

Enforced Expression of a Truncated Form of Bax- α (tBax) Driven by Human Telomerase Reverse Transcriptase (hTERT) Promoter Sensitizes Tumor Cells to Chemotherapeutic Agents or Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL)

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Abstract. *Background:* We have recently demonstrated that a truncated form of the pro-apoptotic molecule Bax- α (tBax) at the NH₂-terminus is more potent in inducing cell death than wild-type (wt) Bax. *In the present study, whether efficient cell death is induced by tBax expression from human telomerase reverse transcriptase (hTERT) promoter, which is highly active in tumor but not normal cells, was examined. Materials and Methods:* Cell death was assessed by luciferase reporter assay and the annexin staining method. *Results:* Enforced expression of tBax resulted in cell death to a greater extent than wt Bax in two types of tumor cells: osteogenic sarcoma MG-63 and squamous cell carcinoma MIT7. The tBax sensitized these tumor cells to death induced by chemotherapeutic agents. Moreover, tBax enhanced cell death induced by the tumor necrosis factor-related apoptosis-inducing ligand to a high level, compared with wt Bax. Furthermore, tBax efficiently induced death of the MG-63 cells overexpressing Bcl-x_L, compared with wt Bax. *Conclusion:* tBax alone, or in combination with chemotherapeutic agents, would be a promising candidate for human gene therapy in the setting of carcinoma, especially for tumors containing high levels of Bcl-x_L.

Apoptosis, programmed cell death, is an evolutionarily conserved cell death that regulates tissue homeostasis. The cellular changes that lead to resistance to apoptosis induction play a crucial role in the initiation and/or progression of

tumors. Tumor gene therapy, using cytotoxicity-inducing genes via induction of apoptosis, has been carried out with some success both *in vitro* and *in vivo* (1, 2). The apoptosis-inducing genes include Bax- α , tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), Fas-ligand (CD95-L) and p53 (3, 4). Bax- α is a member of the Bcl-2 family of proteins comprising pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-x_L and Bcl-2) molecules (5, 6). We and others have recently demonstrated that the introduction of the truncated form of Bax- α (tBax), driven by CAG promoter, induces cell death more efficiently than wild-type (wt) Bax- α (7, 8). The tBax-mediated cell death was not restored by Bcl-x_L, whereas Bcl-x_L substantially rescued Bax- α -mediated toxicity. Moreover, it has been reported that the combination of Bax- α with chemotherapeutic agents (9, 10), irradiation (11), or TRAIL (12) was effective for the treatment of tumors in an additive or synergistic manner. However, Bax, under the control of ubiquitous promoters such as CAG and SV-40, could be toxic to normal cells as well as tumor cells, resulting in unfavorable effects. Thus, development of the specific expression of cytotoxic molecules in tumor cells is of critical importance.

The human telomerase reverse transcriptase (hTERT) promoter has been reported to be highly active in most tumors, but not normal somatic cells, and has been employed for the targeted expression of genes of interest in a variety of tumor cell lines (13-16). For example, the hTERT promoter caused a strong tumor-specific expression of cytotoxic genes such as caspases (14) and Bax- α (13, 17), resulting in cell death. Whether a plasmid vector expressing the tBax gene driven by the hTERT promoter would induce greater cell death than wt Bax, and whether the combined treatment of tBax with chemotherapeutic agents or TRAIL would result in increased cell death compared with either of the latter two alone, were investigated.

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Materials and Methods

Cell culture. The human osteosarcoma cell line MG-63 (Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan), MG-63-overexpressing Bcl-x_L (18) and the human squamous carcinoma cell (SCC) line MIT7 (19) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 µg/ml kanamycin, at 37°C in humidified air with 5% CO₂. At each passage, the cells were harvested as single cell suspensions using trypsin/EDTA (Invitrogen, Carlsbad, CA, USA).

