Expression Profile of Apoptosis Related Genes and Radio-sensitivity of Prostate Cancer Cells

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Radio-resistant or recurrent prostate cancer represents a serious health risk for approximately 20%–30% of patients treated with primary radiation therapy for clinically localized prostate cancer. In the present study, we investigated the expression profiles of 84 genes involved in various apoptosis pathways in two prostate cancer cell lines LNCaP (P53+ and AR+) and PC3 (P53− and AR−). We also studied the effect of monensin, an apoptosis inducing reagent, in X-ray-induced cell killing. Comparison of gene expressions between unirradiated LNCaP and PC3 cells revealed distinguished gene expression patterns. The data showed a significantly higher expression level of genes involved in the caspase/card family and the TNF ligand/receptor family in PC3 cells, whereas, LNCaP cells exhibited higher expressions in the p53 related genes. At 2 and 4 hrs post a 10 Gy X-ray exposure, changes of gene expressions were detected in a significant fraction of the genes in LNCaP cells, but no significant changes were found in PC3 cells. There was no significant apoptosis-inducing effect of X-rays (up to 10 Gy) in both cell lines; however, monensin was shown to be effective in inducing apoptosis in LNCaP, but not in PC3 cells. In addition, the effect of combined treatment of monensin and X-rays in LNCaP cells appeared to be synergistic. Our results suggest that monensin may be effective for both cancer cell killing and radiosensitizing, and the different expression profiles in apoptosis related genes in cancer cells may be correlated with their sensitivity to apoptosis inducing reagents.

INTRODUCTION

Although early prostate cancer diagnoses and early intervention changed the epidemiology with an increasing number of diagnosed patients with organ-confined disease,1,2 and falling overall death rates in many industrialized countries,3 it has been estimated that 217,730 new cases of prostate cancer will occur in the United States (US) during 2010.3 As the most frequently diagnosed cancer in men, prostate cancer is the second-leading cause of cancer death in men in US with an estimated 32,050 deaths in 2010.3 Treatment options vary depending on the age, stage and grade of the cancer. Most patients with organ-confined disease undergo radical prostatectomy or radiation therapy,3,4 whereas in the case of metastatic disease, no curative treatment is available. Radioresistant or recurrent prostate cancer represents a serious health risk for approximately 20%–30% of patients treated with primary radiation therapy for clinically localized prostate cancer.

The survival and growth of prostate cancer cells are initially dependent on the presence of androgens, and virtually all prostate cancer patients respond when first treated with androgen ablation. However, resistance to hormone blockade ultimately results in the recurrence of highly aggressive and metastatic prostate cancer that is androgen independent.5 Two human prostate cancer cell lines, LNCaP and PC3, have been extensively used as cell models for prostate cancer studies and are generally assumed to represent early and late stages of prostate cancer, respectively.6 LNCaP was established from a lymph node metastatic lesion of human prostate adenocarcinoma.7 These cells express the androgen receptor and exhibit androgen sensitive cell growth. PC3 cell line was established from a prostatic adenocarcinoma metastatic to bone and do not respond to androgen.8 LNCaP and PC3 cells not only have different
responses to androgen, but also express different levels of angiogenic factors, pro-inflammatory cytokines and prostate cancer markers.9–11

Although X-rays or gamma rays induce anti-proliferative effect and cause significantly reduced cell survival fraction analyzed by clonal survival assay on both LNCaP and PC3 cells,12,13 these two cell lines are still considered as radio-resistant compared with other types of tumor cells.14,15 It suggests that other than the status of androgen-receptor and p53, multiple mechanisms may be involved in the radio-resistance of these two cell lines. One possible mechanism is the overexpression of various anti-apoptotic genes in Bcl2 family, such as Bcl2 and Bcl-XL.14,16 Downregulated expression of these genes has successfully sensitized the cells to radiation mediated cell killing.14,17

