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ORIGINAL ARTICLE

A Potential Role for Green Tea as a Radiation Sensitizer for Prostate Cancer

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Abstract Prostate cancer (PCa) is the most common non-cutaneous cancer in the United States. There is currently a lack of safe and effective radiosensitizers that can enhance the effectiveness of radiation treatment (RT) for Pca. Clonogenic assay, PCNA staining, Quick Cell Proliferation assay, TUNEL staining and caspase-3 activity assay were used to assess proliferation and apoptosis in DU145 Pca cells. RT-PCR/IHC were used to investigate the mechanisms. We found that the percentage of colonies, PCNA staining intensity, and the optical density value of DU145 cells were decreased (RT/GT vs. RT). TUNEL + cells and the relative caspase-3 activity were increased (RT/GT vs. RT). Compared to RT, the anti-proliferative effect of RT/GT correlated with increased expression of the anti-proliferative molecule p16. Compared to RT, the pro-apoptotic effect of RT/GT correlated with decreased expression of the anti-apoptotic molecule Bcl-2. GT enhances RT sensitivity of DU145 by inhibiting proliferation and promoting apoptosis.

Keywords Green tea · Prostate cancer · Radiation therapy

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Introduction

Prostate Cancer (PCa) is the most common type of cancer in men in the United States following skin cancer [1, 2]. It is the third-leading cause of death from cancer in the U.S. behind lung cancer and colorectal cancer [1, 2]. 161,360 new cases of PCa, as well as 26,730 deaths from this disease, are estimated in the U.S. for 2017 [1]. Radiation therapy (RT), along with surgery, chemotherapy, and hormone therapy, is a common and established treatment for PCa. However, PCa remains quite radioresistant, limiting the effectiveness of low-dose radiation and leads to high levels of damage to surrounding tissue when used at the necessary higher doses. Safe and effective radiation-sensitizing molecules are needed to decrease the therapeutic dose of radiation while maximizing the apoptotic-inducing effects of radiation on PCa.

Green tea extract (GT) contains several bioactive polyphenolic compounds that have been shown to possess many beneficial health effects, including anti-inflammatory, endocrine, neurodegenerative, cardiovascular, and anti-cancerous effects [3]. Our previous studies have shown potential roles for natural chemicals in sensitizing PCa to radiation [4]. Several studies have shown that GT has a powerful antitumor character in PCa [5–10]. GT has been shown to localize to prostatic tissue after oral administration [11]. Thus, it is reasonable to hypothesize that the anti-proliferative effects of GT will work synergistically with RT for the sensitization and enhanced destruction of PCa cells.

The present study is designed to test the hypothesis that GT enhances radiation sensitivity in PCa by altering cell proliferation and apoptosis. Additionally, we report the mechanisms underlying the cellular changes observed when GT is used in combination with RT.

Materials and Methods

Tumor Cell Line

The human PCa cell line that we used in this study, DU145, was obtained from Dr. Lubans' lab at the University of Missouri. Cells were expended in DMEM, supplemented with 1% penicillin-streptomycin and 10% heat-inactivated FBS at 37 °C in CO_2 humidified incubators (Fisher Scientific, Pittsburgh, PA, USA). 70% confluent cells were then used for the designed experimental treatment plans.

Treatment with GT and RT

Based on our pilot experiments to determine optimal treatment conditions [12–15], 70% confluent DU145 cells were treated with 50 μg/ml green tea extract (Badmonkey Botanicals) for 24 h, followed by either 4 Gy RT at room temperature in 75 cm^2 culture flasks, or mock radiation treatment [12, 16, 17]. All RT was completed using an XRAD 320 Biological Irradiator (Precision X-ray, North Brandford, CT, USA), which performed the RT treatment at a rate of 280 cGy/min. Other key technical parameters are listed here: 320 Kv, 12.5 mA, and 50 cm focus-to-surface. Cells were cultured for an additional 48 h after RT.

Clonogenic Survival Assay

The detailed information for clonogenic survival assay was described previously [4, 12, 16]. The number of colonies was expressed as a percentage of total colonies compared to controls.

Immunohistochemistry (IHC)

IHC staining for PCNA, p16, and Bcl-2 was completed as previously described [15, 18]. MetaMorph version 6.3r6 (Molecular Devices Analytical Technologies, Sunnyvale, CA, USA) was used to quantify the number of PCNA(+) cells by manually counting all cells in 3–5 randomly selected high power fields. MetaMorph image analysis software was also used to measure the average staining intensity for proteins within the area covered by cells. Results are expressed as the average integrated immunostaining intensity of 3 slides ± SEM relative to that in control cells.

