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Our previous studies in prostate cancer cells suggested that *Egr*-1 is required for the growth-inhibitory and apoptotic response to ionizing radiation in the prostate cancer cell line PC-3 which lacks wild-type *p53* functional protein. The effect of *Egr*-1 is mediated through the upregulation of TNF-α protein.. These results underscore the need to formally study the functional relevance of EGR-1 expression in radiation treated prostate cancer cells. It is hypothesized that radiation induces EGR-1 protein expression in prostatic carcinoma cells leading to the upregulation of TNF-α protein resulting in apoptosis and cell death. To test this hypothesis the following specific aims are proposed: A. Determine the functional and regulatory role of *Egr*-1 in radiation-inducible apoptosis using prostate cancer cell lines exhibiting a wild-type (LNCaP) and a mutant *p53* (DU-145) background. B. Determine the basal and radiation-inducible expression levels of *Egr*-1 and its target gene TNF-α protein as a function of radiation. Data obtained on *Egr*-1 and its target genes will be compared to those corresponding to clonogenic survival, growth inhibition and apoptosis profiles. In this way the functional role of *Egr*-1 in radiation treated prostate cancer cells can be elucidated. To translate these results in a clinical perspective, we will also analyze *Egr*-1, p53 and TNF-α expression levels and genomic *Egr*-1 mutations in untreated prostatic tumor specimens.

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INTRODUCTION

Prostate cancer is the second most common cause of cancer-related death in men. An analysis of the molecular indicators of cell proliferation and apoptosis may lead to identification and management of prostate cancer patients with inherent resistance to hormone or radiation therapy. Androgen ablation in normal prostate or exposure to radiation causes induction of *Egr*-1, which is a zinc finger transcription factor gene. Our previous studies in prostate cancer cells suggested that *Egr*-1 is required for the growth-inhibitory and apoptotic response to ionizing radiation in the prostate cancer cell line PC-3 which lacks wild-type *p53* functional protein. The effect of *Egr*-1 is mediated through the upregulation of TNF-α protein.. These results underscore the need to formally study the functional relevance of EGR-1 expression in radiation treated prostate cancer cells. It is hypothesized that radiation induces EGR-1 protein expression in prostatic carcinoma cells leading to the upregulation of TNF-α protein resulting in apoptosis and cell death. To test this hypothesis the following specific aims are proposed: A. Determine the functional and regulatory role of *Egr*-1 in radiation-inducible apoptosis using prostate cancer cell lines exhibiting a wild-type (LNCaP) and a mutant *p53* (DU-145) background. B. Determine the basal and radiation-inducible expression levels of *Egr*-1 and it's target gene TNF-α protein) as a function of radiation dose by immunohistochemistry, Western blot and reverse transcription-polymerase chain reaction. Data obtained on *Egr*-1 and its target genes (basal and radiation-inducible) will be compared to those corresponding to clonogenic survival (analyzed by colony-forming assay), growth inhibition (analyzed by [^{3}H] thymidine incorporation assay) and apoptosis (analyzed by TUNEL and flow cytometry) profiles. In this way the functional role of *Egr*-1 in radiation treated prostate cancer cells can be elucidated. To translate these results in a clinical perspective, we will also analyze *Egr*-1, p53 and TNF-α expression levels and genomic *Egr*-1 mutations in untreated prostatic tumor specimens. If this hypothesis is correct, this study will suggest that androgen depletion (hormone) treatment should be combined with radiation therapy to enhance cell-killing response in prostatic tumors exhibiting wild-type functional *Egr*-1 gene.

BODY

In this section, a detailed description of the research accomplishment will be described as per tasks outlined in the approved statement of work. The following tasks pertains to the first year of the granting period:

TASK-1. Radiobiological profile and *Egr*-1 gene characterization in DU-145 and LNCaP cells, months 1- 6:

a. Radiobiological characteristics of DU-145 and LNCaP cells (colony formation, growth inhibition and apoptosis) and radiation-induced *Egr*-1 protein analysis by Westerns and immunocytochemistry. Months 1-4.

b. Characterization of *Egr*-1 gene in DU-145 and LNCaP cells (FISH, mutation analysis and sequencing of whole gene). Months 5-6.

TASK-2. Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cells (DU-145 and LNCaP), months 7-18:

a. Transfections using constructs such as empty vector (pCB6+), the chimera WT1-EGR1 and EGR-1 cDNA will be performed in DU-145 and LNCaP cells. CAT assays will be performed using EBS-CAT construct. Months 7-13.

Radiobiological profile and *Egr*-1 gene characterization in DU-145 and LNCaP cells

Purpose : The present study was undertaken to determine the radiobiological characteristics of prostate cancer cell lines DU-145 and LNCaP and also to determine the structure and functional status of *Egr*-1 gene in these cell lines. This was accomplished by: (1) studying the effect of ionizing radiation on the growth of these cells; (2) analyzing the allelic and mutational status of *Egr*-1 in DU-145 and LNCaP cells; and (3) studying the radio-induction potential of *Egr*-1 protein and also it's target gene TNF-α mRNA DU-145 and LNCaP cells.

Methods: The effect of different doses of radiation on the growth of the prostate cancer cell lines, PC-3, and DU145 were determined by colony forming assays. Radiation-induced early and late stage apoptosis changes were determined TUNEL assay. Allelic integrity of *Egr*-1 was determined by flourescent *in-situ* hybridization (FISH) using *Egr*-1 genomic probe and the presence of mutations in the "hot-spots" trinucleotide (5' region) and mononucleotide (3' region) repeat regions of *Egr*-1 gene was determined by PCR-SSCP analysis. Radiation-induced EGR-1 protein expression was determined by immunocytochemistry and Western blot analysis using an EGR-1-specific antibody. Radiation-induced *Egr*-1 target gene mRNA expression was determined by using ^{32}P-RT-PCR analysis.

Results: <u>Radiation induces clonogenic inhibition in the prostate tumor cell lines DU-145:</u> A dose-response study was performed by exposing DU-145 cells to varying doses of ionizing radiation. Using colony-forming assay, the survival fraction of exponentially growing irradiated DU-145 cells at the clinically relevant dose of 2 Gy (SF_2) was found to be 0.432 and the mean lethal dose D_0 value was 293 cGy with a 'n' value of 1.001. The survival curve in the form of single hit multi-target (SHMT) model for DU-145 is shown in Figure 1 and Table 1. These values suggests that DU-145 cells are relatively more resistant than PC-3 cells (1). The LNCaP cells failed to form colonies after three repeated experimental attempts. It has been previously reported that LNCaP cells have very poor colony-forming potential and thus radiation survival was investigated in the shapes of regrowth curves (2). This study found that the SF_2 value for LNCaP was 0.512. When this value was compared to their data on PC-3 (SF_2=0.32) and DU-145 (SF_2=0.598), LNCaP cells were highly resistant than PC-3 cells and the grade of resistance lie toward

DU-145 degree of resistance. Thus, irrespective of wild or mutant p53 status, both the prostate cell lines are highly radio-resistant as per radiobiology grade scale.

Radiation induces apoptosis in the prostate tumor cell lines DU-145 and LNCaP cells. To determine whether ionizing radiation causes apoptosis in DU-145 and LNCaP cells, these cells were exposed to 5 Gy dose of radiation, TUNEL assay was performed after 24h. In both the cell lines, radiation caused a marginal increase in TUNEL positive cells (Figure 2). These data indicate that DU-145 and LNCaP cells are relatively resistant to radiation-induced apoptosis than PC-3 cells (1).

Molecular status of *Egr*-1 gene in PC-3 and DU-145 cells : DU-145 and LNCaP cells have a complex. Since *Egr*-1 is mapped to the long arm of chromosome 5 (5q23-31.1), it was necessary for us to determine the physical status of *Egr*-1 gene in these cells. This was analyzed by two independent approaches - FISH analysis of *Egr*-1 alleles and PCR analysis of partial *Egr*-1 sequence. FISH analysis performed by using a spectrum orange-labeled *Egr*-1 probe, showed that in DU-145 the proportion of nuclei with three signals for the *Egr*-1 gene was 90-95%, and that the proportion of nuclei with more than three signals was 5% to 10% (Figure 3). Previous cytogenetic report on DU-145 cells indicated that this cell line has three intact chromosome 5 copies (3) and our FISH analysis of *Egr*-1 confirms the presence of three intact alleles of Egr-1. Data on LNCaP cells are being confirmed as preliminary results suggested that these cells carry only one allele.

