Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2008, Article ID 541678, 7 pages
doi:10.1155/2008/541678

Research Article

Analysis of Gene Expression in Normal and Cancer Cells Exposed to γ-Radiation

M. Ahmad Chaudhry

Department of Medical Laboratory and Radiation Sciences, University of Vermont, 302 Rowell Building, Burlington, 05405 VT, USA

Correspondence should be addressed to M. Ahmad Chaudhry, mchaudhr@uvm.edu

Received 27 September 2007; Accepted 8 January 2008

Recommended by Claude Bagnis

The expression of many genes is modulated after exposure to ionizing radiation. Identification of specific genes may allow the determination of pathways important in radiation responses. We previously identified modulation of the expression of several genes in response to ionizing radiation treatment. In the present study, we monitored the expression of RGS1, CC3, THBS1, vWF, MADH7, and a novel gene encoding a secreted protein in irradiated Jurkat, TK6, HeLa, and HFL1 cells. The RGS1 is involved in G-protein signaling pathway, CC3 belongs to the complement system, THBS1 is a component of the extracellular matrix, vWF takes part in blood coagulation, and MADH7 is a member of the TGF-β signal transduction pathway. Our objective was to find similarities and differences in the expression of these genes in ionizing radiation-exposed diverse cell types. RGS1 was downregulated in Jurkat cells but was upregulated in TK6 and HFL1 cells. The expression of CC3 was repressed in Jurkat and HFL1 cells but was induced in TK6 and HeLa cells. THBS1 was downregulated in irradiated TK6 and HFL1 cells. vWF was induced in radiation-exposed HeLa cells, but its expression was downregulated in Jurkat cells. The expression of MADH7 was induced in all the cell types examined. These results indicate cell specific modulation of gene expression and suggest the involvement of different pathways in cellular response to radiation treatment in different cells.

1. INTRODUCTION

Ionizing radiation (IR) is known to potentially interfere with cellular functions at all levels of cell organization.The path from irradiation of the cells to the induction of biological effects comprises several complex steps provoking alterations to a variety of cellular components. The radiation-induced cellular effects include sister-chromatid exchanges, chromosome aberrations, apoptosis, micronucleation, transformation, mutations, and gene expression alterations. The critical DNA lesions generated by IR are double-strand breaks, which are repaired by homologous recombination and nonhomologous end joining. Depending on the structure of broken DNA ends, some of the single-strand breaks may be repaired by long-patch base excision repair (BER) pathway. Oxidative DNA damage is taken care of by short-patch BER [1]. The accuracy of DNA repair depends on the complexity of the DNA lesion [2] and on the fidelity of the DNA repair machinery itself.

The cellular response to IR is mediated via genes that control complex regulatory pathways. In response to IR exposure, several signal transduction pathways (e.g., ERK1/2, JNK, p38, and ATM) and transcription factors (e.g., AP1, NFκB, GADD153, and p53) are activated [3]. The ionizing radiation-induced stress response is very complex and involves altered expression of many genes. Ionizing radiation-induced alterations in the expression of many genes have been reported. In addition to classical radiation responsive genes, new studies have discovered the involvement of other genes as well. Apo1, nuclease sensitive element binding protein 1, syntaxin (a membrane integrated protein involved in exocytosis), cyclin G1, hNOP56, paraoxonase (hydrolyze oxidized lipids), and glutathione peroxidase were overexpressed after irradiation [4]. Activating transcription factor 3 (ATF3) was induced after X-irradiation [5]. IR exposure results in the induction of clusterin (associated with apoptosis) gene expression [6]. CXC chemokines and other secretory products (secretogranin II, thrombospondin type I

domain containing 2, amphiregulin, and interleukin-6) have shown to be modulated by IR [7]. The hematopoietic zinc finger protein, Hzf, was shown to be upregulated in response to genotoxic stress [8]. The isolation and characterization of a novel ionizing radiation-induced gene, apoptosis enhancing nuclease (AEN) has been described. AEN protein acts as DNase and enhances apoptosis following irradiation [9]. The modulation of connexin43 (associated with gap junctions) expression has been shown after exposure to IR [10]. The expression of chromatin assembly factor 1 (CAF-1) and proliferating cell nuclear antigen (PCNA) is dramatically induced as a result of DNA damage by the radiomimetic drug bleocin (a bleomycin compound) or by IR [11]. The expression of inducible nitric oxide synthase (iNOS) in X-ray irradiated mouse skin at both the mRNA and protein levels was recently published [12]. The expression and activation of MMP -2, -3, -9, -14 are induced in rat colon after abdominal X-irradiation [13].

