TNF provokes cardiomyocyte apoptosis and cardiac remodeling through activation of multiple cell death pathways

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Transgenic mice with cardiac-restricted overexpression of secretable TNF (MHCsTNF) develop progressive LV wall thinning and dilation accompanied by an increase in cardiomyocyte apoptosis and a progressive loss of cytoprotective Bcl-2. To test whether cardiac-restricted overexpression of Bcl-2 would prevent adverse cardiac remodeling, we crossed MHCsTNF mice with transgenic mice harboring cardiac-restricted overexpression of Bcl-2. Sustained TNF signaling resulted in activation of the intrinsic cell death pathway, leading to increased cytosolic levels of cytochrome c, Smac/Diablo and Omi/HtrA2, and activation of caspases -3 and -9. Cardiac-restricted overexpression of Bcl-2 blunted activation of the intrinsic pathway and prevented LV wall thinning; however, Bcl-2 only partially attenuated cardiomyocyte apoptosis. Subsequent studies showed that c-FLIP was degraded, that caspase-8 was activated, and that Bid was cleaved to t-Bid, suggesting that the extrinsic pathway was activated concurrently in MHCsTNF hearts. As expected, cardiac Bcl-2 overexpression had no effect on extrinsic signaling. Thus, our results suggest that sustained inflammation leads to activation of multiple cell death pathways that contribute to progressive cardiomyocyte apoptosis; hence the extent of such programmed myocyte cell death is a critical determinant of adverse cardiac remodeling.

Introduction

Transgenic mice with cardiac-restricted overexpression of TNF initially develop a concentric hypertrophic cardiac phenotype that segues to a dilated phenotype as the mice age, thus recapitulating the classic transition to failure that has been reported in many experimental models of cardiac decompensation (1–3). Recently we have shown that the development of LV wall thinning and adverse cardiac remodeling in mice with cardiac-restricted overexpression of secretable TNF (MHCsTNF mice) correlates with the prevalence of cardiomyocyte apoptosis (4). Another interesting observation that arose from these studies was that the prevalence of cardiomyocyte apoptosis increased in concert with an age-dependent decrease in the myocardial protein levels of Bcl-2, a critical member of the apoptosis-regulating protein family that suppresses cell death. This raised the intriguing possibility that sustained inflammatory signaling may have provoked progressive cardiomyocyte apoptosis as a result of the loss of one or more cytoprotective proteins. Two independent pathways may lead to cardiomyocyte apoptosis (reviewed in refs. 5, 6). The extrinsic (type I) pathway is initiated by ligands that bind to death receptors such as TNF receptor 1, whereas the intrinsic (type II) pathway is governed by the release of various proapoptotic proteins from the mitochondria. Whereas the inhibitors of apoptosis (IAPs) and cellular FLICE-like inhibitory protein (c-FLIP) prevent activation of the extrinsic pathway, Bcl-2 is a major regulator of mitochondrial membrane permeability and hence prevents activation of the intrinsic pathway. Although there is not complete agreement, the aggregate data suggest that the intrinsic, rather than extrinsic, death pathway is critical for cardiomyocyte fate decisions during heart failure (reviewed in refs. 5, 6).

The present study was undertaken in order to further delineate the role of cardiac apoptosis and cardiac remodeling in the MHCsTNF mice. Accordingly, we crossed the MHCsTNF mice with a previously reported line of mice harboring cardiac-restricted overexpression of human Bcl-2 (7) in order to determine whether overexpression of Bcl-2 in the heart would prevent cardiomyocyte apoptosis, as one would predict if myocytes behave as “type II cells.” In mice with MHCsTNF mice loss of antiapoptotic proteins contributes to the activation of both intrinsic as well as extrinsic cell death pathways. Cardiac-restricted overexpression of Bcl-2 in the MHCsTNF mice attenuated cardiomyocyte apoptosis and LV remodeling by blocking the intrinsic cell death pathway, but did not prevent cardiac myocyte apoptosis secondary to activation of the extrinsic cell death pathway.