Determination of Proliferation with the Quick Cell Proliferation Assay Kit

Cell proliferation was also examined by using a Quick Cell Proliferation Assay Kit, a commercial kit from BioVision. In this method, the proliferation of viable cells leads to an increase in the activity of mitochondrial dehydrogenases. Consequently, this will result in an increase in the amount of formazan dye which can be detected using spectrophotometry. The detailed information has been described in our previous studies [12, 16, 17].

RT-PCR

DU145 PCa cells exposed to all treatment conditions were washed and homogenized in TRIzol (Invitrogen). RNA was extracted from these cells and 1 μg of RNA was reverse transcribed as described previously [15, 18]. Primer sequences used in this study have been described previously [15, 18] and GAPDH was used as an internal control.

TUNEL Staining

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay was used to visualize apoptosis as previously described [12, 17]. All cells in 5–6 randomly selected fields at a magnification of 400× were counted using MetaMorph to quantify the number of apoptotic cells. TUNEL (+) cells were expressed as a percentage of total cells.

Measurement of Caspase-3 Activity

Cellular apoptosis was also measured by using caspase-3 activity of DU145 cells. A caspase-3/CPP32 colorimetric assay kit from BioVision was used as described before [12, 17].

Statistics

All experiments were repeated at least three times. Statistical analysis of data was performed using an unpaired two-tailed Student's t test. A P value <0.05 was considered significant.

Results

Effect of RT/GT on Inhibition of Survival of DU-145 PCa Cells

The DU145 PCa cells were used to investigate the effect of RT/GT on PCa. 70% confluent cells were treated for 24 h with GT (50 μg/ml), followed by RT at 4 Gy or mock treatment. 48 h after RT, clonogenic survival assay was first used to evaluate survival of DU-145 PCa cells. The percentage of colonies of DU145 was significantly lower after RT/GT treatment compared to controls treated with medium alone, or RT alone (Fig. 1a, $p < 0.05$). PCNA staining was also used to evaluate the anti-proliferative effect of RT/GT on DU145 cells (Fig. 1, b-c) and the results are consistent with the findings from the clonogenic survival assay. These findings were further strengthened by OD values when using a Quick Cell Proliferation Assay Kit to analyze cell proliferation (Fig. 1,

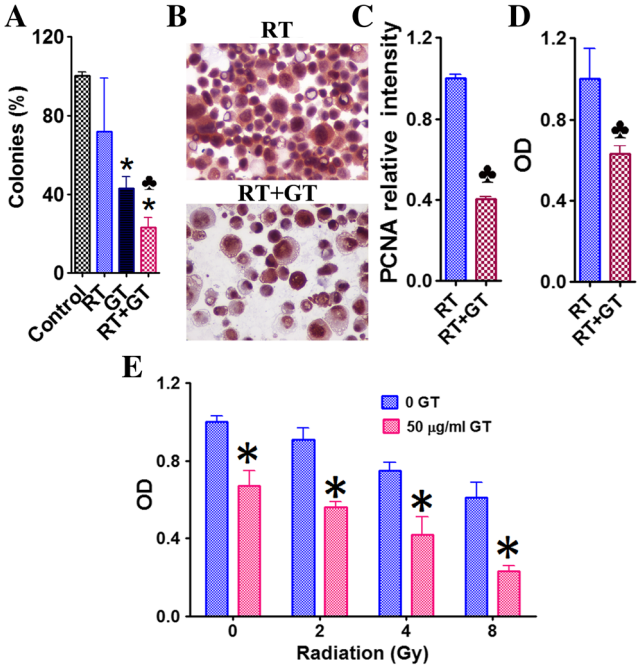

Fig. 1 Effect of RT/GT on survival of DU145 cells. **a:** Clonogenic survival assay of DU145 cells treated with or without RT in the presence or absence of GT. The number of colonies was counted and expressed as a percentage of total colonies in controls (medium alone). **b:** Representative IHC results for PCNA of DU145 cells treated with RT/GT or RT alone. **c:** PCNA+ cells (red) in 5–6 randomly selected high power fields of three slides were counted using MetaMorph software and summarized. **d and e:** Relative OD value determined with a proliferation kit. Results are expressed as the mean OD + SEM in each group, and are representative of two independent experiments. A significant difference in the percentage of colonies in each group compared to that in controls is indicated by the asterisk ($p < 0.05$). A significant difference in the percentage of colonies, PCNA+ cells or OD in RT/GT vs. RT group is indicated by the club or asterisk ($p < 0.05$). Original magnification in B: X400

d). The synergistic growth inhibition effect of GT was also found at different dosages of radiation (2–8 Gy, Fig. 1e). Taken together, these results strongly indicate that GT synergizes with RT to inhibit the survival of DU145 cells.

