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Resveratrol mediated cell death in cigarette smoke transformed breast epithelial cells is through induction of p21Waf1/Cip1 and inhibition of long patch base excision repair pathway

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ABSTRACT

Cigarette smoking is a key factor for the development and progression of different cancers including mammary tumor in women. Resveratrol (Res) is a promising natural chemotherapeutic agent that regulates many cellular targets including p21, a cip/kip family of cyclin kinase inhibitors involved in DNA damage-induced cell cycle arrest and blocking of DNA replication and repair. We have recently shown that cigarette smoke condensate (CSC) prepared from commercially available Indian cigarette can cause neoplastic transformation of normal breast epithelial MCF-10A cell. Here we studied the mechanism of Res mediated apoptosis in CSC transformed (MCF-10A-Tr) cells in vitro and in vivo. Res mediated apoptosis in MCF-10A-Tr cells was a p21 dependent event. It increased the p21 protein expression in MCF-10A-Tr cells and MCF-10A-Tr cells-mediated tumors in xenograft mice. Res treatment reduced the tumor size(s) and expression of anti-apoptotic proteins (e.g. PI3K, AKT, NFκB) in solid tumor. The expressions of cell cycle regulatory (Cyclins, CDC-2, CDC-6, etc.), BER associated (Pol-β, Pol-δ, Pol-ε, Pol-η, RPA, Fen-1, DNA-Ligase-I, etc.) proteins and LP-BER activity decreased in MCF-10A-Tr cells but remain significantly unaltered in isogenic p21 null MCF-10A-Tr cells after Res treatment. Interestingly, no significant changes were noted in SP-BER activity in both the cell lines after Res exposure. Finally, it was observed that increased p21 blocks the LP-BER in MCF-10A-Tr cells by increasing its interaction with PCNA via competing with Fen-1 after Res treatment. Thus, Res caused apoptosis in CSC-induced cancer cells by reduction of LP-BER activity and this phenomenon largely depends on p21.

Introduction

Breast cancer is the most common cause of cancer-related death in women (Russo et al., 2001) and cigarette smoking among women is one of the leading causes for breast cancer development. It is well studied that cigarette smoke contains carcinogens that can cause or influence mammary tumor growth in animal models (Hecht, 2002; Phillips et al., 2001). Cigarette smoke extract (CSE) is a complex mixture of highly genotoxic substance capable of transforming normal breast epithelial cells to a cancerous state (Narayan et al., 2004). Because of the unavailability of proper in vitro model system it was difficult to study the cellular process of breast epithelial cell transformation by cigarette smoke carcinogen. Recently, we have established a novel cigarette smoke condensate (CSC) induced-transformed cell line model that could offer a suitable system to study the mechanism of cellular transformation caused by chemical carcinogens and also could help to investigate the mechanism of potential anticancer molecules against aggressive and transformed breast cancer cell types (Mohapatra et al., 2014). The transformed cell line was developed by repeated and continuous exposure to a single dose of cigarette smoke condensate prepared from commercially available Indian cigarette to normal breast epithelial cells, MCF-10A. CSC transformed cells (MCF-10A-Tr) were capable of anchorage-independent growth, and their anchorage dependent growth and colony forming ability were higher compared to the non-transformed MCF-10A cells. The study explained a higher anchorage dependent growth and colony forming ability in transformed MCF-10A cells (MCF-10A-Tr) compared to their parental lines. We noticed an increased expression of representative biomarkers of oncogenic transformation (NRP-1, Nectin-4), and anti-apoptotic markers (PI3K, AKT, NFκB) in the MCF-10A-Tr cells. Short tandem repeat (STR) profiling of MCF-10A and MCF-10A-Tr cells revealed that transformed cells

Abbreviations: CSC, cigarette smoke condensate; MCF-10A-Tr, MCF-10A transformed cells; Res, resveratrol; MTT, [3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide]; DMSO, dimethyl sulfoxide; STR, short tandem repeat; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; SP-BER, short patch base excision repair; LP-BER, long patch base excision repair; NHEJ, non-homologous end joining; PCNA, proliferating cell nuclear antigen; Fen-1, Flap endonuclease; Pol, DNA polymerase.

