Pergamon

European Journal of Cancer, Vol. 34, No. 11, pp. 1802–1806, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00199-3

Original Paper

A Newly Developed Adenovirus-mediated Transfer of a Wild-type *p53* Gene Increases Sensitivity to *cis*-diamminedichloroplatinum (II) in *p53*-deleted Ovarian Cancer Cells

Y. Kanamori,[1] J. Kigawa,[1] Y. Minagawa,[1] T. Irie,[1] T. Oishi,[1] M. Shimada,[1] M. Takahashi,[1] T. Nakamura,[2] K. Sato[2] and N. Terakawa[1]

[1]Department of Obstetrics and Gynaecology; and [2]Department of Molecular Biology, Tottori University School of Medicine, 36-1 Nishimachi, Yonago 683, Japan

A new recombinant adenovirus carrying a wild-type *p53* gene (AxCAp53) was developed and the combination effect of *p53* gene transfer and *cis*-diamminedichloroplatinum (II) (CDDP) was examined in an ovarian cancer cell line, SK-OV-3, with deletion of the *p53* gene. AxCAp53 showed a high efficiency of gene transduction and increased sensitivity to CDDP in the SK-OV-3 cells. It was found that the sensitivity of the cells to CDDP correlated with the amount of infectious units of virus per cell of AxCAp53 which correlated with p53 protein expression. The results suggest that the combination of CDDP and AxCAp53 may be a potential strategy for the therapy of CDDP–resistant ovarian cancer.

Key words: ovarian cancer, CDDP, *p53* gene transfer, recombinant adenovirus
Eur J Cancer, Vol. 34, No. 11, pp. 1802–1806, 1998

INTRODUCTION

CYTOREDUCTIVE SURGERY followed by platinum-based combination chemotherapy is currently the standard treatment for patients with ovarian cancer. *Cis*-diamminedichloroplatinum (II) (CDDP) is a key drug in chemotherapy for ovarian cancer. However, the success of treatment is limited by the development of resistance to CDDP [1, 2]. A new approach for treatment is necessary to improve the prognosis of patients with ovarian cancer.

The *p53* gene encodes a cell cycle checkpoint protein that functions in the G1 phase of the cell cycle and has a pivotal role in inducing apoptosis [3]. The p53 protein is a transcriptional regulator of cellular response to DNA damage [4]. Several studies have shown the critical role of the *p53* gene in triggering cell death in response to cytotoxic drugs, and that mutations in the *p53* gene have been associated with the lack of response to these agents [5, 6]. The *p53* gene mutation has been found in more than 50% of advanced ovarian cancer [7] and survival was poor in patients with the *p53* gene mutation [8].

Correspondence to J. Kigawa.
Received 23 Oct. 1997; revised 3 Mar. 1998; accepted 5 Mar. 1998.

Although several studies have shown the potential of *p53* gene transfer in cancer gene therapy, the combined treatment of *p53* gene transfer and CDDP has not been examined in ovarian cancer cells. We developed a new adenovirus vector carrying the wild-type *p53* gene and examined the combination effect of *p53* gene transfer and CDDP in ovarian cancer cells with deletion of the *p53* gene.

MATERIALS AND METHODS

Development of p53 *expression adenovirus vector*

The procedure to construct a recombinant adenovirus carrying the wild-type *p53* gene (AxCAp53) is summarised in Figure 1. Briefly, a 1.95 kb, BamH I-EcORI fragment containing the full-length cDNA for human wild-type *p53* was isolated from a plasmid pProSp53 (Health Science Research Resources Bank, Tokyo, Japan). The *p53* coding sequence was inserted into a cassette cosmid pAxCAwt. This cassette cosmid contains human adenovirus type 5 (Ad5) genomic DNA lacking the E1A, E1B and E3 regions and the CAG promoter. The promoter consists of the cytomegalovirus immediate early enhancer, the chicken β-actin promoter, and the rabbit β-globin polyadenylation signal [9]. According to the method described by Miyake and colleagues [10], the

