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Adenovirus-Mediated Specific Expression of the *Bax* Gene Using the *Cre/loxP* System to Induce Apoptosis in Small Cell Lung Cancer Cells

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ABSTRACT

Small cell lung cancer (SCLC) is initially sensitive to chemotherapy or radiotherapy but relapses in a resistant form with a poor prognosis. For this reason, novel therapeutic approaches must be explored. In the present study, an adenovirus-mediated *Cre/loxP* system and the progastrin-releasing peptide (proGRP) gene promoter was used to achieve sufficient cell-type-specific expression of transgenes in proGRP-producing SCLC cells. First, SCLC cell-specific *lacZ* gene expression was achieved in proGRP-expressing SBC-5 cells by co-infection with a *Cre*-expressing adenoviral vector with proGRP promoter (AdGRPCre) and a *lacZ*-expressing vector with a chicken β-actin promoter separated by a pair of *loxP* sequences (AxCANLacZ). Next, SCLC-cell- specific apoptosis was induced with the *Bax* gene transferred by another adenoviral vector containing *loxP* sequences (AxCALNLhBax-alpha) and AdGRPCre. Antitumor effects were confirmed *in vivo* by suppression of tumor growth in nude mice inoculated with SBC-5 cells and co-infected with AdGRPCre and AxCALNLhBax-alpha. In conclusion, a cell-type-specific promoter combined with the *Cre/loxP* system can achieve selective expression of therapeutic genes in SCLC tumor cells. (Jikeikai Med J 2004 ; 51 : 77-89)

Key words : progastrin-releasing peptide, Bax, Cre/loxP system, small cell lung cancer, gene therapy

INTRODUCTION

Lung cancer is a leading cause of cancer death whose incidence is increasing worldwide[1]. Small cell lung cancer (SCLC), a histopathologic subtype accounting for 20% of lung cancers, is characterized by rapid growth and early dissemination[2]. SCLC is initially sensitive to chemotherapy and radiotherapy[2-4] but relapses in a resistant form with a poor prognosis[2,3]. Several novel treatment strategies for SCLC, such as immunoconjugates and antisense oligonuleotides against growth factors, are being studied at the preclinical and clinical levels[2,5]. In addition, a variety of molecular defects in SCLC have been reported, including the *p53* and *Rb* genes, which are mutated or lost in as many as 90% and 70% of tumor cells, respectively[2,6]. In the context of these molecular abnormalities, the use of gene therapy to replace or cause overexpression of tumor suppressor genes has therapeutic potential[2].

SCLC is a neuroendocrine tumor that can be divided into classic and variant phenotypes on the

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basis of the degree of neuroendocine cell differentiation[7]. Seventy percent of SCLCs are of the classic phenotype. These tumor cells have dense-core neurosecretory granules and produce large quantities of neuroendocrine growth factors, such as dopa decarboxylase, neuro-specific enolase, and bombesin-like peptides[8]. Among the bombesin-like peptides, progastrin-releasing peptide (proGRP) is a reliable tumor marker of SCLC[9] which can be used for histopathologic subclassification of lung cancers and to evaluate the efficacy of chemotherapy and radiotherapy for SCLC[9,10]. Mature gastrin-releasing peptide (GRP) is a 27-amino acid neuropeptide present in such tumors as SCLC, carcinoid, and medullary carcinoma of the thyroid[11–14]. Furthermore, GRP may function as an autocrine growth factor for SCLC cells through its binding to bombesin-like peptide receptors[15].

In gene therapy, tissue-specific promoters are effective tools for achieving selective expression of foreign genes in cancer cells. However, the transcriptional activity exerted by most cell-type-specific promoters is lower than that exerted by such promoters as Raus sarcoma virus-long terminal repeats (RSV-LTR), cytomegalovirus immediate early promoter, and chicken β-actin (CAG) promoter[16–18]. To overcome this problem, the *Cre/loxP* system has been used to achieve sufficient expression of therapeutic genes[19–21]. For example, this system has been applied to tumors producing α-feto protein[19], carcinoembryonic antigen, or thyrogrobulin[20,21] to efficiently induce cancer-cell-specific expression of transferred genes. *Cre* recombinase, which is derived from bacteriophage P1, is a 38-kD protein that mediates site-specific excisional deletion of a DNA sequence flanked by a pair of 34-nucleotide *loxP* sites[22].

In the present study, we constructed a *Cre*-expressing adenoviral vector driven by the proGRP promoter and co-infected proGRP-producing SCLC cells with additional gene-expressing adenoviral vectors to achieve cell-specific expression of the transgenes. As a therapeutic transgene, we used *Bax*, a well-characterized proapoptotic gene[23]. Several recent *in vitro* and *in vivo* studies have shown that exogenous proapoptotic genes have antitumor effects in a variety of cancers[24,25]. Of these genes, *Bax*, when overexpressed, could induce apoptosis with or without additional stimuli[26–29]. Furthermore, expression of *Bax* gene at high levels enhances the efficacy of chemotherapy and radiation therapy against cancers and improves clinical outcomes[30]. However, as a strongly proapoptotic gene *Bax* may also induce apoptosis in normal cells when overexpressed[31]. Thus, targeted expression of the gene is desirable.

Here we describe the successful cell-specific gene transfer to SCLC cells by using the 5′-flanking region of the human *proGRP* gene to achieve SCLC cell-targeted apoptosis and antitumor effects *in vitro* and *in vivo*.

