Mechanism of action of interleukin-2 (IL-2)–Bax, an apoptosis-inducing chimaeric protein targeted against cells expressing the IL-2 receptor

Rami AQEILAN, Rotem KEDAR, Ahmi BEN-YEHUDAH and Haya LORBERBOUM-GALSKI

Department of Cellular Biochemistry and Human Genetics, Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

The chimaeric protein interleukin-2 (IL-2)–Bax was designed to target and kill specific cell populations expressing the IL-2 receptor. However, it is not well understood how IL-2–Bax causes target cells to die. In the present study, we investigated the pathway of apoptosis evoked by IL-2–Bax and the possible involvement of endogenous Bax in this process. We report here that, upon internalization of IL-2–Bax into target cells, it is localized first mainly in the nucleus, and only later is it translocated to the mitochondria. Similarly, endogenous Bax is also partially localized in the nucleus, and accumulates mainly in this compartment soon after physiological triggering of apoptosis. Despite the fact that Bax has no nuclear localization sequence, our data suggest that Bax has one or more physiological roles and/or substrates within the nucleus. Indeed, a dramatic repression of nuclear Tax protein expression was induced following treatment of HUT-102 cells with IL-2–Bax, similar to what occurs following serum deprivation of these cells. Unexpectedly, induction of apoptosis using IL-2–Bax was preceded by enhanced expression of newly synthesized Bax protein and suppression of Bcl-2. This imbalance between the pro- and anti-apoptotic genes was associated with p53 induction, although IL-2–Bax activity was also evident in cells lacking p53 expression. By studying the mechanism of action of IL-2–Bax, we were able to follow the intrinsic events and their cascade that culminates in cell death. We have shown that the ability of IL-2–Bax to affect the intracellular apoptotic machinery within the target cells, and to cause the cells to die, uses a mechanism similar to that induced following a normal apoptotic signal.

Key words: apoptosis, Bax, chimaera, interleukin-2 receptor, nucleus, targeted therapy.

INTRODUCTION

The development of specific and selective targeted agents remains a major goal in the treatment of human diseases. In recent years, chimaeric proteins have been developed, by gene fusion techniques, to selectively recognize and kill cell populations expressing specific surface molecules, while not harming neighbouring healthy cells. Generally, these molecules carry a bacterial or plant toxin that destroys the unwanted cells. In previous studies it was shown that chimaeric proteins in which interleukin-2 (IL-2) serves as a targeting moiety could eliminate activated lymphocytes [1–3]. IL-2-based chimaeric proteins recognize the high-affinity IL-2 receptor (IL-2R) that is normally not expressed on resting lymphocytes. One example is IL-2–PE40 (where PE is Pseudomonas exotoxin A), which has shown therapeutic potential in many in vivo models. In addition, the a chain of the IL-2R has been successfully targeted by the murine or humanized anti-Tac antibody in cancer patients and in patients after transplantation [4,5]. Another chimaeric protein, DAB389–IL-2 (where DAB is diphtheria toxin), has been approved for the treatment of patients with cutaneous T-cell lymphoma [6,7].

The major obstacle in the clinical application of such chimaeric proteins is their immunogenicity (mainly towards the toxin moiety) and non-specific toxicity, as they carry bacterial toxins such as Pseudomonas exotoxin or Diphtheria toxin. In order to prevent immunological responses that are raised mainly towards the bacterial toxin component, we suggested taking advantage of the human pro-apoptotic protein Bax as a novel killing moiety, since it is of human origin.

Bax is a central protein in the apoptotic cascade, and has been shown to promote cell death in different pathways. It co-immunoprecipitates with Bcl-2 from various cell lines [8], Bax can heterodimerize with Bcl-2, and the ratio of Bcl-2 to Bax determines susceptibility to cell death following an apoptotic stimulus [8,9]. However, it was also found that Bax is able to regulate apoptosis independently of Bcl-2 [10,11]. Bax can form ion channels in lipid membranes under neutral/physiological conditions [12,13] and thus may exert its pro-apoptotic activity by deranging the ionic homoeostasis. The mitochondrial localization of the Bax protein seems to be essential to its function, as it was demonstrated that Bax moves from the cytosol to the mitochondria during apoptosis [14,15] and promotes cell death by triggering the release of cytochrome c from the mitochondria [16–18]. Recent work has demonstrated that the Bax protein, similar to other members of the Bcl-2 family, undergoes post-translational modifications that alter its function [19]. One such modification is proteolytic cleavage. N-terminal cleavage of the Bax protein generates a potent pro-apoptotic 18 kDa fragment, which enhances its cell death function [20,21].

As the first prototype of an apoptosis-inducing chimaeric protein, we utilized IL-2 as the targeting moiety and the Bax protein as the killing component. This chimaeric protein, termed IL-2–Bax, specifically targets IL-2R-expressing cells and induces cell-specific apoptosis within target cells. However, it is not well understood how IL-2–Bax induces apoptosis within IL-2R-bearing cells [22].

Here we investigated the mechanism of IL-2–Bax-mediated apoptosis in target T cells, and compared it with that of endogenous Bax protein. We found that, upon internalization of IL-2–Bax into target cells, most of the internalized chimaera appears first in the nucleus, and only later is translocated to the

Abbreviations used: DAPI, 4,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HTLV-I, human T-cell leukaemia virus type I; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; PARP, poly(ADP-ribose) polymerase; PHA, phytochaemagglutinin; RT-PCR, reverse transcription–PCR.