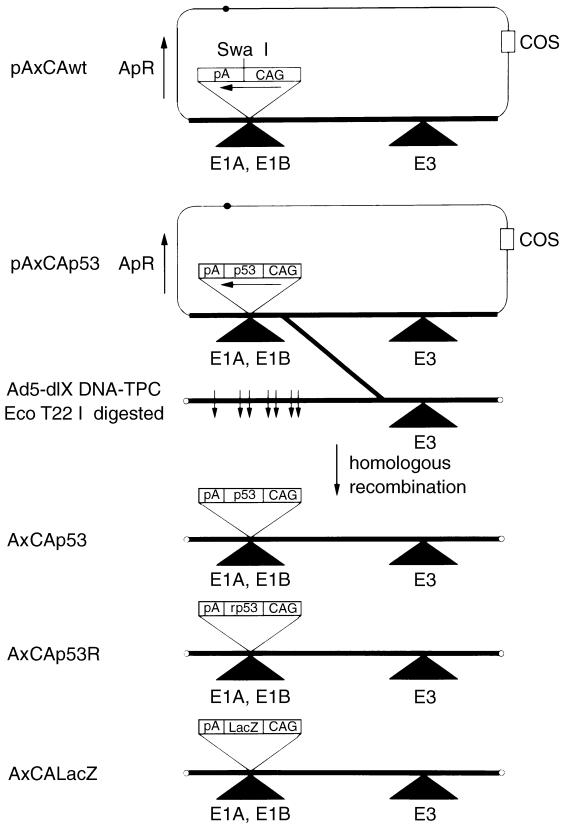

Figure 1. The procedure to construct a recombinant adenovirus carrying the wild-type *p53* gene (AxCAp53), the reverse inserted *p53* gene (AxCAp53R) or the bacterial *LacZ* gene (AxCALacZ). CAG, CAG promoter; pA, polyadenylation signal; ▲, deletion; rp53, reverse inserted *p53* gene; ApR, ampicillin-resistance gene; COS, cos site of λ phage.

p53-bearing cassette cosmid and EcoT22 I-digested DNA–terminal protein complex (TPC) of Ad5-dlX were co-transfected into the 293 cell line (American Type Culture Collection, Rockville, Maryland, U.S.A.) which was a human embryonal kidney cell line transformed by Ad5 E1A and E1B genes. AxCAp53 was generated by homologous recombination between the cosmid and EcoT22 I-digested Ad5-dlX DNA–TPC in the 293 cells. As a control, a recombinant adenovirus which had a reverse inserted *p53* gene (AxCAp53R) was also developed. The procedure for generating AxCAp53R was the same as mentioned above.

A recombinant adenovirus AxCALacZ, encoding for the bacterial *LacZ* gene under the control of the CAG promoter, was used to assess the efficiency of adenovirus-mediated gene transfer.

These three recombinant adenoviruses can replicate only in the 293 cells including the Ad5 E1 region in chromosomal DNA. They were grown and propagated in the 293 cells, maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37°C.

Recombinant adenoviruses were purified and concentrated by sequential centrifugation in CsCl step gradients, according to the method described by Kanegae and associates [11]. The infectious titres were determined by a modified endpoint cytopathic effect assay [11].

Assessment of adenovirus-mediated gene transfer

Cell line. A human ovarian adenocarcinoma cell line, SK-OV-3, with homozygous deletion of the *p53* gene, was obtained from the American Type Culture Collection. The cells were maintained in minimum essential medium (Nissui) with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C.

Examination of transduction. To determine the efficiency of the recombinant adenovirus to transduce SK-OV-3 cells, the cells were infected with AxCALacZ and the transduced cells detected by β-galactosidase staining. Briefly, SK-OV-3 cells were seeded in an 8-well chamber slide (Nunc, Roskilde, Denmark). The number of seeding cells was 30 000 cells/well and the pre-incubation time was 4 h. The cells were then infected with AxCALacZ at 6.25, 12.5, 25, 50, 100, and 200 multiplicities of infection (MOI). After 48 h, the cells were rinsed with phosphate-buffered saline (PBS), fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS. The sample was incubated with X-Gal solution consisting of 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml X-Gal in PBS for 1 h at 37°C. The magnitude of staining was quantitated by counting the percentage of blue cells.

