Overexpression of Bax Enhances Antitumor Activity of Chemotherapeutic Agents in Human Head and Neck Squamous Cell Carcinoma

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ABSTRACT

Overexpression of the Bax protein in human head and neck squamous cell carcinoma A253 cells was reported to result in an increased sensitivity to various chemotherapeutic agents in vitro (Guo et al., Oncol. Res., 11: 91–99, 1999). In the present study, the relationship between Bax expression and response to chemotherapy was further investigated in vitro and in vivo model systems. For in vitro study, A253, A253/Vec (pcDNA3 vector transfectant), and A253/Bax (pcDNA3/Bax transfectant, expressing 50-fold higher Bax protein than A253 and A253/Vec) cells were exposed to various concentrations of raltitrexed (a specific thymidylate synthase inhibitor) and SN-38 (a topoisomerase I inhibitor) for 2 h, and cell growth inhibition was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide clonogenic assay. Compared to A253/Vec, A253/Bax cells exhibited 9.5- and 13.8-fold increases in sensitivity to raltitrexed and SN-38, respectively. For in vivo study, A253/Vec and A253/Bax tumor xenografts were established by s.c. injection of tumor cells into nude mice. The antitumor activity and toxicity of raltitrexed (i.v. push daily for 5 days) and irinotecan (a prodrug of SN-38; i.v. push daily for 3 days) were evaluated. The maximum tolerated doses of raltitrexed and irinotecan were 30 and 100 mg/kg/day, respectively. At the maximum tolerated doses, minimal antitumor activity was observed with raltitrexed, although irinotecan was more active than raltitrexed against A253 or A253/Vec tumors. In contrast, both raltitrexed and irinotecan were significantly more active against A253/Bax xenografts than against A253/Vec xenografts; the yield for complete tumor regression (cure) was 40% and 100% with raltitrexed and irinotecan, respectively, with no significant toxicity. Furthermore, the observed increase of antitumor activity in A253/Bax tumors was associated with an enhanced induction of apoptosis in vivo. The in vivo results demonstrated a proof of the principal concept that selecting up-regulation of the proapoptosis gene Bax can provide the basis for a greater therapeutic efficacy to a variety of chemotherapeutic agents with different structures and mechanisms of action.

INTRODUCTION

Apoptosis is a major mode of cell death induced by chemotherapy. Proteins of the Bcl-2 family are the key regulators of apoptosis (1). Whereas Bax and other accelerators of apoptosis promote apoptosis, Bcl-2 and other repressors inhibit apoptosis. It is suggested that Bax heterodimerizes with Bcl-2, and the Bax:Bcl-2 ratio determines the onset of apoptosis (2). The role of the Bcl-2 family of proteins in chemoresponse has been evaluated extensively in vitro models. Although the overexpression of Bcl-2 and Bcl-XL has been shown to induce drug resistance (3–5), the overexpression of Bax resulted in an increased drug sensitivity in various cell lines (6–8). Thymidylate synthase and topoisomerase I are considered important targets for chemotherapy (9, 10). Raltitrexed (ZD1694), a water-soluble, nonnephrotoxic quinazoline, is a potent highly specific thymidylate synthase inhibitor (11). Raltitrexed is moderately active against human HNSCC (15). A253 and FaDu xenografts in vivo and is being investigated in Phase I and II clinical trials for a number of cancers including head and neck cancer (12, 13). Irinotecan (CPT-11), a topoisomerase I inhibitor, is a new cytotoxic agent with a broad spectrum of clinical activity. It is active against head and neck cancer with a response rate of ~20% (14). Human HNSCC A253 cells are p53-null and express low levels of Bax and Bcl-2 proteins (15). We have previously reported that the overexpression of Bax in A253 cells results in an increased in vitro sensitivity to various chemotherapeutic agents (6). Increased drug response was associated with an enhanced induction of apoptosis in A253/Bax cells (6). In the present study, we further evaluated the role of Bax expression associated with the response to raltitrexed and irinotecan and the induction of apoptosis in the human head and neck tumor xenografts.

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3 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CR, complete tumor regression; PR, partial tumor regression; MTD, maximum tolerated dose.
MTT assay. Cells were treated with the drug for 2 h, washed, and cultured in drug-free medium for 5 days and analyzed by MTT assay. Bar, SD. The data points (mean ± SD) are the average of three to four separate assays in triplicate. There is a significant difference in cell growth inhibition between A253/Bax and A253/Vec or A253 cells with both raltitrexed and SN-38 treatment (P < 0.01).