1 To whom correspondence should be addressed (e-mail hayalg@md2.huji.ac.il).
mitochondria. Similarly, endogenous Bax, which is known as a cytoplasmic protein, is also localized both in the nucleus and in the cytoplasm, accumulating mainly in the nucleus shortly after physiological triggering of apoptosis. Another major finding emerging from the present study is that, following internalization of IL-2–Bax into target cells, enhanced activation of intracellular early-responding pro-apoptotic proteins (such as Bax) and suppression of anti-apoptotic proteins (such as Bcl-2) occurs, leading to cell death. We also found that IL-2–Bax activity involves activation of p53 in certain cell types, although the chimaera was also able to induce cell-specific apoptosis in cells lacking p53 expression, as well as in those harbouring mutant Bax. Apoptosis mediated by IL-2–Bax in HUT-102 cells was also accompanied by repression of Tax, similar to what occurs upon serum starvation. A chimaeric protein such IL-2–Bax is a physiological means of delivering large amounts of Bax protein into specific target cells. As IL-2–Bax behaves like naturally occurring Bax, then theoretically chimaeric porteins such as IL-2–Bax can assist us in studying intracellular proteins and their involvement in cellular events such as apoptosis.

MATERIALS AND METHODS

Cell lines and cell culture

Human T-cell lymphoma HUT-102 cells were kindly provided by Tom Waldman (NIH, Bethesda, MD, U.S.A.); human T-cell leukaemia Jurkat cells were kindly provided by Hanna Ben-Bassat (Hadassah Hospital, Jerusalem, Israel), and mouse T-cell lymphoma 2B4 cells were kindly provided by Rina Guy (The Hebrew University, Jerusalem, Israel). Both HUT-102 and 2B4 cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 5 mM Hepes buffer solution and 10 µM β-mercaptoethanol. Jurkat cells were grown in RPMI 1640 supplemented with 20% (v/v) heat-treated fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 5 mM Hepes buffer solution and 10 µM β-mercaptoethanol. Jurkat cells were grown in RPMI 1640 supplemented with 20% (v/v) fetal calf serum. For activation, cells were incubated with 5 µg/ml phytohaemagglutinin (PHA) for 24 h. All cells were maintained in flasks and grown in a humidified atmosphere of 5% CO2 at 37 °C. Media and supplements were purchased from Biological Industries (Beit Ha’emek, Israel).

Preparation of fresh activated human lymphocytes

Human peripheral blood lymphocytes from healthy donors were isolated on a Ficoll–Isopaque gradient (d 1.077) and used immediately. Lymphocytes were grown in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 µM β-mercaptoethanol and PHA (5 µg/ml, total protein concentration) for 3 days. The activated cells were washed and resuspended in the presence of 10 units/ml recombinant IL-2 (PeproTechEC Ltd., London, U.K.) to maintain cell viability.

Construction and expression of chimaeric proteins

Plasmid pAY1, encoding the protein IL-2–Bax, and plasmid pAY2, encoding mutated IL-2–Bax (named IL-2–BaxS184D), in which Ser-184 in the Bax protein is replaced by Asp-184 [23], were constructed as described in [22]. In brief, human IL-2 cDNA was fused to cDNA encoding human Bax-z or mutated Bax-z (kindly provided by Amotz Nechushtan and Richard J. Youle, NIH), under the control of the T7 promoter. The chimaeric genes were expressed in Escherichia coli strain BL21. Subfractionation of the cells revealed that both chimaeric proteins were highly enriched in the insoluble fraction. The insoluble fraction was denatured and then renatured as described previously [22]. The partially purified proteins, enriched in IL-2–Bax chimaeric proteins, were used in all experiments.

Confocal microscopy

Control cells or cells treated with IL-2–Bax (5 µg/ml, total protein concentration) for various periods of time were collected and allowed to adhere to glass coverslips pretreated with 10% (w/v) poly(t-lysine) (Sigma Chemical Co., St. Louis, MO, U.S.A.). Except where noted, cells were incubated with 250 nM of a mitochondrion-specific dye (Mitotracker Red CMXRos; Molecular Probes Inc., Eugene, OR, U.S.A.). After a 30 min incubation, the cells were washed with PBS and fixed in 3.7% (v/v) paraformaldehyde, permeabilized with 0.2% (v/v) Triton X-100 and blocked with 1% (w/v) BSA. The slides were incubated with antibodies against human Bax (Pharmingen, San Diego, CA, U.S.A.; dilution 1:200), human cytochrome c (Pharmingen; 1:100) or human IL-2 (Serotec, Raleigh, NC, U.S.A.; 1:100) for 40 min at 25 °C and then washed with PBS. Secondary antibodies conjugated to FITC (Jackson Immunoresearch Laboratory Inc., West Grove, PA, U.S.A.) were then used to detect Bax, cytochrome c and IL-2 immunoreactivity. Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole; 1 µg/ml). The slides were examined and photographed with a Zeiss LSM 410 confocal laser scanning system attached to a Zeiss Axiovert 135M inverted microscope with a 63 x 1.2 C-achromatphere water-immersion objective lens (Carl Zeiss, Thornwood, NY, U.S.A.). A krypton/argon laser at 488 and 568 nm was used for fluorescence excitation of FITC and Mitotracker Red CMXRos respectively. A 364 Innova Enterprise Ion Laser was used for DAPI staining.

For isolation of nuclei, treated or control cells were centrifuged at 300 g for 5 min, resuspended in 0.5 % KCl and left for 10 min at room temperature. The reaction was ended by the addition of 1.0 ml of cold (−20 °C) fixation solvent (methanol/acetic acid, 3:1, v/v). Cells were spun down at 300 g for 5 min, resuspended in 5.0 ml of fixation solvent while vortexing continuously, left for 10 min at room temperature and then spun down as before. This was repeated once more. Nuclei were then resuspended in fixation solvent and kept at 4 °C. Nuclei thus isolated were stained with suitable antibodies and visualized by confocal microscopy at the appropriate wavelength (see above).