Results

Characterization of mouse models

Figure 1A shows that exogenous human Bcl-2 protein was readily detectable in cytosolic and mitochondrial extracts of hearts from Bcl-2 mice. Human Bcl-2 protein was also present in the myocardial cytosolic and mitochondrial extracts of the bitransgenic MHCsTNF/Bcl-2 mice, although at lower levels compared with Bcl-2 mice, consistent with the observation that TNF enhances Bcl-2 degradation (8). Human Bcl-2 protein was not detectable in myocardial extracts from littermate control mice. Thus, human Bcl-2 protein was only detectable in cytosolic and mitochondrial extracts from Bcl-2 mice, consistent with its detection in bitransgenic mice. Of note, the human Bcl-2 protein was present in the cytosolic and mitochondrial extracts of transgenic controls (Fig. 1A).

Nonstandard abbreviations used in this report are as follows: c-FLIP, cellular FLICE-like inhibitory protein; c-FLIPL, c-FLIP long isoform; c-FLIPs, c-FLIP short isoform; c-IAP-1, cellular inhibitor of apoptosis 1; Endo G, endonuclease G; IAP, inhibitor of apoptosis; LVEDD, LV end-diastolic diameter; MHCsTNF mice, mice with cardiac-restricted overexpression of secretable TNF; r/h, ratio of LV radius to LV wall thickness; t-Bid, Bid cleaved to its truncated form.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J. Clin. Invest. 117:2692–2701 (2007). doi:10.1172/JCI29134.
Effect of Bcl-2 on LV remodeling

We have shown previously that cardiomyocyte apoptosis contributes to the LV wall thinning in the MHCsTNF mice as they transition from a concentric hypertrophic phenotype at 4 weeks of age to a dilated phenotype at 12 weeks of age (1, 4). To determine whether overexpression of Bcl-2 was sufficient to attenuate adverse cardiac remodeling in the MHCsTNF mice, we examined cardiac structure using standard morphometric analyses and 2D-directed M-mode echocardiography in 4- and 12-week-old mice.

Table 1 shows that the heart weight–to–body weight ratios of the MHCsTNF and MHCsTNF/Bcl-2 mice at 4 and 12 weeks of age were both significantly greater than age-matched littermate control and Bcl-2 mice (4 wk, n = 10/group; 12 wk, n = 12/group) (P < 0.001). Although the heart weight–to–body weight ratio decreased significantly (P < 0.02) in the MHCsTNF and MHCsTNF/Bcl-2 mice from 4 to 12 weeks, there was no significant difference in the heart weight–to–body weight ratio between the MHCsTNF and MHCsTNF/Bcl-2 mice at either 4 or 12 weeks (P > 0.54).

The increase in the heart weight–to–body weight ratio of the MHCsTNF and MHCsTNF/Bcl-2 relative to littermate control and Bcl-2 mice was not secondary to selective differences in body weight as the body weights were not significantly different between groups of the same age (P > 0.51).

Figure 2A depicts representative echocardiograms for each group of mice at 12 weeks of age, whereas Figure 2B–E, summarizes the results of group data (4 wk, n = 6/group; 12 wk, n = 9/group). Consistent with our prior observations (1, 4), the LV end-diastolic diameter (LVEDD) increased by 20% (P < 0.001) in the MHCsTNF mice from 4 to 12 weeks (Figure 2B). Although LVEDD increased in the MHCsTNF/Bcl-2 mice, this increase was smaller when compared with the change in LVEDD in the MHCsTNF mice. Thus at 12 weeks of age, the LVEDD in MHCsTNF/Bcl-2 mice was significantly lower (P = 0.02) than in the MHCsTNF mice. There was no difference in LVEDD between or within the littermate control and Bcl-2 mice at 4 and 12 weeks. Figure 2C shows that at 4 weeks of age, LV wall thickness in both MHCsTNF and MHCsTNF/Bcl-2 mice was significantly increased (P < 0.001) when compared with littermate control and Bcl-2 mice, although there was no difference between the MHCsTNF and MHCsTNF/Bcl-2 mouse groups. However, the salient finding shown by Figure 2C is that in MHCsTNF mice, LV wall thickness decreased by 16% (P = 0.02) from 4 to 12 weeks, whereas in the MHCsTNF/Bcl-2 mice there was no significant change in LV wall thickness during the same time period.