Expression of p53 protein in SK-OV-3 cells. Cells were infected at 25, 50, and 100 MOI of AxCAp53. The expression of the p53 protein was monitored by Western blot analysis at various time points up to 14 days after infection. The cells were washed twice with PBS on ice and lysed in a cell lysis buffer consisting of 50 mM Tris–HCl pH 7.4, 125 mM NaCl, 0.1% Nonidet P-40, 0.1% sodium dodecyl sulphate, 5 mM ethylene diamine tetra-acetic acid (EDTA), 50 mM NaF, 0.3 mM phenylmethylsulphonyl fluoride (Sigma Chemical Co., St Louis, Missouri, U.S.A.), and proteinase inhibitors. The cell lysate was sonicated and centrifuged (15 000***g*** for 5 min at 4°C). The total protein concentration in the supernatant was measured using Bradford's method [12].

The samples of 20 μg protein/well were separated by electrophoresis on a 12.5% polyacrylamide gel. The separated proteins were then transferred to a polyvinylidence difluoride membrane (Millipore Co., Bedford, Massachusetts, U.S.A.). The membrane was incubated for 2 h with a primary antibody, mouse anti-human p53 monoclonal antibody DO-7 (Dako Co., Carpinteria, California, U.S.A.), and exposed to a biotinylated rabbit anti-mouse immunoglobulin G (Nichirei Co., Tokyo, Japan) as a secondary antibody for 30 min and then exposed to a horseradish peroxidase-conjugated streptavidin (Nichirei) as a third antibody for 30 min. The membrane was exposed to enhanced chemiluminescence detection reagent (Amersham International, Buckinghamshire, U.K.) for 1 min.

Effect of AxCAp53 on the growth of SK-OV-3 cells. The growth of SK-OV-3 cells after infection with AxCAp53 or AxCAp53R was examined using the 3-(4,5-dimethylthiazol-2yl)-2, 5-dphenyltetrazolium bromide (MTT) assay according to the procedure described by Mosmann [13]. Briefly, SK-OV-3 cells (2,500 cells/well) were seeded in 96-well tissue culture plates (Corning, New York, New York, U.S.A.) and pre-incubated at 37°C for 4 h. The cells were then infected with either AxCAp53 or AxCAp53R and incubated for 20, 44, 68, 92 and 116 h. Infectious units of each virus per cell were 25, 50 and 100 MOI. Twenty microlitres of MTT solution (2.5 mg/ml in PBS) was added to each well and the

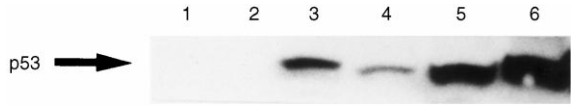

Figure 2. Western blot analysis for the p53 protein in SK-OV-3 cells 48 h after infection with AxCAp53 or AxCAp53R. Lane 1, no infection control; lane 2, cells infected with AxCAp53R; lane 3, positive control of p53 protein; lane 4, cells infected with AxCAp53, 25 MOI; lane 5, 50 MOI and lane 6 100 MOI. The cells infected with AxCAp53R did not express the p53 protein. The expression of p53 protein correlated with the level of infectious units of virus per cell of AxCAp53. We used KF cells, the ovarian cancer cell line with a wild-type *p53* gene, exposed to VP-16 to induce p53 protein as a positive control of p53 protein expression.

plates were further incubated for 4 h. One hundred microlitres of dimethyl sulphoxide were added and the plates were vigorously shaken on a plate-shaker to solubilise the MTT–formazan product. The absorbance at 570 nm was measured with a microplate reader Model 450 (Bio-Rad, Richmond, California, U.S.A.). Absorbance results were expressed as the mean of cell numbers by comparing each absorbance of six replicate wells with the absorbance of a standard curve prepared from a known number of cells.

Combination effect of AxCAp53 and CDDP

The combination effect of AxCAp53 and CDDP was evaluated using the MTT assay. SK-OV-3 cells (10 000 cells/well) were seeded in 96-well plates and pre-incubated for 4 h. The cells were then infected with either AxCAp53 or AxCAp53R. Infectious units of each virus per cell were 25, 50, or 100 MOI for each virus. CDDP was added 1 h after infection with the virus and the cells incubated for 68 h. Concentrations of CDDP ranged from 0.9 to 22.2 μM. The dose–response curve was plotted on a semi-log scale as a percentage of the control cell number obtained from the sample without exposure to CDDP in each MOI for each virus.

