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Brief Report: Increased Apoptosis in Advanced Atherosclerotic Lesions of *Apoe*-/- Mice Lacking Macrophage Bcl-2

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Abstract

Objective—Macrophage apoptosis plays important roles in atherosclerosis. Bcl-2 is a key cell survival molecule, but its role in macrophage apoptosis in atherosclerosis is not known. The goal herein was to determine the effect of macrophage-targeted deletion of Bcl-2 on macrophage apoptosis in atherosclerotic lesions of *Apoe*-/- mice.

Methods and Results—*Bcl2$_{flox}$-LysMCre* mice were created as a model of macrophage Bcl-2 deficiency. Macrophages from these mice were more susceptible to apoptosis than those from control *Bcl2$_{WT}$-LysMCre* mice. The mice were bred onto the *Apoe*-/-background and fed a Western-type diet for 4 or 10 weeks. Apoptotic cells were equally very rare in the lesions of both groups of the 4-wk-diet mice, and there was no difference in lesion area. However, *Bcl2$_{flox}$-LysMCre*;*Apoe*-/- plaques from the 10-wk-diet protocol had a 40-45% increase in apoptotic cells and, in female mice, a ~25% increase in plaque necrosis ($P<0.05$) compared with *Bcl2$_{WT}$-LysMCre* lesions.

Conclusions—Macrophage Bcl-2 plays a protective role against macrophage apoptosis specifically in advanced atherosclerotic lesions of *Apoe*-/- mice.

Keywords

Atherosclerosis-Pathophysiology; Apoptosis; Macrophage; Animal models of human disease

Macrophage apoptosis can be a critical event in atherosclerosis and occurs at all stages of disease development.[1] In early lesions, macrophage apoptosis is associated with a decrease in lesion cellularity and plaque progression,[2, 3] which could be due to rapid phagocytic clearance or egress of the apoptotic cells and/or decreased influx of macrophages. In advanced lesions, however, clearance of apoptotic cells is defective, which leads to secondary necrosis of apoptotic cells.[4-6] Accordingly, we have proposed that macrophage apoptosis in advanced lesions leads to plaque necrosis, which is thought to promote plaque disruption and acute clinical events in humans.[7] In support of this idea, vulnerable, necrotic human plaques have increased macrophage apoptosis.[8] Moreover, genetic or pharmacological manipulations that decrease macrophage apoptosis in advanced murine lesions decrease plaque necrosis, and *vice versa*[9].

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Disclosures None.

Bcl-2 has been on the forefront of cell survival signaling since its discovery more than 20 years ago, yet there is an absence of *in-vivo* causation studies assessing the role of Bcl-2 in atherosclerosis using genetically altered mouse models. This point is critical, because there are a number of other cell survival molecules in lesional cells, including Bcl-xL, apoptosis inhibitor expressed by macrophages (AIM; SPα), Mcl-1, and members of the inhibitor-of-apoptosis (IAP) family, and so redundancy might negate the effect of deletion of any one cell survival molecule. Because Bcl-2 is expressed in other lesional cell types and holo-*Bcl2*-/- mice have multiple abnormalities at birth[10], we created macrophage-targeted Bcl-2-deficient mice and studied the effect of this mutation on atherosclerosis in Western diet-fed *Apoe*-/- mice. Our studies indicate that macrophage Bcl-2 plays a protective role against macrophage apoptosis specifically in advanced atherosclerotic lesions. Moreover, in the more advanced lesions of female mice, macrophage Bcl-2 also has a modest protective effect against plaque necrosis.

Materials and Methods

Materials and methods related to cultured macrophages, genetically altered mice, plasma lipid analysis, laser capture microdissection, quantification of atherosclerotic lesions, and statistics appear in Supplementary Materials.

