A Possible Role for Perforin and Granzyme B in Resveratrol-Enhanced Radiosensitivity of Prostate Cancer

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ABSTRACT: Perforin and granzyme B are expressed primarily by activated lymphocytes (cytotoxic T cells, natural killer cells, and natural killer T cells) and function together to induce apoptosis of target cells. Typically, these proteins are not expressed in tumor cells. In the present study, we established the constitutive expression of perforin and granzyme B by the PC-3 and DU145 prostate cancer (PCA) cell lines with reverse transcription polymerase chain reaction, immunohistochemistry, Western blot, or a combination of techniques. The combination of radiation and resveratrol (XRT/RSV) additively/synergistically decreased survival of PCA because, at least in part, of increased apoptosis. We further demonstrated that treatment with RSV up-regulated the expression of both perforin and granzyme B, whereas treatment with XRT up-regulated the expression of granzyme B, but not that of perforin. Combined XRT/RSV treatment of PCA cells further increased the expression of both perforin and granzyme B compared with RSV or XRT alone. Thus, increased radiosensitivity of prostate cancer cells induced by RSV correlated with up-regulation of perforin and granzyme B, demonstrating a possible mechanism for tumor apoptosis. These findings might be helpful in devising new strategies for treating PCA. Key words: . J Androl 2012;33:000–000

Apoptosis of cancer cells is an active process of cell death mediated by the sequential activation of a series of caspases (Thompson, 1995; Wajant, 2002). In response to extrinsic or intrinsic stimuli, changes in the balance of pro- and antiapoptotic proteins may induce apoptosis (Ju et al, 1995; Thompson, 1995; Wajant, 2002; Brown and Attardi, 2005; Fesik, 2005; Fang et al, 2008; Fang et al, 2010; Rousalova and Krepela, 2010; Hanahan and Weinberg, 2011). The intrinsic pathway of apoptosis is triggered by various types of intracellular stress, including growth factor withdrawal, DNA damage, and unfolding stresses in the endoplasmic reticulum, whereas the extrinsic pathway is triggered by death receptor ligation or activation by cytotoxic granules, such as perforin and granzyme B (Ju et al, 1995; Thompson, 1995; Wajant, 2002; Brown and Attardi, 2005; Fesik, 2005; Fang et al, 2008, 2010; Rousalova and Krepela, 2010; Hanahan and Weinberg, 2011).

Perforin and granzyme B must be present together for cytotoxic granules to induce apoptosis (Masson and Tschopp, 1985; Podack et al, 1985). Perforin, a pore-forming protein, does not have the ability to induce apoptosis, whereas granzyme B, a caspase-like serine protease, requires pore formation to enter target cells (Trapani and Sutton, 2003). Inactive perforin and granzyme B are safely packaged in cytotoxic granules in cytotoxic T cells (CTL), natural killer (NK) cells, and NKT cells (Cullen and Martin, 2010). With the formation of the immunological synapse, cytotoxic granules fuse with the tumor cell membrane releasing perforin and granzyme B into the synapse, ultimately resulting in apoptosis of the tumor cell (Barry and Bleackley, 2002; Trapani and Smyth, 2002).

In recent years, the notion of restricted granzyme B expression has been challenged as cell types other than leukocytes have been shown to express granzyme B (Namekawa et al, 1998; Hirst et al, 2001; Rissoan et al, 2002; Horiiuchi et al, 2003; Hernandez-Pigone et al, 2006; Kim et al, 2007; Strik et al, 2007; Tordjmann et al, 1998; Tschopp et al, 2006; Wagner et al, 2004, 2008). Malignant cells such as breast carcinoma, lung carcinoma, urothelial carcinoma, and oral squamous cell carcinoma have been shown to express granzyme B (Kontani et al, 2001; Hu et al, 2003; Costa et al, 2010; D’Eliseo et al, 2010). Perforin expression has been reported to be restricted only to CD4+ T cells, neutrophils, and macrophage precursors, in addition to CTL, NK, and NKT cells (Li et al, 1994; Appay et al, 2002; Wagner et al, 2004, 2008). Perforin expression by tumor cells has not previously been reported.