To assess apoptosis, SK-OV-3 cells (100 000 cells/well) were seeded in 28 cm^2 dishes and pre-incubated for 4 h. The cells were then infected with 25 MOI AxCAp53. CDDP was added 1 h after infection with the virus and the cells incubated for 68 h. Apoptotic cells were assessed morphologically by staining with Hoechst 33258 (Calbiochem-Novabiochem San Diego, California, U.S.A.) using cells fixed with Clarke fixative (ethanol:acetic acid 3:1). The apoptotic index (AI) was defined as: AI (%) = 100×apoptotic cells/200 cells.

Statistical analysis

All assays were performed in triplicate. Means for all data were compared by Fisher's one-way *post hoc* test (Microsoft Excel software on a Power Macintosh 7,500/100). $P < 0.05$ was considered statistically significant. To distinguish between a synergistic effect and an additive effect due to the combination of *p53* gene transfection and the administration of CDDP, the mathematical models employed were according to Finney [14]. For linearisation of curves of cell suppression, the value of the CDDP concentration was derived from the following formula: $-\ln[1/(1+\exp(\text{CDDP concentration})]$ and then a comparison of two regression slopes was performed.

RESULTS

The efficiency of the recombinant adenovirus to transduce SK-OV-3 cells was 89% for 6.25 MOI, 94% for 12.5 MOI, and 100% for 25–200 MOI. p53 protein was expressed at 24 h and disappeared 14 days after infection with AxCAp53. The peak of expression was 48 h after infection in each MOI. The expression of p53 protein correlated with the level of infectious units of virus per cell of AxCAp53 (Figure 2). The cells infected with AxCAp53R did not express p53 protein.

Figure 3 shows changes in the growth curve of SK-OV-3 cells after infection with either AxCAp53 or AxCAp53R. The suppression rate was calculated as: suppression rate (%) = 100 − 100× the number of infected cells (the number

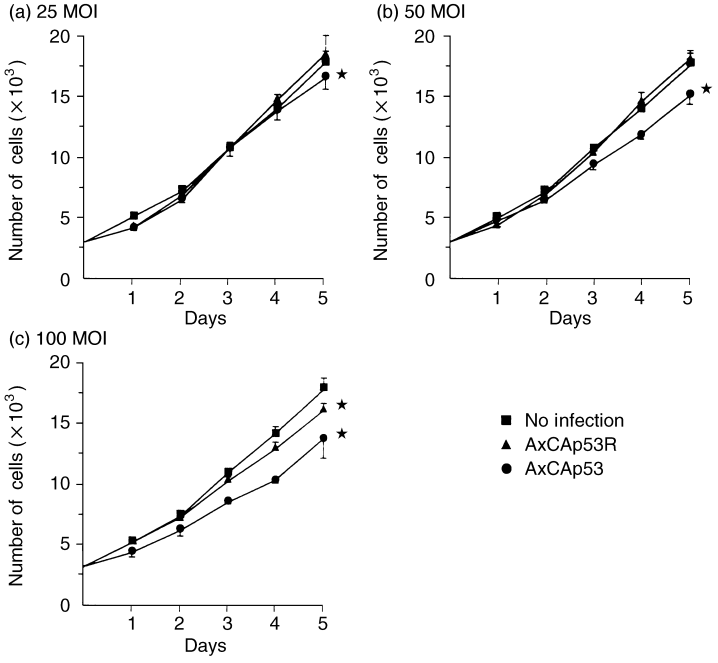

Figure 3. Changes in the cell growth curve of SK-OV-3 cells after infection with AxCAp53 or AxCAp53R. (a) shows the growth curve at 25 MOI, (b) at 50 MOI, and (c) at 100 MOI. AxCAp53 significantly suppressed cell growth at each MOI. Cell growth was not affected by less than 100 MOI of AxCAp53R. Mean ± S.D. *$P < 0.05$.

