J. Radiat. Res., 48, 435–441 (2007)

Regular Paper

Identification of Proteins that Regulate Radiation-induced Apoptosis in Murine Tumors with Wild Type p53

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Radiosensitivity/Apoptosis/Murine tumor/Proteomics.

In this study, we investigated the molecular factors determining the induction of apoptosis by radiation. Two murine tumors syngeneic to C3H/HeJ mice were used: an ovarian carcinoma OCa-I, and a hepatocarcinoma HCa-I. Both have wild type p53, but display distinctly different radiosensitivity in terms of specific growth delay (12.7 d in OCa-I and 0.3 d in HCa-I) and tumor cure dose 50% (52.6 Gy in OCa-I and > 80 Gy in HCa-I). Eight-mm tumors on the thighs of mice were irradiated with 25 Gy and tumor samples were collected at regular time intervals after irradiation. The peak levels of apoptosis were 16.1 ± 0.6% in OCa-I and 0.2 ± 0.0% in HCa-I at 4 h after radiation, and this time point was used for subsequent proteomics analysis. Protein spots were identified by peptide mass fingerprinting with a focus on those related to apoptosis. In OCa-I tumors, radiation increased the expression of cytochrome c oxidase and Bcl2/adenovirus E1B-interacting 2 (Nip 2) protein higher than 3-fold. However in HCa-I, these two proteins showed no significant change.

The results suggest that radiosensitivity in tumors with wild type p53 is regulated by a complex mechanism. Furthermore, these proteins could be molecular targets for a novel therapeutic strategy involving the regulation of radiosensitivity.

INTRODUCTION

Ionizing radiation has long been used as a major cancer therapy. While such therapy has contributed to improved tumor control, increasing the tumor response to radiation has been a major challenge to the field of radiation oncology.

Accumulation of knowledge of cellular and molecular biology has been associated with an increased understanding of how cells respond to radiation and the possibility of manipulating the radioresponse of cells. While mammalian cells show a diversity of responses from cytoprotective to cytotoxic over a range of radiation doses, higher doses of radiation predominantly generate death signals. In addition to reproductive cell death, apoptosis is a major mode of cell killing by ionizing radiation.[1,2)]

There have been a number of reports supporting the proposal that apoptosis plays an important role in determining radiosensitivity.[3–6)] Loss of apoptotic pathways contributes to the development of tumors by enhancing survival of cells, and can also result in resistance to antitumor treatment. Radiation-induced apoptosis varies among different tumors and positively correlates with the antitumor efficacy of radiation, making apoptosis a potential predictor of therapeutic outcome after radiotherapy.[7)] Furthermore, regulation of the induction of apoptosis might be used to improve the therapeutic index through either increasing the apoptotic response of tumors or inhibiting that of the normal tissues.

Apoptosis is regulated by a number of genes.[8–10)] One key molecule, p53, is an important determinant in apoptosis induction by radiation, and hence regulates cellular susceptibility to radiation.[10,11)] Numerous reports have shown that p53 mutation is associated with poor treatment outcome; however, the overall results are heterogeneous with many contradictory reports, suggesting that a complex regulatory mechanism might exist downstream of p53.[12)]

Radiosensitivity is a multifactorial characteristic, and searching for molecules significantly associated with radiosensitivity using proteomics might be expected to yield a complex list of proteins. However, when the analysis is limited to radiation-induced apoptosis, the interpretation is simpler and more meaningful. In this study, we used proteomics technology to investigate proteins that determine the level of

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This work was presented at the 52nd Annual Meeting of the Radiation Research Society Denver CO, Oct 16-19, 2005.
doi:10.1269/jrr.07015

radiosensitivity in terms of induced apoptosis in murine tumors with the same p53 status but with contrasting radiosensitivity.

MATERIALS AND METHODS

Animals and tumors

The study has been reviewed and approved by the committee that oversees the ethics of research involving the use of animals and the welfare of the animals. The study involved 8–10 week old male C3H/HeJ mice that were bred in our specific pathogen-free mouse colony in the Division of Laboratory Animal Medicine, Yonsei University College of Medicine. The temperature and humidity were maintained at 22°C and 55%, respectively, with water and food *ad libitum*. The care and use of animals was based on the guidelines and regulations of Yonsei University. Two murine carcinomas syngeneic to C3H/HeJ were used: an ovarian carcinoma OCa-I, and a hepatocarcinoma HCa-I. These two tumors have been reported to have the same wild type p53[13] but distinctly different radiosensitivity; In OCa-I and HCa-I, the specific growth delays were 12.7 days and 0.3 days,[3] and TCD50s were 52.6 Gy and > 80 Gy, respectively.[14] Solitary tumors were generated by inoculating 1×10^6 viable tumor cells into the muscles of the right thigh of the mice. Tumor cell suspensions were prepared by mechanical disruption and enzymatic digestion of non-necrotic tissue.[15]