MATERIALS AND METHODS

1. *Cell culture*

The human SCLC cell line SBC-5 (JCRB0819), which constitutively expresses proGRP[32], was obtained from Human Science Research Resources Cell Bank (Osaka). The human adenocarcinoma cell line A549 (ATCC CCL-185) and the human embryonal kidney cell line 293 (ATCC CRL-1573) were obtained from the American Type Culture Collection (Manassas, VA, USA). SBC-5 was cultured in minimum essential medium, and A549 and 293 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin, respectively (all reagents were purchased from Invitrogen Corp., Carlsbad, CA, USA). All experiments were performed when cells were 70% to 80% confluent.

2. *Construction of plasmid expression vectors*

Two segments of the 5′-upstream of the proGRP gene (−1,201 and −807 to +106 relative to the transcription start site, respectively) were amplified with the polymerase chain reaction (PCR) and *Taq* DNA polymerase (Sawady Technology, Tokyo) with genomic DNA from a healthy person as a template. The longer segment contains a SCLC-specific enhancer element located between −1,128 and −793[33]. The sense primers used were 5′-GGCGACGCGTGAAGATGTATTGAATCAGCAG-3′ and 5′-AGT-

GACGCGTGGCAGCCTAGGGAAACTGGCAT-3′. For the antisense primer, 5′-TATTCTCGAGTCCCGACGGGAAGCCCTTGGAGAT-3′ was used. The sense primers were designed to contain a *Mul*I restriction sequence at the 5′ end, whereas the antisense primer contained a *Xho*I sequence. For PCR amplification, a cycle of sequential incubations for denaturation at 94°C for 2 minutes, annealing at 55°C for 2 minutes, and extension at 72°C for 3 minutes was repeated 30 times. The PCR products were then digested with *Mul*I or *Xho*I, inserted into a firefly luciferase expression plasmid, pGL2-Basic vector (Promega, Tokyo), to generate pGRP1201L and pGRP807L, respectively. The longer fragment (−1,201 to +106) was also inserted into a *Cre*-expression plasmid pBS185 (Invitrogen) at the *Mul*I and *Xho*I sites to construct a proGRP-promoter-driven *Cre*- expression vector, pGRP-Cre.

3. *Transfection of cells and dual luciferase assay*

SBC-5 and A549 cells (1.0×10^5 cells/well) were cultured in 12-well plates. For transfection, 4 μl of Lipofectin (Invitrogen) was added to 1 ml of Optimen (Invitrogen), and mixed with 500 ng of each of the individual firefly luciferase reporter plasmids and 10 ng of pRL-CMV (Promega, Madison, WI, USA), which contains the *Renilla* luciferase gene, as an internal control. The mixtures were incubated at room temperature for 30 minutes to form a liposome-DNA complex. Before transfection, cells were washed twice with phosphate-buffered saline (PBS), after which 1 ml of liposome-DNA complex was added to cells. After 24 hours' incubation, the medium was replaced with the growth medium and incubated for an additional 72 hours. Luciferase activity was evaluated with the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Lumat LB 9501, Berthold Japan, Tokyo) according to the manufacturers' instructions. The levels of firefly luciferase expression of each plasmid were normalized by that of *Renilla* luciferase.

4. *Construction of recombinant adenovirus vectors*

The GRP promoter-*Cre* fragment was excised from the pGRP-Cre vector and ligated into a *Swa*I site of an adenovirus cassette cosmid of pAxcw (Takara, Tokyo) to produce AdGRPCre. Briefly, the recombinant replication-defective adenovirus was constructed with the cosmid-adenoviral DNA terminal protein complex method[34]. The *Cre*-expressing adenoviral vector with the CAG promoter (AxCANCre) and the *lacZ*-expressing adenoviral vector with the CAG promoter (AxCANLacZ) were provided by Dr. Izumu Saito (Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo). The *lacZ*-expressing vector containing the CAG promoter and the neomycin-resistance gene separated by a pair of *loxP* sequences (AxCALNLNZ), and another adenoviral vector for *Bax* gene expression with *loxP* sequences (AxCALNLhBax-alpha) were obtained from the RIKEN DNA Bank (Tsukuba, Japan)[35].

5. *β-galactosidase expression in cells after infection with recombinant adenoviral vectors*

To evaluate SCLC-cell-specific expression of the transduced *lacZ* gene by double infection with the *Cre*- expressing and *lacZ*-expressing adenoviral vectors, SBC-5 and A549 cells (1.0×10^5 cells/well) were infected with AxCANCre or AdGRPCre and AxCALNLNZ at multiplicity of infection (MOI) of 10 : 10 or with AxCANLacZ as a positive control. First, 72 hours after infection cells were fixed with 0.2% glutaraldehyde, stained with in PBS for 15 minutes at 4°C, washed with PBS, and incubated in the X- gal staining solution[36] (2 mM $MgCl_2$, 1 mg/ml X-gal, 5 mM $K_3Fe_9(CN)_6$, and 5 mM $K_4Fe_9(CN)_6$ in PBS) for 3 hours at 37°C. Stained cells were then examined under a light microscope.

6. *Evaluation of Bax-α mRNA transcripts in cells after double infection with adenoviral vectors*

Levels of *Bax-α* mRNA transcripts were evaluated with reverse transcription (RT) followed by PCR amplification in SBC-5 and A549 cells co-infected with AdGRPCre or AxCANCre and AxCALNLhBax-alpha. First, total RNA was extracted with the guanidinium/cesium chloride centrifugation method[37], after which each RNA sample was converted to single-stranded cDNA with RT and Moloney murine leukemia virus-reverse transcriptase with oligo (dT)

primers (both from Promega). The obtained cDNA was then PCR-amplified with the primer set for the *Bax* gene (sense: 5′-ATGGACGGGTC-CGGGGAG-CAG-3′, antisense 5′-TCACGGTCTGCCACGTGGG-CGT-3′)[38]. The condition of PCR amplification was 20 cycles of denaturation at 94°C for 1 minute, annealing at 64°C for 1 minute, and extension at 72°C in a thermocycler for 1 minute.