Using PCR-SSCP analysis, we analyzed the 5' and 3' regions of *Egr*-1 gene in PC-3 and DU-145 cells. Specifically, mutations were screened in these cell lines at two hot spot regions of the *Egr*-1 gene: the transactivation domain and the 3'UTR. These regions were selected since the transactivation domain harbors six trinucleotide AGC repeats at two regions in nucleotide positions 454-471 and 505-522 ($AGC_{6/33/6}$) and the 3'UTR has polyadenines at nucleotides 2407 to 2422 (3'UTR A_{16}). The transactivation domain activates transcription 100 fold and the 3'UTR region may have a role in mediating selective mRNA degradation of *Egr*-1 (4). This structural pattern implies that the *Egr*-1 gene may be particularly subject to microsatellite instability (MIN) because of nucleotide repeat sequences in three exon regions. The occurrence of repeat sequences among exons of known genes is extremely rare. Recently, Markowitz et al (5) demonstrated frameshift mutations within small repeated DNA sequences of TGF-β RII gene. Thus, these repeat sequences in the *Egr*-1 gene may make it a favorable target for MIN-directed mutation.

Primers were designed flanking these two hot spots based on the cDNA sequence of the *Egr*-1 gene (GenBank accession no. X52541). PCR was performed in these two regions using the DNA samples from these tumor cell lines. The products resulting from both the regions were subjected to SSCP analysis for the detection of mutations. In both the cell line, no variation in the mobility of the band pattern was detected in the $AGC_{6/33/6}$ PCR (5' region) (Figure 4) and 3'UTR A_{16}. PCR Sequencing of the whole gene was performed using primers listed in Table 2. Using RT-PCR products for sequencing, no obvious presence of mutation was detected in both the cell lines. In summary, DU-145 cells have three alleles with no obvious presence of mutation and LNCaP cells may have potentially one allele with no obvious presence of mutation in *Egr*-1 gene.

Elevated basal levels of EGR-1 protein in DU-145 cells. To determine whether EGR-1 is also inducible by radiation in DU-145 and LNCaP cells, the cells were exposed to 5Gy dose of radiation and total proteins were analyzed by Western blot analysis at different time points after radiation treatment for EGR-1 protein expression levels. To ascertain equal loading of extracted protein, the blot was subsequently probed with an antibody to β-actin. As shown in Figure 5A, EGR-1 was expressed at a higher constitutive level in the untreated cells and these levels were slightly increased at 5 Gy reaching peak level (2 folds) around 15 min. Similarly, immunocytochemistry results showed that EGR-1 was expressed at a higher constitutive level in the untreated DU-145 cells and these levels were slightly increased at 5 Gy (Figure 5B). EGR-1 protein was completely absent in untreated LNCaP cells and radiation failed to induce EGR-1 protein

(Figure 6). Day et al (6) reported that protein kinase activator (TPA) induces apoptosis in LNCaP cells by upregulating *Egr*-1 (NGF1-A) and c-fos mRNA. It was found that LNCaP cells had no basal mRNA levels of *Egr*-1 and only 0.1nM of TPA was able to upregulate but not at 0.01nM or less concentration of TPA (6). Thus, in this analysis, radiation dose might not be enough to up-regulate the EGR-1 protein in LNCaP cells.

Radiation induces TNF-α, an Egr-1 target gene in PC-3 and DU-145 cells

To enhance the detection sensitivity of steady-state mRNA level, we used ^{32}P-RT-PCR to detect the TNF-α transcripts in DU-145 cells. Primers flanking the human cDNA sequence of the TNF-α gene were designed (sense primer at nucleotides 839-860 and antisense primer at nucleotides 1039-1060 ref: 7) were radio-labeled with γ-^{32}P ATP. Using these radio-labeled primers, RT-PCR was performed in untreated and irradiated (5 Gy) total RNA samples (isolated at different time points). The same reverse transcribed samples from these cell lines were also subjected to RT-PCR using the radio-labeled primers for β-actin, as a normal internal control gene. The resulting TNF-α expression was normalized with β-actin cDNA products densitometrically to understand the kinetics of radiation-induced TNF-α mRNA expression. Radiation caused an induction of TNF-α mRNA in DU-145 cells, but the levels were below the levels observed in PC-3 cells (1) (Figure 7).

Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in DU-145 cells: Transfections using constructs such as empty vector (pCB6+), the chimera WT1-EGR1 and EGR-1 cDNA will be performed in DU-145 cells

Purpose: To directly understand the functional and regulatory role of *Egr*-1 expression in the radiation-inducible signaling pathway in prostate cancer cells, we describe here the effect of *Egr*-1 overexpression or inhibition of *Egr*-1 function on radiation-induced growth inhibition and apoptosis in the DU-145 prostate cancer cell line.

Methods: The parent human prostate tumor cells DU-145 were stably transfected using the full-length cDNA for *Egr*-1 placed under the control of cytomegalovirus promoter of vector pCB6+ or a dominant negative mutant of EGR-1 from a construct containing the WT-1-EGR-1 fusion protein [as described by us (8) in Mol. Cell. Biol. 15, 682-692; 1995]. Transfections were performed in DU-145 cell line as described previously (1). The ability of the chimera (dominant-negative mutant) to transregulate via the 'GC'-rich, EGR-1 binding site was confirmed in parental DU-145 cells using transient transfection and CAT assays. Transfected cells, DU145/WT1-EGR1.L1 or DU145/WT1-EGR1.L2 expressing the chimera, DU145/EGR1.L1 or DU145/EGR1.L2 expressing the *Egr*-1 cDNA, and DU145/vector.L1 or DU145/vector.L1 expressing the pCB6^{+} vector; were selected by treatment with 300μg/ml of geneticin (G418; GIBCO) in standard growth medium. In particular, the transfection of cDNA for *Egr*-1 was confirmed by Western blot analysis. The transfectant cell lines DU145/WT1-EGR1.L1 (is referred as DU145/WT1-EGR1.1130.L1 or L2), DU145/EGR1.L1 (is referred as DU145/EGR1.930.L1 or L2) and DU145/vector.L1 (is referred as PC3/vector.1131.L1 or L2) will be used for further studies pertaining to clonogenic inhibition and apoptosis.

Results: *DU-145 cells showed low transactivation activity of EGR-1 binding site mediated through either radiation-induced EGR-1 expression or CMV-EGR-1 driven EGR-1 expression, as determined by chloramphenicol acetyltransferase (CAT) assays.* DU-145 cells showed basal levels of EGR-1 protein, with three alleles of *Egr*-1 gene and a potential mutation in the transactivation domain. In addition, these cells also showed low levels of *Egr*-1 target gene, TNF-α mRNA and they were found to be more resistant to exogenous recombinant TNF-α induced growth inhibition (9) and as well as radiation induced growth inhibition. Based on these factors, it was imperative to determine whether the EGR-1 protein is functional in DU-145 using CAT-reporter assays. In NIH 3T3 fibroblast cells, an EGR-1 expression plasmid construct, CMV-EGR1, has been shown (10) to transactivate a CAT reporter plasmid construct, EBS-CAT, which contains three

tandem EGR-1 binding sites placed in the upstream of minimal *c-fos* promoter and CAT cDNA. Using these same constructs, DU-145 cells were transiently transfected with either EBS-CAT alone or EBS-CAT + CMV-EGR1 (in duplicate - one being untreated and the other was irradiated at 5 Gy). In the first set of experiments, we determined whether the radiation-induced EGR-1 protein could cause transactivation of EBS-CAT in DU-145 cells. And in the second set of experiments, we also ascertained whether the EGR-1 expressed by the construct CMV-EGR1 could cause transactivation of EBS-CAT in DU-145 cells. In addition, as a control for CAT assays, we used the promoter of the *gro* gene placed upstream to CAT cDNA. This gene is strongly induced by IL-1 (11). Thus, the *gro*-CAT was also transfected transiently in DU-145 cells (one untreated and the other treated with IL-1) to ascertain efficient functioning of the reporter gene cDNA CAT. As shown in Figure 8, that there was very minimal CAT activity (1.5%) in irradiated DU-145 cells transiently transfected with EBS-CAT, whereas, CMV-EGR1 could transactivate 10 times more than radiation alone. The control experiments with *gro* promoter demonstrated efficient CAT activity. This data indicate that radiation-mediated transactivation of EBS-CAT is very low when compared to CMV-EGR1 mediated transactivation.

Parental DU-145 cells were stably transfected with CMV-EGR-1 or CMV-WT1-EGR1 or empty vector pCB6$^+$ for further studies to understand EGR-1 mediated radiation-induced clonogenic inhibition.

Stable transfectants of DU-145 overexpressing empty vector pCB6+ or overexpressing CMV-EGR-1 were checked by Western blot analysis to confirm the overexpression of EGR-1 protein in DU-145. In Figure 9, one set of selected clones show that DU-145/EGR.L1 show high basal levels of EGR-1 protein than DU-145/Vector.L1. Currently, more selected clones are screened in order to have atleast two stable cell lines for each transfection category. These stable transfectants will be used for CAT assays, growth inhibition and apoptosis assays.