Analysis of apoptosis

Animals with tumors were given a single dose of 25 Gy radiation using clinical linear accelerator (Varian Co. Milpitas, CA). Tumor samples were collected and apoptosis was assessed in tissue sections. The tumors were immediately excised and placed in neutral buffered formalin. The tissues were embedded in paraffin blocks and 4-μm sections were cut and stained with the Apoptag staining kit (Chemicone, Temecula, CA, USA). Apoptotic cells were scored on coded slides at 400X magnification according to the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method. TUNEL-positive cells were considered apoptotic only when associated with apoptotic morphology as previously described and illustrated.[16] Ten fields of non-necrotic areas were selected randomly across each tumor section and in each field apoptotic bodies were expressed as a percentage of 1000 nuclei.

Protein preparation and analysis by 2-DE (2 dimensional electrophoresis)

Protein preparation and 2-DE were performed according to the method previously described.[17] In brief, aliquots of tumor proteins in sample buffer were applied to immobilized pH 3–10 nonlinear gradient (IPG) strips (Bio-Rad). The first dimensional isoelectric focusing (IEF) was performed at 90,000 Vh after which the strips were equilibrated for 10 min in equilibration buffer containing 6 M urea, 2.5% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v) DTT, 5 mM tributylphosphine, 50 mM Tris-HCl (pH 6.8) and 20% (v/v) glycerol (Sigma). The second dimensions were analyzed on 9%–18% linear gradient polyacrylamide gels using the Protean XL system (Bio-Rad) at 20°C. After electrophoresis, the gels were fixed in 40% methanol and 5% phosphoric acid, and stained with Coomassie blue G 250 (Bio-Rad) for 24 h.

Table 1. Radiation-induced apotosis in murine tumors following a single dose of 25 Gy.

Time after radiation (h)	0	4	12	24
OCa-I	1.8 ± 0.6%	16.1 ± 0.6%	8.3 ± 1.2%	3.7 ± 1.1%
HCa-I	0.1 ± 0.0%	0.2 ± 0.0%	0.2 ± 0.1%	0.0 ± 0.1%

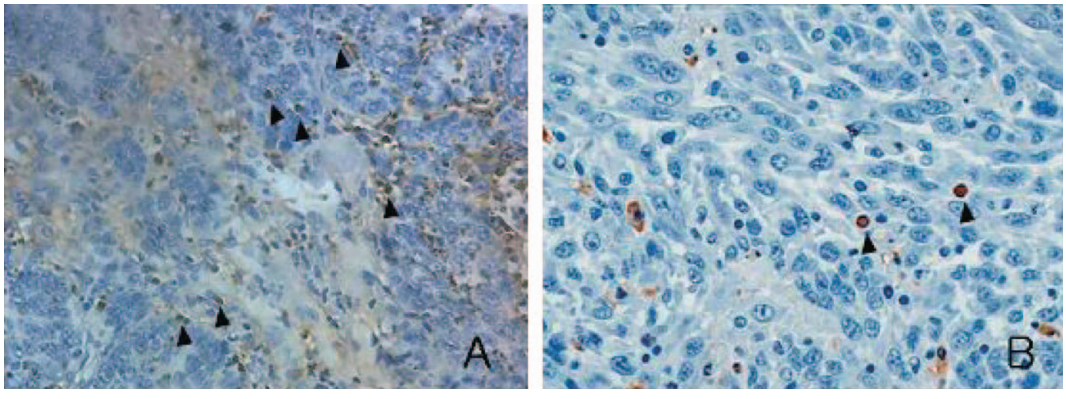

Fig. 1. Radiation-induced apoptosis in murine tumors, OCa-I (A) and HCa-I (B), detected by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method. The arrows indicate representative apoptotic cells.

