Hypoxia Inhibits Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis by Blocking Bax Translocation

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Abstract

The hypoxic environment in solid tumors results from oxygen consumption by rapid proliferation of tumor cells. Hypoxia has been shown to facilitate the survival of tumor cells and to be a cause of malignant transformation. Hypoxia also is known to attenuate the therapeutic activity of various therapies in cancer management. These observations indicate that hypoxia plays a critical role in tumor biology. However, little is known about the effects of hypoxia on apoptosis, especially on apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a potent apoptosis inducer that has been shown to specifically limit tumor growth without damaging normal cells and tissues in vivo. To address the effects of hypoxia on TRAIL-induced apoptosis, HCT116 human colon carcinoma cells were exposed to hypoxic or normoxic conditions and treated with soluble TRAIL protein. Hypoxia dramatically inhibited TRAIL-induced apoptosis in HCT116 cells, which are highly susceptible to TRAIL in normoxia. Hypoxia increased antiapoptotic Bcl-2 family member proteins and inhibitors of apoptosis proteins. Interestingly, these hypoxia-increased antiapoptotic molecules were decreased by TRAIL treatment to the levels lower than those of the untreated conditions, suggesting that hypoxia inhibits TRAIL-induced apoptosis via other mechanisms rather than up-regulation of these antiapoptotic molecules. Additional characterization revealed that hypoxia significantly inhibits TRAIL-induced translocation of Bax from the cytosol to the mitochondria in HCT116 and A549 cells, with the concomitant inhibition of cytochrome c release from the mitochondria. Bax-deficient HCT116 cells were completely resistant to TRAIL regardless of oxygen content, demonstrating a pivotal role of Bax in TRAIL-induced apoptotic signaling. Thus, our data indicate that hypoxia inhibits TRAIL-induced apoptosis by blocking Bax translocation to the mitochondria, thereby converting cells to a Bax-deficient state.

Introduction

Apoptosis, or programmed cell death, is a genetically conserved physiologic event in multicellular organisms (1–3). Apoptosis plays a major role in the elimination of injured or unwanted cells in many physiologic and pathologic conditions, such as normal development, homeostasis, defense mechanism against viral infection, dysregulated immune disease, and uncontrolled cell growth. Thus, dysregulation of apoptosis may be related to serious human pathologies, including neoplasia, degenerative disorders, and autoimmune diseases (4, 5).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo-2L, is a membrane-bound cytokine molecule that belongs to the family of tumor necrosis factor. Studies using TRAIL knockout mice have demonstrated that TRAIL plays a critical role in suppressing tumor initiation and metastasis (6). In addition to these functional roles in normal physiology, TRAIL has been shown to be a potent inducer of apoptosis in a wide variety of cancer cells in vitro and has been shown to limit tumor growth efficiently in vivo, without damaging normal tissues (7–9). This unique feature of selectivity for cancer cells has drawn considerable attention to TRAIL as a potential therapeutic modality to manage human cancers.

Once activated by TRAIL, TRAIL receptors recruit a cytosolic adaptor molecule, Fas-associated death domain, which in turn recruits and activates caspase-8 as an initial event of caspase cascade. Studies have demonstrated that a single death receptor-mediated apoptotic signal separates to two main signaling pathways at the point of caspase-8 (10, 11). One apoptotic signaling pathway, termed the mitochondria-independent pathway, comprises a direct activation of executioner caspases (caspase-3 and -7) by caspase-8. The other apoptotic signaling pathway, termed the mitochondria-dependent pathway, refers to the signaling process occurring through the mitochondria to activate the executioner caspases (caspase-3 and -7). The mitochondria-dependent pathway is initiated by Bid, a proapoptotic Bcl-2 family member protein. Bid, cleaved by caspase-8 (truncated Bid), translocates to the mitochondria and induces cytochrome c release (10, 12). Released cytosolic cytochrome c binds to Apaf-1 and participates in caspase-9 activation (13, 14). The activated caspase-9 then is able to activate caspase-3 (13). The mitochondrial events also include the release of Smac (15, 16), apoptosis-inducing factor (17), and other factors (18) from the mitochondria.

The apoptotic mitochondria-dependent and -independent signal pathways meet again at the point of executioner caspase activation. Studies have demonstrated that the mitochondrial events “amplify” apoptotic signals. Thus, without the engagement of the mitochondria-dependent pathway, the activation of the executioner caspases by only the mitochondria-independent pathway is inadequate and results in limited apoptosis in many cell types.

