Caffeine Suppresses Apoptosis of Bladder Cancer RT4 Cells in Response to Ionizing Radiation by Inhibiting Ataxia Telangiectasia Mutated-Chk2-p53 Axis

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Abstract

Background: Caffeine suppresses ataxia telangiectasia and Rad3 related and ataxia telangiectasia mutated (ATM) activities; ATM is the major kinase for DNA damage detection. This study aimed to investigate the effects of caffeine on DNA damage responses in cells from the bladder cancer cell line RT4 those were exposed to ionizing radiation (IR).

Methods: Immunofluorescent staining was performed to investigate changes in the proteins involved in DNA damage responses with or without caffeine. A mouse xenograft model was used to study the effects of caffeine on the DNA damage responses. Western blotting was used to investigate the effects of caffeine pretreatment on the ATM-Chk2-p53-Puma axis, while real-time polymerase chain reaction (RT-PCR) assessed changes in messenger RNA levels of p53 and downstream targets responding to IR. Finally, terminal deoxynucleotidyl transferase-dUTP nick end labeling assay. Western blotting and colony formation assay were used to measure the effects of caffeine on radiation-related apoptosis. All of the data were analyzed with a two-tailed Student’s t-test.

Results: Immunofluorescent staining showed that caffeine pretreatment profoundly suppressed the formation of γH2AX and p53-binding protein 1 foci in RT4 cells in response to irradiation. Cellular and animal experiments suggested that this suppression was mediated by suppression of the ATM-Chk2-p53-Puma DNA damage-signaling axis. RT-PCR indicated caffeine also attenuated transactivation of p53 and p53-inducible genes. The colony formation assay revealed that caffeine displayed radioprotective effects on RT4 cells in response to low-dose radiation compared to the radiosensitization effects on T24 cells.

Conclusion: Caffeine may inhibit IR-related apoptosis of bladder cancer RT4 cells by suppressing activation of the ATM-Chk2-p53-Puma axis.

Key words: Apoptosis; Ataxia Telangiectasia Mutated; Bladder Cancer; Caffeine; p53

Introduction

Caffeine, a xanthine analogue, is a natural stimulatory compound that exists in many plants, including cocoa beans, caffeine beans, and tea.1 Although the functions of caffeine have been extensively investigated, many of its effects on apoptosis and proliferation appear to be ambiguous. Caffeine inhibits activation of ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and Rad3 related (ATR) proteins. These repair proteins initiate appropriate damage responses to double-stranded and single-stranded DNA breaks.2-4 Caffeine has a greater potency against ATM than ATR. Specifically, caffeine exhibits an IC50 of 0.2 mmol/L for ATM and 1.1 mmol/L for ATR,2-4 indicating that it may suppress DNA damage-induced apoptosis. While caffeine has been shown to potentiate p53-dependent apoptosis, clinical studies in European countries have indicated that heavy coffee consumption had a positive association with bladder cancer among nonsmokers.5,6 Similarly, coffee and caffeine were...
associated with an increased risk of bladder cancer among males who were either former smokers or had never smoked. Nevertheless, the mechanisms are largely unknown.

Bladder cancer is the eighth leading cause of cancer-related death in American males. Tobacco smoking is the most important bladder cancer risk factor. It produces DNA-damaging compounds, including polycyclic aromatic hydrocarbons, aromatic amines, and N-nitroso compounds. These genotoxic compounds can lead to several forms of DNA damage, including adducts, single-stranded breaks (SSBs), double-stranded breaks (DSBs), and modified bases in the bladder epithelium, thereby activating DNA damage responses. DNA damage responses prevent detrimental effects of DNA damage and lead to three possible outcomes: DNA repair, cell cycle regulation or apoptosis. Defects in DNA damage responses can cause cell proliferation, cell survival, increased genomic instability, and tumor progression. Not only do DNA damage responses have preventive functions against the initiation of cancer, but they are also active in adjuvant treatments for bladder cancer, such as radiotherapy and chemotherapy. Both radiotherapy and chemotherapy use DNA as a target by activating the DNA damage response pathways.

