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Reduced Macrophage Apoptosis Is Associated With Accelerated Atherosclerosis in Low-Density Lipoprotein Receptor–Null Mice

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Objective—The majority of apoptotic cells in atherosclerotic lesions are macrophages. However, the pathogenic role of macrophage apoptosis in the development of atherosclerosis remains unclear. Elevated expression of Bax, one of the pivotal proapoptotic proteins of the Bcl-2 family, has been found in human atherosclerotic plaques. Activation of Bax also occurs in free cholesterol–loaded and oxysterol-treated mouse macrophages. In this study, we examined the effect of Bax deficiency in bone marrow–derived leukocytes on the development of atherosclerosis in low-density lipoprotein receptor–null (LDLR−/−) mice.

Methods and Results—Fourteen 8-week-old male LDLR−/− mice were lethally irradiated and reconstituted with either wild-type (WT) C57BL6 or Bax-null (Bax−/−) bone marrow. Three weeks later, the mice were challenged with a Western diet for 10 weeks. No differences were found in the plasma cholesterol level between the WT and Bax−/− group. However, quantitation of cross sections from proximal aorta revealed a 49.2% increase ($P=0.0259$) in the mean lesion area of the Bax−/− group compared with the WT group. A 53% decrease in apoptotic macrophages in the Bax−/− group was found by TUNEL staining ($P<0.05$).

Conclusions—The reduction of apoptotic activity in macrophages stimulates atherosclerosis in LDLR−/− mice, which is consistent with the hypothesis that macrophage apoptosis suppresses the development of atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2005;25:174-179.)**

Key Words: apoptosis ■ atherosclerosis ■ macrophage ■ Bax ■ smooth muscle cell

Apoptosis, also called programmed cell death, plays a critical role in tissue development and maintenance of homeostasis within multicellular organisms. Two different pathways have been identified: mitochondrial pathway and death receptor pathway. The multidomain Bcl-2 family members, consisting of antiapoptotic (Bcl-2 and Bcl-x_L) and proapoptotic (Bax and Bak) proteins, play pivotal roles in the mitochondrial apoptotic pathway.[1] Activation of Bax or Bak has been reported to be the essential gateway to mitochondrial dysfunction and cell death.[2,3] In healthy cells, Bax is predominantly a cytosolic monomer. On stimulation, it undergoes conformational changes and translocates to the outer mitochondrial membrane or endoplasmic reticulum where it oligomerizes.[4–6] In the well-described mitochondrial death pathway, Bax or Bak permeabilize the outer mitochondrial membrane, allowing the efflux of cytochrome *c* and other apoptosis regulatory proteins into the cytosol.[1,7,8] Suggestive of the central physiological role for Bax in programmed cell death, Bak-null mice fail to show any developmental defects[9]; however, Bax-deficient mice displayed hyperplasia of thymocytes and B cells as well as abnormalities in the development of the reproductive system.[10]

During the past 10 years, apoptosis in atherosclerotic lesions has been broadly reported. All 3 of the major cell types found in atherosclerotic lesions (eg, macrophages, smooth muscle cells [SMCs], and endothelial cells) can undergo apoptosis. Given the complexity of atherosclerotic plaque, apoptosis in these different cell types may play different roles in atherogenesis.[11,12]

Macrophages are found in all stages of atherosclerosis. The expression of a variety of genes and their proteins which affect lipoprotein metabolism and cholesterol accumulation has been shown to influence the progression of atherosclerosis.[13,14] On activation, macrophages generate a large number of cytokines and growth factors that regulate lesion development in both paracrine and autocrine manners.[14–16] The majority of apoptotic cells in atherosclerotic lesions are macrophages localized near the necrotic areas of advanced lesions.[17] Overexpression of Bax and other related apoptotic proteins has been found in human atherosclerotic plaques.[18–20] Bax activation has been shown to occur during apoptosis resulting from free cholesterol loading of macrophages[21] or after treatment of macrophages with oxysterols,[22] which are cytotoxic components of oxidized low-density lipoprotein (LDL).[23] Induction of apoptosis by oxysterols in a macrophage cell line is greatly attenuated when Bax is subjected to small interfering RNA knockdown regardless of the normal expression of Bak.[22]

