Cell Killing and Radiosensitizing Effects of Atorvastatin in PC3 Prostate Cancer Cells

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Statin/Radiosensitivity/Prostate cancer/PC3 cells/Gamma rays.

Recent studies have indicated that autophagy may be one of the important pathways induced by ionizing radiation. Atorvastatin (statin), an inhibitor of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase, may exhibit anticancer effects as an autophagy inducer. In our study, the cell killing and radiosensitizing effects of statin were analyzed in PC3 cell line. Activation of the autophagy pathway was analyzed using the GFP-LC3 assay and western blot to determine LC3-II expression. The radiosensitivity of PC3 cells was determined using the clonal survival assay, TUNEL assay, and the Annexin V apoptosis assay. The expression profiles of autophagy related genes were analyzed using a pathway specific real-time polymerase chain reaction (PCR) array. Autophagic response was induced in PC3 cells after exposure to statin and/or gamma rays. Inhibition of the autophagic process using small interfering RNAs (siRNA) targeting Atg7 and/or Atg12 significantly reduced radiosensitivity of PC3 cells. Statin also exhibited a significant apoptosis-inducing effect in PC3 cells, which can be partially suppressed by Atg7 siRNA. Cells treated with statin and gamma irradiation showed significantly reduced colony forming efficiency and increased number of Annexin V positive early apoptotic cells. Analysis of autophagy and its regulatory gene profile showed that the expressions of 22 genes out of 86 genes assessed were significantly altered in the cells exposed to combined treatment or statin alone. The data indicate that activation of the autophagy pathway may be responsible for apoptosis inducing effect of statin. Furthermore, combined treatment with radiation and autophagic inducer, such as statin, may be synergistic in inducing cell death of PC3 cells.

INTRODUCTION

As the most frequently diagnosed cancer in men, prostate cancer is the second leading cause of cancer death in men in the US with an estimated 32,050 deaths in 2010.1) Treatment options for prostate tumors vary depending on the patient’s age, and the stage and grade of the cancer. Most patients with organ-confined disease undergo radical prostatectomy or radiation therapy,1) whereas in the case of metastatic disease, no curative treatment is available. Radioresistant or recurrent prostate cancer represents a serious health risk for about 20–30% of patients treated with primary radiation therapy for clinically localized prostate cancer.

Human prostate cancer cell line PC3 (p53− and PTEN−) was established from a prostatic adenocarcinoma metastatic to bone and does not respond to androgen.2) Although gamma irradiation has an anti-proliferative effect and causes a significantly reduced cell survival fraction as analyzed by clonal survival assay on PC3 cells, these cells are still considered radioresistant compared with other types of tumor cells3,4) PC3 cells are also resistant to many chemotherapy drugs and apoptosis inducers.5,6) The poor response of PC3 cells to radiotherapy is caused not only by their p53 null status, but also by other mechanisms, such as an overexpression of various anti-apoptotic genes in the Bcl2 family and the lack of core machinery of cell death pathways.3-5) Recent studies have revealed that macroautophagy, often referred to as autophagy, a process by which degradation of macromolecules and organelles occurs under stress conditions, is induced by chemicals and radiation and may contribute to the tumor cell killing effects of these agents.2-9) Statin, an HMG-CoA reductase inhibitor, has been widely used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase.10) Epidemiological studies have
shown that use of statin use decreases the incidence of advanced prostate cancer.\textsuperscript{11} Recently, several reports revealed that autophagy and autophagy-associated cell death induced by statin may contribute to its anticancer effect in various cancer cells.\textsuperscript{12,13}