As a result, the absolute LV wall thickness at 12 weeks of age was 32% higher (P < 0.001) in MHCsTNF/Bcl-2 mice when compared with MHCsTNF mice. The increase in LVEDD and LV wall thinning resulted in a 44% increase (P < 0.001) in the ratio of LV radius to LV wall thickness (r/h) in the MHCsTNF mice from 4 to 12 weeks (Figure 2D), consistent with adverse cardiac remodeling. By comparison, the r/h ratio remained relatively unchanged in the MHCsTNF/Bcl-2 from 4 to 12 weeks, and thus, at 12 weeks of age the r/h ratio of MHCsTNF/Bcl-2 mice was 29% lower (P < 0.001) than that of the MHCsTNF mice, suggesting that overexpression of Bcl-2 prevented adverse cardiac remodeling in the MHCsTNF mice. In addition, while the percent of fractional shortening (%FS)
in MHCsTNF mice remained significantly lower ($P < 0.01$) than age-matched littermate controls from 4 to 12 weeks, the %FS in MHCsTNF/Bcl-2 mice increased significantly ($P < 0.05$) during the same time period (Figure 2E). Thus, at 12 weeks of age the %FS in MHCsTNF/Bcl-2 mice was 33% higher ($P < 0.01$) than in the MHCsTNF mice and was not significantly different from littermate control and Bcl-2 mice, suggesting that overexpression of Bcl-2 prevented the decrease in contractile dysfunction observed in the MHCsTNF mice.

**Cardiomyocyte apoptosis**

We have shown that the prevalence of cardiomyocyte apoptosis increases in a time-dependent manner in the MHCsTNF mice and that this increase in apoptosis is accompanied by a progressive loss of myocardial Bcl-2 and a concomitant increase in the release of cytochrome $c$ from the mitochondria into the cytosol (4). Given that TNF enhances Bcl-2 degradation (8), these initial observations suggested a role for TNF-induced activation of the intrinsic cell death pathway in the adverse cardiac remodeling that we observed in the MHCsTNF mice. To determine whether cardiac overexpression of Bcl-2 was sufficient to attenuate apoptosis in the MHCsTNF/Bcl-2 mice, we compared the prevalence of apoptotic cardiac nuclei in 12-week-old MHCsTNF/Bcl-2 mice to MHCsTNF mouse hearts using the in situ DNA ligase technique (4, 9). Figure 3 shows that the prevalence of cardiomyocyte apoptosis was 60% lower (4).

**Mechanisms for cardiomyocyte apoptosis: intrinsic pathway**

*Myocardial cytochrome $c$ release.* To investigate the mechanism(s) responsible for the Bcl-2–mediated attenuation of cardiomyocyte apoptosis we first examined mitochondrial release of cytochrome $c$ in the hearts of 12-week-old MHCsTNF and MHCsTNF/Bcl-2 mouse hearts. Representative Western blots of mitochondrial and cytosolic levels of cytochrome $c$ are depicted in Figure 4, A and B. Reprobing the membranes for both GAPDH and cytochrome $c$ oxidase subunit IV showed that the cytosolic and mitochondrial protein extracts were relatively pure and that protein loading was equal. Analysis of the group data shown in Figure 4, A and B, showed that in MHCsTNF mouse hearts, mitochondrial cytochrome $c$ levels were significantly lower ($P < 0.01$) and cytosolic cytochrome $c$ levels were significantly higher ($P < 0.01$) than the corresponding cytochrome $c$ levels in littermate control and Bcl-2 hearts ($n = 12/group$). Importantly, in MHCsTNF/Bcl-2 hearts, mitochondrial cytochrome $c$ levels were significantly higher ($P < 0.01$) and cytosolic cytochrome $c$ levels were significantly lower ($P < 0.05$) than in MHCsTNF hearts and were not different from the corresponding levels in littermate control and Bcl-2 mice.

