Environmental Mutagens & Carcinogens 22-3 : 142-148 (2002)

Exploiting cDNA Microarray-Based Approach Combined with RT-PCR Analysis to Monitor the Radiation Effect: Antioxidant Gene Response of *ex vivo* Irradiated Human Peripheral Blood Lymphocyte

Myung-Hui Sung[1], Hyun-Jung Jun[2], Seung-Yong Hwang[1], Jae-Hoon Hwang[1], Jong-Hoon Park[3], Mi-Young Han[4], U-Youn Lee[2], Eun-Mi Choi[2] and Young-Mee Park[2]

[1]*Department of Biochemistry, Hanyang University, Ansan 425-791, Korea*
[2]*Division of Chemistry and Biology, University of Incheon, Incheon 402-749, Korea*
[3]*Department of biological science, Sookmyung Womens University, Seoul 140-742, Korea*
[4]*Green Cross Institute of Medical Genetics, Seoul 135-260, Korea*

(Received August 2, 2002 / Accepted September 10, 2002)

ABSTRACT : **Although ionizing radiation (IR) has been used to treat the various human cancers, IR is cytotoxic not only to cancer cells but to the adjacent normal tissue. Since normal tissue complications are the limiting factor of cancer radiotherapy, one of the major concerns of IR therapy is to maximize the cancer cell killing and to minimize the toxic side effects on the adjacent normal tissue. As an attempt to develop a method to monitor the degree of radiation exposure to normal tissues during radiotherapy, we investigated the transcriptional responses of human peripheral blood lymphocytes (PBL) following IR using cDNA microarray chip containing 1,221 (1.2 K) known genes. Since conventional radiotherapy is delivered at about 24 h intervals at 180 to 300 cGy/day, we analyzed the transcriptional responses *ex-vivo* irradiated human PBL at 200 cGy for 24 h-period. We observed and report on 1) a group of genes transiently induced early after IR at 2 h, 2) of genes induced after IR at 6 h, 3) of genes induced after IR at 24 h and on 4) a group of genes whose expression patters were not changed after IR. Since Biological consequences of IR involve generation of various reactive oxygen species (ROS) and thus oxidative stress induced by the ROS is known to damage normal tissues during radiotherapy, we further tested the temporal expression profiles of genes involved in ROS modulation by RT-PCR. Specific changes of 6 antioxidant genes were identified in irradiated PBL among 9 genes tested. Our results suggest the potential of monitoring post-radiotherapy changes in temporal expression profiles of a specific set of genes as a measure of radiation effects on normal tissues. This type of approach should yield more useful information when validated in *in vivo* irradiated PBL from the cancer patients.**

Key words: Radiation therapy, cDNA microarray, Peripheral blood lymphocyte, Antioxidant

Introduction

Ionizing radiation (IR) has been used for nearly a century to treat human cancer (Hall *et al.*, 2000). Since the normal tissue complications are the limiting factor of cancer radiotherapy treatment (Peters *et al.*, 1996), the objective of IR therapy is to deliver a lethal dose to cancer cells but attenuate the toxic effects of IR on adjacent normal tissue (Vijayakumar *et al.*, 1997). Radiobiologists have been seeking reliable tests aimed to monitor the degree of radiation exposure to normal tissues during radiotherapy.

In order to monitor non-invasively the normal tissue response to IR, various methods have been developed. There are five major groupings of indicators that change following IR (Walden *et al.*, 1989). The five groups are physical symptomology of the prodromal syndrome, changes in lymphocyte numbers, alterations in serum composition, urinary composition, and chromosomal aberrations. Measurement of chromosomal aberrations in human peripheral blood lymphocytes (PBL) has long been considered as a possible tool for *in vivo* biodosimetry during radiotherapy (Ekstrand *et al.*, 1982; Bender *et al.*, 1962). Unfortunately, however, attempts to correlate

*To whom all correspondence should be addressed

radiation-induced chromosomal aberrations in PBL with the normal tissue damage of the radiation therapy treatment have not been successful (Bentzen *et al.*, 1999). It has been noted that conventional metaphase analysis is not reliable to monitor normal tissue damage (Lloyd *et al.*, 1973), mainly because radiation-induced cell cycle delay produce a selection in the population harvested at the first mitosis following exposure (Yamada *et al.*, 2000; Durante *et al.*, 1999).