Autophagy is a tightly regulated process that is involved in maintaining a balanced microenvironment between the synthesis, degradation, and subsequent recycling of cellular products in cell growth, development, and homeostasis.\textsuperscript{59} Autophagosome formation requires a set of proteins called autophagy related gene (Atg) proteins and is initiated by class III phosphoinositide 3 kinase (PI3K) and Atg6 (Beclin-1).\textsuperscript{14–16} Regulation of autophagy is a highly complex, with inputs from the cellular environment through the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway,\textsuperscript{17} members of the Bcl2 family,\textsuperscript{18} p53,\textsuperscript{19} and death-associated protein kinases.\textsuperscript{20} Although autophagy and apoptosis cell death pathways are predominantly distinct from each other, many studies have demonstrated that extensive crosstalk exists between the two.\textsuperscript{18,21} During nutrient deficiency, autophagy functions as a pro-survival mechanism; however, excessive autophagy may lead to cell death, a process morphologically distinct from apoptosis.

Autophagy has drawn much more attention recently for its controversial role in cell survival and cell death, particularly in cancer treatment.\textsuperscript{22,23} Data reported in the literature indicate that whether autophagy enables cells to survive or enhances their death depends on many factors, including the genotype and phenotype of the tumor cells, stress factors, and status of the apoptotic machinery.\textsuperscript{22} In PC3 cells, both the apoptosis and the autophagy pathways are proposed to be impaired, especially in response to radiotherapy, because of the cells’ p53\textsuperscript{−} and PTEN\textsuperscript{−} status. The latter causes the activation of mTOR, which subsequently inhibits the autophagic response. Although a previous report has suggested that an alteration of the autophagic process changes the radiosensitivity of PC3, the mechanism of radiation-mediated activation of autophagy has to be investigated. Furthermore, more effective combined treatment with radiation, targeting both apoptosis and autophagy pathways, has to be further developed.

In the present study, we investigated the response of PC3 cells with inhibition or enhancement of autophagic pathways to radiation exposure. It has indicated that the autophagy pathway has an important role in radiation and statin induced cell death. Treatment with autophagy inducers combined with irradiation may be synergetic to reduce cell survival and to increase cell apoptosis.

MATERIAL AND METHODS

Cell culture and gamma irradiation

PC3 prostate carcinoma cells were purchased from ATCC (Manassas, VA) and maintained in GluMax MEM with 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) in a 37°C humidified incubator supplied with 5% CO\textsubscript{2}. Cells were irradiated acutely using a Cs\textsuperscript{137} gamma-ray irradiator at NASA Johnson Space Center (Houston, TX).

**GFP-LC3 assay**

PC3 cells were transiently transfected with the pCMV GFP-LC3 vector or GFP control vector (Cell Biolabs, San Diego, CA) with 70% transfection rate. After 48 hr of transfection with or without 24 hr of statin treatment, cells were irradiated with 2 Gy or 6 Gy of gamma rays. At different intervals post irradiation, cells were fixed with 4% paraformaldehyde, counterstained with 4’,6-diamidino-2-phenylindole (DAPI), and examined under a fluorescence microscope (Zeiss, Thornwood, NY). A total of 600 GFP-positive cells from 3 regions of each sample slide were evaluated and the number of autophagic cells that demonstrated GFP-LC3 puncta (≥ 4 puncta/cell) was determined. The data were presented as mean ± SD of 3 readings from different regions.

**siRNA transfection**

The siRNAs (Sigma, St. Louis, MO) were transfected using Lipofectamine 2000 reagent (Invitrogen) and the knock down efficiency was determined using the protocol described previously.\textsuperscript{24} In subsequent irradiation experiments, 80 nM of each siRNA or a combination of 40 nM of each Atg12 and Atg7 siRNA were transfected into PC3 cells. 24 hr post transfection, cells were subjected to gamma irradiation.

