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Proteomics Analysis of Apoptosis-regulating Proteins in Tissues with Different Radiosensitivity

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Radiation/Proteomics/Apoptosis/Radiosusceptibility.

The aim of this study was to identify of radiosusceptibility proteins in tissues with different radiosensitivity. C3H/HeJ mice were exposed to 10 Gy. The tissues were processed for proteins extraction and were analyzed by 2-dimensional electrophoresis. The proteins were identified by matrix-assisted laser desorption ionizing time-of-flight mass spectrometry and validated by immunohistochemical staining and Western blotting. The peaks of apoptosis levels were 35.3 ± 1.7% and 0.6 ± 0.2% in the spleen and the liver, respectively, after ionizing radiation. Analysis of liver tissue showed that the expression level of ROS related proteins such as cytochrome c, glutathione S transferase, NADH dehydrogenase and peroxiredoxin VI increased after radiation. The expression level of cytochrome c increased to 3-fold after ionizing radiation in both tissues. However in spleen tissue, the expression level of various kinds of apoptosis regulating proteins increased after radiation. These involved iodothyronine, CD 59A glycoprotein precursor, fas antigen and tumor necrosis factor -inducible protein TSG-6nprecursor after radiation. The difference in the apoptosis index between the liver and spleen tissues is closely associated with the expression of various kinds of apoptosis-related proteins. The result suggests that the expression of apoptosis-related protein and redox proteins play important roles in this radiosusceptibility.

INTRODUCTION

Radiosensitivity is observed in a tissue-specific manner according to different level of differentiation, rate of proliferation and regulation mechanism of the particular tissue.[1,2] Radiosensitive responses have shown to be higher in tumor tissues than in normal tissues, in undifferentiated cells than in highly differentiated cells, and in epithelial cells than in mesenchymal cells.[3] Responses by the same cells and tissues, however, are reported to vary due to various factors such as the amount of radiation absorbed dose by the cells, oxygen partial pressure, biological and environmental conditions of cells.[4] Genetic conditions such as the chromosome instability syndrome, ataxia-telangiectasia (AT), fanconi anaemia (FA) and bloom syndrome have been reported to be highly sensitive to ionizing radiation.[5,6]

The liver is resistant to ionizing radiation: the liver cells do not proliferate during the steady state but does so when stimulated by radiation or toxic agent.[7] On the other hand, the pluripotent stem cells of the spleen undergo proliferation and differentiation to produce various committed progenitor cells.[8] Therefore, the spleen appears to be comparatively more radiosensitive than the liver.[9]

In this study, we used the proteomics approach for the identification of proteins that are regulated according to the degree of radio-susceptibility in the tissue following radiation. The observed patterns indicate apoptotic proteins that may be ultimately correlated with radiosensitivity.

MATERIAL AND METHODS

Animals and Irradiation

Animals and Irradiation: 8 to 10-week-old male C3H/HeJ mice were used in this study. They were bred and maintained with five animals per cage, in our specific pathogen-free mouse colony at the Division of Laboratory Animal Medicine. The temperature (22°C) and humidity (55%) were constantly maintained, and water and diet were supplied *ad libitum*. The care and use of animals in the present study were based on the Guidelines and Regulations for the Use and Care of Animals at Yonsei University.

Animals were irradiated using a linear accelerator (Varian Co. Milpitas, CA) with a dose rate of 0.25 Gy/min without anesthesia. Whole body irradiation was done with a single dose of 10 Gy. For the collection of liver and spleen tissues, animals were killed at 0, 4, 8, and 24 after radiation treatment. Each spleen or liver was cut into two halves: one

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half was fixed in 10% neutral formaline, paraffin-embedded and 4 μm sections were stained with apoptoisis analysis. The other half of the organ was analyzed proteomics assay.

Analysis of apoptosis

Five mice were irradiated in each experiment with different times of radiation. The Apoptosis cells were visualized in terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay.[10.11] The tissues were immediately excised and placed in neutral buffered formalin after radiation treatment. The tissues were embedded in paraffin blocks and four-micrometer sections were then cut and stained in previously methods.[11] Apoptotic cells were scored on coded slides at 1,000X magnification. Apoptosis index is percent number of apoptosis body per 1,000 nuclei. Statistical significance was assessed using Student's *t* test ($p < 0.05$).

