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Hypoxia Inhibition of Apoptosis Induced by Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)[1]

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Hypoxia is a common environmental stress. Particularly, the center of rapidly growing solid tumors is easily exposed to hypoxic conditions. Thus, tumor cell response to hypoxia plays an important role in tumor progression as well as tumor therapy. However, little is known about hypoxic effect on apoptotic cell death. To examine the effects of hypoxia on TRAIL-induced apoptosis, human lung carcinoma A549 cells were exposed to hypoxia and treated with TRAIL protein. Hypoxia significantly protected A549 cells from apoptosis induced by TRAIL. Western blotting analysis demonstrated that hypoxia increased expression of antiapoptotic proteins such as Bcl-2, Bcl-XL, and IAP family members. The increase of these antiapoptotic molecules is believed to play an hypoxia-mediated protective role in TRAIL-induced apoptosis. Our findings suggest that an increase of antiapoptotic proteins induced by hypoxia may regulate the therapeutic activity of TRAIL protein in cancer therapy.

Key Words: **TRAIL; hypoxia; apoptosis; Bcl-2.**

Local growth of malignant tumors is largely dependent upon adequate nutrient and oxygen supply. Oxygen is absolutely indispensable for optimal energy metabolism in mammalian cells, and cancer cells are no exception. The center of a rapidly growing solid tumor is easily exposed to hypoxic conditions or even anoxic conditions (1, 2). Thus, tumor cell responses to hypoxia are important for tumor progression as well as tumor therapy. Recent findings showed that tumor cells exposed to hypoxia increase the antiapoptotic potentials via many dysregulations in apoptosis signaling pathways (3, 4).

Apoptosis is an active cell death process that is genetically regulated. This process plays an important role in the development and homeostasis of multicellular organisms (5). Among apoptosis-inducing proteins, the best characterized are the ligand-type cytokine molecules of the TNF family. TNF family member proteins such as TNF-α, Fas ligand and TRAIL are type II transmembrane molecules that trigger the apoptotic signal cascade by binding to cognate receptors displayed on the cell surface (6, 7).

TRAIL is known to induce apoptosis in a variety of tumor cells but not in most normal cells. Recent preclinical studies showed that repeated systemic administration of biologically active recombinant TRAIL protein limited tumor growth without detectable toxicity (8, 9). These results indicate that TRAIL is a promising therapeutic to treat human cancers.

Several studies have demonstrated that solid tumor cells exposed to hypoxia are resistant to both radiotherapy and most commonly used anticancer drugs (3, 10). However, little is known about the effects of hypoxia on TRAIL-induced tumor cell apoptosis. To assess these effects, we exposed A549 cells to hypoxia and treated them with recombinant TRAIL protein. Here we report that hypoxia-treated A549 cells are significantly resistant to TRAIL-induced apoptosis. Hypoxia increased expression of antiapoptotic proteins such as Bcl-2, Bcl-XL, and IAP family members. Our data suggest that hypoxia-induced upregulation of these molecules may modulate the apoptotic activity of TRAIL protein.

MATERIALS AND METHODS

Cell culture. Human lung carcinoma A549 cells were obtained from ATCC and maintained in F-12K culture medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 μg/ml genta-

Abbreviations used: TRAIL, TNF-related apoptosis-inducing ligand; DR, death receptor; TRAIL-R, TRAIL receptor; IAP, inhibitor of apoptosis protein.

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mycin and 100 μg/ml penicillin–streptomycin). Hypoxia (1% O_2) was induced by maintaining cells inside an air-tight chamber with inflow and outflow valves that were infused with a mixture of 1% O_2.

Cell viability. A549 cells plated in 12 wells were exposed to hypoxia. After 24 h, cells were coincubated with recombinant TRAIL protein (11) for 4 h under the same conditions. As a control, A549 cells were exposed to normoxia for 24 h and treated them with recombinant TRAIL protein for 4 h under normoxia. Cell morphology was photographed under the microscope, and cell viability was determined by the crystal violet staining method (11). 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 to the controls.