Effect of RT/GT on the Expression of pro- and Antiproliferative Molecules in DU145 Cells.

To explore the possible molecular mechanisms for this effect, RT-PCR was used to determine mRNA expression levels of major pro-proliferative and anti-proliferative molecules in the RT/GT group as well as the RT group (Fig. 2). The mRNA expression of all molecules was comparable in both groups ($p > 0.05$) except for the anti-proliferative molecules p16 and p27. mRNA expression levels of p16 were significantly higher in the RT/GT group vs. the RT group (Fig. 2, $p < 0.05$). We further investigated and confirmed this finding at the protein level via p16 IHC staining. Staining intensity was much stronger in the RT/GT vs. the RT group (data not shown). Unexpectedly, mRNA expression levels of p27 were

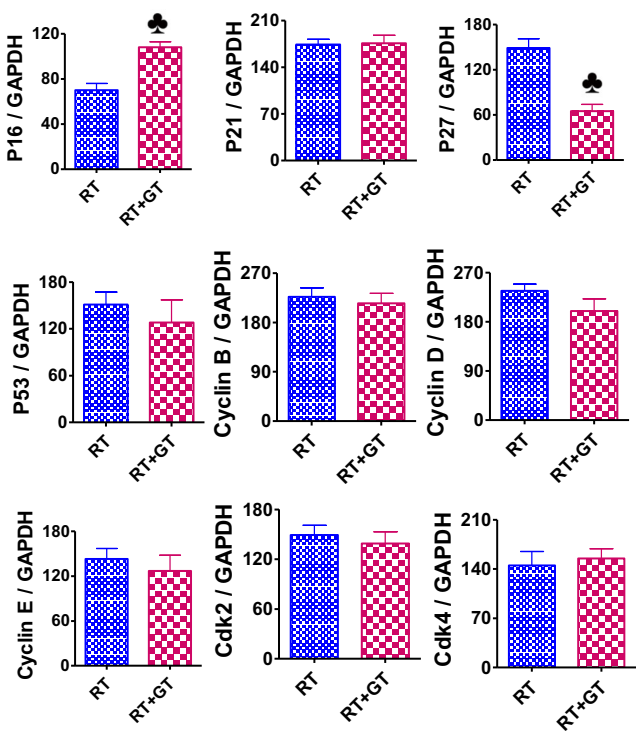

Fig. 2 Effect of RT/GT on expression of pro- and anti-proliferative molecules evaluated by RT-PCR Experiments were done in triplicate and results are expressed as the mean ratio of molecule densitometric Units/GAPDH + SEM (×100). Results are representative of two independent experiments. A significant difference in mRNA expression in RT/GT group vs. RT group is indicated by the club ($p < 0.05$)

significantly lower in the RT/GT group vs. the RT group (Fig. 2, $p < 0.05$). As an anti-proliferative molecule, a lower level of p27 would favor cancer cell growth, suggesting that p27 was not a key molecule which contributed to the growth inhibition effect of RT/GT vs. RT. Thus, upregulation of p16 correlated with the inhibitory effect of RT/GT on the survival of DU145 prostate cancer cells.

RT/GT Promoted Apoptosis of DU145 Cells

One of the mechanisms for tumor development and progression was evasion of cellular apoptosis. When tumor growth was inhibited, it was possible that there was increased apoptosis of tumor cells. To evaluate this possibility, TUNEL staining was performed and data were shown (Fig. 3a). A higher number of TUNEL (+) cells was found in the RT/GT group vs. the RT group and the difference in TUNEL (+) cells was significant (Fig. 3b, $p < 0.05$). To further investigate the role of apoptosis in RT/GT-mediated cell growth inhibition, we used a caspase-3 activity kit to evaluate relative caspase-3 activity in DU145 cells. The caspase-3 activity was significantly higher in the RT/GT group vs. the RT group (Fig. 3c). Thus, RT/GT induces apoptosis of DU145 cells which provides an additional explanation for the inhibitory effect of RT/GT on the survival of DU145 cells.

