REGULATION OF THE *P21* GENE PROMOTER IN RESPONSE TO CLINICALLY LOW DOSE RADIATION

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ABSTRACT

Tumor suppressor protein p53 is a transcription factor that plays important roles in biological responses to a wide range of ionizing radiation doses. Elucidation of the molecular mechanisms for its function after actually relevant doses of radiation is therefore crucial for the accurate understanding of the biological effects of radiation. p21 is one of the p53 target genes, functioning in cell cycle checkpoint regulation. In this article, a comprehensive analysis of the p21 gene promoter after clinically relevant doses of radiation is reviewed, especially emphasizing that a transcription factor Oct-1 plays a cooperative role in p53-mediated regulation of the p21 gene after irradiation. In addition, a potential application of the p21 gene promoter in the development of a low-dose radiation inducible vector for cancer gene therapy is also pointed out.

Keywords: Clinically low dose radiation, Gene regulation, p53, p21, Adeno-associated virus vector, Gene therapy

1 INTRODUCTION

By the recent development of novel technology for transcriptome analysis, such as the cDNA microarray, a multitude of radiation inducible or reducible genes has been identified (Yin, Nelson, Coleman, Peterson, & Wyrobek, 2003; Amundson, Grace, McLeland, Epperly, Yeager, Zhan, et al., 2004), and these radiation responsive genes are considered to be more or less involved in the regulation of cellular sensitivity to radiation. Actually, some of the transcription factors that regulate radiation responsive genes are important biological markers in determining individual radiation sensitivity. Especially, the importance of p53 (Fei & El-Deiry, 2003), which is also known as the guardian of the genome, has been emphasized. Identification of transcription factors regulating each of the various radiation-responsive genes thus may lead to the discovery of new markers of radiation sensitivity and also to the discovery of novel targets for cancer gene therapy.

Figure 1 shows the dose-range that was focused on in this review article. In the usual clinical application of radiation, including radiotherapy of solid tumors and total body irradiation prior to bone marrow transplantation, 2 Gy of dose per fraction is typically utilized. Further, according to the definition by UNSCEAR (UNSCEAR, 2000), the upper limit of so-called “low-dose-radiation” is 0.2 Gy. Thus, we arbitrarily defined “clinically low dose range” as the dose range from 0.2 to 2 Gy. We have previously investigated regulation of the *p21* gene after exposure to radiation in this dose range because this gene is highly responsive to radiation depending on p53 (Fan, el-Deiry, Bae, Freeman, Jondle, Bhatia, et al., 1994; Fei & El-Deiry, 2003). The *p21* is a Cyclin-dependent kinase inhibitor, functioning in inhibition of cell cycle progression. That is, induction of the *p21* gene by radiation results in G_1/S and G2/M cell cycle arrest (Harper, Adami, Wei, Keyomarsi, & Elledge, 1993). Importantly, this correlates with radioresistance. For example, xenografts established from *p21*-deficient tumor cells have been shown to be significantly radiosensitive (Waldman, Zhang, Dillehay, Yu, Kinzler, Vogelstein, et al., 1997). In addition, treatment of cancer cells with antisense oligonucleotides targeting *p21* inhibited radiation-induced *p21* expression and resulted in the loss of G_1 arrest and enhanced apoptosis (Tian, Wittmack, & Jorgensen, 2000).

It was previously shown that p53 functions in the regulation of some of its target genes by cooperating with other transcription factors. For instance, Sp1 (Koutsodontis, Tentes, Papakosta, Moustakas, & Kardassis, 2001),

