Low-dose radiation response of the *p21^{WAF1/CIP1}* gene promoter transduced by adeno-associated virus vector

Mitsuru Neno^{1,5}, Kazuhiro Daino²,
Sachiko Ichimura¹, Shin-Ichiro Takahashi³
and Teruo Akuta⁴

¹Radiation Effect Mechanisms Research Group
National Institute of Radiological Sciences
9-1, Anagawa-4-chome, Inage-ku
Chiba 263-8555, Japan

²Laboratoire de Cancerologie Experimentale
Departement de Radiobiologie et Radiopathologie
Commissariat a l'Energie Atomique
92265 Fontenay-aux-Roses, cedex, France

³Departments of Animal Sciences and
Applied Biological Chemistry
Graduate School of Agriculture and Life Sciences
The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku
Tokyo 113-8657, Japan

⁴Departments of Microbiology and Molecular Cell Functions
Graduate School of Medical and Pharmaceutical Sciences
Kumamoto University

1-1-1 Honsou, Kumamoto 860-8556, Japan

⁵Corresponding author: Tel, 81-43-206-3082;
Fax, 81-43-255-6497; E-mail, m_neno@nirs.go.jp

Accepted 21 September 2006

Abbreviations: CEA, carcinoembryonic antigen; GCV, ganciclovir; HAT, histone acetyltransferase; HSVtk, herpes simplex virus type-1 thymidine kinase; IR, ionizing radiation; ITR, inverted terminal repeat; rAAV, recombinant adeno-associated virus

Abstract

In cancer gene therapy, restriction of antitumor transgene expression in a radiation field by use of ionizing radiation-inducible promoters is one of the promising approaches for tumor-specific gene delivery. Although tumor suppressor protein p53 is induced by low doses (< 1 Gy) of radiation, there have been only a few reports indicating potential utilization of a p53-target gene promoter, such as that of the *p21* gene. This is mainly because the transiently transfected promoter of p53-target genes is not much sensitive to radiation. We examined the response of the *p21* gene promoter to low-dose radiation when transduced into a human breast cancer cell line MCF-7 by use of recombinant adeno-associated

virus (rAAV) vectors. It was shown that the *p21* gene promoter transduced by rAAV vectors was more highly radiation-responsive than that transiently transfected by electroporation. A significant induction of the *p21* gene promoter by radiation of low doses down to 0.2 Gy was observed. When cells were transduced with the *p21* gene promoter-driven HSVtk gene by rAAV vector, they were significantly sensitized to repetitive treatment with low dose radiation (1 Gy) in the presence of the prodrug ganciclovir. It was therefore considered that the *p21* gene promoter in combination with a rAAV vector is potentially usable for the development of a low-dose radiation-inducible vector for cancer gene therapy.

Keywords: cyclin-dependent kinase inhibitor p21; de-pendovirus; dTMP kinase; gene therapy; promoter regions (genetic); radiation; simplexvirus

Introduction

There has been increasing interest in the use of gene therapy for the treatment of malignant tumors. Gene therapy for cancer is based on tumor-specific delivery of genes encoding proteins that are directly toxic to tumor cells, induce anti-tumor immune responses, or sensitize tumor cells to prodrugs. A number of strategies have been developed to localize therapeutic genes to tumor cells, such as those using tissue-specific promoters/enhancers. For example, a MUC1 gene promoter-driven transgene could be preferentially expressed in MUC1-positive breast cancer cells (Kurihara *et al.*, 2000). Similarly, a carcinoembryonic antigen (CEA) promoter could be utilized in preferential expression of transgenes in CEA-producing colon and lung cancer cells (Konishi *et al.*, 1999). Tumor-specific gene delivery by use of restricted replication-competent viruses has also been developed, such as E1B55K-deficient adeno-virus selectively targeting p53-mutated tumor cells (Kirn, 2000). An alternative approach for tumor-specific gene delivery is using ionizing radiation (IR)-inducible promoters (Hallahan *et al.*, 1995). Transgenes under the control of an IR-inducible promoter are expressed selectively in the radiation field. When combined with a stereotactic irradiation technique, expression of transgenes can be spatially and temporally controlled. The most widely utilized promoter for this purpose is that of the early

growth response 1 gene (*Egr-1*). Manome *et al.* have reported that expression of the β -galactosidase gene under the control of the *Egr-1* promoter is enhanced 3-fold after irradiation with 2 Gy of IR in glioma cells (1998). In addition, Marples *et al.* investigated the synthetic promoter using isolated CArG elements of the *Egr-1* promoter (2000), and have shown that the synthetic promoter was more effective than the wild-type promoter in up-regulating expression of the reporter gene after IR in MCF-7 breast adenocarcinoma and U87-MG glioblastoma-astrocytoma. TNFerade, a replication-deficient adenovector that expresses human tumor necrosis factor alfa (TNF α) under the control of the *Egr-1* promoter, was developed, and its safety in combination with radiation therapy was recently evaluated by a phase I study (Weichselbaum *et al.*, 2002; Mundt *et al.*, 2004; Senzer *et al.*, 2004).

