deregulation, such as mutational inactivation of SOC1, should also be investigated.

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BAG3 protein regulates stress-induced apoptosis in normal and neoplastic leukocytes

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TO THE EDITOR

Co-chaperone proteins that share the Bcl-2-associated athanogene (BAG) domain are characterized by their interaction with a variety of partners, such as heat shock proteins (Hsp), steroid hormone receptors, Bcl-2, Raf-1 and others, involved in regulating protein folding and a number of cellular processes, including proliferation and apoptosis.[1–2] Among BAG family members there is BAG3, also known as CAIR-1 or Bis.[2–6] BAG3 forms a complex with Hsp70,[1,2,4,6] a protein able to modulate apoptosis by interfering with cytochrome *c* release, apoptosome assembly and other events in the death process.[7–15] In addition, BAG3 polypeptide binds to phospholipase C-γ (PLC-γ)[4] or Bcl-2 protein.[3,5] Due to such interaction with more than one apoptosis-modulating factor, BAG3 can participate in apoptosis regulation. Indeed, its hyperexpression can decrease apoptosis induced via Bax or Fas in the human epithelial cell line HeLa[3] or by IL-3 deprivation in the murine hematopoietic cell line 32D.[5] Furthermore, we recently showed that BAG3 downmodulation enhances the apoptotic response to chemotherapy in human primary B chronic lymphocytic leukemia cells.[16]

Oxidative stress can induce apoptosis by the effect of reactive oxygen species (ROS) on the permeability of the mitochondrial membrane, with subsequent release of cytochrome *c*, formation of the apoptosome complex and activation of caspases.[9,17] The activation of p53 and stress kinases also contributes to the apoptotic response.[17] A vast series of antineoplastic compounds generate stress signals, evoking apoptotic responses in neoplastic cells.[18] Owing to its interaction with Hsp70,[1,2,4,6] BAG3 could interfere at several levels in this cascade of events, possibly modulating the death process.

We investigated whether BAG3 levels could influence the apoptotic response of human leukocytes to oxidative stress. For this purpose, we first analyzed the apoptotic response of the human myeloid cells U937 to the glutathione- depriving agent diethylmaleate (DEM).[19] To interfere with BAG3 protein synthesis, we used BAG3-specific antisense phosphorothioate oligodeoxynucleotides (ODN) (TGCATCATGGGCGAGTGGG-TGGCGG, antisense 1; GCTCATGCTGGGTTGGGGTCTG, antisense 2; ATTAAAGGCGGGGGTGACGTGG, antisense 3; TGCATCATGGGCGAGTGGGTGGC, antisense 4);[16] control nonsense (TTATATTCTATTATATTTATGAACTCC, nonsense 1; CCTCGTAACCACCGACCTCAAT, nonsense 2; GCTTATGGAG-GATTGAGGTTGG, nonsense 3; GCTTATGGAGGATTGA-GGTTGGG, nonsense 4) ODN were used as controls.[16] As shown in Figure 1, addition of the antisense, but not control nonsense, ODN appreciably downmodulated BAG3 protein levels. On the other hand, ODN did not appear to influence the levels of the unrelated protein Bcl-2 (Figure 1). The ODN were added to cultures of U937 cells, in the presence or absence of DEM, for 20 h and apoptosis was analyzed. DEM-stimulated cells showed $<36\%$ of apoptosis; a similar percentage was observed in cultures with control ODN, while apoptotic cells were $>60\%$ in cultures with BAG3-antisense ODN (Figure 2).

Similar results were obtained in experiments with primary peripheral blood mononuclear cells (PBMC) from normal donors. As verified by intracellular immunofluorescence, the addition of antisense, but not control nonsense, ODN reduced by $>50\%$ the mean intensity of fluorescence observed with BAG3-specific antibody. As a control, the intensities of fluorescence obtained with anti-α-tubulin antibody were comparable in cultures with or without ODN. When PBMC were cultured with DEM, alone or in combination with ODN, for 24 h, apoptosis induced by DEM was increased (from 20% up to 37%) by the addition of antisense, but not control, oligos (Figure 3). Similar results were obtained in experiments with

