

Bax Induction Activates Apoptotic Cascade via Mitochondrial Cytochrome *c* Release and *Bax* Overexpression Enhances Apoptosis Induced by Chemotherapeutic Agents in DLD-1 Colon Cancer Cells

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Cancer cells express different levels of apoptosis-promoting *Bax* protein. The present study evaluated whether induction of *Bax* initiates apoptosis and whether *Bax* overexpression enhances apoptosis induced by several chemotherapeutic agents in DLD-1 colon cancer cells, which originally express a high level of endogenous *Bax* protein and a low level of *Bcl-2* protein. To investigate these two points, parental DLD-1 cells were transfected with the Tet-On *Bax* induction system (*pTet-On* and *pTRE-Bax* plasmids), and stable transduced cells were obtained. Induction of *Bax* by the Tet-On system initiated cytochrome *c* release from mitochondria, caspase-3 activation, and apoptosis to some extent in DLD-1 cells. Apoptosis induced by a chemotherapeutic agent, 5-fluorouracil, mitomycin C, paclitaxel, doxorubicin, or cisplatin, was enhanced by *Bax* overexpression. These findings suggest that *Bax*-overexpression-based gene therapy combined with chemotherapy would be effective in the treatment of colon cancer.

Key words: *Bax* — Tet-On system — DLD-1 colon cancer cells — Mitochondrial cytochrome *c* release — Chemotherapeutic agents

Doses of chemotherapeutic agents are limited by various toxicities. Combined therapies with different mechanisms may be a more effective approach to improve the outcome of chemotherapy and bring survival benefit. Anti-cancer effects induced by various chemotherapeutic agents are considered to be based on induction of apoptosis and are mediated through a final common pathway; activation of caspase-3 and subsequent DNA fragmentation.^{1–4} With the aim of enhancing chemotherapeutic agent-induced apoptosis, we have focused on the function of the apoptosis-promoting gene, *Bax*. It has been reported that *Bax* induction alone induces apoptosis in Jurkat cells,⁵ which have a *Bax* gene mutation.⁶ Strobel *et al.* have reported that *Bax* overexpression enhances apoptosis induced by paclitaxel, vincristine and doxorubicin, but not by carboplatin, etoposide or hydroxyurea in ovarian cancer cells.⁷ Wagener *et al.* have reported that *Bax* induction alone does not induce apoptosis of breast cancer cells, but sensitizes the cells to epirubicin-induced apoptosis.⁸ We have shown that *Bax* overexpression sensitizes K562 erythroid leukemia cells to apoptosis induced by β -D-arabinofuranoside, doxorubicin, and SN-38 (irinotecan), but not to etoposide.⁹ These findings suggest that the effects of *Bax* overexpression to induce apoptosis and to enhance chemotherapeutic agent-induced apoptosis are different among different cell lines and chemotherapeutic agents.

Patients with advanced colorectal cancer are often treated with 5-fluorouracil-based regimens; however, the responses are partial and no survival benefit has been demonstrated.¹⁰ Mutation of the tumor suppressor gene *p53* is frequent in colon cancer cells,¹¹ and in a clinical treatment of lung cancer, wild-type *p53* gene delivery to cancer cells has been performed.¹² *Bax* gene is considered to be one of the downstream genes regulated by *p53*, and the promoter of *Bax* has a *p53* binding domain.¹³ *Bax* gene mutation has been reported in hereditary non-polyposis colorectal cancer, but not in other types of colorectal cancer.^{14–16} In addition, there is no report about the sensitization of colon cancer cells to chemotherapeutic agents by *Bax* overexpression. In the present study, we examined whether *Bax* induction alone initiates apoptosis in DLD-1 colon cancer cells and whether *Bax* overexpression sensitizes DLD-1 colon cancer cells to apoptosis induced by chemotherapeutic agents, 5-fluorouracil, mitomycin C, paclitaxel, doxorubicin, and cisplatin.

MATERIALS AND METHODS

Cell culture DLD-1 cells were obtained from the American Type Culture Collection (Rockville, MD), and were maintained in RPMI-1640 medium supplemented with 10% Tet-system approved fetal calf serum (Clontech, Palo Alto, CA) under air containing 5% CO₂ in an incubator at 37°C.