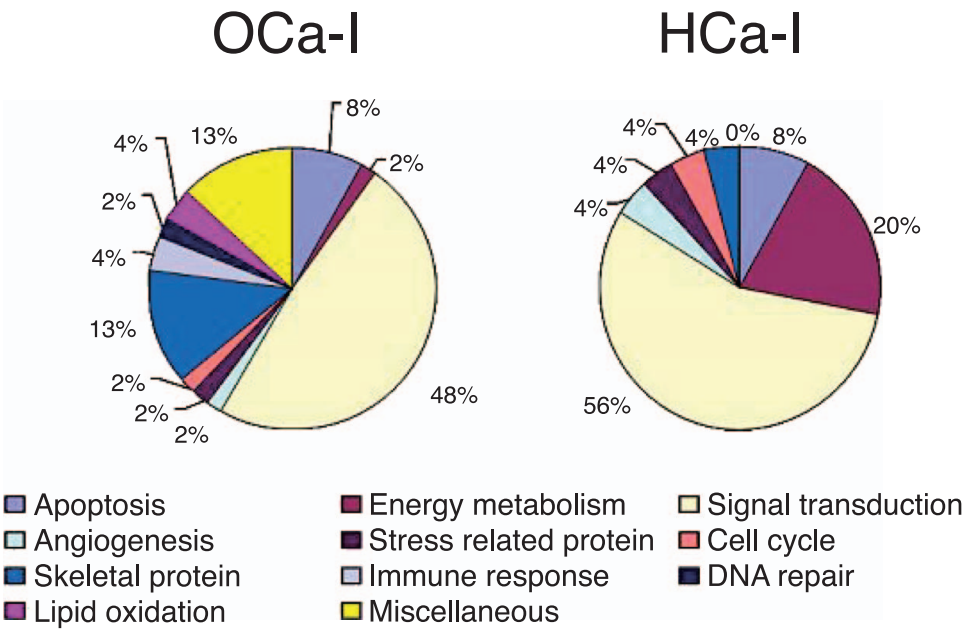

Fig. 2. Categorization of proteins by function in murine tumors, OCa-I and HCa-I at 4 h after 25 Gy radiation. The quantitative proportions are shown.

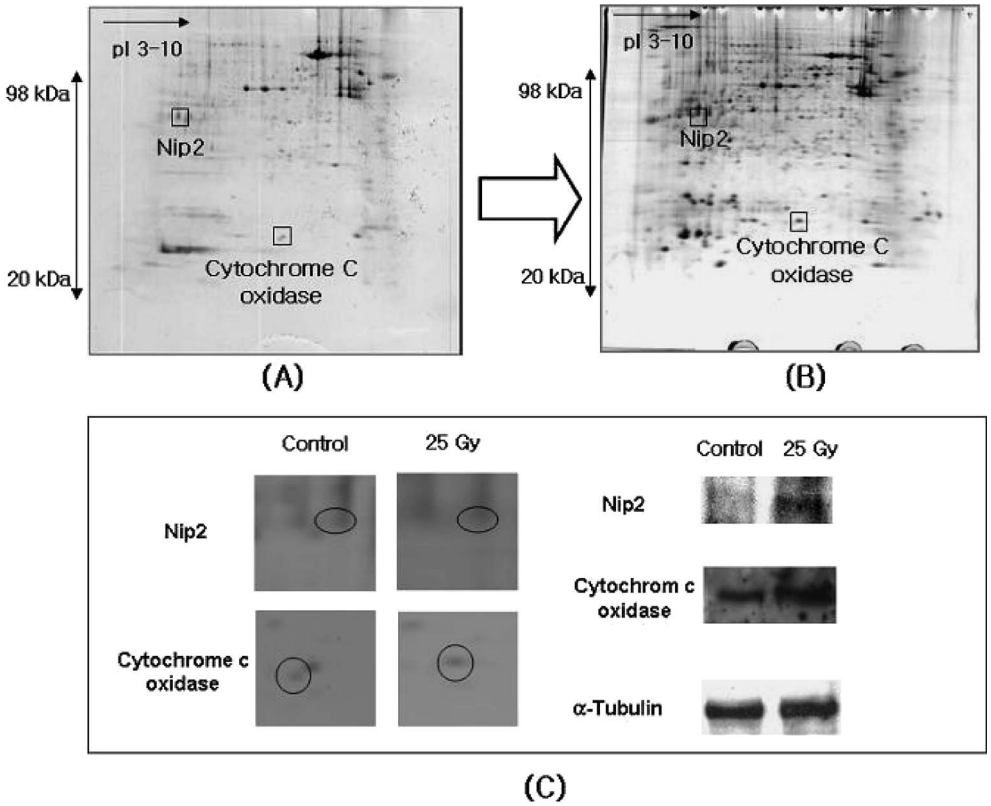

Fig. 3. (A) 2-DE (pI 3-10) image in OCa-I control (left) and (B) OCa-I at 4 h after 25 Gy radiation (right) and (C) validation by western blotting for Nip2 and cytochrome c oxidase. Partial 2-DE images for Nip2 and cytochrome c oxydase are shown. The proteins from the OCa-I tumor tissue was extracted and separated on pH 3–10 nonlinear immobilized pH-gradients strip, follwed by 9–18% polyacrylamide gel. The gel was stained with Coomassie brilliant blue G250.

