1 of 1

ROLE OF X-RAY-INDUCED TRANSCRIPTS IN ADAPTIVE RESPONSES FOLLOWING X-RAYS

Progress Report-Year 2.

A. SPECIFIC AIMS.

From previous data in our lab and others, certain human cells can clearly carry out adaptive survival responses (ASRs) following ionizing radiation. Our research goals are to better understand how some human cells can establish ASRs, and why other cells fail to do so. From our work in the first one and one-half years of this granting period, we have discovered a number of gene transcripts and gene products whose expressions are altered during the establishment of ASRs or following a high dose challenge of ionizing radiation. We are now beginning research which will investigate possible functions of these genes and their relationship to radioresistance. We will use subtractive and/or differential hybridization to isolate known and unknown gene products which might be involved in establishing ASRs, or which might be involved in radioresistance and cell cycle regulation of adapted human cells following a high dose ionizing radiation challenge. The completion of this research will require long-term support from the Department of Energy. Since the last progress report, which was approximately 8 months ago, we have made significant progress on our final two specific aims, Specific Aim #2 and #3. These Specific Aims were:

Specific Aim #2. to use subtractive and/or differential cDNA cloning hybridization techniques to select for, isolate, sequence, and characterize new mRNA transcripts induced following repeated low doses of ionizing radiation as compared to unirradiated or high dose-irradiated human melanoma cells. DNA sequences of the isolated low dose-induced cDNA clones will be obtained, compared to previously sequenced genes via GenBank analyses (including the previously cloned transcripts, xip1-12), and their temporal induction levels will be characterized.

Specific Aim #3. Once low-dose induced cDNA clones have been isolated, we will investigate their function(s) via antisense gene knock out experiments. The effects of such specific gene down-regulation on cell survival recovery (i.e., PLDR), adaptive responses, physical DNA break repair (using pulsed field gel electrophoresis (PFGE) and alkaline and neutral filter elution techniques), and cell cycle regulation will be examined. Antisense cDNA constructs corresponding to two of the twelve xip clones (i.e., xip5 and xip12-related transcripts), which demonstrated low dose induction in Specific Aim #1, will be constructed and utilized to determine their involvement in survival recovery, DNA repair, and/or in radioresistance due to adaptive survival responses (ASRs). Conventional plasmid transfections using the pNUT expression vector (Appendix) will be utilized for these experiments. Northern blot and nuclear run-on RNA analyses will be utilized to ensure that specific gene down-regulation (corresponding to a specific gene) has occurred. Once gene down-regulation has been established, its effects on DNA repair, cell cycle distribution, and overall radioresistance will then be investigated. Candidate cDNA clones thought to be involved in DNA repair will be used in complementation studies (via cDNA clone transfection into various cancer-prone cells) to investigate the possibility of correcting (probably partial correction will result) individual DNA repair defects in cancer-prone cells. We will also individually overexpress genes which show an involvement in ASRs in human cells which are devoid of demonstrating an adaptive survival phenomenon. In this way we may discover a gene or genes which regulate ASRs.

MASTER

B. OVERVIEW OF RESEARCH PROGRESS AND FUTURE PLANS.

I will describe our recent data in which we have extracted and purified a sufficient amount of RNA from primed and unprimed U1-Mel cells to begin the search for new genes which are modulated by priming or high dose irradiation during the establishment and/or challenge of adapted cells, respectively. In this, the final one and one-half years of this granting period, I will demonstrate the long-term nature of this research. Our Specific Aims were originally scheduled to be completed in a period of 5 years. I will describe our recent efforts (under "Preliminary Data") to analyze the function(s) of those gene products which are altered during the establishment of ASRs. Gene transcripts which are altered during ASRs now include alterations in xip5 (a gene with homology to human growth hormone), xip12 (a gene with homology to human angiogenesis factor and a gene which may be involved in apoptosis due to its possible RNase activity), cyclin A (which is altered in primed cells only after a high dose of ionizing radiation), cyclin B (which is also altered in a similar manner as cyclin A), p53 (a tumor suppressor gene involved in cell division control in G₁ following ionizing radiation), and glutathione S transferase-pi (a gene product which has been demonstrated to be involved in DNA repair and redox cycling). In contrast, the remaining xip cDNA clones [i.e., xip1-4,6-11, which were isolated following high dose ionizing radiation exposure to human U1-Mel cells], Prad-1 (a gene involved in cell cycle controlling events at the G₁ portion of the cell cycle), 36B4 (a gene involved in homeostasis), and cdc2 (a gene involved in the regulation of the S-phase portion of the cell cycle), were not altered following ionizing radiation, either during the establishment or challenge of adapted human cells.

C. INTRODUCTION AND BACKGROUND.

1. DNA Repair Following Ionizing Radiation. DNA repair processes which are stimulated by X-ray-induced DNA damage are poorly understood. Two such repair processes, PLDR and sublethal damage repair, may be the same manifestations of a series of common enzymatic steps. PLDR, defined as the survival enhancement observed after subculture delay of cells following ionizing radiation, has two distinct phases based upon DNA lesion repair and survival recovery studies (1). The first phase of PLDR occurs very quickly ($t_{1/2}$: 2-20 mins) to increase the survival of X-irradiated cells and to mend the vast array of DNA lesions created by ionizing radiation. It is associated with a rapid resealing of single-, and later on, double-stranded DNA lesions which are either created initially by X-rays or produced as a result of the repair of various types of base damage (1). The second slower phase of PLDR proceeds much later (i.e., >1-2 hrs) following X-irradiation, during which the remaining double-stranded DNA breaks are completely repaired. This second phase of repair closely corresponds to the restructuring of gross chromosomal damage, and can be partially blocked in some human cells by inhibiting protein synthesis (1,2). This slower phase of PLDR correlated with a rapid decline in X-ray-induced transformation of normal cells.