Western blotting. To prepare whole-cell lysates, cells were harvested, resuspended in lysis buffer (25 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM DTT, protease inhibitor mixture) and sonicated. Proteins were separated on an 8–15% SDS gel and analyzed by Western blotting as described previously (11). DR4 (AAP-420) and DR5 (AAP-430) were probed with antibody obtained from Stressgen (Victoria, Canada); Bcl-2 (sc-492), Bcl-XL (sc-7195), and cIAP-2 (sc-7944) were from Santa Cruz (Santa Cruz, CA); cIAP-1 (556533) was from BD Pharmingen (San Diego, CA); and XIAP (AF-822) was from R & D Systems (Minneapolis, MN).

RESULTS AND DISCUSSION

Hypoxia Inhibits TRAIL-Induced Apoptosis

Tumor cells exposed to hypoxia have been shown to be resistant to radiotherapy as well as chemotherapy (3, 10). Studies have demonstrated that TRAIL selectively kills tumor cells without damaging normal tissues *in vivo* (8, 9). Despite considerable attention on TRAIL as a promising anticancer therapy, no experimental data linking hypoxia and TRAIL-induced tumor cell apoptosis are available.

To investigate the effects of hypoxia on TRAIL-induced apoptosis, we exposed human lung carcinoma A549 cells to hypoxic or normoxic conditions for 24 h and treated them with biologically active recombinant TRAIL protein (11) for 4 h under the same conditions. As shown in Fig. 1, TRAIL induced apoptotic cell death in cells exposed to normoxia, an oxygenated conditions. In contrast, hypoxia-treated cells were significantly resistant to TRAIL-induced apoptosis under a wide range of TRAIL concentrations (50–200 ng/ml). Cell morphology examination also supported an inhibitory role of hypoxic conditions in TRAIL-induced apoptosis (Fig. 2). To rule out the possibility that hypoxic conditions directly affected TRAIL protein activity, we incubated TRAIL protein for 4 h under hypoxic conditions and examined the apoptotic activity of TRAIL protein. Hypoxia-treated TRAIL protein was as efficient as untreated control TRAIL protein in killing HeLa cells, which indicated that hypoxia did not affect the apoptotic activity of TRAIL protein. Therefore, our data suggest that hypoxia may directly regulate apoptotic and/or antiapoptotic signals in A549 cells.

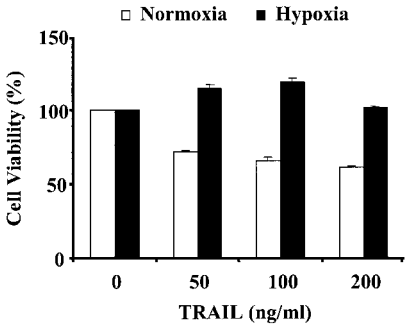

FIG. 1. Effect of hypoxia on TRAIL-induced apoptosis. A549 cells plated in 12 wells were exposed to normoxia or hypoxia for 24 h and coincubated with recombinant TRAIL protein (0–200 ng/ml) for 4 h under the same conditions. Cell viability was determined by crystal violet staining method. Viability of control cells was set at 100%, and viability relative to the control is presented. Experiments were performed in triplicate, at least twice. Bars indicate standard errors.

Hypoxia Increases Expression of Antiapoptotic Proteins

Hypoxia is known to regulate the expression of many genes (12). Nevertheless, little is known about the hypoxia-induced genes that play a direct role in either apoptosis or antiapoptosis. Recently, we (13) and others (14, 15) identified a FADD-activated caspase-8 signaling pathway to be a major signaling pathway in TRAIL-induced apoptosis. Caspase-8 activation cleaved Bid, a Bcl-2 family member, which is involved in mitochondrial events, including cytochrome *c* release (16). These results suggest that cellular factors involved in activation or inhibition of caspases and/or cytochrome *c* release may regulate TRAIL-induced apoptosis. We hypothesized that expression of those factors may be regulated by hypoxia.

