Gamma Irradiation Induces a Caspase-dependent Apoptotic Mechanism in Human Prostate Cancer PC-3 Cells

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Prostate cancer is the most predominant cancer in men and related death rate increases every year. Till date, there is no effective therapy for androgen independent prostate cancer. To investigate the mechanism for cell growth inhibition or apoptosis in human androgen independent prostate cancer PC-3 cells after gamma irradiation. The aim of this study was to examine the potential of gamma irradiation to induce apoptosis in PC-3 cells and to assess the mechanism of gamma irradiation-induced apoptosis. Five different assays were employed in this study: cell proliferation assay, morphological assessments of apoptotic cells, DNA fragmentation analysis, quantification of apoptosis by annexin V (AV) and propidium iodide (PI) staining, and western blot analysis. Cell viability was inversely related to radiation dose. DAPI-positive cells were detected 48 hr after 40 Gy radiation exposure. And nuclear morphological changes of cells were observed by gamma irradiation. DNA ladder patterns in the cells exposed to gamma-radiation were appeared at 24 hr. Also, gamma irradiation induces apoptosis of PC-3 cells via Caspase3, Bax and PARP-dependent fashion.

Key words : Human prostate cancer PC-3 cells, gamma irradiation

Introduction

Death rate of various cancers is increasing more and more worldwide. Prostate cancer is the most predominant cancer in men and related death rate increases every year [2]. Till date, there is no effective therapy for androgen independent prostate cancer. Prostate cancer is lots of occurrence, leading the second of cancer related mortality in American men. But it has been a recent trend in Asia such as Korea and Japan, towards increasing incidences of prostate cancer, reporting a more rapid increase than high risk countries [18]. Unfortunately radical mechanism of prostate cancer is yet not cleared but risk factors with age, race, diet, androgen secretion are associated with this malignancy [10].

Apoptosis is the key biological process for physiological death of eukaryotic cells. It is conserved through evolution and programmed at the genetic level and is necessary for cell recycling, normal tissue homeostasis and regulation of immune system [15]. Morphological changes of apoptosis are usually accompanied by internucleosomal DNA fragmentation [5,24], and produces ladders of DNA fragments that are size of 180-200 bp [8]. Cells have a mechanism converse various extracellular signals to intracellular common signals in apoptotic process. But the mechanism of apoptosis is poorly understood yet, it is generally accepted that many different signal of apoptosis ultimately leads to activation of an endogeneous endonuclease that cuts DNA between the nucleosomes in the linker regions [2,14]. Gamma irradiation has also been shown to inhibit cell proliferation and induce apoptosis in Jurkat T [20] and human erythroleukemia K562 cells [4]. However, the effect of gamma irradiation in human prostate cancer PC-3 cells is still not well understood. Annexin V, belonging to a recently discovered family of proteins, the annexins, with anticoagulant properties has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like phosphatidylserine (PS) in the presence of Ca2+ and shows minimal binding to phosphatidylcholine and sphingomyeline [25]. Changes in PS asymmetry, which is analyzed by measuring Annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost.

Also, one of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The degradation of DNA in the nuclei of apoptotic cells is accom-
plished in a number of ways following activation of caspases [7]. The enzyme poly (ADP-ribose) polymerase, or PARP, was the first protein identified as a substrate for caspases. PARP is involved in repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins [11]. The ability of PARP to repair DNA damage is prevented following cleavage of PARP. The oncogenic protein Bcl-2 which is expressed in membranes of different subcellular organelles protects cells from apoptosis induced by endogenic stimuli. Bax is proapoptotic member of the Bcl-2 family of proteins that is implicated in the pathogenesis of cell death in an increasing number of models of apoptosis both in vivo and in vitro [9]. In particular, Bax has emerged as a mediator of the mitochondrial phase of apoptosis, a process that culminates in the release of cytochrome c from the intermitochondrial space and the activation of effector caspases. Apoptosis is valuable mechanism of the therapy of cancer. Antitumor agents also induce apoptosis in some cancer cells both in vitro and in vivo, indicating that apoptosis plays a very important role in cancer chemotherapy. But reaction mechanism involved in apoptosis induced by a wide variety of cellular stresses was not established yet. The purpose of this study is to elucidate the expression of PARP, Bcl-2, Caspase3, Bcl-2 and Bax in relation to apoptosis induced by gamma irradiation in PC-3 cells.