**Clonogenic assay**

The radiosensitivity of PC3 cells was determined using clonogenic assay. After being pretreated with control, siRNA, or statin, cells were subjected to 0, 2, 4, 6, or 8 Gy gamma irradiation. Immediately after irradiation, cells were reseeded at optimal density in 100mm culture dishes, incubating at 37°C for 14 days to allow colony formation. At the end of culture, cells were fixed and stained by 24% ethanol with 0.1% crystal violet. The fraction of surviving cells was calculated as the ratio of the plating efficiency of irradiated cells to that of non-irradiated cells. The Linear Quadratic Model (LQ) was used to calculate the dose survival curve and to determine the dose enhancement ratio (DER). Survival curve parameters \(D_0\) and \(n\) were fitted to the multi-target single hit equation: \(S = 1 – (1 – e^{-aD})^n\). The survival curve parameters \(\alpha\) and \(\beta\) were fitted to the \(\alpha\)-\(\beta\) model: \(S = \exp(-\alpha D – \beta D^2)\).\textsuperscript{25} DER was calculated as the dose (Gy) for radiation alone divided by the dose (Gy) for radiation plus siRNA or statin treatment, at a surviving fraction of 0.37 (Ln = –1).

**Western blotting**

At 24 hr post irradiation or statin treatment, the cell lysate was isolated using M-PER Mammalian Protein Extraction
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Reagent (Thermo Scientific, Rockford, IL). LC3-II formation was determined using western blotting as described previously. Anti-human LC3 antibody (1:400) for detecting both non-lipidated (LC3-I) and lipidated (LC3-II) forms of LC3, anti-human GAPDH antibody (1:1000) (Abgent, San Diego, CA), and donkey anti-rabbit antibody (1:1000) coupled to horseradish peroxidase were used with an ECL Plus kit (GE Healthcare, Pittsburgh, PA). The image was obtained using a Storm 840 PhosphorImager system and further analyzed using the Imagequant TL software (Molecular Dynamics, Sunnyvale, CA).

RT2 profiler PCR array

At 4 hr post irradiation or/and 28 hr post treatment with statin, the total RNA was isolated using RNeasy Kit and treated with DNase A (Qiagen, Valencia, CA). The quality and integrity of total RNA were analyzed using Agilent RNA 6000 Nano Kit in an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The RT2 Profiler PCR array analysis (Qiagen) for the autophagy pathway was performed as described before. The fold change of gene expression was calculated using the Excel-based PCR Array data analysis program (Qiagen).

Annexin V and TUNEL apoptosis assay

After 24 hr treatment with statin, cells were irradiated with 6 Gy of gamma rays. At 24, 48, and 72 hr post irradiation, cells were labeled with Guava Annexin V reagent or fixed with 4% paraformaldehyde for TUNEL assay according to the manufacturer’s protocol. Cells were then analyzed in a Guava flow cytometry system (Millipore, Billerica, MA). Control or Atg7 siRNAs pretreated cells were also treated with statin and/or 6 Gy of gamma rays. The apoptosis rate was determined using the Annexin V assay.

Statistical analysis

The data were presented as mean ± SD from at least 3 independent experiments. The Student t test was performed to determine whether significant differences \((P < .05)\) were achieved between samples with different treatments.

RESULTS

Activation of the autophagy process in irradiated PC3 cells

A typical autophagic cell induced by gamma irradiation was shown in Fig. 1A with the comparison to a non-autophagic non-irradiated cell. The data shown in Fig. 1B indicated that the number of autophagic cells (27% of transfected cells) was significantly increased at 8 hr post gamma irradiation compared with the non-irradiated controls (14%). The number of autophagic cells peaked at 24 hr post irradiation, reaching 45% of transfected cells, and this peak was followed by a slight decrease at 48 hr post irradiation. At 8 hr post irradiation, the percentages of autophagic cells were similar for cells exposed to 2 and 6 Gy of gamma rays, whereas at 48 hr post irradiation, the percentage was lower in cells irradiated with 2 Gy of gamma rays (Fig. 1B).

