Pro-apoptotic Bax is the major and Bak an auxiliary effector in cytokine deprivation-induced mast cell apoptosis

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The process of apoptosis in immune cells like mast cells is essential to regain homeostasis after an inflammatory response. The intrinsic pathway of apoptosis is ultimately controlled by the pro-apoptotic Bcl-2 family members Bax and Bak, which upon activation oligomerize to cause increased permeabilization of the mitochondria outer membrane leading to cell death. We examined the role of Bax and Bak in cytokine deprivation-induced apoptosis in mast cells using connective tissue-like mast cells and mucosal-like mast cells derived from bax−/−, bak−/− and bax−/− bak−/− mice. Although both Bax and Bak were expressed at readily detectable protein levels, we found a major role for Bax in mediating mast cell apoptosis induced by cytokine deprivation. We analyzed cell viability by propidium iodide exclusion and flow cytometry after deprivation of vital cytokines for each mast cell population. Upon cytokine withdrawal, bak−/− mast cells died at a similar rate as wild type, whereas bax−/− and bax−/− bak−/− mast cells were partially or completely resistant to apoptosis, respectively. The total resistance seen in bax−/− bak−/− mast cells is comparable with mast cells deficient of both pro-apoptic Bim and Puma or mast cells overexpressing anti-apoptotic Bcl-2. These results show that Bax has a predominant and Bak a minor role in cytokine deprivation-induced apoptosis in both connective tissue-like and mucosal-like mast cells.

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Apoptosis is a genetically programmed mechanism for induction of cell death. During an allergic inflammation, the number of inflammatory immune cells including mast cells increases at site. During the resolution phase and termination of the inflammation, the process of apoptotic cell death is critical to regain cellular and tissue homeostasis.1

The intrinsic pathway of apoptosis is activated by stimuli such as growth factor deprivation or cell stress, including DNA damage and is tightly regulated by interactions between pro- and anti-apoptotic members of the Bcl-2 family. The pro-apoptotic BH3-only proteins comprising Bim, Bad, Noxa and Puma are essential for initiation of apoptosis signaling. This group of proteins activates the pro-apoptotic effector proteins Bax and Bak indirectly by binding to the anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and Bfl-1/A1) and thereby titrate them away from Bax and Bak.2 Whether the activation of Bax and Bak occurs only indirectly is, however, actively debated. A model of direct binding and activation of Bax and Bak by the promiscuous BH3-only proteins Bid, Bim and possibly Puma has also been suggested.4–7 Activation of Bax and Bak causes permeabilization of the mitochondrial outer membrane, leading to release of cytochrome c, activation of caspase-9 and ultimately cell demolition.8

Activation of mast cells by aggregation of the high affinity receptor for IgE, FcεRI, is a key event in allergic reactions leading to degranulation and release of pro-inflammatory mediators. Connective tissue-like mast cells (CTLMC) but not mucosal-like mast cells (MLMC) withstand the degranulation process by activation-induced survival,9 a process in which the induction and function of the pro-survival protein A1 is crucial.10 In addition, other Bcl-2 family members such as the pro-survival protein Bcl-XL but also the pro-apoptotic protein Bim are upregulated by FcεRI aggregation in mast cells.11

Experiments with gene-targeted mice have shown that loss of either Bim11 or Puma12 renders mast cells partially resistant to cytokine deprivation-induced apoptosis. Experiments using RNAi-mediated knockdown of gene expression showed that the pro-survival Bcl-2 family member Mcl-1 is critical for...
sustained survival of transformed mast cells. In this study, we have examined the role of the two pro-apoptotic effector proteins Bax and Bak in mast cells, using cells deficient in Bax, Bak or both. We found that Bax has a more prominent role than Bak in cytokine deprivation-induced apoptosis in both CTLMC and MLMC.

