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News and Commentary

Granulocyte apoptosis: death by a secreted lipocalin?

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Granulocytes are terminally differentiated cells generated in the bone marrow under the influence of various cytokines. They are important components of the natural defense system. Under normal circumstances, granulocytes have a very short life span that does not exceed more than 2–3 days. One mechanism to accumulate granulocytes at sites of inflammation is delayed apoptosis, which is at least partially mediated by overexpression of cytokines.[1] In many chronic inflammatory responses, such as lung fibrosis or bronchial asthma, granulocytes (neutrophils and/or eosinophils) cause unwanted tissue damage via the release of toxic mediators. Clearly, induction of granulocyte apoptosis would help to limit tissue damage and to reduce inflammation in these cases. Therefore, investigating the molecular control of granulocyte apoptosis under normal and inflammatory conditions may lead to the identification of new therapeutic drug targets.

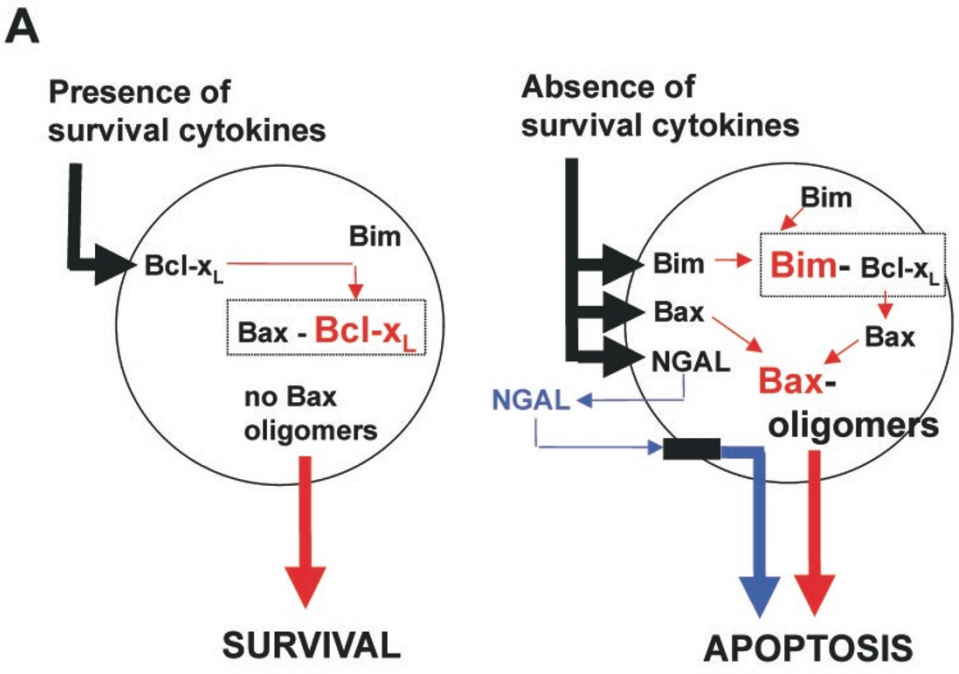
What is the mechanism ultimately responsible for the induction of apoptosis in granulocytes? Several laboratories tried to approach this question by comparing the expression and function of apoptosis-regulating proteins in granulocytes under conditions of cytokine withdrawal and survival factor exposure. These studies revealed that both neutrophils[2] and eosinophils[3] decrease the functional activity of proapoptotic Bax in the presence of survival factors, suggesting that this member of the Bcl-2 family plays a key role in granulocyte apoptosis. Moreover, cytokine withdrawal induced expression of Bax in neutrophils (Figure 1A).[2] Since overexpression of Bax has been shown to induce apoptosis,[4] it is possible that a single granulocyte activates its intracellular death machinery as soon as a certain threshold concentration of Bax is reached.