Based on the pharmacological features of caffeine, we hypothesized that caffeine might affect DNA damage responses in bladder cancer by suppressing DNA damage-signaling pathways. To initiate DNA damage, we used ionizing radiation (IR). This study investigated the effects of caffeine on the ATM-Chk2-p53 axis, the major signaling pathway of DNA damage responses, in bladder cancer RT4 cells.

**Methods**

**Cell culture and reagents**
The human well-differentiated low-grade papillary bladder cancer RT4 cell line and highly malignant bladder cancer T24 cell line were obtained from the American Type Culture Collection (Rockville, MD, USA). RT4 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen). T24 cells harbor mutant p53. Caffeine (Sigma-Aldrich, Germany) were dissolved in dimethyl sulfoxide and stored at −20°C prior to use. Mouse monoclonal anti-Ser15-phosphorylated-p53 (phospho-p53), anti-Chk2, rabbit polyclonal anti-Thr68-phosphorylated-Chk2 (phospho-Chk2), and anti-p53 up-regulated modulator of apoptosis (PUMA) antibodies were purchased from Cell Signaling Technology (Danvers, USA).

**Immunofluorescent staining**
RT4 cells were seeded on 8 mm round coverslips in DMEM medium. After treatment, cells were fixed with 3.5% paraformaldehyde for 20 min and then permeabilized with 0.5% NP-40 buffer for 5 min. Cells were washed and blocked with phosphate buffered saline (PBS) with 3% bovine serum albumin (BSA) for 30 min and then were incubated with anti-γH2AX (1:500) or anti-53BP1 antibody (1:200) for 1 h. After incubation with the primary antibodies, cells were washed and incubated with Alex-488-conjugated goat anti-rabbit IgG antibody (1:500) (Invitrogen). The nuclei were stained with Hoechst 33582 dye (Invitrogen) for 5 min. Cells were visualized with a fluorescence microscope (Olympus, Tokyo, Japan). Fluorescence intensity of the cells was quantified with cell quantification software (FRIIDA, Developed by Department of Pathology, Johns Hopkins University, MD, USA).

**Real-time quantitative polymerase chain reaction**
Total RNA was extracted using TRIzol (Invitrogen). For each sample, 2 μg of RNA was converted to complementary DNA (cDNA) using reverse transcriptase (Invitrogen) according to a standard protocol with poly-T primers. Real-time polymerase chain reaction (RT-PCR) was performed on a Stratagene MX3000P PCR machine using Stratagene SYBR® green master mix (Stratagene, La Jolla, CA, USA). The cDNA was amplified by PCR using the primer pairs as given below. Cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. For quantification, the 2^−ΔΔCT method was used to determine the relative expression levels of the target genes. The details of this method were as previously described. Briefly, the mean of the target gene messenger RNA (mRNA) expression level was calculated from three mRNA measurements. In this study, the data were presented as the relative expression levels of target genes in treated cells normalized to the internal control gene (β-actin) and also relative to the mock-treated cells. The gene-specific primers were used (Table 1).

**Terminal deoxynucleotidyl transferase-dUTP nick end labeling assay**
To detect apoptosis, we performed the terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay (Invitrogen) as described previously. Briefly, RT4 cells were seeded onto a 60-mm dish containing a coverslip. After treatment, cells were fixed with 4% paraformaldehyde in PBS followed by permeabilization with 0.25% Triton X-100. This was followed by the application of 100 μl of the terminal deoxynucleotidyl transferase reaction cocktail per coverslip at 37°C for 1 h. After being washed with PBS, coverslips were incubated with 100 μl of Click-iT reaction cocktail for 30 min. DNA was counterstained with Hoechst 33342 dye.
prior to mounting of the coverslips and TUNEL-positive cells were counted under a fluorescence microscope.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting analysis**

Cells were harvested and lysed with cell lysis buffer (1% Nonidet P-40, 10% glycerol, 135 mmol/L NaCl, 40 mmol/L Tris [pH 7.4], 1 mmol/L PMSF, 1 mmol/L dithiothreitol, and 1% protease inhibitor cocktail [Sigma]). The samples were separated on 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were incubated with antibodies, and the bands were visualized by ECL reagents (GE Healthcare Life Sciences, Buckinghamshire, UK). GAPDH was used as a loading control.