In theory, the fast component of PLDR may be due to constitutively synthesized enzymes [such as DNA ligases, topoisomerases, or polymerases (3)], which act immediately to repair damaged DNA. In contrast, the slow phase of PLDR in human cells may require the *induction* of specific genes and gene products involved in the repair of potentially lethal or carcinogenic DNA lesions, including more complex chromosomal damage. PLDR responses which rely upon inducible genes and proteins may be further enhanced if mammalian cells are "adapted" via prior exposure to low-doses of ionizing radiation. Thus, X-ray-inducible genes and proteins may play a number of roles in ASRs in human cells, including cell cycle control, the regulation of

DNA repair processes, and the suppression of tumor formation through error-correcting, DNA repair proof-reading activities.

2. Are DNA Repair Systems Induced By DNA Damage? A number of experiments with mammalian cells indicate that inducible physiologic responses (i.e., the production of new genes/proteins) may affect radioresistance, mutagenesis, and carcinogenesis, although much of the evidence is indirect (4). The response of a given cell to DNA damage may depend upon a number of factors, including: (a) the necessary time required to repair the entire genome; (b) the ability to induce or constitutively synthesize necessary repair pathways; (c) the extent and type of initial DNA damage created by the cytotoxic agent; and (d) the cell's state of division. Under the influence of repeated low doses of ionizing radiation, cells may respond by demonstrating an "Adaptive Survival Response (ASR)" to higher doses of that same cytotoxic agent.

ASRs are observed when cells are repeatedly treated with low doses of a cytotoxic agent (i.e., primed) and subsequently challenged with higher doses. "Primed" cells survive better than "unprimed" cells following a high dose challenging event. DNA repair enzymes are thought to play key roles in adapting cells to a given DNA damaging agent.

3. X-Ray-Induced Genes and Proteins Synthesized By Mammalian Cells Following Ionizing Radiation. Induced gene products (i.e., proteins) specifically synthesized in response to physiological doses of ionizing radiation in radioresistant human melanoma (U1-Mel) cells, and in a variety of other human normal and cancer-prone cells, were identified by us using two-dimensional gel electrophoresis (1). U1-Mel cells were chosen since they have one of the highest potentially lethal damage repair (PLDR) capacities for survival recovery known. Ten proteins synthesized by U1-Mel cells were identified and characterized. The synthesis of eight of these proteins were specifically *induced* by ionizing radiation and two proteins were *repressed*. Neither heat shock, UV-irradiation, nor bifunctional alkylating agent treatments resulted in the induction of these proteins. The expression of one protein, termed XIP269 to indicate an X-ray-Induced Protein of approximately 269 KDa, correlated very well ($r=0.89$) with PLDR capacity (1). This protein was found to be down-regulated by exposure to caffeine or cycloheximide, under conditions in which both PLDR and subsequent adaptive survival responses were prevented (1, 5, Unpublished Data). XIP269 was induced by as little as 5 cGy. It is, therefore, a good candidate for playing a role in DNA repair processes which might involve adaptive survival responses, and this protein is currently being isolated in sufficient quantity for reverse genetic cDNA cloning.

B. PRELIMINARY DATA

1. Characterization Of X-Ray-Induced cDNA clones (i.e., xip1-12) After An Acute Dose of Ionizing Radiation. The data in this section is background preliminary data which characterizes the induction of xip cDNA clones after acute doses of ionizing radiation. It is included in this Progress Report to clarify the induction characteristic of each of the xip clones.

In addition to characterizing several XIPs, we isolated twelve X-ray-inducible cDNA clones (i.e., xip1-12) from U1-Mel cells via differential hybridization (6). Some of these genes were induced over 20-fold above background levels by as little as 5-20 cGy. Using partial DNA sequence data, we identified three of these cDNA clones as known human genes: DT Diaphorase, tissue-type Plasminogen Activator (6,7), and thymidine kinase. The remaining nine cDNA clones (i.e., xip1,2,4,5,7,8,9,10, and 12) represent unidentified gene transcripts, but have regional homologies to various known genes (6). Table 1 summarizes what we know about these genes at the present time.

We have investigated the kinetics and specificity of induction of the xip transcripts using xip cDNA clones as probes on Northern blots. We investigated the temporal

expression of xip mRNAs following a single dose of ionizing radiation (i.e., 450 cGy). Confluence-arrested U1-Mel cells were treated with ionizing radiation and at various times RNA was extracted. xip mRNA levels were then monitored by RNA dot-blot analyses using individually ³²P-labeled xip cDNA insert probes and appropriate loading standard probes (either β-2-microglobulin or the 36B4 homeostasis gene). The levels of induction for xip mRNA transcripts varied from slightly less than 4-fold for xip11 to greater than 226-fold for xip12 (6). Peak levels of xip mRNA transcripts varied in time from 2 hrs to 14 hrs (Table 1).