Sample preparation

At 8h after radiation, the spleen and liver tissues of five mice were collected. Their tissues were suspended in sample buffer containing 40 mM Tris- HCl, 7 M urea, 2 M thiourea, 4% CHAPS (Bio-Rad, Hercules, CA), 100 mM 1,4-dithioethreitol (DTT) (Sigma, St. Louis, MO), 0.2% (v/v) Bio-Lytes (Bio-Rad) and endonuclease (Sigma). Suspensions were sonicated for approximately 30 sec and centrifuged for 1 h at 100,000 g to remove DNA, RNA and any particulate materials. The supernatants contained the total liver and spleen proteins solubilized in sample buffer. Protein concentrations in the supernatants were determined according to the manufacturer's guidelines, using the Bio-Rad assay system (Bio-Rad). All samples were stored at –70°C until use.

2-DE and Image analysis

2-DE was performed in a Bio-Rad Electrophoresis system.[12] Total protein tissue (1 mg) was used for each electrophoresis. Aliquots of liver tissue 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 100,000 Vh. After IEF, the strips were equilibrated in 6 M urea, 2.5% (w/v) sodium dodecyl sulfate (SDS), 2% (w/v) DTT, 5 mM tributylphosphine, 50 mM Tris-HCl (pH 6.8) and 20% (v/v) glycerol (Sigma) for 10 min. The second dimensions were analyzed on 9–18% linear gradient polyacylamide gels using the Protean XL system (Bio-Rad) at 20°C. Immediately 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. 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 (V. 6.2, Bio-Rad). In these experiments, all samples were run in triplicate.

Protein identification by MALDI-TOF

For mass spectrometry fingerprinting, protein spots were directly cut out of the gels, destained with 50% acetonitrile in 25 mM ammonium bicarbonate and dried in a speed vacuum concentrator (Savant, US). Dried gel pieces were reswollen with 50 mM ammonium bicarbonate (pH 8.0) containing 100 ng/μl trypsin (Promega, Lyon, France) and incubated at 37°C for 17 h. Supernant peptide mixtures were extracted with 50% acetonitrile in 5% trifluoroacetic acid (TFA) and dried in a speed vacuum concentrator. Peptide mixtures were then dissolved in 4 μl of 50% acetonitrile in 0.1% TFA. Aliquots of 0.5 μl were applied to a large disk