**Establishment and treatment of mouse xenografts**

All animal studies were conducted in compliance with the Guide for the Care and Use of Laboratory Animals of Zhejiang University, China. Male athymic BALB/c nude mice (Shanghai Experiment Animal Centre, China), aged 3–4 weeks and weighing 15–20 g, were used and maintained under pathogen-free conditions. RT4 cells (1 × 10^6) in 0.2-ml Matrigel were injected subcutaneously in the flanks of the mice. Tumor growth was monitored every other day, and tumor volumes (TVs) were calculated by measuring the tumor diameter with a sliding caliper and using the following formula: TV = length × (width)^2/2. Treatment was initiated when TV reached 30–75 mm^3. Twenty nude mice were divided into two groups. Ten mice were injected i.p. with caffeine (50 mg/kg) and 10 were injected with vehicle dimethylsulfoxide (DMSO) as a control. One hour after drug treatment, the two groups were subdivided into four groups: Vehicle-treated control, caffeine plus mock treatment, vehicle plus 2-Gy IR and caffeine plus 2-Gy IR. The mice were sacrificed, and the tumors were harvested at 1-h after IR. The excised specimens were fixed with 10% formalin and embedded in paraffin.

**Immunohistochemical staining and image analysis**

After tissue sections were deparaffinized and rehydrated, antigen retrieval was performed by steaming the sections in target retrieval buffer (Dako, Glostrup, Denmark) for 45 min. The tissue sections were treated with 3% hydrogen peroxide, washed with PBS, and incubated with primary antibodies for 45 min at the room temperature. Then, the tissue sections were incubated with the Power-Vision detection system (Leica Biosystems, Newcastle, UK) for 30 min and stained with diaminobenzidine (DAB) (Invitrogen). Nuclei were counterstained with hematoxylin and tissue sections were visualized with a bright-field microscope (Nikon, Tokyo, Japan). Image analysis of the immunohistochemical staining was conducted using FrIDA software. The signal intensity was defined as the sum of the DAB signal divided by the sum of the DAB area and the hematoxylin area.

**Colony formation assay**

Colony formation assay were confirmed in a large format, in which 2.0 × 10^5 cells were treated with caffeine or DMSO for 2 h prior to harvest and suspension in the medium. Then, cell dilutions were plated onto 100-mm culture dishes and irradiated immediately in a Gammacell 40 (Nordion, Ottawa, Canada) ^137^Cs radiator at approximately 0.58 Gy/min. The cells were grown for 12 days and stained with crystal violet. Colonies with >30 cells were scored, and survival fraction (SF) was calculated.

**Statistical analysis**

All of the data were analyzed with a two-tailed Student’s t-test using Excel 2007 (Microsoft, Redmond, WA, USA) and expressed as the mean ± standard deviation (SD). Results were considered statistically significant if *P* < 0.05.

**RESULTS**

Caffeine suppresses γH2AX foci of RT4 cells exposed to ionizing radiation *in vitro*