**Myocardial levels of proapoptotic proteins.** To further explore the effects of Bcl-2 overexpression in the heart, we examined protein levels of several mitochondrial apoptogens. Figure 5A shows representative Western blots for proapoptotic Smac/Diablo, Omi/HtrA2, apoptosis inducing factor (AIF), and endonuclease G (Endo G) in 12-week-old mice. As shown by the corresponding group data in Figure 5B, the cytosolic levels of Smac/Diablo ($n = 16/group$) and Omi/HtrA2 ($n = 8/group$) were significantly higher ($P < 0.001$) in the hearts of the MHCsTNF mice relative to littermate control and Bcl-2 mice. Importantly, overexpression of Bcl-2 in cardiomyocytes led to a significant reduction ($P < 0.01$).
relative to MHCsTNF mice) in the release of these 2 proapoptotic proteins. Although the levels of Omi/HtrA2 were reduced to baseline levels in the MHCsTNF/Bcl-2 mouse hearts, the levels of Smac/Diablo were still greater than those observed in littermate control and Bcl-2 mice. The cytosolic levels of AIF ($n = 16$/group) and Endo G ($n = 12$/group) were not different ($P = 0.3$ and $0.4$, respectively) in the hearts of MHCsTNF, MHCsTNF/Bcl-2, Bcl-2, and littermate control mice.

**Myocardial caspase-3 and -9 activation.** To determine whether cardiac-restricted overexpression of Bcl-2 decreased activation of caspases that have been implicated in the intrinsic cell death pathway, we measured caspase-3– and caspase-9–like activities in the hearts of 12-week-old MHCsTNF and MHCsTNF/Bcl-2 mice. Figure 6, A and B, shows that caspase-3– and caspase-9–like activity were significantly increased ($P < 0.001$) in myocardial extracts from the MHCsTNF mice relative to littermate controls ($n = 8$/group). Although caspase-3– and caspase-9–like activity were both significantly reduced in the MHCsTNF/Bcl-2 mice when compared with the MHCsTNF mice ($P < 0.001$ and $P = 0.03$, respectively), the levels of caspase-3– and caspase-9–like activity were still significantly ($P < 0.05$) greater than those observed in the littermate control and Bcl-2 mice.

**Mechanisms for cardiomyocyte apoptosis: extrinsic pathway**

Given that cardiac-restricted overexpression of Bcl-2 was sufficient to prevent mitochondrial cytochrome c release but did not completely abrogate apoptosis in the MHCsTNF/Bcl-2 mice, we asked whether the extrinsic, Bcl-2–insensitive pathway was activated as well.

**Myocardial caspase-8 activation, Bid, IAPs, and c-FLIP.** Figure 7A shows that caspase-8–like activity was significantly greater ($P < 0.001$) in the MHCsTNF hearts when compared with littermate control and Bcl-2 mice ($n = 8$/group). Importantly, the levels of caspase-8–like activation were not different in the MHCsTNF and MHCsTNF/Bcl-2 mouse hearts ($P = 0.16$). The representative Western blot illustrated in Figure 7B shows that full-length Bid (23 kDa) was present in the cytosolic extracts of the littermate control and Bcl-2 mice, whereas Bid was cleaved to its truncated form (t-Bid; 15 kDa) in MHCsTNF as well in MHCsTNF/Bcl-2 mouse hearts. The group data shown in Figure 7B show that cytosolic full-length Bid was significantly decreased ($P < 0.01$) in the MHCsTNF and MHCsTNF/Bcl-2 mice when compared with the control groups ($n = 8$/group). Moreover, cytosolic t-Bid was significantly increased ($P < 0.01$) in the MHCsTNF and the MHCsTNF/Bcl-2 mice when compared with the control groups ($n = 8$/group). There was no difference in myocardial t-Bid levels between the MHCsTNF and the MHCsTNF/Bcl-2 mice, consistent with the lack of effect of Bcl-2 on caspase-8 activation. Another important finding shown by Figure 7C is that the levels of cellular inhibitor of apoptosis 1 (c–IAP-1) were significantly ($P < 0.001$) reduced in the MHCsTNF mice relative to littermate controls ($n = 12$/group), indicating that sustained expression of TNF induced loss of cytoprotective c–IAP-1.
However, cardiac overexpression of Bcl-2 did not prevent the TNF-induced decrease in the level of c-IAP-1. In contrast, there was no difference (P = 0.43) in the cytosolic levels of c-IAP-2 in the MHCsTNF/Bcl-2 mice relative to levels in the MHCsTNF, littermate control, and Bcl-2 mice (n = 12/group).

