Bak: A Downstream Mediator of Fenretinide-Induced Apoptosis of SH-SY5Y Neuroblastoma Cells

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ABSTRACT

Unlike 13-cis-retinoic acid, the synthetic retinoid fenretinide [N-[4-(hydroxyphenyl)retinamide] induces apoptosis of neuroblastoma cells by mechanisms involving retinoic acid receptors and oxidative stress. After screening a cDNA array for apoptosis-related genes, the Bcl2-related protein Bak was identified as a fenretinide-inducible gene in SH-SY5Y neuroblastoma cells, and this was confirmed by Western blotting and flow cytometry. Although fenretinide acts synergistically in vitro with chemotherapeutic drugs, these drugs did not induce Bak expression. Retinoic acid receptor antagonists did not block the induction of Bak by fenretinide. Conversely, Bak induction was blocked by the antioxidant vitamin C. Overexpression of Bak increased apoptosis in both the presence and absence of fenretinide, whereas expression of antisense Bak inhibited fenretinide-induced apoptosis. Bak expression was also induced in cells overexpressing the stress-induced transcription factor GADD153, but Bak expression was inhibited in cells expressing an antisense GADD153 construct. These results suggest that Bak is a downstream mediator of an oxidative stress pathway leading to apoptosis of SH-SY5Y neuroblastoma cells in response to fenretinide.

INTRODUCTION

Neuroblastoma is responsible for 15% of all pediatric deaths from malignancy (1). 13-cis-Retinoic acid, a compound that differentiates neuroblastoma cells in vitro, increases event-free survival when used to treat residual disease after chemotherapy and bone marrow transplantation (2) and is now included in most treatment regimes for neuroblastoma. However, retinoic acid may also increase the resistance of neuroblastoma cells to chemotherapy (3). One problem might be overcome using retinoids with different biological properties. Fenretinide [N-(4-hydroxyphenyl)retinamide], a retinoid acid analogue, is able to induce apoptosis of neuroblastoma in vitro, unlike retinoic acid (4, 5), and is also synergistic with cisplatin, etoposide, or carboplatin (6) in the induction of neuroblastoma-cell apoptosis. Fenretinide-induced apoptosis in neuroblastoma cells is apparently mediated by RARs (7) and oxidative stress acting together, although the evidence for the involvement of RARs stems only from studies with RAR-specific antagonists (5–7). An increase in reactive oxygen species is detectable within 6 h of treating neuroblastoma cells with fenretinide (5), and this oxidative stress pathway is characterized by an induction of the stress response transcription factor GADD153 (CHOP) via a fenretinide-dependent increase in 12-lipoxygenase activity (7). Both the induction of reactive oxygen species and subsequent apoptosis are blocked by pretreating the cells with antioxidants (5).

Fenretinide-induced apoptosis of neuroblastoma cells is caspase dependent and involves the mitochondrial release of cytochrome c independently of mitochondrial permeability transition (5). Bak, a proapoptotic member of the Bcl2 family, was found to be one of a small number of genes up-regulated in SH-SY5Y cells by treatment with fenretinide (7). In view of the role of Bcl2 family proteins in the control of apoptosis and the possibility that these proteins may be key elements in the synergy between fenretinide and chemotherapeutic drugs, the aim of this study was to determine whether Bak has a functional role in apoptosis induced by fenretinide.

MATERIALS AND METHODS

Cell Culture and Treatments. Human SH-SY5Y neuroblastoma cells and stably transfected derivatives (8) were grown in a 1:1 mixture of DMEM and Ham’s F-12 medium (Life Technologies, Inc., Paisley, United Kingdom), supplemented with 10% fetal bovine serum [Life Technologies, Inc. (culture medium)] and grown in a humidified atmosphere of 5% CO2 in air. For all experiments, cells were seeded into tissue culture flasks (Costar, Bucks, United Kingdom) and allowed to attach overnight before treatment. The seeding density varied according to the type of experiment. For apoptosis and immunofluorescence measurements by flow cytometry, 0.4 × 106 or 2 × 105 cells, respectively, were seeded into 25-cm2 tissue culture flasks in 5 ml of culture medium. For Western blot experiments, 6 × 105 cells were seeded into 75-cm2 flasks in 10 ml of culture medium. Fenretinide (Janssen-Cilag Ltd., Basserdorf, Switzerland) was added to cultures in ethanol, and an equal volume of ethanol (<0.1% of culture volume) was used to treat control cells. Stock solutions of cisplatin (100 mM, freshly prepared in DMSO; Sigma Chemical Co., Poole, United Kingdom) were diluted in culture medium. The antioxidant vitamin C (ascorbic acid sodium salt; Sigma Chemical Co.) was freshly diluted in PBS and added to a final concentration of 100 μM 2 h before 22 h of treatment with fenretinide; cell cultures were washed once with PBS after removal of vitamin C before addition of fenretinide. The RAR/β’γ antagonists CD2665 and Ro 41-5253 were dissolved in DMSO and added to cultures at final concentrations of 1 μM in the presence or absence of fenretinide for 24 h. Construction and characteristics of the clonally selected SH-SHYSY112 cells and SH-SHYSY112 cells stably transfected with sense GADD153 and antisense GADD153 have been described by Lovat et al. (7). The characteristics of stably transfected cells were maintained by periodic culture in the presence of blastidicin (2.5 μg/ml; SH-SHYSY112 cells) or blasticidin (2.5 μg/ml) and zeocin (150 μg/ml; sense and antisense Bak and GADD153 SH-SHYSY112 transfectants).

