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Wild type *p53* gene transfer increases chemosensitivity and apoptotic response of PANC-1 pancreatic tumor cell line

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Abstract
The effect of p53 gene therapy on chemosensitivity and apoptotic response of PANC-1 tumor cells, which express high amount of mutant p53, to cancer chemotherapeutic agents of Etoposide and Doxorubicin was investigated. Comparison of the chemosensitivity of PANC-1 cells to its wild type p53 transfectants showed that wt-p53 expressing transfectants are more sensitive to both Etoposide and Doxorubucin. It further showed that neither the PANC1 cells nor its wild type p53 transfectants arrested at G1 in response to X-irradiation. Furthermore, treatment of both PANC-1 cells as well as its wt-p53 transfectants with etoposide resulted in apoptosis despite the difference in their p53 status, although, the number of apoptotic cells of the wt-p53 transfectants was higher compared to the control cells. This evidence reinforce the view that combining p53 gene therapy with conventional chemotherapeutic agents may yield a more beneficial response than conventional treatments alone in pancreatic tumor cells with high amount of mutant p53.
Keywords: *p53; Pancreatic cancer; Apoptosis.*

INTRODUCTION

P53 mutations are the most commonly observed gene alterations in human cancers. As a central regulator of cell growth, p53 is essential for the maintenance of the normal balance between cell proliferation and cell death. Restoration or overexpression of p53 is known to achieve various antitumor activities, which include induction of apoptosis, inhibition of cell proliferation, suppression of angiogenesis, and repair of damaged genes (Vousden, 2000). These antitumor mechanisms of p53 are known to be dependent in part on the tumor cell type and on the level of p53 expression (Sugrue *et al.*, 1997). Induction of wild type p53 is also known to improve chemosensitivity and radiosensitivity of cancer cells (Ganjavi *et al.*, 2006; Gurnani *et al.*, 1999; Lang *et al.*, 1998; Miyake *et al.*, 1998). Anticancer drugs etoposide and the anthracycline analogue doxorubicin both induce the nuclear accumulation of P53 and apoptosis (Fritsche *et al.*, 1993; Yonish-Rouach *et al.*, 1991). However, resistance to apoptosis severely limits the efficacy of these anti-cancer drugs in certain types of tumors (Dingemans *et al.*, 1999).

Pancreatic tumor cells show a very high frequency of p53 mutation. In our previous work (Hagi Sharifia Taghavi *et al.*, 2006) we showed that gene therapy based on restoration of wild type p53 protein function in pancreatic tumor cell line PANC-1, which expresses high amount of mutant p53, decreased the growth rate of PANC-1 cells and suppressed its potential for tumor formation in nude mice, completely. However, the therapeutic efficacy of this strategy alone may not be sufficient to control cancer growth in cancer patients, and combining this therapy with conventional chemotherapeutic agents may yield a more beneficial response than conventional treatments alone. Therefore, we decided to compare the sensitivity of wt-p53 expressing PANC-1 transfectants to the treatment with chemotheraptic agents, etoposide and doxorubucin to that of untransfected PANC-1 cells. Our results showed that despite higher chemosensitivity of wt-p53 PANC1 cells, neither the PANC1 cells nor its transfectants arrest at G1 in response to X-irradiation. Furthermore, the enhanced sensitivity of wt-p53 transfectants in comparison to the control is the result of increased apoptosis in response to treatment with etoposide.

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MATERIALS AND METHODS

Hoechst 33258 stain, Etoposide and doxorubucin were from SIGMA (Deisenhofen, Germany).

Transfection experiments: Stable transfection of pancreatic cell line, Panc-1, by wild type *p53* was performed using pcNXRS plasmid DNA via polybrene/DMSO-assisted gene transfer according to a procedure described previously (Hagi Sharifia Taghavi *et al*., 2006).

Cell culture: The pancreatic tumor cells PANC-1 were grown in DMEM (10% FBS) (Invitrogen) supplemented with 1mM L-glutamin, 100 U/ml penicillin. All cell cultures were maintained in a humidified incubator at 37°C under an atmosphere of 5% CO_2. Growth medium was replaced every 4 days.

