Ph.D. thesis

The role of PARP-1 induced AKT activation in cytostatic resistance

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Abbreviations

PARP................................poly(ADP-ribose) polymerase

PAR ....poly(ADP-ribose)

PARP-DBD ....N-terminal DNA binding domain of PARP

siRNA...............................small interfering RNA

FCS..................................fetal calf serum

BRCA1/2..........................breast cancer associated gene-1 and -2

FKHR ....forkhead homolog rhabdomyosarcoma transcription factors

JNK..................................c-Jun N-terminal kinase

GFP..................................green fluorescent protein

Akt/PKB ....protein kinase B

GSK ....glycogen synthase kinase

MTT ....3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

ECL ....enhanced chemiluminescence

PAR ....poly(ADP-ribose)

PI3-kinase.........................phosphatidylinositol 3-kinase

MPT.................................Mitochondrial Permeability Transition

Introduction

The nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) is activated in response to DNA damage (1). Single- and/or double-strand DNA breaks induce the production of branched chain ADP-ribose polymers that are covalently attached to numerous nuclear proteins like histones or the PARP itself and this process represents an early event in DNA repair. Although it is well-documented that inhibition of PARP-1 has cytoprotective effects against oxidative stress (2), there is growing evidence suggesting that inhibition of PARP-1 sensitizes cells to DNA-damaging agents (3). This later effect of PARP-1 inhibition is attributed to the DNA-damage sensing function of PARP-1, namely that it responds to single- and/or double-strand DNA breaks, and facilitates DNA repair and cell survival. Furthermore, it was shown that cells deficient in breast cancer associated gene-1 and -2 (BRCA1/2) are extremely sensitive to PARP inhibition because of defective double-strand DNA break repair (4). Based on these data, PARP inhibition is considered a useful therapeutic strategy not only for the treatment of BRCA mutation-associated tumors, but also for the treatment of a wider range of tumors bearing a variety of deficiencies in the homologous recombination DNA repair pathway (5). However, it has also been shown that inhibition of PARP leads to phosphorylation, and thus activation, of Akt in various tissues (6,7,8). It raises the possibility that application of PARP inhibitors in tumor therapy may activate the phosphatidylinositol-3 kinase (PI-3K)-Akt pathway, which initiates processes like the inactivation of glycogen synthase kinase-3, caspase-9, Bad or forkhead homolog rhabdomyosarcoma (FKHR) transcription factors (9) leading to cytostatic resistance.

Paclitaxel (taxol) interferes with the mitotic spindle during mitosis of cells, stabilizing the microtubule by inhibiting tubulin dimerisation and so inhibiting the separation of the sister chromatids (10,11,12). Paclitaxel can affect kinases (13) that play important roles in cell death

processes, and regulate the expression of tumour suppressor genes and cytokines (14). In addition, paclitaxel can induce cytosolic calcium oscillations (15) and mitochondrial permeability transition, as well as elevated generation of reactive oxygen species predominantly at cytochrome oxidase in tumor cells (16). In the paclitaxel-induced cell death process, activation of c-Jun N-terminal kinase (JNK) plays a critical role by suppressing Akt activation and promoting the nuclear accumulation of forkhead-related transcription factor-3a (Foxo3a; 17). Nuclear translocation of Foxo3a can facilitate apoptosis by inducing the expression of Bim, a BH3-only proapoptotic bcl-2 homolog protein (18). It has also been demonstrated that Akt overexpression prevented paclitaxel-induced cell death (19), probably by a mechanism involving Akt dependent phosphorylation of FOXOs that stabilizes their binding to cytosolic 14-3-3 protein and so prevents their translocation to the nucleus, resulting in inhibition of transcription of FOXO dependent genes such as Bim (20).

In the present paper, we provide evidence that inhibition of PARP-1 activity can indeed cause resistance to paclitaxel induced death in tumor cells, and activation of the PI-3K-Akt pathway is significantly involved in this effect. We specially examined the T24 human urine bladder transitional cancer line. Taxane-based chemotherapy is currently the most used remedy for salvage chemotherapy in transitional cell carcinoma of the urothelium. (21). We provide evidence that Akt dependent Bad phosphorilation and presentation of the integrity of mitochondrial membrane systems is a mechanist significantly involved in paclitacel resistance of T24 human urine bladder transitional cancer line.

Objectives

1. We wanted to examine the direct effect of Taxol on mitochondria. We studied the relationship between mitochondrial permeability transition, cytochrome-c release, caspase-3 activation, PARP activation and paclitaxel treatment.
2. We wanted to induce paxlitaxel therapy-resistance in different cell lines via direct attenuation of PARP-1 activation.
3. We wanted to examine the possible mechanism of the citoprotecive effects of PAPR inhibition. We studied specially the NAD^+ and ATP depletion, and the signal transduction pathways.
4. We investigated how important role does the PI3K/Akt signaltrasduction pathway play in paclitaxel resistance in T24 human urine bladder transitional cell line. We looked at the effect of PI3K/Akt pathway activation with reduced PARP level on paclitaxel induced cell death and the effect of PI3K/Akt pathway inhibition with LY294002 on paclitaxel induced cell death.
5. We examined the relationship between the PI3K/Akt signaltrasduction pathway and the mitochondrial apoptotic pathways. We determined the BAD phosphorylation, cytochrome-c release, caspase activation in paclitaxel treated T24 cells.