To address such possibility, we first examined if hypoxia regulates expression of antiapoptotic molecules such as Bcl-2 and Bcl-XL, both of which have been well known to inhibit cytochrome *c* release from the mitochondria (17, 18). A549 cells were exposed to hypoxia for 1, 6, 12, 24, or 48 h and subjected to Western blotting analysis. As shown in Fig. 3A, hypoxia significantly increased Bcl-2 and Bcl-XL expression. The increase of Bcl-2 expression was detected after 6 h of incubation at hypoxic conditions and sustained up to 24 h in a hypoxic setting. The increase of Bcl-XL expression was slightly delayed as detected during the 12-h incubation. Increased expression also lasted through 48 h hypoxia treatment. Despite different profiles in Bcl-2 and Bcl-XL expression, up-regulation of Bcl-2 and Bcl-XL may account for hypoxia-mediated protection in TRAIL-induced apoptosis, because maximal protection by hypoxia in TRAIL-induced apoptosis was observed after 24 h pretreatment with hypoxia (Fig. 1). Our result is also supported by other investigators who have demonstrated that Bcl-2 and Bcl-XL block TRAIL-induced apoptosis (19). Thus, the overex-

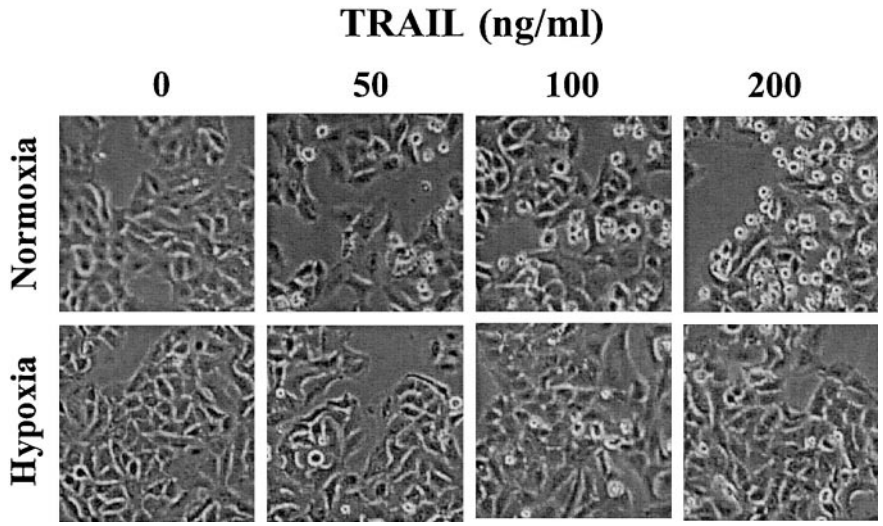

FIG. 2. Morphology of A549 cells treated with TRAIL under hypoxia and normoxia conditions. A549 cells plated in 12 wells were exposed to normoxia or hypoxia for 24 h and coincubated with recombinant TRAIL protein (0–200 ng/ml) for 4 h under the same conditions. Cell morphology was photographed under the microscope.

pression of Bcl-2 family members, Bcl-2 and Bcl-XL may contribute to the protective role of hypoxia.

Next, we examined expression of IAP family members, cIAP-1, cIAP-2 and XIAP, after exposure of A549 cells to hypoxia. IAP family members have been shown to inhibit caspase-9 activation by inhibiting apoptosome formation, which is composed of cytochrome *c*/Apaf-1/caspase-9 (20, 21). The IAP protein family also blocks caspase activation and activity by directly binding active caspases (22, 23). Thus, increase of IAP family proteins may protect TRAIL-induced apoptosis. Hypoxia-treated A549 cells showed increased expression of cIAP-1, cIAP-2, and XIAP. The increase of cIAP-1 expression was rapid, as detected within 1 h after exposure to hypoxia, whereas the increase of cIAP-2 and XIAP expression reached a peak after a 12-h exposure to hypoxia. The expression patterns of cIAP-2 and XIAP were identified to be similar to each other. Expression of IAP family proteins with the exception of cIAP-1, also conceded with the function test results (see Fig. 1). Thus, increased expression of IAP family proteins is also believed to play a hypoxia-induced protective role in TRAIL-induced apoptosis.

