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Expression of Cytoskeletal and Matrix Genes Following Exposure to Ionizing Radiation: Dose-rate Effects and Protein Synthesis Requirements

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Abbreviations:

SHE - Syrian hamster embryo
LET - linear energy of transfer
CHX - cycloheximide
PBS - phosphate-buffered saline
SDS - sodium dodecyl sulfate

ABSTRACT

WOLOSCHAK, G. E., FELCHER, P., and CHANG-LIU, C-M. Expression of Cytoskeletal and Matrix Elements Following Exposure to Ionizing Radiation: Dose-rate and Protein Synthesis Requirements. *Radiat. Res.*

Experiments were designed to examine the effects of radiation dose-rate and of the protein synthesis inhibitor cycloheximide on expression of cytoskeletal elements (γ- and β-actin and α-tubulin) and matrix elements (fibronectin) in Syrian hamster embryo cells. Past work from our laboratory had already demonstrated optimum time points and doses for examination of radiation effects on accumulation of specific transcripts. Our results here demonstrated little effect of dose-rate for JANUS fission-spectrum neutrons when comparing expression of either α-tubulin or fibronectin genes. Past work had already documented similar results for expression of actin transcripts. Effects of cycloheximide, however, revealed several interesting and novel findings: (1) Cycloheximide repressed accumulation of α-tubulin following exposure to high dose-rate neutrons or γ rays; this did not occur following similar low dose-rate exposure. (2) Cycloheximide did not affect accumulation of mRNA for actin genes. (3) Cycloheximide abrogated the moderate induction of fibronectin-mRNA which occurred following exposure to γ rays and high dose-rate neutrons. These results suggest a role for labile proteins in the maintenance of α-tubulin and fibronectin mRNA accumulation following exposure to ionizing radiation. In addition, they suggest that the cellular/molecular response to low dose-rate neutrons may be different from the response to high dose-rate neutrons.

INTRODUCTION

Many recent studies have documented changes in gene expression that accompany exposure to ionizing radiation, with the modulated genes including those encoding cytoskeletal elements (1-3), oncogenes (4,5), cytokines (1,6), protein kinase C (7), and many other cellular proteins (8,9,10). It is believed that this early gene modulation (occurring within the first four hours following exposure) is important in modulating later cellular events such as DNA repair, inhibition of DNA synthesis, changes in cell shape, modulation of later genes, or tumorigenic transformation of cells (8,11,12).

Past work from our group has demonstrated the modulation of cytoskeletal gene expression following exposure to ionizing radiation (1,2,3). From this work, we have documented the similar modulation of expression of α-tubulin and γ- and β-actin genes following exposure to high-LET JANUS fission-spectrum neutrons and low-LET γ rays or X-rays (1). In addition, we had previously examined the effects of dose, cellular proliferative state, and kinetics on the response. In the experiments reported here, we have extended these findings to include dose-rate effects and the effects of the protein synthesis inhibitor cycloheximide on this gene modulation. Cycloheximide has routinely been used as a probe for the role of protein synthesis and labile proteins in cellular responses. Many reports have documented genes induced in response to cycloheximide, including β-actin, c-*fos*, c-*jun*, c-*myc*, histones, and others (13-16). Genes induced by protein synthesis inhibitors are considered to be regulated intracellularly in a negative manner by a labile protein(s). In this report, we also have included studies of the expression of the matrix-associated protein fibronectin

following radiation exposure. We have used doses and time-points shown in previous work to be optimum for studies of gene modulation. Past work also has shown cycling cells to be more sensitive to the effects of ionizing radiation and therefore we have used only cycling cells in this work (2,3).

MATERIALS AND METHODS

Cells and Culture Conditions

These experiments were designed to examine the effects of exposure to ionizing radiation on the expression of specific genes in Syrian hamster embryo (SHE) fibroblasts. These cells are normal, diploid cells that can be neoplastically transformed by ionizing radiation (12,17).

All cell cultures were maintained in Dulbecco's modified Eagle's medium, which contains 10% fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin. Cells were grown to 50% confluence, and 48 h before irradiation they were placed in a medium containing 10% fetal calf serum to maintain them in exponential phase.