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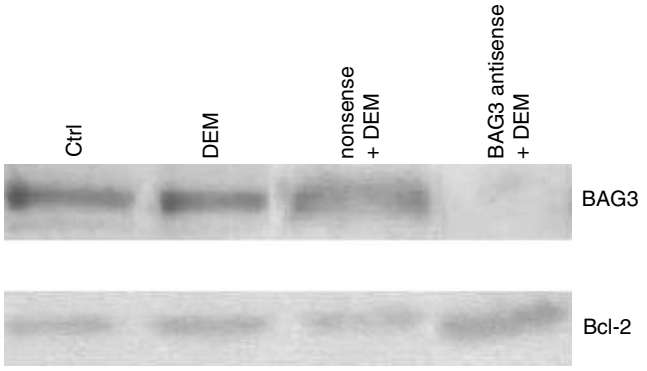

Figure 1 Effect of BAG3-specific antisense oligodeoxynucleotides on BAG3 protein levels in U937 cells. U937 cells were plated at a density of 5×10^5/ml and cultured for 15 h without or with DEM (Sigma, St Louis, MO, USA) (1.2 mM) and/or BAG3 antisense or control nonsense phosphorothioate ODN[16] (5 μM). Then, cell extracts were obtained and 50 μg of protein were analyzed in Western blot with the indicated antibodies.[16,20] Similar results were obtained with any of the antisense or nonsense ODN used.

PBMC from at least three different donors. Therefore, BAG3 downmodulation appeared to enhance DEM-induced apoptosis in either U937 cells or PBMC.

These findings indicate for the first time that apoptosis induced by oxidative stress is regulated by BAG3 protein. Taken together with our previous results, concerning an enhancement of fludarabine-induced B-CLL apoptosis by BAG3 antisense ODN,[16] these data establish an antiapoptotic role of BAG3 in human leukocytes. The present evidence provide two novel pieces of information. First, BAG3 regulates the apoptotic response not only to chemotherapy,[16] but also to oxidative stress, supporting the relation between the two pathways.[18] Second, it regulates cell survival not only in neoplastic,[16] but also in normal leukocytes (PBMC). In this respect, the role of BAG3 in the apoptosis of normal and neoplastic hematopoietic progenitors deserves investigation, to evaluate the potential effects of BAG3-targeting antineoplastic therapies.

Modulation of BAG3 protein levels does not affect basal cell survival, but instead response to a pro-apoptotic stimulus (Figure 2 and Romano *et al*.[16]), hence interfering with molecular events subsequent to apoptosis triggering. BAG3 interacts with Hsp70,[1,2,4,6] a protein able to bind apoptosome components and other elements of the apoptotic cascade,[7–15] strongly suggesting its positive cooperation with Hsp70 in apoptosis downmodulation. Notably, like Hsp70, BAG3 gene expression is stimulated by stress,[20] appearing to be part of a homeostatic loop that controls cell survival in response to stress stimuli.

Besides Hsp70, other potential partners of BAG3 in apoptosis modulation have to be considered. Indeed, BAG domain-carrying proteins can interact with various heat shock proteins, some of which, like Hsp90, Hsp60 and Hsp27, have been reported to interfere with apoptosis events.[15] Furthermore, different proteins, including enzymes, such as phospholipase C, involved in apoptosis control, the antiapoptotic Bcl-2 protein or signal transducers, like Raf, have been reported to bind BAG family members.[1–2] In particular, BAG3 carries a proline-rich domain, able to interact with SH3-type structures[1,6]. Finally, the BAG3/Hsp70 complex has recently been shown to participate in the proteasome – mediated degradation of intracellular proteins.[21] It is therefore likely that BAG3 protein can influence the activity of more than one factor involved in the apoptotic