Results

Characterization of in vitro-produced bax/bak mast cells. To characterize bax/bak mast cells and CTLMC, we examined their surface expression of Kit and the high-affinity IgE receptor FcεRI. Immunofluorescent staining with specific Abs and flow cytometric analysis showed that mast cell populations lacking Bax and Bak expressed levels of Kit and FcεRI similar to their wt counterpart (Figure 1a). We then compared the response of wt and bax/bak MLMC and CTLMC, respectively, to FcεRI crosslinking measuring degranulation using a β-hexosaminidase assay. In response to FcεRI cross-linking both wt and bax/bak MLMC and CTLMC, respectively, responded by degranulation to a similar magnitude (Figure 1b). These results show that the in vitro-produced cells resembled primary mast cells, as confirmed by toluidine blue staining of cytoplasmic granules (data not shown), expression of FcεRI and c-Kit. In these respects, wt mast cells and mast cells lacking Bax and Bak were indistinguishable.

Figure 1  (a) Expression of the receptors Kit and FcεRI on the cell surface of wt and bax/bak CTLMC and MLMC as examined by flow cytometry. The colors black and gray represents wt and bax/bak mast cells, respectively. A dashed line represents the staining with an isotype-matched control Ab, whereas the filled line represents staining with Abs for either Kit or FcεRI. β-hexosaminidase release upon FcεRI cross-linking of wt and bax/bak (dko) CTLMC and MLMC, respectively (b). One representative of two independent experiments is presented.
Expression of Bax and Bak in mast cells. Mast cells were differentiated from mouse embryonic fetal livers. After 4 weeks in culture, pure cell cultures of CTLMC were obtained. We then started by examining the protein and mRNA expression of the two effector proteins Bax and Bak. Analyzing mRNA expression of Bax and Bak in CTLMC by RNase protection assay (RPA) revealed a more abundant expression of bax compared with bak (Figure 2a). Moreover, loss of either bax or bak did not cause any compensatory alteration in the expression of the pro-survival genes bcl-w, a1, bcl-xL, or bcl-2 (Figure 2b). On protein level, both Bax and Bak were detected in wild-type mast cells, although the levels of Bak appeared to be lower than the levels of Bax. Expectedly, no Bax or Bak protein was seen in mast cells from respectively knock-out as well as from double-deficient mast cells (Figure 2c). Furthermore, we investigated if activation of mast cells through FcRI not only alters the expression of pro-survival proteins and BH3-only proteins, but also the effector proteins Bax and Bak. We could observe a minor upregulating effect on Bak but no prominent effect on Bax (Figure 2c), suggesting that IgE receptor activation primarily controls mast cell survival by regulating the levels and function of survival proteins and BH3-only proteins and not the effector proteins.

Loss of Bax protects mast cells from cytokine deprivation-induced apoptosis. Although both Bax and Bak have been shown to have largely overlapping functions in several cell types (e.g., lymphoid cells\textsuperscript{14,15}), it has also been reported that one of these proteins can have the dominant role. For example, Bax has the dominant role in programmed cell death during spermatogenesis\textsuperscript{16} and in NGF deprivation-induced killing of neuronal cells,\textsuperscript{17} whereas Bak is essential for cell death in platelets.\textsuperscript{18} To investigate whether both Bax and Bak are necessary for mast cell apoptosis we assessed cytokine deprivation-induced apoptosis in mast cells deficient in bax, bak or both (bax\textsuperscript{−/−}, bak\textsuperscript{−/−}) and wild type. After 36 h of starvation, more than 60% of bax\textsuperscript{−/−}, but less than 25% of the bak\textsuperscript{−/−} and wt CTLMC were viable. The same pattern was seen in MLMC although somewhat more rapid, with more than 60% viability of bax\textsuperscript{−/−} mast cells and less than 25% of the bak\textsuperscript{−/−} or wt already at 24 h (Figure 3a and b). Notably, only combined loss of both Bax and Bak conferred complete resistance to cytokine deprivation, which demonstrates that Bak does have a role in mast cell apoptosis, albeit a lesser one compared with Bax.

Discussion

In the present study we have analyzed the individual and combined roles of Bax and Bak in cytokine deprivation-induced apoptosis in two mast cell sub-populations, MLMC and CTLMC. We show that Bax has a more prominent role in mast cell apoptosis than Bak, because mast cells lacking Bax have an increased and prolonged survival compared with Bak-deficient mast cells. However, only combined loss of both Bak and Bax conferred complete resistance to cytokine deprivation to a comparable extent as Bcl-2 overexpression or combined loss of Puma and Bim (Figure 3c and d and Alfredsson et al.\textsuperscript{11} and Ekoff et al.\textsuperscript{12}). This shows that Bak does have a function in mast cell apoptosis induced by cytokine deprivation, albeit a lesser one compared with Bax. Both Bax and Bak proteins are expressed in mast cells,
however, the level of Bax is higher than Bak, which could be a plausible explanation for the increased survival in Bax-deficient mast cells when compared with those lacking Bak. In addition, the augmented survival of mast cells deficient in Bak may be an effect of redundancy between the two effector proteins.