Tumor suppressor protein p53 is induced by a low dose of IR, and induces transcription of its target genes (Amundson *et al.*, 2003). We have observed 8-fold increase in *p21^{WAF1/CIP1}* gene transcription and 5-fold increase in *GADD45a* gene transcription after 0.5 Gy of IR in human myeloblastic leukemia cell line ML1 (Daino *et al.*, 2002). However there have been only a limited number of reports showing the potential utilization of a p53-target gene promoter, such as that of the *p21* gene, in the development of IR-inducible vectors for gene therapy (Worthington *et al.*, 2000; 2002; 2004). This situation is mainly due to the fact that the transiently transfected promoters of p53-target genes are less responsive to IR than those of the endogenous p53-target genes. In this study, we show that the *p21* gene promoter transduced by rAAV vectors was remarkably responsive to low-dose IR, much more than that transiently transfected by electroporation. We also show that, when a suicide gene, herpes simplex virus type-1 thymidine kinase (HSVtk), is linked to the *p21* gene promoter followed by transduction by use of rAAV vectors, the transduced cells become sensitized to repetitive treatment with low-dose IR. It is suggested that the *p21* gene promoter in combination with a rAAV vector is potentially usable for the development of a low-dose radiation-inducible vector for cancer gene therapy.

Materials and Methods

Cell culture and X ray-irradiation

MCF-7 human breast adenocarcinoma cells containing wild-type p53 were cultured in RPMI1640 (Life Technologies) supplemented with 10% FBS (JRH Biosciences), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies) at 37°C under 5%

CO₂ in a humidified atmosphere. AAV-293 cells, which were derived from HEK293 human embryonic kidney cells stably expressing the adenovirus E1 gene, were cultured in DMEM supplemented with 10% FBS. X rays were generated from a Pantak unit operating at 200 kVp and 20 mA, with a 0.5 mm copper plus 0.5 mm aluminum filter. A dose rate of 1.0 Gy/min was used.

Northern blot analysis

Northern blot analysis was performed essentially as described (Daino *et al.*, 2002). After irradiation of MCF-7 with IR, total RNA was extracted using TRIzol LS reagent (Invitrogen). RNA was electrophoresed on a standard 1.2% formaldehyde agarose gel and blotted onto GeneScreen nylon filters (New England Nuclear Life Science Products). Filters were hybridized with random primed probes specific to *p21* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA at 60°C for 14 h in 7% SDS, 1% BSA, 0.5M phosphate buffer, 1 mM EDTA. Hybridization signal intensity was measured by a BAS2000 Bio-Imaging Analyzer (Fuji Film).

Construction of plasmids

A 5' flanking region of the *p21* gene containing the p53 recognition sites at -2.2 kb and -1.4 kb was cut out of the plasmid wwp-Luc (El-Deiry *et al.*, 1993) by *Hind*III. A luciferase expression plasmid PLS was constructed by inserting the 5' flanking region of the *p21* gene into the multi-cloning site of a pGL3 basic vector (Promega). The 3'UTR of the *p21* gene, spanning 512 bp/1928 bp relative to the A of the initiation codon, was PCR-amplified with the 5' primer 5'-GTCTAGACTGCAGTCCTGGAAGCGCGAGGG-3' and the 3' primer 5'-GTGGATCCTCGAGAGGTTTACAGTCTAGGT-3', where underlined bases were introduced to facilitate cloning with *Xba*I and *Bam*HI. A luciferase expression plasmid PLP was constructed by substituting the SV40-derived polyadenylation signal in PLS with the PCR-amplified 3'UTR of the *p21* gene. The phRL-TK vector containing the *Renilla reniformis* luciferase gene under the control of the HSVtk promoter was purchased from Promega. HSVtk-encoding sequence was excised from the plasmid pORF-HSVtk (InvivoGen, San Diego) with *Nco*I and *Nhe*I. A HSVtk expression plasmid PtkS was constructed by cutting PLS with *Nco*I and *Xba*I to replace the luciferase-encoding sequence with the HSVtk-encoding sequence prepared as above.

Electroporation

Transfection of MCF-7 cells with luciferase con-

structs was performed as described elsewhere (Nenoi *et al.*, 2001). Briefly, 5×10^6 cells were washed with RPMI1640 without FBS, and were mixed with 10 μ g of either PLS or PLP. The cell/plasmid mixture was transferred into an electroporation cubette with an electrode gap of 4 mm, left on ice for 5 min, and then pulsed at 220 V with a capacitance of 960 μ F. After incubation on ice for 10 min, cells were homogeneously suspended in 48 ml of prewarmed RPMI1640 containing FBS, divided into 5 to 6 flasks (8 ml/flask), and incubated for 48 h until X-ray irradiation. To obtain cells stably expressing the HSVtk gene, PtkS was linearized with *Bam*HI, and introduced into MCF-7 cells by electroporation along with pTK-Hg (Clontech). Stably transfected cells were selected in the presence of 200 μ g/ml of hygromycin.

rAAV vector production

Recombinant adeno-associated virus vector rAAV-PLS was constructed by the triple-transfection method (Matsushita *et al.*, 1998; Xiao and Samulski, 1998) using the AAV Helper Free System (Stratagene). pAAV-MCS, containing a pair of AAV-2 inverted terminal repeats (ITRs), was digested with *Not*I and blunted by use of a DNA Blunting kit (Takara). In addition, the 4.3 kb of *Xho*I/*Bam*HI- fragment containing the 5' flanking region of the *p21* gene, a luciferase-encoding sequence and SV40- derived polyadenylation signal, was excised from PLS and blunted. This fragment was inserted between ITRs of the *Not*I-digested pAAV-MCS resulting in pAAV-PLS. AAV-293 cells (7×10^6) were cotransfected with three plasmids, pAAV-PLS, pAAV-RC encoding AAV-derived *rep* and *cap* genes, and pHelper encoding adenovirus-derived VA, E2A and E4 genes, by a calcium phosphate method using a Profection Mammalian Transfection System (Promega). After a 3-day incubation, the recombinant adeno-associated virus vector, rAAV-PLS, was recovered by 4 rounds of freeze/thaw, followed by centrifugation at 10,000 *g* for 10 min. The viral titer was determined by dot blot hybridization using the standard protocol (Drittanti *et al.*, 2001). MCF-7 cells (10^5) were mixed with 5.5×10^8 physical particles of rAAV-PLS in 12-well microplates, and incubated for 24 h. Then cells were washed with PBS to remove the virus inoculum, and were cultured under the same condition as that for MCF-7 cells. Detection of viral genome in host cells was carried out by PCR using the 5' primer 5'-TCCTGGAGAGTGCCAAAC TCATTCTC-3' and the 3' primer 5'-TTCCAGGAACCAGGGCGTATCTCTTC-3'. pAAV-PtkS was constructed by replacing the luciferase-encoding sequence in pAAV-PLS with the HSVtk-encoding sequence which was excised from