DNA transfection *pTet-On* regulator plasmid (*pTet-On*)¹⁷ and *pTRE* response plasmid (*pTRE*)¹⁸ were purchased

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from Clontech. *Bax* gene was inserted to the *EcoRI* cloning site of *pTRE* (*pTRE-Bax*). *pTet-On* was transfected into DLD-1 cells using Lipofectamine (Life Technologies, Rockville, MD) and stable transfectants (DLD-1-*pTet-On*) were selected in RPMI-1640 medium containing 0.8 mg/ml G418 (Life Technologies) for more than 4 weeks. *pTRE-Bax* and *pTK-Hyg*, which contains hygromycin-resistance gene, were combined in a ratio of twenty to one and ethanol-precipitated. DLD-1-*pTet-On* cells were transfected with *pTRE-Bax/pTK-Hyg*, and stable transfectants were selected in RPMI-1640 medium containing 0.4 mg/ml hygromycin (Sigma, St. Louis, MO).

Western blotting Total cellular protein was extracted with NP-40-based lysis buffer and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.¹⁹⁾ After having been transferred to an Immobilon membrane (Millipore, Bedford, MA), the proteins were incubated overnight with antibodies against Bax (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA), caspase 3 (Santa Cruz), cytochrome *c* (Pharmingen, San Diego, CA), Bcl-2 (DAKO A/S, Denmark), Bcl-X (Santa Cruz) and β -actin (AC-15) (Sigma). The levels of protein were analyzed using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) according to the manufacturer's instructions. To detect the release of cytochrome *c* from mitochondria, cytosol fraction was extracted with mannitol-based lysis buffer,²⁰⁾ and the mitochondria-containing fraction was extracted from the pellet with NP-40-based lysis buffer.¹⁹⁾

Flow cytometry To quantitate apoptotic cells, an apoptosis detection system (fluorescein) (Promega, Madison, WI)

was used according to the manufacturer's instructions. This system measures the fragmented DNA of apoptotic cells and is called terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay.

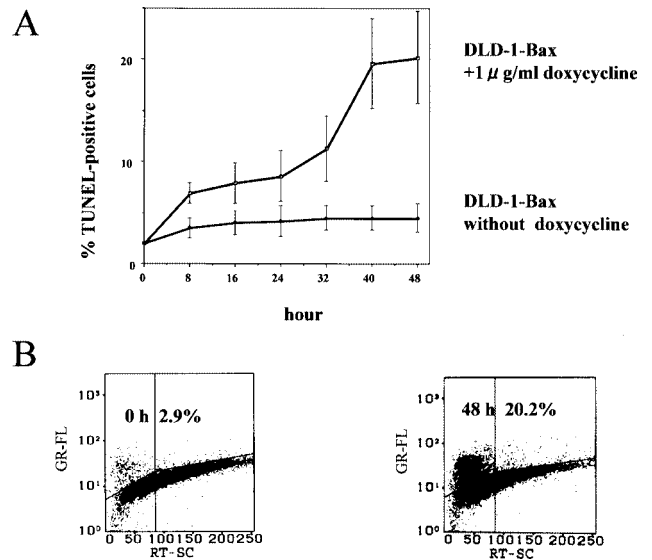


Fig. 1. DLD-1 cells stably transfected with the *Bax*-inducible Tet-On system, DLD-1-*pTet-On/pTRE-Bax* (DLD-1-*Bax*) cells, were used. A. TUNEL-positive cells appeared 6–12 h after addition of 1 μ g/ml doxycycline, and maximum levels were detected at 36–48 h. Data are presented as the average of three independent experiments with standard deviations. B. Flow cytometry of TUNEL assay before and 48 h after addition of 1 μ g/ml doxycycline.