Profiling of mRNAs on cDNA arrays provides a method to identify simultaneously the response of many genes to specific stimuli. Although transcriptional induction of various genes in cancer cells has been reported in response to IR, there exist limited reports on the transcriptional response of normal human PBL. Moreover, these reports generally describe the induction of genes by IR without reference to doses used in radiotherapy or the timing of the induction. Many *in vitro* studies of gene induction were performed under supraphysiologic IR doses, and therefore are of limited value in potential applicability in the clinic.

This study was undertaken to discover the temporal patterns of gene responses of the irradiated normal human PBL using cDNA microarray-based approach. The contents of the cDNA chip (GenoCheck, Ansan, Korea) we used contained genes involved in signal transduction, in cell division, in metabolism, and adaptive/protective responses. A complete list of genes contained in 1,221 (1.2 K) element cDNA chip can be accessed on the web site (www.genocheck.com). Since conventional radiotherapy is delivered at about 24-h intervals at 180 to 300 cGy/day, we monitored the transcriptional responses *ex-vivo* irradiated human PBL at 200 cGy for 24 hr to identify temporal patterns of potential marker gene induction.

Biological consequences of ionizing radiation involve various transient reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Levinson *et al.*, 1966; Walburg *et al.*, 1975; Simic *et al.*, 1989). Generation of the ROS by the interaction of ionizing radiation with water molecules is well recognized. Oxidative stress induced by the ROS can damage important biomolecules (Koh *et al.*, 1999; Cho and Song *et al.*, 2000) in the normal tissues during radiotherapy (Park *et al.*, 2001). In this study, we further tested by RT-PCR the temporal expression profiles of genes involved in ROS modulation.

Materials and Methods

Separation and irradiation of human peripheral blood lymphocytes(PBL)

Human blood samples were obtained from volunteer healthy male donors at the age of 25 to 30 years. The blood was separated with Histopaque 1077 (Sigma, USA) and the buffy coat layers were harvested to obtain PBL. PBL cultures were stimulated *in vitro* for 24 h in RPMI1640 medium supplemented with 1% phytohaemagglutinin (PHA) before IR. The PBL cultures were irradiated (60cGy/min) at room temperature with ^{137}Cs gamma rays (IBL 427 Irradiator, CIS Bio International, France) to achieve the desired 200cGy.

RNA isolation and microarray hybridization

Total RNA was extracted from PBL cultures using TRI reagent (Gibco-BRL, USA). Samples of 50 mg each of total RNA were labeled and hybridized to 1.2 K element cDNA chip manufactured by GenoCheck (Korea). In brief, fluorescently labeled cDNA was prepared from control and irradiated PBL by a single round of reverse transcription in the presence of fluorescent dNTP (Cy3 dUTP or Cy5 dUTP, Amersham Pharmacia, U.K). Probes and targets were hybridized together for 16 h in 3 X SSC at 65°C in the presence of the blockers human COT1 DNA, yeast tRNA and polydeoxyadenylic acid. Hybridized slides were washed at room temperature once in 0.5 X SSC, 0.1% SDS for 5 min, and again in 0.06 X SSC for 5 min.

Scanning and Image analysis

The Cy3 and Cy5 fluorescences were scanned by using Exon scanarray 5000 with a laser confocal microscope (GenePix 5000, USA). Images were analyzed using the GeneSpring program. Briefly, We adjusted the ratios to be normalized by dividing all the ratios by the average ratio around 1, so that the signal intensities can be compared between different cDNA chips. To organize and analyze the data, a modified version of average-linkage method of Wang *et al.*, (Wang *et al.*, 2001) was employed.

RT-PCR analysis

Total cellular RNA was extracted by the modified single step guanidium isothiocyte lysis method (Chomczynski and sacchi *et al.*, 1987). One μg of total RNA isolated from control and irradiated PBL was subjected to RT-PCR as previously described (Baek *et al.*, 1999).