Subcellular fractionation

Cells treated with IL-2–Bax (5 µg/ml, total protein concentration) for various time periods were collected and washed twice with cold PBS, and then subfractionated as described in [24]. Briefly, cells were centrifuged at 500 g for 5 min at 4 °C and the packed cell volume was determined. The packed cells were resuspended in one packed cell volume of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF and 0.5 mM dithiothreitol) and allowed to swell on ice for 15 min. The cells were then lysed by rapidly pushing them through a 26-narrow-gauge hypodermic needle 30 times. The cell homogenate was centrifuged at 2000 g for 10 min to produce a crude nuclear pellet and a cytoplasmic supernatant (cytoplasmic fraction). The crude nuclear pellet was purified further by resuspension in two-thirds of one packed cell volume (determined at the time of cell harvest) of buffer B [20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM dithiothreitol] followed by incubation on ice with

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stirring for 30 min. The lysate was then centrifuged at 4000 g for 60 min. The supernatant contained the nuclear proteins (nuclear fraction), whereas the pellet contained the post-nuclear debris. The subcellular fractions were analysed by SDS/PAGE on 12% or 15% (w/v) acrylamide gels and Western blotted with various antibodies as described below.

Preparation of whole-cell extracts and Western blot analysis
Whole-cell extracts were prepared by washing the cells twice with PBS, then lysing them for 15 min in solubilization buffer (150 mM NaCl, 1% (v/v) Nonidet P40, 0.5% (v/v) deoxycholic acid, 1% (v/v) SDS, 50 mM Tris, pH 8.0, and 1 mM PMSF). The lysates were then cleared by centrifugation for 10 min at 12000 g.

For Western blotting, samples containing equal amounts of protein were analysed by SDS/PAGE on 12% or 15% gels. The proteins were electrophoretically transferred onto nitrocellulose membranes (Amersham Corp., Arlington Heights, IL, U.S.A.), blocked with PBS/0.05% Tween-20 containing 3% (w/v) BSA and then incubated with the specific primary antibodies for 2 h. The antibodies were as follows: anti-Bcl-2 from Dako (Glostrup, Denmark; 1:250 dilution); anti-IL-2 (1:500) and anti-Bax (1:5000) from Pharmingen International; anti-p53 was kindly donated by Igal Haupt (The Hebrew University), and anti-Tax was a gift from Alik Honigman (The Hebrew University). Primary antibody binding was detected by blotting with suitable IgG linked to horseradish peroxidase (Jackson Immunoresearch), followed by band visualization using enhanced chemiluminescence (ECL*) as described by the manufacturer (Amersham Corp.).

RNA extraction and semi-quantitative reverse transcription–PCR (RT-PCR)
Total RNA was isolated from fresh activated human lymphocytes with TriPure Isolation reagent (Boehringer Mannheim) and then reverse transcribed into first-strand cDNA with a reverse transcription system (Promega, Madison, WI, U.S.A.). The cDNAs were used for amplification of one mRNA at a time, by PCR, using the following specifically designed human primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 597 bp), 5′-CCACCATGGCAAATTCATGGCA-3′ (sense) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (antisense); Bax (576 bp), 5′-CGGAACTTGATTTGGGCGGTCGGAGA-3′ (sense) and 5′-CCGGAATCTGGGTCTCAGCAGCCATCTTCTTCTC-3′ (antisense); Bcl-2 (421 bp), 5′-GTGGCCACCTGTGGTCCACCTG-3′ (sense) and 5′-CTCTAATTCTGTGGCCCA-GATAGG-3′ (antisense).

The level of GAPDH gene expression served as an internal control. GAPDH, Bax and Bel-2 mRNAs were amplified using a TGradient Thermocycler (Whatman Biometra, Göttlingen, Germany) for 25, 40 and 26 cycles respectively. Each cycle consisted of 30 s at 95 °C, 30 s at 65 °C and 1 min at 72 °C. Equal amounts of PCR products were run on 2% (w/v) agarose gels. The ethidium bromide-stained gel was analysed using a digital Bio Imaging System 202D (B.L.S.; Pharmacia Biotech). Each RT-PCR reaction was performed in duplicate.

Measurement of apoptosis
The percentage of cells in sub-G1 phase (determined as percentage specific apoptosis) was measured by FACS analysis of propidium iodide-stained nuclei as described in [25]. In brief, after the desired treatment, cells (0.5 × 10^6) were centrifuged at 500 g for 6 min, washed with cold PBS and then resuspended gently in 0.5 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100). The cells were incubated at 4 °C in the dark overnight and then analysed by FACS for DNA content as a function of cell number, using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) and the CellQuest program.

Cell viability
Cells were seeded in 6-well plates (2 × 10^4 cells in 5 ml of culture medium). Chimaeric protein or an equal volume of PBS was added, and the cultures were harvested after various incubation periods. Cells were counted in a haemocytometer and assessed for viability by Trypan Blue exclusion.

Measurement of IL-2–Bax internalization by FACS
Cells treated with IL-2–Bax (5 μg/ml, total protein concentration) for various time periods were collected and prepared for staining with anti-Bax or anti-IL-2 antibodies, and then analysed by FACS as described in [26].

RESULTS
Internalization and subcellular localization of IL-2–Bax within target cells: a kinetic study
We previously described the first prototype chimaeric protein, IL-2–Bax, that specifically targets cells expressing the IL-2R and promotes cell-specific apoptosis in the absence of any additional death stimulus [22]. However, we did not know which of the apoptotic pathways is triggered by IL-2–Bax upon its internalization into the target cells. In the present study, we first examined the kinetics of IL-2–Bax internalization, with the aid of confocal microscopy. The chimaeric protein was visualized with antibodies against human Bax. Very low levels of endogenous Bax were detected in control human HUT-102 lymphoma cells, whereas, upon treatment with IL-2–Bax, augmented amounts of the Bax protein were detected as a function of incubation time (Figure 1A, panels a–e). As anti-Bax binds to endogenous Bax as well (see below), we used an antibody that recognizes the IL-2 moiety of the chimera to determine its fate within the cells. As can be seen in Figure 1A (panels f–h), the concentration of the chimera in treated fresh PHA-activated lymphocytes increased with time, similar to the results obtained when staining with anti-Bax antibodies. Interestingly, within the treated cells, IL-2–Bax appeared to be localized in various compartments at different times of incubation: while it was seen mainly in the nucleus shortly after exposure of the cells, later the chimera was detected mostly in the cytoplasm (Figures 1A and 1B). Moreover, human Bax was detected in mouse 2B4 cells treated with the chimera, using a specific antibody that recognizes only human Bax, indicating that the detected Bax is from an exogenous source, namely IL-2–Bax (Figure 1A, panels i–l).