Materials and Methods

Materials. Phosphatidylinositol-3 kinase (PI-3K) inhibitor LY-294002, poly(ADP-ribose) polymerase (PARP-1) inhibitor PJ-34, protease inhibitor cocktail, and all chemicals for cell culture were purchased from Sigma-Aldrich Kft (Budapest, Hungary). The following antibodies were used: anti-Akt, anti-phospho-Akt, anti-phospho-glycogen synthase kinase-3ß (GSK), anti-phospho Bad, anti-Bad (Cell Signalling Technology, Beverly, MA); anti-mouse IgG and anti-rabbit IgG (Sigma-Aldrich Kft, Budapest, Hungary)

Animals Wistar rats were purchased from Charles River Hungary Breeding Ltd. (Budapest, Hungary). The animals were kept under standardized conditions; tap water and rat chow were provided ad libitum. Animals were treated in compliance with approved institutional animal care guidelines.

Isolation of mitochondria Rats were sacrificed by decapitation and the mitochondria were isolated from the liver and the heart by differential centrifugation as described by a standard protocol (22). The only difference among the organs was in the primary homogenization protocol; the liver was squeezed through a liver press, whereas pooled heart tissue from five rats was minced with a blender. All isolated mitochondria were purified by Percoll gradient centrifuging (22), and the mitochondrial protein concentrations were determined by the biuret method with bovine serum albumin as the standard.

Mitochondrial permeability transition The mPT was monitored by following the accompanying large amplitude swelling via the decrease in absorbance at 540 nm (22) measured at room temperature by a Perkin–Elmer fluorimeter (London, UK) in reflectance mode. Briefly, mitochondria at the concentration of 1 mg protein/ml were preincubated in the

assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, 0.5 mM phosphate) containing the studied substances for 60 s. Mitochondrial permeability transition was induced by the addition of 150 μM Ca^{2+} or of paclitaxel at the indicated concentration plus 2.5 μM Ca^{2+}. Fluorescence intensity changes were detected for 3 min. The results are demonstrated by representative original registration curves from five independent experiments, each repeated three times.

Cell culture. T24 human bladder carcinoma cells and Hela human cervical cancer were from American Type Culture Collection (Wesel, Germany). The cells were maintained as monolayer adherent culture in Minimum Essential Eagle's Medium containing 1% antibiotic-antimycotic solution and 10% fetal calf serum (MEM/FCS) in humid 5% CO_2 atmosphere at 37 °C.

Cell viability assay. The cells were seeded into 96-well plates at a starting density of 10^4 cells per well and cultured overnight before paclitaxel and PJ-34 or LY-294002 were added to the medium at the concentration indicated in the figure-legends for 24 h. The medium was changed to fresh one containing 0.5% of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT^+) for an additional 3 hours, then the MTT^+ reaction was terminated by adding HCl to 10 mM final concentration. Amount of blue formasan dye formed from MTT^+ was proportional to the number of live cells, and was determined with an Anthos Labtech 2010 ELISA reader at 550nm wavelength. All experiments were run in at least 4 parallels and repeated 3 times.

Western blot analysis. Cells were seeded and treated as for the cell viability assay. After the time indicated, cells were harvested in a chilled lysis buffer containing 0.5 mM sodium-metavanadate, 1 mM EDTA and protease inhibitor cocktail in PBS. Immunoblotting was performed exactly as it was described previously (22). All experiments were repeated 3 times.

Caspase-3 activity assay. The cells were treated with paclitaxel in the presence or absence of PJ-34 or the PI3 kinase inhibitor LY294002 for the time indicated. The cells were harvested, and determination of caspase-3 activity was carried out exactly as described previously (22). All experiments were repeated 3 times.

Determination of cytochrome-c level by HPLC method. The analysis of cytochrome-c from the cytosol fraction of T24 cells treated with paclitaxel in the presence or absence of PJ-34 or the PI3 kinase inhibitor LY294002 for 16 h was perfomed exactly as it was described previously (22). Data acquisition was performed from at least three independent experiments.

Statistical Analysis. Data were presented as means ± S.E.M. For multiple comparisons of groups, ANOVA was used. Statistical difference between groups was established by paired Student's *t* test with Bonferroni's correction.

Conclusions

1. We observed that Paclitaxel induces mitochondrial permeability transition with high level of cytochrome-c release. We also detected intensive caspase-3 activation and PARP-1 activation. All these factors together can result in apoptotic cell death.
2. We provided evidence that suppression of PARP-1 activation protected cells from paclitaxel. In all of the examined concentrations of paclitaxel, the control cells were more sensitive to paclitaxel than the ones with decreased PARP-1 activation.
3. We found evidence for undermining the classical view that cytoprotection by PARP inhibitors relies exclusively on the preservation of NAD^+ and consequently the ATP stores in paclitaxel therapy. The PARP inhibition-induced Akt activation was very significantly responsible for the cytoprotective property of PARP inhibitors. We established that the benefit of PARP inhibition is mediated through two different processes: the preservation of energetic of cells and activation of PI3K/Akt as a well-known survival signaltrasduction pathway.
4. Inhibition of Akt activation by specific phosphatidyinositol-3-kinase (PI3K)-Akt inhibitors in a significant extent counteracted the cytoprotective effect of PARP inhibitor, indicating that the PARP-inhibition-induced Akt activation was very significantly responsible for the cytoprotective property of PARP inhibitors.
5. We provide evidence that Akt dependent Bad phosphorylation and preservation of the integrity of mitochondrial membrane systems is a mechanism considerably involved in paclitaxel resistance of T24 human urine bladder transitional cancer cell line.

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List of Publications

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