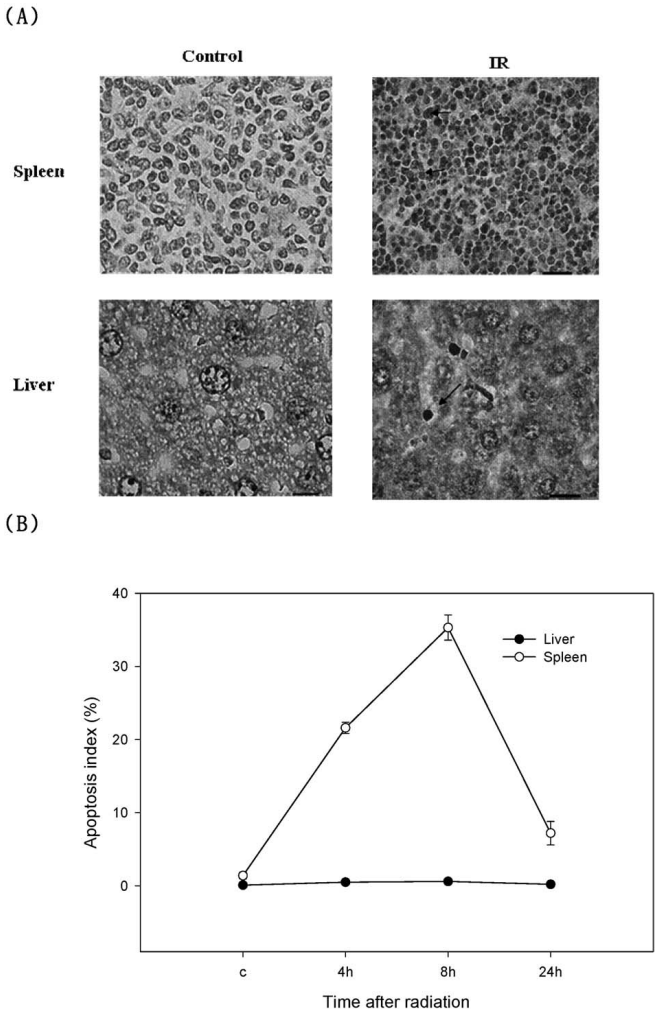


Fig. 1. The apoptosis cells detected by TUNEL assay show at control, 4, 8 and 24 h after ionizing radiation in spleen and liver of C3H/HeJ mice. (A) In TUNEL assay, the apoptosis cells are the brown stained in the nucleus. Magnification, × 1,000. Arrow indicated apoptosis cells. The 10 μm bars are indicated. (B) The peak apoptosis index is percent number of apoptosis body per 1,000 nuclei. Vertical bars are standard deviation of mean.

and allowed to air-dry. The matrix was α-cyano-4-hydroxy-cinnamic acid. Spectra were obtained using a MALDI-TOF mass spectrometer (Micromass, UK). Protein database searching was performed with MS-Fit (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm) using the average molecular weight of the monoisotopic peptide ions. Mass tolerance was allowed within 50 ppm.

Western Blot Analysis

The western blotting was analyzed by according to the method previously described. (10) Antibodies included Bcl-2, cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA), α-Tubulin (Oncogene Science), Bcl-XL_S (BD Biosciences, San Diego, CA) and peroxiredoxin VI (Prx VI, gift from Che HJ). Antibodies were used at the appropriate dilution as recommended by the manufacturer.

Immunohistochemical stain

Immunohistochemical analysis was analyzed by according to the method previously described. (11). Four μm-thick sections of paraffin-embedded tumor tissues were used for immunohistochemical staining with the streptavadin-biotin peroxidase technique (Universal LSAB-HRP kit, DAKO, Carpinteria, CA). The dilution of cytochrome c antibody was done 1:100. The paraffin sections were deparaffinized by xylene and rehydrated by sequential concentrated alcohols. At the final stage, the peroxidase reaction was visualized using 3-amino-9-ethyl-carbazole in *N*, *N*-dimethyl forma-