Transient transfection. wt Bax or tBax was introduced into the expression vector hTERT promoter/pGL3 to create hTERT/tBax or hTERT/wt Bax. Cells (2x10⁵)/6-well plates, allowed to grow with medium alone for 24 h, were transfected with wt Bax, tBax, or control vector alone in combination with the luciferase reporter plasmid using DoFect GT1 (Dojindo, Kumamoto, Japan) or Eugene6 (Roche, Indianapolis, IN, USA), according to the manufacturers' instruction. After incubation for 24 to 48 h, the cells were assayed for luciferase activity using a Dual Luciferase Reporter System (Pomega, Madison, WI, USA). Luciferase activity was monitored with a Lumat LB9507 luminometer (Berthold Technologies GmbH and Co., KG, Wildbad, Germany).

Western blot analysis. Western blot analysis was carried out as previously described (20). Briefly, the blots were incubated with the primary antibodies (Abs), anti-HA Abs (Covance, Berkeley, CA, USA) or anti-actin Abs (Sigma, St Louis, MO, USA), followed by several washes. The blots were developed with a horseradish peroxidase (HRP)-labelled goat anti-mouse IgG or goat anti-rabbit IgG (Cappel, Durham, NC, USA), and ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturers' recommendation.

Measurement of cell death. Cell death was determined by the procedure as described by Nechushtan *et al.* (21). Briefly, the cells were co-transfected with various weights of wt Bax or tBax and 0.2 µg luciferase reporter vector plasmid. After incubation with medium alone for 24 h, the cells were exposed to cisplatin (CDDP), carboplatin (CBDCA), etoposide, recombinant soluble TRAIL (Alexis Biochemicals, Carlsbad, CA, USA), or medium alone for a further 24 h, followed by assay for luciferase activity. In some experiments, the cells were co-transfected with Bax/tBax and the expression vector containing enhanced GFP (EGFP), followed by exposure to the reagents for the indicated times. The cells were assayed for apoptosis using an Annexin V-Cy5 Apoptosis Detection Kit (BioVision Inc., Mountain View, CA, USA), according to the manufacturer's instructions. Briefly, the cells were stained with Annexin V-Cy5, followed by analysis using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). The proportion of the cells undergoing apoptosis from EGFP-positive cells was calculated as a percentage by Cell Quest software (BD Biosciences). The relative apoptotic cell death was calculated by the ratio of the percent apoptotic cell death in the experimental group relative to the control group.

Statistical analysis. The data were expressed as means±SD for each group. Statistics were carried out with a SigmaStat for Windows software package. To determine whether a combination

of Bax/tBax with chemotherapeutic agents or TRAIL has synergistic effects, two-way ANOVA was carried out. Other comparisons were made with the Student's *t*-test. Statistical significance was set at 0.05.

Results

Truncated form of Bax-α induces cell death more efficiently than wt Bax. We have previously demonstrated that the enforced expression of tBax, driven by the CMV promoter, induces cell death to a greater extent than wt Bax in 293T cells (8). Since hTERT is highly active in most tumor cells, we tried to determine whether tBax from the hTERT promoter also induces cell death efficiently in other cell lines. Various concentrations of plasmid DNA encoding tBax or wt Bax were co-transfected with control vector encoding the luciferase reporter gene into the osteogenic sarcoma cell line MG-63 lacking p53 (22), or the squamous cell carcinoma (SCC) MIT7 (19). Cell death was determined by a decline of the expression of the luciferase reporter gene, as previously described (8). A transient introduction of Bax-α (0.5 – 4.5 µg) into the MG-63 cells resulted in substantial levels of cell death 24 h after transfection, compared with the control vector alone, which was further enhanced by tBax (Figure 1A). A similar pattern of tBax-mediated cell death was found in the MIT7 cells.