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Although foam cell apoptosis has long been hypothesized to contribute to the development of lipid core,[17,24] the role of macrophage apoptosis in atherosclerosis remains unclear. Macrophage death may reduce the production of growth factors and inflammatory cytokines, which in turn may impede the development of atherosclerosis. On the other hand, loss of macrophages may promote proatherogenic factors, such as decreased production of apoE and reduced scavenging of toxic substances (ie, oxidized LDL [oxLDL]).[25–27]

The goal of these studies is to investigate the consequences of macrophage apoptosis in atherogenesis. Because of the generally accepted role of Bax in apoptosis as well as the specific observations implicating Bax activation in macrophage apoptosis associated with atherogenesis, we chose to examine the effect of Bax expression on atherosclerosis. Based on previous reports that the expression of LDL receptor in bone marrow–derived leukocytes would not affect the progression of atherosclerosis in LDL receptor–null (LDLR−/−) mice,[28–30] our objective was accomplished by transplanting atherosclerosis-susceptible LDLR−/− mice with Bax-positive or -deficient bone marrow.

Materials and Methods

Mice

Both male LDLR−/− receipt mice (B6.129S7-Ldlr^{tm1Her}) and Bax-null (Bax−/− donor mice (B6.129X1-Bax^{tm1Sjk}) are from The Jackson Laboratory (Bar Harbor, Me) and on C57BL6 background. All mice were maintained in microisolator cages on autoclaved rodent chow containing 4.5% fat (Purina Mills Inc) and autoclaved acidified water. Experimental protocols were performed according to the regulations of the Vanderbilt University Animal Care Committee.

Bone Marrow Transplantation

One week before and 2 weeks after transplantation, all recipient LDLR−/− mice were given acidified water containing 5 mg/L neomycin and 25 000 U/L polymyxin B sulfate (Monarch Pharmaceuticals). Eight-week-old male LDLR−/− mice received 10 Gy whole-body irradiation from a cesium γ source and were transplanted with 5×10^6 bone marrow cells from wild-type (WT) C57BL6 or Bax−/− donor mice as described previously.[25] Three weeks later they were fed a Western diet (21% fat and 0.15% cholesterol; Harlan Teklad) for 10 weeks.

Determination of Chimerism by Western Blotting

Before euthanization, peritoneal macrophages were collected from 4 recipient mice randomly chosen from each group. Protein samples (20 μg each) were separated on NuPage 10% Bis-Tris Gel (Invitrogen) and transferred onto Hybond-P polyvinylidene fluoride membrane (Amersham Biosciences). Bax protein was detected using rabbit polyclonal anti-Bax (Upstate Biotech, Lake Placid, NY). The same blot was stripped and reprobed using rabbit polyclonal anti-β actin (AbCam, Cambridge, Mass).

Serum Cholesterol, Triglyceride, Lipoprotein Analysis, and Peripheral White Blood Cell Count

Blood samples were collected by retro-orbital venous plexus puncture after overnight fasting. Serum was separated by centrifugation at 10 000*g* for 10 minutes at 4°C. Total cholesterol and triglycerides were determined using Cholesterol Reagent and Triglycerides GPO reagent kits (Raichem). Serum from randomly chosen mice was also subjected to fast-performance liquid chromatography (FPLC) analysis of lipoprotein as described.[31]

Blood smears were prepared 12 weeks after bone marrow transplantation and subjected to Quick-Diff staining (Biochemical Sciences). White blood cells were differentiated by scoring a minimum of 200 cells.

Quantitation of Arterial Lesions

After 10 weeks on Western diet, mice were euthanized and perfused with 20 mL of PBS through the left ventricle. Heart and proximal aorta were embedded in OCT and snap-frozen in liquid nitrogen (LN_2). Every other 10-μm cross section was collected starting from the end of the aortic sinus as described.[32] A total of 15 cryosections from each mouse were stained with oil red O (Sigma) and counterstained with Mayer's hematoxylin (Electron Microscopy Sciences). Quantitative analysis of the lesion area was performed using KS300 Imaging System Version 3.0 (Carl Zeiss Vision GmbH).