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acquired allelic variation during transformation, and had become genetically distinct. MCF-10A-Tr cells formed solid tumors when implanted into the mammary fat pads of BALB/C mice (Mohapatra et al., 2014).

p21(waf1/Cip1) is a well established regulator of cell cycle progression that belongs to the cip/kip family of cyclin kinase inhibitors. This protein has a wide variety of properties relating to apoptosis (Fan et al., 2003; Matsushita et al., 1998; Tian et al., 2000), cell proliferation (Weiss et al., 2000; Kavurma and Khachigian, 2003), and DNA damage repair (Chen et al., 1996; Mauro et al., 2012; Oku et al., 1998; Podust et al., 1994; Waga et al., 1994). For a long time p21 was only considered as a downstream molecule of p53 which functions as a G1 cyclin kinase inhibitor (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993), but recently p21 has been described as having function as both a cyclin/cdk assembly factor that is required for G1–S transition and as a survival protein downstream of PI3K (Yu et al., 1998). Many studies also suggest that nuclear accumulation of p21 promotes cell cycle arrest; whereas cytoplasmic p21 inhibits apoptosis (Asada et al., 1999; Zhou et al., 2001). However, the role of p21 in DNA repair remains poorly understood. Recently, Mauro and his group had shown that p21 plays an important role in the promotion of error-free replication during the double strand break (DSB) repair (Mauro et al., 2012). In vitro p21 interferes with the interaction with replication factor C (RFC) (Oku et al., 1998), DNA polymerase δ (Podust et al., 1994; Waga et al., 1994) and Fen-1 (Chen et al., 1996). p21 inhibits DNA mismatch repair (MMR) by displacing MMR proteins from replicating DNA (Guo et al., 2004; Kleczkowska et al., 2001; Masih et al., 2008; Umar et al., 1996). p21 is considered as a regulatory protein which is involved in DNA replication and DNA repair (Waga and Stillman, 1998) which plays a regulatory role in base excision repair (BER) along with APE-1 endonuclease and PCNA (Jaiswal et al., 2002; Tom et al., 2001). Several studies revealed that p21 inhibits PCNA dependent DNA replication by blocking the association of PCNA with DNA polymerases (Podust et al., 1995), Fen-1 (Chen et al., 1996; Warbrick et al., 1997) and DNA ligase-I (Levin et al., 1997). As PCNA interacts with various proteins engaged in long patch BER (Gary et al., 1999) thereby p21 can adversely affect the long patch BER by disrupting repair complexes.

Resveratrol (Res), a natural poly phenolic drug has been attracted the attention of many researchers for its antioxidant, anti-inflammatory, anti-aging and anti-tumor property (Mohapatra et al., 2011). Because of its natural origin Res is considered to be a safe and non-toxic chemopreventive agent (Dorai and Aggarwal, 2004; Le Corre et al., 2005). It is studied that Res reduces Benzo[a]Pyrene induced carcinogenesis by CYP1A1 inhibition (Signorelli and Ghidoni, 2005). Different in vitro analyses revealed that Res directly binds to DNA and RNA (Fukuhara and Miyata, 1998; Usha et al., 2005; Usha et al., 2006). Res also can directly influence the double strand break repair (DSB) in lymphoblastoid cell lines (Gatz and Wiesmüller, 2008) and it was characterized as a class II (catalytic) inhibitor of Topoisomerase II enzyme (Cho et al., 2000; Jo et al., 2005). It is a well established fact that Res can affect all the aspects of DNA metabolism (e.g. DNA replication, recombination, repair, relaxation and telomerase maintenance) (Gatz and Wiesmüller, 2008). Yang et al. showed that Res inhibited, in a dose-dependent manner, APE1/REF1, an essential base excision repair enzyme and rendered melanoma cells more sensitive to dacarbazine treatment (Yang et al., 2005). Several lines of evidences supported that DNA damaging agents increased the p21 expression and increased p21 reduced the cell proliferation in different cancer cell types (Jaiswal et al., 2002). But scarce report is available where Res can cause the apoptosis in cancer cells by induction of p21through DNA damage and repair.