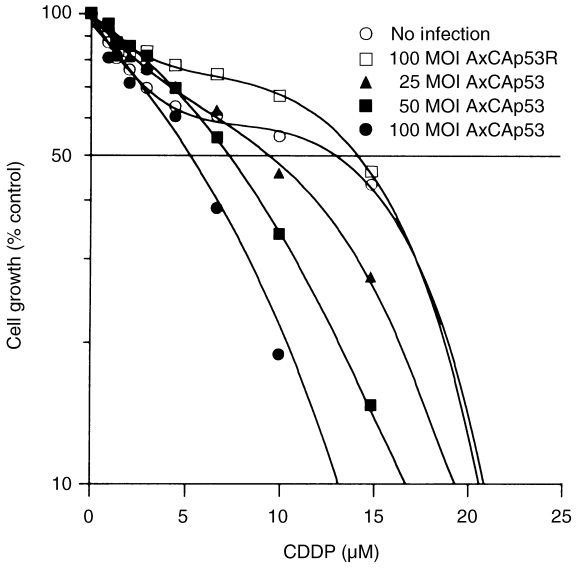

Figure 4. Dose–response curves of *cis*-diamminedichloroplatinum (II) (CDDP) in combination with AxCAp53. Infection with AxCAp53 increased sensitivity to CDDP in the SK-OV-3 cell.

of non-infected control cells). AxCAp53 significantly suppressed cell growth at each MOI. 5 days after infection with AxCAp53, the ratio suppression rate was 6.8% for 25 MOI, 14.6% for 50 MOI, and 23.4% for 100 MOI.

Cell growth was not affected by less than 100 MOI of AxCAp53R, but was significantly suppressed by 100 MOI of AxCAp53R (the ratio was 10.0%).

Dose–response curves of CDDP in combination with AxCAp53 are shown in Figure 4. The IC_{50} to CDDP was 12.9 μM for non-infected SK-OV-3 cells, 9.3 μM for AxCAp53 infected cells at 25 MOI, 7.3 μM for 50 MOI, and 5.2 μM for 100 MOI. The mathematical models showed a synergistic effect for the combination of *p53* gene transfection and the administration of CDDP (Table 1). AxCAp53R did not affect the inhibition of cell growth by CDDP.

The apoptotic cells revealed fragmentated nuclei by Hoechst 33258 staining (Figure 5). The apoptotic index correlated with the suppression rate in each group. The apoptotic index was 3.0% for non-infected SK-OV-3 cells, 13.9% for AxCAp53 infected cells at 25 MOI, 33.4% in 9.3 μM CDDP exposed non-infected cells, and 48.8% in CDDP exposed *p53* transfected cells.

Table 1. *Comparison of regression slopes between* p53 *transfected cells and non-transfected SK-OV-3 cells*

MOI	Regression slope	SEM	*t* value*	*P* value*
200	7.720	0.598	21.029	< 0.05
100	7.998	0.691	23.104	< 0.05
50	7.982	0.730	22.376	< 0.05
25	6.829	0.647	9.818	< 0.05
12.5	6.702	0.717	7.959	< 0.05
Non-transfected	6.004	0.808	–	–

*Compared with controls. MOI, multiplicities of infection; SEM, standard error of the mean.