On the other hand, LNCaP cell line was transfected with these plasmids and currently selection of stable transfectants is underway.

Egr-1 knock-out mef cells exhibited enhanced resistance to apoptosis with consistent down-regulation of _p53_ protein in response to ionizing radiation

In our previous studies, we used tumor cell lines such as wild-type p53 melanoma cells (12) and p53-deficient prostate cancer cells (1) to understand the regulatory role of *Egr*-1 in apoptotic processes. These studies strongly suggested that *Egr*-1 can mediate its apoptotic action irrespective of *p53* status. However, in a recent report, it was found that *Egr*-1 transactivates the promoter of *p53* gene and up-regulates *p53* mRNA and protein levels in response to apoptotic stimuli (13). This prompted us to further investigate the interactive role of *Egr*-1 with *p53* during the process of apoptosis. We sought to investigate this mechanism in normal cell background with varied genomic status for *Egr*-1 gene (cells with both intact *Egr*-1 alleles, homozygous and heterozygous deletion for *Egr*-1 gene). Mouse embryonic fibroblast (MEF) cells were established from homozygous (*Egr*-$1^{-/-}$) and heterozygous (*Egr*-$1^{+/-}$) *Egr*-1 knock-out mice were kindly provided by Dr Jeffrey Milbrandt, Dept of Pathology, Washington University, St.Louis (14). MEFs from normal mice containing intact alleles for *Egr*-1 was established in the laboratory. Using these cells, we demonstrate here that *Egr*-1 is the upstream regulator of apoptosis and this effect is mediated through the upregulation of *p53* protein.

Ionizing radiation causes enhanced cell death in Egr-1$^{+/-}$ cells. MEFs (*Egr*-$1^{-/-}$ and *Egr*-$1^{+/-}$ cells) were left untreated or irradiated at 5 Gy dose of ionizing radiation. TUNEL staining and flow cytometry was performed to determine the incidence of apoptosis. By TUNEL assay, the incidence of apoptosis after 24 hours of radiation was 3.5 % in *Egr*-$1^{-/-}$ cells and 22.8% in *Egr*-$1^{+/-}$ cells (Figure 10). By flow cytometry assay using MC540 and Hoechst 342 staining, the incidence of apoptosis after 48 hours of radiation was 6.2 % in *Egr*-$1^{-/-}$ cells and 53% in *Egr*-$1^{+/-}$ cells (Figure 11). Thus, ionizing radiation caused signifcantly enhanced apoptosis in *Egr*-$1^{+/-}$ cells ($p<0.0001$) when compared to *Egr*-$1^{-/-}$ cells

as demonstrated by TUNEL and flow cytometry assays. These observations suggest that despite the presence of *p53* gene in this normal cell background, MEFs with homozygous deletion of *Egr*-1 were resistant to ionizing radiation-inducible apoptosis.

Ionizing radiation causes induction of EGR-1 protein in Egr-1$^{+/-}$ MEF cells. We examined whether EGR-1 induction was associated with enhanced apoptosis inducible by ionizing radiation in *Egr*-1$^{+/-}$ cells. Western blot analysis confirmed that *Egr*-1$^{+/-}$ and in *Egr*-1$^{-/-}$ cells no detectable basal levels of EGR-1 protein was found. Moreover, after exposure to a 5 Gy dose of radiation, *Egr*-1$^{+/-}$ cells showed induction of EGR-1 expression with peak levels (10 fold) at 30 min (Figure 12) and this was not evident in *Egr*-1$^{-/-}$ cells.

Egr-1$^{+/-}$ MEF cells exhibit EGR-1-dependent transcriptional activation via the GC-rich region in response to ionizing radiation. To ascertain the EGR-1-dependent transactivation process in *Egr*-1$^{-/-}$, *Egr*-1$^{+/-}$ and *Egr*-1$^{+/+}$ MEF cells, we performed transient transfections with (i) only reporter construct EBS-CAT that contains three tandem EGR-1-binding sites; (ii) EBS-CAT and an EGR-1 expression construct CMV-EGR-1; and (iii) EBS-CAT and ionizing radiation. As seen in Fig. 4, CAT activity was completely absent in basal and irradiated *Egr*-1$^{-/-}$ cells, whereas, CMV-EGR-1 elevated the CAT-activity. In *Egr*-1$^{+/-}$ and *Egr*-1$^{+/+}$ cells, ionizing radiation increased relative CAT activity in an allelic-dose dependent manner. A similar situation was observed in terms basal CAT activity in which *Egr*-1$^{+/+}$ cells showed slightly higher basal relative CAT levels when compared to *Egr*-1$^{+/-}$ cells. However, CMV-EGR-1 construct caused an increase in CAT reporter activity irrespective of endogenous *Egr*-1 allelic status (Figure 13). These results confirmed that the EGR-1 protein is necessary for the transactivation of target genes containing EGR-1 binding sites.

Ionizing radiation causes down-regulation of p53 protein in Egr-1$^{-/-}$ cells. The above observations have ascertained the fact that EGR-1 protein is necessary to cause radiation-induced apoptosis. Absence of EGR-1 protein renders enhanced resistance to radiation. Next, we sought to determine the role of *p53* in *Egr*-1$^{-/-}$ radioresistant cells. *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ cells were left untreated or irradiated at 5 Gy and proteins were extracted after each incubated time interval and subjected to Western blotting. *Egr*-1$^{+/-}$ cells showed a peak increase in *p53* levels to five fold in 3-6 hours. However, in *Egr*-1$^{-/-}$ cells, the *p53* protein was down-regulated in 1 hour after radiation and was completely absent in the later time points (Figure 14). Thus, the loss of *p53* transactivation in *Egr*-1$^{-/-}$ cells might have contributed to enhanced resistance to apoptosis. The process of down-regulation and complete absence of *p53* protein in response to radiation remains unclear. However, it may speculated that mdm-2 may be up-regulated upon radiation damage and this protein may interact with nuclear *p53* protein to ubiquitously degrade the *p53* protein so that the cells can move from *p53*-mediated G_1 arrest to S-phase (15). More investigations in this area are warranted to understand the precise role of wild-type *p53* protein upon DNA-damage in cells lacking EGR-1 function.

Radiation causes down-regulation of p53 protein in LNCaP cells: This study and a previous report (13) confirmed that *Egr*-1 upregulates *p53*. To determine the fate of *p53* in prostate tumor cell lines with different EGR-1 protein status, the cells were exposed to 5 Gy dose of radiation and total proteins were analyzed by Western blot analysis at different time points after radiation treatment for *p53* protein expression levels. DU-145 cells showed very high levels of *p53* protein in untreated and irradiated samples with no obvious induction. However, in LNCaP cells, *p53* was down-regulated in 45 minutes and was completely absent in 2 hours (Figure 15). This forms an interesting observation which is similar to the results found in *Egr*-1 knock-out MEFs. Thus, in DU-145 cells, high levels of EGR-1 with elevated levels of mutated p53 gene might have contributed towards enhanced resistance to apoptosis. On the other hand, absence of EGR-1 protein with lack of *p53* up-regulation in LNCaP cells might have contributed towards enhanced resistance to apoptosis.

Egr-1 expression and mutation in untreated and treated primary tumor specimens of prostate cancer

This study was undertaken to determine whether EGR-1 overexpression in the primary tumor correlates with radiation response either in terms of complete local control with no evidence of disease or recurrence / evidence of metastasis. We analyzed 25 pretreated surgically resected paraffin-embedded primary adenocarcinomas of the prostate for the presence of the EGR-1 expression and mutation and correlated with clinical end-points such as serum PSA levels and current clinical status.

EGR-1 expression and mutational analysis in pretreated specimens. Paraffin-embedded prostate tumor samples (obtained from biopsy, radical prostectomy or trans-uretheral resection of the prostate) were obtained before treatment. Twenty two out of 25 patients underwent radical prostectomy as their primary treatment. Two of the patients also received adjuvant radiation therapy for a persistantly elevated PSA post-surgery. In effect seven of 25 patients recieved radiation: two for recurrent disease following surgery, two for adjuvant treatment and three for definitive radiation therapy without surgery.

EGR-1 expression was determined by immunohistochemistry using rabbit polyclonal antibody sc-110 (Santa Cruz Biotechnology, CA) and antigen retrieval system. Using PCR-SSCP analysis, we analyzed the 5' and 3' regions of *Egr*-1 gene in untreated prostate tumor specimens. Specifically, mutations were screened in these tumors at two hot spot regions of the *Egr*-1 gene: the transactivation domain and the 3'UTR. PCR was performed in these two regions using the DNA samples from paraffin-embedded tumor specimens.