Table 1. Locations and nucleotide sequence of the PCR primer pairs for antioxidant and *β-actin*-specific genes

Gene	Nucleotide sequences	Product size (bp)	Locations	Cycle No.
GPx1	5'-CCACCAGGAACTTCTCAAAG-3' 5'-TGGCTTCTTGGACAATTGCG-3'	529	298-317 827-808	35
γ-GCS	5'-GATCGTCTAGAACAGCCCTACGGAGGAAC-3' 5'-GATCGGAATTCCTTCAATGGCTCCAGTTCC-3'	633	413-430 1046-1029	35
Catalase	5'-CAGATGGACATCGCCACATG-3' 5'-AAGACCAGTTTACCAACTGGG-3'	404	617-636 1021-1001	35
CuZn SOD	5'-ATGGCGACGAAGGCCGTGTGC-3' 5'-TTGGGCGATCCCAATTAC-3'	462	65-85 462-445	35
Mn SOD	5'-GGCATCTGCGGTAGCACCAG-3' 5'-TCTCCCTTGGCCAACGCCTC-3'	259	65-84 324-305	35
Prx II	5'-GCTCACGCAGTCATGGCC-3' 5'-CCCCTTCAGAGAGTGGAGGAA-3'	702	68-85 770-750	40
GST π	5'-TCATGGATCAGCAGCAAGTC-3' 5'-CCTACACCGTGGTGTATTTC-3'	483	14-33 497-478	40
PrxI	5'-ATGTCTTCAGAGTGCAAAAATTG-3' 5'-TCACTTCTGTTAGCGAAATACTCT-3'	548	1456-1471 2004-1987	40
GR	5'-TCCTATGACTACCTGGTGATC-3' 5'-TTGATAGATGGCATTCAGGCG-3'	287	165-185 452-432	40
β-actin	5'-GATCGTCTAGATTCCTATGTGGGCGACGA-3' 5'-GATCGGAATTCCAGCGGAACCGCTCATTG-3'	617	226-243 843-826	30

Briefly, RNA concentrations were determined by measuring UV absorption and samples with comparable A_{260}:A_{280} ratios were used for RT-PCR analysis. PCR amplication was performed in a thermocycler as follows: 10 min at 94°C, 1 min at 94°C and 1 min at 55°C. After cycling, there was a DNA extension period of 10 min at 72°C. Cycle number and sequences of the primers to amplify various antioxidant genes are listed in Table 1.

Results

Transcriptional response of the irradiated PBL

Normal PBL was isolated and exposed to IR at 200 cGy. Samples of the total RNA was extracted from control and irradiated PBL at 2 h, 6 h and 24 h after exposure to IR. The RNA obtained at each time point from control and exposed PBL with Cy3 and Cy5 dUTP was hybridized to 1,221 (1.2 K) cDNA chips that was custom-designed and manufactured by GenoCheck (Ansan, Korea). Fluorescence profiles of the representative 1.2 K cDNA chips hybridized with RNA from control and exposed PBL at each time point is shown in Figure 1.

Hierarchical clustering of microarray data

The fluorescent images (Cy3 and Cy5) were then scanned for analysis. Signal was normalized by dividing

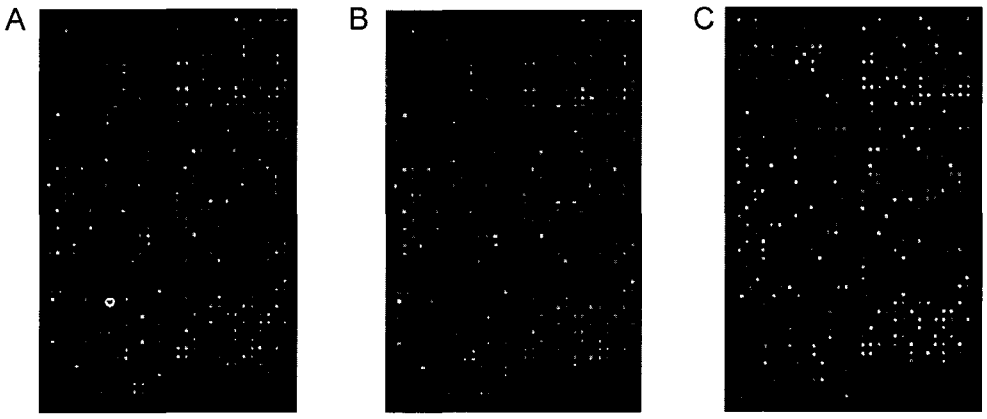

Fig. 1. Fluorescence profiles of 1.2 K cDNA chips hybridized with RNA from control and exposed PBL. Fluorescence patterns of 1.2K cDNA chips obtained from exposed and control PBL at 2hr (panel A), 6hr (panel B), and 24hr (panel C) are shown above.