The histone variant H2AX constitutes up to 25% of the H2A complement in mammalian cells.[10] Upon IR or treatment with DSB-inducing agents such as a topoisomerase II inhibitor, H2AX molecules are rapidly phosphorylated at Ser139 (γH2AX) by ATM, ATR, and DNA-dependent protein kinase in large chromatin domains juxtaposing DSBs.[17] The γH2AX foci, the hallmark of DSBs, can be observed by immunofluorescence microscopy. γH2AX is the initial signal for the recruitment of additional DNA-damage-response proteins.[18] Immunofluorescent staining showed γH2AX foci in RT4 cells in response to IR [Figure 1a]. 2-Gy IR alone produced 18.7 ± 3.5 foci per nucleus, while IR treatment in the presence of caffeine produced 5.7 ± 1.6 foci (*P* < 0.001). 4-Gy IR alone resulted in 24.1 ± 5.0 foci per nucleus compared with 7.9 ± 2.0 foci in the IR and caffeine combinatory treatment (*P* < 0.001). 2-Gy and 4-Gy IR both produced a significantly higher intensity of γH2AX foci in IR alone treated cells compared to IR plus caffeine treated cells (*P* = 0.047 and *P* = 0.003, respectively) [Figure 1a–1c].

Caffeine inhibits γH2AX via suppression of an upstream regulator

The suppressive effects of caffeine on γH2AX were also confirmed by Western blotting. The results [Figure 2a and 2b] showed that caffeine functions in a dose- and time-dependent...
manner. Additionally, it was observed that in the presence of caffeine, ATM, and ATR were suppressed, as was phospho-ATM, the active form of ATM. ATM exists as an inactive dimer and it is transformed to an active monomer by autophosphorylation in response to DSBs. This transformation is of great importance for activating checkpoint kinase 2 (Chk2), a downstream target effector kinase. The Western blotting results also suggested an inhibitory effect of caffeine on Chk2 phosphorylation in the cells treated with IR. 53BP1 is a mediator of Chk2 and p53 activation, and its recruitment depends on phosphorylation of H2AX. The results [Figure 2c] showed that caffeine impeded the induction of 53BP1 foci formation by IR.

Caffeine represses p53 and p53 target gene expression in RT4 cells exposed to ionizing radiation

Following DNA damage, the tumor suppressor protein p53 has a crucial influence on whether cells will survive or die by either stimulating DNA repair or initiating apoptosis if the DNA damage has exceeded a certain threshold. p53 is regulated by phospho-Chk2 and phospho-ATM. RT-PCR was carried out to study whether caffeine affects the expression of p53 and p53-inducible genes, such as p21, PUMA, and Bax. The results showed a relative reduction of mRNA levels in RT4 cells with the combinatorial treatment compared with IR alone [Figure 2d]. Additionally, Western blotting was performed to detect p53 and p53 target genes. The results revealed that the expression of phospho-p53 and p53 target genes decreased in the presence of caffeine [Figure 2b]. It was also observed that protein levels of p53 and PUMA decreased in the presence of KU55933, a specific ATM inhibitor [Figure 2e]. These experiments suggested that ATM inhibition hinders p53 stabilization and p53 transactivation in RT4 cells.

Caffeine suppresses γH2AX response to ionizing radiation in vivo

To further address the suppressive effect of caffeine on the γH2AX response, we conducted a mouse experiment. The xenograft tissue displayed a striking γH2AX response following IR, while caffeine attenuated this response \( P < 0.001 \), Figure 3a. Phosphorylation of H2AX is fully dependent on ATM and the in vitro experiment clearly indicated that caffeine suppresses phosphorylation of H2AX by inhibiting the activation of ATM. The results of the animal experiment are consistent with the in vitro experiment and show that caffeine pretreatment quenches phosphorylation of ATM in response to IR \( P = 0.043 \), Figure 3b.

Figure 1: Suppressive effects of caffeine on the formation of γH2AX foci and 53BP1 foci in response to ionizing radiation. (a) RT4 cells were pretreated with vehicle or 0.2 mmol/L caffeine for 2 h followed by treatment with 2-Gy or 4-Gy ionizing radiation as indicated. After 1 h of irradiation, γH2AX immunofluorescence staining was performed. Scale bar 20 μm. (b) The average number of foci per nucleus is shown in Figure 1a, and the data are expressed as mean ± standard deviation. Columns are the mean of 100 nuclei per sample in triplicate; bars represent standard deviation \( *P < 0.01 \). (c) The average γH2AX foci intensity is shown in Figure 1a, and the data are expressed as mean ± standard deviation. Columns are the mean of 100 nuclei per sample in triplicate; bars represent standard deviation \( *P < 0.01; †P < 0.05 \).
Caffeine abrogates apoptosis of RT4 cells exposed to ionizing radiation