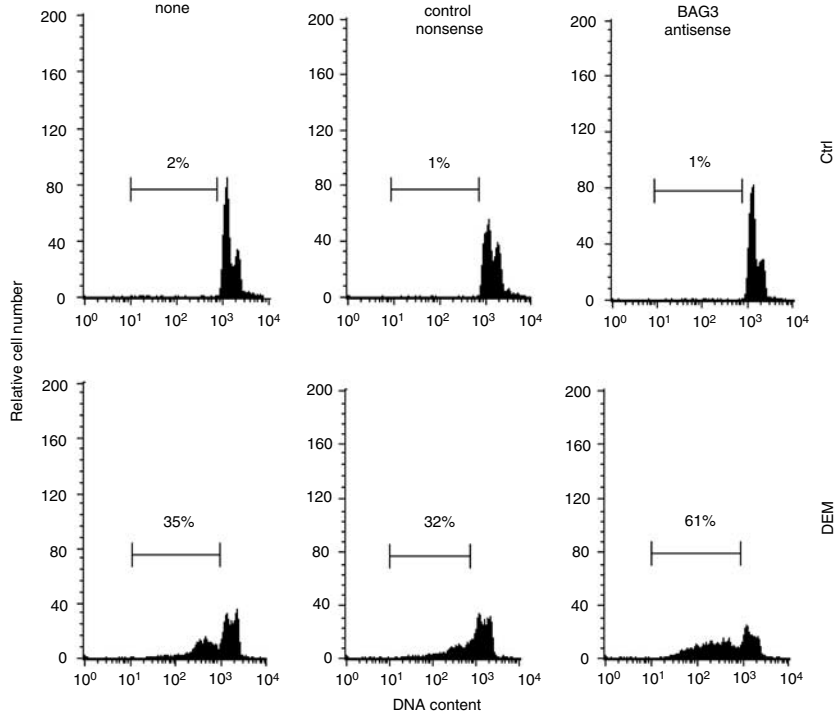

Figure 2 Effect of anti-BAG3 antisense oligodeoxynucleotides on DEM-induced apoptosis in U937 cells. U937 cells were cultured for 20 h with or without BAG3 antisense or control nonsense phosphorothioate oligodeoxynucleotides (5 μM) and treated with DEM (1.2 mM). Then cell apoptosis was analyzed by cell permeabilization and PI staining.[16] Similar results were obtained in atleast four different experiments and with any of the antisense or nonsense ODN used.

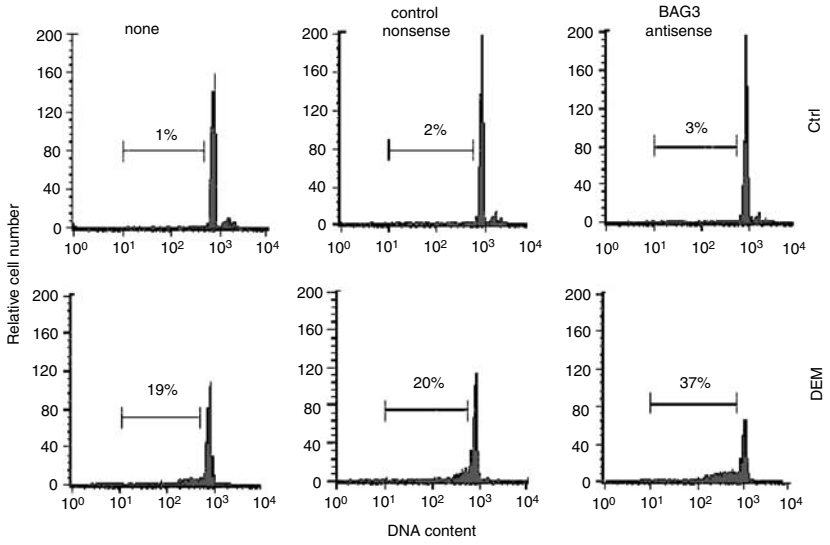

Figure 3 Effect of anti-BAG3 antisense oligodeoxynucleotides on BAG3 protein levels in normal human peripheral blood leukocytes. Human PBMC were obtained from normal donors' heparinized samples by centrifugation through a Ficoll–Hypaque (ICN Flow, Opera, Italy) density gradient at 400 *g* for 30 min. PBMC (1×10^6/ml) were cultured in 10% FCS-RPMI with DEM (1.2 mM) and/or the BAG3 antisense or control nonsense phosphorothioate ODN (5 μM) for 24 h. Then, cell apoptosis was analyzed by cell permeabilization and PI staining. Similar results were obtained in atleast three different experiments and with any of the antisense or nonsense ODN used.

process, and might in this respect constitute to the cell a versatile tool in contrasting death signals.

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