pORF-HSVtk. Production of recombinant adeno-associated virus rAAV-PtkS and transduction of MCF-7 cells were carried out similarly to those for rAAV-PLS. Expression of the HSVtk gene in rAAV-PtkS-transduced cells was checked by RT-PCR using the 5' primer 5'-CGGAGCAGAAAATGCCC ACG-3' and the 3' primer 5'-TGCTGCCCATTAAGGT ACCGC-3'. Expression of actin gene, as a control, was also checked by PCR using the 5' primer 5'-GTAGCCATCCAGGCTGTGTT-3' and the 3' primer 5'-CAGTGAGGCCAGGATAGAGC-3'.

Measurement of luciferase activity

MCF-7 cells either transfected with luciferase-expression plasmids or transduced with rAAV-PLS were X-irradiated or mock-treated. After incubation for various time intervals, cells were washed with PBS, and lysed with a passive lysis buffer (Promega). Luciferase activity in cell lysates was measured by an analytical luminometer (model LB9506; Berthold) using either Luciferase Assay System (Promega) or Dual Luciferase Assay System (Promega) when *Renilla* luciferase expression plasmid pRL-TK was used. The protein content was measured by the pyrogallol red method using a Protein Assay Rapid kit (Wako, Osaka, Japan).

Southern blot analysis

Genomic DNA was isolated from MCF-7 cells transduced with rAAV-PLS by use of DNAzol (Invitrogen). DNA was digested with restriction enzymes, electrophoresed on a 0.7% agarose gel, and blotted onto GeneScreen nylon filters. Filters were hybridized with a random-primed 2.1 kb *Sca*I fragment of PLS which contained the sequence for the *p21* gene promoter as well as a part of the luciferase-coding sequence. Hybridization signal intensity was measured by a BAS2000 Bio-Imaging Analyzer.

Ganciclovir treatment

The cells either transduced with rAAV-PtkS or stably transfected with PtkS plasmids were cultured in 100 μ l of fresh media containing ganciclovir (GCV) in 96-well microplates. The cells were irradiated with 1 Gy of X rays twice a day with an interval of 5 h for the 5 consecutive days. After a 2-day post-incubation in the presence of GCV, the number of survived cells were measured by MTT method using a cell proliferation assay kit provided by Promega.

Figure 1. Induction of mRNA of the *p21* gene in MCF-7 cells after IR irradiation. (A) Total RNA isolated from cells at various time intervals after irradiation with 5 Gy was hybridized with a *p21* gene-specific probe. *GAPDH* was used as a control. (B) Relative levels of *p21* gene mRNA compared to that in unirradiated cells are plotted. (C) Total RNA isolated 4 h after irradiation with various doses of X rays was analyzed. (D) Dose-dependence of the *p21* gene induction was plotted.

Results

IR-response of the endogenous *p21* gene promoter

The *p21* gene is one of the p53-target genes responsive to low-dose IR in a wide variety of cell types. In order to examine the usability of the *p21* gene promoter in the development of a low-dose IR-inducible vector for gene therapy, we first analyzed the IR-response of endogenous *p21* gene expression in human breast cancer cell line MCF-7, which contains wild-type *p53*. Figure 1A shows the Northern hybridization analysis for *p21* gene mRNA levels in MCF-7 cells after irradiation with 5 Gy of X rays. The signal intensity was measured, and its time course change was plotted in Figure 1B. The maximal induction of up to 7.0-fold was observed 4 h after irradiation. Figure 1C shows the Northern hybridization analysis for dose-dependent induction of the *p21* gene. As shown in Figure 1D, the induction of the *p21* gene mRNA was linearly dependent

on IR dose in the dose range lower than 2 Gy. 3.7-fold induction was observed after 1 Gy, and 2.1-fold induction after 0.5 Gy.

IR-response of the *p21* gene promoter transiently transfected by electroporation

In order to examine the IR-response of the *p21* gene promoter in an epichromosomally existing vector, MCF-7 cells were transiently transfected with luciferase expression plasmid PLS (Figure 2A) by electroporation. To remove obscurity due to the variable transfection efficiency, transfected cells were equally divided, and were used in a series of irradiation experiment to assess either time course change or dose-dependence. Figure 2B plots the time course change in luciferase activity per protein content after irradiation with 5 Gy of X rays. In irradiated cells, luciferase activity was transiently induced, reaching a maximum of a 1.9-fold increase 5 h after irradiation. Figure 2C shows the dose-dependence of

Figure 2. IR-response of the *p21* gene promoter in luciferase expression plasmids PLS and PLP transiently transfected into MCF-7 cells by electroporation. (A) Structure of the plasmids PLS and PLP is schematically illustrated. PLP contains the 3'UTR of *p21* mRNA instead of the SV40 derived polyadenylation signal in PLS. (B) MCF-7 cells transfected with PLS were either irradiated with 5 Gy or mock-treated, and luciferase activity in cell lysates prepared at various time intervals after irradiation was measured. Luciferase activity was normalized by protein contents in cell lysates. The relative values of luciferase activity per protein to that in cell lysates prepared at 1 h after mock-treatment is plotted. (C) MCF-7 cells transfected with PLS were irradiated with various doses of X rays. The fold-induction of luciferase activity per protein contents at 5 h postirradiation is plotted. (D) Luciferase activity in cells transfected with PLS and PLP was compared. *Renilla* luciferase activity (R.luc.) from cotransfected pRL-TK was used as a transfection efficiency control. Time course change (E) and dose-dependence (F) of luciferase activity after irradiation in MCF-7 cells transfected with PLP was analyzed, similarly to those in (B) and (C), respectively. Results are the mean of at least two independent experiments. Error bars represent the S.D.