The number of autophagic cells (35% of transfected cells) was also significantly increased at 24 hr post statin treatment compared with that of controls (10%). The combination of statin and 6 Gy of irradiation did not further increase the fraction of autophagic cells. However, at 48 hr post irradiation, cultures treated with statin alone had a much lower number of autophagic cells (28%) than cultures treated both with statin and irradiated (37%) (Fig. 2).

LC3-II formation

LC3-II expression was induced at 24 hr post irradiation in PC3 cells irradiated with either 2 Gy or 6 Gy of gamma rays (Fig. 3). This result provided conclusive proof of the gamma radiation mediated autophagic activation of PC3 cells. 24 hr pretreatment with statin (6 μg/ml) appeared to be able to induce LC3-II in the cells (Fig. 3). However, irradiation of the cells pre-treated with statin did not seem to further increase the LC3-II protein levels (Fig. 3).
Altered radiosensitivity of PC3 cells with statin or siRNA treatment targeting Atg7 and Atg12

To test whether statin is able to sensitize PC3 cells to radiation therapy, cells were pre-treated with Statin or DMSO for 24 hr. Then, cells were subjected to irradiation and analyzed their radiosensitivity using clonogenic survival assay. The result indicated that statin sensitized the cells to gamma irradiation, showing significantly reduced colony forming efficiency by a factor of 2 in response to 8 Gy irradiation (Fig. 4, DER at survival fraction of 0.37: DMSO = 3.05 and statin = 2.38).

70%–80% of the expression of Atg7 and Atg12 was blocked in PC3 cells after 24 hr of siRNA treatment (Fig. 5A). The cells with reduced expression of Atg7, Atg12, or both demonstrated improved survival rate than the cells treated with a control siRNA (Fig. 5B). Compared to the siRNA treatment targeted at Atg7 or Atg12 alone, the treatment targeted at both genes showed a superior effect in protecting cells from radiation induced cell killing by a factor of 5 at doses of 6 and 8 Gy (Fig. 5B).

Apoptosis induced by chemicals and radiation in PC3

Gamma irradiation alone was unable to induce apoptosis efficiently. A significant fraction of 20% in statin treated PC3 cells became apoptotic at 72 hr after treatment analyzed using the TUNEL assay (Fig. 6). Irradiation of statin treated cells did not induce significant difference (about 3%) in the late phase apoptotic cells (Fig. 6A). However, the Annexin V assay showed that statin + IR induced much more early apoptosis cells at a later time point (96 hr after statin treatment/
To determine whether the activated autophagy pathway involves in the statin mediated apoptosis effect on PC3 cells, cells were pretreated with siRNAs targeting Atg7 to knock down the autophagy pathway. After statin and or irradiation treatment, cells were analyzed using Annexin V assay to determine the apoptosis rate. Statin treated cells showed a slightly increased number of early apoptotic cells (15% of the cells vs. 8% of the controls) at 48 hr after treatment with statin (data not shown). A significantly greater fraction of 40% in statin treated PC3 cells became apoptotic at 72 hr after treatment (Fig. 7). Block of autophagy process using Atg7 siRNAs did significantly suppress the apoptosis inducing effect of statin by 30–40%.

The gene expression profiles of autophagy and its regulatory pathways

The analysis of 84 autophagy related genes revealed that 6 of the genes, Arsa, Atg4b, Esr1, Fadd, Ulk1 and Tgm2, were up-regulated at least 2 fold in PC3 cells at 4 hr post 6 Gy irradiation in both two independent experiments (Table 1). Interestingly, the expression of 10 out of 19 genes associated with autophagic vacuole formation showed significant induction, by a factor of 2 to 20, in statin and/or IR treated
Fig. 7. Percentage of apoptotic cells in control siRNA or Atg7 siRNA treated cells after statin (6 μg/ml) and/or gamma irradiation (6 Gy) treatment. The samples were collected at 48 hr post irradiation and analyzed using the Annexin V assay (mean ± SD) (*p < 0.05 compared to control siRNA (CS) treatment).