Results of EGR-1 protein expression, mutation and other clinico-pathological findings for pretreated specimens are shown in Table 3. Out of 25 patients, 18 patients showed expression of EGR-1 (Figure 16). Predominantly, EGR-1 protein was found in the nucleus of basal cell of prostatic acini. Nuclear staining was weak or usually absent in non-malignant regions. Poorly differentiated carcinoma showed the most intense nuclear staining than moderately differentiated tumors (Figure 16). There was no correlation with the Gleason scores. Out of 18 patients with EGR-1 expression, five had mutation in the transactivation domain and one had mutation in both transactivation and 3'UTR region (Figure 17). As compared to EGR-1 overexpression, the incidence of mutation may be underrepresented since a very small area of the gene was analyzed. Information on the clinical outcome of the disease in 7 out 18 EGR-1 positive patients was not available. Out of 11 positive cases with EGR-1 expression, five patients failed (out of which two had mutation) with either clinical recurrence of disease (two cases) or biochemical failure (three cases). No statistically significant correlation was found between the expression of EGR-1 protein and the initial stage, Gleason grade or PSA level. Results of treatment indicate that 9 patients treated with radical prostectomy whose tumors expressed EGR-1 protein, four (45%) developed local recurrence as compared to one out of four with negative expression for EGR-1 (not significant). Of the 7 patients treated with radiation, two out of three (66%) with EGR-1 expression recurred as compared with none out of four patients negative for EGR-1 expression.

KEY RESEARCH ACCOMPLISHMENTS

1. Ionizing radiation caused down-regulation of *p53* protein in MEFs homozygously lacking *Egr*-1 gene. Thus, cells containing wild-type *p53*, *Egr*-1 was found to be the upstream modulator of apoptosis.
2. In prostate tumor cells containing wild-type and mutant *p53*, two important observations were found. In DU-145, higher basal levels of EGR-1 protein might upregulate the mutant *p53* protein leading to enhanced resistance to ionizing radiation. Whereas in LNCaP, potential absence of EGR-1 protein led to down-regulation of wild-type *p53* protein after radiation leading to enhanced resistance to radiation-induced apoptosis. Thus, these two pathways strongly suggest that *Egr*-1 is the upstream modulator of apoptosis and *p53* mediates the effect.
3. In patient samples, *Egr*-1 overexpression (potentially mutant) in tumors correlates with treatment failure.
4. Since our *in vitro* studies suggested an interactive role of EGR-1 with *p53* (in prostate cancer cells with wild-type or mutant p53 status) in the modulation of apoptotic response to ionizing radiation, the differential expression of EGR-1 (potentially in the mutant forms) in the cancerous areas of prostate tissue in patients raises the possibility that these patients may carry radio-resistant tumors. It is necessary, therefore, to determine the relevance of EGR-1 expression (wild and mutant types) by *in-vitro* and *in-vivo* functional studies, and identify the link between particular type of EGR-1 expression in the primary tumor samples of human patients and clinical response to radiation therapy.
5. Thus, in conclusion, these data suggest that: (a) *Egr*-1 plays a key regulatory role in the apoptotic response to ionizing radiation. (b) More studies are warranted to precisely define the role of *Egr*-1 expression in prostate tumors and its use as a marker of radio-resistance.

REPORTABLE OUTCOMES

The following presentations were made in the National Meetings:

1. Ahmed, M.M., Chendil, D., Dey, S., Parekh, S., Mohiuddin, M., Rangnekar, V.M. and Milbrandt, J.D. Ionizing radiation down-regulates *p53* protein in *Egr*-1$^{-/-}$ MEF cells causing enhanced resistance to apoptosis. Presented at 90th Annual Conference of American Society for Cancer Research (1999) **Philadelphia, PA.**
2. Ahmed, M.M., Chendil, D., Dey, S., Parekh, S., Mohiuddin, M., Rangnekar, V.M. and Milbrandt, J.D. Ionizing radiation down-regulates *p53* protein in *Egr*-1$^{-/-}$ MEF cells causing enhanced resistance to apoptosis. Presented at Gordon Research Conference on "Radiation Oncology" (1999) at **Ventura, CA**.

Manuscript under preparation:

Chendil, D., Dey, S., Parekh, S., Mohiuddin, M., Milbrandt, J.D., Rangnekar, V.M. and Ahmed, M.M. Ionizing radiation down-regulates *p53* protein in *Egr*-1$^{-/-}$ MEF cells causing enhanced resistance to apoptosis.

Funding based on work supported by this award:

Base on the work supported by this award, Principal Investigator received RO-1 funding from National Cancer Institute, NIH starting from August 1, 1999 (Critique and Award letter is enclosed in Appendix II). Since there was a significant overlap between the remaining Statement of Work of this award and this new grant, the Grants Officer of USAMRMC approved new Statement of Work for the remaining granting period (Revised Assistance Agreement is enclosed in Appendix II). The new Statement of work beginning from August 1, 1999 is shown below:

TASK-1. Radiobiological profile and *Egr*-1 gene characterization in CWR22R cells, months 1- 4:
   a. Radiobiological characteristics of CWR22R and CWR22 cells (colony formation, growth inhibition and apoptosis) and radiation-induced *Egr*-1 protein analysis by Westerns and immunocytochemistry. Months 1-3.
   b. Characterization of *Egr*-1 gene in CWR22R and CWR22 cells (FISH, mutation analysis and sequencing of whole gene). Month 4.

TASK-2. Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22), months 9-14:
   a. Transfections using constructs such as empty vector (pCB6+), the chimera WT1-EGR1 and EGR-1 cDNA will be performed in CWR22R and CWR22 cells. CAT assays will be performed using EBS-CAT construct. Months 9-11.
   b. Radiobiological characteristics of stable transfectant (CWR22R and CWR22) cells (colony formation, growth inhibition and apoptosis). Months 12-13.
   c. Interpretation of results and preparation of manuscript for publication. Month 14.

TASK-3. Mechanism of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22) by *Egr*-1 mediated up-regulation of the TNF-α gene, months 15-18:
   a. CAT assays using p53 (2.2+1.6)-CAT, pRB-CAT and TNFp-CAT constructs in parental and stably transfected CWR22R and CWR22 cells. Gel-shift assays in parental and stably transfected CWR22R and CWR22 cells. Months 15-17.
   b. Interpretation of results and preparation of manuscript for publication. Month 18.

TASK-4. To understand the mechanism of radiation-induced apoptosis in isogenic mouse embryonic fibroblast (MEF) cells with homozygous (Egr-$1^{+/+}$), heterozygous (Egr-$1^{+/-}$) and null (Egr-$1^{-/-}$) allelic status for Egr-1 gene. Months 5-8.

a. Western blot and RT-PCR analysis of p53, TNF-a and pRB genes in untreated and irradiated MEFs with Egr-1+/+ and Egr-1-/- background. Month 5.

b. CAT assays for p53, TNF-a and pRB. Month 6.

c. Immunblot analysis of p53, mdm-2 and pRB. Month 7

c. Interpretation of results and preparation of manuscript for publication. Month 8.

CONCLUSIONS

Studies using genetically matched *Egr*-1 MEF cells confirmed that *Egr*-1 is the upstream regulator of apoptosis. The role of *p53* is pivotal in *Egr*-1 mediated apoptosis. However, in cells lacking EGR-1 function, the wild-type *p53* may lose its ability to mediate apoptosis. Thus, for a cancer cell to respond to cell-killing agents, both wild-type *Egr*-1 and *p53* are essential to cause cell death. Hence, in prostate cancer, it will be of prime importance to study the interactive role of *Egr*-1 with *p53* in the regulation of cell death.

We present here two prostate cancer cell lines (DU-145 AND LNCaP) which may have two different novel mechanisms towards enhanced resistance to apoptosis. In both the situations, *Egr*-1 was found to be key up-stream modulator regulating cell death. To confirm these mechanisms, more studies are essential at the level of blocking EGR-1 function using a dominant-negative mutant or overexpressing *Egr*-1 cDNA in order to alter apoptotic response to ionizing radiation.

