Modulation of apoptosis may potentiate the sensitivity of tumor cells to chemotherapeutic agents, thus improving the clinical outcome of cancer treatment. Bax, an apoptosis-promoting member of the bcl-2 family, may be a key factor influencing the chemosensitivity of tumor cells, however, its involvement in cellular sensitivity to anti-cancer drugs remains uncertain in squamous cell carcinoma (SCC). To investigate the role of bax gene expression in modulating cisplatin (CDDP)-induced apoptosis in vitro, an established CDDP-resistant human head and neck SCC (IMC-3 cell line) was transfected with bax gene-bearing mammalian expression vector. Overexpression of the bax gene in CDDP-resistant IMC-3 cells elevated the CDDP susceptibility of tumor cells to a level similar to that of the parental IMC-3 cells. In an in vivo study, percutaneous transfer of apoptosis-promoting bax gene by particle-mediated (gene gun) delivery caused overexpression of Bax in SCC, which was confirmed by immunohistochemical staining, and inhibited the growth of mouse CDDP-resistant SCC. Furthermore, combination therapy with bax gene transfer and subcutaneous administration of CDDP at 3-day intervals markedly inhibited the growth of mouse SCC. Thus, overexpression of bax gene in SCC by a gene gun system appears to be a rational approach to improving the efficacy of chemotherapy and treatment outcome. We suggest that exogenous bax expression may have therapeutic applications for enhancing chemotherapy in SCC.

In this report, we investigated the effects of Bax on the sensitivity of H&N SCC to CDDP and whether overexpression of Bax can overcome CDDP-resistance in H&N SCC by gene-transfer technology in vitro and in vivo. Overexpression of bax gene in vitro was induced by an electroporation system. For in vivo transfection of bax gene, the particle-mediated gene delivery system was employed. The particle-mediated gene delivery method using a gene gun utilizes a shock wave to accelerate DNA-coated gold particles to allow them to penetrate target cells or tissues. The gene gun is able to deliver thousands of DNA copies intracellularly into tissues, resulting in high level of transgene expression (Yang and Sun, 1995). Unlike virus vectors, this method is cell surface receptor-independent. Thus, it can successfully deliver genes into a wide variety of mammalian cell types (Cheng et al., 1993). In this work, mouse SCC exhibiting weak Bax expression and resistance to apoptosis induced by CDDP, was transplanted into mice. Then, a gene gun was used for bax gene transfer into the transplanted mouse SCC to examine the effect of Bax overexpression on sensitivity to CDDP or apoptosis of the tumor.

**MATERIAL AND METHODS**

**Cell lines, culture conditions and drugs**

IMC-3 cells, derived from human maxillary squamous cell carcinoma (kindly provided by Dr. S. Komiyama, Kyushu University, Fukuoka, Japan), were maintained in conditioned medium [prepared from RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (GIBCO, Grand Island, NY) at 37°C under a humidified atmosphere of 5% CO₂ in air. Cisplatin (CDDP) was kindly provided by Bristol-Myers Squibb (Tokyo, Japan). The CDDP-resistant IMC-3 cell line (IMC-3CR) was developed in the presence of increasing concentrations of CDDP with repeated subcultures until the cells became fully resistant to CDDP and could grow exponentially in the presence of 0.5 μg/ml of the drug for 2 weeks prior to being returned to.

Grant sponsor: Ministry of Education, Science, Sports and Culture of Japan; Grant number: Encouragement of Young Scientists 09771341; Grant number: Scientific Research (B) 10470354.

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Received 4 January 1999; Revised 26 April 1999.
drug-free medium (Ohtsubo et al., 1997). The drug-resistant cell lines were passed in drug-free medium, and there was no loss of resistance during 6 months of experimentation.