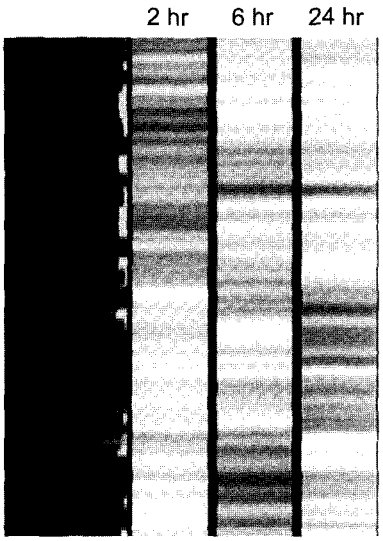

Fig. 2. Hierarchical l clustering of 1.2K cDNA microarray data. The data set was clustered according to the method of Eisen *et al.* (1998) using Gene Cluster software package and visualized by GeneSpring software (Sigenetics, 2001).

all signals by the average signal of the spots on the slide. This resulted in the 'average' spot having a normalized signal of "1". A significant expression was defined as signal two times greater than biological noise, *i.e.*, the average signals from the negative control, *E. coli* genes.

To examine the temporal expression profiles, the data set was clustered and visualized according to the method of Eisen *et al.* (Eisen *et al.*, 1998) by using GeneSpring software (Sigenetics, 2001). As shown in Figure 2, genes were clustered together on the basis of the degree of similarity and their temporal expression profiles.

dentification of a set of potential radiation-response genes

A specific induction of at least 105/1,221 genes were observed in the irradiated PBL in comparison to control cells. Radiation-responsive genes of the PBL identified in this current study include regulatory and signaling genes which in turn lead to the altered expression of multiple down-stream genes that are involved in cell-cycle control, programmed cell death, cellular redox control and in some cases protein degradation. The complete results of the groups of genes can be accessed at the GenoCheck web site at (www.genocheck.com).

Among 1.2K cDNA elements, a peak induction of 62 genes as early as 2 hr after IR (Figure 3A). A second group of 16 genes were transiently induced at 6 hr after IR (Figure 3B). A third group of 27 genes remained elevated at 24 hr after IR (Figure 3C). No significant induction was observed for 251 genes which include house keeping genes (Figure 3D). We were not able to categorize 869 out of 1,221 genes. These genes also revealed a great variability in different donors.

RT-PCR analysis of antioxidant genes expression in the irradiated PBL

Since biological consequences of IR involve oxidative stress generated by the production of ROS, it was of our particular interest to examine the transcriptional responses of genes involved in ROS regulation. To do so, RT-PCR analysis was carried out using primer pairs to amplify genes that encode anti-oxidant enzymes. Our results revealed that the specific temporal patterns of 6 antioxidant genes among 9 genes tested. Having identified the temporal patterns of 6 antioxidant gene responses, we have tested samples of *ex vivo* irradiated PBL from different donors to gauge the reproducibility of the temporal patterns among different individuals. We found that although there was less than 1.5 fold inter-individual variation in the induction levels of these transcripts among different individuals, the temporal patterns of each transcripts were similar among individuals, indicating that distinct ranges of induced transcript levels might be defined for these stress gene mRNA biomarkers for individual patient. Representative results are shown in Figure 4.

Discussion

The rapid discovery of sequence information from the Human Genome Project has increased the amount of data that can be retrieved from biomedical experiments. Gene expression profiling, through the use of microarray technology, is rapidly contributing to an improved understanding of global, cooridnated cellular events in a variety of paradigms. In this study, we explored a cDNA microarray-based approach using 1,221 known genes as an attempt to identify the temporal patterns of gene responses of ex-vivo-irradiated peripheral blood lymphocyte (PBL) from multiple independent doners. It should be noted that the radiation dose used in the current study is in a range relevant to radiotherapy of cancer patient. Our results showed that at least 105/1,221 genes were significantly changed in the irradiated PBL in comparison