Evaluation of chemosensitivity of pancreatic tumor cell line to genotoxic compounds: Chemosensitivity of PANC-1 pancreatic tumor cell line and its wt-p53 transfectants were assessed by treating confluent cells (1×10^5) grown in 100 mm culture with 0, 10, 20, 40 mg/ml of etoposide and 0-10 μg/ml of doxorubucin for 24 h followed by viability measurements.

Cell viability: Viability was estimated as the proportion of cells that excluded 0.2% trypan blue. Cells (1×10^5) were exposed to the dye for 5 min and loaded onto a hemocytometer and scored as either alive or dead using a 10 square count method.

Cell cycle analysis: Cells were exposed to 4Gy X-radiation and incubated at 37°C for various times up to a maximum of 40 h. At the appropriate time intervals the cells were washed in isotonic citrate saline, trypsinized and fixed in 70% ethanol at -20°C. The fixed cells were centrifuged to remove the ethanol, washed in PBS and permeabilized for 10min at room temperature in 1% BSA, 0.5% Triton-X100. The cells were then treated with 100 μg/ml RNAseA and 10 μg/ml propidium iodide and incubated for at least 30 min at room temperature. Cell cycle analyses were performed on a Coulter Epics XL flow cytometer.

Apoptosis: *Cellular staining and microscopy*- To examine apoptotic morphology changes cells were grown onto glass slides followed by staining with 10 μM final concentration of HOECHST 33258 stain (Aldrich Chem.). Chromatin structure was visualized by staining the cells with HOECHST 8-10 h post-treatment with anticancer agents. The cells were examined under a fluorescent microscope (Leica DM IRB, Germany), and phase-contrast and fluorescence pictures were taken.

DNA ladder- Fragmentation of cellular DNA was measured following 24 h treatment with 10 μg etoposide. Adherent and non-adherent cells were pooled, washed with phosphate-buffered saline (PBS), and resuspended in ice cooled buffer containing 0.15 M Nacl, 10 mM Tris (PH 7.4), 2 mM $MgCl_2$, and 1mM dithiothreitol. Nonidet P-40 was added to a final concentration of 0.5% (v/v), and the samples were incubated on ice for 30 min. Nuclei were isolated by centrifugation, resuspended in buffer containing 0.35 M NaCl, 10 mM Tris (PH 7.4), 2 mM $MgCl_2$, and 1mM dithithreitol, and incubated on ice for 15-30 min. The nuclei were then removed by centrifugation, and the DNA was phenol/cloroform extracted. The low molecular weight DNA was recovered by ethanol precipitation. Samples were resuspended in 20 μl of Tris-EDTA and treated with RNAase A for 30 min prior to electrophoresis.

Confirmation of apoptosis- In addition to DNA fragmentation, apoptosis was confirmed morphologically by spinning 15,000 cells onto a microscope slide and staining with Wright/Giemsa stains. One thousand cells were counted and scored as either normal, or apoptotic according to level of chromatin staining (condensed, intense chromatin staining).

RESULTS

Chemosensitivity of PANC-1 tumor cell line and its wt-p53 transfectants to genotoxic compounds: Chemosensitivity of PANC-1 cells was tested using etoposide and doxorubucin (Fig. 1). Following treatment with 0, 10, 20, 40 μg/ml of etoposide for 24 h the viability of PANC-1 cells expressing high amount of mutant p53 reduced to about 65, 45 and 27% at 10, 20 and 40 μg/ml concentration of the drug, respectively. The wt-p53 clone On the other hand displayed relatively higher sensitivity to etoposide and the viability of cells reduced to 40 and 25% at 10 and 20 μg/ml concentration of the drug, respectively. Furthermore, almost all of the cells died following 24 h treatment with 40 μg of etoposide (Fig. 2B). Expression of wt-p53 by the transfections was verified by Western blot (Fig. 2A). The chemosensitivity of PANC-1 cells to doxorubucin was also tested (Fig. 1C). Following treatment with 0-50 μg/ml of doxorubucin for 24 h the viability of PANC-1 cells reduced to 37 and 22% at 5 and 10 μg/ml concentration of the drug respectively, and less than 10% of the cells retained viability after