Other than radiation, some common chemotherapy drugs have been used to treat advanced prostate cancer as a salvage therapy, such as Mitozantrone and Doxorubicin. There are many more chemical reagents that are being investigated in the laboratories or even being through clinical trials. Monensin, isolated from Streptomyces cinnamonensis, is a well-known representative of naturally polyether ionophore antibiotics. It has been shown to be effective in inducing apoptosis in various human myeloma, colon cancer, lymphoma, and leukemia cells.18–22 These studies have demonstrated that monensin is able to induce cell cycle arrest and apoptosis through regulating cell cycle and antiapoptotic gene expression, loss of mitochondria transmembrane potential and increase of caspase-3 activity. Apoptosis can be triggered by monenin through the inhibition of macroautophagy and the induction of mitochondria damage, which is the early event of monensin-induced cell injury.23,24 Monensin has the ability to potentiate the cytotoxic activity of immunotoxins against several human tumor-associated antigens both in vitro and in vivo.25–28 Recently, monensin was identified as one of the selective antineoplastic agents that inhibited LNCaP cell proliferation.29

Most cell deaths after exposure to radiation or toxic chemicals involve expression of genes in the apoptosis pathway. Gene expression analysis of different stages of prostate cancer before and after different therapies may identify genes and pathways that are critical to the progression and their sensitivity to the treatment. Recent studies have revealed the different molecular profiles in different stages of human prostate cancer tissues and cell lines. Genome-wide expression analysis was conducted by Holzbeierlein et al. to compare of the gene expression profiles of normal prostate, primary tumors and LNCaP cells with or without hormone therapy, and androgen independent metastatic tumors.30 It was discovered that the resistance to hormone therapy was associated with a set of genes with differential expression that indicated a reactivation of the androgen response pathway in the absence of exogenous hormone. In an earlier study, Yang et al. identified several genes that differentially expressed in LNCaP and PC3, which are androgen-dependent and independent, respectively.31 Recently, Dozmorov et al. analyzed the differential global gene expression profiles between LNCaP and PC3 cells by using BeadChips containing probes for a total of 24,536 transcripts.32 Thousands of differentially expressed genes have been found between these two cell lines. It suggested that the fundamental differences in gene expression profiles in tissues or cells may largely influence the outcome of various treatments, and thus may be valuable information in designing therapeutic interventions.

In the present studies, we investigated the expression profile of apoptosis related genes (84 genes) in irradiated and control LNCaP and PC3 cells using cDNA PCR array and the differential responses of LNCaP (P53+ and AR+) and PC3 (P53− and AR−) to toxic reagent Monensin and/or X-rays using cell cycle analysis. Our data suggested that the largely differentially expressed genes in various apoptosis pathway between LNCaP cells and PC3 cells may be responsible for the different sensitization effects of monensin in radiation mediated killing.

**MATERIALS AND METHODS**

**Cell lines and cell treatment**

The human prostate cell lines LNCaP and PC3 were obtained from ATCC and grown in RPMI medium or F12 medium respectively supplemented with 10% fetal bovine serum (Invitrogen, Frederick, MD). The day before irradiation, 2 × 10^5–2 × 10^6 cells were seeded in 6-well plate or T75 flask, respectively. For some experiments, monensin (0–20 μM) (Sigma Aldrich, St. Louis, MO) was added to the seeded cells 2 hr prior to irradiation for clonogenic cell survival assays. Cells were irradiated either using X-rays of 250 kVp at a dose rate of 0.86 Gy/min or acutely using a Cs137 gamma-ray irradiator at the NASA Johnson Space Center (Houston, TX). The total doses received by the cells ranged from 2 Gy to 10 Gy.