Materials and Methods

Cell culture and growth condition

Human prostate cancer cell lines, PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained and cultured in DMEM (Hyclone Co., Logan, UT, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Co., Logan, UT, USA), 100 units/ml of penicillin and 100 μl/ml of streptomycin (Hyclone Co., Logan, UT, USA). Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

Cell proliferation assay

PC-3 cells were seeded in 48-well plates at a density of 1×10⁵ cells/well. After 24 hr incubation to allow cell attachment, the cells were exposed to gamma knife radiation. Next, MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-di-phenylterazolium bromide) solution of final concentration 0.5 mg/ml (in phosphate-buffered saline) was added in equal volume (250 μl) to each well and then the plate was incubated for 3-4 hr at 37°C. After the medium was removed, same volume (250 μl) of dimethyl sulfoxide (DMSO) was added to solubilize the MTT-formazan complex. The plate was analyzed on a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm. The cell viability was observed as percentage of control, comparing with one of the untreated cells.

DNA fragment analysis

The apoptotic nature of cells was examined by agarose gel electrophoresis of their nuclear DNA, using the method of Waring [23]. Cells treated with apoptosis inducer in a 100 mm culture dish were lysed in 0.33 ml of buffer containing 5 mM Tris, pH 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X-100. After incubation for 15 mins on ice, samples were centrifuged for 10 mins at 10,000 rpm to separate the intact chromatin (pellet) from the fragmented DNA (supernatant). Pellets were resuspended in 0.33 ml of a buffer containing 10 mM Tris, pH 8.0 and 1 mM EDTA. Pellet and supernatant fractions were separately assayed for DNA content using the diphenylamine reagent containing 1.5% diphenylamine, 1.5% sulfuric acid and 0.008% acetaldehyde in glacial acetic acid. DNA fragmentation was quantified by measuring the ratio of the DNA content in supernatant fraction to the total DNA content (supernatant plus pellet). For visualization of fragmented DNA, the supernatant fraction containing fragmented DNA was extracted two times with phenol and once with chloroform. Extracted DNA fragments was precipitated in 67% ethanol, 0.3 M sodium acetate at -70°C for overnight, and then resuspended in a buffer containing 10 mM Tris, pH 8.0, 1 mM EDTA and 30 μg/ml RNase, prior to electrophoresis in 1.8% agarose gel as described by Jones et al [14].

Morphological changes

DAPI (4’-6-Diamidino-2-phenylindole) stain was used to observe the the apoptotic morphology of PC-3 cells. The cells were cultured in chamber slide for 24 hr at 37°C and adherent cells were exposed to gamma radiation after 48 hr. Next, the cells were fixed with 4% para-formaldehyde for 30 min. Fixed cells were washed with PBS twice and stained with DAPI solution (1 mg/ml) at 4°C for 15 min.
Stained nuclei were visualized using laser confocal microscopy (FV-1000, Olympus, TO, Japan) and the DAPI staining was used to analyze the apoptotic morphological change of cells.

Annexin V bind assay to detect apoptosis cells

After treatment with gamma irradiation, the cells were used to determine the translocation of phosphatidylserine to the outer surface of the plasma membrane during apoptosis using the human phospholipid binding protein, Annexin V, conjugated with fluorescein (Molecular Probes, Inc., Eugene, OR, USA) by flow cytometry as described by the manufacturer. Apoptosis and necrosis were analyzed by quadrant statistics on the propidium iodide-negative, fluorescein isothiocyanate-positive cells, and propidium iodide-positive cells, respectively.

Western blot analysis

Cells were washed three times in cold PBS. Protein content was determined using the Bradford assay. Samples were then diluted with an equal volume of 2× SDS sample buffer and heated for 5 min at 100°C. Samples were loaded to equivalent amount (30 μg/lane) on one-dimensional SDS-polyacrylamide gel and subjected to electrophoresis. Membrane was soaked in methanol for 10 sec and washed in distilled water for 5 min. Blots probed for PARP, Bax, Bcl-2 and Caspase3 were detected with HRP-linked secondary antibodies and enhanced chemiluminescence western blotting reagents (Amersham Pharmacia Biotech, Freiburg, Germany), according to manufacturer’s protocols.