Transfection

The expression vector hBaxpcDNA3 was used with permission of Drs. J.-C. Martinou and R. Brown (Glyco Wellcome, Berkshire, UK). This vector containing a full-length human bax cDNA tagged 3'-57 bp nucleotides subcloned into the mammalian expression pcDNA3 (In Vitrogen, Carlsbad, CA) (Boron, 1996) to distinguish transfected tagged-Bax protein from intrinsic Bax protein. IMC-3CR cells were stably transfected with hBaxpcDNA3 or native pcDNA3 vector using the electroporation method. Five hundred microliters of cell suspension in PBS were added to a sterile cuvette containing 20 µg hBaxpcDNA3, gently mixed, and subjected to electroporation using a GenePulser (Nippon Bio-Rad, Tokyo, Japan). Conditions of 960 µF capacity, 250 V, were typically used. After resting for 5 min at room temperature, cells were returned to the CO2 incubator. After transfection, cells were treated with 10% FBS and returned to the culture medium, and the treatment was repeated every experiment, floating cells in the culture medium were combined with attached cells harvested by trypsinization. After overnight incubation at 4°C in the dark, the propidium iodide content of the individual nuclei was measured using an EPICS flow cytometer (Couler, Hialeah, FL). Cell debris was excluded by appropriately raising the forward scatter threshold. Apoptotic nuclei displayed a decreased DNA content below the G1 peak. Data were analyzed using the Couler flow cytometry software.

Western blot analysis

Cells were washed twice with PBS and sonicated in RIPA buffer, which was PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors (10 mg/ml Leupeptin, 10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1.8 mg/ml iodoacetamide). Lysates were boiled in SDS sample buffer for 3 min and electrophoresed through 12.5% SDS–polyacrylamide gels (30 µg per lane). The gels were transferred onto a nylon membrane (pore size 0.2 µm; Perkin-Elmer, Tokyo, Japan). After transfer, the nylon membrane was blocked with 5% skimmed milk in PBS and probed with 1 µg/ml rabbit anti-human bax polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 2 µg/ml mouse anti-human bcl-2 monoclonal antibody (MAb(clone 124, Dako, Kyoto, Japan) for 1 hr at room temperature. The blot was visualized with the labeled streptavidin-biotin method (Dako), according to the instructions of the manufacturer.

Drug sensitivity test in vitro

Approximately 5 × 10³ cells were seeded in each well of a 96-well plate in triplicate and cultured. After 24 hr, CDDP was added, and the cells were cultured for 24 hr. At that stage, the medium was removed, 10 µl of 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) in PBS was administered and the cells were incubated at 37°C for 4 hr. Two hundred microliters DMSO (Wako, Osaka, Japan) was added, and the absorbance of each well was measured at 540 nm (reference absorbance at 630 nm). The effect of the drugs on cell survival was expressed as a percent viability. The percent viability was calculated using the following equation: (A540 CDDP-treated/A540 CDDP-free) ×100. All values represent the mean ± standard deviation (SD) from triplicate cultures. The test was performed independently 3 times, and 50% inhibitory concentration (IC₅₀) was defined as the concentration of CDDP that killed 50% of the cells.

Flow-cytometric analysis of apoptosis

Apoptosis of cells treated with CDDP was measured by flow cytometry as previously described (Nicoletti et al., 1991). After 24 hr CDDP treatment, the medium was removed and 2 × 10⁶ cells were detached by trypsin treatment, washed with ice-cold PBS and gently resuspended in 300 µl fluorochrome solution (50 µg/ml propidium iodide, 0.1% sodium citrate plus 0.1% Triton X-100). In every experiment, floating cells in the culture medium were

Mice and malignant tumors

Seven to 9 week-old C3H/He (C3H) mice bearing SCC were used in all experiments (Matsumoto et al., 1990). A tumor from the donor mouse was cut into small pieces (about 0.2 g) and one of these pieces was transplanted s.c. into the back of a recipient mouse. When the tumor volume increased to approximately 0.5 mg, gene therapy was started.

Generation of mouse bax expression vector

Mouse bax cDNA was prepared by reverse transcription (RT)-PCR of total RNA from kidney of C3H mouse using a 5’ primer (5’-GCCGGGACGGGCGGTGAT-3’) and a 3’ primer (5’-CTCACGC- CACATCTTTTCAGAT-3’), designed according to the published sequence (Oliva et al., 1993). The PCR product of 595 bp was subcloned into the mammalian expression vector pcR3.1 (In Vitrogen), and the nucleotide sequence was confirmed by a Hitachi DNA sequencer SQ-5500 (Hitachi, Tokyo, Japan). The lacZ expression vector was prepared using an eukaryotic TA cloning unidirectional kit (InVitrogen).