Noting a recent report that showed that TNF-mediated activation of JNK resulted in ubiquitination and degradation of c-FLIP long isoform (c-FLIP <sub>L</sub>), an inhibitor of caspase-8 (10), we sought to determine whether there were differences in the level of c-FLIP <sub>L</sub> in the hearts of the MHCsTNF and/or MHCsTNF/Bcl-2 mice. Figure 8A depicts representative Western blots and resulting group data for the protein levels of cardiac c-FLIP <sub>L</sub> and of its isoform c-FLIP short isoform (c-FLIP <sub>S</sub>) in MHCsTNF and MHCsTNF/Bcl-2 mice. As shown, both c-FLIP <sub>L</sub> and c-FLIP <sub>S</sub> protein levels were significantly (P < 0.01) reduced in MHCsTNF mice relative to littermate control and Bcl-2 mice (n = 8/group). Moreover, protein levels for c-FLIP <sub>L</sub> and c-FLIP <sub>S</sub> were not different in the MHCsTNF and the MHCsTNF/Bcl-2 mice (P = 0.8 and 0.2, respectively). To determine whether the decrease in c-FLIP <sub>L</sub> levels related to decreased c-FLIP <sub>L</sub> mRNA levels, we performed RNase protection assays. As shown in Figure 8B, there was no significant difference (P = 0.29) in the mRNA levels of c-FLIP <sub>L</sub> in the hearts of the littermate control, MHCsTNF, MHCsTNF/Bcl-2, and Bcl-2 mice (n = 8/group).

Figure 5
Myocardial levels of proapoptotic proteins. (A) The release of mitochondrial Smac/Diablo, Omi/HtrA2, apoptosis-inducing factor (AIF), and Endo G into the cytosol was determined by Western blotting in littermate control, MHCsTNF, MHCsTNF/Bcl-2, and Bcl-2 mouse hearts at 12 weeks of age. (B) Group data for the corresponding protein levels (normalized to GAPDH) are expressed as the ratio of experimental (transgenic) to control (wild-type) mouse groups. *P < 0.05 between MHCsTNF and MHCsTNF/Bcl-2 groups; **P < 0.05 compared with LM.

Discussion
Cardiac remodeling contributes to the development and progression of heart failure (reviewed in ref. 11). However, the mechanisms that contribute to the structural changes that underlie progressive cardiac remodeling are only partially understood. Recent studies have shown that forced expression of components of either the extrinsic or intrinsic cell death pathways provokes a dilated cardiac phenotype in the adult heart (12, 13), suggesting that programmed myocyte cell death may contribute to progressive cardiac decompensation. In support of this point of view, studies that have used broad-based caspase inhibitors to prevent myocyte apoptosis have demonstrated that cardiac remodeling is attenuated in lines of transgenic mice with forced cardiac overexpression of G<sub>αq</sub> and TNF (4, 14). However, caspases can degrade myofibrillar proteins and transcription factors responsible for the expression of muscle genes in myocytes (15, 16). In addition, caspases can inhibit the ubiquitin proteasome system that is responsible for recycling peptides obtained from degraded myofibrillar proteins (17). Thus, the aforementioned studies with caspase inhibitors...
cannot be used as unambiguous evidence in support of the contribution of programmed cell death to progressive cardiac remodeling.

Here we have taken a genetic approach to inhibit cardiomyocyte apoptosis in mice overexpressing TNF in the cardiac compartment. Results from the present study show for the first time that cardiac overexpression of Bcl-2 not only reduces the prevalence of TNF-induced cardiomyocyte apoptosis in bitransgenic MHCsTNF/Bcl-2 mice (Figure 3) but also prevents the LV wall thinning, the adverse cardiac remodeling (increased r/h ratio), and the decrease in fractional shortening that occurs in these mice (Figure 2) from 4 to 12 weeks of age. From a pathophysiological standpoint, the increase in LV wall thinning that attends progressive cardiomyocyte apoptosis would be expected to contribute to increased LV wall stress (i.e., afterload mismatch) and hence sustained unfavorable loading conditions of the heart, thereby contributing to progressive cardiac decompensation. Indeed, using a similar transgenic approach we have previously shown that Bcl-2 overexpression prevented cardiac hypertrophy and improved cardiac function in desmin-null mice that develop cardiomyopathy (18).