Besides Bax, another pro-apoptotic member of the Bcl-2 family, Bim, has recently been implicated in apoptosis mediated by growth factor withdrawal in a mouse IL-3 dependent cell line as well as in primary mouse fetal liver cells.[5] Whether Bim plays a role in the induction of spontaneous apoptosis of granulocytes remains to be investigated. Granulocytes may increase Bim levels in the absence of survival factors leading to the neutralization of pro-survival members of the Bcl-2 family (Figure 1A).[5] Interestingly, both Bim[5] and Fas ligand[6] are under the control of forkhead transcription factor FKHR-L1. However, there is only little evidence to suggest that Fas ligand is upregulated in granulocytes cultured in the absence of survival factors *in vitro*.[7]

The presence of Fas ligand and Fas receptors on the surface of granulocytes still implied the possibility that granulocytes initiate a death pathway at the cell surface.[8] This idea was supported by observations made in T cells. Activated T cells express high levels of functional Fas ligand and Fas receptor (CD95) leading to the induction of apoptosis in an autocrine or paracrine manner,[9] at least in the absence of T cell survival factors.[10] Therefore, molecular Fas ligand/Fas receptor interactions have also been implicated as initial events responsible for the so-called 'spontaneous' apoptosis in these cells.[8,11] However, recent work suggests that this hypothesis must be rejected in the granulocyte system.[12–14] Nevertheless, although Fas receptor activation does not appear to be required for spontaneous apoptosis, it is associated with additional induction of neutrophil[15] and eosinophil[16] apoptosis both in the presence and absence of survival cytokines *in vitro*. The nature of Fas ligand/Fas receptor molecular interactions affecting granulocyte apoptosis *in vivo* remains to be determined.

A new potential mechanism responsible for the initiation of apoptosis in granulocytes has recently been suggested by LR Devireddy *et al*.[17] By using DNA microarrays, they searched for death-promoting genes that are transcriptionally activated in IL-3-deprived mouse pro-B lymphocytic FL5.12 cells. Eight hours following IL-3 withdrawal, they observed a 12.6-fold increase in 24p3, a gene which belongs the lipocalin family.[18] By using Northern blot analysis, 24p3 gene expression was detected within 2 h following IL-3 withdrawal in FL5.12 cells, mouse 32D cells and mouse primary bone marrow cells.[17] Interestingly, conditioned medium of IL-3-deprived FL5.12 and bone marrow cells induced apoptosis in a variety of primary cells including neutrophils (Figure 1A). That 24p3 protein is able to induce apoptosis in target cells was demonstrated by several means: (1) Overexpression of 24p3 induced apoptosis in COS-7 monkey kidney and FL5.12 cells. (2) Recombinant 24p3 induced apoptosis in FL5.12 and bone marrow cells. (3) 24p3 phosphorothioate antisense oligonucleotides prevented death of FL5.12 cells after IL-3 withdrawal. (4) Neutralization of 24p3 protein using an anti-24p53 antibody blocked apoptosis of IL-3-deprived bone marrow cells. Taken together, these data suggested that 24p3 protein is a death factor, which is generated by cytokine-dependent cells following growth factor withdrawal.

The identification of a released cellular product that can induce apoptosis in susceptible cells including neutrophils implies that this mechanism may also play an important role in spontaneous granulocyte apoptosis. Immature neutrophils have been shown to generate Neutrophil Gelatinase-Associated Lipocalin (NGAL),[19] the likely