MLMC and CTLMC overexpressing Bcl-2, or cells deficient in the two BH3-only proteins Bim and Puma, resemble Bak/Bax double-deficient mast cells in their profound resistance to cytokine deprivation-induced apoptosis. By affecting critical Bcl-2 family proteins in each of the three levels of the model of indirect activation of apoptosis (Figure 3 and Willis et al.3, Chen et al.19 and Willis et al.20) cytokine deprivation-induced apoptosis in mast cells can be inhibited. According to this model, pro-apoptotic BH3-only proteins activate Bak and Bax by neutralizing pro-survival proteins such as Bcl-2, and not by direct interaction between Bak/Bax and the BH3-only proteins (Figure 3e). Our previous findings together with the data presented here indicate that the two BH3-only proteins Bim and Puma are essential for cytokine deprivation-induced apoptosis in mast cells and that Bim and Puma together interact with and neutralize all pro-survival proteins involved. Furthermore, overexpressing Bcl-2 in mast cells is sufficient for profound resistance to cytokine deprivation-induced apoptosis, suggesting that Bcl-2 keeps both effector proteins in check. In mouse embryo fibroblasts, Bak is restrained by the pro-survival proteins Mcl-1 and Bcl-xL, but not Bcl-2,20 whereas Bax is probably inhibited by the pro-survival proteins Bcl-2, Bcl-w, Bcl-xL and Mcl-1.3 Our data about Bax having a more prominent role compared with Bak in cytokine deprivation-induced apoptosis in mast cells might conduce to explain why mast cells overexpressing Bcl-2 are totally resistant to apoptosis.

The data presented here are in accordance with a previous report in which IL-3 derived bone marrow mast cells from bax−/− mice were shown to have an increased survival upon cytokine deprivation.21 They also found an increase in mast cell numbers in the stomach mucosa and a minor increase of mast cells in back skin of bax−/− mice compared with wild-type mice. This indicates that for mast cell homeostasis, Bax has a more prominent role in mucosal mast cells compared with connective tissue mast cells. By contrast, our in vitro data reveal no difference in the importance of Bax for the induction of apoptosis in MLMC and CTLMC, suggesting other additional mechanisms in vivo.

Mast cells are implicated in a number of different diseases where reducing their numbers would be beneficial for reducing the severity of the symptoms, and in the best scenario contribute to cure the disease. These diseases include mast cell-driven diseases like mastocytosis22 and allergic reactions, but also chronic inflammations such as atherosclerosis and autoimmune diseases23–25 and tumors.26

In summary, in this paper we have analyzed the roles of the two pro-apoptotic proteins Bak and Bax in cytokine deprivation-induced apoptosis of CTLMC and MLMC. We report that Bax has a more prominent role than Bak in this process; however, both are needed because only bax−/−, bak−/− cells were totally resistant to cytokine deprivation-induced apoptosis. Our results contribute to the work of deciphering the role of the Bcl-2 family members in the regulation of mast cell survival and apoptosis and have implications for designing new therapeutic protocols for treatment of mast cell-associated disorders.