It is now well established that the mitochondrial events are regulated by Bcl-2 family member proteins, consisting of proapoptotic and antiapoptotic members (19). Bax, a proapoptotic Bcl-2 family member protein, is known to play a key role in the mitochondrial events. For example, Bax is a critical functional partner for the action of truncated Bid in the release of cytochrome c from the mitochondria (20, 21). Recent studies also have demonstrated that a deletion of Bax from HCT116 human colon carcinoma cells results in an abrogation of mitochondrial events, leading to complete resistance to TRAIL-induced apoptosis (22–24).

Local growth of malignant tumors largely depends on adequate nutrient and oxygen supply. As a result of the rapid proliferation of tumor cells, the center of solid tumors is under hypoxic or even anoxic conditions (0–20 mm Hg oxygen tension; 24–66 mm Hg for normal tissues; Refs. 25, 26). Studies have demonstrated that tumor hypoxia plays an important role in a variety of cellular responses, including apoptosis, growth factor signaling, pH regulation, angiogenesis, me-
tastasis, immortalization, and genetic instability of tumor tissues (27–29). Recent findings showed that hypoxia increases antiapoptotic potentials in tumor cells by regulating the molecules involved in the apoptosis signaling pathways (27, 28). These effects of hypoxia render tumor cells resistant to various cancer therapies and facilitate the survival of tumor cells (30–33). Thus, tumor cell responses to hypoxia are important for tumor progression and tumor therapy.

Despite the critical significance of hypoxia in tumor biology general and therapeutic applications, little is known about the mechanism by which hypoxia regulates apoptosis. Here, for the first time, we demonstrate that hypoxia inhibits TRAIL-induced apoptosis by blocking Bax translocation from the cytosol to the mitochondria. Our data suggest that blockade of Bax translocation may be a mechanism by which hypoxic tumor cells survive from tumor therapies and an antitumor immunity that induces tumor apoptosis.

Materials and Methods

Cell Culture. Bax-containing (Bax+/+) and Bax-deficient (Bax−/−) HCT116 human colon carcinoma cell lines (34) were obtained from Dr. Ben Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). These cell lines were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum and antibiotics. A549 cells were maintained in F-12K culture medium as described previously (35).

Treatment with Hypoxia. HCT116 or A549 cells were exposed to hypoxia as described previously (35). In brief, hypoxia was applied to the cells by maintaining the cells inside an airtight chamber with inflow and outflow valves that were infused with a hypoxic gas (1% O2, 5% CO2, and 94% N2).

Measurement of Cell Viability. HCT116 cells, plated in 12 wells, were pre-exposed to normoxic or hypoxia conditions for the indicated times and further treated with soluble recombinant human TRAIL protein (0–200 ng/ml; Refs. 35, 36) for 4 h. Cell viability was determined by the crystal violet staining method as described previously (35, 36). Briefly, cells were stained for 10 min at room temperature with staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde), washed four times with water, and dried. Cells were lysed with 1% SDS solution and measured at 550 nm. Cell viability was calculated from relative dye intensity and compared with the controls.

Subcellular Fractionation. HCT116 or A549 cells, pre-exposed to normoxic or hypoxia conditions for 24 h, were further treated with TRAIL protein (200 ng/ml) for 4 h. Cells were washed with PBS, resuspended in an isotonic mitochondrial buffer [210 mM sucrose, 70 mM mannitol, 1 mM EDTA, and 10 mM HEPES (pH 7.4)], and broken by six passages through a 25-gauge needle fitted to a syringe. Unbroken cells and nuclei were removed by centrifugation at 700 × g for 10 min at 4°C. The saved supernatant was further centrifuged at 10,000 × g for 30 min at 4°C to collect the heavy membrane fraction in which mitochondria are enriched. The resultant supernatant was used as a cytosolic fraction, and the heavy membrane pellet was resuspended in 50 μl mitochondrial buffer and used as a mitochondrial fraction.

Western Blot Analysis. Whole cell lysates, prepared as described previously (36, 37), were resolved on a 15% SDS gel and transferred onto the nitrocellulose membrane. Western blot analyses were performed as described previously (36, 37) using antibody for Bax (sc-493; Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (sc-492; Santa Cruz Biotechnology), p-Akt (68601N; BD PharMingen, San Diego, CA), or cytochrome c (65971A; BD PharMingen).

