Epigenetic Modifiers Exacerbate Oxidative Stress in Renal Proximal Tubule Cells

EDIT NADAS1, JEB S. CLARK2, ISTVAN SZANYI3, TIMEA VARIAS4, ISTVAN EMBER4, RADHAKRISHNA BALIGA2 and ISTVAN ARANY2

1Quintiles Hungary Ltd, Budapest, Hungary; 2Department of Pediatrics, Division of Pediatric Nephrology, University of Mississippi Medical Center, Jackson, MS, U.S.A.; Departments of 3Ear, Nose and Throat, and 4Public Health and Preventive Medicine, University of Pecs, Hungary

Abstract. Background: Increased production of reactive oxygen species (ROS) by anticancer drugs has been described in patients with various malignancies, which might attribute to their nephrotoxicity. Materials and Methods: The effects of two epigenetic modifiers – trichostatin A (TSA) and 5-aza-deoxycytidine (5AZA) – on ROS production and cell injury alone or in combination with mild oxidative stress were studied in mouse renal proximal tubule cells. Results: Both agents increased mitochondrial ROS production and consequent lactate dehydrogenase (LDH) release either alone or in combination with a low dose of H2O2. The antioxidant N-acetyl-cysteine (NAC) abolished LDH release. It was also found that CREB-mediated transcription, vital for survival of proximal tubule cells, is attenuated by these anticancer agents. Conclusion: The ROS-inducing activity of TSAI and 5AZA might explain the in vivo nephrotoxicity of epigenetic modifiers. The mechanisms that are responsible for this injury could involve attenuation of pro-survival signaling and/or activation of death signaling pathway(s) associated with mitochondrial ROS release.

Epigenetic modifications such as DNA methylation and histone acetylation have a crucial role in cancer diagnosis, prognosis and therapy (1). Promoter methylation of genes, especially tumor suppressors, diminish their activity and confer growth advantage to transformed cells (2). Histone (de)acetylation plays an important role in chromatin remodeling which affects transcriptional activity of key genes in growth control (3). Thus, reversal of these tumor-associated epigenetic changes could restore tumor suppression (4). The histone deacetylase (HDAC) inhibitor trichostatin A (TSA) and the DNA methyltransferase inhibitor 5-aza-deoxy-cytidine (5AZA) alone (5, 6) or in combination (7) are promising agents in human cancer therapy. However, nephrotoxicity is a known side-effect of various anticancer drugs (8, 9). Renal tubular dysfunction has been reported in patients that underwent 5AZA therapy (8, 10, 11). Nephrotoxicity of TSA in vivo is less known; however, a pro-apoptotic role in renal proximal tubules in vitro has been suggested (12).

Certain anticancer drugs can generate reactive oxygen species (ROS) that cause oxidative stress and apoptosis of cancer cells (13): treatment of patients with HDAC inhibitors (14) or 5AZA (15) increased ROS production. Increased ROS production, however, might represent an increased risk to the proximal tubules of the kidney, which are targets of oxidative injury (16). It is important to note that increased ROS production has been observed in various cancer cells (17, 18) resulting in a generalized pro-oxidative milieu in patients (19). Therefore, it is plausible that epigenetic modifiers also exacerbate existing oxidative stress in cancer patients and as such represent an increased risk for renal tubular injury.

Accordingly, the aim of the present study was to evaluate the effects of two epigenetic modifiers (TSA and 5AZA) on ROS production, cell injury and survival signaling alone or under mild oxidative stress in renal tubular cells in vitro.

Materials and Methods

Cell culture and treatment. The TKPTS cell line was established from proximal tubule explants dissected from the 8Tg(SV40E)Br7 mouse kidney by Ernest and Bello-Reuss (20). Cells were grown in 5% CO2 atmosphere at 37°C as described earlier (21). Oxidative stress was induced by treatment of semi-confluent cells with 200 μM
H$_2$O$_2$ for 24 hours similar to the model described earlier (22). In certain experiments cultures were pretreated with TSA (50 nM, Sigma–Aldrich, St. Louis, MO, USA) or 5AZA (100 nM, Sigma–Aldrich) 24 hours prior to treatment with H$_2$O$_2$.