To verify that the convenient luciferase assay correlates with the annexin staining method, MG-63 cells were co-transfected with tBax, Bax, or the control vector in combination with the EGFP construct. Following incubation with medium alone for 24 h, the cells were assayed by the annexin V method. The introduction of Bax into the MG-63 cells resulted in an enhanced rate of apoptotic cell death, compared with the control, and this was further increased by the introduction of tBax (Figure 1B). Next, it was verified that exogenous Bax or tBax protein is expressed in the cells after transfection. The Bax or tBax cDNA was transfected into MG-63 or MIT7 cells, followed by assessment using Western blotting. Although both Bax and tBax were found in both cell lines, the tBax levels were considerably lower than Bax (Figure 1C), as previously reported (8), implying that tBax is more toxic to cells than wt Bax. These findings suggest that tBax induces cell death more efficiently than wt Bax.

tBax sensitizes tumor cells to the chemotherapeutic agents CDDP, CBDCA, or etoposide. We have recently reported that both MG-63 and MIT7 cells undergo apoptosis in response to CDDP or etoposide (19, 23). To address whether a transient administration of Bax results in an increased cell death in combination with chemotherapeutic agents, the MG-63 or MIT7 cells were co-transfected with tBax, wt Bax, or the control vector alone,

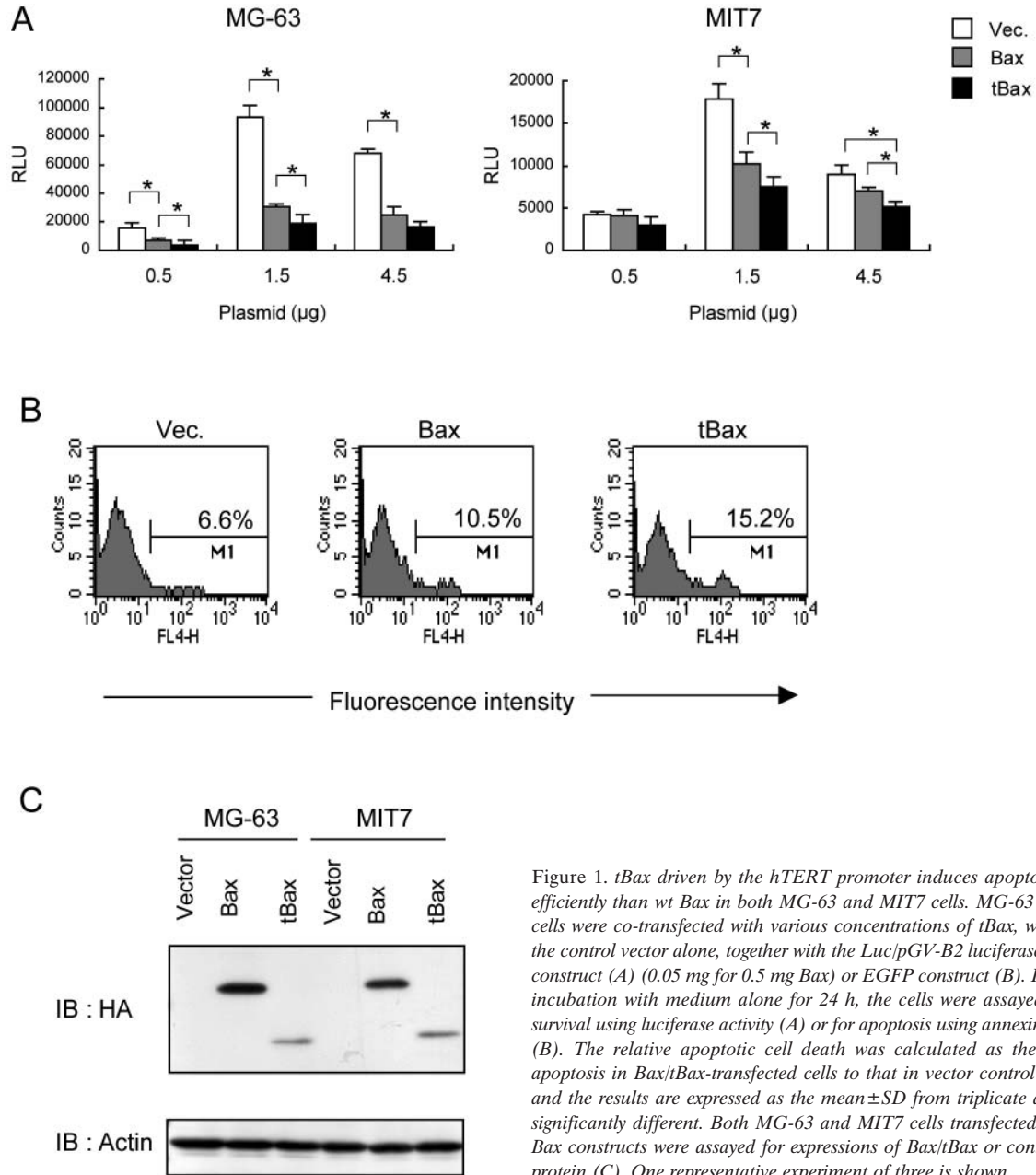