induction at 5 h postirradiation in a low-dose range. Induction was only 1.3-fold after 1 Gy, and 1.1-fold after 0.5 Gy. Thus, a remarkably small IR-response

of the *p21* gene promoter in PLS transfected by electroporation was observed compared with the induced accumulation of the mRNA for the endo-

genous *p21* gene (Figure 1). Lepik *et al.* have reported that electroporation leads to p53 activation (2003). Therefore an elevated basal activity of the *p21* gene promoter in PLS should be one of the reasons for its poor IR-response. However this effect is considered to be small because we did not observe a detectable reduction of growth rate due to the elevated *p21* gene expression 48 h after electroporation.

Influence of the 3'UTR of the *p21* gene

The *p21* mRNA has been shown to be stabilized after various stimuli including retinoid (Li *et al.*, 1996), phenylephrine (Liu *et al.*, 2000), growth factors and ultra violet light C (UVC) (Giles *et al.*, 2003). It was reported that the 3'UTR of *p21* mRNA, spanning 571 bp/829 bp, plays a role in stabilizing the mRNA for a reporter gene after UVC exposure in MDA-468 human breast cancer cell line (Giles *et al.*, 2003). To examine whether the weak IR-response of the *p21* gene promoter in PLS transfected by electroporation can be enhanced by introducing the 3'UTR of *p21* mRNA, MCF-7 cells were transfected with plasmid PLP (Figure 2A) in which the SV40-derived polyadenylation signal in PLS was replaced by the 3'UTR of *p21* mRNA spanning 512 bp/1928 bp. As shown in Figure 2D, the basal level of luciferase expression in PLP-transfected cells at 48 h posttransfection was remarkably reduced compared to that in PLS-transfected cells (0.086-fold), demonstrating that the 3'UTR of *p21* mRNA acted in destabilizing the mRNA in unirradiated cells. When PLP-transfected cells were irradiated with 5 Gy of X rays, the *p21* gene promoter was induced up to 1.9-fold 5 h after irradiation as shown in Figure 2E. Figure 2F shows that induction in the low-dose range was 1.3-fold after 1 Gy, and 1.2-fold after 0.5 Gy. Thus, both the time course change and the dose-dependence of the IR-response of the *p21* gene promoter in PLP were very similar to those observed in experiments using PLS, demonstrating that the 3'UTR did not function in stabilizing the mRNA after IR.

IR-response of the *p21* gene promoter transduced by rAAV-PLS

A recombinant adeno-associated virus rAAV-PLS was produced, the genome of which contains a 5' flanking region of the *p21* gene, a luciferase-encoding sequence, and the SV40-derived polyadenylation signal inserted between a pair of inverted terminal repeats (ITRs) (Figure 3A). After transduction of MCF-7 cells with rAAV-PLS using a multiplicity of infection (MOI) of 5.5×10^3 , basal luciferase activity without radiation drastically decreased

until 40 days of posttransduction as shown in Figure 3B. In contrast, Figure 3C shows that response to 5 Gy of X rays was conversely enhanced during posttransduction incubation. Miao *et al.* have reported that when mice were infused with rAAV, the rAAV genome was randomly integrated into the chromosomes of hepatocytes after incubation for several weeks (1998), and that expression of the transgene was observed only in 5% of hepatocytes. This report indicates that rAAV vectors slowly integrate transgenes into the chromosomes of a small portion of the host cells. Thus, we consider that the strong luciferase expression immediately after transduction of MCF-7 cells with rAAV-PLS was contributed by the episomally existing viral genome (Duan *et al.*, 1998; Vincent-Lacaze *et al.*, 1999), and that those cells containing the episomal rAAV-PLS genome were removed from the cell population until 40 days after transduction. This may be due to dilution of the viral genome in proliferating cell population. Cells containing the viral genome in their chromosomes are thought to have remained as a minor part of the cell population after 40 days of postincubation. Figure 3C shows that the *p21* gene promoter in rAAV-PLS was insensitive to IR immediately after transduction. This is likely due to the fact that the majority of the viral genome existed as an episome at this time, which is a similar situation to that for transfected PLS. The enhanced IR-response of the *p21* gene promoter after postincubation is most likely attributable to the transgene status in the transduced cells. That is, integration of the rAAV-PLS genome into chromosomes may have enhanced the IR-response.

The time course change and dose-dependence of the IR-response of the *p21* gene promoter in rAAV-PLS were determined using cells cultured for 66 days after transduction. Figure 3D shows that induction after 5 Gy of X rays reached a maximum of 6.0-fold at 7 h postirradiation, which was remarkably higher than that observed in cells transiently transfected with plasmid PLS (1.9-fold). The IR-response in the low-dose range was also enhanced, as shown in Figure 3E, with induction of 2.1-fold after 1 Gy, and 1.7-fold after 0.5 Gy. It should be noted that the IR-response of the *p21* gene promoter in rAAV-PLS was very similar to induced accumulation of the endogenous *p21* gene mRNA (Figure 1), suggesting that the intrinsic regulation mechanisms of the endogenous *p21* gene promoter functioned in regulating the transduced promoter.