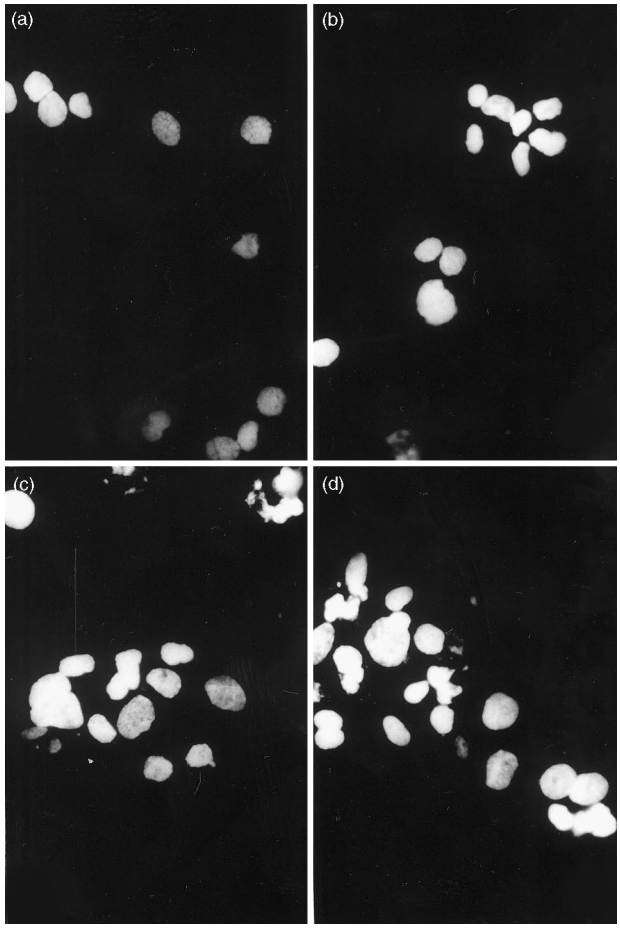

Figure 5. The apoptotic cells revealed fragmentated nuclei by Hoechst 33258 staining (×1,000). (a) Non-infected SK-OV-3 cells; (b) AxCAp53 infected cells at 25 MOI; (c) *cis*-diamminedichloroplatinum (II) (CDDP) exposed non-infected cells; (d) CDDP exposed p53 transfected cells.

DISCUSSION

A new recombinant adenovirus carrying a wild-type *p53* gene, AxCAp53, which has a relatively high efficiency of gene transduction was developed. This recombinant adenovirus showed 100% transduction efficiency to SK-OV-3 cells at 25 MOI. It has been shown that 100 MOI of the ACN53, a different type of recombinant adenovirus carrying the wild-type *p53* gene, was necessary to achieve more than 98% transduction efficiency to SK-OV-3 cells [15]. Our adenovirus vector also showed superior transduction efficiency to HeLa cells. AxCAp53 at 50 MOI had a 100% transduction efficiency, while 100 MOI of Ad5CMV-p53 was needed to achieve 100% transduction efficiency [16]. The CAG promoter was used as the expressor of our adenovirus vector. This promoter seems to be a high expressor, because an expression plasmid vector containing the CAG promoter has shown the highest levels of reporter gene expression among nine vectors containing different promoters such as the cytomegalovirus enhancer/promoter and the SO40 enhancer/promoter [17]. It is, therefore, likely that the CAG promoter induced the higher efficiency of gene transduction for our adenovirus vector.

Although AxCAp53 significantly suppressed cell growth in each MOI, the effect of introducing wild-type *p53* into *p53*-negative cells was relatively poor. The suppression rate was

only 6.8% at 25 MOI. In another study, *p53* gene transfer to *p53* gene-deleted cells did not always induce apoptosis [18]. A further study is necessary to clarify the underlying mechanism of this phenomenon. Cell growth was not affected by less than 100 MOI of AxCAp53R, containing an inserted *p53* gene in reverse, and transduced cells did not express the p53 protein.

The findings suggest that AxCAp53 is useful for examining the combination effect of *p53* gene transfer and CDDP. Loss of the *p53* gene contributes to resistance to CDDP that causes DNA strand breaks followed by apoptosis [19, 20]. SK-OV-3, a *p53*-deleted ovarian cancer cell line, showed low sensitivity to CDDP (IC_{50} 12.9 μM), but infection with AxCAp53 at 100 MOI increased sensitivity to CDDP in the cells (IC_{50} 5.2 μM). It has also been shown that a recombinant adenovirus-mediated transfer of the wild-type *p53* gene to a lung cancer cell line with homozygous deletion of the *p53* gene markedly enhanced sensitivity to CDDP [21].

We demonstrated that sensitivity of cells to CDDP correlated with the amount of infectious units of virus per cell of AxCAp53 and p53 protein expression correlated with the concentration of AxCAp53. The combination of CDDP and AxCAp53 may be a potential strategy for the therapy of CDDP-resistant ovarian cancer. In this study, CDDP was added 1 h after infection with AxCAp53 and a synergistic effect was observed. Recent literature has indicated that the timing of CDDP administration and *p53* gene transfer is critical: CDDP administration simultaneous with or subsequent to *p53* gene transfer was less effective than CDDP-first sequential treatment in lung cancer cells [22]. Future studies are necessary to clarify the most effective gene therapy strategy for ovarian cancer using sequential CDDP administration and *p53* gene transfer.