Flow Cytometry. Apoptosis was evaluated by flow cytometry of PI-stained cells as previously described (7). Cells for immunofluorescence flow cytometry were detached by trypsinization and washed with 2 ml of PBS before fixation in 500 μl of 4% paraformaldehyde in PBS for 10 min at room temperature. After washing twice with PBS, cells were permeabilized with 500 μl of 0.5% Triton X-100 (Sigma Chemical Co.) in PBS for 2 min at room temperature. The cells were then washed twice with PBS and incubated for 1 h at room temperature in the presence or absence of a polyclonal goat antihuman Bak antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100. The binding of antibody was visualized with a rabbit antigen FITC-conjugated antibody (Molecular Probes, Leiden, the Netherlands), and 20,000 events were acquired for flow cytometry as described previously (7).
Western Blotting. Total protein was extracted from SH-SY5Y cells treated in the presence or absence of fenretinide for 2–48 h. 25 μg of total protein were separated by electrophoresis through 12.5% SDS-PAGE gels and blotted onto nitrocellulose (5). Bak was identified with the same antibody used for flow cytometry experiments diluted 1:500 and detected by chemiluminescence (5) using a horseradish peroxidase-conjugated goat anti-IgG (Jackson ImmunoResearch Laboratories Inc.) diluted 1:500. Bak was detected with a mouse monoclonal antibody (Santa Cruz Biotechnology), and Bcl-xl was detected with a rabbit polyclonal antibody (Santa Cruz Biotechnology), all diluted 1:1000 and detected by chemiluminescence as described for Bak. The protein loading in each track was compared using a mouse monoclonal anti-β-tubulin antibody (Sigma Chemical Co.) diluted 1:1000. Band intensities were compared by densitometry using ImageMaster software (Amersham PLC, Little Chalfont, United Kingdom).

**RESULTS AND DISCUSSION**

Immunofluorescence flow cytometry and Western blotting experiments confirmed that Bak protein was induced in SH-SY5Y cells after 24–48 h in response to fenretinide (Fig. 1). Because fenretinide synergizes with cisplatin, etoposide, or carboplatin to give increased levels of apoptosis (6, 9), we asked whether these chemotherapeutic drugs also induce Bak expression. However, after treating SH-SY5Y cells with concentrations of cisplatin (Fig. 1), carboplatin, or etoposide (data not shown) that induce 30–40% apoptosis, there was no detectable induction of Bak.

Fenretinide-induced apoptosis is inhibited by antagonists to RARβ/γ receptors, but not by RARα antagonists (5). Although these reagents are effective in inhibiting retinoic acid-induced gene expression in SH-SY5Y cells (7), neither the RARα antagonist Ro 41-5253 nor the RARβ/γ antagonist CD2665 had any apparent effect on the induction of Bak in response to fenretinide (Fig. 1). Conversely, pretreating SH-SY5Y cells with the antioxidant vitamin C before a 24-h exposure to fenretinide blocked the induction of Bak (Fig. 1). These results suggest that Bak was induced in relation to an oxidative stress pathway of fenretinide action that is independent of RARs.

To demonstrate a functional role of Bak in the fenretinide-induced apoptosis of SH-SY5Y cells, Bak cDNA was cloned into a tetracycline-inducible vector in both sense and antisense orientations and stably transfected into SH-SY5Y cells. Stably transfected cells selected by zeocin and blasticidin tend to be more resistant to drug-induced apoptosis, and therefore higher concentrations of fenretinide were used to induce apoptosis in these cells compared with wild-type cells. Relative to control, uninduced cells, the induction of Bak with tetracycline increased cell death in the absence of fenretinide from 10% in the control, untreated cells to nearly 70% after induction of Bak with tetracycline (Fig. 2). Although the cell death response to fenretinide treatment was not increased, this was already at 90% with the fenretinide concentration used. Western blot data demonstrated that Bak was induced 4-fold by tetracycline in the transfected cells (Fig. 2). In contrast, the induction of antisense Bak with tetracycline did not increase apoptosis relative to untreated control cells (Fig. 3). Furthermore, although fenretinide induced death in nearly 70% of cells (Fig. 3E), this response to fenretinide was effectively blocked by the induction of antisense Bak with tetracycline (Fig. 3). Similar results were obtained using RNA interference (data not shown).

