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para-Phenylenediamine-induced autophagy in human uroepithelial cell line mediated mutant p53 and activation of ERK signaling pathway

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ABSTRACT

para-Phenylenediamine (*p*-PD) is a major aromatic amine that is a widely used commercial oxidative-type hair dye. Some epidemiologic studies have suggested that the use of *p*-PD-based hair dyes might be related to increased risk of human malignant tumors including bladder cancer. However, the effects of *p*-PD on autophagy in human uroepithelial cells (SV-HUC-1) is still unclear. In this study, we demonstrate that *p*-PD can activate the extracellular signaling-regulated protein kinase 1/2 (ERK1/2) signaling pathway in SV-HUC-1 cells. In addition, we observed that autophagosomes increased in *p*-PD-treated SV-HUC-1 cells as shown by electron microscopy. Our results showed incremental increase of the concentrations, Beclin-1 and microtubule-associated protein light chain 3B (LC3B), which are important regulators of autophagosomes. In contrast, the MEK inhibitor (U0126) was suppressed autophagy and the effect of *p*-PD on ERK1/2, Beclin-1 and LC3B proteins expression, except for mutant p53. In this study, we demonstrated that inactivation of p53 induces a potent autophagy response. Finally, our results suggest that *p*-PD can activate the ERK1/2 signaling pathway and mutant p53, leading to the stimulation of autophagy in SV-HUC-1 cells. These results provide us with new insights for the understanding of the mechanism of *p*-PD-induced cell death in urothelial cells.

1. Introduction

para-Phenylenediamine (*p*-PD), also called 1,4-diaminobenzene or 1,4-phenylenediamine, is a colorless, slightly pink, grey or yellow crystalline substance in the form of lumps or powder (Thyssen et al., 2007). *p*-PD is a major aromatic amine that is widely used as a component of engineering polymers and commercial oxidative type hair dyes (Lawrence et al., 2001). It has been reported that *p*-PD can produce severe allergic dermatitis; a delayed cell-mediated immune response that causes skin erythema and edema (Smith Pease, 2003). Although the results of rodent carcinogenicity studies conducted by the National Toxicology Program showed no evidence that dietary administration of *p*-PD was carcinogenic in either F344 rats or B6C3F1 mice (NTP, 1979), *p*-PD was considered as a mutagenic moiety of many mutagenic azo dyes (Chung and Cerniglia, 1992). It could increase the risks of tumors in the kidneys, liver, thyroid gland and urinary bladder in rats (Sontag, 1981). Moreover, an *in vitro* study also showed *p*-PD could induce DNA damage and expression of mutant p53 and COX-2 proteins in urothelial cells (Huang et al., 2007).

Autophagy is derived from Greek roots: *auto*-"self", and *phagy*-"eating" that prolongs survival for a short time under starvation conditions. It is sometimes thought of as a synonym for self-cannibalism. Autophagy is used to degrade components of the cytoplasm and functions as a cell survival mechanism during nutrient deprivation. Cytoplasmic components are degraded within the lysosome by microautophagy, chaperone-mediated autophagy and macrophagy (Cuervo and Dice, 1998; Dunn, 1994; Kim and Klionsky, 2000). In mammalian cells, microautophagy has not been well characterized, and chaperon-mediated autophagy is a secondary response that temporally follows macroautophagy. Autophagosomes are double-membrane cytoplasmic vesicles that are designed to engulf various cellular conditions, including cytoplasmic organelles (Eskelinen et al., 2005). The protein that is essential for autophagosome formation is Beclin-1, a 60-kDa coiled-coil protein encoded by *beclin-1* gene. It binds to class III PI3K, which regulates autophagosome formation (Kihara et al., 2001). In addition,

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Beclin-1 is monoallelically deleted in human breast and ovarian cancers and is expressed at reduced levels in these tumors (Aita et al., 1999; Liang et al., 1999). Previous studies have shown that the inactivation of p53 induces a potent autophagy response (Crighton et al., 2007; Liang et al., 1999; Maiuri et al., 2009; Tasdemir et al., 2008).

The wild-type p53 was first discovered in 1979 (Chang et al., 1979; Kress et al., 1979). Normally, the wild-type p53 is maintained at very low concentrations by its relatively short half-life (about 20 min) (Levine, 1997), whereas it is prolonged to hours by cellular stress or DNA damage (Maltzman and Czyzyk, 1984). The wild-type p53 protein plays a pivotal role in maintaining genome integrity by inducing growth arrest for the purposes of repairing DNA damage (Schwartz and Rotter, 1998; Sionov and Haupt, 1999). Most of the mutations within the *TP53* gene are missense mutations, resulting in the expression of full-length mutant p53 protein (Hussain and Harris, 1998). About 50–55% human tumors have a mutation or loss in the p53 gene (Hollstein et al., 1994). Overexpression of mutant p53 protein and the mutation of p53 have also been shown in a high percentage of urinary bladder carcinomas (Bush et al., 1991; Nakopoulou et al., 1998). However, the mitogen-activated protein kinases (MAPK) signal pathway consists of three main membranes: the extracellular signaling-regulated protein kinases (ERKs), the c-Jun N-terminal kinases or stress-activated protein kinase (JNK/SPAK), and the p38. The MAPK family are involved in multiple cellular pathways and functions in response to a variety of ligands and cell stimuli, including growth factors, cytokines and carcinogens. ERK1/2 are activated by MAPK/ERK kinase (MEK). In many cell types, the ERK1/2 are key kinases in the intracellular signal transduction pathways from membrane receptors into the nucleus to stimulate cell proliferation. Aberrant or inappropriate functions of the MAPK family have now been identified in diseases, inflammatory conditions and cancer.