Radiation therapy (XRT) is commonly used to treat localized prostate cancer (PCA) (Leith, 1994). Resveratrol (RSV), a natural product in grapes, peanuts, and
other plant species (Jang et al, 1997), enhances PCA radiosensitivity and increases cell death (Scarlati et al, 2007). Our unpublished data shows the increased cell death is the result of increased apoptosis, in part mediated through the up-regulation of FAS and TRAILR1. Perforin and granzyme B also play a role in the extrinsic pathway of apoptosis, but little is known about their roles in response to XRT, RSV, or combination XRT/RSV therapy. This study was performed to investigate whether the expression of perforin and granzyme B correlated with the increased apoptosis of PCA cells treated with XRT/RSV.

Materials and Methods

Tumor Cell Line

PC-3 cells and DU145 cells, derived from human PCA, were kindly provided by Drs Susan L. Deutscher, Jessica R. Newton, Lubahn B. Dannis, and Albert G. Jackson (University of Missouri, Columbia, Missouri). Cells were maintained in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, California), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Cultures were incubated at 37°C in a humidified 5% CO₂ incubator (Fisher Scientific, City, State). Cells were grown until they reached 70–80% confluency, at which time they were subjected to experimental treatment.

Treatment with RSV and Radiation of PC-3 Cells

To investigate the effect of RSV on PC-3 and DU145 cell radiosensitivity, 70%–80% confluent PC-3 and DU145 cells were treated with RSV at variable concentration (0–50 μM) for 24 hours, followed by XRT at 2, 4, and 8 Gy or mock treatment. The dosage of RSV and XRT was based on previous studies (Su et al, 2005; Scarlatti et al, 2007; Diaz et al, 2009). All XRT was carried out with an XRAD 320 Biological irradiator (Precision X-ray, North Branford, Connecticut) at 320 kV, 12.5 mA, and 50 cm focal distance from target, with filter 1 (280 cGy/min). Cells were irradiated at room temperature in 75-cm² culture flasks. After XRT, cells were cultured for 24 hours for most experiments and for 72 hours for apoptosis studies.

Clonogenic Survival Assay

Twenty-four hours after XRT, cells were detached from 75-cm² culture flasks with TrypLE express (Invitrogen), suspended in phosphate-buffered saline (PBS) and counted with a hemocytometer. Clonogenic survival assay was performed by plating 1000 cells into 60-mm petri dishes (Corning, City, State) in triplicate and incubating at 37°C in a humidified 5% CO₂ incubator. Fresh media was added at day 5 after seeding. Nine days after incubation, cells were fixed with 10% formaldehyde and stained with 0.04% crystal violet. The number of colonies was counted and expressed as a percentage of total colonies in controls.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End Labeling

Apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay using an ApopTag kit (Chemicon, City, State) as previously described (Fang et al, 2008). Quantification of apoptosis was performed by manually counting cells in 5–6 randomly selected high-power fields (magnification: ×400) with MetaMorph image analysis software. TUNEL-positive (TUNEL+) cells were expressed as a percentage of total cells.

Measurement of Caspase-3 Activity

Cellular caspase-3 activity of PC-3 cells recognizing the sequence DEVD (Asp-Glu-Val-Asp) was measured with a caspase-3/CPP32 colorimetric assay kit (BioVision, City, State) as previously described (Fang et al, 2010). The fold increase in caspase-3 activity (relative caspase-3 activity) was determined by comparing the absorbance of pNA from samples treated with RSV alone or XRT alone or XRT/RSV to that from samples in controls. Assays were performed in triplicate.

Reverse Transcription Polymerase Chain Reaction

Cells were washed with PBS, centrifuged, and homogenized in TRIzol (Invitrogen). RNA was extracted, and its concentration was determined. RNA (1 μg) was reverse transcribed as previously described (Fang et al, 2007, 2008). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to verify that the same amount of RNA was amplified. Primers for GAPDH were sense: 5’-TGCCGCTCTAGAAAAACCTGC-3’, antisense: 5’-ACCCGTGTTGCTAGCCAAA-3’. Perforin primers were sense: 5’-CAGTACGCTTCAGCAGACTGAC-3’, antisense: 5’-ATGAAGTGGGTGCCTTAGTG-3’. Granzyme B primers were sense: 5’-TGCAAGGAAGATCGAAGTGCG-3’, antisense: 5’-GAGGCATGCACTTTCTGTC-3’.