Immunohistochemistry

Serial 5-μm cryosections from proximal aortas were fixed in acetone at −20°C for 10 minutes and stained with either rat anti-mouse macrophages/monocyte marker MOMA-2 (Accurate Chemical & Scientific Corp, Westbury, NY) and biotinylated mouse anti-rat IgG_{2b} antibody (BD Biosciences Pharmingen, San Diego, Calif), or smooth muscle α-actin epitope-specific rabbit antibody (Laboratory Vision, Fremont, Calif) and biotin labeled goat anti-rabbit IgG polyclonal antibody (BD Biosciences Pharmingen). After the inactivation of endogenous peroxidase in 0.3% H_2O_2/methanol for 30 minutes, the sections were incubated with streptavidin–horseradish peroxidase complex (BD Biosciences Pharmingen), developed with diaminobenzidine-enhanced liquid substrate system (Sigma), and then counterstained with methyl green (Vector Laboratories). The staining was analyzed using AxioVision 3.1 connected with Axioplan2 imaging microscope (Carl Zeiss Vision GmbH).

TUNEL Studies

An in situ cell death detection POD kit (Roche Applied Science) was used with slight modification. Five-micron cryosections were pretreated with 3% citric acid, fixed, and labeled according to manufacturer's instruction. After development using diaminobenzidine, all sections were counterstained with methyl green. Four serial sections from each mouse were stained. The same protocol was used for TUNEL of peritoneal macrophages cultured in Laboratory-Tek chamber slides (Nalge Nunc International). TUNEL-positive cells were counted in 10 fields under light microscope (×400).

Caspase-3 Activity Assay

Peritoneal macrophages were isolated 3 days after IP injection with 1.5 mL of 6% thioglycollate. After overnight incubation in DMEM supplemented with 5% FBS, cells were treated with 10 μg/mL 7-ketocholesterol (7-KC) for 12 hours. The caspase-3 activity was assayed using CPP32/caspase-3 fluorometric protease assay kit from Chemicon International Inc and read by Fusion microplate reader (Perkin–Elmer). The relative fluorescent unit was normalized to the protein concentration of the sample.

Statistics

Nonparametric Mann–Whitney test was used to measure the statistical differences in lesion area. Student *t* test assuming 2 samples with equal variances was used in other analyses. $P<0.05$ was considered to be statistically significant.

Results

Bax-Deficient Mouse Peritoneal Macrophages Display Reduced Levels of Apoptosis In Vitro on Stimulation by Oxysterols or Staurosporine

Expression of the multidomain proapoptotic proteins, Bax and/or Bak, are required for the activity of the mitochondrial apoptosis pathway.[2] Various oxysterol components of oxLDL have been shown to largely account for the apoptotic activity of oxLDL and have been hypothesized to play a role in atherogenesis.[23] Oxysterol-induced apoptosis proceeds through the mitochondrial death pathway.[22,23]

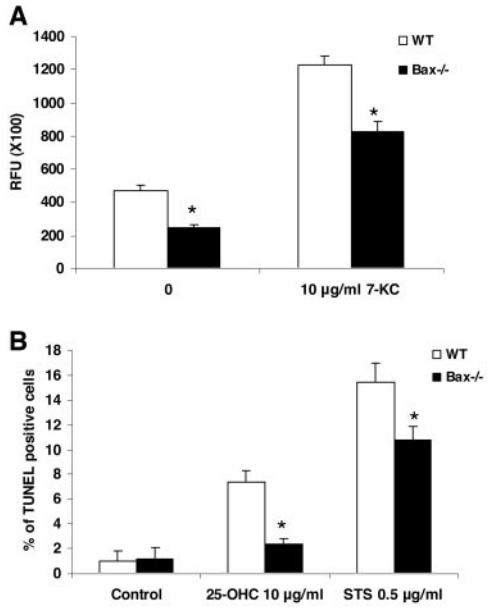

Figure 1. Bax-deficient mouse peritoneal macrophages display reduced apoptosis in vitro. A, Caspase-3 activity assay after 12-hour treatment with 7-KC. Relative fluorescent unit (RFU) was normalized to protein concentrations. Data represent 3 independent experiments. B, TUNEL staining after the treatment with 25-hydroxycholesterol (25-OHC) for 40 hours or staurosporine (STS) for 21 hours. *$P<0.05$ compared with the WT group.