Various studies have examined the radiation-induced gene expression profiles in diverse cell types. Microarray approach has identified genes regulated in response to IR [14–18]. The differential expression of apoptosis-related BAX and BCL-XL [19] and a dose-response relationship for the induction of CDKN1A, MDM2, GADD45A, ATF3, and BAX has been reported [20]. Early gene expression profile in mouse brain after exposure to IR identified change in the expression of JAK3, DFFB, NSEP1, and TERF1 genes [21]. IR was shown to enhance the expression of the nonsteroidal anti-inflammatory drug-activated gene (NAG1) [22]. Microarray analysis of the transcriptional response to single or multiple doses of IR has also been reported [23]. Based on microarray studies, we previously identified the induction of complement component 3 (CC3), von Willebrand factor (vWF), mothers against decapentaplegic homolog 7 (MADH7), regulator of G-protein signaling 1 (RGS1), thrombospondin 1 (THBS1), and a novel gene encoding a secreted protein (Sec Pro) genes in irradiated cells. In the present study, we examined the expression of CC3, vWF, MADH7, RGS1, THBS1, and Sec Pro genes in irradiated normal and tumor cells of various types. Our aim was to investigate variations in the response of these genes in diverse cell types and to identify any genes that are universally induced after exposure to ionizing radiation.

2. MATERIALS AND METHODS

2.1. Cell culture

Human normal lung fibroblast cell line HFL1 was purchased from American Type Culture Collection (ATCC) (Manassas, Va, USA). These cells were cultured as a monolayer in Ham's F12 K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate (Invitrogen, Carlsbad, Calif, USA) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin). Cells were maintained at a density of 2×10^5 cells per mL after trypsin treatment of adherent cells. HeLa cells (kindly provided by Dr. Markus Thali) were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) with 10% fetal calf serum, 1% penicillin, and streptomycin. Subconfluent cultures were trypsinized in 0.25% trypsin, 0.03% EDTA solution to obtain a density of 2×10^5 cells per mL, and grown as a monolayer.

The TK6 human B lymphoblast cells (kindly provided by Dr. Susan Wallace) were grown in suspension in RPMI 1640 medium (Cellgro/Mediatech Inc., Herndon, Va, USA) supplemented with 10% fetal calf serum (Invitrogen, Grand Island, NY, USA) at 37°C in 5% CO_2 and 100% humidity. Cell densities were maintained at 1–10 $\times$ 10^5 cells/mL. Lymphoblast acute T cell leukemia cell line Jurkat (kindly provided by Dr. Markus Thali) was grown in suspension in RPMI 1640 medium supplemented with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. The cell culture medium was supplemented with 10% fetal bovine serum. Cultures were maintained in suspension at a cell concentration between 1×10^5 and 1×10^6 viable cells/mL and the cell density was not allowed to exceed 10^6 cells/mL.

2.2. Ionizing radiation treatment

Exponentially growing cells were irradiated with 2 Gy of γ-radiation using a ^{137}Cs irradiator (Nordion International ISO 1000, Model B) at dose rate of 0.12 Gy/s at the Red Cross facility (Burlington, Vt, USA). The control cells did not receive any radiation and were mock irradiated. The irradiated and control cells were incubated at 37°C for various periods of times before isolating total RNA.

2.3. RNA isolation and cDNA synthesis

Total RNA from cells was isolated by using Trizol reagent (Invitrogen) according to the protocol provided by the supplier. The concentration of samples was determined by measuring the OD at 260 nm wavelength in a nanodrop spectrophotometer. The quality of the RNA was assessed on Bioanalyzer 2100 (Agilent) with a nanochip. 10 μg of total RNA was converted to cDNA with Superscript II reverse transcriptase (Invitrogen) and an oligo-$(dT)_{12–18}$ primer (Invitrogen) according to the manufacturer's recommendations. 1 μL of the cDNA was used as a template for amplification in the PCR.