Figure 1. tBax driven by the hTERT promoter induces apoptosis more efficiently than wt Bax in both MG-63 and MIT7 cells. MG-63 or MIT7 cells were co-transfected with various concentrations of tBax, wt Bax, or the control vector alone, together with the Luc/pGV-B2 luciferase reporter construct (A) (0.05 mg for 0.5 mg Bax) or EGFP construct (B). Following incubation with medium alone for 24 h, the cells were assayed for cell survival using luciferase activity (A) or for apoptosis using annexin staining (B). The relative apoptotic cell death was calculated as the ratio of apoptosis in Bax/tBax-transfected cells to that in vector control cultures, and the results are expressed as the mean \pm SD from triplicate assays. *, significantly different. Both MG-63 and MIT7 cells transfected with the Bax constructs were assayed for expressions of Bax/tBax or control actin protein (C). One representative experiment of three is shown.

together with a luciferase reporter construct. Following incubation with the medium alone for 24 h, the cells were exposed to various concentrations of CDDP or etoposide for a further 24 h, followed by assay for luciferase activity. The Bax- α - or the tBax-mediated cell death was further enhanced by CDDP or CBDCA in both the MG-63 and MIT-7 cells in a dose-dependent manner (Figures 2A and 2C). Similar to CDDP or CBDCA, etoposide enhanced the Bax- or tBax-mediated cell death in MG-63 cells, but

not in MIT-7 cells (Figures 2B and 2D). These results suggest that chemotherapeutic agents sensitize tumor cells to tBax-mediated cell death.

tBax- α in combination with TRAIL induces an increased level of cell death compared with either agent alone. TRAIL has been demonstrated to provide a co-operative mode of cell death in conjunction with chemotherapeutic agents (23-25), which function as mitochondrial-damaging agents. Thus,