In addition to clarifying the role of cardiomyocyte apoptosis with respect to cardiac remodeling, the results of this study provide several new insights into the mechanisms that contribute to programmed cardiomyocyte cell death in vivo. As noted at the outset, we have observed an increased prevalence of apoptosis in the MHCsTNF mice as they age from 4 to 12 weeks. As reported previously, apoptosis in the MHCsTNF mice was primarily observed in cardiomyocytes, in contrast to other studies wherein cardiac-restricted overexpression of TNF was shown to lead to apoptosis in interstitial and inflammatory cells (2–4). This increased prevalence of apoptosis cannot be explained by increasing levels of myocardial TNF in the MHCsTNF mice with age; in fact we have previously reported decreased levels of myocardial TNF in the older MHCsTNF mice, perhaps related to α-myosin heavy chain promoter activity (1). A more likely explanation is that sustained TNF signaling in some way allows the myocytes to become more sensitive to the proapoptotic effects of TNF over time. Several lines of evidence support this intriguing point of view. First, sustained TNF signaling resulted in progressive depletion of cytoprotective proteins that prevent the activation of both the intrinsic (Bcl-2) and the extrinsic (c-FLIP, c–IAP-1) cell death pathways. Second, progressive loss of Bcl-2 in the MHCsTNF mice contributed to the mitochondrial release of cytochrome c, Smac/Diablo, and Omi/HtrA2 into the cytosol (Figures 4 and 5). In keeping with this point of view, overexpression of Bcl-2 restored cytosolic levels of cytochrome c and Omi/HtrA2 to those observed in littermate control mice. The mitochondrial release of Smac/Diablo as well as levels of caspase-9 activity were significantly reduced in the MHCsTNF/Bcl-2 mice, even though their levels were not reduced to baseline levels. Although the reason for this finding is not known, one possibility is that the levels of Bcl-2 in MHCsTNF/Bcl-2 mice were inadequate to normalize the release of mitochondrial apoptogens such as Smac/Diablo (Figure 5). Elevated levels of cytosolic Smac/Diablo would be expected to promote amplification of the intrinsic and extrinsic cell death pathways by preventing the IAPs from blunting caspase-3 activation (see Figure 9) (19). Alternatively, other Bcl-2-independent mechanisms such as the extrinsic apoptotic pathway may have contributed to the observed cardiomyocyte cell death. Recent studies have demonstrated that the TNF-induced extrinsic cell death pathway involves the formation of 2 sequential signaling pathways.
complexes termed “complex I” and “complex II” (see Figure 9) (20). Engagement of TNF receptor superfamily, member 1 (TNFR1) by TNF ligand leads to recruitment of the death domain–containing proteins TNFR1-associated via death domain (TRADD) and TNFR-interacting serine-threonine kinase 1 (RIP1), which can then participate in either complex I or complex II. Complex I, which contains TNFR-associated factor 2 (TRAF2), binds to the cytoplasmic tail of TNFR1 and promotes the activation of the cytoprotective transcription factor NF-κB as well as activation of JNK. The balance between NF-κB and JNK activation is critical for cell fate.

Complex II is assembled when TRADD and RIP1 are ubiquitinated and localize to the cytosol, where they associate with Fas-associated death domain (FADD) and caspase-8. When NF-κB is activated by complex I, complex II harbors the caspase-8 inhibitor c-FLIP, and the cell survives. However, when c-FLIP is degraded, such as by JNK activation (10), and is absent from complex II, cell death proceeds through processing of caspase-8, with resultant activation of distal effector caspases such as caspase-3. The decreased levels of c-FLIP protein in the MHCsTNF and MHCsTNF/Bcl-2 mice may have allowed for the activation of caspase-8 within complex II as well as the cleavage of Bid to t-Bid (Figure 7) with subsequent mitochondrial release of proapoptotic factors that amplified the activation of the downstream intrinsic pathway (21). Nonetheless, it should be recognized that the data linking decreased levels of c-FLIP to increased activation of the extrinsic cell death pathway is correlative and therefore should be regarded as provisional.