Table 1. SUMMARY OF X-RAY-INDUCED CLONES AND CORRESPONDING TRANSCRIPTS.

xip cDNA Clone	Northern MW ¹ (Kb)	X-Fold ²		mRNA Induction Peak ³		RELATIVE ⁴ ABUNDANCE	DNA ⁵ HOMOLOGY
		Inducibility U1-Mel	GM2936B	U1-Mel	GM2936B		
xip 1	5.0	50	13	10	12	Low	Cyto. p450-EtOH Inducible (67)
xip 2	8.0	230	5	2	4	High	UNKNOWN
xip 3	4.0	38	3	4	2-4	Medium	DT Diaphorase (100)
xip 4	2.5	47	3	4	4	Medium	UNKNOWN
xip 5	8.0	30	4	4	4	Medium	UNKNOWN
xip 6	3.2	150	5	14	4-6	High	t-PA (100)
xip 7	7.5	11	3	12	4-12	Low	RAG-1 (62) ⁶
xip 8	7.0	18	3	10	12	Low	hap prot. mRNA (49)
xip 9	4.6	83	2	3	3	Low	c-fps/fes (60)
xip 10	6.5	35	4	4	4	Medium	UNKNOWN
xip 11	1.2	8	2	3	1-2	Low	Thym. kinase (100)
xip12	9.0	9	3	6-8	4-6	Low	Anglogen. Factor (55)

¹ RNA Northern blot, dot-blot, and scanning densitometric analyses were performed on Zeta-probe strips containing total RNA derived from unirradiated as compared to irradiated (450 cGy) confluence-arrested normal human nonfetal fibroblasts (GM2936B) or U1-Mel human melanoma cells as described in (6).

² Induction levels (i.e., X-fold) were obtained from RNA dot-blot analyses at times when peak levels of mRNA were noted (6). Relative mRNA ratios were calculated by dividing the levels of xip mRNA transcripts by the mRNA levels of a loading standard, either 36B4 or β-2-microglobulin as described in (6). Ratios obtained from X-irradiated RNA were compared to those obtained from RNA of unirradiated cells. Ratios were confirmed by Northern RNA blot analyses when xip mRNA transcripts were visible.

³ Confluence-arrested GM2936B or U1-Mel human cells were treated with or without ionizing radiation (450 cGy) and RNA was harvested at various times post-irradiation as described (6). xip mRNA levels were then analyzed via dot-blot. Peak xip mRNA levels, and times of appearance, were derived from data presented (6).

⁴ Represents a subjective measure of the relative abundance of xip mRNAs using t-PA as a relatively abundant transcript following ionizing radiation, and thymidine kinase as a relatively rare mRNA transcript in unirradiated or X-irradiated cells.

⁵ DNA sequence data was obtained from both the T₇ and T₃ primers located on the 3'- and 5'- ends, respectively, of each xip pBluescript cDNA clone and analyzed as described in (6). The homology reported (noted in the parentheses next to the genes of homology) represents the percentage of similar DNA sequences to known genes at both ends of the cDNA inserts.

⁶ Recombinant Activating Gene-1.

Without exception, all of the xip mRNA transcripts were transiently induced following ionizing radiation exposure, although their patterns of peak induction varied.

With the exception of an early peak of xip6 (i.e., t-PA), all of the X-ray-induced transcripts were blocked by post-X-ray treatments with actinomycin D (5 µg/ml) and all of the X-ray-induced transcripts demonstrated only one peak over time. As previously described (7), analysis of t-PA mRNA levels revealed two induction peaks, an early peak (appearing between 30 mins and 1 hour) which was found to be the result of stabilization of its mRNA, and another much larger peak (at 12-14 hours) of t-PA mRNA which was found to be the result of new transcription (7).

Times of peak mRNA induction for xip transcripts in confluence-arrested U1-Mel cells and in normal human fibroblasts are summarized in Table 1, and ranged from 1 to 14 hours. Many of the X-ray-inducible transcripts (i.e., xips2, 3, 4, 5, 9, 10, and 11) were expressed within the first 4 hours in both U1-Mel and GM2936B cells following X-ray treatment. Since xip clones were selected from a cDNA library made from U1-Mel cells 3 hours after X-ray exposure and since cDNA probes used in differential hybridization were made from 3 hour time points, it is not surprising that the majority of xip transcripts isolated were expressed at peak levels within the first 3-4 hours after irradiation.

The expression of xip transcripts was also examined following various doses of ionizing radiation (i.e., dose-response curves for xip expressions were constructed). Confluence-arrested U1-Mel cells were exposed to various doses of ionizing radiation and RNA was extracted at periodic intervals thereafter, corresponding to the optimal expression times of each xip transcript. xip transcript expression was subsequently monitored. xip transcripts hybridizing to xips1-8, 11, and 12 were induced maximally at a dose of 500 cGy. Many of these and other xip transcripts (i.e., xips1, 2, 4, 5, 6, 9, 10, and 12) were induced by as little as 5 cGy. Other xip transcripts (i.e., xip3, 7, 8, and 11) were not induced unless U1-Mel cells were exposed to a dose of at least 200 cGy.

We also examined whether or not the xip transcripts were induced by other cytotoxic agents (6). Confluence-arrested U1-Mel cells were treated with various doses of ionizing radiation and other cytotoxic agents, including UV-irradiation, PMA, and heat shock. U1-Mel cells were exposed to: X-irradiation doses of 200, 500, and 700 cGy; UV-irradiation doses of 1, 10, and 30 Joules/M²; PMA doses of 1, 10, and 100 nM; and heat shock at 44 °C for 1 hr. The heat shock dose chosen was previously shown to result in the synthesis of several known heat shock proteins (1,8). These doses of UV-irradiation and PMA were chosen because they result in the induction of high levels of t-PA (i.e., xip6) (7). After exposure to these cytotoxic agents, RNA was extracted in a temporal fashion from U1-Mel cells at 0 mins, 10 mins, 30 mins, 2 hrs, 4 hrs, 8 hrs, 12 hrs, 24 hrs, and 48 hrs. After dot-blotting onto nylon membranes, the RNAs were probed with PCR amplified, ³²P-labeled xip cDNA inserts. Exposure of U1-Mel cells to ionizing radiation provided the greatest stimulus for the production of xip mRNA transcripts (6). Only t-PA mRNA levels were induced to greater overall levels by UV-irradiation and PMA exposure than by ionizing radiation. mRNA transcripts corresponding to xip4, 7, and 12 were only induced by ionizing radiation. xip1, 2, 10, and 11 were, for the most part, greatly induced by ionizing radiation and only slightly increased following UV-irradiation. X-ray-inducible transcripts were either not affected at all, or only slightly induced by PMA or heat shock treatments during the time frames examined. xip3, 5, and 6 (i.e., t-PA) were induced by all of the cytotoxic agents tested, except for heat shock. This is an important finding with respect to ASRs, since xip5 was one gene whose expression dramatically increased during low dose priming exposures. Since its expression increased in response to several cytotoxic agents, this gene may play a role in the establishment of other forms of ASRs to other agents (i.e., cross-resistances following ionizing radiation to other cytotoxic agents may be stimulated). Both xip3 (i.e., DT diaphorase) and xip11 (i.e., thymidine kinase) were induced by ionizing radiation and

