Stable expression of temperature-sensitive p53: A suitable model to study wild-type p53 function in pancreatic carcinoma cells

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Received January 24, 2006; Accepted March 7, 2006

Abstract. Pancreatic adenocarcinoma is an extremely aggressive malignancy with a dismal prognosis. Inactivation of the p53 tumor-suppressor gene occurs in approximately 50% of primary tumors and is thought to account for a failure of the tumor cells to undergo growth arrest and apoptosis in response to chemotherapy. Hence, it is of interest to study the consequences of the restoration of wild-type (wt) p53 function in pancreatic carcinoma cells. Therefore, we retrovirally transduced temperature-sensitive (ts) human p53 into the p53-null pancreatic carcinoma cell line AsPC-1. ts p53 has a mutant phenotype at 37.5°C, and a wt conformation at 32.5°C. Stable expression of p53 in wt conformation caused upregulation of the p53 responsive gene p21Waf1/Cip1, and G1 growth arrest, but failed to induce Bax expression or apoptosis. In addition, we examined the effect of wt p53 expression on DNA damaging treatment. Interestingly, the doxorubicin- and radiation-induced S-/G2-phase arrests were suppressed by p53 in wt conformation. These results demonstrate that the ts p53/AsPC-1 model is suitable to investigate the effect of wt p53 restoration in pancreatic carcinoma cells.

Introduction

Adenocarcinoma of the pancreas is an extremely aggressive malignancy with a dismal prognosis (1). Development of pancreatic cancer is a multi-stage process resulting from the accumulation of somatic DNA mutations in normal cells. The major genetic alterations affect the K-ras oncogene, which is frequently activated by somatic mutations, and the tumor-suppressor genes p53, p16 and DPC4, which are inactivated in pancreatic cancer. Inactivation of the p53 tumor-suppressor gene is observed in approximately 50% of primary pancreatic carcinomas, and in almost all of these cases this inactivation occurs by loss of heterozygosity (LOH) associated with an intragenic mutation of the remaining allele (2). However, p53 gene mutations are much more overrepresented in established pancreatic carcinoma cell lines. Moore et al recently found 21 out of 22 cell lines to harbour p53 gene mutations (3). p53 responsive genes, such as p21Waf1/Cip1 and Bax, induce cell cycle arrest or apoptosis (4). Therefore, the development of chemoresistance and tumor progression is largely attributed to the disruption of the normal p53 function. However, it is well documented that pancreatic carcinomas with wild-type (wt) p53, are extraordinarily aggressive malignancies and exhibit considerable chemoresistance (5). The lack of cell lines with wt p53 requires ectopic expression of wt p53 to study p53 functions in pancreatic carcinoma cell lines. Unfortunately, as mentioned above, most pancreatic carcinoma cell lines synthesize mutated p53 proteins, which potentially suppress the function of ectopically expressed wt p53 protein by their dominant negative activity (6). Therefore, only a p53-null pancreatic carcinoma cell line would represent a suitable model to investigate the consequences of ectopic wt p53 expression. The pancreatic carcinoma cell line AsPC-1 fulfils this requirement. AsPC-1 cells harbour two mutated p53 alleles, and completely lack p53 mRNA transcripts (7). Previously, Kimura et al reintroduced wt p53 in AsPC-1 cells by retroviral infection (8). Due to the permanent p53-mediated growth inhibition, this approach required the tedious expansion of cell clones in long-term cultures. This step might have led to the selective expansion of subclones not representative of the original cell line (9). To avoid long-term culturing of cells permanently expressing wt p53 we employed a temperature-sensitive (ts) human p53 gene (10). ts p53 has a mutant phenotype at 37.5°C (restrictive temperature), and a wt conformation at 32.5°C (permissive temperature). In this study we show that AsPC-1 cells retrovirally transduced with ts p53 are suitable to investigate p53 functions in clones isolated without selective pressure of permanent wt p53 expression. By using this novel cellular model we analysed the impact of wt p53 on cell cycle regulation and apoptosis of AsPC-1 cells following doxorubicin treatment and γ-irradiation.