Transgene status in cells transduced by rAAV-PLS

It was speculated that an enhanced IR-response of the *p21* gene promoter in rAAV-PLS was due to the

Figure 3. IR-response of the *p21* gene promoter transduced into MCF-7 cells with a recombinant adeno-associated virus vector. (A) Structure of the genome of rAAV-PLS is schematically illustrated. (B) Luciferase activity normalized by protein contents in unirradiated cells was measured at various time intervals after transduction. A representative result is shown. (C) Transduced cells were irradiated with 5 Gy of X rays at various time interval after transduction. The fold-induction of luciferase activity per protein at 5 h postirradiation compared to that in unirradiated cells is plotted. A representative result is shown. (D) Cells cultured for 66 days after transduction with rAAV-PLS were either irradiated with 5 Gy or mock-treated, and luciferase activity in cell lysates prepared at various time intervals after irradiation was measured. The fold-induction of luciferase activity per protein contents is plotted. Results are the mean of at least two independent experiments. Error bars represent the S.D. (E) Cells cultured for 66 days after transduction with rAAV-PLS were irradiated with various doses of X rays. The fold-induction of luciferase activity per protein contents at 6 h post-irradiation is plotted. Results are the mean of at least two independent experiments. Error bars represent the S.D.

ability of rAAV vectors to integrate transgenes into the chromosomes of host cells. We examined the transgene status in cells cultured for 66 days after transduction with rAAV-PLS. First, the copy number of the viral genome per haploid was determined by semi-quantitative PCR using the primers shown in Figure 4A. Figure 4B shows that approximately 0.015 copy of the transgene per 1 copy of the endogenous *p21* gene was present (the ratio corresponding to the difference in 6 cycles of PCR). This result demonstrating that the transgene was

present only in a minor part of the cell population is consistent with the observation by Miao *et al.* (1998). Next, we analyzed the genomic DNA of transduced cells by Southern blotting using a probe specific to the *p21* gene promoter (Figure 4A). The probe must hybridize with both the rAAV-PLS genome and the endogenous *p21* gene promoter. However, as shown in Figure 4C, only a single band derived from the endogenous *p21* gene promoter was observed when the genomic DNA was digested with *Eco*RI or *Bam*H1. This result suggests that the rAAV-PLS

Figure 4. Transgene status in cells transduced by rAAV-PLS. (A) Schematic illustration of the endogenous *p21* gene and the rAAV-PLS genome. Primers used in PCR-amplification of the endogenous *p21* gene and the rAAV-PLS genome are indicated by arrows. A horizontal bar represents the region used as a probe for the genomic Southern blot analysis. The unique *Bgl*II site in the rAAV-PLS genome is indicated. (B) Semi-quantitative PCR was carried out specifically to amplify the endogenous *p21* gene (top) and the rAAV-PLS genome (bottom) by using genomic DNA isolated from cells cultured for 66 days after transduction with rAAV-PLS. It can be seen that amplification of the rAAV-PLS genome requires 6 more cycles of PCR to produce a comparable amount of products as those derived from the *p21* gene. (C) Genomic DNA from cells cultured for 66 days after transduction with rAAV-PLS was digested with *Eco*RI, *Bam*HI or *Bgl*II, and hybridized with the *p21* gene promoter-specific probe schematically illustrated in (A). A weak band at 5 kb observed in the *Bgl*II-digested sample is indicated by an arrow.

genome was randomly integrated in the chromosomes. When the genomic DNA was digested with *Bgl*II, a faint band was observed at 5 kb in addition to an endogenous *p21* gene-derived band at >10 kb. This fragment is likely to correspond to a linear double-stranded monomer of the rAAV-PLS genome. As *Bgl*II cuts once near the 5' end of the rAAV-PLS genome (Figure 4A), this result demonstrates that the rAAV-PLS genome exists as a head-to-tail concatamer.

Enhanced cytotoxic effect of low dose radiation on cells transduced with rAAV-PtkS

To evaluate the potential usability of the *p21* gene promoter in combination with a recombinant adeno-associated virus vector in cancer gene therapy, we tested rAAV-PtkS, which contains the suicide gene HSVtk under the regulation of the *p21* gene promoter, as shown in Figure 5A. The HSVtk, in combination with a prodrug GCV, has been shown to be effective for controlling tumor growth in animal models (Hasenburger *et al.*, 2000; Makinen *et al.*, 2000). MCF-7 cells were transduced with rAAV-PtkS,

and were cultured for longer than 66 days to stabilize the transgene. First inducibility of the HSVtk gene by X rays was checked by RT-PCR. As shown in Figure 5B, an elevated expression of the HSVtk gene after irradiation with 5 Gy of X rays was observed. Next the transduced cells were irradiated with low dose (1 Gy) of X rays twice a day with an interval of 5 h for the 5 consecutive days in the presence of GCV (Figure 5C). Based on the observation that the fold-induction after irradiation is linearly dependent on X-ray dose (Figure 3D and E), a significant induction of the HSVtk gene after irradiation with 1 Gy was expected. Figure 5D shows the cell survival, that is calculated as the ratio of the number of survived cells irradiated or mock-treated in the presence of 10 μ g/ml of GCV relative to that of cells similarly treated in the absence of GCV. It can be seen that GCV has a minimum effect on control cells either irradiated or mock-treated. However a slightly stronger cell killing effect of GCV was observed on cells transduced with rAAV-PtkS. It is considered to be caused by the basal expression of HSVtk. The killing effect of GCV was remarkably enhanced when cells were irradiated, indicating that

