Modification of the Radiosensitivity of Human Cells to which Simian Virus 40 T-antigen was transfected

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Effects of the introduction of the Simian virus 40 T-antigen (SV40 T-Ag) gene to cultured human cells were examined in relation to radiosensitivity. Two relatively radioresistant tumor cell lines (T98 and G361) became significantly radiosensitive after the introduction of SV40 T-Ag, whereas radiosensitive tumor cell lines did not show a change in radiosensitivity. In contrast, a human fibroblast cell line became radioresistant after SV40 T-Ag introduction. T98 cells which have a mutation at codon 237 in the p53 gene were unable to form a complex between p53 protein and SV40 T-Ag, whereas G361, which became radiosensitive by a SV40 T-Ag introduction, formed the complex. This indicates that the status of p53 is independent of the change in radiosensitivity in the cell lines studied.

INTRODUCTION

There is a wide variation in the radiation sensitivity of human tumor cells, and we have little information on the intrinsic factors that control cellular radiation sensitivity. Recently, transfection with certain viral-transforming genes and/or oncogenes has been found to cause changes in cellular radiation sensitivity. The introduction of the simian virus 40 T-antigen (SV40 T-Ag) gene enhanced the radioresistance of human diploid skin fibroblasts1,2) and human testicular cancer cells3).

Su and Little4) suggested that the SV40 T-Ag/p53 complex may function in the determination of the radioresistant phenotype. In addition, Lee and Bernstein5) reported that p53...
mutations in bone marrow cells from p53-transgenic mice enhanced resistance to ionizing radiation. We therefore examined the relationship between the modification of radiosensitivity on introduction of the SV40 T-Ag and the role of p53 gene in radiosensitive and radioresistant human tumor cell lines that originate from glioblastomas and melanomas.

MATERIALS AND METHODS

Cells and Culture Conditions

Established human glioblastoma cell lines (T98 and SAN), human melanoma cell lines (G361 and MeWo), and human fibroblast cells (KD and W138) were used. G361 cells were provided by Dr. H. Utsumi (Kyoto University). MeWo cells and W138 cells were obtained from the Japanese Cancer Research Resources Bank. All the cells were cultured at 37°C with 95% air and 5% CO2 in Dulbecco's modified Eagle's minimum essential medium (Nikken, Kyoto) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

Plasmids and Transfection

The plasmid pZneo1 was provided by Dr. T. Tsukada (National Cancer Center Research Institute). This plasmid has a SV40 T-Ag gene originating from the pZ189 plasmid6, which was ligated to pRSVneo containing a Rous sarcoma virus long terminal repeat promoter (RSV-LTR) and a neomycin resistance gene (neo'). pRSVneo was used for the negative control experiments.

Approximately 10^6 cells and 5 μg of pZneo1 or pRSVneo in 0.2 ml of PBS(—) were placed in a electroporation chamber (electrodes 3 mm apart, ZA-1200, PDS Inc., Madison, WI) then transfected using an electric pulse (800 V). The cells were kept on ice for 30 min then plated in 100 mm dishes and cultured. The medium was changed to selection medium containing geneticin (400–800 μg/ml) after 48 hr. Cultures were maintained for 2 to 3 weeks until the geneticin-resistant colonies appeared. Results of northern7 and western8 blotting confirmed that cells selected by geneticin expressed SV40 T-Ag.

X-irradiation and Cell Survival

Conditions for X-irradiation and the analysis used for cell survival have been described elsewhere9,10. Five replicate plates were used to determine each survival point, and experiments were repeated at least three times. Survival curves were fitted by the linear-quadratic model. Changes in the survival of the cells of these lines after transfection with or without SV40 T-Ag were analyzed statistically using the two-way analysis of variance and the t-test for unpaired data.

PCR-SSCP and Sequencing Analyses

We used a simple and rapid method for the detection of point mutations (the polymerase chain reaction) followed by analysis for single-strand conformation polymorphism (PCR-SSCP)11,12. W138 cells were used as the controls in the PCR-SSCP analysis.