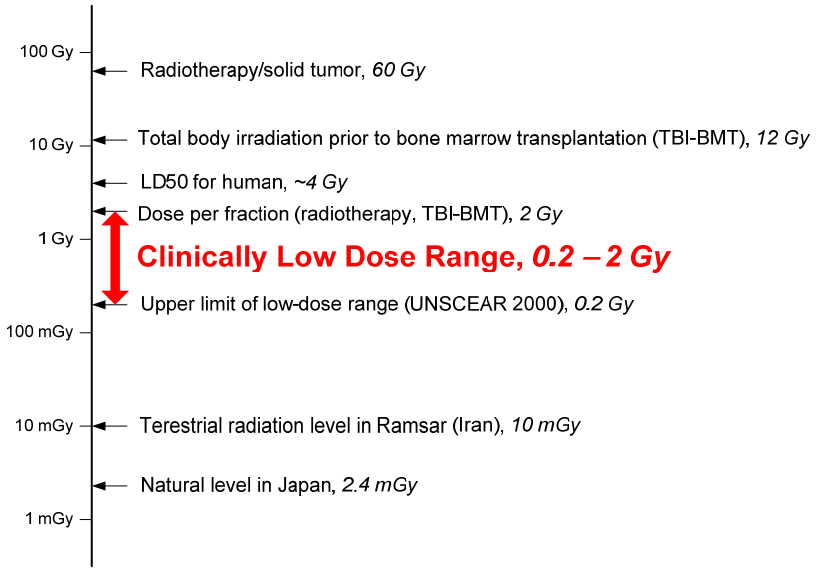

Figure 1. "Clinically low dose range" arbitrarily defined in this review

GKLF (Zhang, Geiman, Shields, Dang, Mahatan, & Kaestner, 2000), Ets1 (Xu, Wilson, Chan, De Luca, Zhou, & Hertzog, 2002), and IRF1 (Tanaka, Ishihara, Lamphier, Nozawa, Matsuyama, & Mak, 1996) have been reported to be involved in the transcriptional activation of *p21* following exposure to UV light or ectopic overexpression of p53 (Figure 2). However, the cooperating transcription factors, involved in induction of *p21* after irradiation, have not yet been elucidated. Identification of such cooperating transcription factors may lead to the discovery of novel targets of radiation-mediated cancer gene therapy. We previously tried to identify the transcription factors that cooperate with p53 to regulate the *p21* gene induction after exposure to clinically low dose radiation (Nenoi, Daino, Nakajima, Wang, Taki, & Kakimoto, 2009).

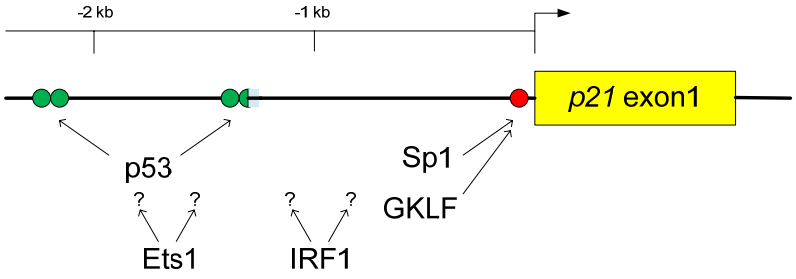

Figure 2. Transcription factors cooperating with p53 in regulation of the *p21* gene after exposure to UV light or ectopic overexpression of p53

2 REPORTER GENE ANALYSIS

In order to identify the cooperating transcription factors, we constructed the reporter gene vector rAAV-PLS as shown in Figure 3. In the figure, "p21 promoter" is a DNA segment located upstream of the *p21* gene, regulating its transcription. The luciferase gene is transcribed under the control of the *p21* promoter, and the produced luciferase causes light emission by reacting with luciferin and ATP. Therefore the activity of the *p21* gene promoter can be monitored by luciferase activity, which can be measured as the light intensity. The human breast cancer cell line MCF-7 was transduced with the reporter vector rAAV-PLS by use of the adeno-associated

virus vector, the cells were irradiated, and the luciferase activity was measured. The dose dependent increase in lucifease activity was confirmed. The reason why we used the adeno-associated virus vector was that the radiation-responsiveness of this vector had been observed to be remarkably elevated compared to the case in which a simple transfection method such as electroporation was used (Nenoi, Daino, Ichimura, Takahash, & Akuta, 2006).

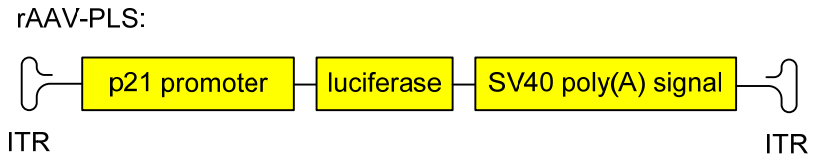

Figure 3. A reporter gene construct containing luciferase gene under the control of the *p21* gene promoter. SV40 poly(A) signal is necessary to produce the functional mRNA containing poly(A) tail at the 3' end. ITR represents the inverted terminal repeat which is required for integration of the viral genome into the chromosomes of the host cells.