Radiation and Cycloheximide Treatments

Cells plated in 100-mm Petri plates containing 10 ml medium were irradiated with ^{60}Co γ rays or fission spectrum neutrons (0.85 MeV) from the JANUS reactor. The actual design of the JANUS reactor and its dosimetry have been reported previously (18). All irradiations were performed at room temperature on cycling cells (50% confluence); equitoxic doses of neutrons and γ rays were selected on the basis of survival data (17). Doses and dose-rates were chosen on the basis of previous work examining cytoskeletal gene expression in SHE cells (2,3). Control cells were taken to the radiation chamber, but not exposed to radiation. Fifteen minutes before

irradiation, 100 µg/mL of cycloheximide (CHX) in PBS was added to the CHX-treated group of cells, while an equal volume (1 mL) of PBS was added to the controls. Sixty minutes after irradiation, the cells were harvested.

Purification of RNA and Northern Blots

RNA was prepared by isolation in 6 M guanidine isothiocyanate, extraction with phenol, and precipitation from 3 M sodium acetate, pH 6.0 (1,2,3). Poly(A)+ RNA was isolated by oligodeoxythymidylate cellulose chromatography until no unbound material absorbing at 254 nm was detected. RNA was stored as an ethanol precipitate at -20 °C. Routinely we found that poly(A)+ RNA represented 5-10% of the total RNA fraction.

RNA was separated by using formaldehyde agarose gel electrophoresis as described previously (1,2,3). Poly(A)+ RNA samples were denatured in a solution of 50% formamide, 1.9 M formaldehyde, 0.2 M 3-(N-morpholino)propanesulfonic acid, 50 mM sodium acetate, and 1 mM disodium EDTA (pH 7.5) for 15 min at 55 °C and then separated on 1.2% agarose gels in a solution of 0.2 M 3-(N-morpholino)propanesulfonic acid, 50 mM sodium acetate, 1 mM disodium EDTA, and 2.2 M formaldehyde. Parallel lanes containing rRNA (*Escherichia coli* and mouse) and RNA ladder markers (BRL Laboratory, Bethesda, MD) were stained with ethidium bromide and photographed under UV light for use in sizing.

Northern blot transfers were performed as described previously (1,2,3). Blots were hybridized to ^{32}P-nick-translated or oligo-labeled cDNA probes. Hybridization conditions were 50% deionized formamide, 0.75 M NaCl, 75 mM sodium citrate, 25-50 mM sodium phosphate (pH 6.5), 0.2% SDS, 0.2% bovine serum albumin, 0.2% ficoll, 0.2% polyvinylpyrrolidone, and 50% µg/mL sonicated denatured herring sperm DNA at 43 °C. Prior to hybridization, all labeled probes were heat denatured at 90 °C for 5 min. After hybridization, nonspecific binding was reduced by washing the hybridized blots three times for 1 h each at 43 °C in 45 mM sodium citrate (pH 7.4), 0.45 M NaCl, 0.2 % ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 µg/mL sonicated denatured herring sperm DNA, and 0.1% SDS. The blots were then dried and exposed to x-ray film at -20 °C.

In some experiments, the same blot was washed and hybridized successively to several different probes. Each probe was eluted by washing for 24 h in distilled, deionized water at 43 °C, and blots were checked for total removal of the labeled probes by 24-h exposure to x-ray film. Blots were washed three times in hybridization buffer before rehybridization to a different probe. Relative quantitation of the hybridized probe was determined by using a Hirschmann microdensitometer. All results were averaged from three independent observations.

Although equal amounts by weight, as determined by spectrophotometry, of RNA were loaded into each well of a given gel, we found sufficient variation from one preparation to another to make poly(A)+ analysis essential; mRNA analysis systems measuring the molar concentration of RNA with 3'poly(A) tails (Molecular Genetics Resources, Tampa, FL) were used for all poly(A)+ RNA preparations, and only RNA

samples showing equimolar concentrations of poly(A)+ RNA were loaded onto the same gel. All results reported here are the composite of three independent determinations.

cDNA Clones

We gratefully acknowledge the gifts of the following clones made available to us: isotype-specific actin cDNA clones (pHFβA-3'ut, pHFγA-3'ut) were obtained from Dr. L. Kedes (Stanford Univ., Palo Alto, CA), non-isotype specific actin cDNA clones from Dr. A. Minty (Pasteur Institute, Paris), α-tubulin from Dr. C. Veneziale (Mayo Clinic, Rochester, MN), and fibronectin clone from the American Type Culture Collection (Rockville, MD).

RESULTS

Dose-rate effects.