For the sequencing analysis, we amplified p53 cDNA by reverse transcription-PCR (RT-
PCR), in which p53 cDNA was divided into three fragments; F1(nucleotides 165–621), F2 (572–1036), F3 (991–1510). The primers for each fragment were

UF1, 5′-TGACACGCTTCCCTGGATTG-3′; LF1, 5′-TGGCAAAACATCTTGTTGAG-3′;
UF2, 5′-AAGTCTGTGACTTGCACGTA-3′; LF2, 5′-AACATGCACCTCAAAGCTGT-3′;
UF3, 5′-CTCCAGTGTAATCTACTTG-3′; and LF3, 5′-GCTTCTGACGCACACCTATT-3′.

After the blunting of the ends by T4 DNA polymerase, the PCR products were phosphorylated by T4 DNA kinase (Takara Shuzo, Otsu, Shiga). The products were cloned into the EcoRV site of pBluescript SK (+) vector (Strategene, La Jolla, CA), and multiple independent clones isolated. The cloned plasmids were purified, and the nucleotide sequences of the inserted fragments analyzed from either side with a Taq DyeDeoxy Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the oligonucleotide primers, 5′-AACAGCTATGACCATG-3′ and 5′-GTAAAACGACGGCCAGT-3′, in an automated DNA Sequencer 373A (Applied Biosystems). At least four independent clones per fragment were analyzed.

**Immunoprecipitation**

Subconfluent cells were washed in cold PBS and proteins extracted using 1 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P40, 2 mM phenylmethylsulfonyl fluoride, and 100 units aprotinin). The lysates were kept on ice for 1 hr then centrifuged at 14000 g, and the supernatants collected. The protein concentration was measured by the Bio-Rad protein assay as adapted from the method of Bradford. Lysates containing 2 mg of protein were mixed with anti-p53 monoclonal antibody solution (PAb421, Oncogene Science, Cambridge, MA) or pre-immune serum and kept overnight at 4°C. Protein A-agarose (Oncogene Science) then was added, and the mixture incubated for 2 hr at room temperature. Immunocomplexes collected by centrifugation at 3000g were washed 5 times in the lysis buffer then resuspended in SDS-PAGE sample buffer. After boiling them, the immunocomplexes were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybound-C Super, Amersham LIFE SCIENCE, Buckinghamshire, UK). The membrane was blocked with 5% skim milk then incubated for 16 to 20 hr at 4°C with anti-SV40 T-Ag monoclonal antibody solution (PAb416, Oncogene Science). The membrane next was washed with 0.1% Tween20 in PBS, incubated for 1 hr at room temperature with horseradish peroxidase-conjugating goat anti-mouse immunoglobulin G (Amersham LIFE SCIENCE), then developed using ECL western blotting detection regents (Amersham LIFE SCIENCE) according to the instructions of the manufacturer.

**RESULTS**

**Characteristics and Radiation Sensitivity of SV40 T-Ag Introduced Cells**

Two human glioblastoma cell lines (T98 and SAN), two human melanoma cell lines (G361 and MeWo), and human fibroblasts (KD) were compared for the effects of plasmid transfection on radiation sensitivity. The doubling times and saturation densities for the cell lines are shown in Table 1. The morphology and growth rates of the glioblastoma and melanoma cell lines did
not change after transfection with pZneol or pRSVneo. KD cells transfected with pZneol (KD-neo-SV) grew in multiple layers with piled-up regions, whereas both the pRSVneo transfected cells (KD-neo) and parental KD cells grew as monolayers (data not shown). Saturation density at the plateau phase for the KD-neo-SV cells was almost 6 times that in KD cells. The durations of the cell cycle phases of all the cell lines in the exponentially growing cells, as measured by a flow cytometric analysis of DNA content (by propidium iodide staining) (Coulter Electronics Ltd., Luton Beds, UK) were not significantly different after transfection (data not shown).

Figure 1 shows the X-ray dose-survival curves for KD, SAN, T98, MeWo, and G361 cells after transfection with the pZneol or pRSVneo plasmid. T98 and G361 cells were markedly radioresistant as compared to the other three cell lines. After the introduction of SV40 T-Ag, KD cells became radioresistant against doses of more than 6 Gy (Fig. 1A, P<0.001), whereas T98 and G361 cells became significantly radiosensitive against doses of more than 6 Gy (Fig. 1B and 1D, P<0.001). SAN and MeWo cells showed no significant change in radiosensitivity after transfection (Fig. 1C and 1E). Transfection with pRSVneo plasmids caused no significant change in radiosensitivity in any of the cell lines. The $D_0$, $D_{10}$, and $D_1$ values for each cell line are given in Table 2.