2.4. Relative quantitative RT-PCR

Relative quantitative RT-PCR was performed using a commercially available kit (Ambion Inc., Calif, USA). This procedure modulates the amplification efficiency of a PCR template without affecting the performance of other targets in a multiplex PCR. The multiplex RT-PCR uses β-actin as an endogenous standard combined with Ambion's Competimer Technology. The actin competimers are modified at their 3′ ends to block extension by DNA polymerase. By mixing actin primers with actin competimers, the PCR amplification efficiency of actin can be reduced without the primers becoming limited and without loss of relative quantification. The actin primer pair and actin competimer are supplied at a concentration of 5 μM. The β-actin primer: competimer mixtures were prepared as three different ratios of 1 : 9, 2 : 8, and

3 : 7, and were examined for multiplex PCR with gene specific primers to establish the optimum concentration. A ratio of 2 : 8 actin primers to competimers is appropriate for most genes. A ratio of 1 : 9 is required for rare messages, and a ratio of 3 : 7 works best for relatively abundant messages. A primer: competimer ratio of 2 : 8 was found to be optimum for all the genes amplified in a multiplex format in this study. The PCR was set up using first-strand cDNA, gene specific primers, and β-actin primer: competimer mixtures and Platinum Taq DNA polymerase (Invitrogen) according to manufacturer's recommendations. All the PCR primers used in this study were designed with the Primer 3 software and were synthesized and purified by Operon Biotechnologies. The sequence of all the primers is shown in Table 1. The PCR amplification products were electrophoresed in 1% agarose gels stained with ethidium bromide (0.5 μg/mL). The gel images were captured with a Kodak digital imaging system and quantified with NIH Image software. The resultant data was plotted to show the alterations in the expression of these genes after exposure to ionizing radiation.

3. RESULTS

Exponentially growing TK6, Jurkat, HeLa, and HFL1 cells were irradiated with 2 Gy of γ-radiation and after incubation at 37°C for 4-, 8-, 12-, and 24-hour, total RNA was isolated and converted to cDNA. We examined the differential gene expression of six genes coding for CC3, vWF, MADH7, RGS1, THBS1, and a "Sec Pro" in Jurkat, TK6, HeLa, and HFL1 cells. We employed relative quantitative RT-PCR to examine differential gene expression. The relative quantitative RT-PCR in a multiplex format was done by using actin/modified actin primers (competimers) in combination with gene-specific primers. Competimers are specially modified primers of the same sequence as the normal β-actin primers that cannot be extended. By adjusting the ratio of β-actin competimers to normal β-actin mRNA primers, we were able to attenuate the signal for β-actin mRNA to the level of rare messages. In separate experiments, all cDNAs corresponding to the genes under investigation were amplified with 1 : 9, 2 : 8, and 3 : 7 ratios of actin primers to actin competimers in a multiplex format (data not shown). The ratio of 2 : 8 resulted in optimum amplification of all the genes and actin cDNAs.

We first monitored the radiation-induced expression of RGS1, CC3, THBS1, vWF, MADH7, and Sec Pro genes in Jurkat cells (Figure 1). The overall expression of RGS and CC3 was lower in these cells as compared to the expression levels of THBS1, vWF, MADH7, and Sec Pro. The expression of RGS1 and CC3 remained unaltered up to 12 hours (Figure 1) but was downregulated at 24-hour time point. A similar downregulation of vWF at the 24-hour time point was also seen. The most prominent gene induction was seen for MADH7, which was induced after 4 hours of radiation exposure of Jurkat cells and remained upregulated up to 24 hours (Figure 1).

The expression of variousgenes in irradiated TK6 cells at various time points is shown in Figure 2. RGS1, CC3, and Sec Pro were all induced after radiation treatment. These three genes were induced at the 4-hour time point and remained upregulated till 24 hours. THBS1 showed two peeks of gene downregulation, one at 4 hours and the other at 12 hours. An opposite effect was seen for the expression of MADH7 gene where an upregulation was seen at 4 and 12 hours after irradiation while the expression of this gene returned to normal levels at 8 and 24 hours. The overall expression of vWF was lower as compared to all the other genes and did not appear to be altered after radiation treatment (Figure 2).

Figure 3 shows the gene expression pattern in HeLa cells after irradiation. The CC3 gene was induced after irradiation and its expression level peeked at 12 hours. After this time point, a decline in the expression of this gene was observed. A similar pattern of gene induction was seen for vWF where the expression of this gene reached the maximum level at 12 hours before showing a decline at the 24-hour time point. In case of Sec Pro, the expression was the highest after at 8 hours after irradiation. An interesting pattern of gene expression was seen for MADH7, where an upregulation was seen at 4-hour postirradiation and then a dramatic gene repression was seen at all the other time points, suggesting a complete shut down of this gene.