Previous work had demonstrated that accumulation of β- and γ-actin mRNA was not affected by the dose-rate at which JANUS fission-spectrum neutrons were administered (2). To determine whether similar results occurred for expression of other cytoskeletal elements (α-tubulin) and for expression of the matrix element fibronectin, experiments were performed using similar doses and dose-rates of JANUS neutrons. In all experiments cycling cells were used as in previous experiments (2). Northern blots were performed and analyzed by microdensitometry; results for both α-tubulin and fibronectin hybridizations are presented in Table I. These results demonstrate little difference, however, between the effects of high- vs. low-dose-rate neutrons for α-tubulin or fibronectin mRNA accumulation. Induction of α-tubulin mRNA was evident following JANUS n_0 exposure. On the other hand, at higher doses, decreased accumulation of fibronectin-mRNA was evident. Due to the different exposure times and kinetics of the response, the dose-rate-dependence of this is unknown.

Effects of Cycloheximide.

Experiments were designed to compare the effects of cycloheximide, a protein synthesis inhibitor, on accumulation of β- and γ-actin mRNA following exposure to equitoxic doses of JANUS neutrons and γ rays (12 cGy neutrons vs. 50 cGy γ rays) administered at high or low dose-rates. These conditions of cycloheximide exposure (100 μg/mL) in SHE cells were shown to be appropriate for induction of c-*fos* mRNA (Figure 1), a transcript previously shown to be cycloheximide responsive (13,14).

Table II presents the results of microdensitometric analyses of Northern blots probed with β- or γ-actin to determine relative expression of these transcripts. From these results, it is apparent that cycloheximide has little effect on accumulation of β-actin mRNA before or after radiation exposure in SHE cells. The moderate inhibition of γ-actin mRNA accumulation that accompanied γ-ray exposure was abrogated by cycloheximide treatment, but these differences were slight and may be peculiarities of this cell system. It should be noted that levels of rRNA were the same under all experimental conditions (Table III), thus showing equal loading/transfer of RNA.

Further Northern blot experiments aimed at examining effects on α-tubulin and fibronectin expression are presented as microdensitometric results in Table IV. These experiments revealed that cycloheximide diminished the level of α-tubulin-specific mRNA following low dose-rate exposure. For fibronectin mRNA expression, moderate induction was observed following exposure to high dose-rate neutrons or high- or low-dose-rate γ rays. Low dose-rate neutrons had no effect. However, cycloheximide provided for induction following low dose-rate neutron exposure but diminished the response following high dose-rate neutrons or high or low dose-rate γ rays. This suggests that the cellular response to low dose-rate neutrons may be different than the response following high dose-rate neutrons or high or low dose-rate γ rays.

DISCUSSION

Past work from our group has documented the modulation of cytoskeletal gene expression that accompanies radiation exposure, examining doses and kinetics as well as cell cycle effects (1,2,3). We have demonstrated similar modulation of β- and γ-actin and α-tubulin mRNA following exposure to high-LET JANUS neutrons and to low-LET γ rays (1,2). In the work reported here, we extended previous results by examining dose-rate effects for α-tubulin and fibronectin mRNA expression and by examining the effects of the protein synthesis inhibitor cycloheximide on accumulation of transcripts specific for actins, α-tubulin, and fibronectin.

Studies done *in vitro* to determine the effects of ionizing radiations on cellular transformation have shown differences in transformation efficiencies between cells exposed to ionizing radiations administered at either high or low dose-rates (19,20). In this study, we set out to determine whether effects of low vs. high dose-rate radiations would be evident in the molecular response of the cell to the radiation exposure. Our results demonstrated little effect of neutron dose-rate on accumulation of α-tubulin and fibronectin mRNA. This is consistent with past results in which similar expression patterns of β- and γ-actin mRNA following high- and low-dose-rate neutrons and γ rays were detected.

In further studies aimed at determining the role of labile proteins in the molecular response of the cell to ionizing radiations, we examined the effects of cycloheximide on these low and high dose-rate responses. These experiments determined that while cycloheximide had no effect on expression of the actin transcripts, the inhibitor caused repressed accumulation of α-tubulin mRNA following

high dose-rate neutrons or γ rays; a similar response did not occur following low dose-rate exposures. In addition, cycloheximide abrogated the moderate induction of fibronectin mRNA which occurred following exposure to γ rays and high dose-rate neutrons. These results suggest the involvement of some unidentified labile protein in the maintenance of α-tubulin and fibronectin mRNA levels following exposure to ionizing radiations. In addition, the data suggest that the cellular/molecular response to low and high dose-rate neutrons and γ rays is quite different and involves different intracellular mechanisms. Further experiments will be required to dissect these responses.

It should be noted that under the conditions reported here, we did not observe an induction of β-actin mRNA following exposure to cycloheximide as had been reported by other groups (13,14). We believe that this is peculiar to SHE cells since we have demonstrated induction of β-actin in other fibroblast cell types (HeLa) following inhibition of protein synthesis (Libertin, Panozzo, and Woloschak, unpublished observations). However, the fact that c-*fos* (Figure 1) and c-*jun* (not shown) are induced in this system demonstrates that the cycloheximide concentrations used here are appropriate for the analyses.