UV-irradiation, but not by PMA or heat shock treatments. Only xip8 was induced in response to ionizing radiation and PMA, but not by UV-irradiation. Exposure of U1-Mel cells to heat shock did not result in an increased production of any of the xip mRNA transcripts. Instead, the basal control levels of xip4, 7, 11, and 12 were decreased following heat shock. The remaining xip mRNA transcripts were not affected by heat shock.

Could the induction of these genes and their respective protein products following DNA damage be directly or indirectly involved in DNA repair? Are these gene products also responsible for ASRs? In the first year of this grant we investigated these possibilities and identified additional gene transcripts and products which may play a role(s) in mammalian DNA repair processes, which ultimately lead to ASRs.

2. PRELIMINARY DATA ADDRESSING THE SPECIFIC AIMS.

Hypothesis and Specific Aims. *The hypothesis that we are testing is that specific gene transcripts are either induced or stabilized following low doses of ionizing radiation. These transcripts result in the production of new gene products which give rise to an adaptive survival response (ASR), ultimately leading to increased radioresistance as a result of increased DNA repair processes.*

a. Adaptive Responses In Different Human Cells. In the first year of this granting period we achieved the goals of Specific Aim #1. Initially, we asked whether or not an adaptive response could be achieved in all, or a select few, human cells.

Table 2. ADAPTIVE RESPONSES IN DIFFERENT HUMAN CELLS¹.

CHALLENGE DOSE (cGy)	UNIRRADIATED CELLS		LOW DOSE-IRRADIATED CELLS	
	-CHM	+CHM	-CHM	+CHM
Radioresistant U1-Mel Tumor Cells				
0	100 ±2.2%	79 ±3.3%	91 ±3.4%	82 ±5.2%
450	24 ±1.6%	9 ±0.9%	66 ±2.1%	12 ±1.5%
HEp-2 Tumor Cells				
0	100 ±3.1%	85 ±4.2%	88 ±3.8%	85 ±1.4%
400	21 ±0.6%	10 ±0.2%	51 ±1.2%	12 ±0.1%
HTB-152 Tumor Cells				
0	100 ±4.9%	79 ±3.1%	89 ±2.3%	71 ±2.9%
400	36 ±1.3%	19 ±0.4%	37 ±0.3%	12 ±1.0%
GM2936B Normal Cells				
0	100 ±1.5%	82 ±3.4%	93 ±5.8%	87 ±3.2
300	31 ±1.9%	13 ±0.6%	26 ±0.9%	20 ±1.1
GM2907A Normal Cells				
0	100 ±7.2%	93 ±5.2%	90 ±4.9%	78 ±5.2%
350	38 ±3.2%	15 ±2.1%	32 ±1.1%	16 ±2.1%

¹Confluence-arrested human cells were exposed to 5.0 cGy each day for 4 days in the presence or absence of 5.0 µg/ml cycloheximide (CHM). CHM exposures lasted 6 hours after each low dose irradiation, and was removed. Cells were then refed with fresh medium not containing CHM. Control cells were fed in a similar manner. Low dose-exposed human cells, or human cells which were not primed with low dose exposures, were then challenged with a high dose of ionizing radiation. The challenging high doses of ionizing radiation were chosen based upon previous equitoxic measurements (1). Survival levels, as measured via limited dilution colony forming ability, were then performed. Experiments were performed eight times, each in duplicate.

Two normal human fibroblasts (GM2936B and GM2907A) as well as three human tumor cell lines (U1-Mel melanoma, HEP-2 laryngeal carcinoma, and HTB-152 lung carcinoma) were examined for their abilities to establish ASRs, and for the prevention of ASRs in these cells using cycloheximide treatments to inhibit protein synthesis (Table 2).

These results indicated that following a series of low dose exposures, U1-Mel and HEP-2 cells were able to survive better (i.e., they adapted) to a higher challenging dose of ionizing radiation. Confluence-arrested U1-Mel cells, which were exposed to 4 doses of 5.0 cGy over 4 days, had greater than two times higher the surviving fraction of cells following 450 cGy than did a similar population of U1-Mel cells which were not "primed" with prior exposures to low doses of ionizing radiation. Cycloheximide treatments (at a dose of 5.0 µg/ml), given each day for 6 hours after low dose priming exposures of ionizing radiation, prevented this adaptive response in primed U1-Mel cells. Similar inhibition of the adaptive response in U1-Mel cells was observed following continuous cycloheximide exposure (0.1 µg/ml) of low dose-irradiated cells. Prolonged exposure of U1-Mel cells to cycloheximide did, however, result in a high degree of cytotoxicity. These results (now performed eight times, in duplicate) indicate that U1-Mel cells are able to "adapt" to enhanced survival after repeated challenges to ionizing radiation damage. HEP-2 cells demonstrated a similar adaptive response as that observed with U1-Mel cells. HTB-152 cells, however, did not demonstrate an adaptive response.