Materials and Methods

Mice and cell cultures. The generation and genotyping of bax−/−, bak−/−, bax−/−bak−/− and bim−/−puma−/− as well as vav-bcl-2-transgenic mice have been described previously.14,16 Fetal liver-derived mast cells from wt C57BL/6 mice or mice of bax−/−, bak−/− and bax−/−bak−/− were differentiated into CTLMC or MLMC using previously described protocols.5 Briefly, fetal livers from E14 mouse embryos were dissected and kept on ice in KDS BSS (10% PBS). Livers were homogenized, cells washed in PBS and cultured at 3 × 10^5 cells per ml under conditions promoting the development of CTLMC (RPMI-1640 supplemented with 25 ng/ml rSCF and 1 ng/ml rIL-4 (Peprotech EC Ltd, Rocky Hill, NJ, USA)) or MLMC (DMEM supplemented with 25 ng/ml rSCF and 100 U/mI IL-3 (cell supernatant from X630 hybromia stably transfected with an IL-3 expression vector), 5 ng/ml rIL-9 and 1 ng/ml rTGF-β1 (both from R&D systems, Minneapolis, MN, USA) respectively. All cells were cultured 4 weeks before being used. Similarly, bone marrow cells from wild-type, vav-bcl-2-transgenic and bim−/−puma−/− were differentiated into CTLMC and MLMC using protocols described.8 All experiments with animals were performed according to the guidelines of the Royal Melbourne Hospital Research Foundation Animal Ethics Committee or the Animal Ethics Committee in Stockholm.

Detection of Kit and FcεRI on mast cell surface. The cells were examined by flow cytometric analysis for expression of Kit and FcεRI, using FITC-anti-mouse CD117 (Kit) mAb 2B8 or FITC-conjugated rat IgG2b isotype control (both from BD Pharmingen, San Diego, CA, USA), FITC-conjugated anti-mouse FcεRI mAb MAR-1, or FITC-conjugated Armenian hamster IgG isotype control (both from eBioscience, Hatfield, UK).

In vitro cell activation by IgE receptor crosslinking. CTLMCs and MLMCs were suspended at 5 × 10^5 cells per ml. For FcεRI stimulation, mast cells were sensitized for 90 min using a monoclonal murine IgE anti-TNP antibody (IgE1-b4, ATCC), supplied as a 15% hybridoma supernatant. The cells were washed twice in PBS and then challenged with 10 ng/ml TNP-BSA (coupling ratio 9, Biosearch Technologies Inc, San Francisco, CA, USA) for the time periods indicated. Cell viability was determined by propidium iodide exclusion (5 μg/ml, Sigma-Aldrich, Steinheim, Germany) and flow cytometric analysis using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).9

N-acetyl-d-p-hexosaminidase release assay. Cells to be used in the N-acetyl-d-p-hexosaminidase assay were resuspended in RPMI 1640 medium supplemented with 0.2% BSA (Sigma-Aldrich) before the cells were activated by IgE receptor crosslinking. For detection of the granular enzyme β-hexosaminidase, an enzymatic colorimetric assay was used as described previously.10 Briefly, 80 μl of supernatant was transferred to a 96-well plate and mixed with an equal volume of substrate solution (7.5 mM p-nitrophényl-N-acetyl-d-p-glucosamind dissolved in 80 mM citric acid, pH 4.5). The mixture was incubated on a rocker platform for 2 h at 37 °C. After incubation, 120 μl of glycine (0.2 M, pH 10.7) was added to each well, and the absorbance at 405 and 490 nm was measured using an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis. CTLMCs (1.5 × 10^6 cells) were activated by IgE receptor cross-linking and harvested after 5 h, washed in ice-cold PBS, and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche, Mannheim, Germany). Lysates from mouse embryonic fibroblasts (MEF) were included as control for expression of Bax and Bak. Protein, 40 μg, was dissolved in SDS loading buffer and size-fractionated on 12% Tris-glycine gels (Invitrogen, Carlsbad, CA, USA). Western blotting was performed using polyclonal rabbit anti-Bak antibodies (1 : 1000; Sigma-Aldrich) or a monoclonal mouse anti-Bax antibody (1 : 1000; Sigma-Aldrich), followed by horseradish peroxidase-conjugated sheep anti-rabbit IgG or sheep anti-mouse IgG antibodies (1 : 5000; Chemicon, Temecula, CA, USA). Bound antibodies were visualized by enhanced chemiluminescence (ECL) and exposure to Hyper ECL film (Amersham Biosciences, Uppsala, Sweden).
Conflict of interest

The authors declare no conflict of interest.