Results

Gamma irradiation reduces cell viability on human prostate cancer cells

To assess cell death or proliferation effect of gamma irradiation, it was observed by MTT assay. The PC-3 cells were exposed to different radiation dose (10-50 Gy) of gamma irradiation. As shown in Fig. 1, gamma irradiation significantly reduced cell viability in a radiation dose-dependent manner and its IC 50 (50% inhibitory concentration) value was measured approximately 40 Gy, after 48 hr. The results presented in Fig. 2 revealed that gamma radiation at 10, 20, 30, 40, and 50 Gy reduced proliferation and survival of PC-3 cells in a post-radiation time-dependent fashion. Significant decreases of gamma radiation-induced cell growth were observed at the radiation dose of 30, 40 and 50 Gy. Also, significant decreases of gamma radiation-induced cell growth were observed at the 48 and 72 hr post-radiation time (Fig. 2). Therefore, gamma radiation treatment of PC-3 cells decreased cell viability in a radiation dose-dependent and post-radiation time-dependent decreased manner. Finally, these data suggested that gamma radiation is potently inhibits the growth of PC-3 cells.
Gamma irradiation affects morphological changes of PC-3 cells

Mode of the PC-3 cells death induced by gamma irradiation was assessed by characteristic morphological criteria. After irradiated with 40 Gy radiation dose of gamma radiation after 48 hr, morphology changes of prostate cancer cells were observed by light microscopy. The control PC-3 cells appeared normal configuration with the spherical, typical adherent and homogeneous cells (Fig. 3A). But, the PC-3 cells exposed to gamma radiation of 40 Gy, after 48 hr showed the characteristic microscopy changes, such as reduction of cell volume, non-adherence and increases of death number (Fig. 3B). To confirmed morphological changes (fragmentation) of PC-3 cells by gamma irradiation, 4'-6-Diamidino-2-phenylindole (DAPI) staining assay was performed. Apoptotic morphological changes were detected by confocal microscopy after DAPI was visualized to be typical apoptotic morphology such as chromatin condensation, nuclear fragmentation and brightness (Fig. 3D).

Gamma irradiation induces apoptosis in PC-3 cells

Using Annexin-V staining, classical early events of apoptotic cell death were detected the translocation of phosphatidylserine (PS) from inner side of cytoplasm membrane to the outer side by determining. As shown in Fig. 4, the PC-3 cells were exposed to 10, 20, 30, 40, and 50 Gy of gamma irradiation and stained with both Annexin V-FITC and propidium iodide at 20°C for 20 min. Then, the significantly changes were observed between the control and gamma irradiation exposed cells by flow cytometry analysis. The dual parameter fluorescent dot plots showed the viable cell population in the lower left quadrant (Annexin V/PI), the cells at early apoptosis in the lower right quadrant (Annexin V'/PI), and the ones at the late apoptosis in the upper right quadrant (Annexin V'/PI'). As indicated in Fig. 4, control cells were abundant in Annexin V/PI negative cells, whereas a lot of exposed cells changed to early and late apoptosis in a radiation dose-dependent manner. As a result, it is suggest that gamma irradiation induces apoptosis phenotypes. DNA fragmentation during apoptosis in the cells irradiated to gamma radiation is shown in Fig. 5. After treatment with 40 Gy gamma radiation, cells were incubated for various times. DNA ladder pattern exposed to gamma radiation was shown at 24 hr.

![Fig. 3. Gamma-radiation induces apoptotic morphological changes in PC-3 cells. The morphological changes were detected by light microscopy (A: control cells, B). After exposed to gamma irradiation (40 Gy), the PC-3 cell showed reduced volume, non-adherence, shirrinkage and increased death number of cells (B). Apoptotic morphological changes were observed by DAPI staining. The control cells (C) were not changed but exposed cells exhibited nuclear change, nuclear fragmentation and brightness by confocal microscopy.](image-url)
Apoptosis related proteins were detected by western blot analysis.