The TUNEL assay showed that IR produced more apoptotic cells compared to those that received the combinatory treatment of caffeine and IR. IR alone generated significantly more TUNEL-positive cells at 48 h after irradiation \( P = 0.014 \), Figure 4a and 4c. Finally, Western blotting indicated that caffeine blocked the activation of caspase-3 and PUMA at a later stage after irradiation [Figure 4b].

Caffeine has radioprotective effects on RT4 in response to low-dose ionizing radiation

The dose-response curves shown in Figure 5a indicated radioprotective effects of caffeine treatment on RT4 cells in response to IR. Exposure to 2-Gy decreased SF to 0.077 ± 0.015 (standard error of the mean), whereas exposure to 0.2 mmol/L caffeine plus 2-Gy potentiated SF to 0.117 ± 0.028 \( (P < 0.05) \). Interestingly, significant differences of SF between the control group and caffeine group in response to 6-Gy IR were observed, suggesting radiosensitization effects of caffeine on RT4 cells in response to high-dose IR. We also conducted colony formation experiments with T24 cells. As indicated in Figure 5b, caffeine functioned as a radiosensitizer for T24 cells whether in response to low-dose IR or high-dose IR. Representative images of colony formation on 2-Gy with and without caffeine treatment were included [Figure 5c].

**DISCUSSION**

DNA is the main target of genotoxic agents, including IR, ultraviolet light, and alkylating compounds. Chemotherapy usually targets DNA via cytotoxic anticancer drugs that react either directly with the DNA through metabolites or indirectly by incorporating into the DNA or blocking topoisomerases and polymerases necessary for DNA structural maintenance. Radiotherapy causes DSBs, which activate the DNA damage response.

The DNA damage-signaling pathway has traditionally been divided into two major parts: The ATM/Chk2 and...
Normally, DSBs activate the ATM/Chk2 pathway and SSBs activate the ATR/Chk1 pathway. There is also cross-talk between the ATM/Chk2 and ATR/Chk1 pathways. Cells cope with DNA damage using several strategies, including initiation of repair, tolerance of the DNA damage insult, and induction of apoptosis in the cells harboring the DNA damage. The strategies adopted depend on the proteins involved in the DNA damage response and the severity of the damage. If the damage is beyond the capacity for repair, the affected cells undergo apoptosis; however, the components involved in the damage responses and signaling pathways, ATM-Chk2-p53 axis, are even more important in determining the cell strategy used than the DNA damage response.

**Figure 3:** Inhibitory effects of caffeine on the phosphorylation of H2AX and ataxia telangiectasia mutated in vivo. Nude mice were pretreated with caffeine or vehicle for 1 h and then treated with 2-Gy ionizing radiation or mock. After 1 h of exposure, the xenograft tumor was harvested for (a) γH2AX immunohistochemical staining and (b) phospho-ataxia telangiectasia mutated staining, original magnification ×40. The graph on the right of the staining shows the signal intensity. All values represent the mean ± standard deviation of three independent counts (*P < 0.01; †P < 0.05).

**Figure 4:** Suppressive effects of caffeine on the apoptosis of RT4 cells exposed to ionizing radiation. RT4 cells were pretreated with 0.2 mmol/L caffeine for 2 h and then treated with 4-Gy ionizing radiation. After 48 h and 72 h of exposure, (a) terminal deoxynucleotidyl transferase-dUTP nick end labeling assay, and (b) Western blotting were performed. Scale bar 20 μm. (c) The graph shows the apoptotic index, the percentage of terminal deoxynucleotidyl transferase-dUTP nick end labeling–positive cells in 500 cells. All values represent the mean ± standard deviation of three independent counts (*P < 0.05).
It has been reported that ATM-Chk2-p53 is constitutively activated in human bladder cancer and functions as an anticancer barrier. However, ATM and p53 are frequently mutated in human cancers, and their aberrant expression levels are 9% and 29%, respectively. Deficiencies in the ATM-Chk2-p53 pathway may result in tumor progression, survival and increased genomic instability, and are thought to contribute to tumorigenesis by enhancing mutations that allow for the accrual of genetic lesions and the development of predispositions toward malignant transformation.