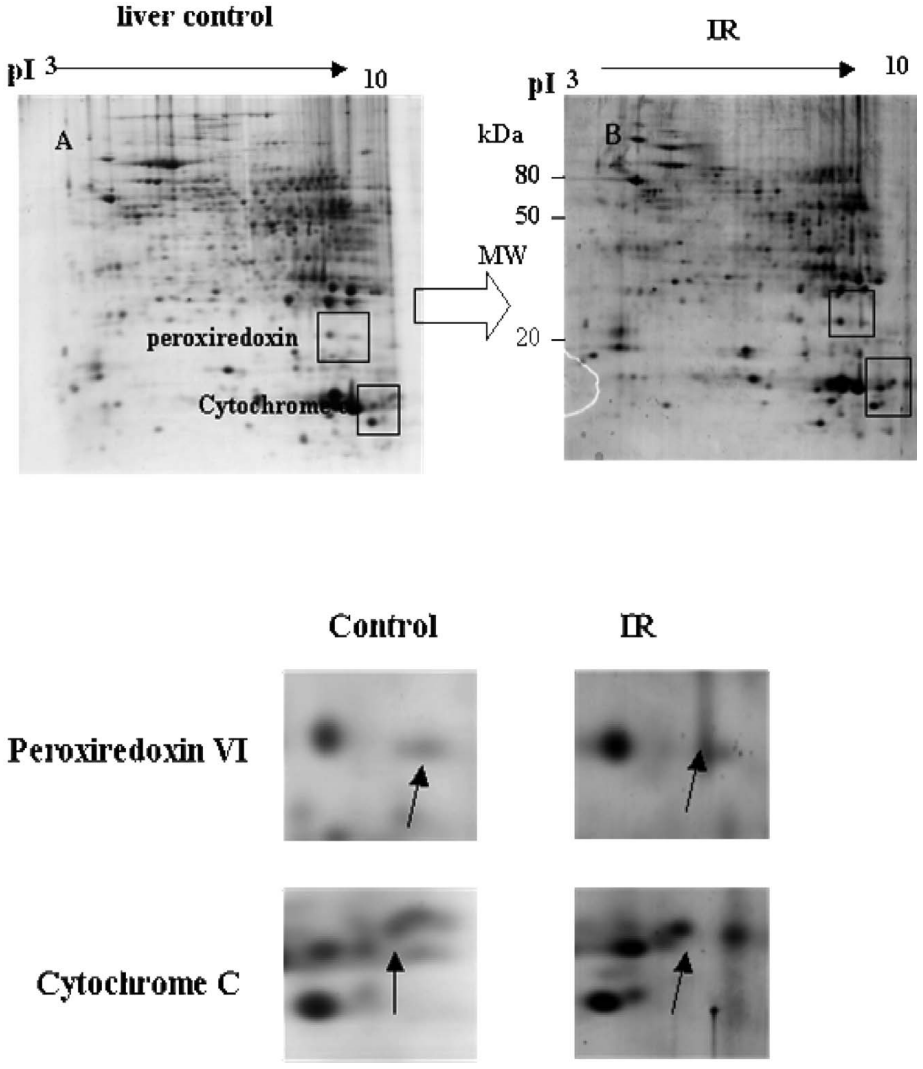


Fig. 2. 2D-Gel Electrophoresis of C3H/HeJ mouse liver proteins. (A) In liver tissues, partial 2-DE images for cytochrome c and Prx VI are shown. The proteins from the whole liver were extracted and separated on pH 3 to 10 nonlinear immobilized pH-gradients strips, followed by 9% to 18% polyacrylamide gel. The gel obtained for each 8 mice liver tissue was stained with Coomassie brilliant blue G250. Proteins shown are those differentially changed more than 3 fold after ionizing radiation.

mide (AEC) for 310 min and the slides were counterstained by Mayer's hematoxylin and mounted.

RESULTS

Level of radiation-induced apoptosis in liver and spleen tissues

To explore the effect of ionizing radiation response in tissues showing different levels of radiosensitivity, the level of radiation-induced apoptosis was analyzed by TUNEL staining. TUNEL clearly revealed distinct staining of apoptotic nuclei. After 10 Gy radiation, the peak level of induced apoptosis was 35.3 ± 1.7% at which decreased to 7.2 ± 1.6% at 24h in the spleen. In the liver tissues, the level of induced apoptosis was 0.6 ± 0.2%, which then decreased to 0.2 ± 0.1%. The apoptosis index of the spleen and liver tissues showed different levels of radiation-induced apoptosis ($p < 0.05$, Fig. 1).

Proteomics Analysis of proteins in the Irradiated Liver

2-DE gels were done to analyze the alteration of proteins by radiation in mouse liver. In the image analysis, an average of 800 protein spots were detected and localized in pI 3–10, with the molecular mass range of 10 – 100 kDa. Through the PDQUEST program the proteins were compared and eventually 28 proteins were detected that showed 3-fold change after irradiation. Those proteins were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry and data base search (Fig. 2). Both the pI and MW of each identified protein were determined by comparison to the standard 2-DE gels of the Swiss-2D PAGE database (http://kr.ExPaSy.org).

In the irradiated liver, the identified proteins involved G-type proteins, metabolism-related proteins and reactive oxygen species (ROS)- related proteins. Four proteins such as protocadherin, hemoglobin A, phosphatidyl-ethanolamine and the ras related protein showed a decrease in their expression levels after ionizing radiation. In contrast, 24 proteins showed an increase in their expression level after radiation. Especially, the proteins related to the ROS metabolism including gluthathione S- tranferase Pi (GSTP), NADH dehydrogenase and Prx VI were increased following ionizing radiation (Table 1). A partial 2-DE image for Prx VI, as shown in Fig. 2, indicated that its expression level increased 3-fold after ionizing radiation. Western blotting analysis showed that the expression of Prx VI was significantly upregulated (Fig. 4A).

Proteomics Analysis of the Irradiated Spleen Proteins

In the spleen, 60 protein spots were significantly increased in their level of expression following radiation (Fig. 3).