Results and Discussion

Although TRAIL has been recognized as a key factor in innate immune surveillance against tumor development and metastasis, as well as a potential cancer therapy, little is known about the effects of hypoxia on TRAIL-induced apoptosis. We recently demonstrated that hypoxia significantly inhibits TRAIL-induced apoptosis in the human lung carcinoma cell line A549 (35). In that study, hypoxia was shown to up-regulate antiapoptotic Bcl-2 family member proteins and inhibitor of apoptosis proteins (IAPs) but not proapoptotic TRAIL receptors. Up-regulation of these antiapoptotic molecules was suggested to associate with hypoxia-induced inhibition of TRAIL-induced apopto-

Figure 1. Effects of hypoxia and Bax deficiency on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. A. Bax+/+ and Bax−/− cells were pre-exposed to normoxia or hypoxia for 24 h and further incubated with recombinant TRAIL protein (0–200 ng/ml) for an additional 4 h under the same conditions. Viable cells were stained with crystal violet. The experiments were performed in triplicate at least twice and presented is a representative. B. Bax+/+ and Bax−/− cells were treated with TRAIL under the normoxic or hypoxic conditions as described in A and analyzed for cell viability using crystal violet staining method. Viability of control cells was set at 100%, and viability relative to the control was estimated. The experiments were repeated twice in triplicate; bar, SE.
First, to determine whether hypoxia attenuates TRAIL-induced apoptosis in HCT116 cells, as TRAIL does in A549 cells, Bax+/− and Bax−/− cells were exposed to hypoxia or normoxia for 24 h, followed by treatment with TRAIL (0–200 ng/ml) for an additional 4 h under the same incubation conditions. As shown in Fig. 1, hypoxia significantly inhibited TRAIL-induced apoptosis in Bax+/− cells that were observed to be highly susceptible to TRAIL in normoxia. In contrast, Bax−/− cells were completely resistant to TRAIL-induced apoptosis in normoxia and hypoxia. In terms of TRAIL susceptibility, hypoxia converts Bax+/− cells to Bax−/− cells left in normoxia (compare Bax+/− hypoxia with Bax−/− normoxia; Fig. 1A). In other words, inactivation of Bax function by hypoxia in Bax+/− cells is a key mechanism by which hypoxia inhibits TRAIL-induced apoptosis. Thus, our data suggest that Bax itself or a mechanism inhibiting the Bax function is a target mechanism through which hypoxia inhibits TRAIL-induced apoptosis.

Functional inhibition of Bax can be achieved by two mechanisms: down-regulation of Bax and up-regulation of Bax inhibitors. Up-regulation of Bax inhibitors, such as antiapoptotic Bcl-2 family members Bcl-2 and Bcl-XL, attenuates the function of Bax through a protein-protein interaction. We previously reported that hypoxia up-regulates Bcl-2 and Bcl-XL in A549 cells (35). Thus, we examined the effects of hypoxia on expression of Bax and also these antiapoptotic Bcl-2 family members in HCT116 cells. As observed in A549 cells, hypoxia increased the protein levels of Bcl-2 (Fig. 2A), Bcl-XL, and IAPs (data not shown) in Bax+/− and Bax−/− cells, although increase of Bcl-2 was observed to be greater in Bax+/− than in Bax−/− cells (Fig. 2A). Contrarily, hypoxia treatment did not significantly affect the total expression levels of Bax and active Akt (p-Akt, phosphorylated Akt; Fig. 2A), a key factor mediating cell survival signals. These observations suggest that up-regulation of antiapoptotic Bcl-2 family member proteins and IAPs may play an important role in the mechanism by which hypoxia increases antiapoptotic potential.

Therefore, we next examined their functional role in hypoxia inhibition of TRAIL-induced apoptosis. As a simple approach to evaluate their inhibitory role in our experimental settings, in which cells first pre-exposed to hypoxia then are treated with TRAIL under hypoxic conditions, we first questioned whether the increased levels of hypoxia up-regulated antiapoptotic molecules are maintained even after TRAIL treatment. Interestingly, hypoxia-increased Bcl-2 was decreased by TRAIL to the levels lower than those of the untreated conditions (Fig. 2B). Similarly, a decrease of Bcl-XL and IAPs also was observed (data not shown), whereas the levels of Bax were not affected by TRAIL treatment (Fig. 2B). It is important to note that despite this dramatic decrease of hypoxia-increased antiapoptotic molecules, TRAIL failed to induce apoptosis in hypoxia-treated Bax+/− cells (Fig. 1). Our data suggest that hypoxia inhibits TRAIL-induced apoptosis via other Bax-compising mechanism(s) than up-regulation of the identified antiapoptotic molecules.