Table 1. The list of genes that was up- or down-regulated for a factor of 2 by radiation (IR) or the combination of statin and radiation (Statin + IR) in both two independent experiments. The data were presented as the average of fold changes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Statin</th>
<th>IR</th>
<th>IR + Statin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophagy Machinery Components</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arsa</td>
<td>20.8</td>
<td>2.8</td>
<td>17.4</td>
</tr>
<tr>
<td>Atg16l1</td>
<td>4.3</td>
<td>–</td>
<td>3.7</td>
</tr>
<tr>
<td>Atg4b</td>
<td>5.7</td>
<td>2.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Atg4d</td>
<td>2.1</td>
<td>–</td>
<td>2.7</td>
</tr>
<tr>
<td>Atg9a</td>
<td>4.0</td>
<td>–</td>
<td>4.6</td>
</tr>
<tr>
<td>Atg9b</td>
<td>–</td>
<td>–</td>
<td>2.4</td>
</tr>
<tr>
<td>Gabarap1</td>
<td>6.1</td>
<td>–</td>
<td>5.3</td>
</tr>
<tr>
<td>Map1lc3a</td>
<td>3.9</td>
<td>–</td>
<td>3.1</td>
</tr>
<tr>
<td>Rgs19</td>
<td>2.9</td>
<td>–</td>
<td>3.3</td>
</tr>
<tr>
<td>Ulk1</td>
<td>19.6</td>
<td>2.3</td>
<td>19.1</td>
</tr>
<tr>
<td>Co-Regulators of Autophagy and Apoptosis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bcl2</td>
<td>–</td>
<td>–</td>
<td>2.9</td>
</tr>
<tr>
<td>Cln3</td>
<td>2.2</td>
<td>–</td>
<td>2.4</td>
</tr>
<tr>
<td>Eif2ak3</td>
<td>3.9</td>
<td>–</td>
<td>3.1</td>
</tr>
<tr>
<td>Fadd</td>
<td>2.9</td>
<td>2.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Fas</td>
<td>–</td>
<td>–</td>
<td>–2.0</td>
</tr>
<tr>
<td>Sqstm1</td>
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<td>–</td>
<td>3.8</td>
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<tr>
<td>Tgm2</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>Tgrfb1</td>
<td>2.4</td>
<td>–</td>
<td>2.8</td>
</tr>
<tr>
<td>Trp73</td>
<td>2.7</td>
<td>–</td>
<td>2.4</td>
</tr>
<tr>
<td>Cdkn1b</td>
<td>2.4</td>
<td>–</td>
<td>2.6</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ifna4</td>
<td>3.6</td>
<td>–</td>
<td>3.6</td>
</tr>
<tr>
<td>Esr1</td>
<td>5.1</td>
<td>2.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Mapk14</td>
<td>2.9</td>
<td>–</td>
<td>3.0</td>
</tr>
<tr>
<td>Hsp90aa1</td>
<td>–</td>
<td>–</td>
<td>–2.5</td>
</tr>
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These genes were Arsa, Atg16l1, Atg4b, Atg4d, Atg9a, Atg9b, Gabarap1, Map1lc3a, Rgs19, and Ulk1. Furthermore, the expression of genes encoding 9 regulators of autophagy and apoptosis were also significantly up- or down-regulated (Table 1).

