (Figure 1B), indicating that the Fas/FasL system was not involved in VSMC apoptosis induced by vascular injury.

Development of Lesions in FasL- or Fas-Deficient Mice

Four weeks after the injury, a concentric neointimal lesion was found where the large wire had been placed in all mice examined (Figure 2).32 FasL- and Fas-deficient mice developed lesions similar to those developed by wild-type mice (n=6 per group, Figure 2). There was no significant difference in the degree of the neointima (intima/media area ratio) was as follows: wild type 1.4±0.3, gld 1.0±0.2, and lpr 1.3±0.2) or in the vessel size (circumference of external elastic lamina was as follows: wild type 1.00±0.08 mm, gld 1.07±0.08 mm, and lpr 1.07±0.05 mm) among the 3 groups.

Staining by TdT-Mediated dUTP Nick End-Labeling

The 4% paraformaldehyde–fixed sections (5 μm) were deparaffinized and rehydrated. The tissue was then treated with 20 μg/mL protease K for 30 minutes. Terminal deoxynucleotidyl transferase (TdT) enzyme and fluorescein-dUTP were added to the tissue sections in accordance with the instructions provided by the manufacturer (In Situ Death Detection Kit, Roche Molecular Biochemicals). Nuclei were counterstained with Hoechst 33258 (Sigma Chemical Co) and mounted with Vectashield mounting media (Vector Laboratories, Inc). Specimens were examined and photographed by using a Provis AX80 microscope (Olympus) equipped with an epifluorescence optical lens.

Morphometric Analysis

Cross sections (5 μm) were deparaffinized, stained with hematoxylin and eosin, and mounted with mounting media (Mount Quick, Daido). The image was digitized by a Fujix Digital Camera (HC-300/OL, Fujifilm Co) on a Provis AX80 microscope (Olympus). Morphometric analysis was performed by using software for image analysis (LIAS2 for Windows 95, version 0.372, Kazukiko Yamamoto) on a PC computer (Presario 2295, Compaq). All data are expressed as the mean±SEM.
The neointima was exclusively composed of smooth muscle cells in wild-type, gld (FasL−/−), and lpr (Fas−/−) mice, as determined by immunostaining for α-smooth muscle actin (Figure 3). In all mice, the luminal side of the intima was almost completely reendothelialized at 4 weeks, as determined by anti-CD31 staining (Figure 3). No capillary formation was detected in the intima, excluding the possibility that neointimal formation had resulted from recanalized thrombosis. Macrophages were detected in the adventitia and occasionally in the neointima. The accumulation of macrophages in the adventitia was more prominent in gld and lpr mice than in wild-type mice.

Chronic VSMC Apoptosis in the Vascular Lesion in the Absence of FasL/Fas Interaction

TUNEL staining revealed that at 4 weeks after injury, a small fraction of the VSMCs was undergoing apoptotic cell death in the neointima as well as in the media (Figure 4). The

Figure 1. Wire-induced injury causes medial cell apoptosis in FasL- and Fas-deficient mice at 4 hours after injury. A large spring wire was inserted into the femoral artery of C3H/HeJ (wild-type), C3H-gld (FasL−/−), and C3H-lpr (Fas−/−) mice. The wire was left in place for 1 minute to denude and expand the artery. Four hours after injury, the injured artery was perfusion-fixed with 4% paraformaldehyde and carefully excised. A, Total nuclei and apoptotic nuclei were stained by use of Hoechst 33258 (top, blue) and by the TUNEL technique (bottom, green), respectively. B, The number of TUNEL-positive nuclei were counted and expressed as a percentage of the total number of nuclei (n = 4 per group).

Figure 2. Neointimal formation in wild-type, gld (FasL−/−), and lpr (Fas−/−) mice 4 weeks after the injury. The injured femoral artery of C3H, C3H-gld, and C3H-lpr mice was perfusion-fixed 4 weeks after the injury. A, Paraffin-embedded sections were deparaffinized and stained with hematoxylin and eosin. Arrows indicate the internal elastic lamina. Bar=100 μm. B, The digitized images were analyzed morphometrically by using software for image analysis. The intima/media area ratio was calculated. All data are expressed as the mean±SEM (n=6 per group). C, The circumference of the external elastic lamina (EEL) was measured to evaluate the vessel size. All data are expressed as the mean±SEM (n=6 per group).