This study indicated that the incidence of EGR-1 overexpression is high in the prostate adenocarcinoma. This is supported by a previous report that high levels of *Egr*-1 mRNA expression was detected in 12 out of 12 intraprostatic adenocarcinomas by PCR and differential display assays (16). Recently, a study reported by Eid et al indicated that EGR-1 mRNA was expressed at significantly higher levels in cancer than in normal prostate (17). The presence of EGR-1 in prostate tumors may be potentially due to many reasons. A mutation in the transactivation domain may lead to the loss of transcriptional activity of *Egr*-1 and eventually shut off the downstream gene induction pathway (for eg via p53 or TNF-α) that normally leads to growth arrest or cell death caused by various treatment protocols. Or, if *Egr*-1 gene amplified and if the tumor contains *p53* mutation, then overexpressed wild-type EGR-1 protein may drive mutant *p53* gene causing the cell to be highly resistant to DNA-damaging agents. In conclusion, based on the small number of samples studied, EGR-1 overexpression (in mutant form or in amplified form with mutant *p53*) may provide an indication of clinical failure.

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Appendix I
(Three Tables and Seventeen Figures)

TABLES

TABLE 1. Estimates of radiation inactivation of DU-145 using 'SHMT' formalisms. Error bars represent standard error of the mean of two separate experiments.

	Radiation formalism SHMT		
	SF_2	"n" value	D_0 (cGy)
DU-145	0.432	1.001	293

TABLE 2. Sequences of Primers used for the sequencing of Egr-1 gene in prostate cancer cell lines DU-145 and LNCaP.

Nucleotide Location	Primer Name	Sequence	Product Size
157 - 730	Upper	5'-TCC CCG CGC CCC GCA TGT AA-3'	573 bp
	Lower	5'-GGG CTC GGG CCA CAA GGT GT-3'	
630 - 1525	Upper	5'-TTA CCC CAG CCA AAC CAC TCG ACT-3'	895 bp
	Lower	5'-GTG GAT CTT GGT ATG CCT CTT GCG T-3'	
1000 - 1838	Upper	5'-AAG GGT GGC TTC CAG GTTCCC ATG A-3'	838 bp
	Lower	5'-TGA AGG AGT TGG TGA CAG CTG AGG A-3'	

TABLE 3. Clinico-pathological findings of prostate adenocarcinomas

CODE	GRADE	Treatment		Egr-1			STATUS
		Surg	RT	Mutation 5'	3'	Expression	
PS-05	7	RP	For Rec	M	N	+++	Recurrence
PS-06	7	RP	For Rec	F	F	Negative	NED
PS-15	4	T	D	N	N	+++	Metastasis
PS-19	5	T	D	M	M	++	NED
PS-22	6	T	D	N	N	Negative	NED
PS-25	7	RP	A	N	N	Negative	NED
PS-32	8	RP	A	N	N	Negative	NED
PS-40	7	RP	-	N	N	+	NA
PS-41	7	RP	-	M	N	+++	NED
PS-42	6	RP	-	M	N	+++	Recurrence
PS-43	7	RP	-	N	N	+++	NED
PS-44	7	RP	-	N	N	+++	Recurrence
PS-45	7	RP	-	N	N	+++	NED
PS-46	6	RP	-	N	N	Negative	NED
PS-47	8	RP	-	N	N	+++	Recurrence
PS-48	6	RP	-	N	N	+++	NED
PS-49	6	RP	-	M	N	++	NA
PS-50	6	RP	-	N	N	++	NA
PS-51	6	RP	-	N	N	++	NA
PS-52	8	RP	-	N	N	Negative	NA
PS-53	6	RP	-	N	N	++	NA
PS-54	8	RP	-	M	N	+++	NA
PS-55	8	RP	-	N	N	+	NA
PS-56	PIN	RP	-	N	N	Negative	NA
PS-57	PIN	RP	-	N	N	++	NA

Grade: Gleason score; PSA: serum PSA levels at the presentation of disease; NED: no evidence of disease; Surg: Surgery; RT: radiation therapy; RP: radical prostectomy; For rec: radiation therapy for recurrence; T: TURP; PIN: prostatic intra-epithelial neoplasia; D: definitive; A:adjuvant; +++:strong immunoreaction; ++:moderate immunoreaction; +:low immunoreaction; N: Normal for mutation; M:mutated for *Egr*-1; F: failed to amplify product in PCR assay.

Figure 1

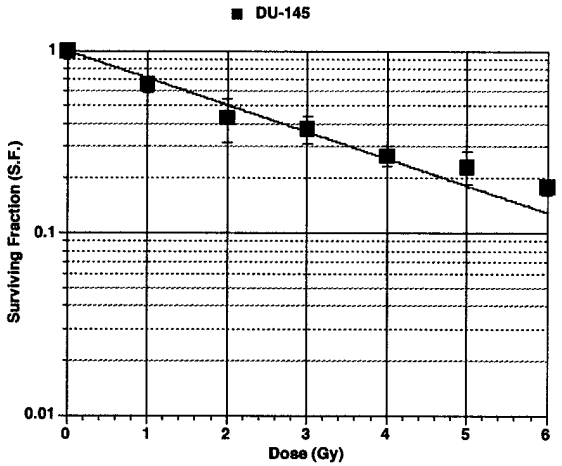


Figure 1. Effect of ionizing radiation on the surviving fraction of prostate tumor cell line DU-145. Cell survival curve DU-145 cells following radiation as assayed by colony-forming ability is shown in the form of Single Hit Multi-Target (SHMT) model.

Figure 2

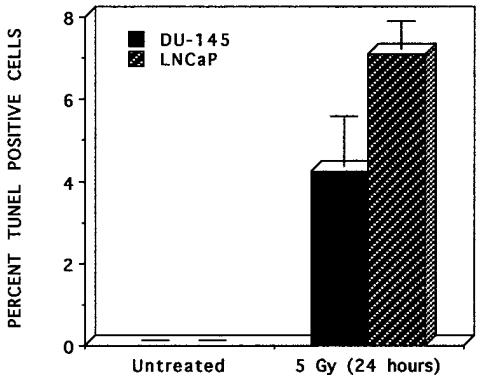
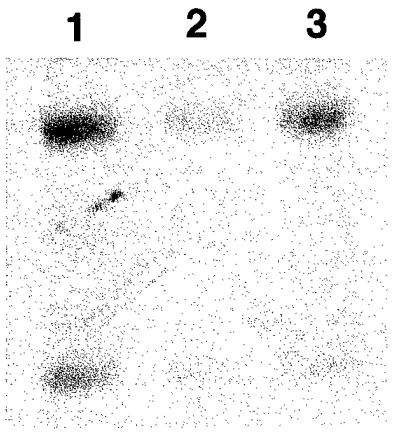


Figure 2. Quantification of apoptosis by TUNEL assay. To quantify apoptosis, the ApopTagTM in situ apoptosis detection kit (Oncor, Gaithersburg, MD) was used. The ApopTag kit detects the DNA strand breaks in single cells by terminal transferase-mediated dUTP-digoxigenin-end labelling (TUNEL). Cells were seeded in chamber slides and 24 h later they will be exposed to 5 Gy dose of radiation. The DNA was then tailed with digoxigenin-dUTP and conjugated with anti-digoxigenin fluorescein. The cells were stained with antifade. The stained cells were observed in triple band-pass filter using Nikon-microphot epifluorescence microscope. To determine the percentage of cells showing apoptosis, a total of 500 cells were counted for each experiment. Bar graph showing the percentage of TUNEL-positive cells. Background levels in untreated were normalized over treated. Data represent a mean of two experiments. The error bars represent standard deviation.

FIGURE 3

FIGURE 3. EGR-1 FISH ANALYSIS IN PROSTATE TUMOR CELL LINE DU-145. FISH analysis was performed to detect the Egr-1 copy number by using a spectrum orange-labeled Egr-1 probe. Arrows indicate Egr-1 signal (red dots). Interphase and metaphase plates showing three alleles in DU-145 cells.

FIGURE 4



1 : Normal Placenta
2 : LNCaP
3 : DU-145

FIGURE 4. MUTATION ANALYSIS OF THE EGR-1 GENE IN DU-145 and LNCaP cells. MDE gel autoradiograph of PCR SSCP analysis of the Egr-1 gene in the transactivation domain.