7. Detection of Bax protein in lung cancer cells after double infection with adenoviral vectors

Expression of Bax protein was investigated with immunoblot analysis[39]. After infection of SBC-5 or A549 cells (3×10^6 cells each) with adenoviral vectors, cells were washed with PBS, suspended in 150 μl of the lysis buffer[39] (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 200 μM sodium orthovanadate, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin [all chemicals from Sigma Chemical Co., St. Louis, MO, USA]), and incubated for 30 minutes on ice. The supernatants of the samples were collected after centrifugation at 15,000 rpm for 30 minutes. Protein concentrations were measured with the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Aliquots of the lysates (60 μg of protein) were mixed with the SDS sample buffer in reduced condition (final concentration: 30 mM Tris [pH 6.8], 1.2% SDS, 6% glycerol, 3% 2-mercaptoethanol) and boiled for 5 minutes. Proteins were separated with SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (BioRad, Hercules, CA, USA). After blocking in TBS-T buffer (25 mM Tris-HCl [pH8.0], 150 mM NaCl, and 0.1% Tween 20) with 3% skim milk (BD Diagnostic Systems, Sparks, MD, USA), the membranes were treated with a Bax-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Amersham Biosciences K.K., Tokyo) to detect Bax protein. The expression of β-tubulin was used as a loading control, detected with an anti-β-tubulin antibody and an HRP-conjugated secondary antibody (Santa Cruz Biotechnology). The membrane was analyzed with the ECL Detection System (Amersham Biosciences) according to the manufacturer's instructions.

8. Evaluation of cell viability with the methylthiotetrazol assay after introduction of the Bax-α gene

To determine the optimal ratio of the *Cre*-expressing adenoviral vector to the *Bax*-expressing vector, SBC-5 and A549 cells were grown in 24-well plates (1.0×10^5 cells/well), co-infected with AdGRPCre or AxCANCre and AxCALNLhBax-alpha at various ratios of MOI ranging 50:0 to 0:50, respectively. Seventy-two hours after infection, cell viability was evaluated with the methylthiotetrazol (MTT) assay[40] and a MTT substrate (10 mg/ml, Sigma). Each sample was measured at a wavelength of 590 nm with a microwell plate reader (BioRad).

9. Detection of cellular apoptosis after SCLC cell-specific introduction of the Bax-α gene

Bax gene-induced apoptosis in lung cancer cells was evaluated morphologically by staining with Hoechst 33342[39]. To achieve this, 3×10^6 cells of SBC-5 or A549 were infected with AdGRPCre or AxCANCre plus AxCALNLhBax-alpha, incubated for 48 hours, and stained with 10 μM of Hoechst 33342 in the presence of propidium iodide (both from Sigma). Induction of apoptosis under each condition was then studied with a fluorescent microscope (Carl Zeiss, Oberkochen, Germany) as described previously[41].

Next, the activity of caspase-3 was investigated with a Caspase-3 Cellular Activity Kit PLUS (BIOMOL International, Plymouth Meeting, PA, USA)[42]. SBC-5 or A549 cells were infected with AdGRPCre plus AxCALNLhBax-alpha or AxCANCre plus AxCALNLhBax-alpha at an MOI ratio of 10:10 as before. At various time points after infection, cell lysates were prepared with the lysis buffer provided in the kit. Cells were washed with PBS, lysed in the cell lysis buffer (50 mM [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 100 mM NaCl, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate, 10 mM DTT, and 1 mM ethylenediaminetetraacetic acid) and centrifuged for 10 minutes at 10,000 g. Protein concentrations in the lysate supernatant were determined as described pre-

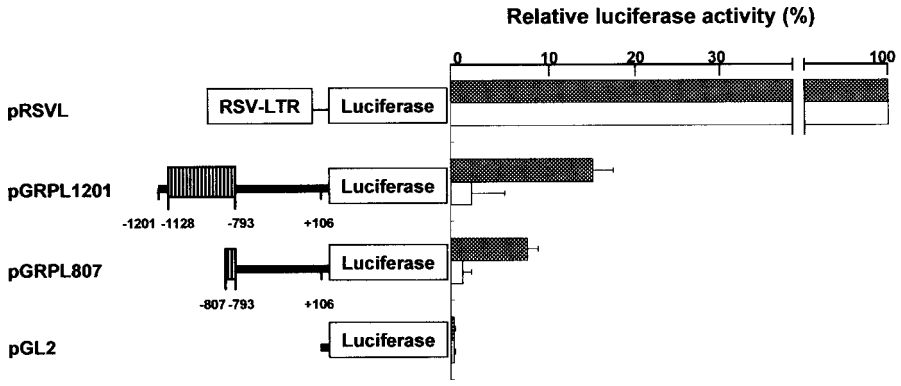

Fig. 1. SCLC-cell-specific promoter activity of the 5′-flanking region of the *proGRP* gene.
Normalized levels of luciferase expression by the plasmid constructs of the 5′-flanking region of the *proGRP* gene and a firefly luciferase reporter gene are shown relative to the expression of the positive control, pRSVL (defined as 100%), in SBC-5 (shaded bar) and A549 (open bar). pGL2, a promoterless luciferase plasmid, served as a negative control. The luciferase activity of pGRPL1201, which contains the SCLC-specific enhancer element (striped box) located upstream of the promoter, was 17.1±3.5% in SBC-5 cells and 2.4±1.0% in A549 cells ($p<0.0001$), whereas the luciferase activity of pGRPL807 was 6.2±2.6% in SBC-5 cells and 1.3±0.5% in A549 cells ($p<0.0001$). The presence of the SCLC-specific enhancer significantly enhanced the promoter activity in SBC-5 cells ($p<0.005$). Each datum is the average of 4 independent experiments.

viously. Into a reaction volume of 100 μl, aliquots of the lysate (20 μg of protein) buffer and caspase-3 substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-*p*-nitroaniline (Ac-DEVD-pNa, final concentration 200 mM) were added and incubated at 37°C. Release of pNa was monitored by recording the OD_{405nm} at 30-minute intervals[42].

