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

Jinsil SEONG1,2,3*, Hae Jin OH1,2, Jiyoung KIM1,2, Jeung Hee AN1,2 and Wonwoo KIM1,2

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 radiresistance of cells. While mammalian cells show a diversity of responses from cytotoxic 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...
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 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 × 10⁶ 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 (Chemicon, 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, 50 mM Tris-HCl (pH 6.8) and 20% (v/v) glycercol (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 apoptosis 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 (pl 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 oxidase are shown. The proteins from the OCa-I tumor tissue was extracted and separated on pl 3–10 nonlinear immobilized pH-gradients strip, followed by 9–18% polyacrylamide gel. The gel was stained with Coomassie brilliant blue G250.

J. Radiat. Res., Vol. 48, No. 5 (2007); http://jrr.jstage.jst.go.jp
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-dimensional 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 oxidase (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).

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**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, followed by 9–18% polyacrylamide gel. The gel was stained with Coomassie brilliant blue G250.
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 ± 0.6% for OCa-I and 0.2 ± 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 Reintroduction 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. 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-signal 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. 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. 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 and is also involved in the intrinsic apoptotic pathway. 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 deenergization 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, radiosensitivity 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.

REFERENCES

1. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C. and Hooper, M. L. (1993) Thymocyte apoptosis is induced by p53-dependent and independent pathways. Nature 362: 849–852.
2. Lowe, S. W., Schmitt, H. E., Smith, S. W., Osborne, B. A. and Jacks, T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362: 847–849.
3. Stephens, L. C., Ang, K. K., Schultheiss, T. E., Milas, L. and Meyn, R. E. (1991) Apoptosis in irradiated murine tumors. Radiat. Res. 127: 308–316.
4. Meyn, R. E., Stephens, L. C., Hunter, N., Ang, K. K. and Milas, L. (1994) Reemergence of apoptotic cells between fractionated doses in irradiated murine tumors. Int. J. Radiat. Oncol. Biol. Phys. 30: 619–624.
5. Story, M. D., Voeringer, D. W., Malone, C. G., Hobbs, M. L. and Meyn, R. E. (1994) Radiation-induced apoptosis in sensitive and resistant cells isolated from a mouse lymphoma. Int. J. Radiat. Biol. 66: 659–668.
6. Bristow, R. G., Benchmol, S. and Hill, R. P. (1996) The p53 gene as a modifier of intrinsic radiosensitivity; implication for radiotherapy. Radiother. Oncol. 40: 197–223.
7. Meyn, R. E., Stephens, L. C., Ang, K. K., Hunter, N. and Milas, L. (1993) Heterogeneity in the development of apoptosis in irradiated murine tumors of different histologies. Int. J. Radiat. Biol. 64: 583–591.
8. Korsmeyer, S. J. (1992) Bcl-2: a repressor of lymphocyte death. Immunol. Today 13: 285–288.
9. Wyllie, A. H. (1992) Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. Cancer Metastasis Rev. 11: 95–103.
10. Kastan, M. B., Canman, C. E. and Leonard, C. J. (1995) p53, cell cycle control and apoptosis: implications for cancer. Cancer Metastasis Rev. 14: 3–15.
11. Fei, P. and El-Deiry, W. S. (2003) p53 and radiation responses. Oncogene 22: 5774–5783.
12. Mashima, T., Ob-hara, T., Sato, S., Mochizuki, M., Sugimoto, Y. and Yamazaki, K. (2005) p53-defective tumors with functional apoptosome-mediated pathway; a new therapeutic target. J. Natl. Cancer Inst. 97: 765–777.
13. Seong, J., Hunter, N. and Milas, L. (1997) Induction of apoptosis and expression of apoptosis-related gene products in response to radiation in murine tumors. J. Korean Soc. Ther. Radiol. Oncol. 15: 187–195.
14. Milas, L., Wike, J., Hunter, N., Volpe, J. and Basic, I. (1987) Macrophage content of murine sarcomas and carcinomas: associations with tumor growth parameters and tumor radio- curability. Cancer Res. 47: 1069–1075.
15. Milas, L., Hunter, N. and Mason, K. (1974) Immunological...
resistance to pulmonary metastases in C3Hf/Bu mice bearing syngeneic fibrosarcoma of different sizes. Cancer Res. 34: 61–71.