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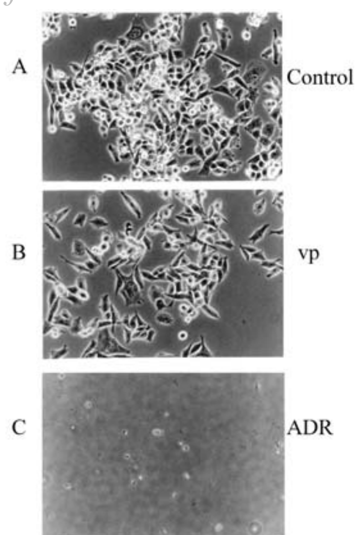

Figure 1. Evaluation of chemosensitivity of PANC-1 tumor cell line to anticancer drugs, etoposide and doxorubicin. A, control, B, PANC-1 cells treated with 20 μg/ml etoposide for 24 h and C, PANC-1 cells treated with 10 μg/ml doxorubicin for 24 h.

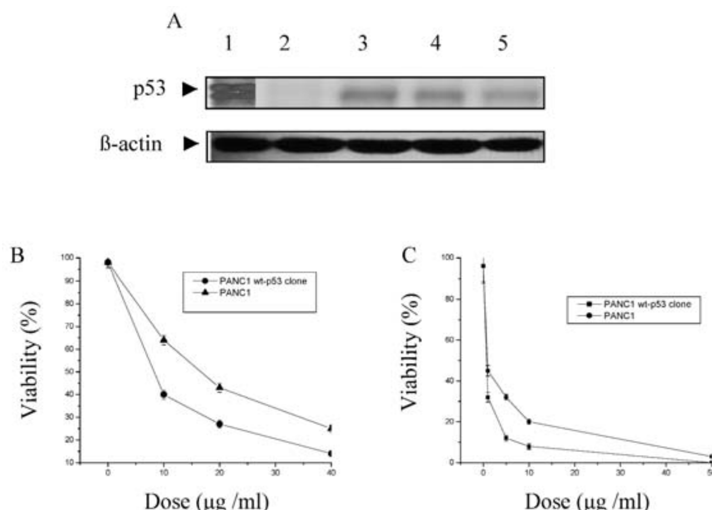

Figure 2. A, Analysis of wild type p53 expression in PANC-1 cells and its wtp53 transfectants by western blotting using wild type p53 antibody (1620 antibody): Lane 1, PANC-1 cells expressing mutant p53 (detected by 1801 antibody), lane 2, PANC-1 negative control (1620 antibody), lanes 3, 4 and 5 wild type p53 expressing transfectants. B and C, Comparison of chemosensitivity of PANC-1 tumor cell-line and its wtp53 transfectants to anticancer drugs treated with etoposide or doxorubicin, respectively.

24 h exposure to 50 μg/ml of the drug (Fig. 2C). The viability of wt-p53 cells reduce to about 12, 8 and 0% at 5, 10 and 50 μg/ml concentration of the drug respectively. Therefore, introduction of wild type p53 to PANC-1 cell line led to enhanced sensitivity to both of the drugs (Figs. 2B and C).

The *p53* gene transfer on PANC1 does not affect G1 arrest: A major mechanism whereby abnormalities of P53 contribute to the development and progression of tumors may be the abrogation of the normal pathways, i.e. G1 arrest or apoptosis. G1 phase is the first checkpoint in the cell cycle regulation before DNA replication. In response to DNA damage by irradiation, p53 mediates G1 arrest by induction of *p21* gene expression to protect genetic stability of the cells. Thus the effect of irradiation on cell cycle of wild type p53 transfected PANC-1 cells was compared to the parent, PANC-1, by flowcytometry. Irradiated PANC1 cells did not show a significant change in G1 phase of wtp53 transfectants compared to PANC1 cells (Table 1), which agrees with previous findings (Ng *et al.*, 1999).

Etoposide triggers apoptosis in PANC-1 tumor cells: Modulating intrinsic chemosensitivity or drug resistance by increasing the susceptibility of tumor cells to apoptosis may be an important aim in future cancer research. There is no evidence for occurrence of P53-dependent apoptosis in pancreatic tumor cells, which are carrying *p53* gene mutation. To find out whether abnormalities of P53 have any effect on apoptotic response to DNA damage in PANC-1 cells with mutant p53, they were exposed to cancer chemotherapeutic agent Etoposide shown to induce nuclear accumulation of P53 (Fritsche *et al.*, 1993). As visualized by staining with bis-benzimide (Hoechst), overnight treatment of PANC-1 tumor cells (Fig. 3B) and its wt-p53 transfectant (Fig. 3C) with 10 μg etoposide resulted in apoptotic cells which contained condensed chromatin and fragmented nuclei. Untreated cells used as negative control did not exhibit any apoptosis

Table 1. Comparison of cell cycle phases of PANC1 cells to its wild Type p53 transfectant.