10. In vivo evaluation of inhibition on tumor growth by intratumoral injection of Bax-expressing adenoviral vectors

Human lung cancer xenografts were established in Balb/c nude mice (5 to 7 weeks old, CLEA Japan, Tokyo) through subcutaneous inoculation of 8×10^6 SBC-5 or A549 cells into the dorsal flank. The size of tumors was measured 3 times a week, and the tumor volume was calculated by multiplying the large and small diameters[43]. When the surface area of xenografted tumors was greater than 30 mm^2, each mouse was given intratumoral injection of 100 μl of PBS or adenoviral vectors (4×10^8 PFU) with a 27-gauge needle directed to 3 sites for 3 consecutive days. The ratios of AxCANCre : AxCALNLhBax-alpha and AdGRPCre : AxCALNLhBax-alpha were both set at 1 : 1, and thereafter regression of grafted tumors was observed for 4 weeks.

11. Statistical analysis

All data are expressed as the mean±standard error of the mean. Student's *t*-test was used to evaluate the significance of differences.

Results

1. The 5′-flanking region of the ProGRP gene for cell type-specific promoter activity in SCLC cells

The relative luciferase activity with pGRPL1201, which contained the upstream SCLC-specific enhancer element of the *proGRP* gene, was 17.1±3.5% and 2.4±1.0% in SBC-5 and A549 cells, respectively, compared to that of the RSV-LTR promoter, which was defined as 100% (Fig. 1). In contrast, the luciferase activity with pGRPL807 was 6.2±2.6% and 1.3±0.5% in SBC-5 and A549 cells, respectively, demonstrating less efficient but still SCLC cell-dominant promoter function compared to that of pGRPL1201. Thus, the 5′-flanking region of the *proGRP* gene showed low, but cell-specific promoter activity in proGRP-producing SCLC cells in the presence of the upstream SCLC-specific enhancer element.

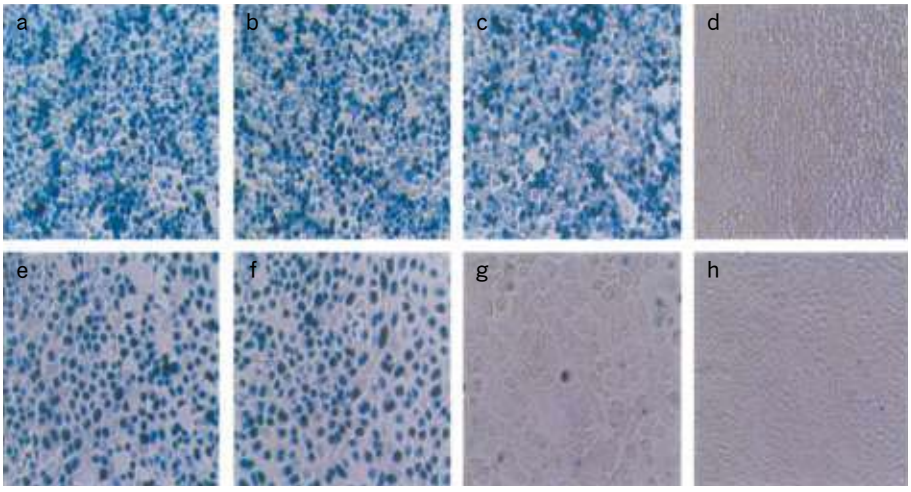

Fig. 2. Evaluation of *lacZ* gene expression in SCLC and NSCLC cells after co-infection with adenoviral vectors. Data shown are for SBC-5 (a-d) and A549 (e-h) cells. a and e: Infected with AxCANLacZ alone (MOI ratio=10); b and f: co-infected with AxCANCre and AxCALNLNZ (MOI ratio=10:10); c and g: co-infected with AdGRPCre and AxCALNLNZ (MOI ratio=10:10); and d and h: uninfected control. Note SCLC-cell-type-specific β-galactosidase expression in SBC-5 cells after double infection with AdGRPCre and AxCALNLNZ (panel c).

