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Development of a mechanistically-based genetically engineered PC12 cell system to detect p53-mediated cytotoxicity

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Abstract

The human wild type p53 gene, key for apoptosis, was introduced into the pheochromocytoma (PC12) cell line, to create a mechanistically-based *in vitro* test model for the detection of p53-mediated toxicity. Expression of the wt p53 gene was regulated by a system, which allowed or blocked expression p53 by absence or presence of tetracycline in the culture media. Western blot analyses confirmed an inducible and tetracycline-dependent expression of the wt p53 protein. Functionality of the p53 protein was verified by camptothecin treatment, known to induce p53-dependent apoptosis. Results showed that p53-expressing cells were significantly more sensitive to camptothecin induced cytotoxicity compared to non-expressing cells, and presented a significantly higher incidence of apoptosis. A screening study on 31 metal compounds, showed that the classified human carcinogens ($NaAsO_2$, $CdSO_4 \cdot 8H_2O$, $Na_2CrO_4 \cdot 4H_2O$, $MnCl_2$, $(NH_4)_2PtCl_6$) significantly increased cytotoxicity in p53-expressing cells compared to non-expressing cells, suggesting that their cytotoxicity was p53-mediated. Finally, acute and subchronic treatment with methyl mercury showed no significant differences in cytotoxicity and the percentage of apoptosis or necrosis between p53-expressing and non-expressing differentiated cells, suggesting that methyl mercury cytotoxicity was p53-independent.

Keywords: *In vitro*; p53; Apoptosis; Camptothecin; Metals; Mercury; Arsenic

1. Introduction

The p53 tumor suppressor gene is known to induce apoptosis in reaction to cellular stress or damage (May and May, 1999; Striteska, 2005). There is accumulating evidence that p53 is perturbed in the central nervous system in a number of neurodegenerative disorders (Miller et al., 2000). In particular, the involvement of p53- dependent apoptosis have been shown in neurodegenerative diseases such as Parkinson, multiple sclerosis and Alzheimer (Biswas et al., 2005; Ohyagi et al., 2005; Wosik et al., 2003). Furthermore, the p53 tumor suppressor protein was identified to be a critical mediator of programmed cell death in response to DNA damage and genotoxic carcinogens (Kaiser and Bodey, 2000; Schulte-Hermann et al., 1999), and has been shown to be involved in the carcinogenic effect of metals (Valko et al., 2005).

Previous studies showed that toxicity of chemicals can be dependent on the interaction with the p53 gene (Chen et al., 2006; Vanlandingham et al., 2005). Low levels of toxicity may affect p53-expression which can lead to apoptosis,

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EPA, environmental protection agency; GCCP, good cell culture practice; PC12, human p53 genetically engineered rat PC12 cell line; IARC, International Agency for Research on Cancer; MeHgCl, methyl mercury chloride; MTT, (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PC12, pheochromocytoma 12; PI, propidium iodide; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene; REACH, Registration Evaluation and Authorisation of Chemicals; RPMI, Roswell Park Memorial Institute; TRE, tetracycline responsive element; wt, wild type.

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while high toxicity levels affect homeostasis and finally induce necrosis (Nicotera, 1996). As a consequence, p53-mediated apoptosis could be used as a sensitive and early endpoint for *in vitro* toxicity of chemicals at low concentrations (Anselmi et al., 2002). Perhaps for that reason several biotechnology companies show interest in focusing on ways to control apoptosis with new therapeutics (Potera, 1998).

The recent proposal for a new European Union policy on chemicals, i.e. the REACH Regulation, will require information on the human health effects of around 30,000 existing chemicals currently marketed in volumes greater than 1 ton per year. If alternative methods are not used, this could result in a substantial increase of animal use for toxicity testing (Hofer et al., 2004). Integrated testing strategies including genetically engineered cell lines and more complex *in vitro* systems could play an important role within this new regulatory context such as for the assessment of neurotoxicity (Coecke et al., 2002, 2006).

As a consequence, the present study aimed at developing a genetically-modified rat pheochromocytoma cell line (PC12) to detect p53-mediated toxic effects induced by chemicals. The PC12 cell line is known to respond to nerve growth factor (NGF) by extending long, branching neuron like processes and is commonly used in neurobiological research (Greene and Tischler, 1976). This cell line also constitutes a useful model for studying the mechanisms of apoptosis, its prevention or induction (Wang et al., 2005; Raza and John, 2006; Zhao et al., 2002).