	%			
	G0/G1	S	G2/M	SubG1
PANC1 cells	53.1	23.3	12.8	10.8
PANC1 wtp53 transfectants	48.3	24.9	13.9	12.9

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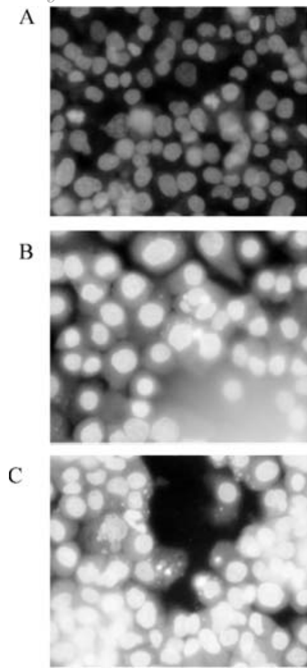


Figure 3. Induction of apoptosis in PANC-1 tumor cells and its wtp53 transfectants by etoposide. A, untreated PANC-1 cells, B, PANC-1 cells treated with etoposide, C, PANC-1 wtp53 transfectant treated with etoposide.

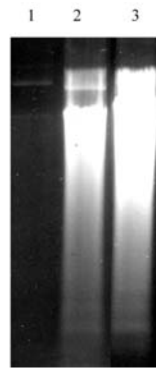
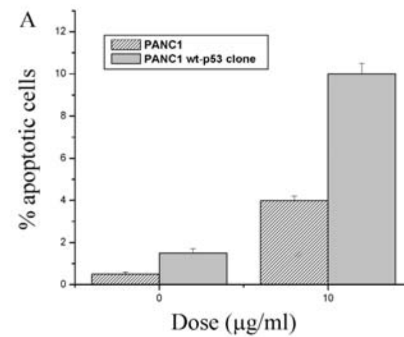


Figure 4. Analysis of apoptosis in PANC-1 cells and its wtp53 transfectant by DNA ladder. Cells were incubated with 10 μg etoposide for 24 h. Lane 1, untreated PANC-1, lane 2 PANC-1 treated with etoposide, lane 3, wtp53 transfectant treated with etoposide.



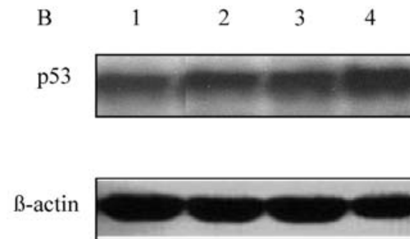


Figure 5. A, the proportion of morphologically apoptotic cells, PANC1 cells are compared to its wild type p53 transfectant for 8-10 h. B, Western blot analysis of p53 protein in cells treated with 10 μg/ml etoposide, lane 1 PANC1, lane 2 PANC1/etoposide, lane 3 PANC1 wtp53 transfectant, lane 4 PANC1 wtp53 transfectant/etoposide.

(Fig. 3A). Furthermore, etoposide treated PANC-1 tumor cells and its wtp53 transfectant exhibited DNA degradation, detected by typical DNA ladder consisting of 180bp fragments on agarose gels (Fig. 4), which was routinely observed 24 h post-treatment with 10 μg of etoposide. These data indicate that PANC-1 cells, which express high amount of mutant p53 protein, undergo apoptosis in response to etoposide-treatment. The remaining question was if the introduction of wild type p53 protein by gene transfer in PANC-1 cancer cells can increase the extent of apoptotic in response to the anticancer drug. As it is shown in (Fig. 5A) the number of apoptotic cells increased in wtp53-transfectants compared to control in agreement with higher level of DNA laddering observed for the wt p53 transfected cells (Fig. 4). Moreover, etoposide treatment of the Panc-1 cells resulted in higher p53 protein level of p53 transfected cells but not the cells lacking wtp53 protein (Fig. 5B).