Fig. 6 represents the western blot analysis of the expression of PARP, Caspase3, Bax, and Bcl-2 proteins in PC-3 cells exposed to gamma irradiation for 12, 24, 48 or 72 hr. Gamma irradiation of 40 Gy induced Caspase3 cleavage and activation from 24 hr. Intact 32 kDa Caspase3 and its 20 kDa cleaved products are indicated. As shown in Fig. 6, treatment of the cells with 40 Gy gamma irradiation after 48 hr increased the cleavage of PARP to its 89 kDa active forms in the cells. After treatment of gamma irradiation the PC-3 cells, the increase of the cleavage of the PARP protein by activated Caspase3. The expression of Bcl-2 proteins was persisted until 24 hr with elevated level, and thereafter was disappeared after 48 hr incubation.

These results suggested that Caspase3 or PARP cleavage in the cells affected to apoptosis. The representative apoptogenic factor Bax level increased, but expression of survival factor Bcl-2 was decreased. These results suggest that gamma irradiation induced apoptosis of PC-3 cells via Caspase3-dependent fashion, and this apoptosis is related to disappearance of Bcl-2 proteins.

Discussion

Various physical and chemical agents can induce cell...
death offering some promising strategies for the treatment of cancer. One of the physical agents is gamma-radiation. Numerous studies have focused on the targeted induction of apoptotic cell death in order to control the cancer cells. Gamma irradiation has also been shown to inhibit cell proliferation and induce apoptosis in human leukemic HL-60 and MOLT-4 cells [1]. However, the effect of gamma irradiation in human prostate cancer PC-3 cells is still not well understood. Thus, our study intends to examine the effect of gamma radiation in PC-3 cells. Our results show that gamma radiation treatment of PC-3 cells decreased cell viability in a radiation dose-dependent and post-radiation time-dependent decreased manner. In this study, gamma irradiation produces a significant radiation dose-dependent decrease in cell proliferation and induced an apoptotic-type cell death.

Apoptosis, as described by Wyllie et al. [24], involves the condensation of chromatin, restructuring of the cytoplasm, blebbing of cytoplasmic membranes, and finally fragmentation of the cells into apoptotic bodies that are phagocytosed by neighbouring cells, and features that distinguish the process from necrotic cell death. Apoptosis is initially characterized by morphological changes of dying cells. A number of morphological and biochemical features are typical of apoptosis [27]. Even though the nucleosomal DNA ladders are not always associated with apoptosis, and the morphological changes during apoptosis are associated with double strand cleavage of nuclear DNA at the linker regions between nucleosome. Agarose gel electrophoresis displays the inter-nucleosomal DNA from apoptotic cells in a typical ladder pattern, whereas the DNA cleavage in necrotic cells is random and is seen as a smear [2]. DNA ladder was shown in HL 60 cells after they were treated with 250 J/m² UV [26]. Gamma irradiation has been reported to induce apoptosis in human glioma cells [11,12] and esophageal squamous cell carcinoma lines [21]. DNA ladder by various concentrations of gamma irradiation was observed. In the present study, DNA ladder induced by treatment with gamma irradiation in PC-3 cells was shown at 24, 48 or 72 hr.

Apoptosis can be triggered in various ways [17]. The caspases have cleverly targeted the proteins within signaling pathways that will assist in their purpose to destroy the cell [9]. Thus death receptor mediated caspase cleavage of substrates can engage the mitochondria to amplify the pro-apoptotic signal [6]. Caspase3 had been perceived as the principle enzyme responsible for clearing PARP [15,16]. PARP plays the active role of "nick sensor" during DNA repair and apoptosis, when it synthesizes ADP-ribose from NAD⁺ in the presence of DNA strand breaks. Moreover, PARP becomes a target of apoptotic caspases, which originate two proteolytic fragments of 89 and 24 kDa [19]. Gamma irradiation treatment demonstrated that the expression of Caspase3 and PARP cleaved in post radiation time-dependent manner. Bax proteins is involved in apoptosis and prevents the accumulation of chromosomal damage. It has been reported that Bax proteins expression was increased in a benign disease and apoptosis. In this study, gamma irradiation treatment induced the increase of Bax proteins. Also, Overexpression of Bcl-2 is known to convey resistance to apoptosis induced by many agents [11,16]. Considering previous reports by others and the present data, the Bcl-2 proteins, when overexpressed, are able to block apoptosis. The results presented here indicate that gamma irradiation is capable of killing PC-3 cells through apoptosis mechanism. In conclusion, all observations indicate that gamma irradiation may provide a superior therapeutic index and advantage in the clinic for the treatment of PC-3 cells.

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