Materials and methods

Cell line and culture conditions. The human pancreatic cancer cell line AsPC-1 was obtained from the American Type Culture Collection (Rockville, MD, USA) and routinely maintained in RMPI-1640 supplemented with 10% FCS, 2 mM
glutamine and 1 mM sodium pyruvate (Life Technologies, Inc., Eggenstein, Germany) without penicillin or streptomycin (11). Retrovirally transduced cells expressing ts p53 received 700 μg/ml geneticin (Sigma, Deisenhofen, Germany) in addition. All cells were kept in a water-saturated atmosphere containing 5% CO2 at 37.5°C, and pre-warmed medium was used in all medium changes. For treatment with DNA damaging agents, the cells were seeded at 70% confluence in 6-well plates and were allowed to adhere overnight at 37.5°C. Cells were then incubated for an additional 8 h at either 37.5 or 32.5°C and subsequently exposed to 0.1 μg/ml doxorubicin (Pfizer, Switzerland), 8 Gy of γ-irradiation (Gammarcell 40, Anatomic Energy of Canada Ltd.) or remained untreated for 24 h.

**Generation of retroviral vectors and retroviral infection.** The cDNA for the human temperature-sensitive p53 mutant, p53Val-138 (amino acid 138, Alanine is changed into Valin), was kindly provided by Dr N. Tsuchida (Tokyo Medical and Dental University, Japan) (10). The ts p53 cDNA was released from pCMVnb with BamHI followed by polishing of both ends with Klenow fragment and was sub-cloned in sense orientation into the Pmel site of the retroviral vector TJBa5bMoLink-neo (TJM). This construct was co-transfected into human embryonic kidney 293T producer cells, along with retroviral packaging vectors, as described previously (12). Conditioned medium from 293T cells containing retroviral particles was used to transduce AsPC-1 cells in an overnight incubation.

**Immunological reagents.** The mouse monoclonal antibody (mAb) NCL-53-DO1 against human p53 was purchased from Novocastra Laboratories (Newcastle, UK). NCL-53-DO1 likewise detects p53, and ts p53 in wild-type and mutant conformation. The mouse mAb against human p21Waf1/Cip1 (clone 70) was purchased from Novocastra Laboratories (Newcastle, UK). NCL-53-DO1 against human p53 was purchased from BD Transduction Laboratories (Hamburg, Germany). Anti-β-actin (clone AC-15) antibody was obtained from Sigma, (St. Louis, Missouri, USA), and the rabbit polyclonal antibody against human Bax was purchased from Pharmingen (Hamburg, Germany). Anti-Bax was purchased from Pharmingen (Hamburg, Germany) and the rabbit polyclonal antibody against human Bax was purchased from Pharmingen (Hamburg, Germany). Anti-Bax was purchased from Pharmingen (Hamburg, Germany) and the rabbit polyclonal antibody against human Bax was purchased from Pharmingen (Hamburg, Germany).

**Western blotting.** For Western blotting, 30 μg of total cellular protein (measured with BCA Protein Assay Reagent, Pierce Chemical Co., IL, USA, according to the manufacturer’s instructions) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF) (Immobilon-P, Millipore, Eschborn, Germany). Membranes were blocked with PBS containing 5% non-fat dry milk overnight at 4°C, washed several times with PBS containing 0.2% Tween-20 (PBS/Tween) and then incubated with the primary antibody. After washing with PBS/Tween, blots were incubated with appropriate secondary antibody. Peroxidase-conjugated secondary antibodies against primary anti-p53 mAb and p21Waf1/Cip1 mAb were developed with the chemiluminescent detection kit ECL (Amersham). The detection of β-actin demonstrates equal protein loading.