Next we studied the localization of IL-2–Bax following internalization, hoping that this would provide some insight into intrinsic suicide events and their cascade, culminating in the elimination of target cells. To follow the intracellular pathway of IL-2–Bax, we examined the localization of Bax at different time points, with the aid of confocal microscopy. The nuclei were detected using the DNA-intercalating agent DAPI (blue staining). Bax protein was detected using a FITC-coupled secondary antibody against a polyclonal anti-Bax antibody (green staining). To identify the mitochondria, we treated the...
Figure 1  Internalization and subcellular localization of IL-2–Bax in various target cells

Confocal microscopy was used to study internalization of the chimaeric protein (A) and its subcellular localization over different time scales (B). (A) Cells were exposed to IL-2–Bax for different periods of time. Anti-Bax or anti-IL-2 antibodies were visualized with suitable IgG-FITC-conjugated antibody and examined by laser fluorescence confocal microscopy as described in the Materials and methods section. (a)–(e) Human HUT-102 T cells. The chimaera was detected with anti-Bax antibodies (red) in control untreated cells containing endogenous Bax (a), and in cells exposed to IL-2–Bax (5 μg/ml, total protein concentration) for 30 (b), 60 (c), 120 (d) and 200 (e) min. (f)–(h) Fresh PHA-activated lymphocytes. The chimaera was detected with anti-IL-2 antibodies (red) in control cells (f), and in cells exposed to IL-2–Bax for 3 h (g) or 24 h (h). (i)–(j) Mouse 2B4 T cells. IL-2–Bax was detected with anti-Bax antibodies (green) and counter-stained with DAPI nuclear staining (blue). Shown is a field of control cells (i), and cells exposed to IL-2–Bax for 3 h (j) and for 24 h (k). All fields were photographed with a 63 × 1.2 objective lens, with zoom ×3, except (h) (× 4). (B) HUT-102 cells were incubated with IL-2–Bax (5 μg/ml, total protein concentration) for 3 or 24 h, treated with 250 nM Mitotracker Red CMXros to stain the mitochondria (Mito), fixed and stained with both anti-Bax antibodies (green) and DAPI nuclear marker (blue). Fields were visualized by laser fluorescence confocal microscopy at the appropriate wavelengths as described in the Materials and methods section.
cells with Mitotracker Red CMXros, a specific mitochondrial probe (red staining).

In control cells, Bax protein was localized primarily in the nucleus. At 3 h following IL-2–Bax treatment, Bax was localized mainly both around and inside the nucleus; however, some IL-2–Bax was detected in the cytoplasm and co-localized with the mitochondria (Figure 1B). At 24 h post-treatment, Bax was redistributed to the cytoplasm and co-localized mainly with the mitochondria, as indicated by the yellow staining (Figure 1B). Since anti-Bax recognizes both IL-2–Bax and endogenous Bax, we also used anti-IL-2 antibody to detect the chimaera, and obtained similar results (Figure 1A and results not shown).

Subfractionation and Western blot analysis support the results obtained by confocal microscopy

We also investigated the internalization of IL-2–Bax by Western blotting of whole-cell protein extracts of treated cells. IL-2–Bax was detected within the cells as early as 3 h following treatment, and its total amount increased with time (Figure 2A). This was evident in other tested T-cell types of both human and murine origin (results not shown). In addition, FACS analysis of HUT-102 and 2B4 cells treated with IL-2–Bax showed that both Bax and IL-2–Bax protein levels rose following treatment (results not shown).

To characterize further the subcellular localization of Bax in IL-2–Bax-treated cells, we used a biochemical approach. We treated HUT-102 cells with the chimaera for various periods of time and then subfractionated the cells. Cytoplasmic and nuclear fractions were isolated, and immunoblotted with anti-Bax antibody. As can be seen in Figure 2(B), following 3 h of incubation, IL-2–Bax was detected predominantly in the nuclear fraction, and to a lesser extent also in the cytoplasmic fraction. Accumulation of IL-2–Bax in the cytoplasmic fraction increased over a period of 24 h, while a substantial amount of IL-2–Bax was still observed in the nuclear fraction. The purity of the nuclear and cytoplasmic fractions was determined routinely by detection of the nuclear protein marker PARP and of the cytoplasmic protein α-tubulin (Figure 2C). Moreover, to rule out the possibility that the nuclear fraction was contaminated with mitochondria, we immunoblotted both fractions for the mitochondrial protein porin (voltage-dependent anion channel), and found that the nuclear fraction was completely free of this mitochondrial protein (Figure 2C).

Taken together, both the biochemical approach and the immunofluorescence experiments demonstrated that, upon IL-2–Bax internalization in target cells, changes are observed in its subcellular distribution in relation to the length of incubation. Our results suggests that this redistribution of the IL-2–Bax chimaeric protein is crucial and may play a role in the apoptosis of target cells.

IL-2–Bax triggers cytochrome c release in treated cells

We have shown previously that cells treated with IL-2–Bax die due to apoptosis [22]. To explore the mechanism by which IL-2–Bax causes this effect, we followed changes in the levels of key proteins involved in apoptotic pathways. We focused on the route involving the mitochondria, as we had evidence that IL-2–Bax activity does not involve the cell death receptor pathway (R. Aqeilan and H. Lorberboum-Galski, unpublished work). We therefore examined the release of cytochrome c and the activation of caspases.