In contrast, normal human fibroblast cells were not able to carry out an adaptive response under conditions in which U1-Mel cells demonstrated such a response. Repeated exposures of 5.0 cGy to normal human fibroblasts did not result in an overall increase in the survival of these cells following an equitoxic high dose challenge. Experiments with other normal human fibroblasts (i.e., GM2907A and IMR-90) also did not demonstrate a convincing ASR. In summary, only two out of six cell lines tested have, thus far, demonstrated an ASR. We are in the process of investigating: "Why do U1-Mel and HEP-2 cells demonstrate an adaptive response while normal fibroblasts and HTB-152 cells do not?". The answer may be partially explained by the model we have formulated in the next section, which is based upon our initial preliminary data on gene expression changes following adaptive and challenging doses of ionizing radiation. The conditions of growth, the ability of cells to manufacture needed inducible proteins, the ability of cells to arrest at certain points in the cell cycle, and the dose used to establish an ASR are all determinants which lead to the radioresistance generated through ASRs.

Now that alterations in specific gene transcripts have been identified, we will use probes for these genes to monitor changes in their expression from RNA isolated from U1-Mel, HEP-2 and HTB-152 cells during the establishment and subsequent challenge of adapted cells. We have purified RNA from these cells and we are now screening Northern blots (which have been set up in a similar manner to those illustrated in Figure 1 for U1-Mel cells) for alterations in genes previously observed to change in U1-Mel cells (Figure 1). We will then ask if those human cells which do not demonstrate ASRs also do not have alterations in specific genes, such as xip5, xip12, and cyclin a and p53. In addition, we will ask if human cells that do demonstrate ASRs, such as HEP-2, demonstrate alterations in these genes in a similar manner as U1-Mel cells.

b. Changes In Gene Transcription Following Adaptive and Challenging Doses of Ionizing Radiation. We have identified a number of gene transcripts whose expressions change due to low dose priming (i.e., 5 cGy) exposures and/or after a high dose challenge (i.e., 450 cGy) (ref. 9). We have also expanded our search for

changes in gene expression to include examining protein patterns via two-dimensional gel electrophoresis for changes due to low dose priming and a high dose challenge.

The goals of Specific Aim #1 were to: "test if X-ray-induced cDNA clones already isolated (Table 1, xip1 to xip12) were induced by low doses of ionizing radiation, and if they accumulated in human cells upon repeated exposures to small doses of ionizing radiation. Conventional Northern, dot-blot, and nuclear run-on RNA analyses will be utilized to investigate the overall levels of induction and the rates at which these new transcripts are produced following repeated low doses of ionizing radiation. We will also screen low dose-irradiated human cells with probes corresponding to the cDNA which encodes XIP269. We are currently in the process of cloning this cDNA via reverse genetic approaches, beginning with antibodies produced from purified protein from 2-D gels." The production of an antibody to XIP269 appears to be at least one year away, so the last section of Specific Aim #1 will be somewhat delayed. At the same time, we have discovered a number of changes in gene expression (below) which will require extensive investigation.

Thus far, we have tested "adapted" or "unadapted" U1-Mel cells for changes in the expression of numerous known genes as well as for changes in xip1-12 gene transcripts (6). All twelve of the xip cDNA clones (xip1-12) discussed previously have been examined. In addition, we have looked for changes in the expressions of various known genes involved in cell cycle regulation (e.g., cyclin A, cyclin B, p53, Prad-1, and radioresistance/DNA repair (e.g., small nuclear protein and glutathione-S-transferase-pi). Confluence-arrested U1-Mel cells (grown in two-hundred, 150 mm² tissue culture dishes) were exposed to 5 cGy each day for 4 days to establish a "primed" state. Control cells were treated in an identical manner, but without exposure to ionizing radiation each day. After each priming dose, RNA and protein samples were extracted at 4 hours post-irradiation. We have isolated enough RNA to be used for both Northern and subtractive/differential hybridization experiments. We have also isolated enough protein for two-dimensional gel electrophoretic analysis of protein patterns and for western blot analyses using antibodies to available cell cycle regulated proteins. Unfortunately, antibodies are not yet available which are directed against any of the xip protein products, such as for proteins encoded by xip5 or xip12. Enough RNA has been isolated to identify changes in gene expression using both Northern analyses via known (including the xip cDNA clones) cDNA probes and for the isolation of other, more low abundant, gene transcripts which increase in response to various low and/or high dose ionizing radiation stimuli by subtractive hybridization. Mark Meters (graduate student), Meizhi Wang (technician), Sam W. Lee, Ph.D. (Assistant Professor) and I have performed these experiments. Subtractive and/or differential hybridizations will be performed within the next year and we will then begin to characterize these gene transcripts as we have done with all of the xip transcripts (6).

Flow cytometric analyses and [³H]thymidine-labeled nuclei measurements were made throughout the ASR experiment to examine the state of cell division of U1-Mel cells during the adaptive and challenging doses of ionizing radiation. Confluence-arrested U1-Mel cells were treated as follows (Figure 1): **U₁**, RNA isolated from unirradiated cells on the first day of the experiment; **X₁'**, 5 cGy on day 1; **X₂'**, 5 cGy on days 1 and 2; **X₃'**, 5 cGy on days 1-3; **X₄'**, 5 cGy on days 1-4; **X₄',450**, 5 cGy on days 1-4, followed by 450 cGy on day 5; **X₄',450 + Act D**, 5 cGy each day on days 1-4 in the presence of 5 µg/ml actinomycin D for 5-6 hours post-irradiation, followed by 450 cGy on day 5 in the presence of actinomycin D for 5 hours. **X₄₅₀**, 450 cGy to unadapted cells on day 5; **U₅**, present on only a few Northern blots, RNA from unirradiated confluence-arrested U1-Mel cells on day 5 of the experiment. RNA and protein were extracted from cells under the above conditions at 3 and 4 hours, respectively.