To examine the impact of Bax deficiency on apoptosis in mouse peritoneal macrophages, we assayed the caspase-3 activity in macrophages isolated from WT C57BL6 and Bax knockout mice after 12-hour treatment with 10 μg/mL 7-KC, an important cytotoxic component of oxLDL.[23] The caspase-3 activity was significantly lower in Bax−/− macrophages compared with that in WT macrophages ($P<0.05$; Figure 1A). Consistent with this result, Bax−/− macrophages also showed less TUNEL-positive cells compared with the WT cells after treatment with either another oxysterol, 25-hydroxycholesterol, or staurosporine (STS, Figure 1B). Therefore, consistent with prior studies with Bax knockdown cultured cells,[22] Bax−/− mouse peritoneal macrophages are partially resistant to apoptosis induced by oxysterols. They are also partially resistant to STS, which also acts through the mitochondrial pathway. Partial resistance to the STS induction of apoptosis in Bax-deficient mouse embryonic fibroblast cells has been reported previously.[2]

Reconstitution of LDLR−/− Mice With Bax-Deficient Bone Marrow Does Not Affect the Plasma Cholesterol and Triglyceride Levels, Plasma Lipoprotein Profile, or Peripheral Lymphocyte Counts

As expected, our in vitro studies show that Bax deficiency leads to significantly reduced apoptosis in mouse peritoneal macrophages in response to agents that activate the mitochondrial death pathway. To investigate the role of macrophage apoptosis in the development of atherosclerosis, 8-week-old LDLR−/− mice (male, n=14 in each group) were lethally irradiated and transplanted with 5×10^6 bone marrow cells from WT or Bax−/− donor mice. The reconstitution of recipient mice with donor bone marrow–derived hematopoietic cells was determined by Western blotting 13 weeks after bone marrow transplantation. Bax protein was detected in the peritoneal macrophages isolated from the WT group but not in those from the Bax−/− group (Figure 2). The level of reconstitution was also determined by polymerase chain reaction of the LDLR gene, because both sets of donor bone marrow cells were LDLR-positive. Consistently, 8 weeks after the bone marrow transplantation, the WT LDLR gene was detected in the genomic DNA extracted from the whole blood of all mice from both groups. In addition, the mutant LDLR gene in both groups was below the detectable level after 30 polymerase chain reaction cycles, indicating a complete reconstitution of the recipient hematopoietic cells by the donor bone marrow cells (data not shown).

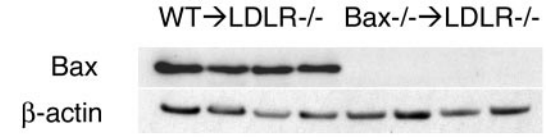

Figure 2. Complete repopulation of recipient mice by donor marrow was confirmed by Western blotting. Whole-cell lysates were prepared from peritoneal macrophages isolated from 4 transplants randomly chosen from each group 13 weeks after bone marrow transplantation. β-actin was probed as the loading control.

To assess the lipid parameters, plasma samples were collected before the start of Western diet as the baseline and every 4 weeks thereafter. Bax deficiency in macrophages did not affect the plasma cholesterol (Figure 3A) and triglyceride levels (Figure 3B). Plasma lipoprotein analysis by FPLC did not reveal any differences between the 2 groups (Figure 3C). Differential white blood cell count 12 weeks after bone marrow transplantation revealed no differences in the peripheral lymphocyte counts (81.3±2.9% in the WT group and 79.5±8.7% in Bax−/− group).