Following exposure of cells to stimuli that trigger apoptosis, cytochrome c is released rapidly from the mitochondria into the cytoplasm, where it activates caspases, such as caspase-9 [17]. This cytochrome c release was shown to be initiated by the protein Bax [16]. As we introduced large amounts of Bax (in the form of IL-2–Bax) into the cells, immunohistochemistry was used to determine whether treatment with IL-2–Bax induces the release of cytochrome c from mitochondria in HUT-102 cells. As shown in Figures 3(A) and 3(B), the immunostaining of cytochrome c was co-localized with the mitochondria in control cells. However, following treatment with IL-2–Bax, cytochrome c diffused throughout the cell as early as 3 h post-treatment (Figure 3A), and an increase in its release was evident with increasing incubation time. Upon prolonged exposure of the cells to IL-2–Bax (24 h), cells demonstrated heavily diffuse cytochrome c staining (Figures 3A–3C). Cells at this late stage of treatment appeared to be apoptotic, since their nuclei were condensed, as assessed by DAPI staining (Figure 3B). These results suggest that IL-2–Bax may have provoked the redistribution of cytochrome c from the mitochondria to the cytoplasm, similar to that following a physiological stimulus for apoptosis. Cytochrome c release following treatment with IL-2–Bax was also evident in Jurkat cells (Figure 3C), and could be detected as early as 3 h post-treatment, similar to the results obtained with HUT-102 cells.

Caspase activation in cells treated with IL-2–Bax

Since IL-2–Bax activity was accompanied by cytochrome c release, we examined whether this induced downstream processes.
Figure 3 IL-2–Bax induces cytochrome c release from mitochondria in HUT-102 cells (A, B) and Jurkat cells (C)

Cells were incubated with IL-2–Bax (5 μg/ml, total protein concentration) for various time periods, treated with 250 nM Mitotracker Red CMXRos to stain the mitochondria, fixed and stained with both antibodies against cytochrome c (Cyt. c) (green; A) and DAPI nuclear marker (blue; B). Fields were visualized by laser fluorescence confocal microscopy at the appropriate wavelength (zoom × 3), as described in the Materials and methods section. Jurkat cells (C) were stained for cytochrome c (green) only.

such as caspase activation. Following secretion of cytochrome c from the mitochondria, it binds to pro-caspase-9 and Apaf-1 (apoptotic protease-activating factor 1), resulting in the activation of caspase-9 and, subsequently, caspase-3 [27]. Therefore HUT-102 cells were treated with IL-2–Bax for various time periods, harvested, subjected to SDS/PAGE and immunoblotted using antibodies against caspase-9 and its substrate caspase-3. As shown in Figures 4(A) and 4(B), the amounts of both caspase-9 and caspase-3 proenzymes increased in treated cells as a function of incubation time. Furthermore, both caspases had undergone activation as early as 3 h post-treatment, as indicated by the appearance of its cleaved products, which were absent from control cells (Figures 4A and 4B).

To learn more about the consequences of caspase activation in IL-2–Bax-mediated apoptosis, we studied the effects of a caspase inhibitor on HUT-102 cells treated with the chimera. We treated the cells with a broad-range, cell-permeable tripeptide inhibitor, Z-VAD-FMK (benzyloxy-carbonyl-Val-Ala-dL-Aspfluoromethylketone) [28], and examined its effects by FACS analysis as well as by a cell viability assay. The inhibitor almost
Apoptotic machinery induced by interleukin-2–Bax

Figure 4 IL-2–Bax triggers activation of caspases in HUT-102 cells

(A, B) Cells were treated with IL-2–Bax (5 µg/ml, total protein concentration) for the indicated times, and subjected to subcellular fractionation as described in the Materials and methods section. Fractions were analysed by SDS/PAGE and immunoblotted with either anti-caspase-9 (A) or anti-caspase-3 (B) antibodies. C, cytoplasmic fraction; N, nuclear fraction. (C) Effect of Z-VAD-FMK on IL-2–Bax activity. Cells were incubated with IL-2–Bax (5 µg/ml, total protein concentration) in the absence or presence of the broad-range tripeptide caspase inhibitor Z-VAD-FMK (50 µM). Specific apoptosis was determined after 24 h, as described in the Materials and methods section. (D) Cleavage of PARP in IL-2–Bax-treated cells and in starved cells. Whole protein extracts were prepared from starved cells (S), control cells (C), and cells treated with IL-2–Bax (T), as described in the Materials and methods section. The proteins were analysed by SDS/PAGE, then immunoblotted with anti-PARP antibody; 116 kDa is the size of uncleaved PARP, and 89 kDa indicates its cleaved fragment.

Figure 5 IL-2–Bax induces the expression of endogenous Bax and suppresses Bcl-2

(A) Changes in Bax protein levels following treatment with IL-2–Bax. HUT-102 cells were treated with 5 µg/ml IL-2–Bax for the indicated times and then lysed as described in the Materials and methods section. Proteins were analysed by SDS/PAGE and then immunoblotted with anti-Bax antibody. The 21 kDa band indicates full-length endogenous Bax, and the 18 kDa band indicates N-terminally truncated cleaved Bax. (B) Subcellular localization of endogenous Bax following treatment with IL-2–Bax. HUT-102 cells were treated with 5 µg/ml IL-2–Bax for 3 or 24 h and subjected to subcellular fractionation as described in the Materials and methods section. Fractions were analysed by SDS/PAGE and immunoblotted with anti-Bax antibody. C, cytoplasmic fraction; N, nuclear fraction. (C) Semi-quantitative RT-PCR. PHA-activated lymphocytes were treated with IL-2–Bax for the indicated times. Total RNA was extracted and reverse-transcribed as described in the Materials and methods section. RT-PCR products were run on agarose gels, demonstrating mRNA expression of the genes encoding Bax, Bcl-2 and GAPDH. (D) Changes in Bcl-2 protein levels following treatment with IL-2–Bax. HUT-102 cells were treated with 5 µg/ml IL-2–Bax for the indicated times, as in (A), and immunoblotted with anti-Bcl-2 antibody. Each RT-PCR reaction was performed in duplicate.

completely protected the cells from apoptosis for up to 72 h after IL-2–Bax treatment (Figure 4C and results not shown), indicating that the activation of caspases indeed plays a role in IL-2–Bax-induced apoptosis. As we did not see complete protection from apoptosis, we cannot exclude the possibility that other proteases, that do not belong to the caspase family, may contribute to IL-2–Bax-mediated apoptosis. One such protease is calpain, which cleaves Bax and thus enhances cell death [20,21].