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1. Serhan CN, Savil J. Resolution of inflammation: the beginning programs the end. Nat Immunol 2005; 6: 1191–1197.
2. Zhivotovsky B, Kroemer G. Apoptosis and genomic instability. Nat Rev Mol Cell Biol 2004; 5: 752–762.
3. Willis SN, Fletcher JJ, Kaufmann T, van Deft MF, Chen L, Czabatar PE et al. Apoptosis initiated when B1H3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 2007; 315: 856–859.
4. Cartron PF, Galienne T, Bougras G, Gautier F, Manero F, Vusio P et al. The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the B3H-only proteins Bid and PUMA. Mol Cell 2004; 16: 807–818.
5. Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, Katz SG et al. BAX activation is initiated at a novel interaction site. Nature 2008; 455: 1076–1081.
6. Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol 2006; 8: 1348–1358.
7. Walensky LD, Pitter K, Morash J, Oh KJ, Barbuto S, Fisher J et al. A stapled BID BH3 helix directly binds and activates BAX. Mol Cell 2006; 24: 199–210.
8. Newmeyer DD, Ferguson-Miller S. Mitochondria: releasing power for life and unleashing the machineries of death. Cell 2003; 112: 481–490.
9. Ekoff M, Strasser A, Nilsson G. FcepsilonRI aggregation promotes survival of connective tissue-like mast cells but not mucosal-like mast cells. J Immunol 2007; 178: 4177–4183.
10. Xiang Z, Ahmed AA, Möller C, Nakayama K-I, Hatakeyama S, Nilsson G. Essential role of the pro-survival bc-2 homolog A1 in mast cell survival following allergic activation. J Exp Med 2001; 194: 1561–1569.
11. Alfredsson J, Puthalakath H, Martin H, Strasser A, Nilsson G. Proapoptotic Bcl-2 family member Bim is involved in the control of mast cell survival and is induced together with Bcl-XL upon IgE-receptor activation. Cell Death Differ 2005; 12: 136–144.
12. Ekoff M, Kaufmann T, Engstrom M, Motoyama N, Villunger A, Jonsson J et al. The B3H-only protein Puma plays an essential role in cytokine deprivation-induced apoptosis of mast cells. Blood 2007; 110: 3209–3217.
13. Alfredsson J, Mayerhofer M, Greiner KV, Krauth MT, Grue A, Pickl WF et al. Identification of MCL1 as a novel target in neoplastic mast cells in systemic mastocytosis: inhibition of mast cell survival by MCL1 antisense oligonucleotides and synergism with PKC412. Blood 2007; 109: 3031–3041.
14. Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shels HS et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 2000; 6: 1389–1399.
15. Rathmell JC, Lindsten T, Zong WX, Cinnili RM, Thompson CB. Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. Nat Immunol 2002; 3: 932–939.
16. Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 1995; 270: 96–99.
17. Putcha GV, Deshmukh M, Johnson Jr EM, BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. J Neurosci Res 1999; 19: 7476–7485.
18. Mason KD, Carpinielli MR, Fletcher JJ, Collinge JE, Hilton AA, Ellis S et al. Programmed anuclear cell death delimits platelet life span. Cell 2007; 128: 1173–1186.
19. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JJ, Hinds MG et al. Differential targeting of prosurvival Bcl-2 proteins by their B3H-only ligands allows complementary apoptotic function. Mol Cell 2005; 17: 393–403.
20. Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JJ et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xl, but not Bcl-2, until displaced by B3H-only proteins. Genes Dev 2005; 19: 1204–1205.
21. Maurer M, Tsai M, Metz M, Fish S, Korsmeyer SJ, Galli SJ. A role for Bax in the regulation of apoptosis in mouse mast cells. J Invest Dermatol 2000; 114: 1205–1206.
22. Metcalfe DD. Mast cells and mastocytosis. Blood 2008; 112: 946–956.
23. Leslie M. Mast cells show their might. Science 2007; 317: 614–616.
24. Benoist C, Mathis D. Mast cells in autoimmune disease. Nature 2002; 420: 875–878.
25. Sun J, Sukhova GK, Wolters PJ, Yang M, Kitamoto S, Libby P et al. Mast cells promote atherosclerosis by releasing proinflammatory cytokines. Nat Med 2007; 13: 719–724.
26. Soucek L, Lawlor ER, Soto D, Shochrs K, Swigart LB, Evan GI. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. Nat Med 2007; 13: 1211–1218.
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