The stained gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). The digitized gel images were normalized and comparatively analyzed using the PDQUEST program (v6.2, Bio-Rad). The percentage spot volume representing a certain protein was determined by comparison with the protein present in the 2-demensional gels.

Protein identification by MALDI-TOF

For mass spectrometry fingerprinting, protein spots were cut out of the gels, destained with 50% acetonitrile in 25 mM ammonium bicarbonate and dried in a speed vacuum concentrator (Savant, Pleasanton, CA). Dried gel pieces were reconstituted with 50 mM ammonium bicarbonate (pH 8.0) containing 100 ng/μl trypsin (Promega, Lyon, France) and incubated at 37°C for 17 h. Supernatant peptide mixtures were extracted with 50% acetonitrile with 5% trifluoroacetic acid (TFA) and dried in a speed vacuum concentrator. The peptide mixtures were then dissolved in 4 μl 50% acetonitrile and 0.1%TFA. Aliquots of 0.5 μl were applied to a large disk and allowed to air-dry. A matrix of α-cyano-4-hydroxycinamic acid (Sigma) in 50% acetonitrile, containing 0.1% TFA was used to obtain the spectra using a MALDI-TOF mass spectrometer (Micromass, Manchester, UK).

Database analysis

A protein database search was performed using MS-Fit (the UCSF mass spectrometry database, ProteinProspector v 4.0.5) and MOSCOT program. The search parameters were set up as follows: the database was Swiss-Prot and NCBInr. 10.21.2003; the mass tolerance was 50 ppm; the number of missed cleavage sites allowed was up to 1; the minimum number of matched-peptides was 4; species selected was mammals; and monoisotope masses were used.

Western blot analysis

Western blotting was performed according to the method previously described using antibodies targeting Nip2 (Santa Cruz, USA) and cytochrome c oxydase (Santa Cruz, USA). Antibodies were used at the dilution recommended by the manufacturer and detectable proteins were quantitated by densitometry (Amersham Pharmacia Biotech, USA) after chemiluminescence detection (Fuji photo film, Japan) using the ECL western blot detection system (Amersham Pharmacia Biotech).