We also investigated cleavage of the DNA repair enzyme PARP, which is one of the first identified cellular substrates of caspase-3 to be cleaved during apoptosis [29]. Treatment of HUT-102 cells with IL-2–Bax led to PARP cleavage, similar to
As shown in Figure 5(A), treatment with IL-2–Bax induced a significant and sustained increase in the levels of endogenous Bax. The steepest rise in the Bax protein level was observed in the first 3 h post-treatment; it continued to increase with time, but more slowly (Figure 5A). We also detected a cleaved Bax product in the treated cells, most probably the potent pro-apoptotic 18 kDa fragment of Bax (Bax/p18).

We next used semi-quantitative RT-PCR to examine whether these changes were also evident at the mRNA level. Surprisingly, as early as 3 h after incubation of PHA-activated lymphocytes with IL-2–Bax, we observed a dramatic increase in BAX gene expression (Figure 5C). Up-regulation of Bax mRNA expression was also evident 24 h post-treatment, but to a lesser extent (Figure 5C). These results also confirm that the augmented amount of endogenous Bax detected following treatment was not the result of IL-2–Bax degradation.

Since we observed elevated expression of BAX, we decided to examine what happens to the counter-gene, BCL-2. Following treatment with IL-2–Bax, a decrease in BCL-2 gene expression was detected as early as 3 h post-treatment, and this trend continued throughout the incubation (Figure 5D). In addition, we also detected a dramatic suppression of Bcl-2 at the protein level (Figure 5D). These results indicate that IL-2–Bax triggers apoptosis in treated cells by modulating the balance between the pro- and anti-apoptotic genes, as well as their respective proteins.

A disturbance in the Bax/Bcl-2 ratio was also observed in HUT-102 cells following starvation, which was used as a normal trigger of apoptosis in these cells (Figure 6A). Thus IL-2–Bax can modulate the Bax/Bcl-2 ratio and, in turn, affect cell fate in a manner similar to that occurring normally when these cells undergo apoptosis following a physiological trigger of cell death.

**IL-2–Bax triggers the subcellular translocation of endogenous Bax**

Since we observed that IL-2–Bax-mediated apoptosis is accompanied by alterations both in its subcellular distribution (Figures 1 and 2) and in the level of endogenous Bax (Figure 5), HUT-102-treated cells were subfractionated to follow changes in the level of endogenous Bax protein in the various cellular compartments. As shown in Figure 5(B), endogenous Bax (p21) was detected in the tested compartments, including the nucleus, of the control cells. Following treatment, there was a significant rise in the level of endogenous Bax, which was detected mainly in the nuclear fraction. As treatment proceeded, Bax appeared mainly in the cytoplasmic fraction. In addition, truncated Bax (p18) was also observed at 24 h in the cytoplasm (Figure 5B).

To examine how endogenous Bax behaves upon normal triggering of apoptosis, we subfractionated HUT-102 cells following 48 h of serum deprivation. We found that, following serum withdrawal, most of the nuclear Bax was translocated to the cytoplasmic fraction (Figure 6A, panel 1). Massive proteolytic cleavage of Bax protein was also observed. In contrast, the level of Bcl-2 protein was repressed dramatically following starvation in HUT-102 cells (Figure 6A, panel 2). These results emphasize the ability of IL-2–Bax to affect the intracellular apoptotic machinery within target cells and to force the cells to die, similar to a physiological apoptotic signal.

**IL-2–Bax represses the expression of Tax protein in treated HUT-102 cells**

To determine how IL-2–Bax affects both the expression and the distribution of endogenous Bax, we studied how Bax protein is regulated in HUT-102 cells. HUT-102 T cells are infected with the human T-cell leukaemia virus type 1 (HTLV-I). The virally encoded Tax protein is strongly linked to the oncogenesis.
there was indeed a time-dependent induction of p53 protein in cells harbour a wild-type treatment with IL-2–Bax. It should be pointed out that HUT-102 examined whether the level of the p53 protein changes following mechanisms independent of its transcriptional activity [33], we also investigated the involvement of p53 in the expression of Effects of IL-2–Bax on level and subcellular localization of p53. To assess further the effects of IL-2–Bax on Tax levels, we employed a biochemical approach. We treated HUT-102 cells with IL-2–Bax for different periods of time and then examined the Tax protein level by Western blot analysis. Whereas untreated control cells showed profound expression of the Tax protein, IL-2–Bax-treated cells displayed a reduced amount of Tax, if any (Figure 6B, panel 2). These results are similar to those obtained when HUT-102 cells were serum-deprived (Figure 6B, panel 1). 

Staining with anti-Tax (red) showed a lower amount of Tax (more greenish) within the nuclei of IL-2–Bax-treated cells (Figure 6B, panel 1, lower panels). Staining with anti-Bax (green) revealed that IL-2–Bax increased the Bax protein level, we showed that IL-2–Bax increased the Bax protein level, we examined its effect on Tax expression. To this end, we treated HUT-102 cells with IL-2–Bax for 3 h and then isolated the nuclei. Staining the nuclei with anti-Bax (green) revealed that there was an increased level of IL-2–Bax within the nuclei of treated HUT-102 cells (Figure 6B, panel 2). These results suggest that IL-2–Bax activity was exclusively p53-dependent.

Effects of IL-2–Bax on level and subcellular localization of p53

We also investigated the involvement of p53 in the expression of both BAX and BCL-2 following treatment with IL-2–Bax. As p53 has been found to regulate the induction of apoptosis via its transcriptional activity, by deregulating the balance between pro- and anti-apoptotic proteins, such as Bax/Bcl-2 [31,32] or by mechanisms independent of its transcriptional activity [33], we examined whether the level of the p53 protein changes following treatment with IL-2–Bax. It should be pointed out that HUT-102 cells harbour a wild-type p53 gene [34]. As shown in Figure 7A, there was indeed a time-dependent induction of p53 protein in IL-2–Bax-treated HUT-102 cells. Next we examined whether this induction also involved changes in the subcellular localization of p53. To this end, we isolated both nuclear and cytoplasmic fractions of treated cells and subjected them to Western blotting. The p53 level remained constant in the cytoplasmic fraction as compared with that in the control cells (Figure 7B). This may indicate that p53 is translocated into the nuclear compartment, where it acts as a transcriptional activator of Bax and a suppressor of Bcl-2, thus constituting an apoptotic signal.