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FIGURE LEGENDS

Figure 1. Northern blot examining c-*fos* mRNA accumulation in untreated SHE cells (0) or in SHE cells following exposure to 12 cGy JANUS neutrons administered at 0.5 cGy/min (Ln_o) or 12 cGy/min (Hn_o) or γ rays administered at 1 cGy/min ($L\gamma$) or 14 cGy/min ($H\gamma$). This was done in the absence or presence (CHX) of 100µg/mL cycloheximide.

Table I. Cycling Cells: α-Tubulin and Fibronectin mRNA Expression Following n_o Exposure[a]

Dose (cGy)	Dose-rate (cGy/min)	Relative α-tubulin mRNA[b]	Relative fibronectin mRNA[c]
0	0	1.0 (.01)[d]	1.0 (.06)
6	0.5	1.6 (.03)*	1.3 (.01)
12	0.5	1.3 (.04)	ND
24	0.5	1.4 (.05)	1.3 (.06)
36	0.5	1.3 (.03)	1.2 (.01)
12	12	1.7 (.04)*	1.4 (.03)
24	12	1.6 (.01)*	1.1 (.04)
48	12	1.6 (.04)*	0.4 (.13)
96	12	1.4 (.07)	0.2 (.05)

[a]All mRNA levels based on cells harvested 1 h following completion of the radiation exposure.

[b]α-tubulin mRNA in untreated cells was set at 1.0. All samples are expressed relative to that.

[c]Fibronectin-mRNA levels in untreated cells were set at 1.0. All other RNAs were expressed relative to that.

[d]Standard deviations are in parentheses.

*Denotes significantly different from untreated (0) controls at $p < .05$.

Table II. Effects of γ rays/JANUS Neutrons on β-actin/γ-actin Expression in Cycloheximide-treated Cells[a]

Treatment	Dose	Relative β-actin mRNA[b]	Relative γ-actin mRNA[c]
Untreated	0	1.0 (.06)[d]	1.0 (.01)
JANUS n_o, 0.5 cGy/min	12 cGy	0.8 (.15)	0.7 (.01)
JANUS n_o, 12 cGy/min	12 cGy	1.2 (.06)	0.8 (.01)
γ rays, 1 cGy/min	50 cGy	1.1 (.10)	0.6 (.03)*
γ rays, 14 cGy/min	50 cGy	1.0 (.17)	0.7 (.01)
CHX[d]	0	0.9 (.12)	0.6 (.03)*
CHX, JANUS n_o, 0.5 cGy/min	12 cGy	1.3 (.06)	1.1 (.03)
CHX, JANUS n_o, 12 cGy/min	12 cGy	1.2 (.05)	0.6. (.01)*
CHX, γ rays, 1 cGy/min	50 cGy	0.9 (.09)	1.3 (.03)
CHX, γ rays, 14 cGy/min	50 cGy	1.0 (.14)	1.3 (.01)

[a]Cycling SHE cells were exposed to doses and dose-rates of γ rays as shown above. 1 h post-exposure RNA was harvested and analyzed by Northern blots.

[b]Relative expression of β-actin in untreated cells was set at 1.0. All other β-actin mRNA levels are expressed relative to that. Values in parentheses are standard deviations.

[c]Relative expression of γ-actin in untreated cells was set at 1.0. All other γ-actin mRNA levels are pressed relative to that. Values in parentheses are standard deviations.

[d]Cycloheximide (100 μg/mL) was given 10 min prior to irradiation, and cells were harvested 1 h after completion of the radiation exposure.

*Denotes significantly different from untreated (0) controls at $p < .05$.

Table III. Effects of γ rays/JANUS Neutrons on rRNA Expression in Cycloheximide-treated Cells[a]

Treatment	Dose	Relative rRNA[b]
Untreated	0	1.0 (.02)[d]
JANUS n_o, 0.5 cGy/min	12 cGy	0.9 (.04)
JANUS n_o, 0.5 cGy/min	12 cGy	0.8 (.01)
γ rays, 1 cGy/min	50 cGy	1.0 (.11)
γ rays, 14 cGy/min	50 cGy	1.0 (.11)
CHX[c]	0	0.9 (.04)
CHX, JANUS n_o, 0.5 cGy/min	12 cGy	1.0 (.15)
CHX, JANUS n_o, 12 cGy/min	12 cGy	0.9 (.09)
CHX, γ rays, 1 cGy/min	50 cGy	1.1 (.02)
CHX, γ rays, 14 cGy/min	50 cGy	0.9 (.10)

[a]Cycling SHE cells were exposed to doses and dose-rates of γ rays as shown above. 1 h post-exposure RNA was harvested and analyzed by Northern blots.