### Table 1. Doubling time and saturation density of cells before and after the SV-40T-Ag introduction

| Cell Lines       | Doubling Time (hr, mean ± SD) | Saturation Density ($\times 10^6$ cells/cm²) | Plating Efficiency (%) |
|------------------|--------------------------------|---------------------------------------------|------------------------|
| KD               | 22 ± 2.0                       | 1.9                                         | 13.9                   |
| KD-neo           | 22 ± 2.0                       | 2.0                                         | 8.0                    |
| KD-neo-SV        | 19 ± 2.0                       | 11.0                                        | 9.9                    |
| SAN              | 28 ± 3.0                       | 5.7                                         | 13.5                   |
| SAN-neo          | 28 ± 3.0                       | 5.6                                         | 12.2                   |
| SAN-neo-SV       | 27 ± 2.0                       | 5.7                                         | 8.9                    |
| T98              | 20 ± 1.5                       | 7.0                                         | 22.4                   |
| T98-neo          | 20 ± 1.5                       | 7.0                                         | 35.4                   |
| T98-neo-SV       | 20 ± 2.0                       | 7.0                                         | 24.6                   |
| MeWo             | 19 ± 1.5                       | 8.7                                         | 45.2                   |
| MeWo-neo         | 19 ± 2.0                       | 8.7                                         | 40.7                   |
| MeWo-neo-SV      | 19 ± 1.5                       | 8.8                                         | 45.6                   |
| G361             | 17 ± 2.0                       | 12.0                                        | 30.8                   |
| G361-neo         | 17 ± 1.5                       | 12.0                                        | 45.1                   |
| G361-neo-SV      | 17 ± 1.5                       | 12.0                                        | 37.3                   |

### Status of p53 Gene

Table 3 shows the status of the p53 gene in the KD, SAN, T98, MeWo, and G361 cells. In the PCR-SSCP analysis, no band-shift in exons 4 to 10 of p53 gene was detected in the KD, SAN and G361 cells. The T98 cells had a mutation in the p53 gene, a G to A transition in the third
Fig. 1. Changes in radiosensitivity after transfection of human cell lines with SV40 T-Ag. Exponentially growing cells were exposed to X-rays. (A) KD, KD-neo, KD-neo-SV; (B) T98, T98-neo, T98-neo-SV; (C) SAN, SAN-neo, SAN-neo-SV; (D) G361, G361-neo, G361-neo-SV; and (E) MeWo, MeWo-neo, MeWo-neo-SV. Error bars represent standard deviation.

Table 2. Do, D10, D1, and n values of cells before and after the SV40T-Ag introduction

| Cell Lines   | Do (Gy) | D10 (Gy) | D1 (Gy) | n    |
|--------------|---------|----------|---------|------|
| KD           | 0.93    | 4.16     | 6.67    | 15.8 |
| KD-neo       | 1.24    | 3.41     | 6.16    | 1.6  |
| KD-neo-SV    | 1.83    | 5.05     | 9.11    | 1.9  |
| SAN          | 1.13    | 3.16     | 3.67    | 3.7  |
| SAN-neo      | 1.12    | 3.15     | 3.66    | 3.7  |
| SAN-neo-SV   | 1.11    | 3.14     | 3.66    | 3.7  |
| T98          | 2.03    | 7.13     | 10.33   | 10.3 |
| T98-neo      | 2.01    | 6.67     | 10.03   | 10.0 |
| T98-neo-SV   | 0.97    | 5.12     | 7.89    | 42.5 |
| MeWo         | 0.91    | 2.84     | 5.34    | 4.8  |
| MeWo-neo     | 1.15    | 2.84     | 5.33    | 2.2  |
| MeWo-neo-SV  | 1.08    | 2.83     | 5.35    | 1.3  |
| G361         | 1.83    | 5.75     | 9.85    | 4.8  |
| G361-neo     | 1.90    | 5.16     | 9.42    | 2.2  |
| G361-neo-SV  | 1.16    | 4.50     | 7.81    | 10.0 |
position of codon 237, whereas the KD and G361 cells had no mutation in that gene. SAN cells had no mutated site in exons 4 to 10 of the p53 gene where most of the mutations are found to take place.