Since p53 is mutated in over 50% of tumours [35,36], there was a possibility that IL-2–Bax activity was exclusively p53-dependent and would not cause apoptosis in cells harbouring a mutated p53 gene. We therefore examined the effects of IL-2–Bax on T cells lacking p53 expression, such as Jurkat cells (Figure 8A and [37]). Treatment of Jurkat T cells with IL-2–Bax induced cell-specific apoptosis (Figure 8B), which was correlated with IL-2R expression. Jurkat T cells express constitutively the intermediate-affinity IL-2R [38]. However, following stimulation with PHA, the cells express the high-affinity receptor, and thus become more sensitive to IL-2–Bax (Figure 8B). Moreover, IL-2–Bax efficiently inhibited the growth of Jurkat T cells and eventually led to cell death (Figure 8C). These results suggest that IL-2–Bax can induce cell-specific apoptosis, most probably through several mechanisms, including p53-dependent and independent pathways, depending on the specific cell type.
Jurkat cells have also been found to harbour a frameshift mutation in the BAX coding sequence [39–41]. However, these cells underwent apoptosis upon treatment with IL-2–Bax via a mechanism that did not require the involvement of endogenous Bax (Figures 8B and 8C). These observations indicate that IL-2–Bax can cause cell-specific apoptosis even when both p53 and Bax are defective. This may imply that there are other intracellular targets for the chimaera.

**DISCUSSION**

IL-2–Bax was the first prototype of a human apoptosis-inducing chimaeric protein to be constructed for the targeted therapy of human diseases [22]. Since IL-2–Bax has great potential to be developed for human use, it was important to investigate how IL-2–Bax induces apoptosis in its target cells. Therefore we explored the activity of IL-2–Bax, determined its intracellular pathway and studied the molecular and biochemical changes that occur within the target cells following treatment.

Our results show that, upon IL-2–Bax internalization, changes are detected in the subcellular distributions both of the chimaera and of endogenous Bax (Figures 1, 2, 5 and 6). Endogenous Bax is located predominantly in the nucleus, and to a lesser extent in the cytoplasm, in control cells. However, following treatment with IL-2–Bax, it was translocated to the cytoplasm (Figure 5B). Similarly, IL-2–Bax itself was found to be shuttled to the nucleus shortly after internalization, while at later stages of incubation it co-localized mainly with the mitochondria (Figures 1B and 2B). These events, observed with both IL-2–Bax and endogenous Bax, may initiate a cascade, involving the release of cytochrome c (Figure 3) and caspase activation (Figure 4), leading to cell death. We also found that treatment with IL-2–Bax dramatically enhanced the expression of newly synthesized Bax protein and suppressed expression of Bcl-2 protein (Figure 5). This IL-2–Bax activity was found to involve p53 activation (Figure 7), at least in certain cell types (Figure 8). In addition, IL-2–Bax efficiently suppressed the expression of Tax protein, which is known to be involved in the transformation and leukaemogenesis of HUT-102 cells (Figure 6B). All experiments were performed while validating the cytotoxic effects of IL-2–Bax on the target cells (results not shown).

To study the behaviour of our chimaeric protein within target cells, we used both biochemical methods of cell fractionation and confocal microscopy. Our results clearly indicate that both IL-2–Bax, upon internalization into the cells, and endogenous Bax are detected mainly in the nucleus of target cells (Figures 1, 2 and 5B), and to a lesser extent in the cytoplasm. It should be emphasized that it is the Bax component of the chimaera that directs IL-2–Bax to the nucleus, and not the IL-2 targeting moiety [3]. The question thus arising is how Bax enters or escapes from the nucleus. According to the literature, Bax lacks a nuclear localization sequence, but has a putative C-terminal transmembrane region [8]. Recently the structure of Bax was resolved, and found to consist of nine α helices [42]. The C-terminal z9 helix of Bax is thought to control its mitochondrial membrane targeting [42]. This implies that Bax has the potential to insert itself into membranes, and perhaps into nuclear membranes as well. Our finding is in agreement with a report by Macho et al. [43], who demonstrated the presence of Bax protein in the nucleus of K562 cells; however, after serum withdrawal it was localized mainly in the cytoplasm. It has also been reported for a number of cell types that Bax is translocated to both the nuclear envelope and the interior of the nucleus when the cells are induced to undergo apoptosis [44,45]. Other reports have confirmed the presence of nuclear Bax using electron microscopy, as well as Western blot analysis, of isolated nuclei [46,47]. It was also shown that Bax can form ion channels in lipid membranes under neutral/physiological conditions [12,13]. It is conceivable, therefore, that Bax regulates ion channels across nuclear membranes in a manner that allows its translocation, and later triggers apoptosis. In addition, a recent study proposed an important role for caspases in disturbing nuclear transport during apoptosis, thus allowing cytoplasmic proteins to enter the nucleus [48]. These observations may also explain how Bax can enter or exit the nucleus. Both our results and others suggest that the cellular venues in which Bax acts should be expanded to include the nucleus. It should be pointed out that our experiments were performed on different T-cell lines as well as primary T cells, which were found to contain nuclear Bax. However, in other different cell types, Bax is localized mainly in the cytoplasm, and only upon an apoptotic signal is it translocated to the mitochondrial membranes [15–18]. These conflicting results indicate that the the behaviour of Bax may vary from one cell to another.