In-situ TUNEL Assays and Plaque Necrosis

Apoptotic cells in atherosclerotic lesions were detected by the TUNEL (TdT-mediated dUTP nick end labeling) technique using the TMR red *in-situ* cell death detection kit (Roche). Nuclei were stained with DAPI. TUNEL-positive nuclei were counted under an Olympus IX-70 inverted fluorescent microscope. For assessment of macrophage co-localization, macrophages were detected using a rabbit anti-macrophage antibody (AIA31240) from Accurate Chemical and Scientific Corporation, and nuclei were stained with Hoechst. Plaque necrosis was quantified by measuring the area of hematoxylin and eosin-negative acellular areas in the intima, as described previously.[11]

Results

Intimal Cell Apoptosis is Increased in Lesions of *Bcl2$_{flox}$-LysMCre*;*Apoe-/-* Mice Fed A Western Diet for 10 Weeks But Not 4 Weeks

Mice with Bcl-2 deficiency in macrophages using the cre-lox strategy (*Bcl2$_{flox}$-LysMCre*) were created as described in Supplementary Materials (Supplementary Results and Figure IA-B). Peritoneal macrophages from these mice were more susceptible than control *Bcl2$_{flox}$-LysMCre* mice to a variety of apoptosis inducers (Supplementary Results and Figure IC-D). The two groups of mice were bred onto the *Apoe*-/- background and fed a Western-type diet for 4 or 10 weeks, and the aortic root was examined. The 4-wk lesions showed similar area between the two groups of mice, and only rare examples of lesional macrophage apoptosis (Supplementary Results).

Background data for the 10-wk-diet study are described in Supplementary Results and Supplementary Figure II. Figure 1A demonstrates and increase in TUNEL-positive nuclei, a measure of apoptosis, in *Bcl2$_{flox}$-LysMCre*;*Apoe*-/- *vs. Bcl2$_{WT}$-LysMCre*;*Apoe*-/-lesions. As expected, the areas of TUNEL staining correlated with macrophage-rich areas in these lesions (Figure 1B). The quantified data for the full cohort of mice is shown in Figure 1C. Examples of hematoxylin and eosin-stained aortic root sections of from female *Bcl2$_{WT}$-LysMCre*;*Apoe*-/- and *Bcl2$_{flox}$-LysMCre*;*Apoe*-/- mice are shown in Figure 2A. Total lesion area *per se* was not affected by macrophage Bcl-2 depletion (Figure 2B). Also not affected by macrophage Bcl2 deficiency were plasma levels of TNFα and IL-6 (data not shown).

Increases in macrophage apoptosis are translated into increases in plaque necrosis only after further lesion progression.[12] In the current study, the female mice as a group had larger and more advanced lesions than male mice, and female *Bcl2$_{flox}$-LysMCre;Apoe*-/- lesions appeared to have more acellular necrotic areas (*black arrows* in Fig. 2A) as well as necrotic areas with cholesterol crystals (*red arrows* in Fig. 2A). Quantification showed a ~25% increase in necrotic area in *Bcl2$_{flox}$-LysMCre;Apoe*-/- *vs. Bcl2$_{WT}$-LysMCre;Apoe*-/- female mice ($p < 0.05$) (Figure 2C). Neither fibrous cap thickness nor lesional collagen content were affected by macrophage Bcl-2 deficiency (data not shown).

The difference in the affect of macrophage Bcl-2 deficiency on plaque necrosis could be due to a direct effect of sex differences, *e.g*., sex steroids, and/or to the fact that female lesions were more advanced (see Fig. 2B).[12] In attempt to sort out these possibilities, individual plaques from the female mice were divided into two sub-groups based on plaque area: <200,000 and >200,000 μm^2. We found a statistically significant difference in necrotic area between the genotypes only in the larger plaque subgroup (*Table in* Figure 2D). This finding is consistent with the conclusion that macrophage Bcl-2 deficiency has a selective effect on more advanced lesions. There were not enough larger plaques among the male lesions for analysis, and so pending future studies with more advanced male lesions, there is also the possibility of a direct sex effect as well. In summary, macrophage Bcl-2 deficiency is associated with increased advanced lesional macrophage apoptosis and, in large lesions in female mice, an increase in plaque necrosis.