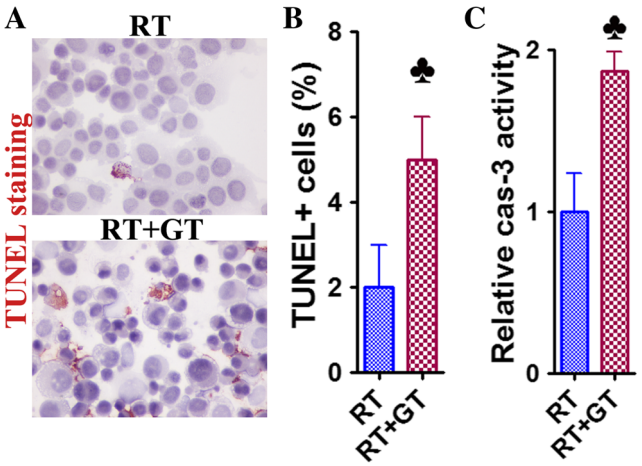

Fig. 3 RT/GT increases apoptosis of DU145 cells. **a:** representative TUNEL staining is shown. **b:** TUNEL+ cells in 3–5 randomly selected high power fields of three slides were counted. **c:** Relative cellular caspase-3 activity. Results are expressed as mean activity relative to RT + SEM. Assays were done in triplicate. A significant difference in the percentage of TUNEL+ cells or relative caspase-3 activity in cells treated with RT/GT and those in RT alone is indicated by the club ($p < 0.05$). Shown are representative of two independent experiments. Original magnification: A: X400

RT/GT Increased the Expression of pro-Apoptotic Molecule Bcl-2 in DU145 Cells

It was of particular interest to further investigate the possible molecular mechanisms by which RT/GT induces increased apoptosis of DU145 cells. To address this, RT-PCR was used to determine mRNA expression levels of pro- and anti-apoptotic molecules. The mRNA expression of these molecules was similar in both groups ($p > 0.05$) except for Bcl-2 and TRAIL. mRNA expression levels of Bcl-2 were significantly lower in the RT/GT group vs. the RT group (Fig. 4, $p < 0.05$). This mRNA data was further echoed with protein analysis. Staining intensity for Bcl-2 via IHC was much weaker in the RT/GT group vs. the RT group (data not shown). Unexpectedly, mRNA expression levels of TRAIL were also significantly lower in the RT/GT group vs. the RT group (Fig. 4, $p < 0.05$). As a pro-apoptotic molecule, a lower level of TRAIL would favor cancer cell growth, suggesting that TRAIL was not a key molecule which contributed to the growth inhibitory effect of RT/GT vs. RT. Thus, downregulation of Bcl-2 in DU145 cells correlated with the increased apoptosis in DU145 cells induced by RT/GT.

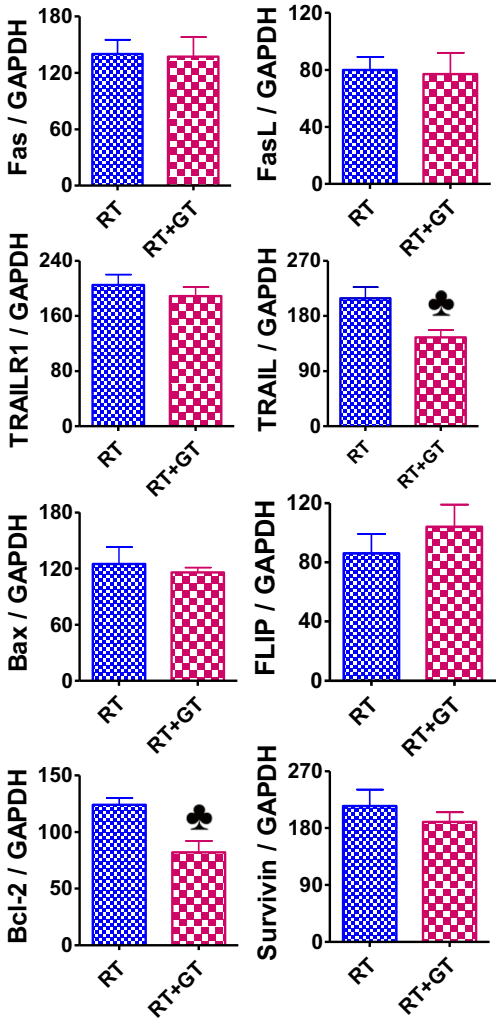

Fig. 4 Effect of RT/GT on expression of pro- and anti-apoptotic molecules evaluated by RT-PCR Results are expressed as the mean ratio of molecule densitometric Units/GAPDH + SEM (×100), and are representative of two independent experiments. A significant difference in mRNA expression in RT/GT group vs. RT group is indicated by the club ($p < 0.05$)