Figure 3. Characterization of neointimal lesions in wild-type, gld (FasL−/−), and lpr (Fas−/−) mice. Paraffin-embedded sections harvested 4 weeks after the injury were deparaffinized and stained with specific antibodies to detect endothelial cells (CD31), macrophages (F4/80 [Mφ]), and smooth muscle cells (α-smooth muscle actin) by using the avidin-biotin complex technique and Vector Red as the substrate. The sections were counterstained with hematoxylin. The neointima was observed to be exclusively composed of smooth muscle cells. The luminal side was almost completely reendothelialized. Macrophages were detected in the adventitia and occasionally in the neointima. The accumulation of macrophages in the adventitia was more prominent in gld and lpr mice than in wild-type mice. No capillary formation was detected in the intima, excluding the possibility that neointimal formation had resulted from recanalized thrombosis. Macrophages were detected in the adventitia and occasionally in the intima, as determined by immunostaining for F4/80. Macrophage accumulation in the adventitia was more prominent in gld and lpr mice than in wild-type mice (Figure 3). However, we observed no macrophage infiltration in the media. Polymorphonuclear cells and T lymphocytes were seldom detected in the intima (data not shown).
frequency of apoptosis in the chronic lesions was markedly smaller than that observed 4 hours after the wire injury. The number of apoptotic cells in gld (FasL−/−) or lpr (Fas−/−) mice was not significantly different from that found in wild-type mice, indicating that a Fas-independent pathway mediates VSMC apoptosis in the vascular lesions induced by acute mechanical injury (Table).

Lack of Fas-Mediated Apoptosis in Fas-Deficient Mice

To demonstrate lack of Fas-mediated apoptosis in lpr mice, wild-type mice and lpr mice were challenged with an agonistic anti-Fas antibody. Consistent with the results of a previous report, the present study found that intravenous injection of the anti-Fas antibody killed all wild-type mice (n = 3) within 3 hours, whereas no lpr mouse died. Histological examination revealed fulminant liver injury with diffuse hemorrhage and massive apoptosis of hepatocytes in the liver of wild-type mice, whereas the liver of the lpr mice appeared intact (data not shown). These findings demonstrate that the lpr mice used in the present study were in fact deficient in Fas-mediated apoptosis.

**Discussion**

In the present study, we evaluated the potential involvement of Fas-mediated signaling in the pathogenesis of proliferative vascular diseases by using a new mouse model of arterial injury that may resemble the balloon angioplasty. There was no significant difference between wild-type mice and mice deficient for Fas or FasL in the number of apoptotic medial cells 4 hours after injury, in the degree of neointimal hyperplasia 4 weeks after injury, or in the number of apoptotic VSMCs in chronic lesions. Our findings provide a crucial insight into the contro-versial role of Fas-mediated signaling in apoptosis, inflammation, and proliferation in the vessel wall.

Numerous studies have shown that Fas is implicated in VSMC apoptosis in vascular lesions. VSMCs abundantly express Fas, whose expression is regulated by cytokines and a tumor suppressor gene, p53. Fas expression was also identified on apoptotic VSMCs in human atherosclerotic plaques. Our results indicate the existence of other molecular pathways that may mediate acute and chronic VSMC apoptosis, at least in lesions induced by the mechanical overexpansion of the artery.

It has been suggested that Fas-mediated signaling contributes to inflammatory responses in vessel walls and, therefore, may promote atherogenesis. It has been demonstrated that the activation of Fas in peritoneal exudate cells provokes inflammatory responses by stimulating the release of interleukin-1. Overexpression of the Fas-associated death domain protein in VSMCs seeded within balloon-injured rat carotid arteries has been shown to result in chemokine expression and the recruitment of macrophages. In the present study, we did not observe any attenuation of inflammatory responses in mice deficient in FasL or Fas after the vascular injury. On the contrary, the accumulation of macrophages in the adventitia was more pronounced in FasL−/− and Fas−/− mice. Moreover, it is well known that Fas-deficient mice spontaneously develop generalized degenerative vascular lesions and necrotizing arteritis, which are preceded by infiltration of mononuclear cells and granulocytes. More recently, it has been shown that FasL-deficient mice display enhanced macrophage and T-cell infiltration and intima hyperplasia during vascular remodeling in flow-restricted vessels with an intact endothelium. These data do not support the hypothesis that the Fas/FasL system plays a major role in promoting inflammatory responses in the vessel wall after acute mechanical injury.

Furthermore, it has been suggested that Fas may promote VSMC proliferation in vascular lesions. Recent studies have demonstrated the role of Fas in the transduction of growth-promoting signals in T cells, cardiomyocytes, fibroblasts, and hepatocytes. Schneider et al have demonstrated that adenovirus-mediated overexpression of exogenous FasL facilitates VSMC accumulation in hypercholesterolemic rabbits, and they have suggested that vascular expression of FasL may contribute to the progression of atherosclerosis. In the present study, mice deficient in Fas or FasL developed neointima hyperplasia similar to that observed in wild-type mice, excluding the possibility that Fas/FasL signaling mediated the VSMC proliferation in the vascular lesions. Moreover, we previously reported that overexpression of FasL induced massive VSMC apoptosis in vitro and that adenovirus-mediated gene delivery of FasL into the balloon-injured artery potently limited the size of the lesion. Taken together, the present findings indicate that endogenous Fas/FasL interaction is not a major determinant for VSMC proliferation, at least in response to mechanical overexpansion of the artery.

In conclusion, the present results indicate that VSMC apoptosis after acute mechanical injury can occur independently of the Fas-death pathway. Fas-independent pathways appear to exist for these phenomena, although further study is required to evaluate the role of the Fas/FasL system in...
apoptosis, proliferation, and inflammation in other types of vascular diseases, such as atherosclerosis and transplantation-associated arteriosclerosis.

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