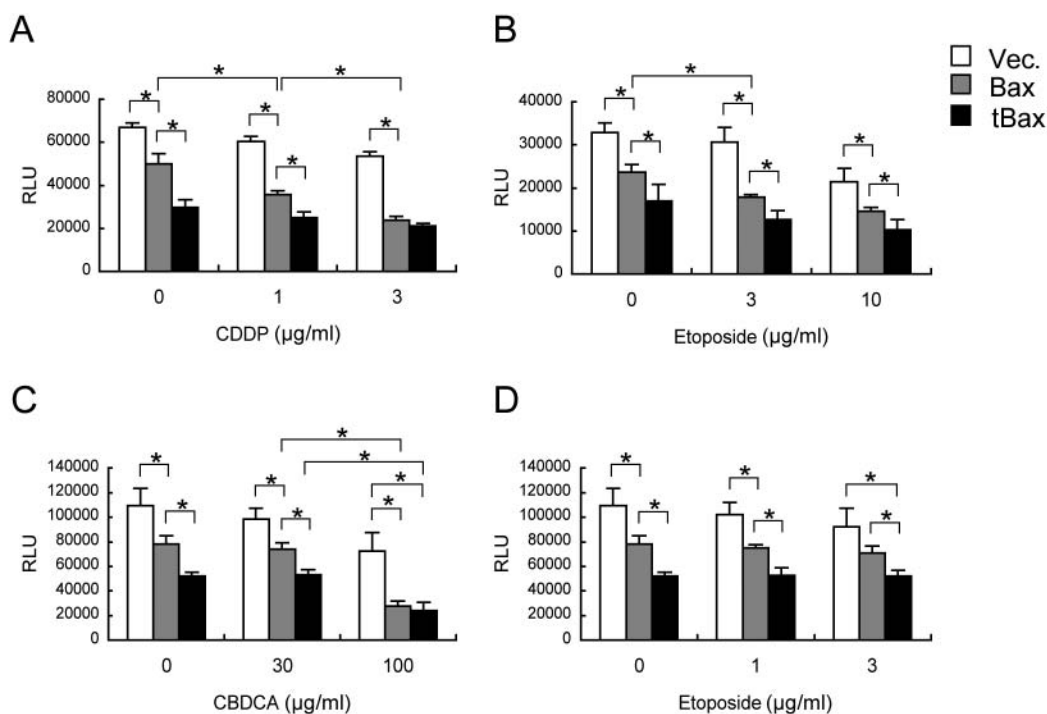


Figure 2. Treatment with tBax in combination with chemotherapeutic agent induces an increased toxicity, compared with either agent alone. MG-63 (A, B) or MIT7 (C, D) cells co-transfected with the Bax constructs, as described in Figure 1, were incubated with medium alone for 24 h, followed by stimulation with CDDP (A), CBDCA (C), or etoposide (B, D) for a further 24 h. The cells were then assayed for cell survival using luciferase activity. The results are expressed as the mean \pm SD from three independent experiments.

either the Bax- or tBax- α -transfected cells, cultured with medium alone for 24 h, were stimulated with TRAIL for a further 24 h. tBax in combination with TRAIL induced a higher level of cytotoxicity compared with either agent alone in MG-63 cells (Figure 3A). In MIT7 cells, the tBax-mediated cell death was substantially enhanced by TRAIL, which had only a slight effect up to 10 ng/ml (Figure 3B). Together, the tBax- α -mediated cytotoxicity was more pronounced than wt Bax, and was potentiated by either chemotherapeutic agent or TRAIL.

tBax induces cell death in MG-63 cells expressing Bcl-x_L. We and others have previously demonstrated that cells overexpressing Bcl-x_L are resistant to multiple chemotherapeutic agents (26-28). To examine whether the tBax-mediated cytotoxicity overcomes Bcl-x_L-mediated cytoprotection, MG-63 cells overexpressing Bcl-x_L were transfected with tBax, Bax, or the control vector alone, followed by an assay for cell survival using luciferase activity. The Bcl-x_L-overexpressing cells were almost resistant to Bax-mediated cell death (20% versus 40% in control MG-63 cells), as recently reported in 293T cells (Figure 4A). Interestingly, however, comparable levels of tBax-mediated cell death were detected in both the Bcl-x_L-

overexpressing and control MG-63 cells. The tBax- or Bax-mediated effects were confirmed by the annexin staining method (Figure 4B). These results suggest that tBax somehow abrogates Bcl-x_L-mediated cytoprotection.

Discussion

Chemotherapeutic agents have contributed to an increased survival rate of patients with most tumors, including osteogenic sarcoma and SCC (29, 30). However, their effect is limited because of inherent and/or acquired resistance to the chemotherapeutic agents (31, 32). Multiple chemotherapeutic agents induce apoptotic cell death at least through the induction of apoptosis (26, 33). Recent developments in gene therapy using cytotoxic genes include Bax, p53 and TNF family members (24, 26, 34). We have recently reported that the introduction of a truncated form of Bax, driven by a CMV promoter, is more potent than wt Bax, and that the tBax induces cytotoxicity against tumor cells overexpressing Bcl-x_L (8). The pro-apoptotic gene Bax, driven by ubiquitous promoters including CMV and SV-40, may induce toxicity against normal cells, resulting in adverse effects. To obtain tumor-selective expression of cytotoxic genes, the hTERT promoter, which is highly active in most