Figure 5. Enhanced radiation sensitivity of cells transduced with rAAV-PtkS. (A) Structure of the genome of rAAV-PtkS is schematically illustrated. (B) RT-PCR was carried out to specifically amplify the HSVtk gene (top) and the endogenous actin gene (bottom) by using total RNA isolated from irradiated or untreated cells. The number of the PCR cycle was indicated. (C) Regimen of treatment of cells with GCV and X rays. (D) Relative cell survival is represented that is calculated as the ratio of survival of cells irradiated or mock-treated in the presence of 10 µg/ml of GCV to that of cells similarly treated in the absence of GCV. Control MCF, MCF/rAAV-PtkS and MCF/PtkS represent the MCF-7 cells introduced with unrelated vectors, MCF-7 cells transduced with rAAV-PtkS and MCF-7 cells stably transfected with PtkS, respectively. (E) The same as in (D) except that 1 µg/ml of GCV was used. Results are the mean of at least two independent experiments. Error bars represent the S.D.

the induced HSVtk gene enhanced the cytotoxic effect of X rays. The similar effect of HSVtk gene was observed when cells stably transfected with PtkS plasmids were used. In this case the effect of HSVtk was observed when 1 µg/ml of GCV was used. It is therefore evident that cells transduced with rAAV-PtkS are significantly sensitized to repetitive treatment of 1 Gy of X rays. In addition, it is suggested that integration of transgene into chromosomes is an important requirement for vector effi-

cacy, based on the observation that the stably transfected cells were similarly sensitized to 1 Gy of X rays.

Discussion

In response to genotoxic stresses, p53 is stabilized and accumulates in nuclei (Caspary, 2000; Fei and El-Deiry, 2003). However, the precise mechanism by

which the accumulated p53 transactivates its target genes, has yet to be elucidated. Recently, plenty of evidence has been reported indicating that the association of p53 with its recognition sequence of DNA is dependent on the chromatin structure. For instance, it was revealed by *in vitro* experiments that native p53 binds with higher affinity to chromatin-assembled DNA than to double-stranded oligonucleotides (Espinosa *et al.*, 2001). *In vivo* DNase I-hypersensitive site analyses showed that p53 recognition sites in the *p21*, *14-3-3 σ* , and *KARP-1* genes are resistant to nuclease digestion even after gene induction by irradiation (Braastad *et al.*, 2003). This finding implies that p53 recognizes and binds to the nucleosomal structure, as a whole, without detectable structural changes. In addition, it was reported that transactivation by p53 requires recruitment of histone acetyltransferase (HAT) p300/CBP (Espinosa *et al.*, 2001), which is considered to function in chromatin remodeling. Assuming that p53-induced transcription of its target genes depends on the chromatin structure and its remodeling, we examined whether the response of the *p21* gene promoter to a low dose of IR could be enhanced when transduced into cells by use of recombinant adeno-associated virus (rAAV) vectors which can stably integrate transgenes into chromosomes. Our data demonstrated that the *p21* gene promoter transduced by rAAV vectors was much more highly IR-responsive than that transiently transfected by electroporation. By analyzing the genomic DNA, it was suggested that the viral genome was randomly integrated into chromosomes of a minor part of the cell population. Thus, it is thought to be critical to provide the chromatin structure for transgenes in order to make a highly IR-responsive vector for gene therapy by use of a p53-target gene promoter. It should be noted that the chromatin structure at the recognition site for transcription factors other than p53 may be also important, because there are multiple pathways for the *p21* gene transcriptional regulation.

By use of rAAV, we observed *p21* promoter induction up to 2.1-fold after 1 Gy and 1.7-fold after 0.5 Gy in the low-dose range. This sensitivity to low-dose IR confers a clinical advantage in optimizing the synergism of anti-tumoric effects caused by radiation and transgene products. In gene therapy using a vector with the *Egr-1* promoter, maximum induction of the transgene is expected to occur several hours after irradiation, by which point most of the early stages of the signal transduction processes triggered by radiation are terminated. Therefore, an optimum synergistic interaction between radiation and the transgene product cannot necessarily be expected. In contrast, by use of a low-dose IR-

responsive vector, induction of transgene expression and radiation therapy can be temporally adjusted, if a priming irradiation with low-dose IR to induce transgene expression is carried out prior to a therapeutic irradiation. By optimizing the length of the time interval between priming irradiation and therapeutic irradiation, the maximum synergism of radiation and transgene products may be expected.

We observed a remarkable basal luciferase expression immediately after transduction, which suggests that rAAV-PLS is leaky when existing as an episome. When the vector system of rAAV-PLS is applied to a clinical use, by substituting the luciferase gene by an appropriate antitumor gene, the leaky expression of the antitumor gene may cause unintentional damages in transduced cells. This effect may be reduced by introducing a transcriptional pause sequence upstream of the *p21* gene promoter. Alternatively, utilization of a transgene which is inactive by itself, such as the *HSVtk* gene, should be another solution. If irradiation and a prodrug treatment are carried out after transgenes are stably integrated into chromosomes, harmful effects by the leaky expression of the transgene can be avoided.

We estimated the copy number of the rAAV-PLS genome in stably transduced cells at 0.015 per haploid. It should be noted that the fraction of the stably transduced cell population was even less than 0.015 because part of the viral genome was present as a concatemer in transduced cells. In addition, as viral genomes seem to be randomly integrated into chromosomes, the transcriptional activity of transgenes is thought to be variable depending on integration loci. Consequently, the fraction of cells contributing transgene expression induced by IR is thought to be remarkably small. In order to overcome this defect of the rAAV vector, such transgenes should be chosen that encode secretable therapeutic products, causing toxic effects on surrounding cells. *HSVtk* and *TNF α* genes are examples of those having such bystander effects. Suitability of *HSVtk* gene was demonstrated in this study by observing that cytotoxic effect of repetitive treatment of low-dose IR was enhanced on cells transduced with rAAV-PtkS.