Immunohistochemistry

Immunohistochemistry (IHC) staining for proliferating cell nuclear antigen, perforin, granzyme B, active caspase-3, and active caspase-8 was performed as described previously (Jang et al, 2007, 2008). Primary antibodies are all obtained from Santa Cruz (City, State), except active caspase-3 (BD Pharmingen, City, State) and active caspase-8 (Imegex, City, State). The dilution used for all primary antibody (Ab) was 1:200 and for secondary antibody, 1:500. As a negative control, primary Ab was replaced with an equal amount of normal rabbit or goat immunoglobulin G. These controls were uniformly negative. To quantify the immunostaining intensity for perforin, granzyme B, active caspase-3, and active caspase-8, 5–6 randomly selected high-power fields of 3 slides from each group were analyzed with MetaMorph software (Fang et al, 2010). Results are expressed as the average integrated immunostaining intensity of 3 slides ± SEM relative to that in control cells.
Western Blot

Expression of perforin was quantified by Western blot with 1:3000 rabbit polyclonal antibody (Santa Cruz) as described above and 1:5000 donkey anti-rabbit secondary antibody (Jackson Laboratory). Protein (30 μg) was added to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel as prescribed previously (Fang et al, 2007).

Statistics

All experiments were repeated at least 2 or 3 times. Statistical analysis of data was performed with an unpaired 2-tailed Student’s t test or the Mann-Whitney rank sum test. P < .05 was considered significant.

Results

Effect of XRT/RSV on Survival of PC-3 and DU-145 Cells

To investigate the effect of RSV on PC-3 cell radiosensitivity, 70%–80% confluent PC-3 cells were treated with RSV at variable concentration (0–50 μM) for 24 hours, followed by XRT at a dose different from that described in “Material and Methods.” The concentration of RSV and dosage of radiation were based on previous studies (Su et al, 2005; Scarlatti et al, 2007; Diaz et al, 2009). Twenty-four hours after XRT, cell survival was evaluated by clonogenic survival assay; the percentage of colonies after treatment is shown in Figure 1A. In the absence of RSV, the percentage of PC-3 cell colonies surviving 2 Gy of XRT and sham treatment was comparable (89% ± 6% vs. 100% ± 3%, P > .05), suggesting that PC-3 cells are resistant to XRT. Survival of PC-3 colonies was inhibited at a dose of 2 μM RSV (73% ± 5% vs. 100% ± 3%, P < .05), and the effect of RSV was more striking at the dose of 50 μM. Of particular interest, the percentage of PC-3 cell colonies treated with the combination of XRT and RSV (50 μM) decreased to 18% ± 3% (2 Gy), 6% ± 3% (4 Gy), and 6% ± 2% (8 Gy), respectively. Consistent with this result, the cell density was much lower in the group treated with XRT/RSV than that in the control group (Figure 1B). These results indicated that XRT/RSV decreased survival of PCA cells, suggesting an increase in the radiosensitivity of PCA cells by RSV. Similar results were obtained when another PCA cell line, DU-145, was used (Figure 1C), suggesting that this effect is not specific to a cell line.

Effect of the Combination of RSV and Radiation on Apoptosis of PC-3 Cells

Decreased survival of PC-3 cells by XRT/RSV treatment might be due to increased apoptosis of PC-3 cells. To examine this possible mechanism, 70%–80% confluent PC-3 cells were treated with RSV (50 μM) for 24 hours,
followed by radiation (8 Gy). Twenty-four hours after XRT, apoptosis was evaluated by TUNEL staining. There were few TUNEL+ cells in each group and there was no significant difference in the percentage of TUNEL+ cells in the group of XRT/RSV compared with that in any other group. Similar results were obtained when caspase-3 activity of PC-3 cells was assayed using the colorometric detection method. However, when apoptosis was evaluated by TUNEL staining 72 hours after XRT, rather than 24 hours after XRT (Figure 2A and B, \( P < .05 \)), differences in induction of apoptosis became evident. Only 4% ± 2%
of cells were TUNEL+ in the radiation group, whereas 48% ± 5% and 73% ± 5% PC-3 cells were TUNEL+ in the RSV alone and XRT/RSV treatment groups, respectively (P < .05 compared with control). Similar results were obtained when caspase-3 activity of PC-3 cells was assayed (Figure 2C). Consistent with the results obtained by TUNEL staining and caspase-3 activity, the staining intensities for active caspase-3 and active caspase-8 were also higher in the RSV group than those in the control group and were the highest in the XRT/RSV group (Figure 2D through G). These results suggest that the decreased survival of PC-3 cells correlates with increased apoptosis, enhanced by the combination treatment (XRT/RSV).