It has been demonstrated that ATM signaling primarily enhances apoptosis in p53 and ATM proficient cancer cells. If this type of cancer cell were treated with conventional DNA-damaging chemotherapy or radiotherapy, the ATM-Chk2-p53-Puma signaling axis should be considered. Our results suggest, as expected, that RT4 cells undergo apoptosis in response to IR by activating this axis in the absence of caffeine. Conversely, in the presence of caffeine, apoptosis is attenuated due to inhibition of this axis, as characterized by a decrease in γH2AX foci and 53BP1 foci. It has been suggested that phosphorylation of H2AX is a predictor of DNA-damage-induced cell death.

Chk2, also a target for ATM in response to DSB, is phosphorylated on Thr68. In turn, it can phosphorylate p53, thus increasing the DNA-binding activity of p53, as well as its stability. p53 can trigger apoptosis by transactivating pro-apoptotic genes such as PUMA and Buxar causes G1-S arrest by up-regulating p21 in the context of low levels of DSBs. Based on the results of RT-PCR and Western blotting, we found that caffeine hindered the p53-mediated apoptotic response of RT4 cells to IR. IR alone, however, resulted in a notable level of apoptosis through the activation of p53 and transactivation of p53-inducible genes.

The inhibition of IR-induced apoptosis by caffeine was also measured by a colony formation assay. Caffeine exhibited radioprotective effects on RT4 cells in response to low-dose radiation; it displayed radiosensitization roles following high-dose radiation. One gray of IR generates approximately 2000 base modifications, 1000 SSBs and 35 DSBs per cell. It is mostly accepted that DSBs are the effective trigger of apoptosis. We speculate that in the context of low levels of DSBs, caffeine makes RT4 cells initiate cell cycle arrest and damage repair rather than apoptosis due to suppression of ATM-Chk2-p53-Puma signaling pathway. However, if DNA repair fails or is overwhelmed by many DNA lesions, cells inevitably undergo death. Treatment with caffeine may reprogram RT4 cells, harboring overwhelming DNA lesions generated by high-dose radiation, cell cycle arrest, and trigger apoptosis due to mitotic catastrophe. Additionally, the colony formation assay indicated that caffeine sensitized T24 cells to IR. In our opinion, T24 cells may effectively launch cell cycle and DNA repair rather than apoptosis in response to IR, due to lack of functional ATM-Chk2-p53-Puma signaling axis. Caffeine stimulates apoptosis in T24 cells by suppressing cell cycle and DNA repair mediated by ATM. This suggests that anti-apoptotic effects of caffeine on RT4 cells in response to IR is mainly dependent on functional ATM-Chk2-p53-Puma signaling axis.

Cumulatively, we speculate that caffeine may initiate bladder tumor formation and accelerate the progression of bladder cancer with wild-type p53 by inhibiting the ATM-Chk2-p53-Puma signaling axis. Loss of p53 in tumor cells dramatically diminishes apoptosis and redirection of ATM signaling in p53 deficient tumor cells mediates a robust cell cycle arrest and DNA damage repair following DNA damage. Thus, the combination treatment of an ATM inhibitor and chemotherapy or radiotherapy can cause synergistically cytotoxic effects in cancer cells with mutant p53. This may explain why caffeine enhances the cytotoxicity of chemotherapy or radiotherapy for high-grade soft tissue sarcoma and osteosarcoma.
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Conflicts of interest
There are no conflicts of interest.

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