Finally, we examined the gene expression in irradiated HFL1 cells (Figure 4). RGS1 was induced in these cells after 8 hours of radiation exposure. The expression level of CC3 did not change up to 8-hour postirradiation and then started to get downregulated. THBS1 gene was first repressed and later returned to normal levels at 12-hour time point. MADH7 was upregulated at 8 hours, and its expression level continued to be increased until 24 hours. vWF and Sec Pro were expressed at reduced levels in irradiated HFL1 cells.

4. DISCUSSION

IR activates both pro- and antiproliferative signal transduction pathways, the balance of which determines cell fate. The initiating and amplifying mechanisms involved in the activation are poorly understood. The cellular response to IR is mediated via genes that control complex regulatory pathways such as cell cycle progression, apoptosis, or DNA repair. The relative contribution of changes in the expression of these genes on signaling pathways is unknown. The mechanisms for the survival of cells after IR exposure may involve the induction of DNA repair, immediate early, cytokine, and growth factor genes. Cells respond to IR with the activation of specific early and later response genes. The products of early response genes may be responsible for regulating downstream genes that are important in the adaptation of cells and tissues to radiation-induced stress.

We investigated the radiation-induced gene expression in Jurkat, TK6, HeLa, and HFL1 cell lines. Jurkat cells are p53 negative and TK6 cells are p53 positive. Both of these cells have lymphoblast origin and are grown as suspension cultures. On the other hand, HeLa (epithelial origin), and HFL1 (fibroblast origin) cell lines are grown as adherent cultures. HeLa cells are p53 negative while HFL1 cells are p53 positive. The examination of radiation-induced gene expression in a variety of cell lines differing in p53 status or growth

Table 1: RT-PCR primers for various genes.

Gene	Accession	Left primer	Right primer	Product size
G-protein signaling 1 (RGSI)	NM_002922	TGCCAGGAATGTTCTTCTCT	CTATTAGCCTGCAGGTCATTTAGA	580
Von Willebrand factor (vWF)	NM_000552.2	GTCCGAGGCTGAGTTTGAAG	TAACGATCTCGTCCCTTTGC	500
Mothers against the decapentaplegic homolog 7 (MADH7)	NM_005904.1	CCAACTGCAGACTGTCCAGA	GTCGAAAGCCTTGATGGAGA	450
The complement component 3 (CC3)	NM_000064.1	GGAAAAGGAGGATGGAAAGC	ACCCAAAGACAACCATGCTC	497
Thrombospondin 1 (THBS1)	NM_003246.2	GGAGACAACAGCGTGTTTGA	GCCAGGTCTCTGGTGAAGAC	500
Secreted protein (Sec Pro)	BF440021.1	GCTTTTCACCGAGGAGGAG	TGCAGGAGATTCAGCACCTA	393

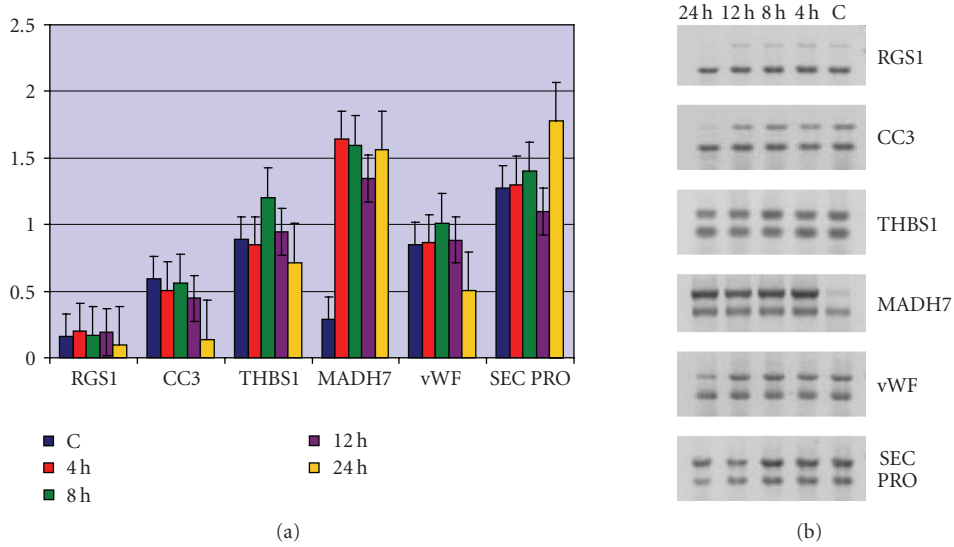

Figure 1: Ionizing radiation-induced gene expression in Jurkat cells. (a) The expression ratios of various genes at 4-, 8-, 12-, and 24-hour time points compared to unirradiated sham control. (b) Agarose gel images of relative quantative RT-PCR for various genes. In all cases, the upper gel band represents gene-specific amplification and the lower gel band indicates the relative amplification of β-actin internal standards.