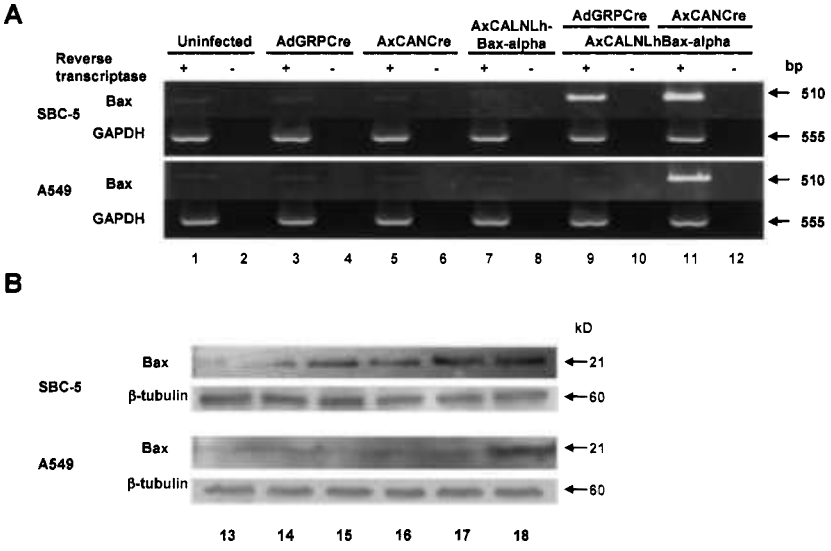

Fig. 3. Expressions of *Bax-α* mRNA and Bax protein in SCLC and NSCLC cells after adenovirus-mediated gene transfer.
Data obtained from SBC-5 cells are shown at the top, and data from A549 cells are at the bottom. GAPDH mRNA transcripts were evaluated as an internal control. Detection of *Bax-α* mRNA by RT-PCR amplification. '−' and '+' indicates the absence and presence, respectively, of reverse transcriptase in the cDNA synthesis reactions. The expected sizes of the PCR products are indicated on the right (510 bp for *Bax-α* transcripts, and 555 bp for GAPDH transcripts). Lanes 1 and 2: uninfected control; lanes 3 and 4: infected with AdGRPCre alone; lanes 5 and 6: infected with AxCANCre alone; lanes 7 and 8: infected with AxCALNLhBax-alpha alone; lanes 9 and 10: co-infected with AdGRPCre and AxCALNLhBax-alpha; and lanes 11 and 12: co-infected with AxCANCre and xCALNLhBax-alpha. Note that augmented expression of *Bax-α* transcripts was observed only in SBC-5 cells infected with both AdGRPCre and AxCALNLhBax-alpha adenoviral vectors, although endogenous *Bax-α*mRNA was detected in both cell lines at low levels. GAPDH mRNA expression was detected in both cell lines. (B) The expression of *Bax* (top) in SBC-5 (above) and A549 (below) cells were examined by immunoblot analysis. Expression of β-tubulin (bottom) was evaluated as a loading control. Lane 13: uninfected control; lane 14: infected with AdGRPCre alone; lane 15: infected with AxCANCre alone; lane 16: infected with AxCALNLhBax-alpha alone; lane 17: co-infected with AdGRPCre and AxCALNLhBax-alpha; and lane 18: co-infected with AxCANCre and AxCALNLhBax-alpha. Note the overexpression of Bax protein only when SBC-5 cells were co-infected with AdGRPCre and AxCALNLhBax-alpha.

2. Cell type-specific LacZ gene expression in ProGRP- producing SCLC cells after double infection with the adenoviral vectors AdGRPCre and AxCALNLNZ

A majority of SBC-5 and A549 cells were stained with X-gal after single infection with the control vector AxCANLacZ (Fig. 2a, 2e) or after co-infection with AxCANCre and AxCALNLNZ (Fig. 2b and 2f). In contrast, only SBC-5 cells were stained with X-gal after co-infection with AdGRPCre and AxCALNLNZ (Fig. 2c), whereas A549 cells were not stained (Fig. 2g). Neither SBC-5 nor A549 cells were stained blue in the uninfected control (Fig. 2d and 2h).

3. Expression of Bax-mRNA and Bax protein after cell-type-specific introduction to SCLC cells with the proGRP promoter and the Cre/loxP system

Endogenous *Bax-α* mRNA transcripts were detected in both SBC-5 and A549 cells at low levels (Fig. 3a). However, after double infection with AdGRPCre and AxCALNLhBax-alpha, expression of exogenously transduced *Bax-α* mRNA was detected in SBC-5 cells but not in A549 cells. In contrast, transduced *Bax-α* mRNA transcripts were present at high levels in both SBC-5 and A549 cells that had been co-infected with AxCANCre and AxCALNLhB ax-alpha. Control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcription and β-tubulin expression were similar in all samples evaluated.

In concordance with mRNA expression, endogenous Bax protein was detected in SBC-5 and A549 cells (Fig. 3b). However, co-infection with AdGRPCre and AxCALNLhBax-alpha transduced Bax protein in SBC-5 cells but not in A549 cells, whereas co-infection with AxCANCre plus AxCALNLhBax-alpha resulted in overproduction of exogenous Bax protein in both cell types.

4. Co-infection with AdGRPCre and AxCALNLhBax-alpha for cell-type-specific cytotoxic effect only on SCLC cells

When SBC-5 cells were co-infected with AdGRPCre and AxCALNLhBax-alpha at various MOI ratios ranging from 50 : 0 to 0 : 50, cell viability was significantly lower at MOI ratios of 40 : 10 to 10 : 40 than at an MOI ratio of 0 : 50 ($p < 0.0001$ for each ratio). The most prominent cytotoxicity was observed at an MOI ratio of 25 : 25, with 76% of cells dead (Fig. 4). In contrast, cytotoxic effects were modest when A549 cells were infected with both adenoviral vectors. Single infection with AdGRPCre slightly decreased the viability of A549 cells.