In vivo gene transfer

In vivo gene transfer was performed according to the manufacturer’s instruction of the Helios Gene Gun System (Nippon Bio-Rad). Plasmid DNA was purified on Qiagen columns (Funakoshi, Tokyo, Japan) and precipitated onto 0.6 µm gold particles. Particles were suspended in 0.25 mg/ml of polyvinyl pyrrolidone in absolute ethanol. This DNA/gold/particle preparation was coated onto the inner surface of Tefzel tubing using a tube loader, and the tubing was cut into 0.5-inch segments, resulting in the delivery of 0.5 mg gold and 2 µg plasmid DNA per transfection. For tumor therapy, the target tumor in the mouse was transfected in vivo with mouse bax or control expression vector starting 7 days after the tumor was s.c. inoculated. Each treatment consisted of 3 transfections (6 µg plasmid DNA/treatment) with a 250 psi helium gas pulse. Three transfections were delivered directly over the tumor site in a triangle pattern. Twenty-four hours after transfection, 1.0 µg/g (body weight of mouse) CDDP or saline was s.c. injected around the tumor.

In vivo evaluation of tumor growth

C3H mice were s.c. inoculated with tumor tissue as described above. Every day, 2 of us (MS and GF) without knowledge of the treatment performed the measurements in 2 dimensions, length (a) and width (b), using calipers. Tumor weight (W) was calculated by the formula W = a²b/2, where a is the longer of the 2 measurements (O’Dwyer et al., 1994).

Histological examination

The mice were inoculated s.c. with tumor tissue as described above. The inoculated site was dissected, fixed in 10% neutral buffered formalin, and embedded in paraffin. Five micrometer sections were then examined. For immunohistochemistry, immunoperoxidase staining was performed using the conventional avidin-biotin-peroxidase complex technique (Tsuzuki et al., 1998). Deparaffinized 5 µm-thick specimens were incubated with rabbit anti-mouse Bax polyclonal antibody (Santa Cruz Biotechnology) at 4°C overnight.
For in situ staining of apoptotic cells, the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method was performed using the ApopTag in situ detection kit (OnCOR, Gaithersburg, MD). The staining procedures were modified based on the manufacturer’s instruction (Lu and Tanigawa, 1997). Detection was performed using a diaminobenzidine substrate, and counterstained with methyl green. The apoptotic index (AI) was expressed as the number of positively staining tumor cells per 1000 tumor cells, as described previously.

For histochemical β-galactosidase assay, the dissected fresh tumors were frozen and cut into 8-µm sections. These sections were fixed and stained for β-galactosidase activity according to the manufacturer’s instruction of the β-Gal Staining Kit (InviTrust) and then counterstained with hematoxylin.

Statistical analysis

All in vitro determinations were made in triplicate, and the results were expressed as the mean ± SD (standard deviation). Significance of differences was determined by Mann-Whitney U test. A p value of 0.05 or less was considered significant.

In vivo experiments, significant differences in tumor weight among the animals in the various groups were determined by repeated measures ANOVA using Stat View software (Abacus Concepts, Berkeley, CA).

RESULTS

Overexpression of bax in stable transfectant of CDDP-resistant IMC-3 cells

After we transfected hBaxpcDNA3 into the CDDP-resistant IMC-3 cells (IMC-3-CR), 6 clones expressing exogenous Bax were obtained and designated CSCR1 to CSCR6. The extent of transfected Bax protein expression is as shown in Figure 1. Twenty-four kDa bands of exogenously transfected tagged-Bax were observed in all cell lines. Exogenous Bax protein were equally observed in all cell lines. Exogenous produced Bax protein were observed in all cells tested.

Measurement of CDDP-induced apoptosis by flow cytometry

To quantitate the extent of apoptosis occurring in IMC-3 cells exposed to CDDP, we determined the fraction of cells expressing sub-G1 DNA by propidium iodide analysis as previously described (Nicoletti et al., 1991)(Fig.2a). As shown in Figure 2b, the percentage of apoptotic cells was calculated by EPICS analysis, the level of spontaneous apoptosis was very low (less than 2%) and similar in the 5 cell lines. CDDP (10 µg/ml) induced apoptosis in 43.7 and 63.2% of CSCR3 and CSCR6 cells, whereas apoptosis was found in only 5.1 and 4.5% of parental IMC-3CR and IMC-3CRCV cells, respectively. The percentage of apoptotic cells in CSCR3 and CSCR6 was significantly higher than that in IMC-3CRCV cells (p < 0.01, respectively). There were no differences in the average of IC50 of CDDP between IMC-3CR and IMC-3CRCV.