I. Changes In xip Transcripts. The expression of xip5 was dramatically altered by low dose ionizing radiation priming. Levels of xip5 mRNA gradually accumulated after each exposure to the 5 cGy priming doses of ionizing radiation (Figure 1). Likewise, a mRNA transcript related to xip12 (a 500 base transcript) gradually built up in response to low dose priming events (Figure 1). The gene transcript corresponding to the original xip12 cDNA clone (i.e., a 10 Kb) did not change, except in response to a high dose of ionizing radiation, and the levels of expression of this gene were not different between primed and unprimed cells receiving 450 cGy. The overall levels of both xip5 and the xip12-related gene transcripts in primed cells following a high dose challenge were not as high as those observed in unexposed U1-Mel cells following a single high dose (i.e. 450 cGy) of ionizing radiation (Figures 1).

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

Figure 1.

* Glutathione-S-transferase-pi

This may signify negative feedback control of gene expression due to low dose exposures. Possibly, low dose primed cells may quickly reach plateau levels of expression of xip5 and xip12-related genes. Primed cells do not, therefore, respond in the same way that unirradiated U1-Mel cells do, by inducing massive quantities of xip5 or xip12-related gene products when exposed to high doses of ionizing radiation.

Treatment of U1-Mel cells with actinomycin D for 5 hours after each exposure to ionizing radiation prevented the expression of xip5 both during priming, and following a high dose challenge. These results indicate that new gene transcription, and not mRNA stabilization, is responsible for the dramatic increases in xip5 expression following exposure to ionizing radiation. Identical results were found for the xip12-related transcript and for alterations in cyclins A and B (data not shown).

Figure 2.

Since xip5 is being characterized and sequenced we can now ask several questions regarding the gene's function, which were previously outlined in Specific Aim #3: -"What is the function of this gene transcript? Does it control DNA repair and ASRs, or is the appearance of this gene simply the result of a vast number of regulatory changes occurring during low dose exposures to 'adapt' the cell for increased repair and survival in response to high dose ionizing radiation exposure?-. We are in the process of constructing antisense oligos and expression vectors (see Appendix) containing antisense xip5 DNA inserts to specifically prevent the low dose induction of this gene. Once we are able to specifically prevent xip5 induction during the adaptive response through antisense RNA expression, we will then observe subsequent changes in survival and DNA repair as outlined in Specific Aim #3.

Similar questions can be asked of the xip12-related gene, only after its transcript is isolated and cloned. Although xip12 was isolated from the RNA of X-irradiated (450 cGy) U1-Mel cells, we did not observe its related transcript, a 500 bp message. Our initial Northern blots were mistakenly cut off at about the 700 bp region, just below that of the 36B4 transcript. We were, therefore, surprised to observe this transcript in these experiments. None of the other xip transcripts have shown low molecular weight-related messages. We plan to generate various probes from the xip12 cDNA clone via restriction enzyme digestion. Once a piece of the xip12-related transcript is found which binds solely to the 500 bp region on the Northern blot presented in Figure 1, we will screen for a full length cDNA clone using an adapted, X-ray-induced U1-Mel cDNA library, which is being constructed from the RNA of U1-Mel cells exposed to priming and challenging doses of ionizing radiation described above. Questions as to the function of the xip12-related cDNA clone will then be asked.

II. Changes in Selected Known Gene Transcripts Following Ionizing Radiation. We have also used the RNA isolated from the ASR experiments above to screen for changes in the expressions of various known cell cycle-related genes (Figure 1). Cyclin A, and to a lesser extent cyclin B levels, were induced in primed cells following a high dose challenge of ionizing radiation (Figure 1). Cyclin genes were examined initially since XIP proteins were known to be cell cycle-regulated and since changes in cyclin B were reported following acute doses of ionizing radiation in HeLa cells (10). Interestingly, changes in the expression of either cyclin A or cyclin B were not observed in untreated (i.e., "unadapted") U1-Mel cells, either exposed or not exposed to a challenging high dose of ionizing radiation. Like xip5 and xip12-related transcripts, the levels of glutathione S transferase-pi gradually increased following low and high dose ionizing radiation exposures. In contrast, p53 transcript levels gradually decreased due to low dose ionizing radiation exposures. Other genes, such as Prad-1, cdc2 (not shown), and small nuclear protein (not shown) did not change, either following adaptive low dose exposures, or following high dose challenging exposures (Figure 1). Ethidium bromide stained gels were included to demonstrate small differences in amounts of RNA loaded. For example, lane 2 was loaded with slight more RNA than the other lanes and small increases in transcript levels in these wells must be compensated for. After densitometric readings of band intensities, cyclin A was found to increase 3-fold, while cyclin B levels only increased 50% in primed cells exposed to 450 cGy compared to unadapted cells treated with 450 cGy. Changes in the expressions of a number of xip and cell cycle-regulated genes have been quantified:

Figure 3. Induced Gene Transcripts During the Establishment of Adaptive Survival Responses (ASRs).

Figure 4. Changes In Cell Cycle-Regulated Gene Transcripts During the Establishment of ASRs and Subsequent High Dose Challenging.

Figure 5. Effect of Actinomycin D Treatment on The Expression of xip5 During the Establishment of ASRs and High Dose Challenging.