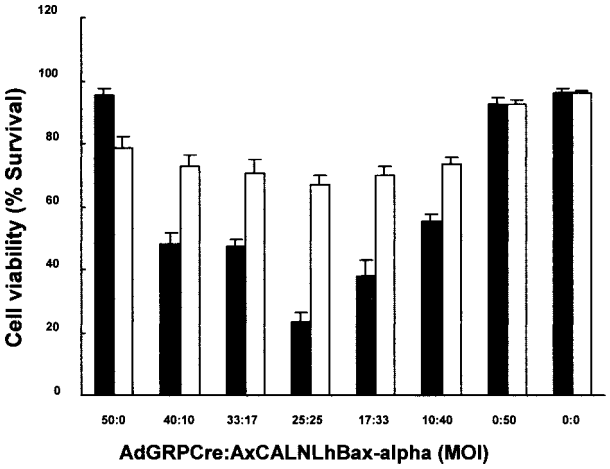

Fig. 4. Evaluation of cell viability after adenovirus-mediated *Bax-α* gene transfer using the *Cre/loxP* system *in vitro*.
SBC-5 and A549 cells were co-infected with AdGRPCre and AxCALNLhBax-alpha at various ratios of MOI in total of 50. Cell viability was determined by the MTT assay. Data shown are from four independent experiments for SBC-5 (shaded bar) and A549 (open bar) cells, respectively. The maximum cytotoxic effect was obtained at the MOI ratio of 25 : 25 in SBC-5 cells (viability : 23.1±3.32%), although less prominent but still significant cytotoxicity was also observed at other vector ratios ($p < 0.0001$ for each MOI ratio compared to that of 0 : 50). A modest decrease in cell viability was observed in the presence of AdGRPCre in A549 cells.

5. Cellular apoptosis and intracellular caspase-3-like protease activity in SCLC cells in a cell-type-specific manner after co-infection with AdGRPCre and AxCALNLhBax-alpha

Shrunken and fragmented nuclei were observed in more than 85% of both SBC-5 and A549 cells after co-infection with AxCANCre and AxCALNLhB ax-alpha (Fig. 5a and 5e). Importantly, nuclear fragmentation was observed in as many as 72% of SBC-5 cells after co-infection with AdGRPCre and AxCAL-

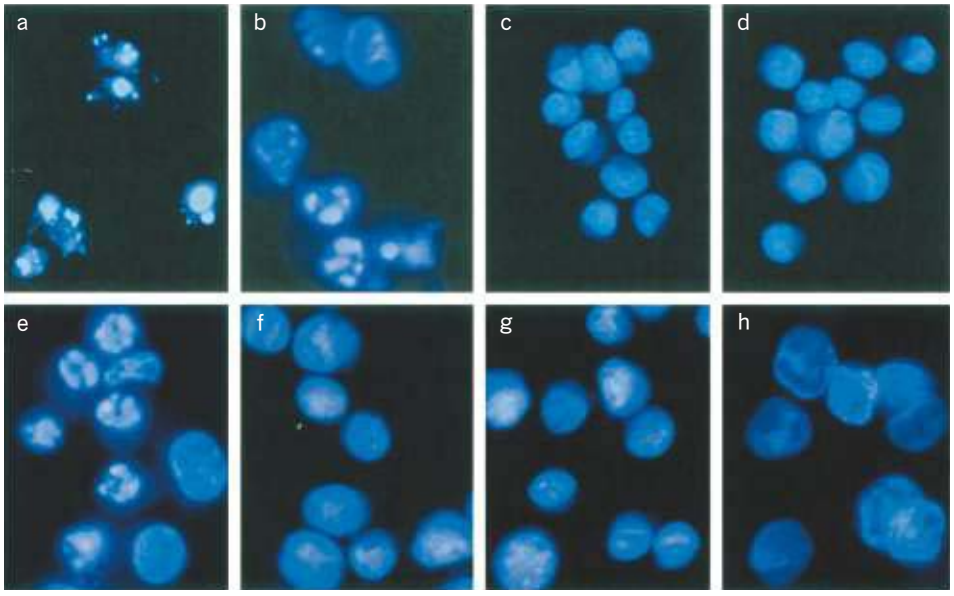

Fig. 5. Detection of apoptosis with Hoechst 33342 dye after adenovirus-mediated *Bax-α* gene transfer. Fluorescent microscopic analysis is shown for SBC-5 (a-d) and A549 (e-h) cells. a and e: co-infected with AxCANCre and AxCALNLhBax-alpha (MOI ratio=10:10); b and f: co-infected with AdGRPCre and AxCALNLhBax-alpha (MOI ratio=10:10); c and g: infected with AxCALNLhBax-alpha alone (MOI=10); d and h: uninfected control. Note that SCLC-cell-specific nuclear shrinkage and fragmentation were achieved by co-infecting cells with AdGRPCre and AxCALNLhBax-alpha (panel b). Magnification: ×100 in both cell lines.

NLhBax-alpha (Fig. 5b), whereas only 9% of A549 cells became apoptotic after co-infection (Fig. 5f). In contrast, most nuclei were intact in uninfected cells and in cells of both types infected with AxCALNLhBax-alpha alone (Fig. 5c, 5d, 5g, and 5h).

No caspase-3 activation was observed in uninfected control SBC-5 cells or in cells infected with AxCALNLhBax-alpha alone (Fig. 6). Intracellular caspase-3-like protease activity at 48 hours in SBC-5 cells was markedly greater after co-infection with AxCANCre and AxCALNLhBax-alpha (2.60±0.27 pmol pNa/min/μg protein) than in uninfected controls (0.76±0.15, $p<0.0001$; Fig. 6a). Likewise, when SBC-5 cells were co-infected with AdGRPCre and AxCALNLhBax-alpha, enzyme activity increased from 0.60±0.15 to 2.16±0.14 pmol pNa/min/μg protein ($p<0.0001$) but peaked 72 hours after infection (Fig. 6a). Similarly, in the NSCLC cell line A549, co-infection with AxCANCre and AxCALNLhBax-alpha significantly increased caspase-3-like activity from 0.58±0.08 (at baseline) to 2.57±0.21 pmol pNa/min/μg protein at 48 hours ($p<0.0001$; Fig. 6b). However, intracellular caspase-3-like activity in A549 cells was not significantly increased by double infection with AdGRPCre and AxCALNLhBax-alpha (0.59±0.12 pmol pNa/min/μg protein at 72 hours; $p=0.58$).

