Enhanced Expression of Apoptin by the Myc–Max Binding Motif and SV40 Enhancer for SCLC Gene Therapy

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Apoptin is derived from chicken anemia virus (CAV) and known to induce tumor specific apoptosis but not normal cells. The aim of this study was to use increased expression of apoptin by the Myc–Max response element (MMRE) and SV40 enhancer in small-cell lung cancer (SCLC) gene therapy. To investigate the possibility of the utilization of the MMRE, apoptin, and SV40 promoter/enhancer in targeted cancer gene therapy, adenovirus vector expressing apoptin controlled by the MMRE, and SV40 promoter/enhancer was constructed. Ad-MMRE-apoptin-enh infected SCLC cells were significantly suppressed and induced apoptosis more than those of Ad-apoptin or Ad-apoptin-enh. Infection with Ad-MMRE-apoptin-enh of normal cells did not increase apoptosis. About 85% of SCLC tumors show overexpression of the myc family, so the increased expression of apoptin by MMRE and SV40 enhancer can be used in targeted SCLC gene therapy. These results indicate that apoptin expression was increased by the MMRE and SV40 promoter/enhancer, and that this strategy can be used in SCLC targeted cancer gene therapy.

Key words: apoptin; Myc–Max response element; SV40 enhancer; small-cell lung cancer; targeted cancer gene therapy

Materials and Methods

Cell culture. Wi-38 (human normal fibroblast) and QBI-293A (Quantum-Appigene, U.S.A.; a human cell line transformed by adenovirus 5 DNA) cells were grown in DMEM (Gibco BRL, Germany) supplemented with 10% FCS (HyClone, Logan, U.S.A.), penicillin (50 units/ml), and streptomycin (50 μg/ml) in the presence of 5% CO₂. NCI-H417 (small-cell lung cancer cells) were grown in RPMI1640 (Gibco BRL, Germany) supplemented with 10% FCS, penicillin (50 units/ml), and streptomycin (50 μg/ml) in the presence of 5% CO₂. All cell lines except QBI-293A were obtained from the American Type Culture Collection (Manassas, VA).

Construction of reporter plasmids. Sense and antisense oligonucleotides for Myc–Max response elements were synthesized (Takara, Japan) as described by Eiseman’s group. Two oligomers were annealed as described by Kishimoto’s group. This annealed fragment includes restriction endonuclease sites of Kpn I and Bgl II at the end of 5’ and 3’ respectively. This fragment has four repeats containing a core sequence CACGTG, which is the binding site of Myc–Max heterodimers (Fig. 1A). pGL3-MMRE-enh, which has induced tumor specific apoptosis, but not normal, diploid cells. Apoptin-induced apoptosis is p53 independent, Bcl-2 dependent, and correlated with the subcellular localization of the protein. Apoptin is found in the cytoplasm of normal cells, whereas it is found in the nucleus of tumor cells. This character makes apoptin a tumor specific killing agent if it is delivered to tumor cells sufficiently.

Because specific therapeutic gene expression is important in cancer gene therapy, targeted cancer gene therapy has the aim of concentrating the target therapeutic gene expression into the specific target tissue. Then it can minimize a secondary effect and maximize the therapeutic index. In this study, it was attempted to increase the expression of apoptin by using the Myc–Max response elements (MMRE) and transcriptional enhancer of SV40 virus in adenoviral vector.
MMRE, SV40 promoter, luciferase gene, and SV40 enhancer was constructed by inserting MMRE fragments into the Kpn I and Bgl II site of pGL3-Control plasmid. Direct dideoxynucleotide sequencing ensured a correct sequence and direction.

Luciferase assay. The selective increased expression of the luciferase gene in SCLC cells by MMRE and SV40 enhancer was determined by luciferase reporter plasmid using FuGENE™6 (Roche, Germany) according to the manufacturer’s protocol. Briefly, $10^5$ cells seeded in a 6-well culture dish were exposed to a transfection mixture containing 2 μg of luciferase reporter plasmids and 0.5 μg of pSV-β-galactosidase control plasmid vector (Promega, U.S.A.) for 48 h at 37°C. Luciferase assays were performed according to the manufacturer’s protocols. (Promega, U.S.A.). Transcriptional activity was measured with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). The Simian virus 40 (SV40) promoter (pGL3-Promoter, pGL3-Control) was used as a positive control. The luciferase activity of pGL3-3Promoter plasmid in each cell line was considered to be 1. β-Galactosidase assay was also performed with the same cell extracts to standardize for transfection efficiency. All of the data shown in this study were obtained from at least three independent experiments.