In addition to blocking the release of apoptotic factors (22), Bcl-2 may be cytoprotective by inhibiting the mitochondrial permeability transition pore (23) and enhancing the rate of oxidative phosphorylation by attenuating the mitochondrial Na⁺-Ca²⁺ exchanger (and hence preventing mitochondrial calcium matrix efflux) (24) as well as by reducing the rate of ATP decline and intracellular acidification during ischemia by directly inhibiting F1F0-ATPase (25). In addition, Bcl-2 exerts antiapoptotic effects through an NF-κB–dependent (26) and/or antioxidant mechanism(s) (27) and by inhibiting proapoptotic JNK activation (28) in ventricular myocytes. Thus, we cannot exclude caspase-independent effects of Bcl-2 in our model. Lastly, since cardiac-restricted overexpression of Bcl-2 alone was not sufficient to abrogate the adverse remodeling in MHCsTNF mice, it is likely there are other mechanisms that contribute to cardiomyocyte apoptosis in the MHCsTNF mice. In parallel studies we have determined that decreased vascularization and/or decreased numbers of proliferating cardiomyocytes
were unclear to explain the adverse remodeling in the MHCsTNF mice (see Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI29134DS1). Moreover, we have shown that the broad-based matrix metalloproteinase inhibitor abrogated LV dilation but had no effect on LV wall thickness in a similar line of mice with cardiac-restricted overexpression of TNF (29). Thus adverse remodeling in the MHCsTNF mice likely represents the consequence of increased wall thinning secondary to apoptosis and matrix degradation secondary to activation of matrix metalloproteinases.

**Conclusion**

The results of this study, in which we have taken a genetic approach to inhibit programmed cell death, suggest that the development of progressive cardiomyocyte apoptosis plays a critical role in the LV wall thinning and adverse cardiac remodeling that occur in the setting of sustained inflammation. This study further suggests that the biological processes that govern cardiomyocyte cell death in vivo are far more complex than have been proposed (12, 13). That is, our results suggest that in vivo, apoptotic cell death does not necessarily occur as a direct result of the activation of the cell death pathways per se but rather that cell death proceeds after sustained TNF signaling renders myocytes competent to die, after one or more antiapoptotic cytoprotective proteins are depleted (see Figure 9). Indeed, our prior observation that progressive loss of Bcl-2 protein correlates with the increasing prevalence of cardiomyocyte apoptosis in the MHCsTNF mice, (4) coupled with the observation in the present study that cardiac overexpression of Bcl-2 is sufficient to partially attenuate apoptosis and abrogate LV wall thinning in the MHCsTNF/Bcl-2 mice, is entirely consistent with this point of view. In this regard, in future studies it will be extremely important to determine whether progressive depletion of the antiapoptotic cytoprotective proteins that regulate the intrinsic and/or extrinsic cell death pathways favors activation of these pathways in a “feed-forward” self-amplifying manner that renders cells progressively more competent to die in the setting of sustained cardiac injury and/or inflammation.

**Methods**

**Characterization of mouse models**

The generation and characterization of the line of transgenic mice with cardiac-restricted overexpression of secretable TNF (referred to as MHCsTNF mice) in a C57BL/6 background has been described in detail (1, 30). Transgenic mice with cardiac-restricted overexpression of human Bcl-2 (Bcl-2 mice) were generated in a FVB/N background as previously described (7). Both mouse lines were hemizygous for their respective transgene. Male MHCsTNF mice were mated with female Bcl-2 mice to generate bitransgenic MHCsTNF/Bcl-2 mice. The resulting F1 offspring was genotyped for each heart, for a total of 30 microscopic fields per heart.