Table 1. List of oxidative stress-related proteins that change in their level of expression after ionizing radiation

Name	Spleen Control→ IR	Liver Control→ IR	Coverage (%)[a]	MW/pI[b]	Accession No.[c]
Gluthathione S transferase P1	–	↑[d]	38	23,537/7.7	P46425
Gluthathione S transferase P2	–	↑	35	23,609/7.7	P19157
Peroxiredoxin VI	–	↑	30	21898/9.1	6755114
NADH dehydrogenase	–	↑	20	51,551/7.4	1334705
Carbonic anhydrase	–	↑	30	29,239/6.5	12834481
RIKEN cDNA	↑	↑	25	17181/4.6	13385208
Stress-induced phosphoprotein 1	↑		20	62640/6.4	P31948
Delta-1-pyrroline-5-Carboxylate Dehydrogenase	↑		16	61752/8.2	P30038
Heat shock protein 1A	↑		21	70550/5.9	P55063

[a] Sequence coverage % (MC data)
[b] Theoretical *pI* and MW
[c] Accession number in Swiss-Prot and NCBI database
[d] ↑ 3 fold increased after radiation

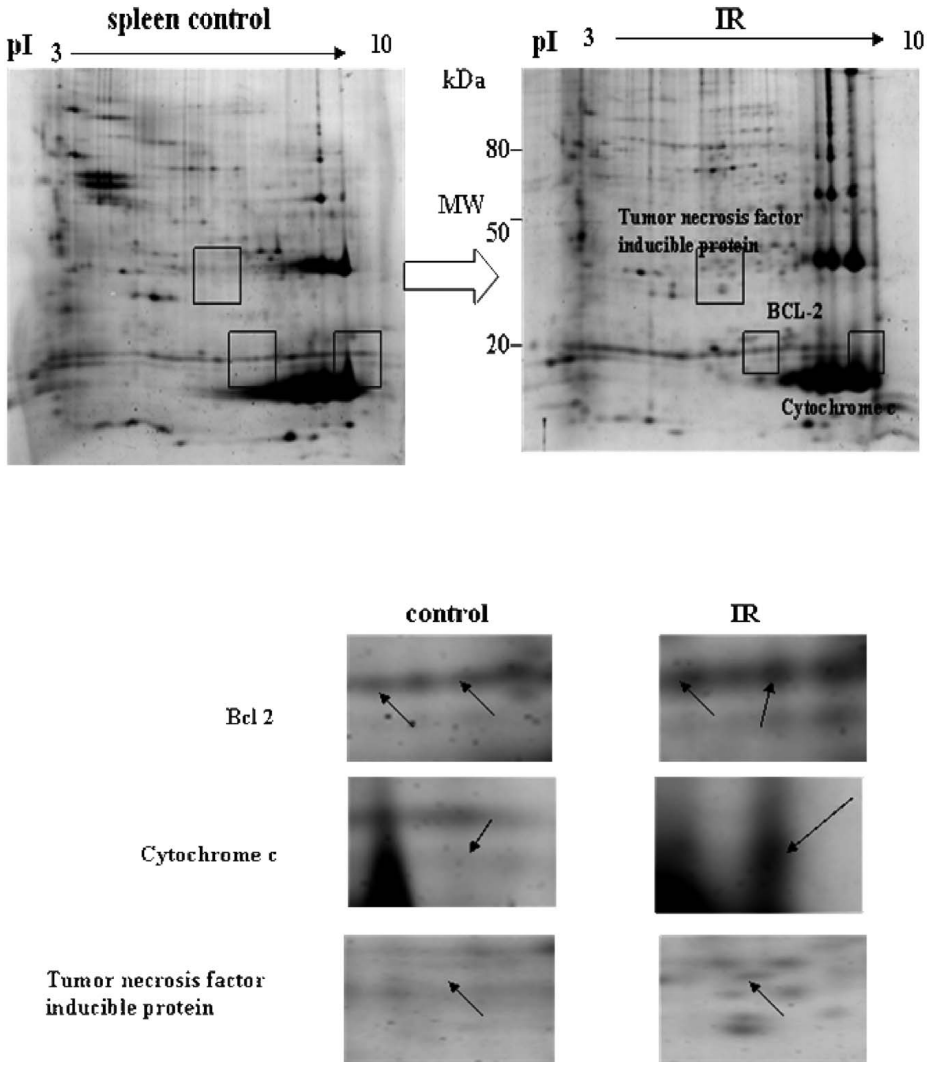


Fig. 3. 2D-Gel Electrophoresis of C3H/HeJ spleen proteins. Partial 2-DE images for Bcl-2, cytochrome c and tumor necrosis factor-inducible protein are shown.