DISCUSSION

Apoptosis, or programmed cell death, is a mechanism by which cells undergo death to control cell proliferation in response to various stimuli. The understanding of apoptosis has provided the basis for novel targeted therapies that can induce death in cancer cells or sensitize them to established cytotoxic agents and radiation therapy. Many pathways and proteins control the apoptosis machinery, the most important of which is p53 pathway (Ghobrial *et al.*, 2005). Functional inactivation of p53 can occur by several mechanisms including direct genetic mutation, binding to viral oncoproteins or cellular factors (e.g. mdm2), or alteration of the subcellular localization of the protein (Ozbun and Butel, 1995; Selter and Montenarh, 1994). Mutations in p53 have been reported in a majority of clinical cancers, and it has been estimated that p53 function is altered in half of all human malignancies (Ozbun and Butel, 1995; Selter and Montenarh, 1994). In general, cancers containing nonfunctional p53 tumor suppressor protein are less sensitive to chemotherapy (Lowe, 1995). Many anticancer agents induce apoptosis via p53-dependent (etoposide, doxorubicin) or p53-independent (paclitaxel) pathways (Wahl *et al.*, 1996; Lowe, 1995). Therefore in theory, the introduction of wild type p53 into cells with nonfunctional p53 protein should induce p53-dependent apoptosis and as a result should enhance their sensitivity to most chemotherapeutic drugs (Ganjavi *et al.*, 2006; Gurnani *et al.*, 1999). This is the most promising aspect of p53 gene therapy and it has the potential to sensitize cells to chemotherapy that are otherwise drug

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resistant. Thus, in this study we combined wt-p53 gene therapy of PANC-1 tumor cell line with etoposide and doxorubucin treatment, which are widely employed in the treatment of different cancers, and examined their chemosensitivity. The choice of these drugs was made due to the well-known fact that they activate intrinsic apoptotic pathway through p53 (Fritsche *et al.*, 1993; Yonish-Rouach *et al.*, 1991). Prior to assessment of the effect of p53 on the chemosensitivity of PANC-1 cells, it was established by western blot analysis (Fig 2A) that the wild type p53 protein was produced in the transfected clones. Then the viability of both PANC-1 cells and its p53 transfectants in the absence and presence of doxorubucin and etoposide was examined. PANC-1 cells, which express high amount of endogenous mutant p53, were found to be sensitive to both etoposide and doxorubucin (Fig. 1). Furthermore, p53 gene transfer increased chemosensitivity of PANC1 cells towards both drugs (Figs. 2B and C). In order to determine whether or not this decrease in the number of viable cells was due to G1 arrest in response to wtp53 gene transfer, the percentage of cells at various stages of cell cycle was determined by flow cytometry. Our data did not show any significant change in cell cycle of wtp53 transfectants compared to the control in response to X-irradiation (Table 1). It can therefore be concluded that the decrease in number of cells is rather due to enhanced cell death than cell cycle arrest. Presence of apoptosis as one of the mechanisms of cell death was examined by the formation of DNA ladder and chromatin condensation. PANC-1 and its wt-p53 transfected cells both underwent apoptosis in response to the drug, although the extent of apoptosis was higher for the p53 transfected cells (Figs. 4 and 5). These results suggest that either mutant p53 in PANC-1 cells is still partially functional or a p53 independent apoptosis is triggered upon etoposide treatment. Furthermore, upregulation of p53 protein in response to etoposide may contribute to enhanced sensitivity of PANC-1 wtp53 clones to chemotherapy (Fig. 5B).

In summary our results showed that wtp53 gene therapy of PANC-1 cells considerably enhanced their sensitivity to both etoposide and doxorubucin. It also indicated that although both PANC-1 cells and its wtp53 transfectants were able to enter programmed cell death upon etoposide treatment, the number of apoptotic cells increased for the wtp53 transfectants. Since the difference between the normal and tumor cells lies in the balance between cell proliferation and cell death, one can speculate that in PANC-1 tumor cells with defective p53 function, this balance was shifted towards cell proliferation. However, restoration of p53 function suppressed tumor formation by forcing more cells to enter p53 dependent cell death. Thus, combining *p53* gene therapy with chemotherapy may hold promise for the treatment of pancreatic tumors that contain high amount of mutant p53. It may also yield a more beneficial response than conventional treatments alone and may be the most effective cancer therapy regimen in the future.

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