**Cell cycle analysis**

At 48 hr after X-ray irradiation, cells were trypsinized and collected. Then, cells were fixed in cold 70% ethanol for at least 1 hr on ice for cell cycle analysis. The fixed cells were washed twice with 1XPBS and then stained for DNA content with the cell cycle reagent (Millipore, Billerica, MA) for 30 min on ice according to the manufacturer’s protocol. The stained cells were processed by using a Guava PCA Flow Cytometer (Millipore). The data were analyzed and the cell population of different cell cycle stage was determined by the software provide by the manufacturer. Each experiment was performed at least twice and the data were presented as percentage ± SD.
4',6-diamidino-2-phenylindole (DAPI) nuclei assay
At 48 hr after X-ray irradiation, cells were fixed in 3:1 Methanol/Acetic Acid fixation for the DAPI nuclei assay. The fixed cells were spread on the slides and kept in 2 × SSC buffer with 0.1%NP40 for two minutes. Then, the cells were stained with DAPI and scored by using a Zeiss Axioplan 2 microscope. The percentage of apoptotic cells were determined by evaluating nuclear morphology from about 400 cells of each sample according to the criteria described before.32,33)

Clonogenic cell survival assay
The radiosensitivity of LNCap and PC3 cells was determined using the clonogenic assay. After 24 h DMSO or Monensin (20 μM) pretreatment, cells were subjected to gamma irradiation. Immediately after irradiation, cells were reseeded at optimal densities in 100 mm culture dishes, and incubated at 37°C for 14 days to allow colony formation. At the end of the culture period, cells were fixed and stained with 24% ethanol containing 0.1% crystal violet. The fraction of surviving cells was calculated as the ratio of the plating efficiency of the irradiated cells to that of non-irradiated cells.

RT2 profiler PCR array assay
Exponentially growing LNCaP and PC3 cells from at least two independent cultures were collected for analysis of the expression of 84 apoptosis related genes using the RT2 Profiler PCR Array technique. At 2 hr and 4 hr post 10 Gy X-ray irradiation or 2 hr post Monensin exposure, the treated and untreated cells were also collected for gene expression analysis. Total RNA was isolated by the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. After DNase treatment, 1 μg total RNA of each sample was used to synthesize cDNA by using a RT2 PCR array first strand kit (SABiosciences, Frederick, MD). RT2 Profiler PCR Array was performed in an ABI7900HT instrument according to the manufacturer protocol. The fold-change for each of the genes between the irradiated sample and its unirradiated control or between LNCaP and PC3 cells was calculated from the original threshold cycle using Excel-based PCR Array data analysis template provided by the company.

Statistical analysis
The data were presented as mean ± SD from at least 3 independent experiments, except for apoptosis identified by the morphology in DAPI stained cell nuclei and for gene expression analysis, in which the data from two independent experiments were presented. The Student t test was performed to determine whether significant differences (P < .05) were achieved between samples with different treatments.

RESULTS
Expression of apoptosis related genes in untreated LNCaP and PC3 cells
To investigate whether there is any expression profile difference between LNCaP and PC3 in various apoptosis pathways, we compared the expression profiles of 84 apoptosis related genes using the PCR array technique. These 84 genes involve in various apoptosis pathways including caspase, IAP, Bcl-2, TNF ligand/receptor, CARD, p53 related and death domain families. Surprisingly, the expression profiles were found to be dramatically different (Fig. 1).

In comparison to PC3 cells, the expressions of p53 and p73 were dominantly higher in LNCaP cells (Table 1 and Fig. 2). PC3 cells showed a significantly higher expression of multiple genes in the TNF family and CARD family. In the TNF family, 5 of the 6 genes that showed differentially expressed exhibited at least 2 fold increased expressions in PC3 cells in comparison to the expressions in LNCaP (Table 1 and Fig. 2). PC3 cells also presented higher expression levels in the Card family genes, including BIRC2, BIRC3, CARD6, CASP1, CASP4 (Table 1 and Fig. 2).

Response of LNCaP and PC3 to X-ray mediated cell killing
At 48 hr post X-ray irradiation, both LNCaP and PC3 cells showed only a slight increase in the sub-G1 population using the cell cycle analysis as shown in Fig. 3A, indicating a limited X-ray mediated cell killing as measured by this technique. In the present study, LNCaP cells were subjected to a set of X-ray doses from 2 to 10 Gy, while the PC3 cells were exposed to only one dose of 10 Gy. For all the doses and both cell lines, the increased percentage of sub-G1 population was less than 6%.