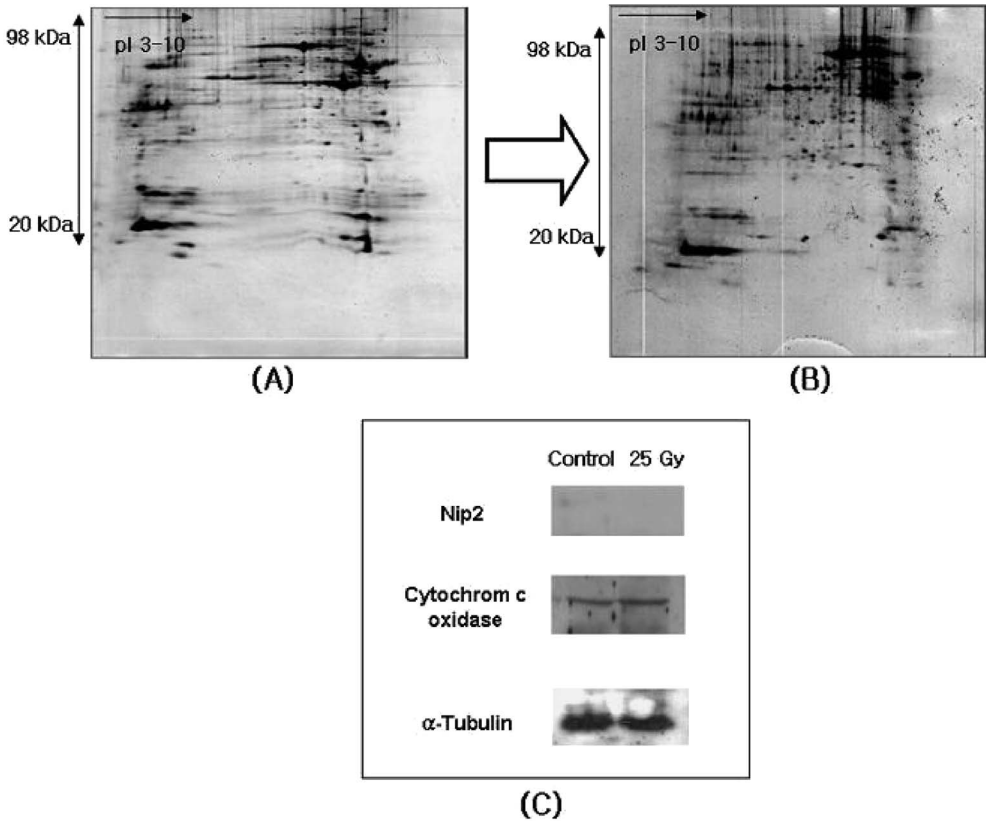

Fig. 4. (A) 2-DE (pI 3-10) image in HCa-I control (left) and (B) HCa-I at 4 h after 25 Gy radiation (right), (C) validation by western blotting for Nip2 and cytochrome c oxidase (Partial 2-DE image in HCa-I, data was not shown). The proteins from the HCa-I tumor tissue was extracted and separated on pH 3–10 nonlinear immobilized pH-gradients strip, follwed by 9–18% polyacrylamide gel. The gel was stained with Coomassie brilliant blue G250.

Table 2. Proteins that showed radiation-induced changes in expression.

	Proteins in signal transduction	
OCa-I :	↓	Growth arrest specific 2
	↓	Zinc-binding protein 1
	↓	Protein phosphatase-1 regulatory subunit 7 beta 1
	↓	Mitogen activated protein kinase p38-2
	↑	Myogenic factor 5
	↑	Nucleotide phosphodiesterase 7 B1
HCa-I :	↓	Lysosomal di-N-acetylchitobiase precursor
	↓	Zinc finger protein 177
	↓	Sorting nexin 12
	↓	Rab-related GTP-binding protein RabJ
	↓	Elk protein
	↓	Testis expressed gene 27
	↑	Ras related protein Rab-12
	↓	Mitogen-activated protein kinase kinase
	↓	BHLHZip transcription factor BIGMAX beta
	↓	ADP-ribosylation factor binding protein
	Proteins in apoptosis and energy metabolism	
OCa-I :	↑	Cytochrome C oxidase
	↓	28S ribosomal protein S9 mitochondrial precursor (MRP-S9)
	↑	Oxysterol binding protein-like protein 3
	↑	BCL2/adenovirus E1B19 kDa protein interacting protein
HCa-I :	↓	Tumor protein 63kDa with strong homology to p53
	↓	Leptin precursor
	↓	Long chain fatty acid CoA ligage
	Proteins in angiogenesis, cell cycle, stress related protein	
OCa-I :	↓	Insulin-like growth factor binding protein 1 precursor
	↓	Cyclin D
	↓	Heat shock protein
HCa-I :	↓	CYR61 protein
	↓	Tumor repeat associated with PCTAIRE 2
	↓	Hypertension-associated protein SA
	Regulatory cytokines and proteins in DNA repair , immune response	
OCa-I :	↓	Biglycan protein
	↓	Vascular endothelial junction-associated molecule
	↓	Xeroderma pigmentosum group A correcting protein
	↓	Phosphateacetidyl transferase
	↑	Ig heavy chain V region
	↓	MHC I class II -A beta q chain beta 1 domain

RESULTS

Radiation-induced apoptosis in murine tumors

Both tumors were positive for wild type p53. The TUNEL assay revealed distinct staining of apoptotic nuclei and showed different levels of radiation-induced apoptosis in the two tumors (Fig. 1). The different response to radiation in these tumor tissues reflected different levels of radiosensitivity. The peak level of apoptosis was $16.1 \pm 0.6\%$ for OCa-I and $0.2 \pm 0.0\%$ for HCa-I, at 4 h after radiation. Therefore, the apoptotic response was dramatically increased at 4 h after radiation in OCa-I but not in HCa-I.