Western blot data for these experiments with the inducible antisense Bak clones confirmed that Bak was induced 2-fold in response to fenretinide, but this induction was blocked by adding tetracycline to induce expression of the antisense Bak cDNA construct (Fig. 3F). Although fenretinide does not alter levels of Bcl-2 in SH-SY5Y neuroblastoma cells (5), it is possible that other Bcl2-related proteins are regulated in response to fenretinide. To investigate this, Western blots of proteins extracted from the sense and antisense Bak cells were probed with antibodies against the antiapoptotic protein Bcl-xL and the proapoptotic Bax (Fig. 4A). However, there was no consistent change
in the expression of Bax or Bcl-xI in response to fenretinide or in response to the induction of sense or antisense Bak with tetracycline. These results clearly demonstrate that the induction of Bak is required for fenretinide-induced apoptosis of SH-SY5Y cells.

The data from these experiments show that Bak was induced in SH-SY5Y neuroblastoma cells in response to fenretinide and was required for fenretinide-induced apoptosis. GADD153 is also induced by fenretinide, but relatively faster, reaching a peak at about 8 h (7). Considerable evidence indicates that GADD153 mediates cellular responses to oxidant injury (10), and mediating ER stress may be one of its main functions (11, 12). Because Bak is associated with the ER (13, 14), neuroblastoma cell apoptosis in response to fenretinide may be mediated by an ER stress pathway in which GADD153 and Bak are major components. GADD153 is a member of the CCAAT/enhancer-binding protein family of transcription factors (15) and may have important regulatory interactions with members of the Bcl2 family (16, 17). Clearly, GADD153 may play a role in fenretinide-induced apoptosis of SH-SY5Y neuroblastoma cells by regulating the expression of proapoptotic proteins such as Bak.

To test the hypothesis that GADD153 is responsible for the induction of Bak, we compared the level of Bak expression in SH-SY5Y*12 cells stably transfected with tetracycline-inducible sense or antisense GADD153 cDNA (7). By immunofluorescence flow cytometry, SH-SY5Y*12 cells showed a 1.6-fold induction of Bak in response to fenretinide (Fig. 4B). A sense GADD153 clone (S7) showed induction of Bak when GADD153 was induced in response to tetracycline alone, and in these cells, Bak was induced to the same level whether treated with tetracycline or fenretinide alone or with both reagents together (Fig. 4B). In SH-SY5Y*12 cells stably transfected with tetracycline-inducible antisense GADD153, Bak was induced by fenretinide in the absence of tetracycline and was not induced by tetracycline alone. Conversely, induction of antisense GADD153 by tetracycline reduced the induction of Bak in response to fenretinide by 50% (Fig. 4B). These results demonstrate that the induction of GADD153 independently of fenretinide leads to the induction of Bak. Therefore, it is possible that GADD153 directly regulates Bak expression. However, because expression of the antisense GADD153 con-

Fig. 2. Transfection of sense Bak cDNA. A–D, flow cytometry profiles of PI-stained SH-SY5Y*12 cells stably transfected with sense Bak cDNA after treatment with control vehicle (A, Ctrl), tetracycline (B, +Tet; 1 μg/ml for 48 h), tetracycline and fenretinide (C, +Tet + FenR; 1 μg/ml tetracycline for 48 h and 10 μM fenretinide for the last 24 h of tetracycline treatment), or fenretinide alone (D, +FenR; 10 μM for 24 h) and analyzed for apoptosis by flow cytometry. E, summary of data for three consecutive experiments with cells treated as described for A–D; error bars represent SE. F, Western blot results for Bak and tubulin (loading control) expression after treatment with ethanol (vehicle control; Ctrl), tetracycline (+Tet), tetracycline and fenretinide (+Tet + FenR), or fenretinide alone (+FenR) as described in A–D and in the same population of cells. After correcting for variation in loading using the tubulin band intensity, Bak was induced 4-fold relative to control cells by tetracycline, 4.5-fold by tetracycline and fenretinide, and 2.6-fold by fenretinide alone.

Fig. 3. Transfection of antisense Bak cDNA. A–D, flow cytometry profiles of PI-stained SH-SY5Y*12 cells stably transfected with antisense Bak cDNA after treatment with control vehicle (A, Ctrl), tetracycline (B, +Tet; 1 μg/ml for 48 h), tetracycline and fenretinide (C, +Tet + FenR; 1 μg/ml for 48 h and 10 μM for 24 h, respectively, as described in the Fig. 2 legend), or fenretinide alone (D, +FenR; 10 μM for 24 h) and analyzed for apoptosis by flow cytometry. E, summary of data for three consecutive experiments with cells treated as described for A–D; error bars represent SE. F, Western blot results for Bak and tubulin (loading control) expression after treatment with ethanol (vehicle control; Ctrl), tetracycline (+Tet), tetracycline and fenretinide (+Tet + FenR), or fenretinide alone (+FenR) as described in A–D and in the same population of cells. After correcting for variation in loading using the tubulin band intensity, Bak was reduced to approximately half (0.49-fold) the level in control cells by tetracycline or tetracycline with fenretinide (0.6-fold) but induced 2-fold by fenretinide in the absence of tetracycline.
Bak-mediated neuroblastoma apoptosis by fenretinide

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