6. *SCLC-cell-specific inhibition of tumor growth by direct injection of adenoviral vectors AdGRPCre and AxCALNLhBax-alpha in vivo*

Growth inhibition and regression of xenograft SBC-5 tumors were achieved with double infection with AdGRPCre and AxCALNLhBax-alpha, as with co-infection with AxCANCre and AxCALNLh Bax-alpha (Fig. 7a). For example, co-injection of AdGRPCre and AxCALNLhBax-alpha with SBC-5 tumor suppressed tumor growth 86% (tumor area) on day 31 compared with uninfected controls ($p<0.005$). However, intratumoral injection of AxCANCre, AdGRPCre, or AxCALNLhBax-alpha alone did not suppress tumor growth. In marked contrast, double infection with AdGRPCre and AxCALNLhBax-alpha did not inhibit the growth of A549 tumors (Fig. 7b). Growth of A549 xenografts was inhibited only by co-infection with AxCANCre and AxCALNLh Bax-

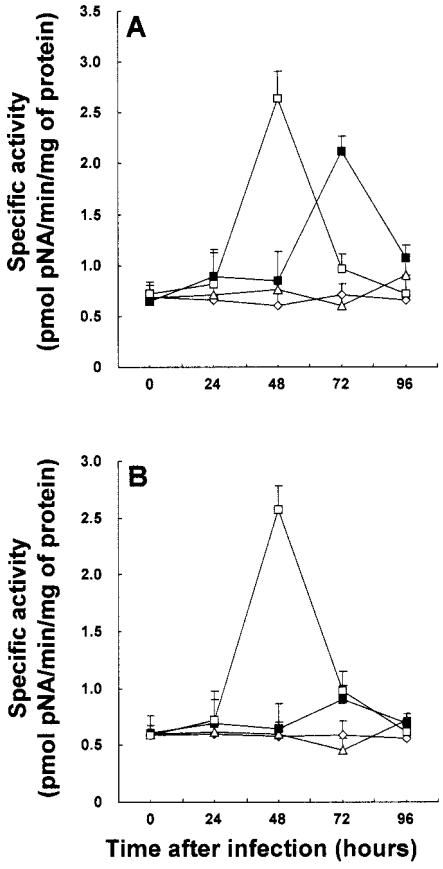

Fig. 6. Evaluation of capase-3 activity in lung cancer cells after adenovirus-mediated *Bax-α* gene transfer.
Data shown are from 4 independent experiments for caspase-3-like protease activity in SBC-5 (A) and A549 (B) cells detected with fluorescent-labeled tetrapeptide cleavage assay. ◇: uninfected control; △: cells infected with AxCALNLhBax-alpha alone; and ■: cells co-infected with AdGRPCre and AxCALNLhBax-alpha; and □: cells co-infected with AxCANCre and AxCALNLhBax-alpha. Note the caspase-3 activity was clearly increased 72 hours after infection with AdGRPCre and AxCALNLhBax-alpha only in SBC-5 cells ($p<0.0001$ compared with the basal level), whereas caspase-3 activity was elevated 48 hours after infection with AxCANCre and AxCALNLhBax-alpha in both SBC-5 cells and A549 cells (both $p<0.0001$ compared with the basal levels).

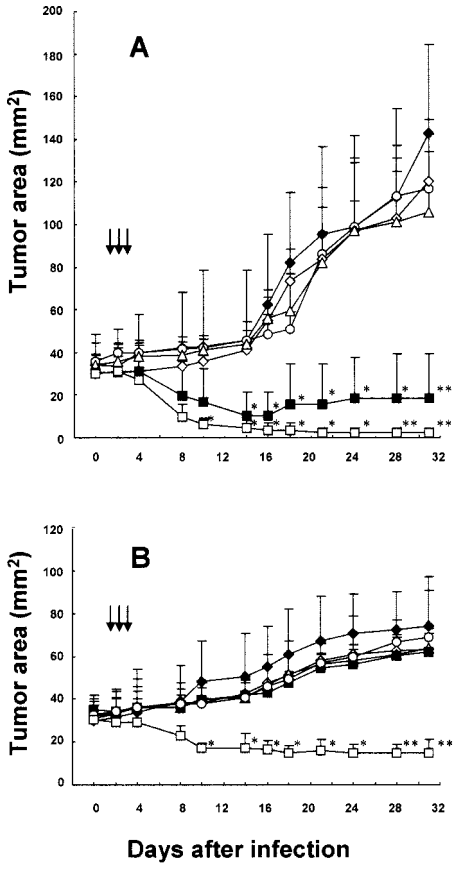

Fig. 7. Evaluation of antitumor effect of adenovirus-mediated *Bax-α* gene transfer *in vivo*.
Data depicted are from 5 mice receiving xenografts of SBC-5 (A) or A549 (B) cells for each time point. Adenoviral vectors (5×10^8 PFU) were administered intratumorally for 3 consecutive days after the average area of the inoculated tumors had reached 30 mm² (arrows). ◆: uninfected control; ○: infected with AdGRPCre alone; ◇ infected with AxCANCre alone; △: infected with AxCALNLhBax-alpha alone; ■: co-infected with AdGRPCre and AxCALNLhBax-alpha; and □: co-infected with AxCANCre and AxCALNLhBax-alpha. Treatment of xenograft tumors with AdGRPCre and AxCALNLhBax-alpha, or with AxCANCre and AxCALNLhBax-alpha caused significant regression of tumor growth compared with the uninfected control at the indicated time points (*: $p<0.01$; **: $p<0.005$).

alpha, not by infection with AxCANCre, AdGRPCre, or AxCALNLhBax-alpha alone.