**Results**

The ts p53 induces p21Waf1/Cip1 following temperature shift to permissive temperature. AsPC-1 cells harbour mutated p53 alleles and lack p53 mRNA transcripts (7). The absence of endogenous p53 protein was confirmed by Western blotting of total cellular protein from parental AsPC-1 cells (AsPC-1-pa) (Fig. 1, top). To investigate the consequences of wt p53 restoration in AsPC-1 cells, we introduced the cDNA of ts p53 into AsPC-1 cells by retroviral transduction, followed by geneticin selection of individual clones. Further experiments were carried out with two clones (AsPC-1-cl.A and -cl.B), which exhibited strong expression of ts p53 (Fig. 1, top). AsPC-1-pa cells and AsPC-1 cells transduced with an empty vector (AsPC-1-v) were employed as controls. The level of ts p53 expression in AsPC-1-cl.A and -cl.B was significantly higher at the restrictive temperature and decreased following a shift to the permissive temperature (Fig. 1, top). Expression of ts p53 in it’s wt conformation (at the permissive temperature) but not in its mutant conformation (at the restrictive temperature) resulted in an upregulation of the p53 responsive
gene p21Waf1/Cip1, indicative of proper ts p53 function (13) (Fig. 1). In contrast, the expression of Bax, a pro-apoptotic gene that has been reported to be transcriptionally induced by p53 (14), remained unaltered following a shift to the permissive temperature (Fig. 1). Consistently, at the permissive temperature, AsPC-1-cl.A and -cl.B cells showed an increase in the G1-cell-fraction when compared to AsPC-1-pa and AsPC-1-v cells (Fig. 2a). In addition, p53 mediates apoptosis by transcriptional activation of a variety of pro-apoptotic genes, such as Bax (4). However, neither AsPC-1-cl.A/-cl.B nor AsPC-1-pa/-v displayed a statistically significant increase in the sub-G1 fraction, indicative of apoptosis, at the permissive temperature (Fig. 2b). Nevertheless, the observed G1-arrest at the permissive temperature clearly demonstrates the functional activity of ts p53 in AsPC-1-cl.A and -cl.B cells (Fig. 2a).

The ts p53 suppresses DNA damage-induced S-/G2-arrests at the permissive temperature. As part of the stress response program wt p53 is upregulated in response to DNA damaging agents at the transcriptional and posttranscriptional level. However, the ability of p53 to induce individual target genes, such as p53AIP1, requires distinct posttranslational modifications (15). DNA damaging drugs and γ-irradiation both stimulate the posttranslational modification of p53, and thus, alter the pattern of expressed p53 responsive genes (15,16). Hence, we exposed the various AsPC-1 derivatives to doxorubicin or γ-irradiation following the temperature shift. A slightly pronounced expression of p21Waf1/Cip1 was observed following doxorubicin treatment of AsPC-1-cl.A and -cl.B at permissive temperature (Fig. 3). Again, expression of ts p53 at the permissive temperature and exposure to doxorubicin or γ-irradiation both failed to cooperatively upregulate Bax expression in AsPC-1-cl.A and -cl.B clones (Fig. 3). Remarkably, regarding the cell cycle distribution, AsPC-1-cl.A and -cl.B exhibited an S-arrest following doxorubicin treatment at the restrictive temperature, which was absent at the permissive temperature. In contrast, AsPC-1-cl.A and -cl.B expressing ts p53 at wt conformation at permissive temperature were all G1-arrested and did not respond to doxorubicin with respect to changes in the cell cycle distribution (Fig. 4a and b). In particular, doxorubicin-treated AsPC-1-cl.A and -cl.B did not show a higher number of apoptotic cells at the permissive temperature (Fig. 4a and b). AsPC-1-cl.A and -cl.B exhibited a G2-arrest following γ-irradiation at the restrictive temperature, which was absent at the permissive temperature (Fig. 4a and b). At the permissive temperature, AsPC-1-cl.A and -cl.B were G1-arrested and did not respond to γ-irradiation in terms of changes in the cell cycle distribution (Fig. 4a and b).