**MATERIALS AND METHODS**

**Drugs.** Raltitrexed was supplied by Zeneca Pharmaceuticals (Macclesfield, United Kingdom). It was dissolved in sterile saline, and the solution was adjusted to a pH of 7.0 with NaOH immediately before use. Irinotecan was supplied by Pharmacia & Upjohn (Kalamazoo, MI) as a ready to use solution. PLX3397 was dissolved in sterile saline, and the solution was adjusted to a pH of 7.0 with NaOH immediately before use. Irinotecan was supplied by Zeneca Pharmaceuticals.

**Cell Lines.** The human HNSCC cell line A253 was purchased from American Type Culture Collection (Rockville, MD). The cells were maintained as a monolayer in RPMI 1640 media supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). The cells were tested for Mycoplasma bimonthly using the GEN-PROBE Mycoplasma T.C. rapid detection system (GEN-PROBE Inc, San Diego, CA) and were found to be consistently negative throughout these experiments. A253/Bax cells were established by transfection of a full-length Bax cDNA, which was constructed into the EcoRI site of the pcDNA3 (Invitrogen) expression vector, and the authenticity of the construct was confirmed by DNA sequencing (6). The pcDNA3/Bax or pcDNA3 empty vectors were transfected into A253 cells using LIPOFECTAMINE Reagent (Life Technologies, Inc.) following the manufacturer’s protocol. G418-resistant clones were selected using a cloning cylinder and were expanded. Bax expression was examined by Western blot analysis (6).

**Animals.** Eight- to 12-week-old female athymic nude mice (nu/nu, body weight, 20–25 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The mice were housed five per cage under specific pathogen-free conditions with water and food ad libitum according to an institutionally approved protocol.

**In Vitro Cell Growth Inhibition Assay.** Cells were seeded in 96-well plates at 400 cells/well in 100 μl of medium. Twenty-four h after seeding, cells were exposed for 2 h to various concentrations of drug and washed and cultured in drug-free medium for 5 days. Cell growth inhibition was assessed by the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) colorimetric assay.

**Establishment of Tumor Xenografts in Vivo.** Tumor xenografts were established by implanting 2 × 10^6 cultured A253/vec and A253/Bax cells into nude mice s.c.

**Isolation of Protein from Tumor Xenografts and Western Blot.** Tumor samples were removed from mice bearing A253/Bax or A253/vec xenografts at various times after implantation. Samples were immediately frozen in liquid nitrogen and stored at −80°C. Tumor tissues (100 mg) were broken into a fine powder, then lysed in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, and 100 μg/ml aprotonin), homogenized mechanically on ice, and centrifuged at 3000 rpm for 15 min. The supernatants were collected and stored at −80°C. Protein concentrations were determined with a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Fifty μg of protein of the lysates were subjected to 12.5% SDS-PAGE. Western blot was performed (16) with polyclonal antihuman Bax antibody (PharMingen) and used the Renaissance Chemiluminescence Reagent Kit (New England Nuclear Life Sciences, Boston, MA).

**Drug Doses and Schedules.** Raltitrexed was administered by i.v. push daily for 5 days at 15 and 30 mg/kg/day. Irinotecan was administered by i.v. push daily for 5 days at 50 and 100 mg/kg/day.

**MTD and Toxicity Evaluation.** The MTD was defined as the maximum dose that caused no drug-related lethality and resulted in a <20% loss in body weight. Drug-induced toxicity (body weight loss, lethality) was determined daily for a minimum of 4 weeks and observed at least twice a week thereafter for up to 3 months.

**Antitumor Activity.** Drug treatments were initiated 11 days after s.c. tumor implantation when tumors reached about 200 mg. Five tumor-bearing mice were assigned randomly to each group. Tumor measurement, calculation, and determination of tumor response were described previously (17). Briefly, two axes of the tumor (L, longest axis; W, shortest axis) were measured with a caliper. Tumor weight was calculated as: \( W = L \times W^2/2 \). Tumor response was defined as PR when tumor weight was temporarily reduced by at least 50% and as CR when the tumor was undetectable by palpation for 3 months after termination of therapy. In general, regrowth of the tumor was only observed in mice with PRs.