Construction of recombinant adenovirus Ad-MMRE-Apoptin-enh. pGL3-MMRE-Apoptin-enh, which has MMRE, SV40 promoter, apoptin gene, and SV40 enhancer, was constructed by replacing the luciferase gene of pGL3-MMRE-enh plasmid with the apoptin gene. CAV DNA sequences encoding apoptin (nt 427~868) were artificially synthesized by the oligo DNA assembly method (Takara, Japan). Then the MMRE-SV40 promoter-apoptin-SV40 enhancer cassette was digested with Kpn I and Sal I and ligated with Kpn I and Sal I digested pShuttle (Ad transfer vector), and named pS-MMRE-apoptin-enh (Fig. 1B). The pS-MMRE-apoptin-enh was then co-transformed with pAdEasy-1 (Quantum-Apppligene, U.S.A.) into BJ5183 E. coli cells using electroporation methods. After selection on kanamycin plates, 40 colonies were selected and screened of recombinants by plasmid size and restriction enzyme analysis. Viral DNAs of candidates were transfected in QBI-293A cells using FuGENE™6 (Roche, Germany) and the manufacturer’s protocol. This constructed adenovirus vector was named Ad-MMRE-apoptin-enh. The constructed adenovirus were then purified from the lysates of infected QBI-293A cells by two rounds of CsCl gradient centrifugation, then the titers of the virus stock were determined by plaque dilution assay. The recombinant virus were stored at −80°C until use. Control recombinant adenovirus Ad-apoptin and Ad-apoptin-enh using pGL3-Promoter and pGL3-Control that do not have SV40 enhancer and MMRE respectively were constructed the same way as above. The negative control vector (Ad5.CMV-Null; Ad-CMV) was purchased from Quantum-Appligene (Quantum-Appligene, U.S.A.). Ad-CMV is an empty vector that contains no coding sequences between the CMV promoter and PA (poly adenylation site).

Cell death detection ELISA. Cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) after induced cell death by Ad-MMRE-apoptin-enh were determined by Cell Death Detection ELISA™PLUS (Roche, Germany). Briefly, the Ad-CMV, Ad-apoptin, Ad-apoptin-enh, and Ad-MMRE-apoptin-
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QBI-293A

Enh infected cell lysates were placed into a streptavidin-coated micro plate. A mixture of anti-histone-biotin and anti-DNA-POD was added and incubated for 2 h at 15~25°C. After removal of unbound antibodies by a washing step, POD was determined photometrically at 405 nm with ABTS as substrate.

Results

Effect of MMRE on the strength of the SV40 promoter/enhancer

To determine if plasmid vectors containing MMRE could produce higher levels of transgene expression than those without it, a MMRE was inserted upstream from the SV40 promoter. As shown in Fig. 2, pGL3-MMRE-enh with a MMRE sequence produced a more than 6-fold higher expression of luciferase than pGL3-Con without a MMRE in QBI-293A cells. To show that c-myc increased the activity of the SV40 promoter with the MMRE sequence, c-Myc was overexpressed in QBI-293A and luciferase assays were performed. In QBI-293A cells, it was observed that the introduction of c-Myc expression vectors (pCMV-c-myc) resulted in more than 6-fold transcription activation. NCI-H417 SCLC cells are known to have overexpressing c-Myc protein.9 As shown in Fig. 2, MMRE increased the activity of the SV40 promoter/enhancer more than 6-fold without them in NCI-H417. These results showed that MMRE sequences activated the SV40 promoter/enhancer and can be used for SCLC treatment.

Ad-MMRE-apoptin-enh confers more cell death than Ad-apoptin or Ad-apoptin-enh to SCLC cells but does not affect normal fibroblast cells

Almost 100% efficiency of viral infection was shown at a MOI of 50 for the NCI-H417 and Wi-38 cells, as determined by the Ad5-CMV-LacZ (Quantum-Applicene, U.S.A., Fig. 3). To determine increased SCLC specific cell death by Ad-MMRE-apoptin-enh, SCLC and normal fibroblast cell lines were infected with Ad-MMRE-apoptin-enh, Ad-apoptin-enh, Ad-apoptin, and Ad-CMV. Two days after infection, the morphology of a minority of the apoptin encoding adenovirus-infected cells had been changed into apoptotic cells. Then after 4 days, a majority of the cells became rounded up and detached from the culture dishes to undergo cell death (Fig. 3). The cytotoxic effect of Ad-MMRE-apoptin-enh was determined by trypan blue dye exclusion assay (Fig. 4). Ad-MMRE-apoptin-enh resulted in about 91% cell death. Ad-apoptin and Ad-apoptin-enh infected cells were killed at about 67%, and 79% respectively. However, normal fibroblast Wi-38 was not affected by Ad-MMRE-apoptin-enh, Ad-apoptin-enh, or Ad-apoptin. The viability of the cells infected with Ad-CMV was 10~20% less than that of the mock infected cells, whereas the change was insignificant compared to the viability of the apoptin encoding adenovirus infected cells (Fig. 4).