The AsPC-1-pa and AsPC-1-v cells exhibited a G2-arrest following doxorubicin treatment at the restrictive temperature, which was also observed, albeit to a lesser degree, at permissive temperature (Fig. 4c and d). In contrast, AsPC-1-cl.A and -cl.B exhibited an S-arrest following doxorubicin treatment at the restrictive temperature. This discrepancy might be explained by a particular, yet aberrant, function of ts p53 in the mutant conformation, which has also been demonstrated for mutant p53. The AsPC-1-pa and AsPC-1-v cells exhibited a G2-arrest following γ-irradiation treatment at the restrictive temperature, which was, to a lesser degree, also observed at the permissive temperature (Fig. 4c and d). A dominant G1-arrest
at permissive temperature was not noted in AsPC-1-pa and AsPC-1-v cells, and the doxorubicin- and radiation-induced G2-arrests were not abolished by the shift to permissive temperature. In summary, the cell cycle distribution of treated and non-treated AsPC-1 cells was similar only in cells expressing ts p53 at wt conformation (Fig. 4a and b). Apparently, wt p53 suppressed DNA damage-induced S-arrest (in the case of doxorubicin treatment) and G2-arrest (in the case of γ-irradiation) by the dominance of the p53-dependent G1-arrest.

Discussion

In the present study we introduced a novel cellular model to investigate the effect of restoration of wt p53 in pancreatic carcinoma cells. We retrovirally transduced a human temperature-sensitive p53 mutant into the p53-null pancreatic carcinoma cell line AsPC-1. In addition, we examined the effect of temperature-regulated wt p53 expression on DNA damaging treatment. A temperature shift to permissive temperature induced the p53 responsive gene $p21^{Waf1/Cip1}$, indicative of proper ts p53 function. This observation is in line with the observed G1 arrest at the permissive temperature. The level of expressed ts p53 decreased significantly following the shift to permissive temperature. This is explained by the conformational change of ts p53 into the wt p53 phenotype, which is more susceptible to Mdm2-dependent degradation (17). Interestingly, K-ras, which is constitutively active in AsPC-1 cells, is known to induce transcriptional upregulation of Mdm2 and subsequent p53 degradation (17,18). In contrast to the induction of $p21^{Waf1/Cip1}$, expression of the p53 responsive gene $Bax$ remained unaltered following the shift to permissive temperature, and the cells did not undergo apoptosis. Previously, Zhao et al. demonstrated that the transcriptional activation of p53 responsive genes depended on the p53 expression level. Limited expression of wt p53 was shown to suffice for $p21^{Waf1/Cip1}$ induction, while transcriptional activation of proapoptotic genes, such as $Pig3$, required high levels of wt p53 (16). Hence, the down-regulation of ts p53 expression levels following the temperature shift might account for the lack of Bax upregulation at the permissive temperature. Notably, to the best of our knowledge, neither the Val-138 nor any other ts p53 mutant has ever been demonstrated to increase endogenous Bax protein expression in human tumor cells. Nevertheless, the ts Val-138 mutant has been shown to induce apoptosis in cell lines other than AsPC-1 (10).

The ability of p53 to induce its responsive genes also depends on posttranslational modifications, like Ser-46 phosphorylation, following exposure to DNA damaging drugs or γ-radiation (15,19). Therefore, we subjected AsPC-1 ts p53 clones to doxorubicin treatment and γ-irradiation, both of...
which failed to induce Bax expression or apoptosis at the permissive temperature. At the restrictive temperature, however, these insults induced S- or G2-arrest. In contrast, AsPC-1 clones being G1-arrested as a result of wt p53 function completely lacked specific doxorubicin or radiation-induced alterations of cell cycle distribution. These results suggest that both mutant as well as wt p53 might influence the response of AsPC-1 cells to DNA damaging treatment in a disadvantageous way. On the one hand, the lack of functionally active p53 renders p53-dependent elimination of G1-arrested cells impossible. On the other hand, the presence of functionally active p53 prevents cells from progressing to S-phase of cell cycle, which is crucial for certain DNA damaging drugs and irradiation to execute their cytotoxicity. In support of this notion, previous studies have indicated that p21Waf1/Cip1-induced cycle arrest renders cancer cells resistant towards DNA damaging agents, such as doxorubicin (20). In summary, retroviral transduction of the human ts p53 mutant into AsPC-1 cells is a suitable model to investigate the function of wt p53 restoration in human pancreatic carcinoma cells.

Acknowledgements

We thank Drs K. Römer and N. Tsuchida for providing the ts p53 cDNA and S. Rathjen for excellent technical assistance. This study was supported by the ‘Deutsche Krebshilfe’ (Grant 10-1457).

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