Effect of XRT/RSV on the mRNA Expression of Perforin and Granzyme B in PC-3 Cells

The balance between pro- and antiapoptotic molecules plays an important role in cell apoptosis (Griffith et al, 1995; Ju et al, 1995; Zhang et al, 2000; Fesik, 2005; Fang et al, 2007, 2011; Hanahan and Weinberg, 2011). The role of Fas, FasL, TRAILR1, TRAIL, and Bax, as well as FLIP, Bcl-2, and survivin, have been investigated as mediators of the additive/synergistic effect of XRT/RSV (Fang et al, unpublished), whereas it is still unknown whether perforin and granzyme B are expressed in PC-3 cells and whether their expression level correlates with increased apoptosis of PC-3 cells by XRT/RSV. To address this, mRNA expression of perforin and granzyme B in PC-3 cells, with or without XRT (8 Gy) in the presence or absence of RSV (50 µM), was determined by reverse transcription polymerase chain reaction (RT-PCR; Figure 3). The mRNA expression of both perforin and granzyme B were detectable in PC-3 cells. RSV up-regulated mRNA expression of both perforin and granzyme B. XRT alone had little effect on the mRNA expression of perforin, although the mRNA expression of granzyme B was up-regulated. Compared with cells treated with XRT alone, the mRNA expression of both perforin and granzyme B was up-regulated in cells treated with XRT/RSV.
Figure 4. Effect of the combination of radiation and resveratrol (XRT/RVS) on the protein expression of perforin and granzyme B in PC-3 cells evaluated by immunohistochemistry (IHC). Western blot, or both. (A) IHC for perforin and granzyme B in PC-3 cells treated with or without XRT (8 Gy) in the presence or absence of RVS (50 μM), ×400. (B) The relative immunostaining intensity for perforin and granzyme B in 5–6 randomly selected high-power fields of 3 slides from each group was analyzed by MetaMorph software. Results are expressed as the average integrated immunostaining intensity of 3 slides plus the standard error of the mean relative to that in control cells. (C) Protein expression level of...
Effect of XRT/RSV on the Protein Expression of Perforin and Granzyme B in PC-3 and DU-145 Cells

Protein expression of perforin and granzyme B in PC-3 cells was also examined by IHC, Western blot, or both. Consistent with the mRNA expression patterns of perforin and granzyme B shown in Figure 3, there was weak staining for both perforin and granzyme B in the control group, whereas, compared with cells treated with XRT alone, the relative immunostaining intensity for both perforin and granzyme B was stronger in cells treated with XRT/RSV (Figure 4A and B). The expression level of perforin was further confirmed by Western blot (Figure 4C). Similar results were obtained when DU-145 cells were evaluated by IHC (see the Supplemental Figure, available online at http://www.andrologyjournal.org). These results indicate that perforin and granzyme B were constitutively expressed in PC-3 and DU-145 cells, and their increased expression correlated with the increased apoptosis of PCA cells by XRT/RSV.

Discussion

The purpose of this study was to investigate a possible role for perforin and granzyme B in RSV-enhanced radiosensitivity of radioresistant PCA cells. In this study, we demonstrated that treatment with combination XRT/RSV decreased the survival of PC-3 and DU-145 cells by enhancing apoptosis. We then demonstrated that perforin and granzyme B were constitutively expressed in PC-3 and DU-145 cells. To our knowledge, this is the first study demonstrating the expression of perforin in human cancer cells and granzyme B in PCA cells (Namekawa et al, 1998; Barry and Bleackley, 2002; Trapani and Smyth, 2002). In addition to demonstrating the presence of both perforin and granzyme B in human PCA cells, we have shown treatment with XRT and RSV can enhance the expression of these proteins.

Our study shows constitutive expression of granzyme B by PCA. Previous studies showing the presence of granzyme B in cancer used clinical samples. In these studies, it is difficult to conclude whether granzyme B is the product of tumor cells or is present as a part of the antitumor response mediated by tumor infiltrating lymphocytes (Masson and Tschopp, 1985). The use of tumor cell lines in our study leads to the clear conclusion that tumor cells are capable of producing granzyme B. Besides its expression in CTL, NK, and NKT cells, perforin expression has been reported only in CD4+ T cells, neutrophils and macrophage precursors (Li et al, 1994; Appay et al, 2002; Wagner et al, 2004, 2008). The data presented in this study further extend the understanding of perforin expression by showing it can be constitutively expressed in human cancer cells. Additionally, we have shown that perforin and granzyme B can be coexpressed in cancer cells.