B

	1	11	21	31
24p3	MALSVMCLGL	ALLGVLQSQA	QDSTQNLIPA	PSLLTVPLQP
NGAL	MPLGLLWLPS	- LLGALHAQA	QDSTSDLIPA	PPLSKVPLQQ

	41	51	61	71
24p3	DFRSDQFRGR	WYVVGLAGNA	VQKKTEGSFT	MYSTIYELQE
NGAL	NFQDNQFQGK	WYVVGLAGNA	I LREDKDPQK	MYATIYELKE

	81	91	101	111
24p3	NNSYNVTSI L	VRDQDQGCRY	WIRTFVPSSR	AGQFTLGNMH
NGAL	DKSYNVTSVL	FRKK - - KCDY	WIRTFVPGCQ	PGEFTLGNI K

	121	131	141	151
24p3	RYPQVQSYNV	QVATTDYNQF	AMVFFRKTSE	NKQYFKITLY
NGAL	SYPGLT SYLV	BVVSTMYNQH	AMVFFKKVSQ	NREYFKITLY

	161	171	181	191
24p3	GRTKELSPEL	KERFTRFAKS	LGLKDDNI IF	SVPTDQCIDN
NGAL	GRTKELTSEL	KENFI RFSKS	LGLPENHIVF	PVPI DQCIDG

Figure 1 (**A**) Hypothetical scheme showing potential key events in granulocyte apoptosis induced by survival factor withdrawal. Granulocytes have been demonstrated to express members of the Bcl-2 family. The pathways involving different Bcl-2 family members are indicated in red. In the presence of survival factors (left panel), Bcl-x_L and Mcl-1 (not shown) expression is maintained or even upregulated and may prevent Bax oligomerization and subsequent insertion into the outer membrane of mitochondria. Therefore, cytochrome *c* release, caspase activation and apoptosis do not occur. Absence of survival factors (right panel) may also induce gene expression in granulocytes. Increases in Bax levels have been demonstrated in neutrophils. Moreover, increased Bim levels have been observed in cytokine dependent cell lines. Bim is believed to be associated with the microtubular motor complex as long as sufficient survival signals are transduced into the cell. In the absence of survival factors, however, Bim is released into the cytoplasma and may neutralize anti-apoptotic members of the Bcl-2 family such as Bcl-x_L (or Mcl-1). Consequently, increasing amounts of Bax molecules get free, can associate, and create pores into the outer membrane of mitochondria to release cytochrome c and other pro-apoptotic factors. In addition to Bax and Bim, induction of the mouse NGAL homolog (24p53) has been seen in cytokine dependent cell lines and in primary bone marrow cells following survival factor withdrawal. 24p53 is secreted and can induce apoptosis in human neutrophils (blue). The 24p53 receptor and its signal transduction pathways remain to be investigated. (**B**) Comparison of mouse 24p53 and human NGAL predicted protein sequences. The sequences were aligned by computer analysis with gaps (indicated by dashes) inserted to maximize identity. Capital letters in red denote identity. Conserved cysteine residues are boxed. The sequence data were obtained from EMBL/GenBank Data Libraries. The accession numbers are as follows: X81627 for 24p53 and P80188 for NGAL

human homolog of 24p53 (Figure 1B). Expression studies in mature neutrophils following *in vitro* culture in the presence and absence of survival factors have not been reported so far. Nevertheless, NGAL is a new candidate, which might largely be involved in the induction of apoptosis via an unknown cell surface receptor in mature granulocytes (Figure 1A). Clearly, the identification of this receptor is a prerequisite for the identification of the death pathway initiated by NGAL.

The fact that 24p53 also induced apoptosis in lymphocytes[17] suggests that this lipocalin might be involved in the homeostasis of leukocytes in general. Perhaps, 24p53/NGAL-mediated apoptosis plays an equally important role as Fas ligand/Fas receptor molecular interactions in the regulation of the immune system. For example, similar to the Fas system, overexpression of 24p53/NGAL has been reported on immunoprivileged sites,[20] supporting its potential role in limiting inflammatory responses.

Although the current data on 24p53 are interesting, much more work has to be performed to clarify its role in granulocyte apoptosis. So far we just know that mouse 24p53 induced apoptosis in human blood neutrophils. The following immediate questions need to be answered: (1) Is it possible to delay granulocyte apoptosis by adding neutralizing anti-24p53/NGAL antibody or phosphorothioate antisense oligonucleotides *in vitro*? (2) Is 24p53/NGAL expressed in granulocytes and how is the kinetic during culture in the presence and absence of survival factors? (3) What is the receptor of 24p53/NGAL and how is it linked to caspase activation? When we have answered these questions, we will be better able to estimate the potential role of 24p53/NGAL as an initiator of spontaneous granulocyte apoptosis.

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