Next, we constructed reporter gene constructs with deletions at various sites in the *p21* gene promoter, and we found that the radiation responsiveness of the cells transduced with vectors containing deletions at -1962bp/-1679bp, -1398bp/-1119bp, and -1118bp/-839bp was remarkably low, where the numbers represent the distance in base-pair from the transcriptional start site. This result suggested that DNA elements required for promoter activation by radiation existed in each of these regions. By analyzing the nucleotide sequence, we found the recognition sequence for a transcription factor Oct-1 in -1118bp/-839bp, and a tandem duplication of the Oct-1 recognition sequence was found in -1962bp/-1679bp. We designated these Oct-1 recognition sequences as Oct-1/-1.1kb and Oct-1/-1.8kb, respectively. An imperfect recognition sequence for p53 was found in -1398bp/-1119bp. We constructed reporter gene constructs in which Oct-1/-1.1kb and Oct-1/-1.8kb were specifically deleted and found that the radiation responsiveness of cells transduced with these constructs was dramatically diminished. This result suggested that Oct-1 plays a role in the *p21* gene induction after irradiation by binding to these sites. In contrast, we observed that the radiation responsiveness of the cells transduced with the vector containing a deletion at -1678bp/-1399bp was more highly responsive to clinically low dose radiation than wild-type rAAV-PLS. This result suggested that a DNA element that antagonizes promoter activation by radiation exists in -1678bp/-1399bp.

Oct-1 is the abbreviated name of Octamer-binding transcription factor 1. It is a ubiquitous transcription factor of the POU subfamily of homeodomain proteins (Herr & Cleary, 1995), regulating the transcription of genes for H2B, small nuclear RNA genes, immunoglobulin, and so on. Oct-1 is induced after exposure to multiple DNA-damaging agents, including UV light, methyl methanesulfonate, etoposide, cisplatin, and camptothecin, and thus, it has been referred to as a "stress sensor" (Tantin, Schild-Poulter, Wang, Haché, & Sharp, 2005). A significant induction of the DNA-binding ability of Oct-1 after exposure to radiation has been reported (Meighan-Mantha, Riegel, Suy, Harris, Wang, Lozano, et al., 1999). The functional activation of Oct-1 has been directly linked to the phosphorylation of its N-terminal transcriptional regulatory domain by the DNA-dependent protein kinase, DNA-PK (Schild-Poulter, Shih, Tantin, Yarymowich, Soubeyrand, Sharp, et al., 2007). The fibroblasts lacking Oct-1 were shown to be hypersensitivity to radiation (Tantin, Schild-Poulter, et al., 2005).

3 DNA BINDING ANALYSIS

We examined whether Oct-1 actually binds to its recognition site by a gel-shift analysis. When a radio-labeled DNA probe containing the Oct-1 recognition sequence at -1.8 kb (Oct-1/-1.8kb) was mixed with nuclear

proteins extracted from 2Gy-irradiated cells, we observed an interaction of the DNA probe with protein factors. As this DNA-protein complex was supershifted by the addition of ani-Oct-1 antibodies, Oct-1 was revealed to be contained in the complex. This result was further confirmed by the observation that the complex disappeared with the addition of an excess amount of unlabeled oligonucleotides containing the Oct-1 consensus sequence. However, binding of Oct-1 to the probe was also observed even when the cells were not irradiated, indicating that the binding was constitutive. The constitutive binding of Oct-1 to the recognition sequence at -1.1 kb (Oct-1/-1.1kb) was also observed. Consistent results on the constitutive binding of Oct-1 to these chromatin sites were also obtained from a chromatin immunoprecipitation (ChIP) analysis.

4 RNA INTERFERENCE ANALYSIS

In order to examine the functional involvement of Oct-1 in *p21* gene regulation, we specifically suppressed the *Oct-1* expression using siRNA. By a Western blotting analysis, it was confirmed that the protein level of Oct-1 was significantly lower in the cells that had been stably transfected with siRNA-expression plasmids. Using these cells, we measured the *p21* gene expression by a Northern analysis. As a result, the basal expression in knockdown cells was significantly reduced compared with that in cells transfected with negative control siRNA plasmids. Also, the radiation-inducible components of expression were also remarkably reduced.

Altogether, it was revealed that transcription factor Oct-1 plays a crucial role in the p53-mediated regulation of *p21* following exposure to clinically low dose (0.2-2 Gy) radiation, further supporting the idea that Oct-1 is an important biological marker of radiosensitivity.

5 RADIATION INDUCIBLE VECTORS FOR CANCER GENE THERAPY

Tumor-specific transcriptional targeting of the suicide gene is one of the fundamental ideas of cancer gene therapy. Figure 4 is a conceptual illustration of radiation-mediated cancer gene therapy. An extensive area of normal tissue would be damaged if a suicide gene is non-specifically expressed, but the damage would be restricted only in tumor volume if a suicide gene under a radiation responsive promoter is used in combination with a precisely localized irradiation technique. TNFerade is a replication-deficient adenovirus vector that expresses tumor necrosis factor alpha (TNF-alpha) under the control of a radiation-inducible Egr-1 gene promoter (Weichselbaum, Kufe, Hellman, Rasmussen, King, Fischer, et al., 2002). Its safety in clinical use has