**Morphological Detection of Apoptosis.** Tumor xenografts were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Apoptosis was evaluated by morphological staining of conventional H&E staining. Apoptotic cells were identified by nuclear condensation and fragmentation.
Apoptosis indices were calculated as the percentage of apoptotic cells among 300 tumor cells in a randomly selected nonnecrotic portion of the tumor. The presence of apoptotic cells was confirmed by terminal deoxynucleotidyl transferase-mediated nick end labeling immunohistochemical assay on a paraffin section (Apop Tag Plus In Situ Detection Kit, Oncor, Gaithersburg, MD) according to the manufacturer’s instructions.

**Immunohistochemical Detection of Bax and Bcl-2.** Formalin-paraffin sections of tumor samples were deparaffinized and rehydrated, treated with 3% H₂O₂, and blocked with 0.03% casein. Then, sections were incubated with polyclonal antihuman Bax antibody (DAKO Corp., Carpinteria, CA) in 1:200 dilution (5.5 μg/ml) overnight at room temperature, followed by the Vectastain Universal Elite ABC Peroxidase Kit (Vector Lab., Burlingame, CA) according to the manufacturer’s instructions. For Bcl-2 detection, sections were pretreated in 6-M urea in a microwave for 10 min, and mouse antihuman Bcl-2 monoclonal antibody (DAKO) in 1:40 dilution (4.5 μg/ml) was applied overnight at room temperature. Sections were counterstained with hematoxylin. Negative controls were normal rabbit serum for Bax and isotype-matched mouse IgG1 at the same concentration as the primary antibody for Bcl-2. Known positive controls were formalin-paraffin sections of Bax-transfected A253 HNSCC cell pellet for Bax and of tonsil for Bcl-2 (mantle zone lymphocytes are positive, germinal center cells are negative).

**Statistical Analysis.** Differences between the mean values were analyzed for significance using the unpaired two-tailed Student’s test for independent samples; \( P \leq 0.05 \) was considered to be statistically significant.

**RESULTS**

**Raltitrexed and SN-38 Inhibition of Cell Growth in Vitro.** A253/Bax cells expressing 50-fold higher Bax protein than A253 and A253/vec (pcDNA3 vector transfectant) cells were established as described in an early report (6). The *in vitro* growth rates of A253, A253/Vec, and A253/Bax cells were similar (6). *In vitro* cell growth inhibition by raltitrexed and SN-38 (the active metabolite of irinotecan) was evaluated in A253, A253/Vec, and A253/Bax cells, and the results are shown in Fig. 1. Growth inhibition was assessed by MTT assay at 5 days after the initial 2-h drug exposure, with 1.0 and 0.1 mM as the drug concentrations required for 50% inhibition of cell growth by raltitrexed and SN-38, respectively. As shown in Fig. 1, no significant difference in cell growth inhibition was observed between A253 and A253/Vec cells with both raltitrexed and SN-38 (\( P > 0.05 \)). However, A253/Bax cells exhibited 9.5- and 13.8-fold increases in sensitivity to raltitrexed and SN-38, respectively (\( P < 0.01 \)).

**Bax and Bcl-2 Expression in Tumor Xenografts.** To evaluate the role of Bax overexpression in drug response *in vivo,*
A253/Bax and A253/vec xenografts were established s.c. in nude mice. Tumors of the two cell lines grew at similar rates, reaching a size of about 150–200 mg at day 11 after implantation. The data in Fig. 2 indicate that a high level of Bax expression was maintained in A253/Bax xenografts in nude mice, and tumor samples were further examined by immunohistochemical staining for Bax and Bcl-2 (Fig. 2). On day 11 after implantation, about 90% of cells in A253/Bax tumor were strongly stained for Bax protein. On day 17, about 50% of the cells lost the high level of Bax staining. In contrast, only about 30% of cells in A253/Vec xenografts were weakly stained by Bax antibody. Bcl-2 was not detected in any of the samples.
There was a slight increase in the Bax level in the A253/Vec tumor on day 17. This is consistent with the early observation in A253 tumor xenografts, which was associated with tumor differentiation (15).

**Antitumor Activity and Toxicity of Raltitrexed and Irinotecan.** To evaluate the impact of Bax overexpression on in vivo response to raltitrexed, mice bearing tumors were treated daily for 5 days with 15 and 30 mg/kg/day (one-half of the MTD and MTD, respectively). As summarized in Fig. 3A and Fig. 4, the antitumor activity of raltitrexed was significantly increased with 40% CR (cure) and 40% PR in mice bearing A253/Bax xenografts when treated at the MTD, whereas no CR or PR was observed in mice bearing A253/Vec xenografts. The mean body weight losses were about 15% at the MTD with both models.