Detection of SV40 T-Ag/p53 complex in SV40 T-Ag Introduced Cells

Figure 2 shows the SV40 T-Ag/p53 complex in pZneo1-transfected cells, as determined by immunoprecipitation with anti-p53 antibody. The SV40 T-Ag/p53 complex is present in the KD-neo-SV, SAN-neo-SV, MeWo-neo-SV, and G361-neo-SV cells, but not in the T98-neo-SV cells. The changes in cellular radiosensitivity may be caused by the introduction of SV40 T-Ag which combines with the to p53 protein affecting its function.

Fig. 2. Detection of p53/SV40 T-Ag complexes in SV40 T-Ag-introduced cells. In the control experiment shown as G361-neo-SV*, immunoprecipitation was done with pre-immune antibody.

Table 3. Status of the p53 gene

| Cell Lines | p53 gene status |
|------------|-----------------|
| KD         | wild-type*      |
| SAN        | wild-type (exon 4–10) |
| T98        | mutant-type (codon 237: ATG→ATA)* |
| MeWo       | wild-type**     |
| G361       | wild-type*      |

* Sequencing results of whole p53 cDNA
** ref. 13
DISCUSSION

We observed that the changes in radiosensitivity presumably caused by the introduction of SV40 T-Ag owing to pZneo1 transfection differed among the human cell lines assayed. Two radioresistant tumor cell lines (T98-neo-SV and G361-neo-SV) became sensitive to ionizing radiation after pZneo1 transfection, whereas human fibroblasts (KD-neo-SV) became radioresistant (Fig. 1).

SV40 transformation of mouse15) and rat16) cells induces the elevated expression of many genes. SV40 T-Ag has a number of biological activities, such as stimulation of cellular DNA replication in quiescent cells17-19), transcriptional activation of some cellular genes16,20, and binding to tumor suppressor proteins21,22). Moreover, transfection with SV40 T-Ag itself leads to transient genetic instability in precrisis cells23,24). The increase in radiosensitivity after the introduction of SV40 T-Ag in the radioresistant T98 and G361 cells may be related to the activities of SV40 T-Ag.

Pardo et al.25) reported that cells transfected with the neo-gene showed clonal diversity in their radioresistance. We analyzed the expression of SV40 T-Ag in the SV40 T-Ag-introduced KD cells, and 5 clones that express SV40 T-Ag showed very similar radiosensitivity (data not shown). We therefore used mixed transfected clones for the analysis of the radiosensitivity of the cell lines after each clone was confirmed to express SV40 T-Ag. We consider that with this method there were no possible errors in analyzing radiosensitivity.

There are several reports that the status of p53 may affect cellular radiosensitivity5,26-28). Lee and Bernstein5) reported that p53 mutations increased the resistance to ionizing radiation in bone marrow cells from transgenic mice. Furthermore, SV40 T-Ag has been shown to be associated with p5321,22). Su and Little4) demonstrated that human skin fibroblast cell lines transfected with SV40 T-Ag mutants defective in nuclear localization or origin binding show increased radioresistance, whereas cell clones transfected with three different p53 binding-defective mutants showed no change in radiosensitivity when compared with the neo gene-transfected control. In our study, the T98 cells had a mutant-type p53, with a G to A transition in the third position of codon 237, whereas the KD, SAN, MeWo and G361 cells had no mutation (Table 3). We could confirm the presence of SV40 T-Ag/p53 complexes in the KD-neo-SV, SAN-neo-SV, MeWo-neo-SV, and G361-neo-SV cells, but not in T98-neo-SV cells (Fig. 2); therefore, there is no clear relation between the status of the p53 gene and changes in radiosensitivity after SV40 T-Ag introduction to the two radioresistant cell lines T98 and G361. Although the mechanism(s) of the change in radiosensitivity is not certain, the fact that radioresistant cells become radiosensitive on the introduction of SV40 T-Ag provides information about the factors that determine radiation sensitivity and the possible involvement of p53 gene functions.
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