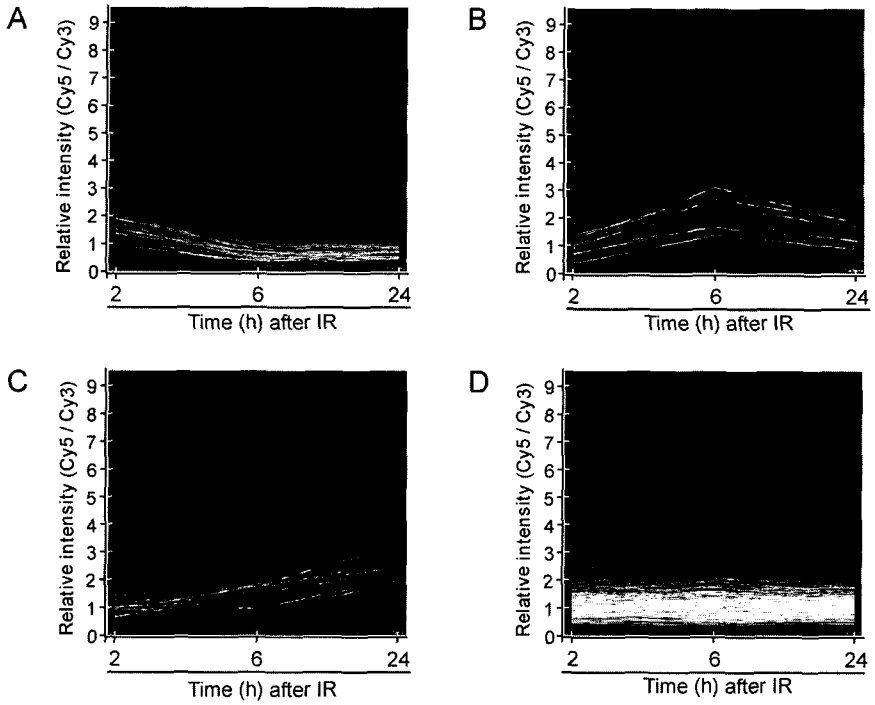

Fig. 3. Identifaication of radiation-responsive genes. Expression profiles of genes at 2 hr, 6 hr, and 24 hr after irradiation are shown above. Panel A shows genes that were maximally accumulated at 2 hr after IR. Panel B shows genes that were maximally induced at 6 hr after IR. Panel C shows genes that remained elevated at 24 hr after IR. Panel D shows genes that were not changed significantly.

to control cells. A specific and reproducible temporal patterns of 6 antioxidant gene responses of ex vivo irradiated PBL from independent doners were also obtained when analyzed by RT-PCR.

One of the major major supposition of the current study is that the transcriptional responses of PBL to ionizing radiation would reflect the effect of ionization to normal tissues during cancer therapy. Although the definitive demonstration is lacking for these "temporal genetic fingerprints" to reflect the normal tissue response during radiotherapy, the present study demonstrate a potential application of PBL-based cDNA microarray approach to monitor the effectiveness of treatment regimen. Since most anticancer drugs cause DNA damage and cell cycle arrest or cell death, the set of genes identified in the current study may also be potentially applicable to other forms of cancer therapy.

We believe that the temporal profiles of 105 genes identifed in irradiated PBL provide a large body of potentially valuable information of the response of the cancer patient to radiotherapy and represents a source of surrogate biomarkers to monitor the side effect of radiotherapy on the normal tissue during radiotherapy. Monitoring the changes in the relative levels of radiation-responsive genes when combined with pre-existing bioassays should serve as a complementary tool to predict the possible normal tissue complications in the cancer patient following radiotherapy.

That the expression levels of the genes examined in 200cGy-irradiated PBL varied only slightly between donors, and that temporal patterns of the induction of these genes were similar in 8 independent donors, supports their potential usefulness as markers to monitor the normal tissue effect during radiotherapy. An approach evaluating patterns of gene sets rather than a single transcript would likely produce more accurate estimate of the side effect of the radiotherapy. It should be possible to establish differences in the temporal induction patterns of genes from patient with or without normal tissue complications after therapy.

The responses to IR and other stresses are complex and are regulated by a number of overlapping molecular pathways. Biological consequences of IR involve various transient reactive oxygen species (ROS). Oxidative stress induced by ROS can damage the normal tissues during radiotherapy. That the specific set of genes that encode