**DISCUSSION**

PC3 cells are radio-resistant and are considered to represent advanced disease. Various types of radiation were found to be ineffective in inducing apoptosis in PC3 cells, as measured by cell cycle, Annexin V, and TUNEL assays (unpublished data). In this study, we provided conclusive evidence on that gamma irradiation failed to effectively induce apoptosis, but it did induce autophagic processes in PC3 cells, shown by both the GFP-LC3 assay and LC3-II western blot analysis. However, even though the GFP-LC3II assay showed that IR alone seemed to be enough to induce autophagic response, both the LC3-II western blot and gene analysis suggested that IR might have only limited effectiveness to induce autophagy. Statin did show strong autophagic activating effect. From GFP-LC3II data, it suggested that IR may help to prolong the autophagic response in the cells pre-treated with statin. Interestingly, the fractions of radiation-induced autophagic cells at 24 hr post irradiation were similar (20%–25%) regardless of the radiation dose (2 Gy or 6 Gy). Similar percentages of radiation induced autophagic PC3 cells were also reported after exposure to 3 Gy of gamma rays. It suggests that a proportion of cells may be more susceptible to radiation/statin mediated autophagy activation.

Various autophagy inducers have been tested to increase the sensitivity of various cancer cells to anticancer agents or radiation, including PC3 cells. Our results showed that statin, as an autophagy inducer, has superior effect in inducing possibly both autophagic and apoptotic cell deaths and, in the meantime, suggested that statin may increase the radiosensitivity of PC3 cells to gamma ray irradiation. Cells treated with a combination of statin and radiation in the present study exhibited a 10%–15% greater number of autophagic cells as well as early apoptotic cells at 96 hr post statin treatment/72 hr post-IR than statin treatment alone, indicating that autophagy induced may contribute to the radiosensitization of PC3 cells.

Though autophagy process affects both cell survival and cell death, the outcome depends on the cell type and the condition of individual cell. We further demonstrated that blockage of autophagy pathways by using siRNAs targeting the Atg7 and Atg12 genes significantly increased the survival fraction of irradiated cells, suggesting that autophagic process contributes more to cell death in PC3 cells. Atg7 is an E1-like enzyme downstream in the autophagy pathway under Beclin-1. It is responsible for conjugating both ubiquitin-like proteins to form complexes, Atg12 to Atg5/Atg16 and LC3.
to phosphatidylethanolamine (PE). Inhibition of the expression of Atg7 alone appeared to be more effective in protecting PC3 cells from radiation exposure than inhibition of Atg12. Our data further indicated that in addition to the LC3/Atg5 conjugation system, the Atg12/Atg5 conjugation system is also important in autophagy mediated cell killing. The combined impairment of expression of Atg7 and Atg12 in the present study showed a synergistic effect in protecting PC3 cells from radiation-induced cell killing. Our data and the results reported by Cao et al. conclusively proved that genes required for autophagosome formation, including Beclin-1, Atg5, Atg7, and Atg12, play a significant role in radiation mediated cell death.

Apel et al. reported that irradiation of cancer cells induced the expression of genes encoding the autophagy machinery components (Atgs) and accumulation of autophagosomes. Analysis of the expression profile of Atgs reveals that PC3 is less responsive than HTB43 to radiation mediated induction of Atg gene expression, indicating that the autophagy pathway in PC3 cells may be suppressed by other mechanisms, such as elevated mTOR activities. In our study, at 4 hr post 6 Gy of gamma irradiation, the expression of Atg4B was increased, by a factor of 2. Atg4B is a cysteine protease, which is essential for the processing of LC3. The mammalian homologs of yeast Atg8, such as LC3 and GABARAP, are specifically cleaved by the Atg4 family of cysteine proteases, allowing their conjugation to phosphatidylethanolamine (PE). Other than Atg4B, the expressions of 3 autophagy regulators, Fadd, Tgm2 and Esr1, were induced in irradiated PC3 cells. While Esr1 is reported as an autophagy regulator possibly through its coactivator PELP1, FADD and TGM2 have been reported to be an autophagy activator and a suppressor, respectively. Thus, even though gamma radiation does induce autophagy in PC3 cells, multiple suppressive mechanisms may contribute to minimize its impact on cell survival.