Discussion

See Supplementary Material for a discussion of the findings in this report in relationship to relevant articles in the literature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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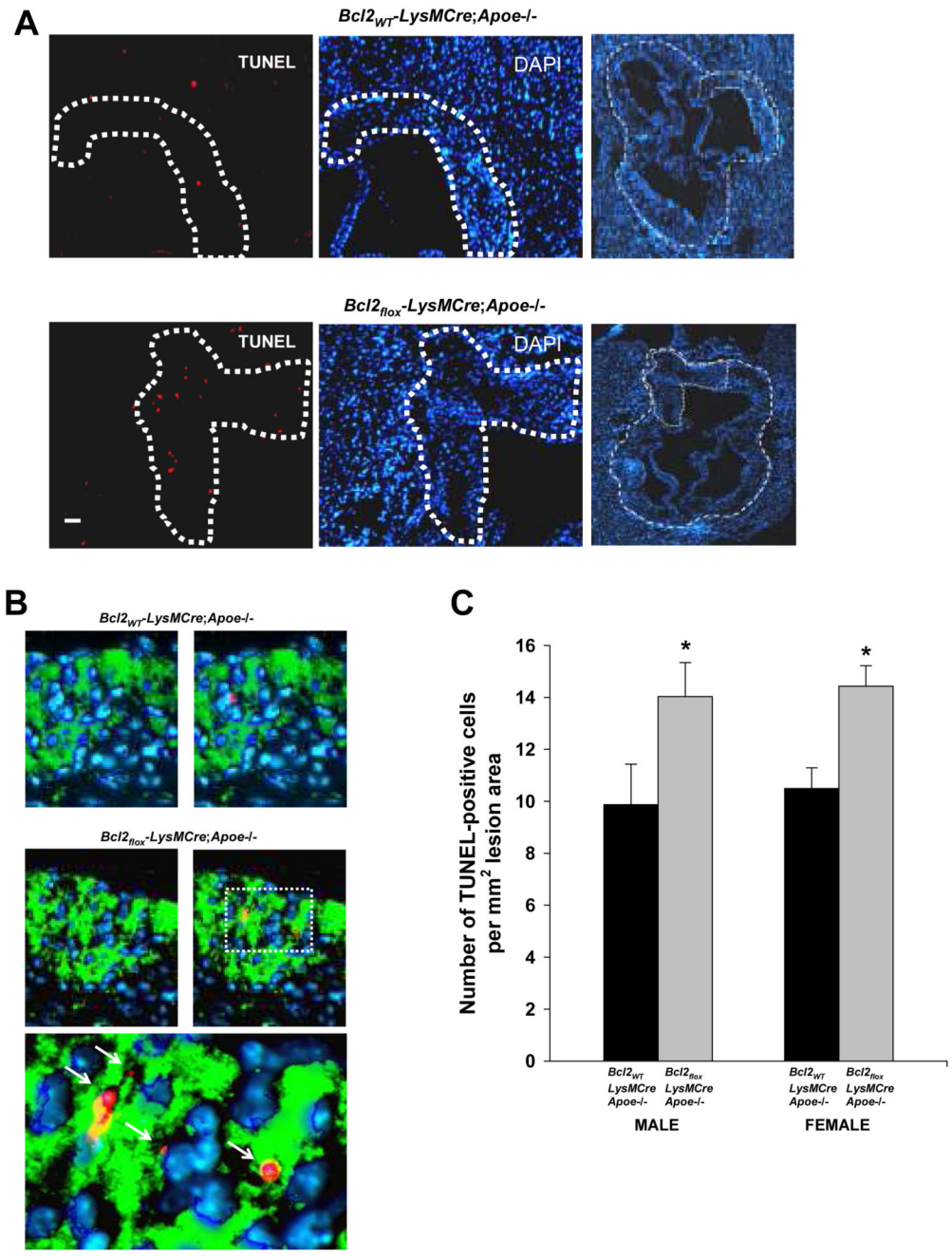
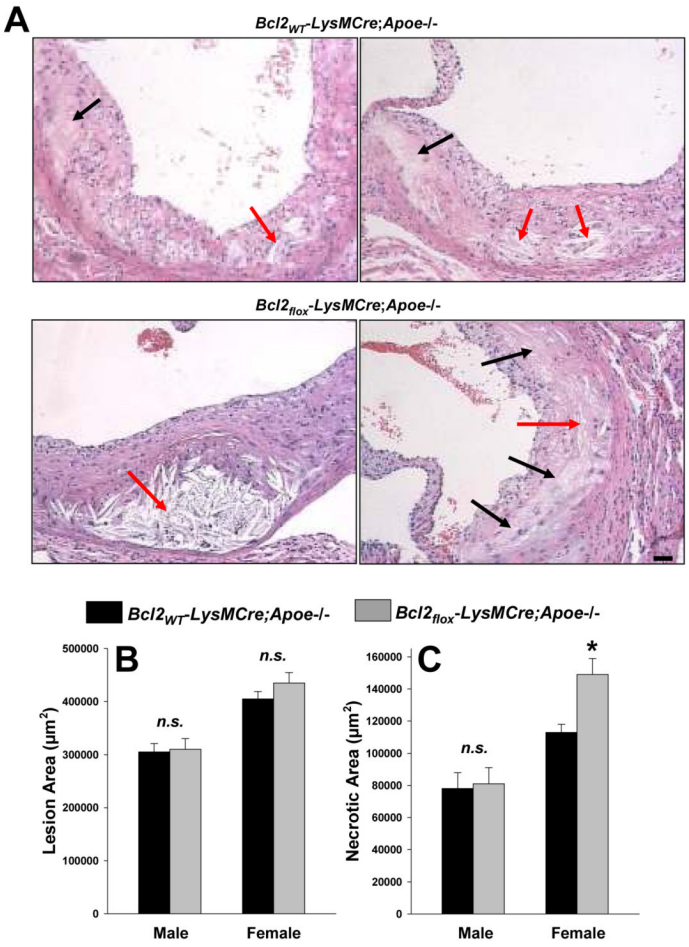