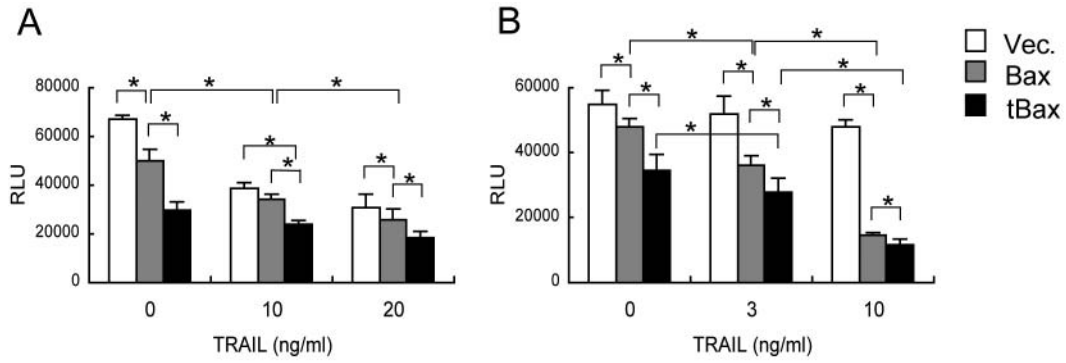


Figure 3. Treatment with tBax in combination with TRAIL induces an increased toxicity, compared with either agent alone. MG-63 (A) or MIT7 (B) cells co-transfected with the Bax constructs, as described in Figure 1, were incubated with medium alone for 24 h and then stimulated with or without TRAIL (3 or 10 ng/ml for MIT7; 10 or 20 ng/ml for MG-63) for a further 24 h, followed by an assay for luciferase activity. The results are expressed as the mean \pm SD from three independent experiments. *, significantly different. One representative experiment out of three is shown.

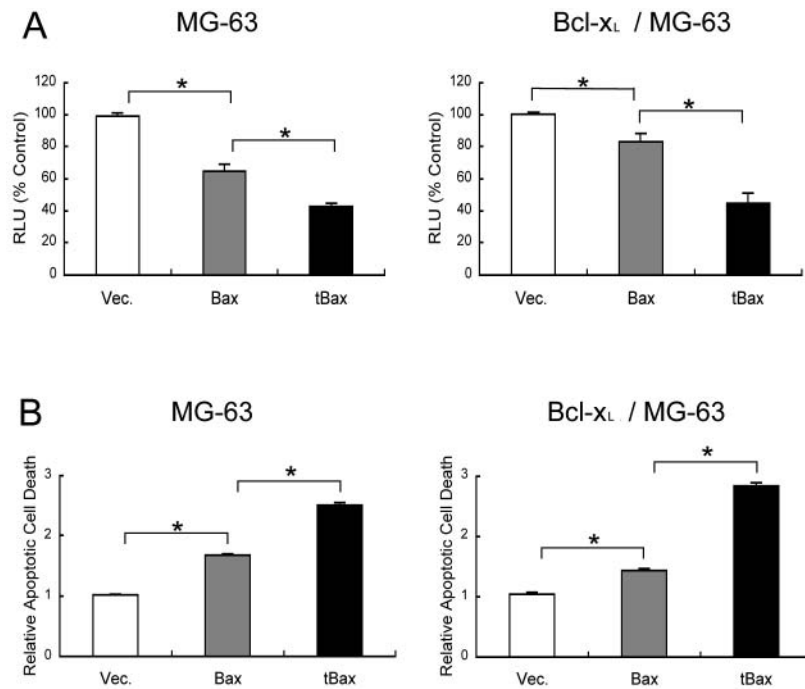


Figure 4. tBax abrogates Bcl-x_L-mediated cytoprotection in MG-63 cells. MG-63 cells overexpressing Bcl-x_L were co-transfected with Bax/tBax, together with the Luc/pGV-B2 luciferase reporter construct. After 24-h incubation with the medium alone, the cells were assayed for luciferase activity (A) or annexin staining (B), as described in Figure 1. The results are expressed as the mean \pm SD from triplicate assays. *, significantly different. One representative experiment out of three is shown.

human cancer cells (35), is employed to induce expression of tBax or Bax genes.