**Assessment of cell injury.** Cell morphology was determined by a phase-contrast inverted microscope (Nikon TS-100F; magnification: ×100). Cell injury was assessed by the CytoTox-One Homogenous Membrane Integrity assay kit (Promega, Madison, WI, USA). Accordingly, 24 hours after treatment, an aliquot of the growth medium was removed and saved. The monolayer was lysed according to the manufacturer’s recommendation and lactate dehydrogenase (LDH) content was determined by a fluorescent substrate both in the medium and cell lysate. LDH release was calculated as percentage of LDH content in the medium compared to the total LDH content (medium+lysat).

**Evaluation of ROS production.** The intracellular generation of ROS was determined following a microplate assay procedure described by Wang and Joseph (23) using the oxidant-sensitive 2',7'-dichlorofluorescein-diacetate (DCFDA-Invitrogen, Grand Island, NY, USA). The NAPDH oxidase inhibitor diphenyleneiodonium chloride (DPI), the xanthine oxidase inhibitor allopurinol (Allo) and the mitochondrial inhibitor roteone (Rot) were purchased from Sigma-Aldrich and used to assess intracellular source of ROS.

**Assessment of survival signaling: Plasmid transfection and luciferase assay.** The pCRELuc plasmid that contains four direct repeats of CRE binding sites (Stratagene, La Jolla, CA, USA) together with a Renilla-luciferase plasmid (Promega) were transiently transfected into TKPTS cells by the Lipofectamine LTX reagent (Invitrogen) as described earlier (24). Briefly, 1 μg of pCRE-Luc and 50 ng of Renilla luciferase (pRL-TK) plasmids were mixed in OPTIMEM medium (Invitrogen) that contained the Lipofectamine LTX as recommended. After 30 minutes’ incubation at room temperature, the mixture was added to semiconfluent cells grown in 6-well-plates and further incubated for 24 hours in CO$_2$ atmosphere at 37°C. Cells were treated with 200 mM H$_2$O$_2$ for 5 hours and luciferase activities were determined by using the Dual-Luciferase Reporter Assay kit as suggested by the manufacturer (Promega). Renilla-luciferase served as internal control to determine the efficiency of transfection. The reporter luciferase (pCRE) activity was measured and was normalized to the activity detected for the co-transfected Renilla luciferase.

**Statistical evaluation.** Statistical differences between the treated and control groups were determined by Student’s t-test. Differences between means were considered significant if p<0.05. All analyses were performed using a SigmaStat 3.5 software package.

**Results**

**TSA and 5AZA treatment induces cell injury alone and exacerbates oxidative stress-mediated injury in renal proximal tubule cells.** Treatment with 200 μM H$_2$O$_2$ resulted in moderate injury as shown by the cell morphology and the LDH release data (Figures 1A-B and 2). The observed loss of cell number is consistent with a growth arrest combined with mild injury at low concentrations of H$_2$O$_2$ (22). Similarly, changes in cell morphology and LDH release were modest in cells treated with TSA (Figures 1C and 2) or 5AZA (Figures 1E and 2) for 24 hours. In contrast, 200 μM H$_2$O$_2$ induced significant changes both in morphology and LDH release in those cells that were pretreated with TSA (Figures 1D and 2) or 5AZA (Figures 1F and 2), respectively. This type of change in cell morphology is consistent with necrotic (oncotic) cell death (22).

**TSA and 5AZA treatment increases oxidative stress in renal proximal tubule cells.** TKPTS cells were treated with either TSA or 5AZA for 24 hours. ROS production was determined using the oxidant-sensitive fluorescent dye DCFDA. As shown in Figure 3, ROS production was significantly higher in TSA or 5AZA-treated cells compared to their untreated counterparts. Importantly, TSA or 5AZA pretreatment exacerbated H$_2$O$_2$-induced ROS production.