Bax Deficiency in Bone Marrow–Derived Leukocytes Stimulates the Development of Atherosclerosis

To examine the influence of Bax deficiency on atherosclerosis, all recipient mice were euthanized after 10 weeks on a Western diet. The atherosclerotic lesions in the proximal aortas were stained with oil red O. Quantitation revealed a 49.2% increase in the mean cross sectional lesion area ($\mu m^2\pm$SEM) in the proximal aortas of Bax−/− group (233 800±20 489 μm^2) compared with the control group (156 700±20 243 μm^2; Figure 4, $P=0.0259$).

To further characterize the atherosclerotic plaque, macrophages and SMCs in serial sections were stained with specific antibodies (Figure 5). Lesions were classified into 2 types based on the prior literature[33–35]: early lesions mainly composed of macrophage-derived foam cells and advanced lesions characterized by the presence of SMCs and collagen in the cap area. Interestingly, 31% of the lesions in the Bax−/− group, compared with only 17% in the control group, were advanced lesions with fibrous caps. These fibrous plaques from both groups were larger in size compared to those without SMC staining in the cap region, indicating they were in a more developed stage. These lesions often contained necrotic cores while observed under

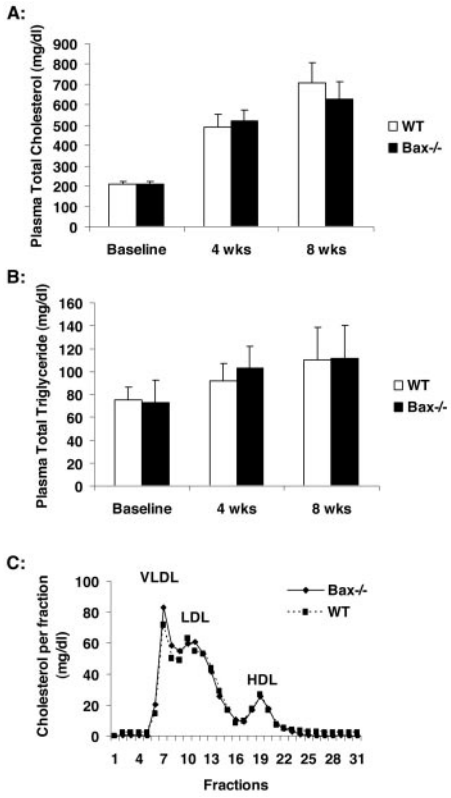

Figure 3. Plasma total cholesterol (A) and triglyceride levels (B) measured before and every 4 weeks after the beginning of western diet. C, Plasma lipoprotein profiles obtained by FPLC after 8-week Western diet.

higher magnification (data not shown). Taken together, our studies show that Bax deficiency in bone marrow–derived cells stimulates the development of atherosclerosis in LDLR−/− mice.

Deficiency of Macrophage Bax Leads to Reduced Apoptosis in Atherosclerotic Lesions

As described above, Bax−/− macrophages display reduced levels of apoptosis in vitro. To investigate whether the increased lesion area in the Bax−/− transplantation group was associated with decreased macrophage apoptotic activity in vivo, modified TUNEL was performed on cross sections of proximal aortas. The majority of apoptotic cells in the lesions were macrophages. Consistent with the proposed role for Bax in macrophage apoptosis, the number of apoptotic macrophages was decreased by 53% in the Bax−/− group (15.3 ± 9.9 per mm^2) compared with that of the control group (32.5 ± 10.8 per mm^2, Figure 6, $P<0.05$). Interestingly, the SMCs in the proximal aorta from Bax−/− group also had reduced apoptosis compared with those from WT group (8.6 ± 6.3 per mm^2 in Bax−/− group and 25.6 ± 10.3 per mm^2 in control group, Figure 6, $P<0.05$).