Figure 1. *In-situ* TUNEL staining of aortic root lesions of *Bcl2$_{WT}$-LysMCre;Apoe*-/-and *Bcl2$_{flox}$-LysMCre;Apoe*-/- mice fed a Western-type diet for 10 wks

A, The *left* and *middle* panels show TUNEL- and DAPI-stained aortic root lesions from 10-wk-diet-fed female *Bcl2$_{WT}$-LysMCre;Apoe*-/- and *Bcl2$_{flox}$-LysMCre;Apoe*-/- mice. The full aortic root sections are shown in the right panel (outlined by the *dashed lines*). *Bar*, 20 μm. ***B,*** Macrophage (*green*) and TUNEL (*red*) staining of female *Bcl2$_{WT}$-LysMCre;Apoe*-/- and *Bcl2$_{flox}$-LysMCre;Apoe*-/- lesions. Hoechst-stained nuclei are *blue*. The two left images show only macrophage and nuclei. The bottom image is a high-magnification of the area designated by the box in the right image from the *Bcl2$_{flox}$-LysMCre;Apoe*-/- lesion. The *arrows* in this image show apoptotic macrophages. ***C,*** Quantification of TUNEL-positive nuclei per mm^2

lesion area. The differences between the two genotypes for both males and females were statistically significant (*asterisks*, $P<0.05$).



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	Smaller Plaque Subgroup			Larger Plaque Subgroup		
	Mϕ Bcl2 WT	Mϕ Bcl2 Deficient	P value	Mϕ Bcl2 WT	Mϕ Bcl2 Deficient	P value
Number of individual plaques analyzed	219	220	n.s.	109	105	n.s.
Mean total area of individual plaques (μm^2)	116,394 ± 2,942	124,467 ± 3198	n.s.	245,099 ± 4,574	243,735 ± 4,893	n.s.
Mean necrotic area of individual plaques (μm^2)	54,160 ± 1,700	53,294 ± 1,547	n.s.	89,921 ± 2,622	113,320 ± 2,182	*P*<0.001

Figure 2. Images and quantifications of lesion and necrotic areas of aortic root lesions of *Bcl2$_{WT}$-LysMCre;Apoe-/-* and *Bcl2$_{flox}$-LysMCre;Apoe-/-* mice fed a Western-type diet for 10 wks

A, Images of hematoxylin- and eosin-stained aortic root lesions from 10-wk-diet-fed female *Bcl2$_{WT}$-LysMCre;Apoe-/-* and *Bcl2$_{flox}$-LysMCre;Apoe-/-* mice. *Black arrows*, acellular necrotic areas; *red arrows*, necrotic areas with cholesteryl crystals. *Bar*, 20 μm. ***B,*** Quantification of cross-sectional lesion area. The differences between female *vs*. male lesions in both genotypes are statistically different by Mann-Whitney analysis ($P<0.05$). ***C,*** Quantification of cross-sectional necrotic area. *Asterisk*, $P<0.05$. ***D,*** Individual plaques in aortic root cross sections from female mice were separated into two groups based on plaque area, <200,000 and >200,000 μm^2, and then analyzed for plaque necrosis For each mouse, six cross

sections were analyzed, and each section had, on average, three individual plaques. The differences in necrotic area between larger and smaller plaques for each genotype were statistically significant ($P<0.001$ by Mann-Whitney).