Another important issue is the role of Bax within the nucleus. We showed here that in HUT-102 cells the levels of both Tax and p53 were modulated upon treatment with IL-2–Bax (Figures 6C and 7). We also showed that Tax is involved in HUT-102 cell death following physiological triggering of apoptosis, such as by serum withdrawal. Additional support for our findings that Tax probably plays an important role in the activity of IL-2–Bax comes from our results with the mutated IL-2–BaxS184D chimaera, that contains non-active Bax (R. Aqeilan and H. Lorberbaum-Galski, unpublished work). Upon internalization of IL-2–BaxS184D into HUT-102 cells, there was indeed no alteration in the Tax level (Figure 6C). This may suggest that Tax protein in HUT-102 cells is a check-point for the decision of the cell to either die or survive. At present we do not know the mechanism by which IL-2–Bax alters the expression of Tax. However, Brauweiler et al. [30] demonstrated that repression of Bax by Tax is mediated through an E-box-containing element in the Bax promoter. In addition, the HTLV-1 Tax protein was shown to induce the expression of Bcl-xL [49] and to inhibit the activation of caspases [50], indicating that Tax-induced oncogenesis involves alterations in the apoptotic process. These observations may suggest that our chimaeric protein, upon its entry into the nucleus of HUT-102 cells, may alter the expression of Tax, which in turn modulates the expression of several target genes, such as apoptotic genes, explaining the rise in the Bax/Bcl-2 ratio.

Another important role of Tax is its involvement in the inactivation of p53 function in HTLV-I-infected cells [51,52]. Our results clearly indicate that, following treatment with IL-2–Bax, an increased amount of p53 protein was detected in the cell nucleus (Figure 7). This finding may also explain the up-regulation of the BAX gene and the down-regulation of the BCL-2 gene, since p53 has been shown to act as a direct trans-activator of BAX and a down-regulator of BCL-2 transcription [31,32]. Additional support for a role for Bax within the nucleus comes from a study that demonstrated the formation of nuclear Bax–p53 complexes in several cancer cell lines when induced to undergo apoptosis [53]. Taken together, these observations suggest that Bax by itself or in the form of IL-2–Bax may play a regulatory role within the nucleus in determining the fate of the cell. It is also possible that the nuclear localization of Bax may have other unknown functions, in HUT-102 cells and other cell types, in addition to a role in apoptosis. Bel-2 was shown to modulate entry into the cell cycle [54,55] and, together with Bax, it may regulate Cdk-2 activity [56]. It is also conceivable that, when certain cells become transformed, they store Bax in the nucleus, distancing it from its target (the mitochondrial membrane) and
thereby bypassing its apoptotic function in balancing cell proliferation and cell death.

As HUT-102 cells are infected with HTLV-I virus and harbour a wild-type p53, we next examined whether IL-2–Bax can kill T cells that are not infected with HTLV-I and whether its activity occurs only via a p53-dependent pathway. To this end, we used human Jurkat T cells, which are not infected and are devoid of p53 expression [37], and found that IL-2–Bax induced cell death via apoptosis in these cells also (Figure 8).

In addition, IL-2–Bax increased the percentage of apoptotic cells in the mouse 2B4 cell line [22]. This finding may support p53-independent activity of IL-2–Bax, since there is no analogous p53-binding site in the regulatory region of murine Bax [35]. Moreover, the Bax promoter has been shown to contain a distinct region that is responsive for the c-Myc oncoprotein [57]. These observations indicate that the pro-apoptotic activity of Bax is presumably involved not only in the p53-induced apoptosis pathway, but also in the c-Myc-induced apoptosis pathway, and maybe in other pathways as well. Cumulatively, our results suggest that IL-2–Bax can act via at least two distinct pathways: p53-dependent and p53-independent. It is conceivable that IL-2–Bax, upon entering its target cells, will utilize or recruit the endogenous apoptotic machinery as it exists in the specific treated cell, i.e. it will affect p53 protein if present, or other endogenous related proteins if p53 is lacking or mutated.

In addition, since Jurkat T cells harbour a mutated Bax [39–41], we suggest that IL-2–Bax activity does not depend merely on enhancement of endogenous Bax. Although Bax is an important pro-apoptotic protein, the redundant Bcl-2 family proteins and other factors that are involved in the apoptotic machinery may also be targets of IL-2–Bax.

We propose a model for the action of IL-2–Bax in HUT-102 cells. First, IL-2–Bax enters the cell and is localized primarily in the nucleus. IL-2–Bax then, by an unknown mechanism, suppresses the level of Tax protein, thus relieving the suppression of both p53 and Bax. Stabilization of the p53 protein causes augmentation of endogenous Bax and, in parallel, suppression of Bcl-2. This modulation of the Bax/Bcl-2 ratio activates other intracellular apoptotic pathways, including the release of cytochrome c and the activation of caspases, leading to cell death. We suggest that the repression of Tax following treatment with IL-2–Bax in these HTLV-I cells may play an important role in activation of the apoptotic machinery. Our data are also in agreement with a recent report by Mahieux et al. [58], who demonstrated that, although multiple apoptotic pathways are inhibited in HTLV-I-infected cells, these effects are reversible.

In conclusion, we present data showing that the IL-2–Bax chimaeric protein causes cell death via the mitochondrial apoptotic pathway using a mechanism similar to that which occurs naturally after a physiological stimulus such as serum withdrawal. Despite the fact that IL-2–Bax is delivered from the exterior of the cell, we showed that it is allocated to the same compartments as endogenous Bax protein, and it can induce apoptosis in a way similar to the normal triggering of apoptosis by, for example, serum withdrawal. Therefore chimaeric proteins such as IL-2–Bax can be used to study the cellular activity of intracellular proteins such as Bax, without the need to overexpress these proteins. Chimaeric proteins may thus provide a unique approach to studying the fate of intracellular proteins within cells. Originally, IL-2–Bax was constructed to kill target unwanted cells involved in various diseases. We show here that it can also be applied to address some basic questions in cellular biology. Therefore the use of chimaeric proteins such as IL-2–Bax appears to be highly promising both for basic research and in the clinical setting.

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