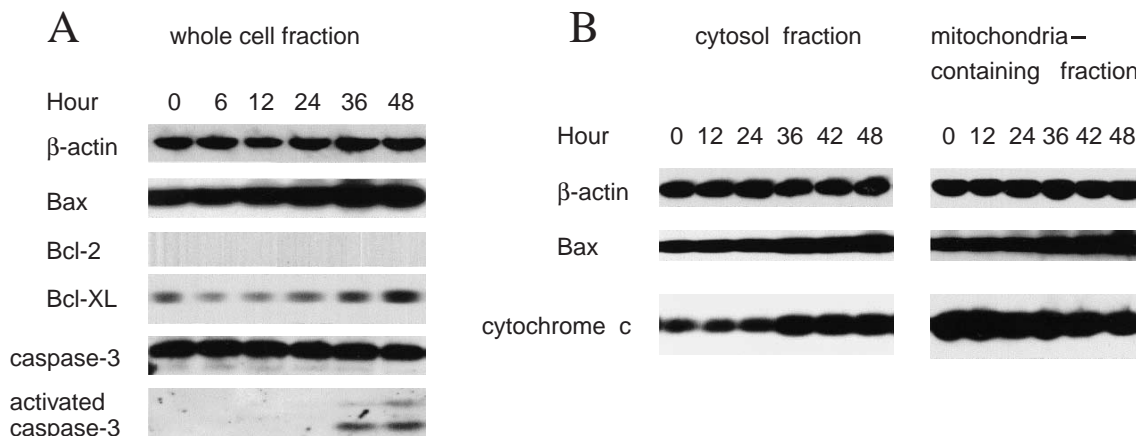


Fig. 2. A. Western blotting of whole cell fraction extracted from DLD-1-*Bax* cells. Caspase-3 activation was observed from 36 h after addition of doxycycline. Bcl-2 was not detected at any time point and Bcl-XL was slightly induced 48 h after addition of doxycycline. B. Western blotting of cytosol fraction and mitochondria-containing fraction. Bax induction was observed in both cytosol and mitochondria-containing fraction. Increase of cytochrome *c* in cytosol fraction was observed 24 h after addition of doxycycline and decrease of cytochrome *c* in mitochondria-containing fraction was observed 36 h after addition of doxycycline.