C. Are Changes In Cyclin A, p53, xip5, and Other Gene Transcripts Responsible For Adaptive Survival Responses (ASRs)? A Proposed Model. Using the data above (Figures 1-5), I have formulated a model of how ASRs may be

established in human cells and how these cells may become resistant to ionizing radiation following repeated exposures of radiation [Figure 6, (ref. 9)]. At present, cyclin A and cyclin B are genes which are known to control the progression of eukaryotic cells through cell division. Yet under the conditions of our experiments, U1-Mel cells did not progress into S-phase as observed by [³H]thymidine-labeled nuclei (Figure 1) and flow cytometric analyses (not shown). The induction of cyclin A under these conditions may indicate an involvement of this gene product in stimulating DNA repair, without a full stimulation of cell division. This could be due in part to a lack of all necessary factors to push U1-Mel cells into cell division (including space limitations) as well as the X-ray-inducible expression of certain genes which might prevent movement through cell division after DNA damage [e.g., the growth arrest and DNA damage-inducible (gadd) genes (11,12)].:

Figure 6. Model Explaining the Establishment of An "Adapted" Condition.

Initially, cells are arrested in G₀ or at some point in G₁. Upon repeated exposures to low doses of ionizing radiation (i.e. 5 cGy per day for 4 days) cells progress to, and arrest at, an "A" (for "Adaptive") arrest point at or near the G₁/S border of the cell cycle, thus explaining the readily inducible levels of cyclin A after high dose ionizing radiation exposure; cyclin A levels begin to appear at the G₀/G₁ border (10). It is possible that gene transcripts which build up slowly in response to priming doses (i.e., xip5 and xip12-related mRNAs) produce proteins that regulate or control those transcripts which appear only in response to a high dose challenge (e.g., cyclin A). **At the "A" point, cells are "poised" to stimulate various DNA repair systems which are not inducible in the initial G₀/G₁-arrested state, but are eventually required to establish an "Adaptive Survival Response".** We will attempt to test this model using sense and antisense techniques as described in the experimental plan outlined in Specific Aim #3.

D. Future Investigations.

1. Continued Studies: Years 2 and 3.

In the second year of our studies we will continue to screen for changes in known genes and xip transcripts using the RNA isolated in Specific Aim #1. Once a gene transcript has been identified which is affected by low and/or high dose exposures of ionizing radiation we will study these genes in terms of the functional goals identified in Specific Aim #3.

In the latter part of Year 1 and the early part of Year 2, we began to isolate total RNA from "primed or adapted" U1-Mel cells either exposed to, or not exposed to, a high dose challenge of 450 cGy ionizing radiation. Our plan is to construct cDNA libraries from this RNA. cDNA libraries from unirradiated U1-Mel cells and from single-dose-exposed (450 cGy) U1-Mel cells will also be constructed for use in subtractive hybridization screening. We will construct subtracted libraries from primed as compared to unprimed confluence-arrested U1-Mel cells and from primed U1-Mel cells exposed to, or not exposed to, a 450 cGy challenging dose of ionizing radiation.

The experiments described in Specific Aim #2 will not, however, be completed in the course of this grant. It will take 3 to 5 years to isolate, identify, characterize, and determine the function of low abundance gene transcripts which are affected by low dose priming and/or high dose challenging exposures of ionizing radiation. The goals of Specific Aim #2 were: to use subtractive and/or differential cDNA cloning hybridization techniques to select for, isolate, sequence, and characterize new mRNA transcripts induced following repeated low doses of ionizing radiation as compared to unirradiated or high dose-irradiated human melanoma cells. DNA sequences of isolated low dose-induced cDNA clones will be obtained, compared to previously sequenced genes via GenBank analyses (including the xip genes 1-12 previously discussed), and their temporal induction levels will be characterized. We will continue this work through years 2 and 3.

The nature of these goals are long-term, and will require additional funding and several years to complete. The experiments are essential to better understand the genes which control ASRs and DNA repair processes following ionizing radiation. We will, of course, begin these experiments during the next one and one-half years but we do not expect new findings for several years. Isolation and characterization of genes controlling the ASR will be required to better understand radioresistance, and how it develops in certain human cells and not in others. Another question that will be addressed in the future is how do these gene products interact with one another to yield develop ASRs. In all probability the establishment of ASRs in human cells requires a very complex interaction of a number of gene products, many of which are as yet unidentified. The proposed experiments above will be useful to better understand this potentially complex gene interaction.

2. Investigation Of The Function(s) Of Gene Transcripts Which Vary During The Establishment Of Adapted Conditions. Now that alterations in the expression of several genes have been discovered during low dose exposures and after high dose challenging events, we may begin to investigate what their functions are during the ASR in U1-Mel cells (Figure 1, Table 1) and in other cells which do not demonstrate ASRs. Certain genes (i.e., xip5, the xip12-related transcript, and GST-pi) build up slowly in response to low priming doses of ionizing radiation. Other genes (e.g., cyclin A and B) are not affected by low adapting doses of ionizing radiation, but then appear in response to a high dose challenge. Since these genes do not appear after single acute doses of ionizing radiation, they may be directly involved in subsequent radioresistance, which accompanies the ASR. As outlined previously, we

will investigate the functions of these genes using the techniques and experimental plan outlined in Specific Aim #3.

The goals of Specific Aim #3 are: once low-dose induced cDNA clones have been isolated, we will investigate the function of these cDNA clones via antisense cDNA constructs. The effects of specific gene down-regulation on cell survival recovery (i.e., PLDR), ASRs, and physical DNA break repair using PFGE will be examined. Antisense cDNA constructs to xip5 and xip12-related transcripts, which demonstrate low dose induction, are being constructed and utilized to determine their involvement in repair and/or radioresistant ASRs. Conventional plasmid transfections will be sufficient for these studies using a new metallothionine-inducible vector (see Appendix, pNUT Expression Vector). Northern blot and nuclear run-on RNA analyses will be utilized to ensure that specific gene down-regulation (corresponding to a chosen xip transcript) has occurred.