Fig. 1. Different expression profiles of apoptosis related genes in LNCaP and PC3 cells.
Table 1. The list of genes that showed at least a 2 fold difference between unirradiated LNCaP and PC3 cells from at least two independent cultures (+ represents at least a 2 fold difference; ++ represents more than a 10 time difference).

| LNCaP | PC3 | CARD Family | LNCaP | PC3 |
|-------|-----|-------------|-------|-----|
| Anti-apoptosis | BCL2 | + | CARD Family | BIRC2 | ++ |
| | BCL2A1 | ++ | | BIRC3 | ++ |
| | BCL2L10 | + | | CARD6 | ++ |
| | BIRC2 | ++ | | CASP1 | ++ |
| | BIRC3 | ++ | | CASP4 | + |
| | BRAF | + | | CRADD | + |
| | IGFIR | + | | | |
| TNF Family | TNFRSF10B | + | p53 related | BCL2 | + |
| | TNFRSF11B | ++ | | CASP3 | + |
| | TNFRSF21 | ++ | | CASP6 | + |
| | TNFRSF9 | ++ | | TP53 | ++ |
| | CD40 | ++ | | TP73 | ++ |
| | CD70 | ++ | | | |
| Caspase | CASP1 | ++ | Others | NOD1 | + |
| | CSAP14 | ++ | | DAPK1 | ++ |
| | CASP3 | + | | TRAF2 | + |
| | CASP4 | + | | TRAF4 | + |
| | CASP6 | + | | | |

Fig. 2. Relative expressions of TP53, TP73, some TNF family and CARD family genes in LNCaP and PC3 cells.
A significant accumulation of G2 cells showing 10–20% more than that of unirradiated controls was observed in both irradiated LNCaP and PC3 cells, as determined by the cell cycle analysis (Data not shown). The damaged population of LNCaP cells was also analyzed using the DAPI nuclei assay for the same doses of X-ray irradiations at 48 hrs after exposure. As shown in Fig. 3B, percentage of apoptotic cells, as scored by the morphological changes with microscopes, appeared to be low and a clear dose response was not observed. 

Gene expressions induced by X-rays in LNCaP and PC3

The gene expression changes in LNCaP and PC3 cells at 2 h and 4 h post 10 Gy of X-ray irradiation were also analyzed by the PCR array for the same 84 apoptosis related genes. Our results, as shown in Fig. 4, showed that X-rays induced the expression changes in a number of apoptosis related genes in LNCaP cells. However, the response of gene expression in PC3 cells was poor, as only one gene displayed a fold change of expression level of 2.0 or greater (Fig. 4 and Table 2).

In LNCaP cells, 9 genes were found to express an increased or decreased expression level by at least 1.7 fold in both 2 h and 4 h post irradiation samples. P53 related genes, such as BAX, FAS and GADD45A, were up-regulated in LNCaP, which is consistent with the p53 status of LNCaP and PC3 cells (Table 2). The induced expression of FAS in LNCaP cells was further verified by FACs analysis (Data not shown).
Cell killing and radiosensitizing effects induced by monensin

The LNCaP cells were treated with monensin of a set of concentrations from 0.2 to 20 μM. The percentage of apoptotic cells, as measured by the sub-G1 population, was apparently not affected by the monensin for a concentration of 0.2 or 1 μM, as shown in Fig. 5A. A noticeable increase in the apoptosis percentage was observed for 5 μM of monensin treatment. At a concentration of 20 μM, monensin was found to induce a significant fraction of 27% of apoptotic cells (Fig. 5A). Similar effects of monensin treatment was also confirmed in the analysis of damaged cells based on the morphological changes, as shown in Fig. 5B, where the cells were analyzed at 48 hrs post irradiation. The data of the expressions of apoptosis related genes in the cells after 2 h Monensin treatment did not show any significant changes (Data not shown).