It has been reported that the frequency of site-specific integration of the AAV-type 2 (AAV-2) into a site on human chromosome 19 called AAVS1 occurs within 96 h post infection (Huser *et al.*, 2002). Thus improvements of the AAV vectors enabling rapid integration of transgenes into the chromosome may be expected. It should be noted that integration of a plasmid containing AAV inverted terminal repeats occurred at high frequency in the presence of Rep proteins (Surosky *et al.*, 1997). It seems promising

that effective AAV vectors would be developed that enable rapid and efficient integration of transgenes by use of co-expression system of Rep proteins and/or other AAV-derived factors.

It was reported that *p53* is mutated in approximately 50% of human cancers (Hollstein *et al.*, 1991). Therefore it is definitely required to check the *p53* status of the tumor before treatment with the low-dose IR-responsive vector utilizing the *p21* gene promoter. However, the *p53* mutation frequency varies depending on the cancer site and the progression stage. For example, PCR-SSCP and immunohistochemical analyses revealed that the frequency of *p53* mutation in prostate cancers is relatively low, and suggested roles for the *p53* mutation in tumor progression in Caucasians and African-Americans (Effert *et al.*, 1992; 1993; Bookstein *et al.*, 1993; Navone *et al.*, 1993). A low frequency of the *p53* mutation in prostate cancers of Japanese was also reported by examining mutations in exon 5-8 (9.5% of 21 primary prostate cancers) by PCR-SSCP analysis (Uchida *et al.*, 1993). Utilization of the low-dose IR-responsive vector may be considered as a higher priority of treatment of such cancers with low frequency of *p53* mutations.

In summary it was demonstrated that a *p21* gene promoter in combination with rAAV vector is potentially usable in developing a low-dose IR-inducible vector for cancer gene therapy. By use of this vector, the maximum synergistic antitumor effects of radiation and transgene products are expected to be attained, resulting in the improved efficacy of cancer gene therapy combined with radiotherapy.

Acknowledgement

A part of this study was financially supported by the Budget for Nuclear Research of the Ministry of Education, Culture, Sports, Science and Technology, based on the screening and counseling by the Atomic Energy Commission.

References

- Amundson SA, Lee RA, Koch-Paiz CA, Bittner ML, Meltzer P, Trent JM, Fornace AJ Jr. Differential responses of stress genes to low dose-rate gamma irradiation. *Mol Cancer Res* 2003;1: 445-52
- Bookstein R, MacGrogan D, Hilsenbeck SG, Sharkey F, Allred DC. *p53* is mutated in a subset of advanced-stage prostate cancers. *Cancer Res* 1993;53:3369-73
- Braastad CD, Han Z, Hendrickson EA. Constitutive DNase I hypersensitivity of *p53*-regulated promoters. *J Biol Chem* 2003;278:8261-8
- Caspari T. How to activate *p53*. *Curr Biol* 2000;10:R315-7
- Daino K, Ichimura S, Neno M. Early induction of CDKN1A (*p21*) and GADD45 mRNA by a low dose of ionizing radiation is due to their dose-dependent post-transcriptional regulation. *Radiat Res* 2002;157:478-82
- Drittanti L, Jenny C, Poulard K, Samba A, Manceau P, Soria N, Vincent N, Danos O, Vega M. Optimised helper virus-free production of high-quality adeno-associated virus vectors. *J Gene Med* 2001;3:59-71
- Duan D, Sharma P, Yang J, Yue Y, Dudus L, Zhang Y, Fisher KJ, Engelhardt JF. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol* 1998;72:8568-77
- Effert PJ, McCoy RH, Walther PJ, Liu ET. *p53* gene alterations in human prostate carcinoma. *J Urol* 1993;150: 257-61
- Effert PJ, Neubauer A, Walther PJ, Liu ET. Alterations of the *p53* gene are associated with the progression of a human prostate carcinoma. *J Urol* 1992;147:789-93
- El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of *p53* tumor suppression. *Cell* 1993;75: 817-25
- Espinosa JM, Emerson BM. Transcriptional regulation by *p53* through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. *Mol Cell* 2001;8:57-69
- Fei P, El-Deiry WS. *p53* and radiation responses. *Oncogene* 2003;22:5774-83
- Giles KM, Daly JM, Beveridge DJ, Thomson AM, Voon DC, Furneaux HM, Jazayeri JA, Leedman PJ. The 3'-untranslated region of *p21WAF1* mRNA is a composite cis-acting sequence bound by RNA-binding proteins from breast cancer cells, including HuR and poly(C)-binding protein. *J Biol Chem* 2003;278:2937-46
- Hallahan DE, Mauceri HJ, Seung LP, Dunphy EJ, Wayne JD, Hanna NN, Toledano A, Hellman S, Kufe DW, Weichselbaum RR. Spatial and temporal control of gene therapy using ionizing radiation. *Nat Med* 1995;1:786-91
- Hasenburg A, Tong XW, Rojas-Martinez A, Nyberg-Hoffman C, Kieback CC, Kaplan A, Kaufman RH, Ramzy I, Aguilar-Cordova E, Kieback DG. Thymidine kinase gene therapy with concomitant topotecan chemotherapy for recurrent ovarian cancer. *Cancer Gene Ther* 2000;7:839-44
- Hollstein M, Sidransky D, Vogelstein B, Harris CC. *p53* mutations in human cancers. *Science* 1991;253:49-53
- Huser D, Weger S, Heilbronn R. Kinetics and frequency of adeno-associated virus site-specific integration into human chromosome 19 monitored by quantitative real-time PCR. *J Virol* 2002;76:7554-9
- Kirn DH. A phase II trial of intratumoral injection with a selectively replicating adenovirus (ONYX-15) in patients with recurrent, refractory squamous cell carcinoma of the head and neck. In: Walther W, Stein U, eds. *Methods in Molecular Medicine*, Vol. 35: Gene Therapy: Methods and Protocols, Totowa, NJ: Humana Press; 2000, 559-74
- Konishi F, Maeda H, Yamanishi Y, Hiyama K, Ishioka S,