Proteomic analysis

Approximately 800 protein spots were visualized on each gel using a modified Coomasie blue stain that permits mass spectrometric analysis of visualized proteins. A total of 149 protein spots were excised by in gel digestion and analyzed by MALDI-TOF MS. The remaining spots were not excised as their expression levels were below the detection limit of MALDI-TOF MS. The MS-Fit database was used to identify the peptide mass data, and the protein spots that showed significant difference between the control and radiation group were identified. Proteins were classified according to their functions: apoptosis, signal transduction, angiogenesis, stress related protein, cell cycle, skeletal protein, immune response, DNA repair, lipid oxidation and energy metabolism (Fig. 2).

For this study we focused on those associated with apoptosis, and noted a significant difference between the control and the radiation group in the expression of 2 proteins; cytochrome c oxidase and Bcl2/adenovirus E1B-interacting 2 (Nip 2) proteins. In OCa-I, radiation increased the expression of cytochrome c oxidase and Nip 2 higher than 3-fold; this change was validated through western blotting (Fig. 3). However in HCa-I, these two proteins showed no significant change(Fig. 4).

DISCUSSION

The results in this study show that tumors with the same wild-type p53 status differ in the regulation of radiation-induced apoptosis. Since p53 is such an important regulator of apoptosis and is frequently mutated in many tumors, a tempting therapeutic approach is to restore wild type p53 function and reverse the resistance to apoptosis. Experimental genetic approaches to restore a normal p53 gene have been attempted through adenovirus-mediated transduction of wild type p53.[18,19] Reinstitution of wild type p53 protein function has also been attempted through pharmacological intervention.[20] However, a number of reports have addressed the presence of p53-independent apoptosis, suggesting complex mechanisms underlying the death pathway.[21–24]

The murine tumor models in the present study represent an example of significantly different levels of apoptosis in tumors with the same p53 status, suggesting that radiation-induced apoptosis in tumors with wild type p53 is regulated through a complex mechanism independent of p53.

Two major pathways have been identified for the induction of apoptosis; the extrinsic or receptor-mediated pathway, and the intrinsic or mitochondrial pathway.[25,26] While the two pathways have independent initiating caspases, they share the effector caspases that execute the final process of cell death. The mitochondrial intrinsic pathway of apoptosis is known to be triggered by stress signals involving DNA damage, hypoxia, and loss of survival signals. Ionizing radiation, a major DNA damaging agent, might elicit induced apoptosis through this pathway. The p53 tumor suppressor gene can activate both the death receptor and mitochondria-signaled forms of apoptotic cell death in response to diverse stimuli.

In this study, the post-radiation increase of cytochrome c oxidase and BNip2 proteins in OCa-I is associated with a high level of radiation-induced apoptosis. BNip2 belongs to a group of recently identified proteins called BNips, pro-apoptotic members of the Bcl-2 family.[27,28] These proteins are central regulators of mitochondrial membrane permeability and induce both apoptotic and necrotic cell death. Proapoptotic activity of the BNip proteins likely occurs through interaction with pro-survival Bcl-2 and Bcl-XL proteins, and integration into the mitochondrial outer membrane. Compromise of mitochondrial permeability may release proapoptotic molecules such as cytochrome c, Apaf-1, and procaspase-9. These molecules form a complex, apoptosome, in the cytosol and autocatalyze caspase-9, ultimately leading to activation of downstream effector caspases.[29] However, the exact mechanisms by which the proapoptotic Bcl-2 family proteins, including BNips, cause cell death remain to be elucidated.

Cytochrome c oxidase is known to be a regulator of mitochondrial respiration[30,31] and is also involved in the intrinsic apoptotic pathway.[32] It has been suggested that oxidative stress induces release and reuptake of Ca2+ from mitochondria resulting in mitochondrial destabilization and ultimately apoptosis. In this process, nitric oxide binds to cytochrome c oxidase and blocks respiration causing mitochondrial de-energization and Ca2+ release.

The proteins identified in this study have not previously attracted attention in relation to radiation-induced apoptosis. As in most diseases involving multiple genes in their molecular mechanism, radioresponsiveness of tumors could be regulated through complex genetic communication. Use of a proteomics approach reveals a spectrum of new molecules, and may help to elucidate this complex molecular network. The significance of the proteins identified in this study with respect to radiosensitivity and apoptosis needs to be investigated further using different cellular systems.

ACKNOWLEDGEMENT

This work was supported by the Korea Science and Engineering Foundation grant R01-2006-000-10084-0 funded by the Korea government (MOST) and the grant 2007-00299 through its National Nuclear Technology Program.

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Received on February 14, 2007
1st Revision received on April 20, 2007
2nd Revision received on May 30, 2007
Accepted on June 29, 2007
J-STAGE Advance Publication Date: August 23, 2007