Here, we have systematically studied the anti-cancer mechanism of bioactive compound Res against CSC induced transformed cells (MCF-10A-Tr) in vitro and in vivo. Res caused apoptosis in MCF-10A-Tr cells by induction of p21 and disruption of LP-BER stimulation. Res-induced p21 inhibits PCNA dependent DNA repair by blocking the association of PCNA with Fen-1.

Materials and methods

Cell culture and reagents. The MCF-10A cells were grown in DMEM/F-12 (50:50, v/v) medium supplemented with 10% FBS, 1% antibiotic (100 U/mL of penicillin, 10 mg/mL of streptomycin in 0.9% normal saline), 0.5 μg/mL of hydrocortisone, 100 ng/mL of cholera toxin, 10 μg/mL of insulin, 10 ng/mL of epidermal growth factor and 1% (w/v) of L-glutamine at 37 °C under a humidified atmosphere and 5% CO_2. The cigarette smoke transformed cells i.e. MCF-10A-Tr cells were grown according to the protocol described earlier (Mohapatra et al., 2014). In brief, cells were grown in DMEM medium supplemented with 10% fetal bovine serum in the presence of 1% antibiotic and 1% (w/v) L-glutamine. Resveratrol (Res) and other chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA). Anti-APE/ref-1, anti-DNA Ligase-I and anti-DNA Pol-β antibodies were purchased from Novus Biologicals, CO, USA. Anti-pol-δ and anti-pol-ε antibodies were obtained from Abcam, MA, USA. All the other antibodies including the mouse IgG used in the study were procured from Cell Signaling Technologies Inc., MA, USA. The p21siRNA (h) (Cat# sc-29427) and control siRNA-A (Cat# sc-37007) were obtained from Santa Cruz Biotechnology Inc. USA. Nuclear and cytoplasmic extraction reagent (Prod# 78833B) was procured from Pierce protein biology products, Thermo Scientific, USA.

Silencing of p21 in MCF-10A-Tr cells. Small interfering RNA (siRNA) transfection against p21 was performed using p21siRNA (h) constructs according to the protocol described earlier (Mohapatra et al., 2012). Briefly, two separate sets of MCF-10A-Tr (2×10^5 cells per well) cells were plated in a six-well plate, in 2 mL normal growth medium supplemented with FBS. Cells were cultured at 37 °C until 60–80% confluency. One set of cells was transiently transfected with 0.25 μg of p21siRNA and equal amount of siRNA-A (scrambled siRNA as transfection control) was transfected in other set according to the manufacturer's protocol. After 8 h post-transfection the media were replaced with normal serum containing media and further incubated for different time periods. After the end of each time period cells were harvested and processed for western blot experiment to check the status of p21 protein expression.

MTT assay. Approximately, $8–10 \times 10^3$ cells/well of MCF-10A cells were plated in 96-well plates and exposed to increasing concentrations of Res for 48 h. In other sets $8–10 \times 10^3$ cells/well of scrambled siRNA-A or p21siRNA pre-transfected MCF-10A-Tr cells were plated in triplicate in 96-well flat-bottom tissue culture plates and then treated with different concentrations of Res for 48 h. After treatment, the media were removed and 100 μL of 0.05% MTT reagent was added to each well and incubated at 37 °C overnight to allow the formation of purple formazan crystals. NP-40 detergent solution (100 μL) was added to each well, and the reaction mixture was incubated in the dark for 1 h at room temperature to dissolve the formazan crystals. The visible absorbance of each well was quantified using a microplate reader at 570 nm (Berthold, Germany). The data obtained was calculated and represented as percentage survival compared to control to calculate LC_{50} (concentration causing 50% growth reduction compared to control) value. Each data point was performed in triplicate, and all assays were performed at least three times.

DAPI nuclear staining. DAPI staining was performed to quantify apoptotic cells with condensed or fragmented nucleus according to the protocol described earlier (Mohapatra et al., 2011, 2012; Preet et al., 2012). The scrambled siRNA-A or p21siRNA transfected MCF-10A-Tr cells were treated with Res with 48 h and then were fixed in methanol: acetone (1:1) and kept at −20 °C for 15 min. After fixation, cells were