- Yamakido M. Transcriptionally targeted *in vivo* gene therapy for carcinoembryonic antigen-producing adenocarcinoma. *Hiroshima J Med Sci* 1999;48:79-89
- Kurihara T, Brough DE, Kovessi I, Kufe DW. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. *J Clin Invest* 2000;106:763-71
- Lepik D, Jaks V, Kadaja L, Varv S, Maimets T. Electroporation and carrier DNA cause p53 activation, cell cycle arrest, and apoptosis. *Anal Biochem* 2003;318:52-9
- Li XS, Rishi AK, Shao ZM, Dawson MI, Jong L, Shroot B, Reichert U, Ordonez J, Fontana JA. Posttranscriptional regulation of p21WAF1/CIP1 expression in human breast carcinoma cells. *Cancer Res* 1996;56:5055-62
- Liu J, Shen X, Nguyen VA, Kunos G, Gao B. Alpha(1) adrenergic agonist induction of p21(waf1/cip1) mRNA stability in transfected HepG2 cells correlates with the increased binding of an AU-rich element binding factor. *J Biol Chem* 2000;275:11846-51
- Makinen K, Loimas S, Wahlfors J, Alhava E, Janne J. Evaluation of herpes simplex thymidine kinase mediated gene therapy in experimental pancreatic cancer. *J Gene Med* 2000;2:361-7
- Manome Y, Kunieda T, Wen PY, Koga T, Kufe DW, Ohno T. Transgene expression in malignant glioma using a replication-defective adenoviral vector containing the Egr-1 promoter: activation by ionizing radiation or uptake of radioactive iododeoxyuridine. *Hum Gene Ther* 1998;9:1409-17
- Marples B, Scott SD, Hendry JH, Embleton MJ, Lashford LS, Margison GP. Development of synthetic promoters for radiation-mediated gene therapy. *Gene Ther* 2000;7:511-7
- Matsushita T, Elliger S, Elliger C, Podsakoff G, Villarreal L, Kurtzman GJ, Iwaki Y, Colosi P. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* 1998;5:938-45
- Miao CH, Snyder RO, Schowalter DB, Patijn GA, Donahue B, Winther B, Kay MA. The kinetics of rAAV integration in the liver. *Nat Genet* 1998;19:13-5
- Mundt AJ, Vijayakumar S, Nemunaitis J, Sandler A, Schwartz H, Hanna N, Peabody T, Senzer N, Chu K, Rasmussen CS, Kessler PD, Rasmussen HS, Warso M, Kufe DW, Gupta TD, Weichselbaum RR. A phase I trial of TNFerade biologic in patients with soft tissue sarcoma in the extremities. *Clin Cancer Res* 2004;10:5747-53
- Navone NM, Troncoso P, Pisters LL, Goodrow TL, Palmer JL, Nichols WW, von Eschenbach AC, Conti CJ. p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. *J Natl Cancer Inst* 1993;85:1657-69
- Nenoi M, Ichimura S, Mita K, Yukawa O, Cartwright IL. Regulation of the catalase gene promoter by Sp1, CCAAT-recognizing factors, and a WT1/Egr-related factor in hydrogen peroxide-resistant HP100 cells. *Cancer Res* 2001;61:5885-94
- Senzer N, Mani S, Rosemurgy A, Nemunaitis J, Cunningham C, Guha C, Bayol N, Gillen M, Chu K, Rasmussen C, Rasmussen H, Kufe D, Weichselbaum R, Hanna N. TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene: a phase I study in patients with solid tumors. *J Clin Oncol* 2004;22:592-601
- Surosky RT, Urabe M, Godwin SG, McQuiston SA, Kurtzman GJ, Ozawa K, Natsoulis G. Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. *J Virol* 1997;71:7951-9
- Uchida T, Wada C, Shitara T, Egawa S, Koshiba K. Infrequent involvement of p53 gene mutations in the tumorigenesis of Japanese prostate cancer. *Br J Cancer* 1993;68:751-5
- Vincent-Lacaze N, Snyder RO, Gluzman R, Bohl D, Lagarde C, Danos O. Structure of adeno-associated virus vector DNA following transduction of the skeletal muscle. *J Virol* 1999;73:1949-55
- Weichselbaum RR, Kufe DW, Hellman S, Rasmussen HS, King CR, Fischer PH, Mauceri HJ. Radiation-induced tumour necrosis factor-alpha expression: clinical application of transcriptional and physical targeting of gene therapy. *Lancet Oncol* 2002;3:665-71
- Worthington J, Robson T, Murray M, O'Rourke M, Keilty G, Hirst DG. Modification of vascular tone using iNOS under the control of a radiation-inducible promoter. *Gene Ther* 2000;7:1126-31
- Worthington J, Robson T, O'Keeffe M, Hirst DG. Tumour cell radiosensitization using constitutive (CMV) and radiation inducible (WAF1) promoters to drive the iNOS gene: a novel suicide gene therapy. *Gene Ther* 2002;9:263-9
- Worthington J, McCarthy HO, Barrett E, Adams C, Robson T, Hirst DG. Use of the radiation-inducible WAF1 promoter to drive iNOS gene therapy as a novel anti-cancer treatment. *J Gene Med* 2004;6:673-80
- Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 1998;72:2224-32