Discussion

In this study, reconstitution of LDLR−/− mice with Bax−/− bone marrow resulted in a significant increase in mean lesion area as compared with the mice reconstituted with WT bone marrow. As expected, fewer apoptotic macrophages were

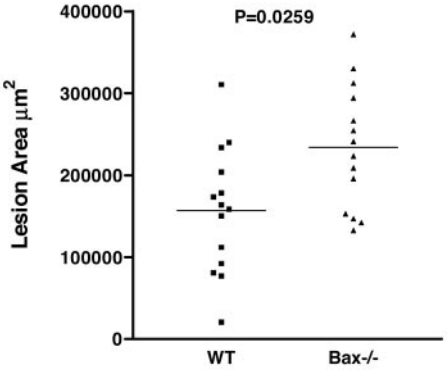

Figure 4. Lesion area quantitation in cross sections of proximal aortas after 10 weeks on Western diet revealed a 49.2% increase in the mean cross-sectional lesion area of Bax−/− group compared with control group. Each data point represents the mean cross-sectional lesion area per mouse; the line represents the mean value of each group, n=14, $P=0.0259$ by nonparametric Mann–Whitney test.

found in the Bax−/− group, consistent with the hypothesis that the increase in lesion size is because of decreased apoptotic activity. In addition, higher numbers of advanced lesions with fibrous caps were found in the Bax−/− group. Our data demonstrate that macrophage apoptosis plays a protective role in the development of atherosclerosis. This is the first study that directly tests the effect of mitochondrial apoptotic pathway on atherogenesis.

Our in vitro studies showed that the induction of apoptosis by oxysterols in Bax-deficient mouse peritoneal macrophages is attenuated. This finding is consistent with our previous studies demonstrating that small interfering RNA knockdown of Bax results in the loss of oxysterol-induced apoptosis.[22] Partial resistance is to be expected, because the functions of Bax have been shown to be redundant with Bak and only cells lacking both Bax and Bak are totally defective in the mitochondrial pathway.[2]

Induction of apoptosis by oxysterol is mediated through the mitochondrial death pathway and probably occurs within atherosclerotic lesions.[23] However, because of the central role that

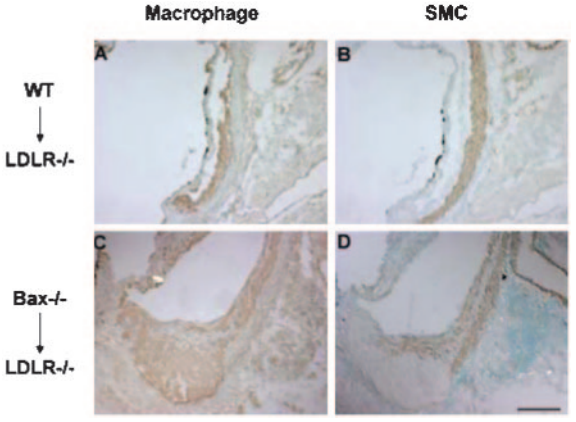

Figure 5. Immunostaining for macrophages in the lesion (A, C) and SMCs in the arterial wall and lesion cap area (B, D) in serial sections from proximal aorta. A and B, Serial sections from control group. C and D, Serial sections from Bax−/− group. Scale bar=200 μm.

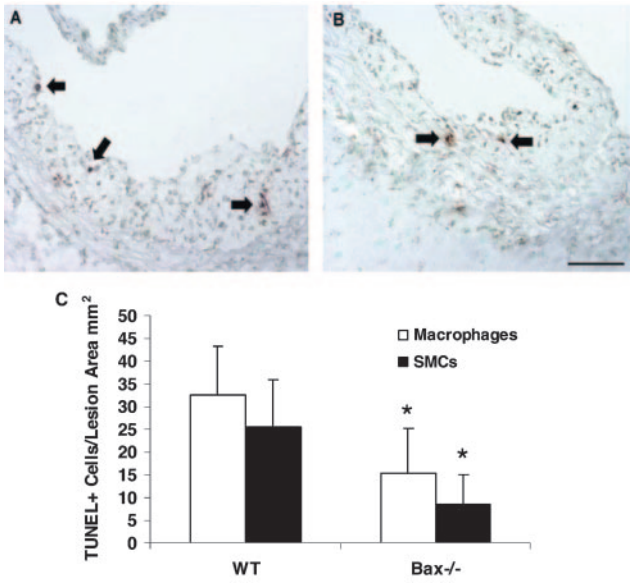

Figure 6. In situ TUNEL staining of proximal aorta sections from the control group (A) and the Bax−/− group (B). Arrows indicate TUNEL-positive nuclei, scale bar=200 μm. C, Quantitation of TUNEL-positive macrophages and SMCs per mm² lesion area, n=14, *$P<0.05$ compared with WT group.