These were identified as proteins associated with signal transduction, apoptosis, cytokines, Ca signaling, stress, cytoskeletal regulation, ROS metabolism, and others (Fig. 3). Also, the expression level of stress related proteins, such as stress-induced phosphoprotein 1, riken cDNA and heat shock proteins 1A, was increased after ionizing radiation (Table 1). The expression levels of cytochrome c and immune related proteins showed similar patterns of change both in the spleen and the liver tissues after ionizing radiation (Table 3.)

Differential expression of proteins between liver and spleen tissues

Since liver and spleen tissues showed different level of radiosensitivity in terms of radiation-induced apoptosis, our 2-DE analysis concentrated on the apoptosis signal regulating proteins. In the liver, which was apoptosis resistant, the partial 2-DE images showed that only the expression of cytochrome c was significantly changed after ionizing radiation (Table 2, Fig. 2). However in spleen, various kinds of apoptosis regulating proteins were increased beside cytochrome c. These involved iodothyronine, CD 59A glycoprotein precursor, fas antigen and tumor necrosis factor (TNF)-inducible protein TSG-6nprecursor (Table 2, Fig. 3). The expression level of calpain, which acts as a p53 inhibitor, was decreased, whereas the expression level of Bcl-2 isozymes was increased after ionizing radiation (Table 2).

The partial 2-DE image for cytochrome c in the two different tissues is shown in Fig. 2 and 3. Its expression level increased to 3-fold after ionizing radiation in both tissues. These changes were validated through immunoblotting analysis and Western analysis, showing significant increase of cytochrome c in the irradiated liver and the spleen tissues (Fig. 4). The expression of Bcl-2 was not significantly

changed, whereas the expression of Bax increased after ionizing radiation (Fig. 4B).

DISCUSSION

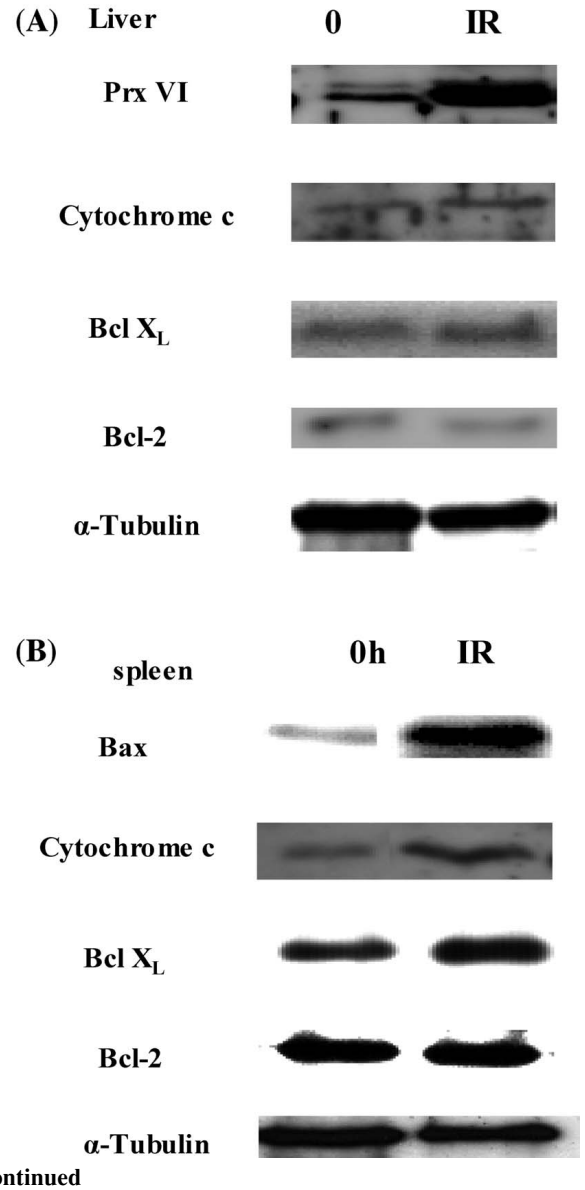
Recent advances in our understanding of cellular and molecular biology has provided new insights into the mechanism of radiation pathogenesis, indicating the usefulness of radioresponse of normal tissue for radiation therapy. Proteomics analysis is a useful tool in investigating biological events, as it provides us with significant information about particular proteomics or gene products, including information about modifications that take place in response to various internal or external factors such as diseases or radiation damage. There is, however, still room for improvement for the analysis of membrane protein, proteins with high molecular weight, very acidic or very basic proteins. In this paper, we describe the identification of the most differentially expressed proteins between the radiosensitive spleen and the radioresistant liver tissues, using 2DE techniques to map out the different protein expression patterns, and MALDI-TOF MS to identify this select group of differentially expressed proteins.