16. Milas, L., Hunter, N. and Kurdoğlu, B. (1995) Kinetics of mitotic arrest and apoptosis in murine mammary and ovarian tumors treated with Taxol (paclitaxel). Cancer Chemother. Pharmacol. 325: 297–303.

17. An, J. H. and Seong, J. (2006) Proteomics analysis of apoptosis-regulating proteins in tissues with different radiosensitivity. J. Radiat. Res. 47: 147–155.

18. Fujiwara, T., Cai, D. W., Georges, R. N., Mukhopadhyay, T., Grimm, E. A. and Roth, J. A. (1993) Therapeutic effect of a retroviral wild-type p53 expression vector in an orthotopic lung cancer model. J. Natl. Cancer Inst. 86: 1458–1462.

19. Manzano, C. G., Fueyo, J., Kyritsis, P., Steck, P. A. and Roth, J. A. (1996) McDonnell, Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma cells via apoptosis. Cancer Res. 56: 694–699.

20. Milner, J. (1995) DNA damage, p53 and anticancer therapies. Natl. Med. 1: 879–881.

21. Yu, Y. and Little, J. B. (1998) p53 is involved in but not required for ionizing radiation-induced caspase-3 activation and apoptosis in human lymphoblast cell lines. Cancer Res. 58: 4277–4281.

22. Eriksson, T. B., West, C. M. L., Cvetskovska, E., Svensson, M., Karlsson, E., Magnusson, B. and et al. (1999) The lack of correlation between proliferation (Ki-67, PCNA, LI, Tpot), p53 expression and radiosensitivity for head and neck cancer. Br. J. Cancer 80: 1400–1404.

23. Hara, S., Nakashima, S., Kiyono, T., Sawada, M., Yoshimura, S., Iwama, T. and et al. (2004) p53-independent ceramide formation in human glioma cells during γ-radiation-induced apoptosis. Cell Death Differ. 11: 853–861.

24. Afshar, G., Jelluman, N., Yang, X., Basila, D., Arvold, N. D., Karlson, A. and et al. (2006) Radiation-induced caspase-8 mediates p53-dependent apoptosis in glioma cells. Cancer Res. 66: 4223–4231.

25. Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. (1999) Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol. 15: 269–290.

26. Hajra, K. M. and Liu, J. R. (2004) Apoptosome dysfunction in human cancer. Apoptosis 9: 691–704.

27. Zhou, Y. T., Soh, Y. J. K., Shang, X., Guy, G. R. and Low, B. C. (2002) The BNIP-2 and cdc42GAP homology/sec14p-like domain of BNIP-St6 is a novel apoptosis-inducing sequence. J. Biol. Chem. 277: 7483–7492.

28. Qin, W., Hu, J., Guo, M., Xu, J., Li, J., Yao, G. and et al. (2003) BNIPL-2, a novel homologue of BNIP-2, interacts with Bcl-2 and cdc42GAP in apoptosis. Biochem. Biophys. Res. Comm. 308: 379–385.

29. Zhang, H. M., Cheung, P., Yanagawa, B., McManus, B. M. and Yang, D. C. (2003) BNips: A group of pro-apoptotic proteins in the Bcl-2 family. Apoptosis 8: 229–236.

30. Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., Van der Meer, R. and Tager, J. M. (1982) Quantification of the contribution of various steps to the control of mitochondrial respiration. J. Biol. Chem. 257: 2754–2757.

31. Yoshikawa, S. (2002) Cytochrome c oxidase. Adv. Protein Chem. 60: 341–395.

32. Kadenbach, B., Arnold, S., Lee, I. and Huttemann, M. (2003) The possible role of cytochrome C oxidase in stress-induced apoptosis and degenerative disease. Biochim. Biophys. Acta. 1655: 400–408.