One approach to study the role of specific genes, such as p53, is to use a tetracycline regulated expression system. Such approach has the advantage to provide with an easily inducible "genetic switch" that tightly regulates gene expression in a reversible and quantitative way (Gossen and Bujard, 1992). The tetracycline-responsive element (TRE) is located upstream of the promoter of minimal immediate early cytomegalovirus (PminCMV) and the gene of interest (Fig. 1). The presence of tetracycline (Tc) blocks the binding of the tetracycline responsive transcriptional activator (tTA) and thereby expression. In the absence of tetracycline the transcriptional activator can bind to the tet-responsive element and thereby activates the transcription of the gene of interest. As a consequence, presence of low and non-toxic tetracycline concentrations suppresses gene expression (Tet-Off), but in the absence of tetracycline, expression is fully re-stored within a few hours (Tet-On).

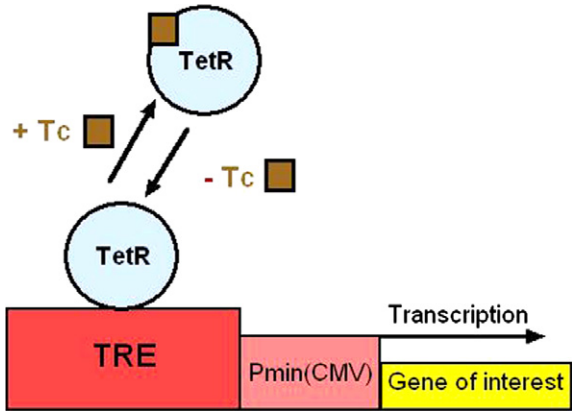

Fig. 1. Gene regulation in the Tet-On/Tet Off gene expression system.

Further to the creation of the genetically modified PC12 cell lines, the tetracycline inducible expression of the wt p53 protein was verified, as well as the functional state of the expressed human wt p53 protein by treating the undifferentiated cells with the topoisomerase I inhibitor camptothecin known to mediate apoptosis via a p-53 mediated mechanism (Lesuisse and Martin, 2002). Cells were then used for screening p53-mediated toxicity with trace metals which could interact with the p53 gene (Valko et al., 2005). In addition the acute and sub chronic toxicity of the well known neurotoxic compound methyl mercury (Clarkson, 2002) were investigated using undifferentiated and neuron-like differentiated PC12 cells.

2. Materials and methods

2.1. Compounds

$AgNO_3$, $AuCl_3$, H_3BO_3, $CdSO_4$, $CoCl_2 \cdot 6H_2O$, $Ga(NO_3)_3 \cdot 6H_2O$, $HgCl_2$, $La(NO_3)_3 \cdot 6H_2O$, LiCl, $KMnO_4$, $MnCl_2$, $NaNO_3$, $(NH_4)_2PdCl_6$, $(NH_4)_2PtCl_6$, $(NH_4)_2PtCl_4$, RbCl, $(NH_4)_3RhCl_6 \cdot H_2O$, Na_2TeO_3, Na_2TeO_4, $TlSO_4$, Ph_4As, $C_5H_{11}AsO_2$, $NaVO_3$, and $ZnSO_4 \cdot 7H_2O$ were from Alfachem, (Cologno Monzese, Milan, Italy); $(CH_3)_2$ AsOOH, K_2MoO_4, $NaAsO_2$, $Na_2CrO_4 \cdot 4H_2O$ were from Fluka (Milan, Italy); $(C_6H_5)_4AsCl \cdot H_2O$, MeHgCl (methyl mercury chloride), and $C_{20}H_{16}N_2O_4$ (camptothecin) from Sigma–Aldrich (Milan, Italy); $SnCl_2$, $Na_2WO_4 \cdot 2H_2O$ were from BDH (Milan, Italy); $CH_3AsO(OH)_2$or MMA (monomethylarsonate), $(CH_3)_3AsCH_2COO$ or DMA (dimethylarsonate) were from Tri Chemical Laboratory (Yamanashi, Japan). Metal compounds were freshly dissolved in water at 30 °C for 1 h at concentrations of 10^{-2} or 10^{-3} M. Aliquots of mother solutions were added to the culture media to reach a final concentration of 100 μM (50 μM in the case of $(NH_4)_2PdCl_6$). Methyl mercury chloride was diluted in culture media at concentrations ranging from 0.01 to 5 μM. Camptothecin was dissolved in dimethyl sulfoxide (DMSO) at concentrations ranging from 0.1 to 150 μM. The final concentrations of DMSO in the medium were 0.01% to prevent cytotoxic effects.

2.2. Cell culture materials and reagents

Cell culture flasks were from Corning (Milan, Italy); RPMI (Roswell Park Memorial Institute Medium), PBS, penicillin G sodium, and streptomycin sulphate were from Gibco, (Milan, Italy); horse serum was from Biochrom (Berlin, Germany); Vitrogen 100 was from Collagen (Ismaning, Germany); fetal calf serum and hygromicin B were from Genzyme (Cinisello Balsamo, Milan, Italy). Restriction