The two tissues showed distinct apoptotic responses after ionizing radiation exposure. The analysis of the responses of different tissues taken from the same C3H/HeJ mice indicates considerable differences in their susceptibility to apoptosis following ionizing radiation. A previous report showed that the apoptosis index of the C57BL/6J (81.0 ± 2.5%) mice was higher than the C3H/HeJ (59.4 ± 4.0%) mice in irradiated thymus. Also, the apoptosis index of the thymus and liver tissues showed different levels the apoptosis in both strain types. In the liver, however, the apoptosis response was no difference in the C57BL/6J and C3H/HeJ mice.[2] These indicate that the two strain mice revealed significant differences in apoptosis response according to different strain types and different tissues of the same animal. In this study, we confirmed the results of the previous report that the difference in the apoptosis index between the liver and spleen tissues of C3H/HeJ mice is closely associated with the expression of various kinds of apoptosis-related proteins.

The radiation-induced apoptosis signal pathway is regulated by the activities of the Fas/fas-ligand,[13] sphingomyelin/ceramide pathway,[14] early expression genes,[15] caspases of cystein protease[16] and TNF-family.[17] We have previously reported that the expression levels of p53, bcl-X and bax were increased in the thymus tissues after ionizing radiation. In the liver, however, the radiation-induced apoptosis was minimal and the expression level of apoptosis related genes was not significant. The results indicate that the difference in radiosensitivity seems to be determined by the difference in the expression levels of apoptosis related genes.

In particular, the expression of cytochrome c has been reported to go through a significant change in the irradiated liver and spleen tissues. According to the report, the intermembrane cytochrome c normally activates the electron transport in the respiratory chain, whereas the cytochrome c in the apoptosis signal pathway triggers the caspase cascade, resulting in the apoptotic cell death.[18,19] In our study, we also observed the overexpression of cytochrome c following radiation in the liver and spleen tissues, and this may play an important role in the radiation induced apoptosis signal pathway.

The Bcl-2 family of proteins includes anti-apoptotic molecules such as Bcl-2 and Bcl-X_L, and pro-apoptotic molecules such as Bax, Bak, Bid, and Bad.[20] Bcl-2 and Bcl- X_L are known to block the disruption of the mitochondria membrane potential as well as the release of AIF and cytochrome



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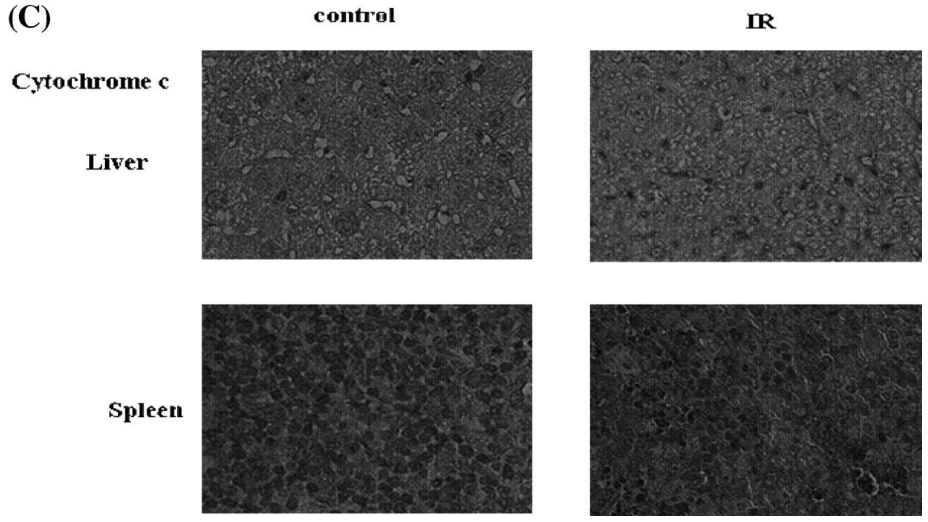

Fig. 4. Protein expressions of irradiated and normal tissue by Western blot analysis and Immunohistochemical stain. (A) The expression level of Prx VI, cytochrome c, Bcl-2 and Bcl- X_L were showed by Western blotting analysis in liver (*IR* at8 h after ionizing radiation). (B) The expression level of cytochrome c, Bcl-2, Bax and Bcl- X_L were showed by Western blotting analysis in spleen (*IR* at 8 h after ionizing radiation). (C) Immunohistochemical stain of cytochrome c is shown in liver and spleen. The results were analyzed by densitometry.