The monensin treated LNCaP cells were further exposed to X-rays of varying doses. As shown in Fig. 5A, for the percentage of apoptosis using the cell cycle method, monensin at a concentration of 0.2 or 1 μM did not boast any effect on the response of LNCaP cells to X-ray exposures at any of the doses from 2 to 10 Gy above the background. At concentrations of 5 and 20 μM, the apoptotic cells were induced not only by monensin alone, but also by the exposure to X-rays (Fig. 5A). Figure 5B shows the percentage of apoptotic cells after the combined treatment of Monensin and X-rays, but analyzed based on the morphological changes. The morphology data were consistent with those from the cell cycle analysis. The PC3 cells were also treated with 20 μM of monensin alone, as well as with a combination of 20 μM Monensin and 10 Gy X-rays. Unlike LNCaP cells, neither treatment scenarios produced an observed increase in the percentage of apoptosis (Data not shown).

The survival data from clonogenic analysis showed that 20 μM Monensin sensitized LNCaP cells to gamma irradiation by a factor of 3, but no sensitizing effect was seen in PC3 cells (Fig. 6). Taken together the results presented in Fig. 5 and 6, the effect of combined treatment of monensin

![Fig. 5](image-url)  
(A) Direct and sensitized X-rays mediated cell killings in LNCaP cells after monensin treatment at the concentration from 0.1 μM to 20 μM. Percentage of sub-G1 cell population is presented as Mean ± SD; (B) Percentage of apoptotic LNCaP cells at 48 hr postmonensin and/or X-ray irradiation by analyzing nuclear morphology. (* p < 0.05 compared to radiation treatment alone; # p < 0.05 compared to monensin treatment alone).

![Fig. 6](image-url)  
Clonogenic efficiency of DMSO or 20 μM Monensin pre-treated (A) PC3 and (B) LNCaP cells after gamma irradiation (* p value < 0.05 compared to DMSO control treatment).
and radiation appeared to be synergistic for LNCaP cells, but not PC3 cells.

**DISCUSSION**

Human prostate cancer cells LNCaP and PC3 have been extensively used as models to study the possible therapeutic methods, the prostate biomarkers and the progression associated different stages of prostate cancer. Being androgen-responsive and p53 positive, LNCaP cells were originally isolated from metastasis lymph node and normally considered to represent early stage of prostate cancer development, which shows androgen-sensitive cell growth. With certain circumstances, LNCaP cells have been proven to be able to progress to AR-independent growth, which is the feature of more advanced stage. PC3 cells are androgen-insensitive and p53 null cells, which were isolated from metastatic bone tumor tissue, thus, they are considered to represent advanced disease. Both cell lines are considered radio-resistant. In the present study, we investigated the response of these two cell lines to X-rays and an apoptosis inducing agent Monensin. Similar to the reported findings, X-rays in the present study were found to be ineffective in inducing apoptosis, as measured by the sub-G1 population or by the cell morphology. However, monensin at high concentrations appeared to effectively induce apoptosis in LNCaP, but not PC3 cells. In addition, monensin was found to radio-sensitize LNCaP cells, as the combined treatment of monensin and X-rays produced a synergistic effect in cell killing. Radio-sensitization of monensin was not found, however, in PC3 cells.

Although there are several common features such as prostate specific antigen (PSA) expressions, different cellular responses to various growth factors, such as transforming growth factor beta (TGFβ), epidermal growth factor (EGF), and fibroblast growth factor (FGF) have been recognized between LNCaP and PC3 cells previously. Furthermore, differences in sensitivity to various apoptosis inducers have been identified among various prostate cancer cell lines, which PC3 cells have been reported to be more resistant than LNCaP cells that maintain many of the typical components of the apoptotic machinery.