**Increased ROS production is responsible for cell injury.** To determine if ROS mediates cell injury, TSA or 5AZA-treated cells were pre-incubated with the antioxidant N-acetylcysteine (NAC) 1 hour prior to adding 200 μM H$_2$O$_2$. LDH release was determined 24 hours later. As shown in Figure 4, NAC significantly attenuated LDH release under the described experimental conditions.
The source of ROS is the mitochondrion. Possible sources of intracellular ROS production are the mitochondria, NADPH oxidase or xanthine oxidase (19). To distinguish between these sources, ROS production was determined in the presence of the xanthine oxidase inhibitor allopurinol (Allo), the NADPH oxidase inhibitor DPI or the mitochondrial electron transport chain inhibitor Rot. Significant attenuation of ROS production was only seen when the mitochondrial inhibitor was applied (Figure 5), suggesting that the primary source of ROS is the mitochondrion.

Increased oxidative stress inhibits pro-survival signaling. One important consequence of increased oxidative stress is the inhibition of pro-survival signaling pathways (25). In renal proximal tubule cells, the CREB-mediated transcription is essential for survival during oxidative stress (26). To determine if the TSA/5AZA-mediated oxidative stress inhibits CREB mediated transcription, TKPTS cells were transiently transfected with the reporter pCRE-Luc plasmid and treated with TSA/5AZA alone or in combination with 200 μM H2O2. As Figure 6 shows, TSA and 5AZA treatment attenuated both endogenous and 200 μM H2O2-associated CRE-Luc activities.

Discussion

Acute kidney injury frequently occurs in cancer patients owing to nephrotoxic drugs (27). Some anticancer agents such as HDAC inhibitors (14) or 5AZA (15) are known to increase ROS production in patients with various malignancies. It is also plausible that accumulation of ROS by cancer cells (17, 18) and the consequent systemic oxidative milieu (19) could result in an increased oxidative stress in the kidney. These events together might enhance oxidative stress in the kidney of cancer patients that have undergone epigenetic modifier therapies resulting in nephrotoxicity.

ROS primarily injure the proximal tubules in the kidney (28); thus, TSA- and 5AZA-mediated nephrotoxicity might be due to their ability to increase ROS production and...
exacerbate pre-existing oxidative stress. Indeed, both TSA and 5AZA increased ROS production in cultured renal proximal tubule cells alone and also exacerbated H2O2-mediated oxidative stress (Figure 3). The increased ROS are, indeed, responsible for cell injury (Figures 1-2) as the antioxidant NAC significantly attenuated LDH release (Figure 4). These studies also demonstrated that the source of the increased ROS production is the mitochondria (Figure 5). This process could therefore lead to mitochondrial depolarization, consequent opening of the mitochondrial permeability transition pore and ultimately cell injury (29). Further studies are needed to explore this angle of nephrotoxicity of epigenetic modifiers.

ROS such as H2O2 could mediate cell injury by either inhibiting pro-survival pathways (21) or up-regulating death-signaling cascades (30). CREB-mediated transcriptional activity is essential for survival of renal proximal tubule cells during oxidative stress (26). Here, it is demonstrated that TSA or 5AZA inhibits pro-survival CREB-mediated transcription (Figure 6). Manipulating the CREB pathway might offer therapeutical means to ameliorate nephrotoxicity of epigenetic modifiers.

TSA and 5AZA have been shown to increase expression of the p66shc adaptor protein (31). The p66shc protein is involved in responses to oxidative stress (32); it inhibits pro-survival signaling during oxidative stress in renal proximal tubule cells (21), or increases cell death via increased ROS production through a mitochondrial pathway (33). Thus, p66shc might be a potential mediator of TSA- and 5AZA-induced tubular toxicity. Further studies are in progress to evaluate the role of p66shc in the observed nephrotoxic events.

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