Bax plays in the mitochondrial apoptosis pathway, the reduction of apoptosis in atherosclerosis lesions observed in mice transplanted with Bax−/− bone marrow would be expected for a broad spectrum of apoptotic inducers. For this reason, our observations are more relevant to the role of macrophage apoptosis in atherogenesis than to the role of any specific physiological apoptotic inducer.

Some prior studies have attempted to determine the role of apoptosis in atherogenesis by means of p53-null mice. Macrophages deficient in the tumor suppresser protein p53 have been reported to enhance atherosclerosis in apoE*3-Leiden transgenic mice, which was correlated with a decrease in apoptosis in the p53-deficient mice.[36] Increased atherosclerosis has also been reported in another study using p53−/−apoE−/− mice, although the authors did not find significant change in apoptotic cell numbers but, rather, an increase in cell proliferation.[37] These contradictory results probably reflect the multiple functions of p53, which acts to integrate a number of cellular signals to regulate either cell cycle arrest or apoptosis rather than acting as a specific regulator of the mitochondrial death pathway.[38,39] In contrast, Bax deficiency directly affects the mitochondrial pathway, which can be stimulated by various signals including the activation of p53.

In another study of the role of apoptosis in atherosclerosis, inhibition of neointimal cell *bcl-x_L* expression, 1 of the antiapoptotic Bcl-2 family members, by transfection with anti–*bcl-x* antisense oligonucleotides was observed to induce apoptosis within intimal cells and acute regression of lesions in carotid arteries of rabbits.[40] However, under these experimental conditions, the cell types that take up the antisense oligonucleotides are uncertain. In contrast, the current study is focused on lesion macrophages derived from Bax−/− donor bone marrow (Figures 5 and 6). Our results more specifically demonstrate the effect of mitochondrial apoptotic pathway in macrophages on the development of atherosclerosis.

Bcl-2 family proteins also affect the survival of lymphocytes.[41,42] Bax-deficient mice have been reported to have selective hyperplasia of lymphoid tissues.[10] In searching for any additional mechanistic explanation for increased lesion area in mice transplanted with Bax−/− bone marrow, we examined the peripheral leukocytes in all recipient mice by Quick-Dif staining. No difference was found in the peripheral leukocyte content between the 2 groups. However, increased inflammation in Bax−/− group cannot be excluded, because lesions in this group contain a relatively higher number of macrophages, one of the mediators of inflammatory response in atherosclerotic lesions.[14,43]

There are a number of proatherogenic aspects of lesion macrophages. Mice deficient in both macrophage colony stimulating factor and apoE (op/apoE) have smaller proximal aortic lesions at earlier stages of lesion progression and decreased blood monocyte differentials.[44] Consistent with this observation, IP administration of antibody for the macrophage colony stimulating factor receptor prevents the initial events of atherogenesis.[45] Therefore, a lowered level of apoptosis in Bax-deficient macrophages would also be expected to be proatherogenic by certain macrophage-dependent processes, such as increased number and/or functions of monocyte-derived macrophages, increased growth factor production, cell proliferation, or foam cell accumulation.[14]

Our observation that mice reconstituted with Bax−/− bone marrow have decreased SMC apoptosis is also of interest. Although we are unable to distinguish the origin of these SMCs, the possibility that these SMCs are derived from Bax-deficient bone marrow cannot be excluded according to previous reports.[46,47] The increased SMC content in the lesions of Bax−/− group may also be associated with the interaction between Bax−/− macrophages and SMCs.

In summary, our studies show that the reduced apoptotic activity in macrophages because of Bax deficiency promotes the development of atherosclerosis, indicating that macrophage apoptosis provides a critical self-defense mechanism in suppressing atherosclerosis. Mitochondria-targeting proapoptotic drugs have been under broad development for a wide range of applications, from cancer chemotherapy, HIV, to organ transplantation.[48,49] Our data indicate that the mitochondrial apoptosis pathway in vascular macrophages may be an attractive target for the prevention and treatment of atherosclerosis.

Acknowledgments

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