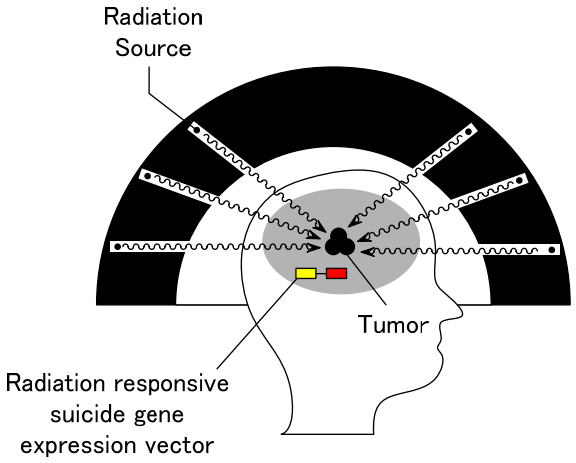

Figure 4. A conceptual illustration of radiation-mediated cancer gene therapy. An extensive area of normal tissue would be damaged if a suicide gene is non-specifically expressed (indicated by grey). However, the damage would be restricted only in tumor volume if a suicide gene under a radiation responsive promoter is utilized in combination with a precisely localized irradiation technique.

been evaluated by a phase I study in the USA (Senzer, Mani, Rosemurgy, Nemunaitis, Cunningham, Guha, et al., 2004). However, we consider it to be a problem that a relatively high dose of radiation is required to induce the Egr-1 gene promoter in combination with adeno virus vectors.

The usability of the *p21* gene promoter in combination with adeno-associated virus vectors was tested as shown in Figure 5 (Nenoi, Daino, et al., 2006). rAAV-PtkS is a vector that expresses Herpes simplex virus type-1 Tthymidine kinase (HSVtk) under the control of the *p21* gene promoter. HSVtk phosphorylates the prodrug ganciclovir (GCV). The triply-phosphorylated GCV blocks DNA synthesis, resulting in cell death. Therefore, the cells transduced with the vector rAAV-PtkS are expected to be sensitized to clinically low dose radiation.

The control MCF7 cells or MCF7 cells transduced with rAAV-PtkS were irradiated with 1 Gy of X rays twice a day for 5 consecutive days in the presence of GCV, and cell survival was measured by MTT assay on day 7. When cells were not irradiated, a slight cell killing effect of GCV was observed in cells transduced with rAAV-PtkS. This effect was considered to be caused by the leaky expression of HSVtk. When the cells were irradiated, the cell killing effect of GCV was remarkably enhanced in cells transduced with AAV-PtkS, indicating that the induced HSVtk sensitized cells to radiation.

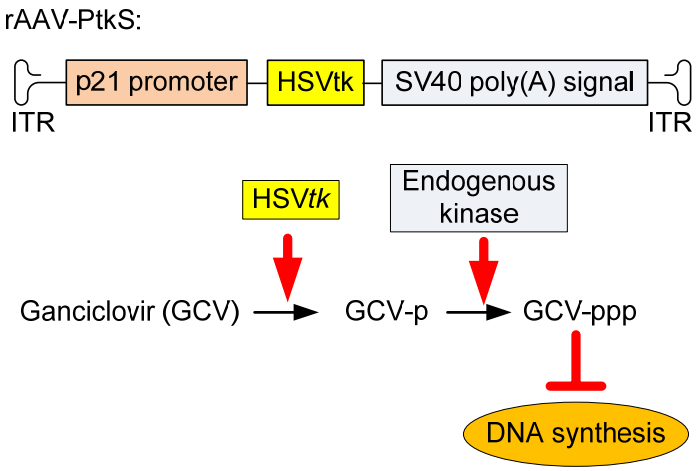

Figure 5. Structure of low dose radiation inducible suicide expression vector for cancer gene therapy

6 CONCLUSION

The transcription factor Oct-1 was revealed to play a crucial role in the p53-mediated regulation of *p21* following exposure to 0.2 - 2 Gy of radiation, further supporting the idea that Oct-1 is an important biological marker of radiosensitivity. It should be noted that the *p21* gene could be regulated through the DNA-PK pathway, based on the previous report that Oct-1 is functionally activated through phosphorylation of the N-terminal transcriptional regulatory domain by DNA-PK. This conclusion is consistent with the fact that induction of *p21* was delayed and attenuated in embryonic fibroblasts of the DNA-PK-deficient *scid* mouse. In addition, it was pointed out that the *p21* gene promoter in combination with a rAAV vector was potentially usable for the development of a low-dose radiation-inducible vector for cancer gene therapy.

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