Figure 5

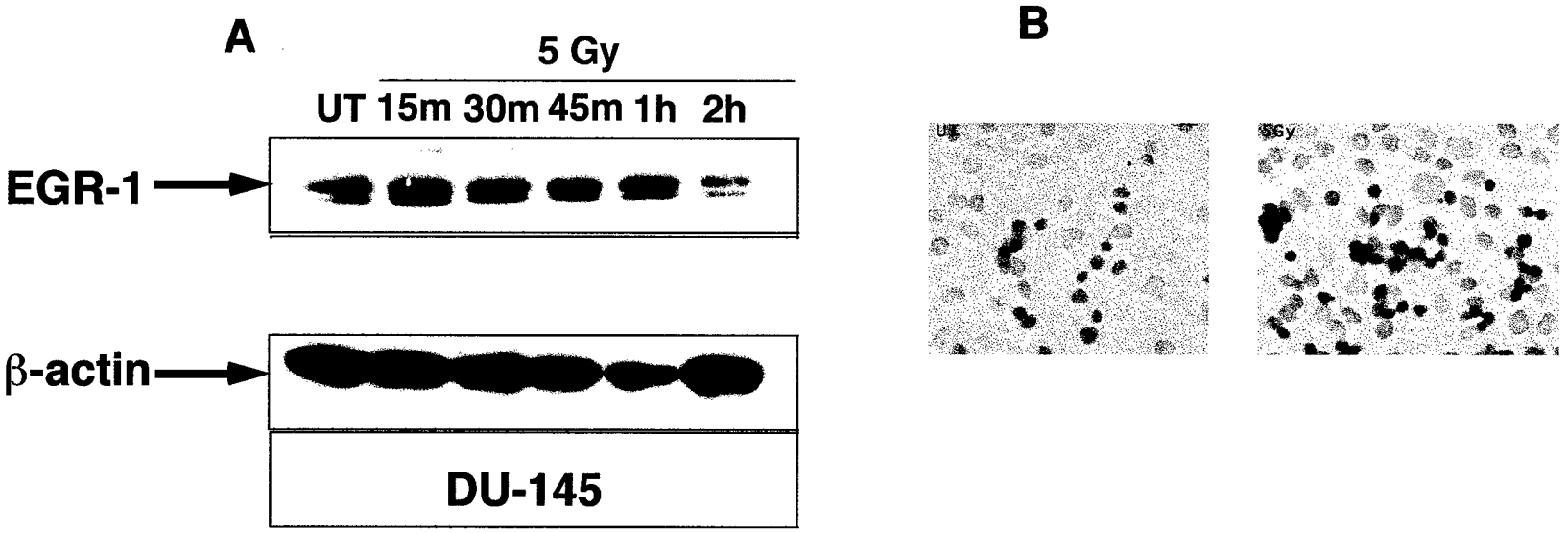


Figure 5. EGR-1 protein expression analysis in DU-145 cells. EGR-1 protein levels were detected by Western blot analysis and immunocytochemistry before and after radiation. (A)Whole cell protein extracts were prepared from DU-145 and LNCaP cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for the time interval indicated and then subjected to Western blot analysis for EGR-1 or β-actin. The blot was subsequently probed with an antibody for EGR-1 (sc-110) or β-actin. (B) Nuclear induction of EGR-1 following exposure to ionizing radiation. DU-145 cells were left untreated (UT) or treated with a 5-Gy dose of ionizing radiation and subjected to immunocytochemistry 45 min after the exposure. EGR-1-positive cells showed strong brown nuclear staining with the diamino benzidene-H_2O_2 substrate.

Figure 6

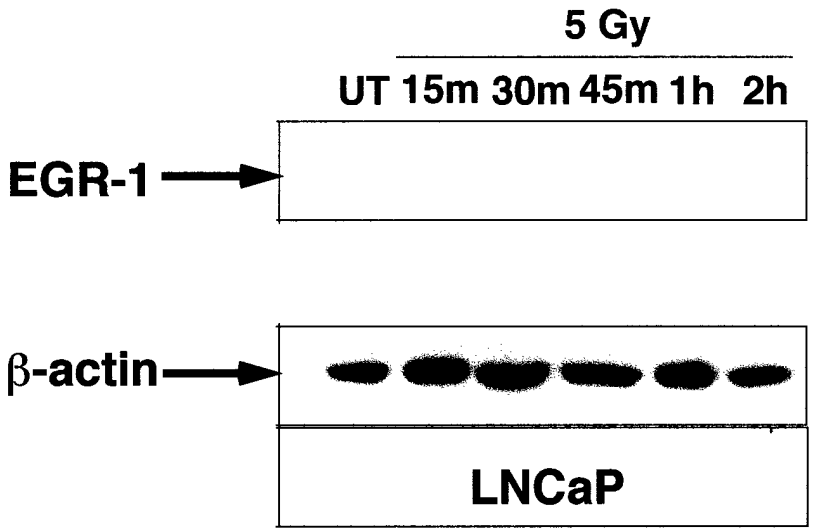


Figure 6. EGR-1 protein expression analysis in LNCaP cells. EGR-1 protein levels were detected by Western blot analysis and immunocytochemistry before and after radiation. Whole cell protein extracts were prepared from DU-145 and LNCaP cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for the time interval indicated and then subjected to Western blot analysis for EGR-1 or β-actin. The blot was subsequently probed with an antibody for EGR-1 (sc-110) or β-actin.

FIGURE 7

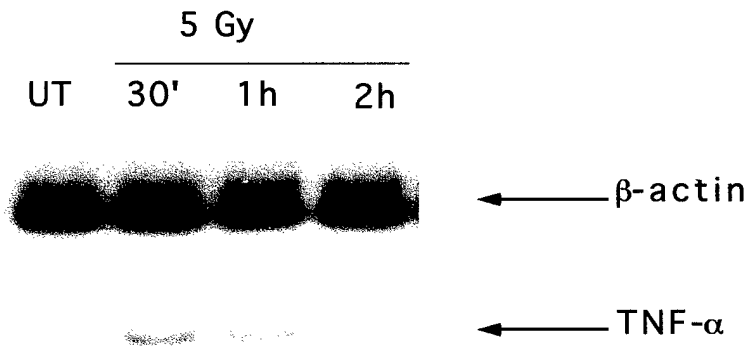


Figure 7. ^{32}P-RT-PCR analysis of mRNA expression of the TNF-α , a target gene of Egr-1, in untreated and irradiated prostate cancer cell line DU-145. PCR was performed by using the products of reverse transcription reaction and the upstream and downstream primers flanking the TNF-α gene. G6PDH RT-PCR was performed as a normal internal gene control.

Figure 8

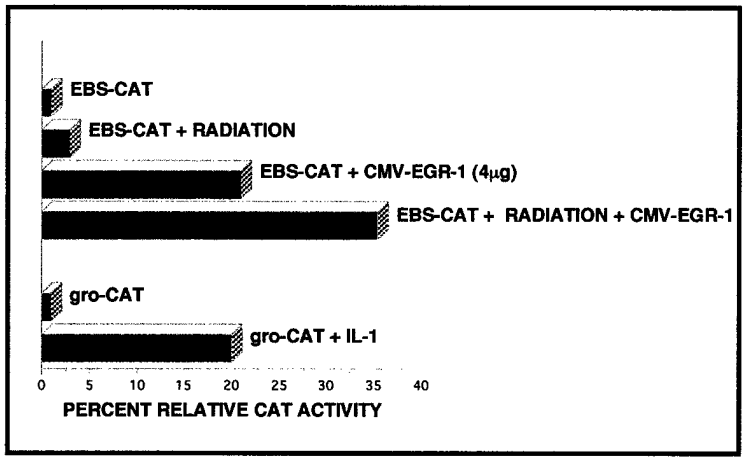
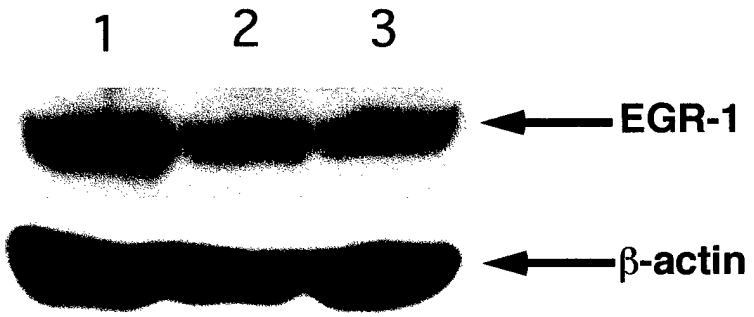


Figure 8. Radiation-induced EGR-1 protein show low transactivation ability of CAT-reporter construct containing EGR-1 binding sites in the DU-145 cells. Parental DU-145 cells were transiently transfected with 4mg of CAT reporter plasmid (EBS-CAT or gro-CAT) alone or plus effector plasmid (CMV-EGR1). The total amount of DNA was made up to 36μg, with vector DNA. DU-145 cells containing either EBS-CAT or EBS-CAT plus CMV-EGR1 were left untreated or irradiated at 5 Gy, and cells containing gro-CAT was left untreated or treated with IL-1. CAT activity was assayed and normalized by determining the percent conversion of [^{14}C] chloramphenicol to acetylated forms using densitometeric ratios.