1. Maggie FM. Cisplatin update. *Semin Oncol* 1991, **8**, 1–4.
2. Kigawa J, Minagawa Y, Ishihara H, Kanamori Y, Terakawa N. Tumor DNA ploidy and prognosis of patients with serous cystadenocarcinoma of the ovary. *Cancer* 1993, **72**, 804–808.
3. Williams GT, Smith CA. Molecular regulation of apoptosis: genetic controls on cell death. *Cell* 1993, **74**, 777–779.
4. Kastan MB, Canman CE, Leonard CJ. p53 cell cycle control and apoptosis: implications for cancer. *Cancer Metastasis Rev* 1995, **14**, 3–15.
5. Kellen JA. Molecular interrelationships in multidrug resistance. *Anticancer Res* 1994, **14**, 433–435.
6. Man S, Bani MR, Adachi D, *et al.* Retroviral insertional mutagenesis as a strategy for the identification of genes associated with cis-diamminedichloroplatinum(II) resistance. *Cancer Res* 1995, **55**, 1139–1145.
7. Righetti SC, Della TG, Pilotti S, *et al.* A comparative study of *p53* gene mutations, protein accumulation, and response to cisplatin-based chemotherapy in advanced ovarian carcinoma. *Cancer Res* 1996, **56**, 689–693.
8. Jonathan J, Herod O, Eliopoulos AG, *et al.* The prognostic significance of Bcl-2 and p53 expression in ovarian cancer. *Cancer Res* 1996, **56**, 2178–2184.
9. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991, **108**, 193–199.
10. Miyake S, Makimura M, Kanegae Y, *et al.* Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc Natl Acad Sci USA* 1996, **93**, 1320–1324.
11. Kanegae Y, Makimura Y, Saito I. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn J Med Sci Biol* 1994, **47**, 157–166.
12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976, **72**, 248–254.
13. Mosmann T. Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983, **65**, 55–63.
14. Finney DJ. Statistical logic in the monitoring of reactions to therapeutic drugs. *Methods Inf Med* 1971, **10**, 237–245.
15. Mujoo K, Maneval DC, Anderson SC, Gutterman JU. Adenoviral-mediated *p53* tumor suppressor gene therapy of human ovarian carcinoma. *Oncogene* 1996, **12**, 1617–1623.
16. Hamada K, Alemany R, Zhang WW, *et al.* Adenovirus-mediated transfer of a wild-type *p53* gene and introduction of apoptosis in cervical cancer. *Cancer Res* 1996, **56**, 3047–3054.
17. Fukuchi K, Hearn MG, Deeb S, *et al.* Activity assays of nine heterogeneous promoters in neural and other cultured cells. In Vitro *Cell Dev Biol Anim* 1994, **30**, 300–305.
18. Polyak K, Waldman T, He TC, Kinzler KW, Volgelstein B. Genetic determinations of p53-induced apoptosis and growth arrest. *Genes Dev* 1996, **10**, 1945–1952.
19. Fritsche M, Haessler C, Brandner G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene* 1993, **8**, 307–318.
20. Perego P, Giarola M, Righetti SC, *et al.* Association between cisplatin resistance and mutation of *p53* gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res* 1996, **56**, 556–562.
21. Fujiwara T, Grimm EA, Mukhopadhyay T, Zhang WW, Owen SLB, Roth JA. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type *p53* gene. *Cancer Res* 1994, **54**, 2287–2291.
22. Nguyen DM, Spitz FR, Yen N, Cristiano RJ, Roth JA. Gene therapy for lung cancer: enhancement of tumor suppression by a combination of sequential systemic cisplatin and adenovirus-mediated *p53* gene transfer. *J Thorac Cardiovasc Surg* 1996, **112**, 1372–1376.

Acknowledgements—We thank Dr Izumu Saito and Dr Yumi Kanegae of the Institute of Medical Science, University of Tokyo, Tokyo, Japan for providing us with pAxCAwt and AxCALacZ, and Dr Junichi Miyazaki of the Institute of Medical Genetics, Kumamoto University Medical School, Kumamoto, Japan for providing the CAG promoter.