Autophagy and apoptosis are closely associated and under elegant molecular controls of which the exact mechanism is still unclear. It has been shown that inducing autophagy by mTOR inhibitors, especially in apoptosis defective cells, promotes tumor cell killing. Furthermore, inhibition of Caspase 3/7 induces autophagy and promotes radiosensitivity in vitro and in vivo. It has been reported that radiation resistant cells derived from prostate cancer cells LNCaP, PC3, and DU145 exhibited higher levels of androgen and epidermal growth factor (EGF) receptors and activation of their downstream pathways, such as Ras-mitogen-activated protein kinase (MAPK) and (PI3K)/Akt and Jak/STAT, indicating that the phenotype of radiation resistant prostate cancer cells may involve a suppressed autophagy pathway and activated cell survival mechanism. In PC3 cells, not only is the autophagic machinery suppressed, but also the apoptosis machinery is apparently unbalanced. In our previous study, analysis of expression profiles of genes involved in various apoptosis pathways revealed that none of the 84 genes analyzed was induced in PC3 cells at 4 hr post 10 Gy of X ray irradiation (data not shown). The exclusive non-response of PC3 cells to radiation may be caused by their lack of a stress responsive mechanism, such as the p53 mediated DNA damage response pathway, as well as their elevated basal expression level of genes in the TNF family and the Card family, which are mainly involved in the extrinsic apoptosis pathway. Therefore, manipulation of both autophagy and apoptosis pathways can be a strategy for improving the effectiveness of radiotherapy.

In our study, 22 autophagy related genes were found to be either up- or down-regulated by a factor of 2 after combined treatment with statin and radiation, whereas radiation alone induced upregulation of only 4 of the 84 genes. Of the 19 genes encoding components of the autophagy machinery, 10 were up-regulated (ArsA, Atg16L1, Atg4b, Atg4d, Atg9a, Atg9b, Gabarapl1, Map1lc3a, Rgs19 and Ulk1). None of the genes encoding components of the proapoptotic machinery, such as BAD, BAK1, BAX, BID, BNIP2, CASP3, or CASP8, were expressed as a result of treatment with statin or radiation or both. These gene expression data suggested that the late apoptosis resulting from statin treatment may be an outcome of activated autophagy and autophagy induced cell death. In contrast, the expression of Bcl2, which is a well known blocker for both autophagy and apoptosis pathways, was elevated after treatment with statin and radiation. Several co-regulators for activating or maintaining the autophagy machinery, such as CLN3, EIF2AK3, FADD, and p63, were also upregulated. Ceroid-lipofuscinosis, neuronal 3 (CLN3), which encodes Battenin protein, is important for the normal function of lysosomes by regulating the size and relative acidity (pH). The protein encoded by EIF2AK3 located in the ER and phosphorylates the alpha subunit of eukaryotic translation-initiation factor 2 (EIF2), leading to its inactivation, causing repression of translational initiation and protein synthesis. It is induced by ER stress, which is one of the factors initiating autophagy. This modulation may indicate the effort of cellular machinery to produce various components to meet the requirement to maintain a highly activated autophagic process. The expression of several other key regulators, Tgfβ1, p27 (Cdkn1b), p73, Esr1, Mapk14, and Hsp90aa1, was elevated as well. These regulators are involved as key factors in various signaling pathways to modulate cell survival, cell death, and cell cycle progression. Further investigations have to be extended to determine whether and how these pathways play roles in statin mediated cell killing and in radio-sensitization.

In summary, our present results provided further evidence on radiation mediated autophagy and on the radio-sensitizing effect from activation of the autophagic process by chemicals in PC3 cells. For the first time, we have reported that statin may be capable of radio-sensitizing PC3 cells, indicating that a combined treatment of radiation and an autophagy...
activator, especially statin, may induce synergistic cell killing effect to certain type of cancer cells. Furthermore, our data also indicated that autophagy may be the primary pathway that is responsible for statin mediated cell killing and radiosensitization. Finally, our gene expression data provided information that enables the development of novel, but more effective, combined therapy by alteration of cellular self-defense responses identified in the present study.

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REFERENCES


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