Ad-MMRE-apoptin-enh induced apoptosis of SCLC cells

The apoptotic cells in the Ad-MMRE-apoptin-enh infected cell lysates were analyzed by Cell Death Detection ELISAPLUS (Roche, Germany). The analysis showed that the Ad-MMRE-apoptin-enh treated SCLC cells had undergone about 2-, 4-fold more apoptosis than that of Ad-apoptin, and Ad-apoptin-enh respectively (Fig. 5). Consequently, the experiment clearly indicated that Ad-MMRE-apoptin-enh increased cell death by apoptosis of SCLC cells.
In cancer gene therapy, restricted expression of the therapeutic gene in the tumor is important. If the therapeutic gene is expressed in all cells, it will affect tumor and normal cells. Use of the tumor specific promoter system will solve this problem. However, true tumor specific promoters are rare, and often these promoters are useful only in the particular types of cancers from which they are derived. The tumor specific cell killing nature of apoptin makes it a new alternative for cancer gene therapy.

In this paper, the generation of increased expression of the apoptin gene by recombinant adenovirus vector with MMRE and SV40 enhancer is described. In previous studies, it has been shown that MMRE increased the expression level of the HSV-TK gene and it can be used in SCLC gene therapy. Therefore, it is expected that increased expression of the apoptin by MMRE and SV40 enhancer in adenovirus vector can be used in SCLC gene therapy. To investigate the possibility of utilizing increased expression of the apoptin gene by MMRE and SV40 enhancer in targeted SCLC gene therapy, adenovirus vector expressing apoptin controlled by the SV40 promoter/enhancer and MMRE for the induction of specific Myc activated SCLC cell

**Discussion**

In cancer gene therapy, restricted expression of the therapeutic gene in the tumor is important. If the therapeutic gene is expressed in all cells, it will affect tumor and normal cells. Use of the tumor specific promoter system will solve this problem. However, true tumor specific promoters are rare, and often these promoters are useful only in the particular types of cancers from which they are derived. The tumor specific cell killing nature of apoptin makes it a new alternative for cancer gene therapy.

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**Fig. 3.** Cytotoxic Effect of Ad-MMRE-Apoptin-enh on NCI-H417.
Wi-38 and NCI-H417 were infected with Ad5-CMV-LacZ (Quantum-Appligene, U.S.A.), Ad-CMV, and Ad-MMRE-apoptin-enh at a moi of 50. Three days after infection, for the estimation of adenovirus infection efficiency, cells were stained with X-gal. Cells expressing β-galactosidase are stained blue.

**Fig. 4.** Exposure of Wi-38 and NCI-H417 Cells in Vitro to Ad-Apoptin, Ad-Apoptin-enh, or Ad-MMRE-Apoptin-enh (50 moi), and Determination of the Number of Viable Cells by Trypan Blue Exclusion Three Days Later.
The cells were seeded at 10^5 cells in a 6-well dish 24 h prior to infection. The means from at least three independent experiments are shown; bars, SD.

**Fig. 5.** Detection of Nucleosomes in the Cytoplasm of NCI-H417 Cells Treated with Ad-MMRE-Apoptin.
NCI-417 cells (10^5 cells) were treated with Ad-apoptin, Ad-apoptin-enh, and Ad-MMRE-apoptin-enh (50 moi) for three days. After lysis, the cells were centrifuged and the supernatant was analyzed by ELISA. Ad-CMV infected cells were used as a negative control. The positive control provided by the manufacturer was used; bars, SD.
death was constructed. This virus was introduced into normal fibroblast and SCLC cancer cell lines. After treatment, the morphology of the majority of the Ad-MMRE-apoptin-enh infected cancer cells changed into apoptotic cells, with rounded-up shapes and detached from the culture dishes, and underwent cell death, but not normal fibroblasts. The growth of Ad-MMRE-apoptin-enh infected cancer cells was much more significantly suppressed than those of Ad-apoptin or Ad-apoptin-enh infected cells (Fig. 3, 4). Using a Cell Death Detection ELISAPLUS, the apoptotic cells infected with Ad-MMRE-apoptin-enh were analyzed, and it was found that the Ad-MMRE-apopin-enh treated cancer cells had undergone about 2-, 4-fold more apoptosis than those of Ad-apoptin and Ad-apoptin-enh infected cells respectively (Fig. 5). Therefore, it was concluded that the adenovirus Ad-MMRE-apopin-enh suppressed tumor cell growth and induced apoptosis and, as such, might be a useful method for suppressing SCLC growth in targeted cancer gene therapy. The existing cancer treatment method has limitations for elevation of the survival rate of cancer patients and has serious side effects on normal tissues and organs because of indiscrimination between normal and cancer cells. Therefore the technology of gene therapy that can kill only tumor cells will be another alternative, which can increase the rate of treatment and the quality of life (QOL) of cancer patients. Adenovirus vector consisting of SV40 promoter/enhancer, MMRE, and apoptin has the feature of maintaining a cancer specific character due to apoptin and increasingly activated expression of apoptin due to MMRE and SV40 enhancer, and it might be useful for targeted SCLC cancer gene therapy.

Acknowledgments

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References