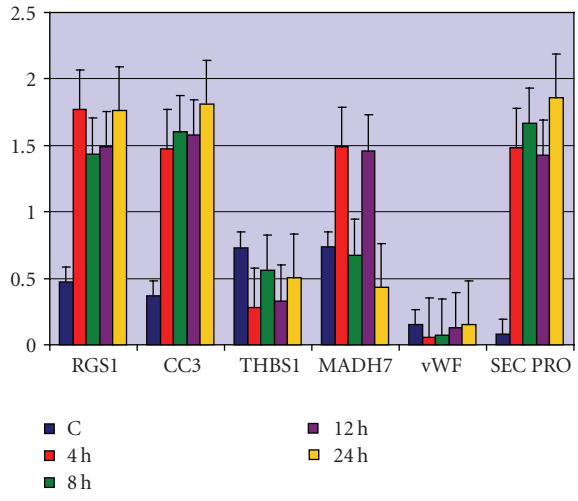

Figure 2: The gene expression in ionizing radiation treated TK6 cells. The expression ratios for various genes at 4, 8, 12, and 24 hours after radiation exposure were determined and compared to the control mock irradiated cells.

conditions could provide information as signature pathways operating in all cell types.

A striking observation was the induction of MADH7 in all the cell lines exposed to ionizing radiation. MADH7 (also known as Smad7) belongs to the category of Smad proteins. These proteins are signaling transducers downstream from TGF-β receptors [24]. Smad7 belongs to the inhibitory Smads category of TGF-β signal transduction pathway and prevents TGF-β signaling. Expression of TGF-β after radiation exposure is well known [25]. It has been suggested that TGF-β is the master switch cytokine, which, once activated after radiation, promotes a cascade of cellular events. Escape from TGF-β-induced inhibition of growth and proliferation may contribute to tumorigenesis. Our data suggests that MADH7 is upregulated in all the cells examined in this study regardless of their p53 status.

vWF was induced in radiation-exposed HeLa cells (Figure 3) but its expression was downregulated in Jurkat cells (Figure 1). However, vWF is a glycoprotein involved in blood coagulation. Increased amounts of vWF in blood plasma are indicative of damaged endothelium. Ionizing

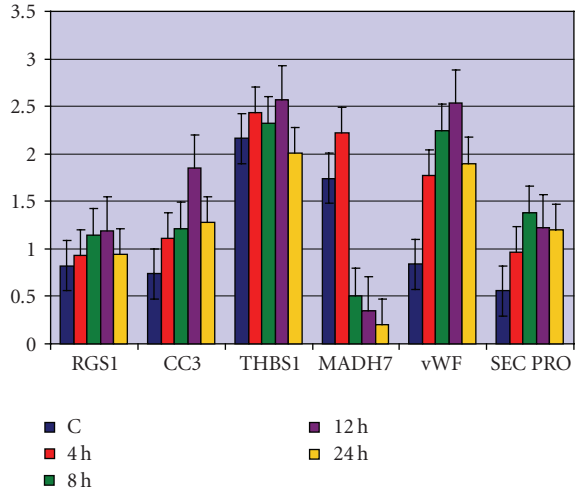

Figure 3: The expression of RGS1, CC3, THBS1, vWF, MADH7, and Sec Pro at various time points in irradiated HeLa cells.