It is well known that Bax translocates from the cytosol to the mitochondria when cells are surged with extracellular apoptotic stimuli, including activation of TRAIL receptors (38). Studies have demonstrated that Bax translocation is a pivotal signaling process for inducing the mitochondrial event that is required for effective induction of apoptosis. Thus, based on our data in which hypoxia and/or TRAIL did not significantly affect the total expression levels of Bax (Fig. 2), we finally examined the possibility that hypoxia inhibits Bax translocation. In normoxic Bax+/− cells, TRAIL treatment induced translocation of a significant amount of Bax to the mitochondria, concomitantly releasing cytochrome c from the mitochondria to the cytosol (compare Lane 1 with Lane 5; Fig. 3A). In a sharp contrast, when Bax−/− cells were treated with TRAIL after being pre-exposed to hypoxia, the majority of Bax was detected in the cytosol (compare Lane 2 with Lane 6; Fig. 3A). In Bax−/− cells significantly resistant to TRAIL-induced apoptosis, TRAIL failed to

Fig. 2. No effects of hypoxia on the protein levels of Bax, Bcl-2, and Akt. A, Bax+/− and Bax−/− cells were exposed to hypoxia for 12, 24, or 36 h and subjected to Western blot analysis. B, Bax+/− cells exposed to hypoxia or normoxia for 24 h were incubated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; 200 ng/ml) as indicated for an additional 4 h and subjected to Western blot analysis.
induce the release of cytochrome c irrespective of hypoxia treatment, confirming the known role of Bax in the release of mitochondrial factors, including cytochrome c. Our data clearly demonstrate that hypoxia protects Bax-dependent cells from TRAIL-induced apoptosis by inhibiting Bax translocation. To address whether inhibition of Bax translocation is restricted to HCT116 cells, we additionally examined A549 cells. As observed in HCT116 cells, hypoxia also inhibited TRAIL-induced Bax translocation, concomitantly blocking the release of cytochrome c from the mitochondria (Fig. 3B). Our data suggest that inhibition of Bax translocation is a general mechanism by which hypoxia attenuates apoptosis.

For the first time, we report here that hypoxia inhibits Bax translocation. Despite critical significance of the Bax function in apoptosis, the biochemical and signaling mechanisms for Bax translocation and conformational changes are not fully understood. A line of evidence has demonstrated that a small antiapoptotic peptide, termed humanin, identified to interact with Bax, controls translocation of Bax from the cytosol to the mitochondria (38). Overexpression of humanin blocked Bax translocation, resulting in inhibition of apoptosis induced by many apoptotic stimuli. Interestingly, however, overexpression of humanin did not inhibit TRAIL-induced apoptosis, suggesting that TRAIL may induce Bax translocation in a manner independent of humanin. Nonetheless, currently, whether humanin is involved as a major factor for hypoxic effects in inhibition of Bax translocation remains to be examined.

In detail characterizations for the mechanism for cytochrome c release, several studies have demonstrated that a double deficiency of Bax and Bak impairs cytochrome c release in hepatocytes (39). However, in HCT116 Bax−/− cells expressing Bak, deficiency of Bax alone was shown to suffice blocking cytochrome c release in many studies (22–24). These results suggest that a requirement of Bak for activation of the mitochondrial events depends on cell types. In our own studies, hypoxia, but not TRAIL, was observed to decrease Bax expression in HCT116 cells. Interestingly, this hypoxia-decreased Bax expression was increased by TRAIL back to the levels of the untreated conditions, suggesting that hypoxia inhibition of TRAIL-induced apoptosis is not affected by the protein levels of Bak, at least, in HCT116 cells. Thus, in HCT116 cells, Bax appears to be a key factor in regulating the mitochondrial events.

Recent studies have suggested Bax deficiency as a possible selection mechanism by which tumor cells act against various tumoral activities (23). In this context, inhibition of Bax translocation by hypoxia, resulting from a functional similarity to the Bax-deficient state, may be a mechanism by which tumor cells survive against tumor therapies and an antitumor immunity that induces tumor apoptosis. Thus, our data suggest that an enhancement of Bax translocation from the cytosol to the mitochondria may be important to increase therapeutic potential of tumor therapies in hypoxic conditions. Our further studies aimed to the elucidation of the signaling and biochemical mechanism for Bax translocation and conformational changes are now ongoing.

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