Monensin is a carboxylic ionophore, which has shown the apoptosis inducing effect on various human tumor cells, such as myeloma, colon cancer, lymphoma, and leukemia cells. Apoptosis can be triggered through monensin through mitochondria damage, which is the early event of monensin-induced cell injury, as well as inhibition of macroautophagy. Our present results not only confirmed the previously reported findings in the cell killing effect of monensin, but also, for the first time, demonstrated the radio-sensitizing effect of the agent in LNCaP cells. This radio-sensitizing effect may be linked to monensin mediated generation of intracellular reactive oxygen species and induction of oxidative stress response in prostate cancer cells. The analysis of expression profile of 84 apoptosis related genes in cells at 2 h post monensin treatment did not show any significant changes, which is consistent with the late cell killing effect of monensin that happens at 48 h to 72 h post treatment. Further studies on later time points are needed to identify the signaling pathways that are involved in Monensin mediated cell killing effects.

PC3 cells’ resistance to monensin does not appear to be unique for this particular compound. In fact, PC3 cells have been reported to have resistant responses to other apoptosis inducers, such as N-(4-hydroxyphenyl) retinamide, staurosporine (STS) and the topoisomerase I inhibitor, camptothecin, whereas, LNCaP cells undergo apoptosis in response to these apoptosis inducers. Previously, we treated both PC3 and LNCaP cells with another compound, PMA, and observed the effects of cell killing and radio-sensitization in LNCaP, but not in PC3 cells, similar to the present results for monensin (Unpublished data).

In the present study, we also investigated the expression of a set of 84 genes involved in the apoptosis pathways using the PCR array in order to understand the different cellular responses of these two cell lines to the treatment. Studies on gene expression analysis of LNCaP and PC3 have revealed that these two cells represent two distinct prostate cancer cell lineages. It has been reported that of a total 24,526 transcripts, 2198 genes were identified to be differentially expressed between LNCaP and PC3 cells. In our present study, the 84 genes can be categorized into anti-apoptosis, p53/DNA damage response, TNF ligand/receptor family, Bcl-2 family, caspase family, IAP family, TRAF family, CARD family, death domain effector domain family, and CIDE domain family.

Consistent with the reports in the literature, our data showed that genes involved in p53 and DNA damage response pathway are dominantly or even exclusively expressed in LNCaP, such as p53, p73, Bcl2 and Casp3. In anti-apoptosis genes, the over-expressions of Bcl-2, Bcl-2 family, caspase family, IAP family, TRAF family, CARD family, death domain effector domain family, and CIDE domain family.

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Due to the dramatic differences in the expression profiles of apoptosis genes between LNCaP and PC3, we further compared gene expression changes in X-ray irradiated cells to identify early changed expressions of the 84 apoptosis related genes induced by X-rays. To our surprise, even though X-ray-induced apoptosis was low in both cell lines, a total of 12 genes showed an expression change of more than 2 fold in LNCaP cells within 4 hr post irradiation, in comparison to 1 gene in PC3 cells. Such a dramatic difference in gene expressions may be a result of their different p53 statuses, since the expressions of BAX, Fas and GADD45A were induced by X-rays in LNCaP with wild type p53. Furthermore, the different basal gene expression levels in several apoptosis pathways between these two cell lines may also influence the response to X-rays. The expressions of Casp14, TNFSF10, CD70, TNFRSF10B and TNFRSF11B were also induced in irradiated LNCaP cells, but not PC3 cells, which have constitutively higher basal expressions of these genes. These data suggest that the higher basal gene expression levels in several apoptosis pathways in PC3 may lead PC3 to be more tolerant to apoptosis inducing reagents and radiation.

In summary, our present study demonstrated that LNCaP cells were considerably more sensitive than PC3 cells to apoptosis inducer monensin, and that the X-ray mediated cell killing was enhanced by monensin. The expression profile of 84 genes in various apoptosis pathways is dramatically different between LNCaP and PC3, especially in TNF and CARD family. Although no significant differences have been observed in X-ray mediated cell death by the cell cycle analysis, there are more gene expression changes in LNCaP cells than in PC3 cells after X-ray exposure. The different responses at both the molecular and the cellular levels between these two cell lines may be caused by their different genetic background and tissue origins. Thus, improving the efficiency of the therapy may require the therapy to be more specific to the distinct gene expression patterns of the prostate tumor in different individuals and tumor stages.

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