[b]Relative expression of rRNA in untreated cells was set at 1.0. All other rRNA levels are expressed relative to that. Values in parentheses are standard deviations.

[c]Cycloheximide (100 μg/mL) was given 10 min prior to irradiation, and cells were harvested 1 h after completion of the radiation exposure.

[d]Standard deviations are in parentheses.

Table IV. Effects of γ rays/JANUS neutrons on α-tubulin and fibronectin mRNA in Expression Cycloheximide-treated Cells[a]

Treatment	Dose	Relative α-tubulin mRNA[b]	Relative fibronectin mRNA[c]
Untreated	0	1.0 (.06)[e]	1.0 (.13)
JANUS n_o, 0.5 cGy/min	12 cGy	1.1 (.06)	1.3 (.02)
JANUS n_o, 12 cGy/min	12 cGy	1.9 (.04)*	1.5 (.04)*
γ rays, 1 cGy/min	50 cGy	1.0 (.06)	1.7 (.02)*
γ rays, 14 cGy/min	50 cGy	1.0 (.04)	1.5 (.04)*
CHX[d]	0	0.7 (.05)	1.5 (.05)*
CHX, JANUS n_o, 0.5 cGy/min	12 cGy	0.9 (.04)	1.9 (.05)*
CHX, JANUS n_o, 12 cGy/min	12 cGy	0.4 (0.1)*	1.1 (.01)
CHX, γ rays, 1 cGy/min	50 cGy	0.9 (.03)	1.1 (.14)
CHX, γ rays, 14 cGy/min	50 cGy	0.5 (.07)*	0.9 (.03)

[a]Cycling SHE cells were exposed to doses and dose-rates of γ rays as shown above. 1 h post-exposure RNA was harvested and analyzed by Northern blots.

[b]Relative expression of α-tubulin in untreated cells was set at 1.0. All other α-tubulin levels are expressed relative to that. Values in parentheses are standard deviations.

[c]Relative expression of fibronectin mRNA in untreated cells was set at 1.0. All other fibronectin mRNA levels are expressed relative to that.

[d]Cycloheximide (100 μg/mL) was given 10 min prior to irradiation, and cells were harvested 1 h after completion of the radiation exposure.

[e]Standard deviations are in parentheses.

*Denotes significantly different from untreated (0) controls at $p < 0.05$.

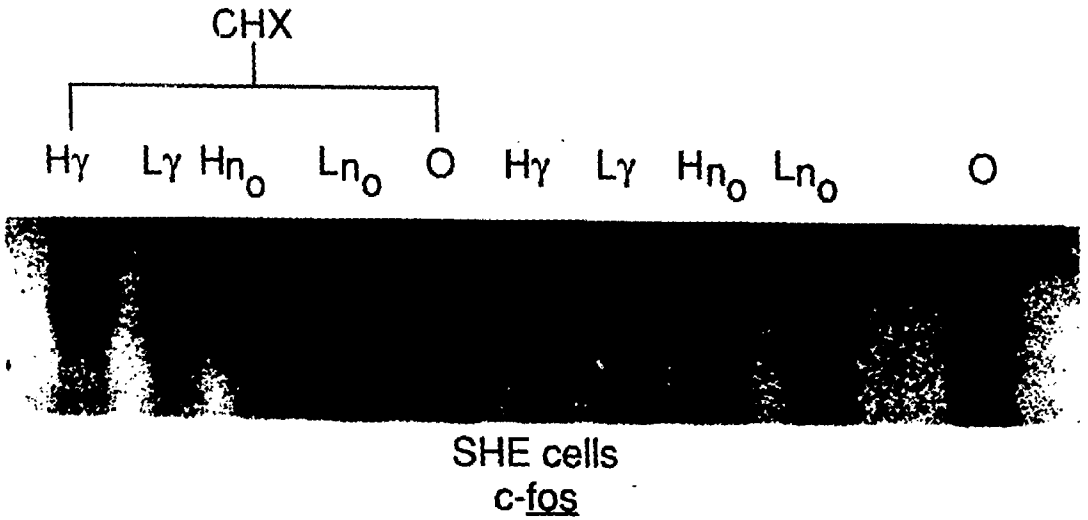

Fig. 1

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ABSTRACT

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