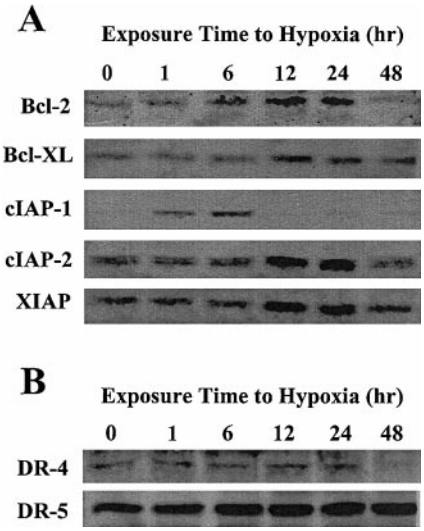

FIG. 3. Expression of TRAIL receptors and antiapoptotic proteins in A549 cells exposed to hypoxia. (A) Expression of antiapoptotic proteins. A549 cells were exposed to hypoxia for 1, 6, 12, 24, or 48 h followed by whole-cell lysate preparation as described under Materials and Methods. Protein samples (40 μg) for each time point were separated on SDS gel, and the corresponding proteins were detected by Western blotting. (B) Expression of TRAIL receptors DR4 and DR5. The same blots prepared as described in A were analyzed for DR4 or DR5 by Western blotting.

It is hypothesized that if hypoxia down-regulates TRAIL receptor DR4 and/or DR5, the TRAIL-R-mediated death signal would be attenuated and result in less apoptosis. Thus, we examined whether hypoxia has a regulatory function in TRAIL receptor expression. Hypoxia did not have a significant influence on DR4 expression (Fig. 3B), even though DR4 expression remarkably decreased after 48 h of hypoxic incubation. However, it is unlikely that this decrease is associated with a protective role of hypoxia in TRAIL-induced apoptosis since the effect of hypoxia on TRAIL-induced apoptosis was tested after 24 h of hypoxic incubation and lasted for 4 h longer. In contrast, hypoxia slightly increased DR5 expression (Fig. 3B). However, this increase did not enhance TRAIL-induced apoptotic cell death (see Fig. 1). Thus, DR5 increase may not be sufficient to counteract antiapoptotic functions driven by antiapoptotic molecules upregulated under hypoxic conditions.

Our data demonstrate that hypoxia protects cells from TRAIL-induced apoptosis. Other studies also showed

that tumor cells under hypoxic conditions are resistant to radiotherapy and chemotherapy (10). These results suggest that hypoxia may be a common resistance mechanism by which tumor cells escape tumoricidal activity of cancer therapies or natural defense systems *in vivo*. Even though the detailed mechanisms are not fully understood, our data suggest that an increase of antiapoptotic proteins may be one of the key mechanisms. Thus, for a successful anticancer therapy, it is imperative to develop that a strategy against hypoxic tumor cells. Combination therapies may be more effective than TRAIL protein alone. For example, TRAIL protein plus a chemotherapeutic drug that is selectively toxic to hypoxic tumor cells such as mitomycin C (24), triapazamine (25) or AQ4N (26), may be a good combination therapy for hypoxic tumor cells. TRAIL protein plus an inhibitor of the antiapoptotic proteins increased under hypoxic conditions may also be a possible consideration for use in a combination therapy for hypoxic tumor cell killing. Antisense of Bcl-2 may also increase TRAIL-induced apoptotic activity under hypoxic conditions. Another avenue to consider would include whether SMAC/DIABLO, a protein that directly binds and inactivates IAP family members induced by hypoxia, enhances TRAIL-induced apoptosis under hypoxia.

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