Table 2. List of apoptosis related proteins that exhibit differential expression in liver and spleen tissues after ionizing radiation

Name	Spleen Control→ IR	Liver Control→ IR	Coverage (%)[a)]	MW/pI[b)]	Accession No.[c)]
Cytochrome c oxidase	↑	↑	45	13,695/9.3	2144362
M-calpain	↓	–	20	37,809/4.7	P43367
Bcl-2- related protein A1	↑	–	31	19,914/5.2	Q07440
CD59A glycoprotein precusor	↑	–	17	13648/7.5	O55186
Bcl 2l2 protein	↑	–	34	19,119/6.9	25955645
Iodothyronine deiodinase	↑	–	13	31,070/7.6	4009517
Fas antigen	↑	–	23	36,103/6.6	4996371
Tumor necrosis factor-inducible protein TSG-6 precursor	↑	–	31	31,232/6.5	1351315

[a)] Sequence coverage % (MC data)
[b)] Theoretical *pI* and MW
[c)] Accession number in Swiss-Prot and NCBI database
[d)] ↑ 3 fold increased and ↓ fold decreased after radiation

Table 3. List of proteins showing similar pattern of change in spleen and liver tissues after ionizing radiation

Name	Changed In pattern	Coverage (%)[a)]	MW/pI[b)]	Accession No.[c)]
Cytochrome c oxidase	↑	45	13,695/9.3	2144362
RiKen cDNA	↑	25	17,181/4.6	13385208
Imunoglobuline kappa light chain variable region	↑	44	9538/8.0	11137493
Imunoglobuline heavey chain variable region	↑	50	9552/8.9	11137493
Imunoglobuline heavey chain variable region	↑	53	13,606/6.9	4530543

[a)] Sequence coverage % (MC data)
[b)] Theoretical *pI* and MW
[c)] Accession number in Swiss-Prot and NCBI database
[d)] ↑ 3 fold increased after radiation

c into the cytosol.[21)] Our proteomics results showed that the expressions of Bcl-2 isoforms were increased in the irradiated spleen tissues. The reason for this may be that the Bcl-2 family of proteins such as Bcl-Xs and Bax is concerned with the cytochrome c release. But the Western-blotting results showed that the expressions of Bcl-2 and Bcl- X_L were not changed after ionizing radiation.

In this study, the expression of tumor necrosis factor (TNF-α) inducible protein TSG-6 precursor was increased after the irradiation of the spleen. The sphingomyelin pathway has been shown to be important role in TNF-α and fas-mediated apoptotic signal transduction.[17)] Our data show that the increased fas antigen expression is correlated with the enhanced apoptosis response. However, we observed that the expression level of m-calpain was decreased after the radiation treatment. As a calcium-activated cystein protease, m-calpain has been reported to suppress the p53 protein activity[22)]; the reduction of calpain may therefore increase p53 activity, thus enhancing the induction of p53-dependent apoptosis, and increase radiosensitivity.

In this study, the expression levels of apoptosis regulated proteins were significantly changed after radiation in the apoptosis-sensitive spleen. In contrast to the apoptosis-resistant liver, we found increased expressions of proteins related to the ROS metabolism after ionizing radiation. Particularly, the expression levels of antioxidant enzymes such as GSTP, Prx VI and NADH dehydrogenase were increased after the irradiation of the liver.

Prx has emerged as an interesting molecule that affects the cellular radiation response.[23,24)] Lee *et al*. showed that the expression levels of Prx II and I were increased in the mouse testis 6 hrs after ionizing radiation.[23)] Also, Park *et al*. showed that levels of human Prx II were associated with the resistance of cells to radiation therapy.[25)] Our results showed overexpression of Prx VI following radiation in the liver tissues, which suggests activation of the protecting mechanism against radiation.

Our data suggest that apoptosis related proteins and redox proteins play important roles in radio susceptibility of different tissues.

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