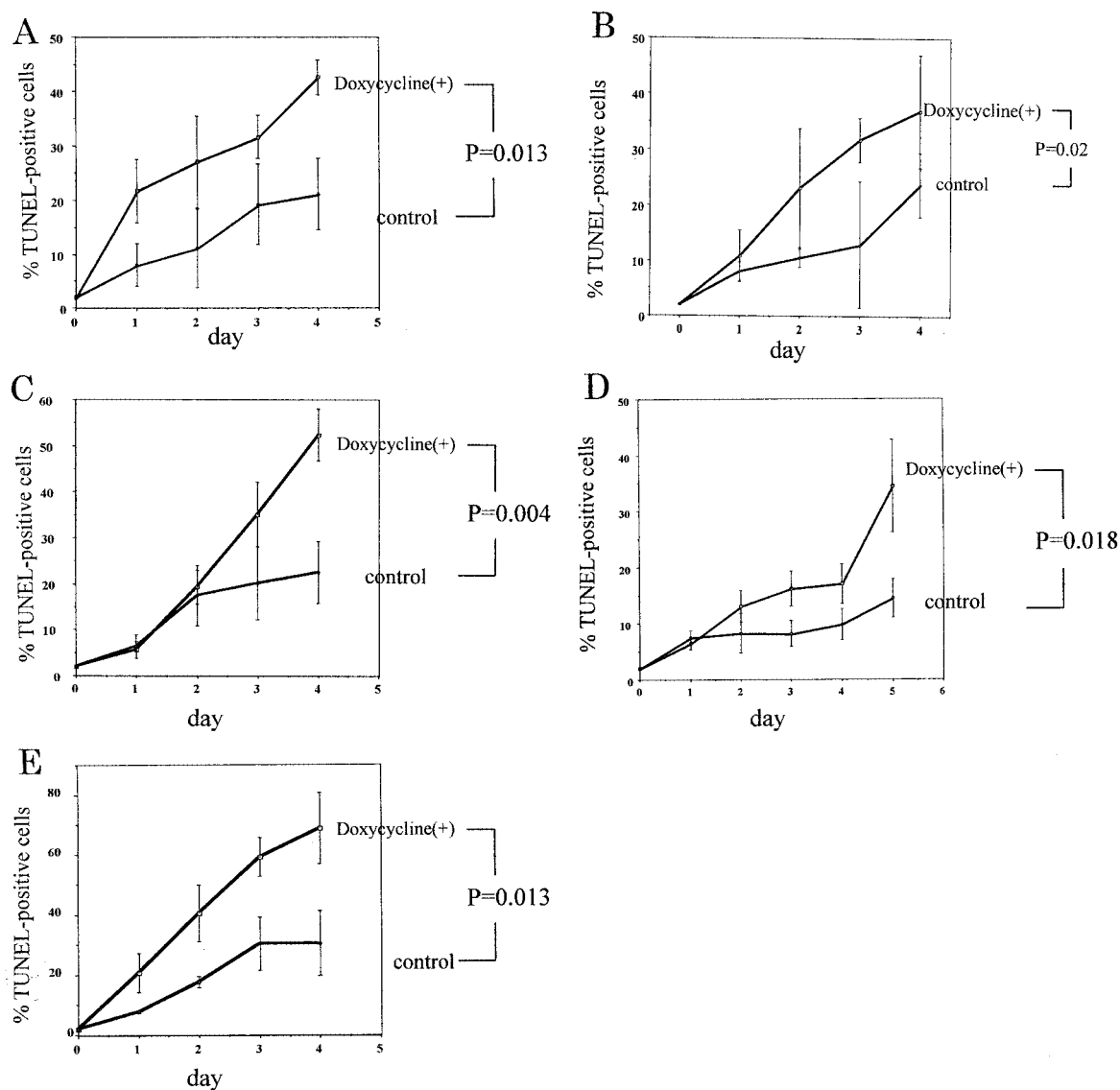


Fig. 3. TUNEL-positive cells among DLD-1-Bax cells with or without induction of *Bax* after treatment with various chemotherapeutic agents. A. 25 $\mu\text{g/ml}$ 5-fluorouracil. B. 2 $\mu\text{g/ml}$ mitomycin C. C. 1 $\mu\text{g/ml}$ paclitaxel. D. 1 $\mu\text{g/ml}$ doxorubicin. E. 10 $\mu\text{g/ml}$ cisplatin. Data are presented as the average of three independent experiments with standard deviations.

RESULTS

Establishment of a stable cell line transfected with Tet-On system To evaluate apoptosis induced by *Bax* overexpression in DLD-1 colon cancer in the absence of any cytotoxic signals, a stable cell line (mixed population of stable transfectants), DLD-1-*pTet-On/pTRE-Bax/pTK-Hyg* (DLD-1-*Bax*), was established.

Detection of apoptosis and western blotting after induction of *Bax* TUNEL-positive cells increased after exposure to 1 $\mu\text{g/ml}$ doxycycline and the highest percent-

ages of TUNEL-positive cells were observed at the 36 h and 48 h time points (Fig. 1). Continuous exposure of these cells to 1 $\mu\text{g/ml}$ doxycycline did not result in total cell death but resulted in establishment of *Bax*-overexpressing stable cell lines (data not shown). Western blotting using whole cell fraction revealed *Bax* induction (Fig. 2A) and caspase-3 activation (Fig. 2A) 36 h after exposure to doxycycline. Bcl-2 protein expression did not show any change and Bcl-XL was slightly elevated 36–48 h after exposure to doxycycline (Fig. 2A). *Bax* induction was observed in both cytosol and mitochondria-containing

fractions (Fig. 2B). Cytochrome *c* release from mitochondria to cytosol was detected 36 h after exposure to doxycycline.