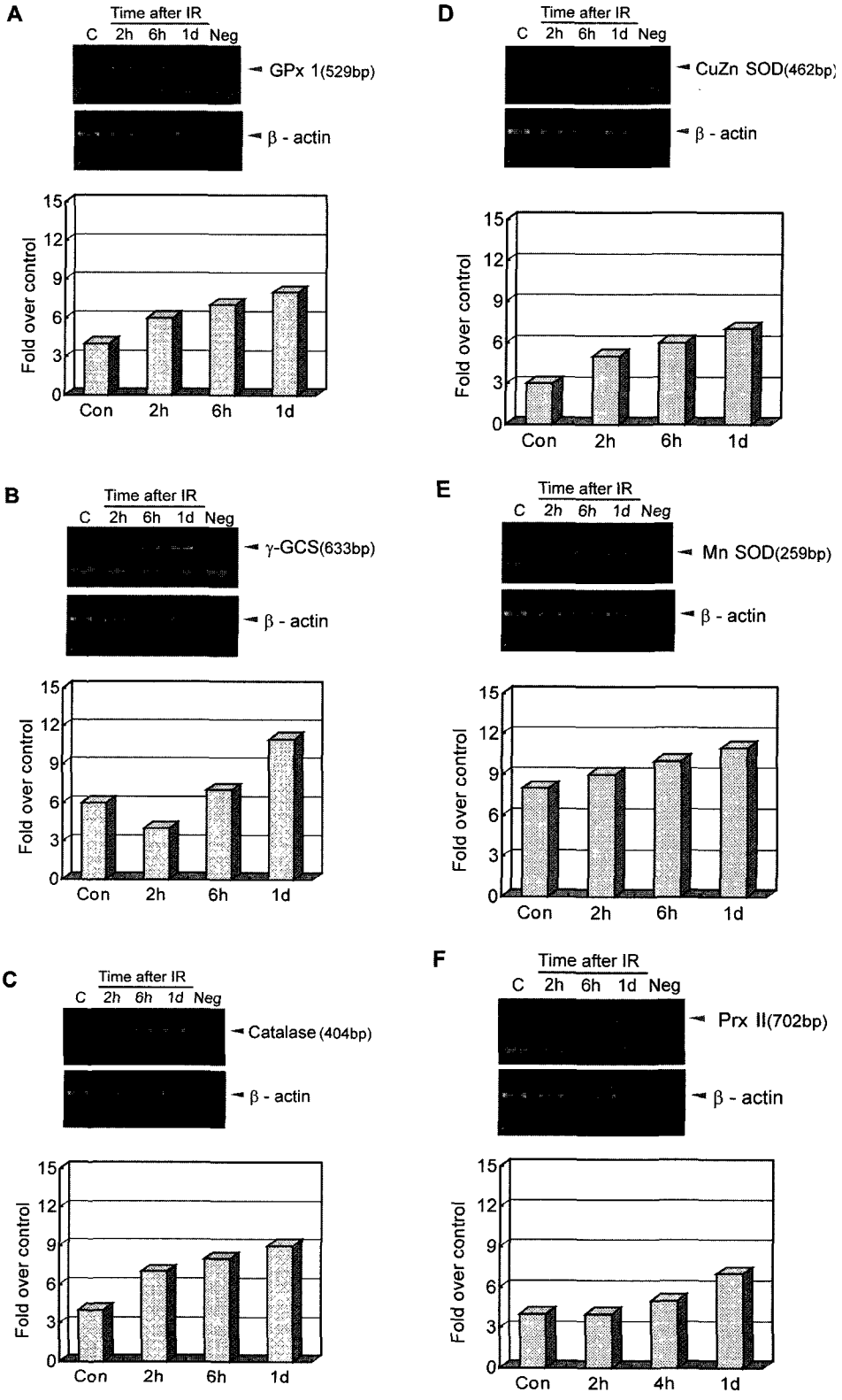

Fig. 4. RT-PCR analysis of antioxidant enzymes in the control and irradiated PBL. One μg each of total RNA was reverse transcribed and amplified with *anti-oxidant enzyme* specific primers or *β-actin*-specific primers. Results are representative of at least five independent experiments.

antioxidant enzymes was induced in irradiated PBL appears to substantiate this view. It may be possible to exploit these differences in anti-oxidant enzyme gene responsiveness as molecular markers through the use of a combined informatics and functional genomics approach. Perturbations in radiation-responsive gene function is also likely to have important implications in radiation-induced secondary carcinogenesis as well as in cancer treatment. Characterization of these radiation-responsive genes using a functional genomics approach and elucidation of their *in vivo* and *in vitro* functions deserve a further in-depth study.

Most of the studies that define the molecular markers for radiation response of tumor cells rely on the observation of gene expression using immunostaining, Northern blot, or Western blot analysis if a single or several genes. The results vary among different studies, and some results are contradictory. However, the study agrees that the change in expression of the genes affect the radiation response. Accurate monitoring of normal tissue response to radiotherapy and concomitant chemoradiation would be an important tool to assist the physician in making appropriate recommendations for cancer treatment of the individual patient. Further prospective studies are required to correlate the temporal expression profiles of a specific set of genes identified in the current study with the degree of normal tissue complications of the cancer patients after radiotherapy. The validation of a specific gene set would enable more rapid, accurate, and non-invasive testing of potential normal tissue complications.