The coexpression of perforin and granzyme B is critical to induce apoptosis of target cells. Granzyme B–induced apoptosis is dependent on the presence of perforin because granzyme B cannot initiate binding to lipid cell membranes (Besenicar et al, 2008); granzyme B internalization into target cells requires perforin-mediated (Hoves et al, 2010) membrane pore formation (Masson and Tschopp, 1985; Podack et al, 1985; Young et al, 1986; Uellner et al, 1997). In this study, RSV alone induced apoptosis of PCA cells, whereas XRT alone had little effect on apoptosis. RSV and XRT both up-regulated expression of granzyme B, whereas RSV, but not XRT, up-regulated expression of perforin. Increased apoptosis correlated with the increased expression of perforin and granzyme B, which could explain, at least in part, the ability of RSV to increase the radiosensitivity of PCA.

In cells in which the intrinsic pathway of apoptosis is nonfunctional, as commonly occurs in cancer cells, coexpression of perforin and granzyme B could denote another mechanism for apoptosis of tumor cells (fraternal suicide; Brown and Attardi, 2005; Fesik, 2005; Rousalova and Krepela, 2010; Hanahan and Weinberg, 2011). In our study, the up-regulation of both perforin and granzyme B by XRT/RSV correlated with increased apoptosis of PCA cells, suggesting these proteins might play a role in mediating the additive/synergistic effect of XRT/RSV on PCA apoptosis. Our results are consistent with data that show z-Ala-Ala-Asp(OMe)-CH2F, a granzyme B inhibitor, partially inhibited apoptosis induced by tumor necrosis factor alpha and XRT (Kimura and Gelmann, 2002). However, correlation of increased expression of perforin and granzyme B with increased apoptosis of PC-3 cells suggests, but does not prove, that these proteins mediate the enhanced apoptotic effect of XRT/RSV. The true

perforin by Western blot; 30 μg of protein was loaded in each lane. A significant difference in immunostaining intensity or protein expression level in each group compared with that in controls is indicated by the asterisk ($P < .05$). A significant difference in immunostaining intensity or protein expression level between the group treated with XRT/RSV and that in the group treated with XRT alone is indicated by the club ($P < .05$). Shown are representative data of 2 or 3 independent experiments.
role of perforin and granzyme B in this addition/synergism awaits further investigation.

Other cellular processes might contribute to the decreased survival of PCA cells induced by XRT/RSV. In this regard, we have found that XRT/RSV inhibited proliferation and promoted senescence of PC-3 cells (Fang et al, unpublished). We have also found a role for Fas, TRAILR1, and Bcl-2 family proteins in apoptosis induced by XRT/RSV (Fang, unpublished), suggesting multiple mechanisms are involved in the inhibition of cancer cell survival. Thus, we postulate it is not an alteration of 1 specific pro- or antiapoptotic molecule that determines whether PCA cells undergo apoptosis after XRT/RSV, but changes in the balance between pro- and antiapoptotic mechanisms that determine cell survival. Perforin and granzyme B might be involved in this process.

This study does not exclude the possibility that increased tumor expression of perforin and granzyme B might promote tumor growth and progression in vivo by mediating apoptosis of immune cells and normal stromal cells, such as endothelial cells and fibroblasts. It has been suggested that perforin and granzyme B might be involved either in promoting or suppressing tumor growth and progression in vivo (Rousalova and Krepefa, 2010). Further in vivo studies are needed to address this issue as well.

Although our study underscores the role of RSV as a radiation sensitizer of PCA, no data currently substantiates a therapeutic effect in humans (Vang et al, 2011). In animal models, RSV appears to be well tolerated. RSV is safe for human consumption but optimal dosing has yet to be established, and the side effects at therapeutic levels are unknown. Orally dosage RSV in humans above 2.5 g/d is commonly associated with gastrointestinal discomfort or diarrhea (Vang et al, 2011). Side effects are less common at lower doses. Studies examining RSV dosing, side effects, and therapeutic benefits in humans are ongoing (Vang et al, 2011).

In summary, we are the first group to report that perforin and granzyme B are both constitutively expressed in PCA cells, and their up-regulation correlates with the increased radiosensitivity of PCA cells by RSV. These findings support a possible role of perforin and granzyme B in tumor apoptosis, growth, or both, and further investigation might prove helpful in devising new strategies for treating PCA.

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