We also evaluated the role of Bax expression on the in vivo antitumor activity of irinotecan. Tumor-bearing mice were treated at one-half of the MTD and MTD (50 and 100 mg/kg/day, respectively) daily for 3 days. Compared to raltitrexed, irinotecan was more active against A253/Vec xenografts in vivo, a result that is consistent with the in vitro data shown in Fig. 1. As summarized in Fig. 3B and Fig. 4, a dramatic increase in the antitumor activity of irinotecan was observed in A253/Bax tumors, with 100% of animals achieving CR at one-half of the MTD. In contrast, only a 20% CR was achieved with the MTD of irinotecan in A253/Vec tumors. The mean body weight losses were about 15% at the MTD with both models.

**Induction of Apoptosis in Vivo.** To determine whether the observed increase in antitumor activity was associated with an increased level of drug-induced apoptosis, mice bearing A253/Vec and A253/Bax tumors were treated by an i.v. push with a single dose of irinotecan (100 mg/kg). Tumors were removed 24 h after treatment, and drug-induced apoptosis was examined by H&E staining. About a 4-fold increase of apoptosis was observed in irinotecan-treated A253/Bax tumors, but no significant increase was observed in A253/Vec tumors (Fig. 5).

**DISCUSSION**

We have previously reported that the overexpression of Bax resulted in increased in vitro drug sensitivity to various chemotherapeutic agents in A253 cells (6). Whether increased drug sensitivity seen in vitro can be achieved in vivo with greater antitumor efficacy needs to be determined. As the data in the present study indicate, the overexpression of Bax in vivo resulted in a significantly increased tumor response (cure rate) to raltitrexed and irinotecan, two active drugs in the treatment of head and neck cancer, with different mechanisms of action. Kinetically, increased levels of Bax expression corresponded with increased levels of apoptosis and preceded the observed increase of in vivo antitumor activity.

Expression of a high level of Bax protein was maintained up to 11 days after the implantation of A253/Bax cells. Beginning on day 11 after implantation and thereafter, a fraction of the tumor cells started to lose Bax expression. On day 17, about 50% of tumor cells maintained a high level of Bax protein, and 50% had only basal levels of Bax. Lack of the selective agent (G418) in the in vivo environment may be responsible for the gradual loss of Bax expression. When A253/Bax cells were cultured in vitro without G418 for 2 weeks, a significant portion...
Fig. 5  Detection of apoptosis. Tumors of A253/Vec and A253/Bax specimens were removed 24 h after irinotecan treatment (100 mg/kg, single i.v. push) and fixed in formalin. Paraffin sections of the tumors were examined for apoptosis by H&E staining. A, morphological detection of apoptosis in untreated (1, A253/vec; 3, A253/Bax) and irinotecan-treated tumors (2, A253/vec; 4, A253/Bax). Arrows, apoptotic cells. B, apoptotic index was calculated as described in “Materials and Methods.” Data in each column represent the mean ± SD of three independent samples. No significant difference was observed in the apoptotic index between the untreated control and irinotecan-treated groups in A253/Vec tumors. The difference in the apoptotic index between the untreated control and the irinotecan-treated group was statistically significant in A253/Bax tumors ($P < 0.01$).
of the cells lost Bax expression (data not shown). Thus, the presence of a selective pressure from G418 is required for maintaining the Bax protein level. However, this does not exclude the possibility that other factors in the in vivo environment may also contribute to the loss of Bax expression.

A253 tumor cells are sensitive in vitro to raltitrexed and irinotecan, but they are relatively resistant to these agents in vivo. The result generated herein clearly demonstrated that the deliberate alteration of the molecular make up of tumor in favor of programmed cell death, e.g., overexpression of the proapoptotic gene Bax, can result in improved therapeutic efficacy and selectivity to chemotherapy, such as the result generated by raltitrexed and irinotecan. With the overexpression of Bax protein in tumor tissue, nontoxic but therapeutically effective low doses of chemotherapeutic agents can be administered effectively and safely.

In conclusion, these results with human HNSCC indicate that the overexpression of Bax significantly increased drug sensitivity in vitro and in vivo. Furthermore, the observed higher antitumor efficacy in tumors with a high level of Bax (A253/Bax) was associated with an enhanced induction of apoptosis. The data clearly demonstrate a strong correlation between high level Bax expression, induction of apoptosis, and antitumor efficacy to chemotherapeutic agents evaluated in this tumor model system. The specific and selective alteration of the molecular make up of tumor tissue can lead to an improved therapeutic efficacy of chemotherapeutic agents with different structures and mechanisms of action.

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