Although it has been demonstrated that *p*-PD could induce apoptosis in Mardin-Darby canine kidney cells (MDCK) via p53 (Chen et al., 2006), the induction of autophagy in *p*-PD-treated human cells has not yet been reported. Therefore, we intended to investigate the mechanism of *p*-PD induced autophagy in SV-40-immortalized human uroepithelial cells (SV-HUC-1).

2. Materials and methods

2.1. Cell culture and treatment

SV-HUC-1 cells, a SV-40-immortalized human uroepithelial cell line obtained from the American Type Culture Collection (ATCC, USA) were grown in 25-cm^2 flasks (initial density 1×10^5 cells/ml). Cells were cultured in Ham's F-12 medium (HyClone, USA) supplemented with 10% FBS (HyClone, USA), 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C and 5% CO_2 incubator.

Subcultures for experiments were set up the day before treatment. Approximately, 2×10^5 cells at logarithmic growth phase were treated with *p*-PD (Sigma, St. Louis, MO, USA). The cells were pretreated with 5 μM U0126 for 24 h and stimulated with *p*-PD for 24 h. The final concentrations for *p*-PD were 0.018, 0.046 and 0.37 mM (Huang et al., 2007). Cells were incubated at 37 °C in 5% CO_2 incubator for 24 h.

2.2. Immunocytochemistry (ICC)

Cells grown in flasks (Nunc) were pelleted by cytospins, after fixation with ethanol for 30 min. Antigen retrieval was accomplished by immersing the slides in 0.1 M citrate buffer (pH 6.0), followed by heating in autoclave. The slides were then allowed to cool at room temperature. After washing with Tris buffer solution, blocking followed for 20 min in 5% BSA. The mouse monoclonal primary antibody used was p53 PAb240 from Novocastra (Newcastle, UK) and the rabbit polyclonal antibody against total ERK1/2, p-ERK1/2, Beclin-1 and LC3B were from Cell Signaling Technology Inc. (Beverly, MA, USA) (Table 1). Primary antibody was incubated at room temperature for 1 h and biotinylated second antibody and peroxidase-conjugated streptavidin from the DAKO REAL EnVision Detectin System (DAKO, Denmark) were applied for 30 min each. Finally, sections were incubated in DAB (DAKO, Denmark) for 5 min and hematoxylin as a nuclear counterstain. The labeling index (LI), percentage of immunostained cells, was determined by counting 1000 cells in 4–6 high-power fields of densest immunostaining areas using a light microscope at 400× magnification. Negative controls were obtained by replacing the primary non-immune serum in ICC and Western blotting.

Table 1
Antibodies, incubation conditions and positive control for immunohistochemistry and Western blotting.

	Immunocytochemistry (antibody dilutions/incubation time/temperature)	Western blotting (antibody dilutions/incubation time/temperature)	Positive control
Mutant p53	50×/1 h/RT	400×/overnight/4 °C	A341 cells
Beclin-1	100×/1 h/RT	1000×/overnight/4 °C	Rat brain tissue
p-ERK1/2	550×/1 h/RT	500×/overnight/4 °C	Human colon carcinoma
T-ERK1/2	–	500×/overnight/4 °C	Rat brain extract
LC3B	300×/1 h/RT	1000×/overnight/4 °C	Human brain tissue

RT, room temperature.
T-ERK1/2, total ERK1/2.

2.3. Western blotting

Cells were harvested with lysis buffer (500 mM NaCl, 20% Glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM HEPES-Na) and protease inhibitors (Sigma, St. Louis, MO, USA) for 1 h. Protein concentrations were determined by a Bradford assay (Bio-Rad, Hercules, CA), and standard curves constructed with a series of protein standards using bovine serum albumin (BSA). The samples were loaded onto a 10% SDS gel. After protein separation, the samples were transferred to a polyvinylidene-difluoride (PVDF) membrane and the membrane was incubated for 2 h in Tris-Tween 20 containing 5% nonfat milk. The primary antibodies against p53, total ERK1/2, p-ERK1/2, Beclin-1 and LC3B were added and detected with secondary antibody conjugated with horseradish peroxidase. The p53, total ERK1/2, p-ERK1/2, Beclin-1 and LC3B was visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, England). In separate experiments, SV-HUC-1 cells were exposed to various concentrations of *p*-PD for 24 h to determine the effect of *p*-PD on these protein expressions. As measured by densimeter, the densitometry data are "fold change" as compared with controls.

2.4. Transmission electron microscopy (TEM)

After incubation with *p*-PD or pretreatment with 5 μM U0126 for 24 h, the cells were harvested by trypsinization, washed twice with PBS, and fixed with ice-cold 2% paraformaldehyde + 2.5% glutaraldehyde with 0.1 M phosphate-buffered saline and stored at 4 °C until embedding. The cells were postfixed with 1% phosphate-buffered osmium tetroxide, and embedded in Spurr resin. After polymerization, thin sections (0.12 μm) were cut with a