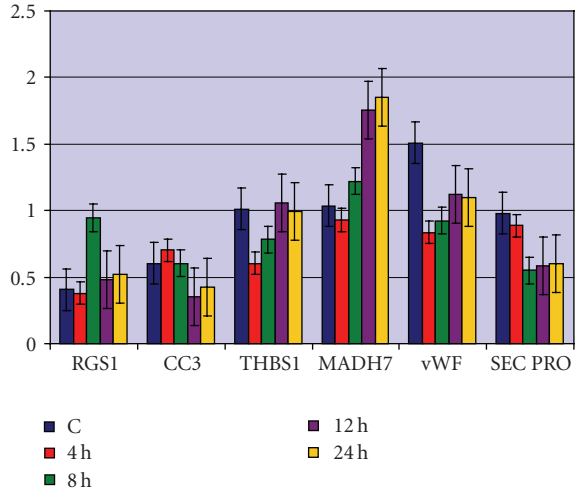

Figure 4: The expression of RGS1, CC3, THBS1, vWF, MADH7, and Sec Pro at 4-, 8-, 12-, and 24-hour time points in irradiated HFL1 cells.

irradiation damage to the vasculature results in an increase in procoagulant activity of endothelial cells, including elevated vWF secretion [26]. The induction of vWF in brain after IR exposure has been reported [27].

RGS1 was downregulated in Jurkat cells (Figure 1) but was upregulated in TK6 (Figure 2) and HFL1 cells (Figure 4). G-protein-coupled receptors (GPCRs) play a central role in the regulation of cell communication and encompass a wide range of functions that includes the rhodopsin-like GPCRs, the secretin-like GPCRs, the cAMP receptors, the pheromone receptors, and the metabotropic glutamate receptor subfamilies as well as nuclear receptors. A recent in vivo study examined the effect of acute and fractionated low-dose radiation and sex differences in the expression of the GPCRs in the murine muscle tissue [28]. Regulators of G-protein signaling (RGS) play a critical role in GPCR signaling in mammalian cells and could be involved in radiation-induced signal transduction pathways.

The expression of CC3 was repressed after exposure to IR in Jurkat cells (Figure 1) and HFL1 cells (Figure 4). On the contrary, this gene was induced in TK6 and HeLa cells (Figures 2 and 3, resp.). The complement system has long been regarded as consisting of proinflammatory proteins circulating in the peripheral blood and tissue fluids. The complement system is an important mediator of natural and acquired immunity. It consists of approximately 30 proteins that can exhibit catalytic activity, function as regulators, or act as cellular surface receptors [29]. These components normally circulate in inactive forms and are activated by the classical, alternative, or lectin pathways. CC3 plays a central role in all three activation pathways where it exerts several important biological roles. Different cell groups of immune system give different responses in individuals exposed to long-term ionizing radiation. Recently, the effect of occupational exposure to low levels of IR were investigated in selected indices of cellular and humoral immunity in radiology workers and were found to be weaker in radiation workers [30].

The expression of THBS1 was found to be downregulated in irradiated TK6 (Figure 2) and HFL1 cells (Figure 4). THBS1, a matricellular glycoprotein, is a component of the extracellular matrix predominantly during active or subacute processes [31]. THBS1 is released by platelets during the formation of a hemostatic plug. In addition, macrophages, monocytes, fibroblasts, vascular smooth muscle cells, tumor cells, and endothelial cells also secrete THBS1 or their function is modulated by THBS. THBS1 is also an endogenous angiogenesis inhibitor. Our studies are consistent with those of others showing THBS1 to be downregulated by ultraviolet B light [32] and by IR [7].

Many studies have reported the modulation of gene expression in IR-treated cells. The results of such studies are mixed and perhaps are not surprising, given the complexity of the response to damage and the multitude of factors that contribute to the diversity of cellular sensitivity. Additional difficulties to interpret data involve variations in growth conditions of cells under investigation and a variety of IR doses and quality employed. The ionizing radiation-induced gene expression could be cell-type-specific. The expression of connexin43 after exposure to IR has shown to vary in different cell lines [10]. The variation in the response has not been fully explained and may be related to intrinsic biologic capability of the cells. The investigation of the regulation of the gene expression in multiple cells of various origins exposed to IR is necessary for an understanding of the generality of gene expression response.

The identification of radiation responsive genes in various cell types will permit an understanding of the molecular mechanisms underlying radiation-induced physiological responses and an ability to predict the radiation susceptibility of normal tissues in radiotherapy patients. The ability to detect altered gene expression has prompted a search for molecular markers that could predict tumor response to therapy. Advances in the knowledge of how cells respond to IR will provide opportunities for the development of new approaches that selectively enhance radiotherapy of

tumors. Additionally, the availability of biomarkers to monitor potentially exposed individuals after a radiological accident would be extremely valuable. The investigation of gene expression after exposure to IR could serve as a potential molecular marker for such biodosimetry.

ACKNOWLEDGMENT

This work was supported by a research incentive grant from the College of Nursing and Health Sciences, University of Vermont.

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