Sensitivity to CDDP in each stable transfectant

As shown in Table 1, the average of IC50 of CDDP against IMC-3, IMC-3CR, IMC-3CRCV, CSCR3 and CSCR6 cells from 3 independent experiments were 8.36, 41.00, 39.40, 6.80 and 5.43 µg/ml, respectively. The CDDP cytotoxicity to bax transfectants, CSCR3 and CSCR6 was significantly higher than that to IMC-3CRCV (p < 0.01, respectively). To examine whether this enhancement of chemosensitivity in bax transfectants is limited to CDDP, we also investigated sensitivity to 5-FU in these cell lines. However, there were no differences in the sensitivity to 5-FU between CSCR3 or CSCR6 and IMC-3CRCV (data not shown).

Tumor regression following bax gene therapy

We examined the antitumor effect of bax gene transfer and CDDP in C3H mice bearing SCC. Combination therapy comprised of control vector transfer and CDDP administration demonstrated a slight antineoplastic response in this animal system, compared with that in control vector transfer plus saline administration until day 7 (Fig.3). However, there were no differences in the mean tumor weight between the 2 groups on day 14 (control vector plus CDDP vs. control vector plus saline, 4.09 ± 1.26 mg vs. 5.33 ± 0.92 mg, mean ± SEM), suggesting that this C3H mouse might be an animal model for testing in vivo CDDP resistance. The mean tumor weight in mice treated with bax gene transfer plus saline was smaller than

| Cell lines | IC50 (µg/ml) |
|------------|-------------|
| IMC-3      | 8.36        |
| IMC-3CR    | 41.00       |
| IMC-3CRCV  | 39.40       |
| CSCR3      | 6.80        |
| CSCR6      | 5.43        |

*Significantly decrease of IC50 (p < 0.01) compared with IMC-3CRCV cells.
that of mice treated with control vector plus saline on day 14 (3.10 ± 0.86 mg, \( p < 0.05 \)). Moreover, marked inhibition of tumor growth was found in mice treated with \( bax \) gene transfer plus CDDP (1.11 ± 0.41 mg, \( p < 0.0001 \) compared with that in mice treated with control vector plus saline). The combination of \( bax \) gene transfer and CDDP at 3-day intervals resulted in complete ablation of the tumor in 1 of 7 animals, and in 4 other animals, the tumor weight was less than 1 mg on day 14. In mice given \( bax \) gene

![Figure 2](image-url)

**Figure 2** – Measurement of apoptosis by flow cytometry. (a) DNA fluorescence histogram of propidium iodide-stained cells after treatment with 10 µg/ml CDDP for 24 hr or without treatment (control). Apoptosis is characterized by a decrease in the nuclear DNA content. As data from 3 independent experiments were highly reproducible, representative data are shown. Bar: apoptotic cells. (b) Percentage of apoptotic cells after treatment with 10 µg/ml CDDP for 24 hr were calculated by EPICS analysis. Results are represented as mean ± standard deviation of 3 independent experiments. *\( p < 0.01 \).
transfer plus CDDP only once a week, the growth of the SCC was not significantly prevented (data not shown).

Expression of Bax in mouse SCC following bax gene therapy

We immunohistochemically confirmed overexpression of the Bax protein in the target sites on the tumors as a result of bax gene transfer. The mouse SCC exhibited weak expression of intrinsic Bax protein (data not shown); bax gene transfer using a gene gun increased expression of the Bax protein (Fig. 4a, b), but there were no changes in the Bax protein expression level in tumors from the control vector transfer group (Fig. 4c, d). The activity of β-galactosidase directed by control vector bearing LacZ gene transfected by the gene gun system was significantly positive in tumor cells and the surrounding tissues visualized using X-gal as a substrate (Fig. 4e). The percentage of positive stained tumor cells was 37.6 ± 27.9% (mean ± SD, in selected 5 high-powered fields, with each field containing more than 200 tumor cells, counted the number of positive cells). Bcl-2 expression is constitutively very low in mouse SCC, and it was not affected by bax gene transfer (data not shown).