Acknowledgement

This study was supported by a grant from Ministry of Science and Technology.

References

Baek, S.H., Han, M.Y., Lee, S.H., Chio, E.H., Park, Y.M. (1999): Hypoxic microenviromental control of stress protein and erythropoietin gene expression. *J. Biol. Chem.* **32**, 112-118.

Bender, M.A., Gooch. P.C. (1962): Types and rates of X- ray-induced chromosome aberrations in human blood irradiated *in vitro*. Proc. Nat. Acad. Sci. USA. **48**, 522-532.

Bentzen, S.M., Hendry, J.H. (1999): Variability in the radiosensitivity of normal cells and tissues. *Int. J. Radiat Biol.* **75**, 513-517.

Cho, Y. and Song, K.B. (2000): Effect of g-irradiation on the molecular properties of bovine serum albumin and b-lactoglobulin. *J. Biochem. Mol. Biol.* **33**, 137-137.

Chomczynski, P. and Sacchi, N. (1987): Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Durante, M., Furusawa, Y., Majima, H., Kawata, T., Gotoh, E. (1999): Association between G2-phase block and repair of radiation-induced chromosome fragments in human lymphocytes. *Radiat. Res.* **151**, 670-676.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D. (1998): Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA.* **95**, 14863-14868.

Ekstrand, K.E., Dixon, R.L. (1982): Lymphocyte chromosome aberrations in partial-body fractionated radiation therapy, *Phys. Med. Biol.* **27**, 407-411.

Hall, E.J., (2000): A radiation biologist looks to the future. *Int. J. Radiat. Oncol. Biol. Phys.* **46**, 1-2.

Koh, Y.H., Yoon, S.J. and Park, J.W. (1999): Inactivation of copper, zinc superoxide dismutase by the lipid peroxidation products malondialdehyde and 4-hydroxynonenal. *J. Biochem. Mol. Biol.* **32**, 440-444.

Levinson, W. (1966): Toxic effects of X-irradiated medium on chick embryo cell. *Exp. Cell. Res.* **43**, 398-413.

Lloyd, D.C., Purrott, R.J., Dolphin, G.W. (1973): Chromosome aberration dosimetry using human lymphocytes in simulated partial body irradiation. *Phys. Med. Biol.* **18**, 421-431.

Park, E.M., Park, J.S., Kim, Y.J., Sung, J.S., Hwang, T.S., Kim, W.C., Han, M.Y., Park, Y.M. (2001): Role of oxidative stress in the radiation-induced lung pathogenesis in mice. *J. Biochem. Mol. Biol.* **34**, 544-550.

Peters, L.J. (1996): Radiation therapy tolerance limits. *Cancer.* **77**, 2379-2385.

Simic, M.G., Taylor, K.A., Ward, J.F. and von Sonntag C. (1989): Radioprotection of mice with ascorbic acid, desferal and mercaptoethylamine; in *Oxygen Radicals in Biology and Medicine*, Singh A, Singh H and Henderson, J, pp. 587-590.

Vijayakumar, S., Hellman S. (1997): Advances in radiation oncology. *Lancet.* **349**, 1-30.

Walburg, H.E. Jr. (1975): Radiation-induced life-shortening and premature aging in *Advances in Radiation Biology*, Lett, JT and Adler, DH, **5**, New York, pp. 145-179.

Walden, T.J. Jr., Farzaneh, N.K., (1989): Biological assessment of damage. In: Walker RI, Cervaney TJ, eds. Textbook of military medicine. Volume 2: Medical consequences of nuclear warface, Falls Church, VA: TMM Publications, Office of the Surgeon Geneal, 85-103.

Wang, X., Ghosh, S., Guo, S.W. (2001): Quantitative quality control in microarray image processing and data acquisition. *Nucleic. Acids. Res.* **29**, 1-8.

Yamada, S., Durante, M., Ando, K., Furusawa, Y., Kawata, T., Majima, H., Tsujii, H. (2000): Complex-type chromosomal exchanges in blood lymphocytes during radiation therapy correlate with acute toxicity. *Cancer. Letters.* **150**, 215-221.