Discussion

In the present study, we have shown through measurement of levels of mRNA and protein that SCLC-cell-specific transduction of the proapoptotic molecule Bax was achieved. We have also shown that Bax transduced by means of the proGRP promotr combined with the *Cre/loxP* system through adenoviral-mediated gene transfer induced SCLC-specific

activation of intracellular caspase-3 and subsequent apoptosis *in vitro* and induced tumor regression *in vivo* in a subcutaneous tumor xenograft model in mice.

ProGRP is a specific product present in the cancer cells of two thirds of patients with SCLC and has been used as a therapeutic marker[7-9]. SCLC-cell-specific expression of the proGRP gene is thought to be modulated by upstream enhancer sequences located at −1,128 to −793 relative to the transcription start site[33]. This specific control was confirmed by our finding of higher promoter activity in the presence of the proGRP gene in the SCLC cell line. A tissue-specific promoter is an effective tool for inducing selective expression of foreign genes in cancer cells[19-21]. However, the transcriptional activity exerted by such promoters is believed to be lower than that exerted by other potent promoters[16-18]. In fact, the 5′-flanking region of the proGRP gene exhibited low promoter function, approximately 15% of that of the RSV-LTR, despite the presence of the SCLC-specific enhancer. To overcome this limitation of tissue-specific but low-level activity of the proGRP gene promoter to drive expression of the foreign therapeutic gene in SCLC cells, the *Cre/loxP* regulating system was used with adenoviral vectors as a vehicle for efficient gene transfer. When the bacterial *Cre* recombinase gene was introduced to SCLC cells in a cell-specific manner with the proGRP promoter by using AdGRPCre, only SCLC cells were transduced to express either the bacterial *lacZ* gene with AxCALNLNZ or the *Bax-α* gene with AxCALNLhBax-alpha *in vitro* and *in vivo*.

In gene therapy for patients with SCLC, the possibility of early metastasis to the brain, bones, and liver should be considered[2]. In this context, it is rational to aim systemic delivery of the gene therapy vector containing the therapeutic gene although the local implanted model exhibited an excellent response in this study. Because of the toxicity and limitations of current adenoviral vectors, new, efficient, and less-toxic vectors are needed[44]. When an ideal vector becomes available, a systemic approach would be a more promising for treating SCLC tumors regardless of distant metastases. Another issue in the use of a proGRP promoter is that GRP is normally present in neurons of the brain and gastrointestinal tract and in Kulchitsky cells, albeit at low levels[11,32]. To avoid phenotypic changes in normal cells due to transgene expression, several approaches may be used. First, if overexpression of the transgene is harmful to cancer cells but not to normal cells, cancer-cell-specific gene therapy could be achieved[32]. Second, because GRP is expressed in normal tissue cells at low levels, the transferred regulatory unit containing the proGRP promoter might not function efficiently in normal GRP-producing cells, thus causing no severe adverse effects. Third, a gene therapy vector might be delivered only to cancer cells by means of cancer-cell-specific receptor-mediated gene transfer[2].

Transferring an appropriate therapeutic gene is important in cancer gene therapy. In this regard, Bax is a well-characterized proapoptotic gene which can induce apoptosis and thus exerts potent antitumor effects against many cancer cell lines[27-30]. Overexpression of Bax enhances intracellular accumulation of chemotherapeutic agents and may improve the effectiveness of chemotherapy and radiation therapy of cancers[28-30,45]. The *Bax* gene promoter contains P53 binding sites, and expression of the *Bax* gene might be upregulated by P53[24]. However, *Bax*-mediated apoptosis appears to be independent of p53 status and of levels of endogenous Bcl-2 and Bax[45-47]. Importantly, naked DNA containing the *Bax*-expression cassette kills 70% to 90% of transfected tumor cells, whereas naked DNA containing the *p53*-cassette kills only 40% cells, suggesting that Bax may be more toxic than p53[24]. These reports clearly demonstrate that *Bax* gene alone can be used to induce apoptosis in SCLC cells, although more than 80% of SCLC tumors have an abnormality of the *p53* gene and the Bcl-2/Bax ratio is higher than in NSCLC cells[2,6,46,47].

As shown in the present study, introduction of the *Bax* gene into cancer cells activates caspase-3, further confirming the proapoptotic property of Bax and the subsequent Bax-associated intracellular apoptotic pathway[48]. Interestingly, the activity of caspase-3 in SBC-5 SCLC cells peaked 72 hours after double infection with AdGRPCre and AxCALNLhBax-alpha and

48 hours after co-infection with AxCANCre and AxCALNLhBax-alpha. This time difference in caspase activity is likely due to the different potency of the CAG and proGRP promoters used in this experiment.

In summary, our data have shown that combined intratumoral administration of two types of adenoviral vectors can induce SCLC-tumor-specific growth suppression *in vivo*. Adenoviruses are useful vectors for efficient expression of transgenes, and preclinical studies of intratumoral administration of adenoviral vectors have shown promising results[25,49,50]. As discussed previously, intravenous or intra-arterial administration of therapeutic vectors is considered the most efficient method for treating patients with advanced SCLC, which tends to metastasize[2]. Importantly, however, currently available vectors, including adenovirus and gene transfer systems, have certain limitations regarding efficient gene transfer and safety[44,51]. Therefore, treating the primary tumor and metastatic tumors with systemic gene delivery remains challenging. Further development of novel and improved vectors is needed for treating SCLC.

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