The administration of Bax alone (13, 36, 37), or together with chemotherapeutic agents or TRAIL (3, 10, 12, 38), has been demonstrated to induce the efficient death of tumor cells. In the present study, it was confirmed that Bax, driven by the hTERT promoter, induced cell death in sarcoma and

SCC cells, and further demonstrated that tBax (corresponding to amino acids 29 – 192), driven by the hTERT promoter, is more effective than Bax in the induction of cell death. The tBax- α -mediated cytotoxicity was further enhanced by both the chemotherapeutic agents (CDDP or etoposide) or TRAIL. Moreover, hTERT-driven tBax gene expression induced cytotoxicity against Bcl-x_L-

overexpressing MG-63 sarcoma cells more efficiently than wt Bax. Thus, tBax could be a candidate for potential human gene therapy against tumors.

The Bcl-2 family of proteins, comprising pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-x_L, Bcl-2) members, play a crucial role in the induction of apoptosis (26, 33), which is reported to be involved in the initiation and/or progression of a variety of tumors including MG-63 and SCC (18, 19, 27). Most chemotherapeutic agents induce cell death *via* induction of apoptosis, probably through a fall in the mitochondrial membrane potential. Tumor cells overexpressing Bcl-x_L showed resistance to multiple chemotherapeutic agents including CDDP and etoposide (26-28). tBax lacking the N-terminal 28 amino acids was more effective in the induction of apoptosis of Bcl-x_L-overexpressing MG-63 cells than wt Bax (Figure 4B). Consistent with our findings, the NH₂-terminal truncated Bax- α (DN Bax; corresponding to amino acids 112–192) was a more potent inducer of cell death than wt Bax (7). tBax contains the BH3 domain and associates with Bcl-x_L (8), while DN Bax does not contain this domain (7), although both agents showed resistance to Bcl-x_L-mediated cytoprotection. The molecular mechanism(s) underlying the distinct agents inducing efficient cell death remain unresolved at present.

The combined treatment of tumor cells with mitochondrial-damaging chemotherapeutic agents and TRAIL has been effective for the induction of additive/synergistic apoptotic cell death in some tumors including sarcoma and SCC (23-25, 39). Consistent with this observation, tBax in combination with TRAIL resulted in an increased cell death in an additive/synergistic manner (Figures 3A and 3B), suggesting that this mitochondria-initiated intrinsic death pathway is amplified by a distinct receptor-mediated extrinsic pathway including TRAIL-receptors (TRAIL-Rs). The TRAIL-Rs are expressed in diverse cell types, and the interaction of TRAIL with TRAIL-Rs results in apoptosis of many transformed cell types, but not of normal cells (40). Thus, tBax driven by the hTERT promoter together with TRAIL would be effective for tumor cells with minimum effects on normal cells.

p53 mutations have been demonstrated to confer chemoresistance in some cell types (41, 42). Since one of the targets of p53 is Bax- α (43), it can be anticipated that Bax-mediated gene therapy would be effective even in tumor cells with p53 mutations. Indeed, substantial cell death mediated by wt Bax or tBax was found in MG-63 cells lacking p53 (22), either alone or in combination with chemotherapeutic agents (Figures 1A, 2A and 2B). Thus, tBax would be effective in the induction of apoptosis in p53-negative cells.

In the present study, it was shown that hTERT-driven tBax gene expression alone, or in conjunction with TRAIL or chemotherapeutic drugs, resulted in efficient apoptotic cell death in tumor cells including sarcoma and SCC. The

findings suggest that tBax gene therapy, under the control of the hTERT promoter, is a promising approach for patients with tumor, especially chemotherapy-resistant tumors harboring Bcl-x_L.

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