Discussion

With the screening of PSA in men, more patients are diagnosed with PCa although the tumors are relatively smaller and with lower grading in differentiation. Radiation therapy is the first recommended treatment for these patients [4, 19]. However, side effects such as bowl problems, bladder problems, and erectile problems significantly affect the living quality for these patients when they are treated with higher and longer dosages of radiation [4, 19]. An effective radiosensitizer which will lower the dosage and duration of radiation would significantly benefit these patients. In this study, we show that a combination of GT with RT synergistically inhibits the growth of DU145 PCa cells by inhibition of cellular proliferation and induction of cellular apoptosis. Mechanistically, the anti-proliferative effect of RT/GT correlated with increased expression of the anti-proliferative molecule p16, and the pro-apoptotic effect of RT/GT correlated with decreased expression of the anti-apoptotic molecule Bcl-2. To the best of our knowledge, this is the first study to show mechanistic evidence of RT/GT-mediated inhibition of prostate cancer cell growth.

It is well established that the regulation of the eukaryotic cell cycle is extremely delicate and balanced between known pro- and anti-proliferative molecules [20]. p16 is a well-known anti-proliferative molecule (also known as a tumor suppressor protein) responsible for inhibiting cell cycle dependent kinases cdk4 and cdk6 [21]. p16 can inhibit cells progressing from the G1 to S phase [22, 23]. Decreased levels or a deficit of p16 has been shown to be involved in many cancer types, especially melanoma and pancreatic cancer [22, 23]. Upregulation of this protein will arrest the cell cycle and thus result in growth inhibition. In our study, RT/GT significantly increased p16 which was demonstrated by both mRNA and protein levels. Thus, p16 might be the main target of GT. Similar to the tight regulation of the cell cycle, cellular apoptosis is a programmed cell death which is also tightly regulated by balancing known pro- and anti-apoptotic molecules [13–15, 24–26]. Bcl-2 inhibits apoptosis, while the Bcl-2 family protein Bax promotes apoptosis through regulation of mitochondrial voltage-dependent anion channels [27]. Bcl-2 is the first identified anti-apoptotic molecule [28] and it is involved in many cancer types including melanoma, lymphoma, PCa and nasopharyngeal cancer [28–34]. In our study, inhibition of DU145 cell proliferation correlated with a reduction in the expression of Bcl-2 seen with RT/GT. p16 and Bcl-2 represent just two proteins in the milieu of cell-signaling molecules known to be responsible for controlling cell cycle progression and apoptosis. These two molecules are the target molecules for GT in this study. It is possible that some other molecules might also play a critical role in this growth inhibition effect of RT/GT in PCa. Nonetheless, these two molecules help explain our finding of reduced proliferation and increased apoptosis of DU145 cells in response to RT/GT treatment.

We unexpectedly found that treatment of DU145 cells with RT/GT decreased the expression of two pro-apoptotic molecules, p27 and TRAIL. The decrease in p27 and TRAIL may be part of an adaptive response to cell injury to prevent cells from further damage. Similar scenarios have also been noticed by our previous studies [4, 12, 16, 17]. It is also possible that there could be unidentified pro- and anti-proliferative and/or pro- and anti-apoptotic molecules that play some role in DU145 cell proliferation and apoptosis. It is reasonable to conclude that it may not be just one or two specific pro- or anti-proliferative and/or pro- or anti-apoptotic molecules that are responsible for the fate of DU145 cells, but rather the balance between the complex milieu of pro- and anti-proliferative and pro- and anti-apoptotic molecules that determines the cellular fate.

Green tea is known to be safe to human beings since it has been used as a drink for a couple thousand years, although the optimal dosage for maximal therapeutic benefit for GT has not been well investigated or documented. As mentioned before, green tea polyphenols have been shown to localize to prostatic tissue when administered orally [11], highlighting a particularly prescient role for GT as a radiation sensitizer for prostate cancers. Studies are ongoing to determine proper dosage for localization of therapeutic levels of green tea polyphenols in prostatic tissue.

In summary, GT synergistically enhances the anti-proliferative and pro-apoptotic effects of RT in DU145 PCa cells. These data suggest a potential role for GT as a radiation sensitizer for PCa. Further animal model and clinical trials are necessary to determine the detailed efficacy and side effects of this combination. Such a study might be helpful in development of new radiosensitizers.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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