Figure 9



1 : DU145/EGR-1.L1
2 : DU145/WT1-EGR-1.L1
3 : DU145/Vector.L1

Figure 9. EGR-1 protein expression analysis in DU-145 transfectant cells. EGR-1 protein levels were detected by Western blot analysis and immunocytochemistry before and after radiation. Whole cell protein extracts were prepared from DU-145 transfectants that were left untreated (UT) or exposed to a 5 Gy dose and incubated for the time interval indicated and then subjected to Western blot analysis for EGR-1 or β-actin. The blot was subsequently probed with an antibody for EGR-1 (sc-110) or β-actin.

Figure 10

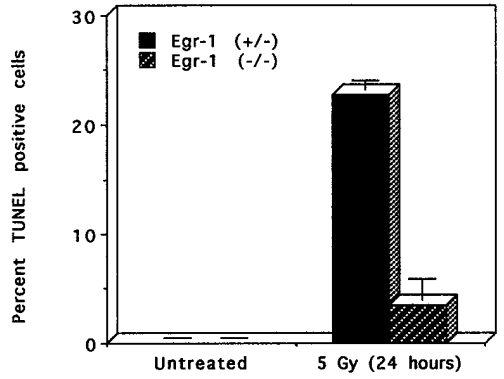


Figure 10. Quantification of apoptosis by TUNEL assay. To quantify apoptosis, the ApopTagTM in situ apoptosis detection kit (Oncor, Gaithersburg, MD) was used. The ApopTag kit detects the DNA strand breaks in single cells by terminal transferase-mediated dUTP-digoxigenin-end labelling (TUNEL). Cells were seeded in chamber slides and 24 h later they will be exposed to 5 Gy dose of radiation. The DNA was then tailed with digoxigenin-dUTP and conjugated with anti-digoxigenin fluorescein. The cells were stained with antifade. The stained cells were observed in triple band-pass filter using Nikon-microphot epifluorescence microscope. To determine the percentage of cells showing apoptosis, a total of 500 cells were counted for each experiment. Bar graph showing the percentage of TUNEL-positive cells. Background levels in untreated were normalized over treated. Data represent a mean of two experiments. The error bars represent standard deviation.

Figure 11

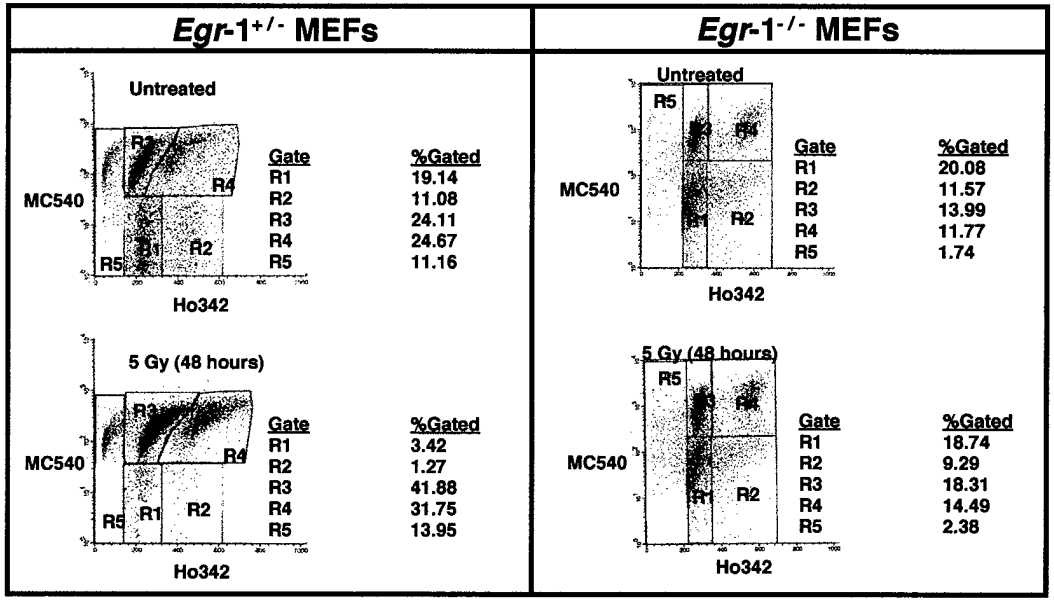


Figure 11. Quantitation of apoptosis by Hoechst 33342 and merocyanin 540 staining : Cells were irradiated at 5 Gy and after 48 hours stained with Hoechst (Ho342) and merocyanine (MC540). The gates were set so as to analyze cell cycle and apoptosis stages. Ho342 is a DNA-specific dye that measures DNA content and MC540 binds to membrane phospholipids that are exposed on the outside of the membrane during the process of apoptosis. These two dyes separate five distinct populations of tumor cells : viable resting cells, 2n DNA content and MC540-unstained (R1); viable cycling cells, >2n DNA content and MC540-unstained (R2); viable resting cells undergoing apoptosis, 2n DNA content and MC540-stained (R3); viable cycling cells undergoing apoptosis, >2n DNA content and MC540-stained (R4); and late stage apoptotic cells that are MC540-stained but Ho342-unstained indicating DNA fragmentation (R5). The data shown are representative of two independent experiments. The untreated population contained MC540-stained cells owing to spontaneous apoptosis that occurred during cell culture. The percent increase (mean + standard deviations from two experiments) in apoptotic cells (i.e., MC540-stained cells in the R3, R4, and R5 compartments) in the irradiated population over the untreated population was 6.18+1.02 in Egr-1-/- cells and 52.97+2.32 in Egr-1+/- cells.

Figure 12

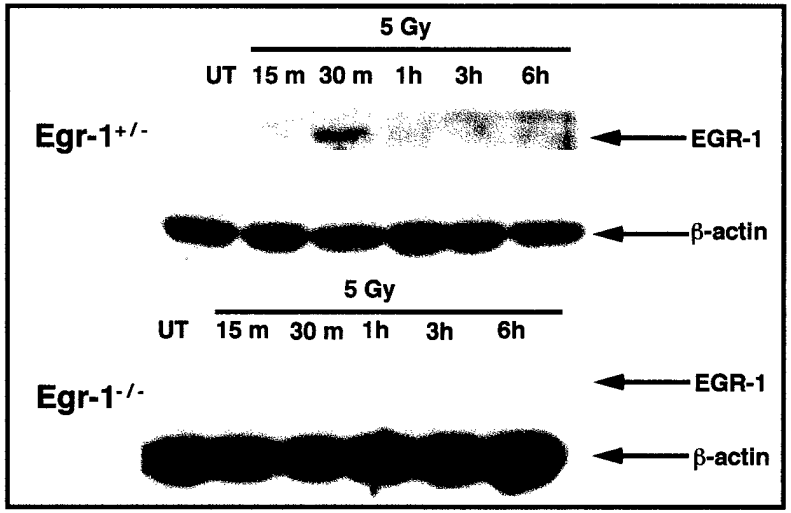


Figure 12. EGR-1 is induced by ionizing radiation. EGR-1 protein induction detected by Western blot analysis. Whole cell protein extracts were prepared from Egr-1+/- and Egr-1-/- cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for the time interval indicated in min and then subjected to Western blot analysis for EGR-1 or β-actin. The blot was subsequently probed with an antibody for EGR-1 or β-actin.

Figure 13

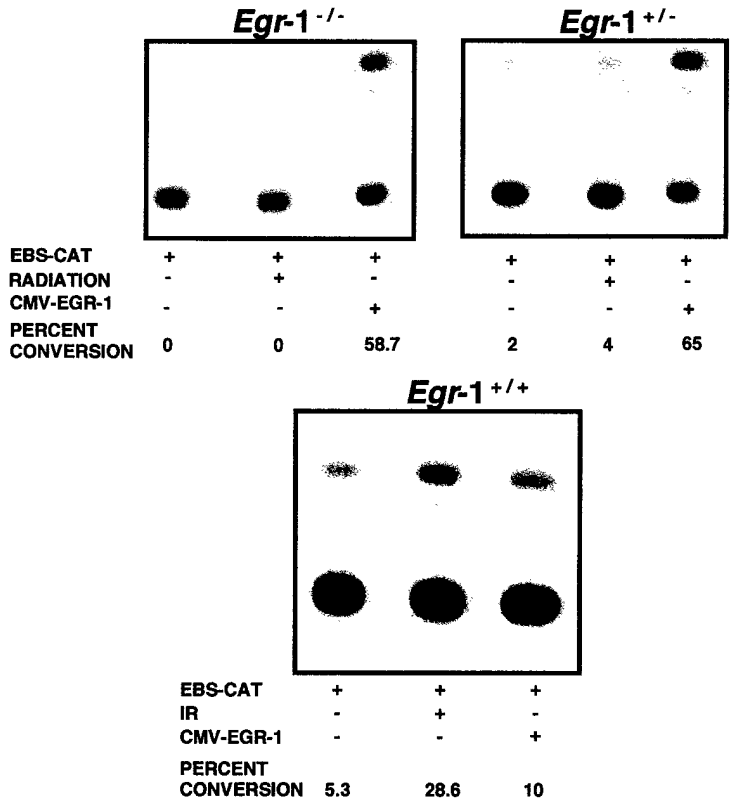


Figure 13. Ionizing radiation and CMV-EGR-1 transactivates EBS-CAT reporter construct containing three tandem repeat of EGR-1- binding sites. MEF cells were transiently cotransfected with 4 mg of EBS-CAT or of and 4 mg of CMV-EGR-1. Next, the cells were either left unexposed or exposed to a 5 Gy dose of radiation and CAT activity was assayed and expressed as percent conversion of 14C-chloramphenicol to acetylated forms.