Augmented CDDP-induced apoptosis in vivo by bax gene therapy

Since combination therapy with bax gene transfer plus CDDP significantly inhibited tumor growth, we investigated whether bax gene transfer affected apoptosis induced by CDDP in vivo. Repeated bax gene transfer and CDDP treatment clearly increased the number of apoptotic cells demonstrated by TUNEL staining within the tumor sections from these mice (Fig. 4f, g). In the bax transfer plus CDDP group, the apoptotic index (AI) was 60.7 ± 12.5 (mean ± SD, Fig. 5), whereas in the control vector plus saline group, this value was only 6.1 ± 5.7 (p < 0.01). No such an increase in AI was observed in mice treated with control vector transfer plus CDDP (5.1 ± 2.8), although the AI was significantly increased in the bax gene transfer plus saline group (32.3 ± 8.7, p < 0.01 compared with the AI in the control vector plus saline group, p < 0.01 compared with the AI in the bax plus CDDP group).

DISCUSSION

In this study, we have found that overexpression of Bax significantly enhances in vitro sensitivity to CDDP in established...
FIGURE 4. BAX GENE TRANSFER REDUCES SCC
transfectants, a delicate imbalance in cellular expression levels of Bax and Bcl-2 (Bax > Bcl-2) relative to case was an increase in CDDP-induced apoptosis. As shown in Figure 1, parental IMC-3 cells express relatively high levels of endogenous Bax, as compared with exogenous tagged-Bax in bax transfectants. Of interest, the ratio of Bax/Bcl-2 is unchanged in IMC-3 cells as compared to CDDP-resistant IMC-3CR, despite their difference in sensitivity to CDDP. Therefore, intrinsic Bax might be nonfunctional to induce apoptosis because of its full heterodimerization to Bcl-2. Thus, bax transfectants were more sensitive to CDDP-induced apoptosis, although the expression of exogenous tagged-Bax is much lower than the endogenous Bax expression.

In an in vivo study, we have examined the efficacy of a protocol consisting of 4 repeated treatments with bax gene and CDDP at 3-day intervals. All mice treated with control vector and saline died within 21 days after therapy was started. Clear differences were found in the mean tumor weight between mice treated with bax gene transfer plus CDDP and those treated with control vector transfer plus saline (p < 0.001). The by-stander effect might play a role in this gene gun-mediated in vivo delivery of bax cDNA, however, we have no data about this point. In the group treated with bax gene transfer plus CDDP therapy, the transplanted SCC started to grow again within a couple of days after the final treatment. However, the mice that underwent bax gene transfer demonstrated extended survival compared with that of mice receiving control vector transfer and saline [surviving mice in each group on day 21; control vector plus saline 0% (0/7), control vector plus CDDP 12.5% (1/8), bax gene plus saline 14.3% (1/7), bax gene plus CDDP 50% (4/8)]. The reason why bax gene transfer by the gene gun system was only effective in suppressing tumor growth remains unclear. Similar results have been reported by others (Yin et al., 1997; Bargou et al., 1996; Krajewski et al., 1995), suggesting that Bax affects several microenvironmental factors and induces cell death in vivo. Repeated bax gene transfer and CDDP treatment clearly increased the number of apoptotic cells as detected by TUNEL staining within tumor sections from these mice, consistent with the in vitro findings.

Our present data provide evidence that overexpression of Bax in SCC, which was achieved by bax gene transfer in vitro and in vivo, can enhance chemosensitivity by increasing of chemotherapy-induced apoptosis. Thus, the combination of bax transfection strategy and chemotherapy/radiation may improve local tumor control in the cases of non-resectable tumors or tumors that are non-responsive to chemotherapy or radiation alone. Furthermore, percutaneous transfer of a variety of apoptosis-promoting genes by a gene gun into superficial tumors may be a therapeutic concept worth pursuing. Our present trials suggest that gene gun-mediated in vivo delivery of bax cDNA warrants further investigation as a valid clinical approach to human cancer gene therapy. Further studies are needed to determine how significant is the contribution of apoptosis to anticancer therapy in vivo and for identifying other mechanisms that control apoptosis.

ACKNOWLEDGEMENTS

We thank Drs. T. Saito, T. Ohtsubo, G. Tsuda, I. Noda, N. Tanaka and T. Ito (Dept. of Otorhinolaryngology, Fukui Medical University) for critical review of this work. We also thank Drs. Z.J. Zhang and I. Funatsu for skillful technical assistance.

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