**Myocardial cytochrome c release**

Myocardial sections with anti–desmin-rhodamine as previously described (4). Stained apoptotic myocyte nuclei were detected using a fluorescence microscope (×400; Nikon Eclipse E800; Nikon Instruments). Images were digitally photographed (SPOT II; Diagnostic Instruments) and analyzed with software that enabled us to enumerate cell nuclei (MetaView; Universal Imaging). Only those nuclei that were labeled with the ligase technique and were identified as cardiomyocytes were included in our quantification of cardiomyocyte apoptosis. In each microscopic field, the number of cardiomyocyte nuclei labeled by the in situ DNA ligation technique was divided by the total number of DAPI-stained nuclei. To quantify the prevalence of apoptosis, 3 LV cross-sections at the level of the papillary muscles were analyzed for each heart, for a total of 30 microscopic fields per heart.

**Mechanisms for cardiomyocyte apoptosis: intrinsic pathway**

Myocardial cytochrome c release. Whole hearts from 12-week-old mice were harvested, weighed, and immediately frozen. Cytosolic and mitochondrial proteins were isolated using a commercially available mitochondrial/cytosol fractionating buffer system (BioVision). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad). Equivalent amounts of mitochondrial (20 μg) and cytosolic (50 μg) myocardial protein extracts were separated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Western blot analysis was performed using a polyclonal anti-mouse cytochrome c antibody (Santa Cruz Biotechnology Inc.) followed by a peroxidase-labeled secondary antibody. The antigen-antibody complexes were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech). Membranes were incubated at 70°C for 10 minutes in stripping buffer (10 mM Tris-HCl, pH 6.8, 2% SDS, 0.7% β-mercaptoethanol) then reprobed for GAPDH (Advanced Immunochemical) and COX IV (Molecular Probes) for loading normalization. Original films exposed to ECL (Hyperfilm, ECL; Amersham Pharmacia Biotech) were scanned on a Personal Densitometer S1 (Molecular Dynamics), and band density (measured in arbitrary units) was evaluated using ImageQuaNT 4.2a (Molecular Dynamics). Each sample value was normalized for loading errors and then
Mechanisms for cardiomyocyte apoptosis: extrinsic pathway

Caspase-8 activation. Caspase-8–like activity was measured exactly as described above for caspase-3, except that we used Ac-DEVD-afc as a fluorogenic substrate and Ac-IETD-CHO as the specific inhibitor (Biomol Research Laboratories).

Bid and IAPs. Cytosolic protein extracts were subjected to Western blotting using a polyclonal anti-mouse Bid antibody that detected both full-length and b-Bid and polyclonal anti-mouse c-IAP-1 and c-IAP-2 (all from Santa Cruz Biotechnology Inc.).

c-FLIP. Cytosolic protein extracts were subjected to Western blotting using polyclonal anti-mouse c-FLIP, and c-FLIP, antibodies (Kamiya Biomedical Co.). Total RNA was isolated from whole hearts using TRIzol (Reagent (Invitrogen)), and levels of c-FLIP, mRNA were determined by a denaturing gel protection assay system using a custom-designed probe, according to the manufacturer’s protocol (RiboQuant; BD Biosciences—Pharmingen) and described previously (33). Signals were quantified through the use of ImageQuaNT 4.2a (Molecular Dynamics Inc.) and were normalized to L32 expression.

Statistics

All data are expressed as mean ± SEM. Two-way ANOVA was used to evaluate the age- and mouse-dependent differences of LV dimensions, posterior wall thickness, and heart weight-to-body weight ratios. One-way ANOVA was used to test for mean differences in the prevalence of cardiomyocyte apoptosis, abundance of pro- and antiapoptotic proteins, and caspase-3–, caspase-8–, and caspase-9–like activities. Post-hoc testing (Student-Newman-Keuls method) was performed when appropriate. A P value of less than 0.05 was considered statistically significant.

Acknowledgments

We thank Thuy Pham and Claudia Aguillon for expert technical review. We thank Mark Entman for his critical review of the manuscript. This research was supported by research funds from the Veterans Administration and the NIH (P50 HL-06H, RO1 H1LS8081-01, RO1 HL61543-01, and HL-42250-15/15 to D.L. Mann; AG-17899 to G.E. Taffet).

Received for publication September 18, 2006, and accepted in revised form May 29, 2007.

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