Using antisense oligos for cyclin A, cyclin B, Prad-1, p53, and cdc2, as well as for xip5 and the xip12-related cDNA, we hope to begin the experiments outlined in Specific Aim #3. The cDNA clones generated in Specific Aim #2 will also be investigated using this experimental plan when these cDNA clones have been isolated, sequenced and their induction characteristics have been investigated.

E. Previous Time Frame of Investigations.

As a reminder, I have included my original time frame of experimentation in the initial grant proposal. We have made great progress in the first one and one-half years of this proposal, yet we have a long way to go in understanding the regulation and function(s) of genes involved in establishing an ASR in certain human cells. We will follow the previously described experimental plan to carry out the goals outlined in the figure below (Figure 7).

The following diagram outlines the general research plan of attack described in this proposal for investigating the involvement of transcripts induced by low doses of ionizing radiation in ASRs:

Figure 7.

Since we have discovered changes in the expression of a number of gene transcripts during the establishment of ASRs and after high dose ionizing radiation exposures, we have more focused the research plans. It will require a number of years to clone XIP269, therefore, we have eliminated this part of the Specific Aims until the transcript which encodes this gene is cloned.

In addition to the Specific Aims outlined in Figure 7, we have investigated whether or not topoisomerase I (Topo I) levels are altered in any way during the establishment of ASRs, or following subsequent high dose challenges. These experiments were performed because recent evidence from our lab indicated that Topo I was involved in DNA repair (3,14) and because Topo I assays are routinely performed in my lab. We have not detected any changes in Topo I expression or activity following ionizing radiation exposures during the establishment of ASRs or following high dose ionizing radiation exposures. In the Appendix to this report you will find the results of these experiments, which have subsequently not been continued.

Summary. The overall goal of this proposal is to clone low dose X-ray-induced genes via molecular biology cloning techniques. I have been successful at identifying several genes whose expressions are altered during the establishment of, and actual processes occurring in, the adaptive response of U1-Mel cells. I will, in the next year or so, investigate the kinetics of expression of these genes, and whether or not other environmental stresses affect the expressions of these genes via Northern blot analyses. Finally, I will begin to investigate the function(s) of these genes within the cell, either under normal conditions of growth or following X-irradiation, using antisense expression technologies. Antisense RNA analyses and DNA repair endpoints (i.e., survival recovery, alkaline/neutral filter elutions, and PFGE) will be combined to examine the effects of specific gene "knock out" experiments on ASRs in those human cells which demonstrate this response. We are constructing mammalian expression vectors containing cyclin A, cyclin B, p53, or xip5, and xip12 (when full-length cDNAs are available) in the "sense" orientation to overexpress these genes in human cells which did not demonstrate ASRs, such as HTB-152 or a variety of other human fibroblasts. Since many of the xips identified by 2-D gel electrophoresis were cell cycle regulated (1), the factors turning them on or off within a normal cell cycle may be clearly related to the events occurring in human cells following a genetic insult. This theory has been strengthened by our recent findings that cyclin A, cyclin B, and p53 transcripts change in human cells in response to low dose priming and high dose challenging exposures of ionizing radiation.

F. Human Subjects. None.

G. Vertebrate Animals. None in this proposal.

H. Consultants/Collaborators. None.

I. Consortium/Contractual Arrangements. None.

J. Literature Cited.

1. Boothman DA, Bouvard I, and Hughes EN. Cancer Res. 49: 2871-2878, 1989.
2. Ronai ZA, Lambert ME, Weinstein IB. Europ. Environ. Occup. Cancer. Cell Biol. Toxicol. 6: 105-126, 1990.
3. Boothman DA, Trask DK, and Pardee AB. Cancer Res. 49: 605-612, 1989.

4. Stein B, Rahmsdorf HJ, Schonthal A, Busher M, Ponta H, and Herrlich P. In: Mechanisms and Consequences of DNA Damage Processing, New Series, (E. Friedberg and P. Hanawalt, Eds.) Alan R. Liss, Inc. New York, NY, 1988.
5. Hughes EN, and Boothman DA. Radiat. Res. 125: 313-317, 1991.
6. Boothman DA, Meyers M, Fukunaga N, and Lee SW. Isolation and characterization of X-ray-induced transcripts from radioresistant human melanoma cells. Mol. Cell. Biol. (In Preparation), 1991.
7. Boothman DA, Wang W, and Lee S. Cancer Res. 51:5587-5595, 1991.
8. Lucke-Hule, C., and Herrlich, P. Int. J. Cancer 39: 94-98, 1987.
9. Meyers M, Schea R, Seabury H, Petrowski A, McLaughlin WP, Lee I, and Boothman DA. Role of X-ray-induced proteins and gene transcripts in adaptive survival responses in radioresistant human melanoma cells. In: "Low Dose Irradiation and Biological Defense Mechanisms", (Takashi Aoyama, Ed.) Elsevier Science Publishers 1992; (In Press).
10. Muschel RJ, Zhang HB, Iliakis G, and McKenna WG. Cancer Res. 51:5113-5117, 1991.
11. Fornace AJ, Jr., Zmudzka B, Hollander MC, and Wilson SH. Mol. Cell. Biol. 2: 851-853, 1989.
12. Fornace AJ, Jr., Alamo I, Jr., and Hollander MC. Proc. Natl. Acad. Sci., USA 85: 8800-8804, 1988.
13. Boothman DA, Geller AI, and Pardee AB. FEBS Letters 258: 159-162, 1989.
14. Boothman, DA, Wang, M, Schea, RA, Burrows, HL, Strickfaden, S, and Owens, JK. Posttreatment exposure to camptothecin enhances the lethal effects of X-rays on radioresistant human malignant melanoma cells. Int. J. Radiat. Oncol. Biol. Phys. 24: 939-948, 1992.

**DATE
FILMED**

10 / 14 / 93

END