Apoptosis induced by chemotherapeutic agents Concentrations of anticancer agents used in this study are based on previous reports,^{7,9} ten times higher concentrations than the maximum plasma levels in clinical use and preliminary dose-setting experiments. DLD-1-*Bax* cells with or without *Bax* induction were treated with a chemotherapeutic agent, 25 $\mu\text{g}/\text{ml}$ 5-fluorouracil, 2 $\mu\text{g}/\text{ml}$ mitomycin C, 1 $\mu\text{g}/\text{ml}$ paclitaxel, 1 $\mu\text{g}/\text{ml}$ doxorubicin, or 10 $\mu\text{g}/\text{ml}$ cisplatin, and apoptosis was evaluated by TUNEL assay (Fig. 3). In the preliminary experiments, DLD-1-*Bax* cells were incubated with 1 $\mu\text{g}/\text{ml}$ doxycycline 6 h or 24 h before addition of chemotherapeutic agents, but there was no difference in apoptosis between 6 h and 24 h. Therefore, 1 $\mu\text{g}/\text{ml}$ doxycycline was added 6 h before chemotherapeutic agent treatments. Four or five days after treatment with chemotherapeutic agents, more-than-additive numbers of apoptotic cells were observed in chemotherapeutic agent-treated DLD-1-*Bax* cells with *Bax* overexpression compared with those lacking *Bax* induction (Fig. 3).

DISCUSSION

It has been reported that *Bax* induction alone induces apoptosis in Jurkat cells,⁵ but Jurkat cells have a *Bax* gene mutation and do not express *Bax* protein.⁶ Cancer cells express different levels of proapoptotic *Bax* protein.⁶ The present study demonstrated that *Bax* induction alone initiated cytochrome *c* release from mitochondria, activation of caspase 3 and apoptosis to some extent in DLD-1 cells, which originally express a high level of proapoptotic *Bax* protein. Recently, a pathway between *Bax* induction and caspase-3 activation has been reported. Overexpressed *Bax* moves to mitochondria²¹ and induces cytochrome *c* release from mitochondria.^{20, 22} Released cytochrome *c* binds to Apaf-1 and caspase-9, and the complex subsequently activates caspase-3.^{23, 24} The release of cytochrome *c* occurs independently of changes of mitochondrial membrane potential^{20, 25} and caspase activation^{20, 22} and is inhibited by *Bcl-XL*.^{20, 26} In the present study, after *Bax*

induction, *Bcl-XL*, one of the antagonists of *Bax*, was slightly elevated, which might alleviate the *Bax*-induced apoptosis in DLD-1 cells. Cytochrome *c* release was observed at the same time as caspase-3 activation, supporting the sequential activation of the apoptotic cascade already described.^{20–24} We have recently shown that *Bax* overexpression sensitizes KATO-III gastric cancer cells to chemotherapeutic agent-induced apoptosis through enhancing the release of cytochrome *c* from mitochondria.²⁷ In the present study, apoptosis induced by all chemotherapeutic agents tested was enhanced by *Bax* overexpression, suggesting that the target of *Bax* overexpression is the final common pathway. It has been reported that dimerization of mitochondrial *Bax* is also associated with increased drug response in *Bax*-transfected human head and neck squamous cancer cells.²⁸ On the other hand, Strobel *et al.* reported that intracellular accumulation of paclitaxel was enhanced by *Bax* overexpression.²⁹ We assume that enhancement of chemotherapeutic agent-induced apoptosis by *Bax* overexpression is mediated both by the common pathway of mitochondrial cytochrome *c* release and by the specific mechanism of each chemotherapeutic agent.

It has been reported that wild-type *p53* does not induce apoptosis in wild-type *p53*-bearing cancer cells. The present study showed that overexpression of *Bax* itself induced apoptosis in DLD-1 colon cancer cells to some extent, and that *Bax* overexpression sensitizes DLD-1 cells to chemotherapeutic agent-induced apoptosis. In the case of 5-fluorouracil, which is a key drug in the treatment of colorectal cancer,¹⁰ enhancement of apoptosis by *Bax* was observed at an early time. Thus, *Bax* overexpression-based gene therapy may be an effective approach for the treatment of colon cancer, in combination with chemotherapy.³⁰

ACKNOWLEDGMENTS

This study was supported in part by grants (No. 11140229 and No. 10670944) from the Ministry of Education, Science, Sports and Culture, Japan.

(Received July 28, 2000/Revised September 18, 2000/Accepted September 25, 2000)

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