Figure 14

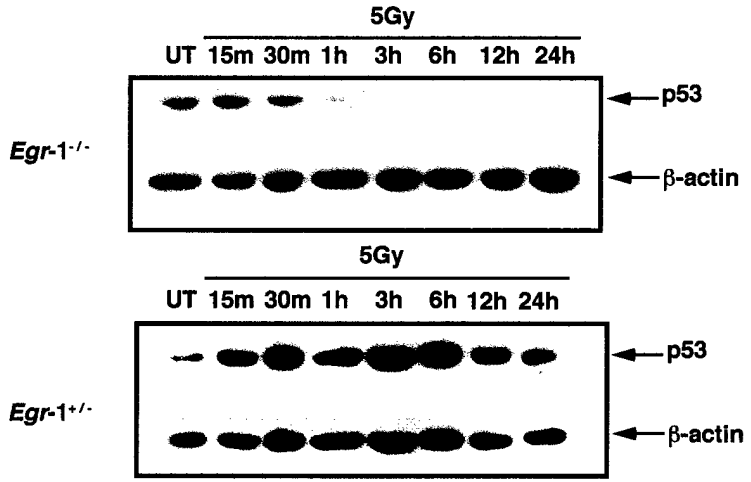


Figure 14. p53 protein is down-regulated by ionizing radiation in Egr-1-/- cells. p53 protein levels were detected by Western blot analysis before and after radiation. Whole cell protein extracts were prepared from Egr-1+/- and Egr-1-/- cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for the time interval indicated and then subjected to Western blot analysis for p53 or β-actin. The blot was subsequently probed with an antibody for p53 (DO-1) or β-actin.

Figure 15

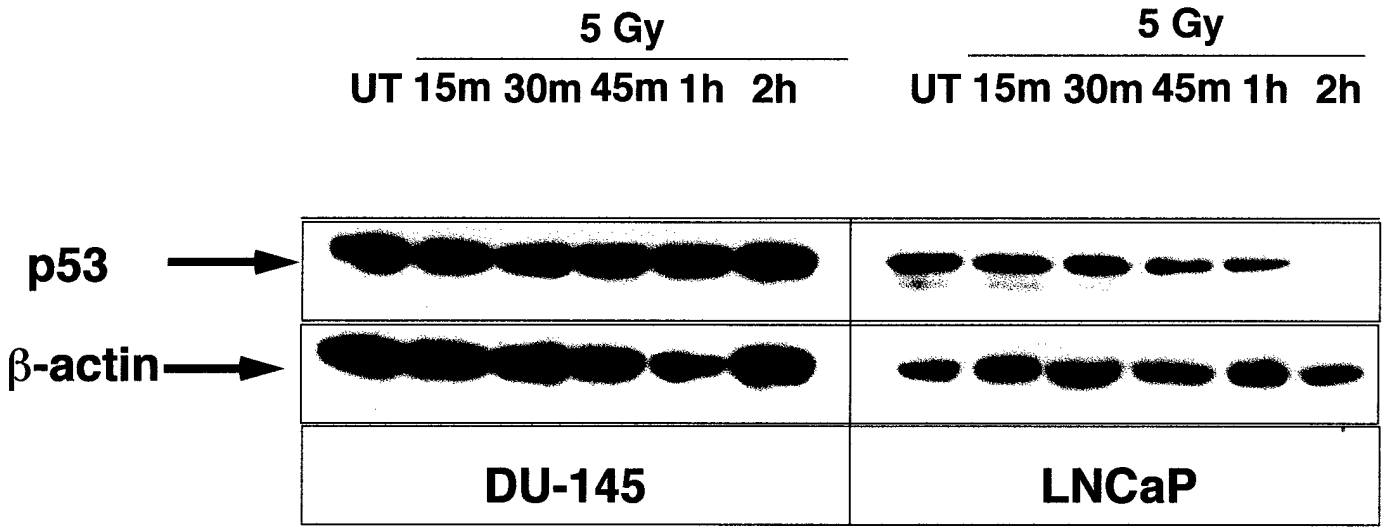


Figure 15. p53 protein expression analysis in DU-145 and LNCaP cells. p53 protein levels were detected by Western blot analysis before and after radiation. Whole cell protein extracts were prepared from DU-145 and LNCaP cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for the time interval indicated and then subjected to Western blot analysis for p53 or β-actin. The blot was subsequently probed with an antibody for p53 (DO-1) or β-actin.

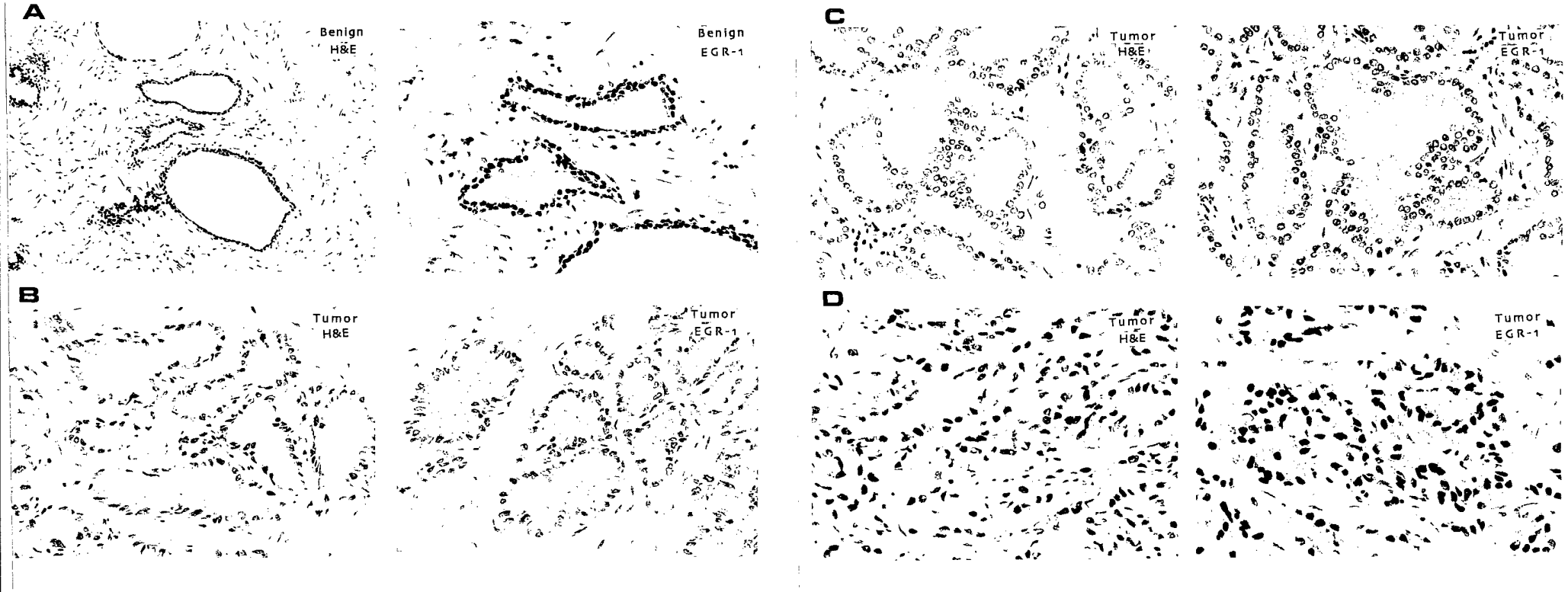


Figure 16. EGR-1 GENE EXPRESSION IN PROSTATE CANCER. Radical prostectomy samples of patients PS-56 benign region (A), PS-52 moderately differentiated tumor (B), PS-53 moderately differentiated tumor (C) and moderate to poorly differentiated tumor PS-54 (D). showing H&E and EGR-1 expression staining (magnification 20x).

Figure 17

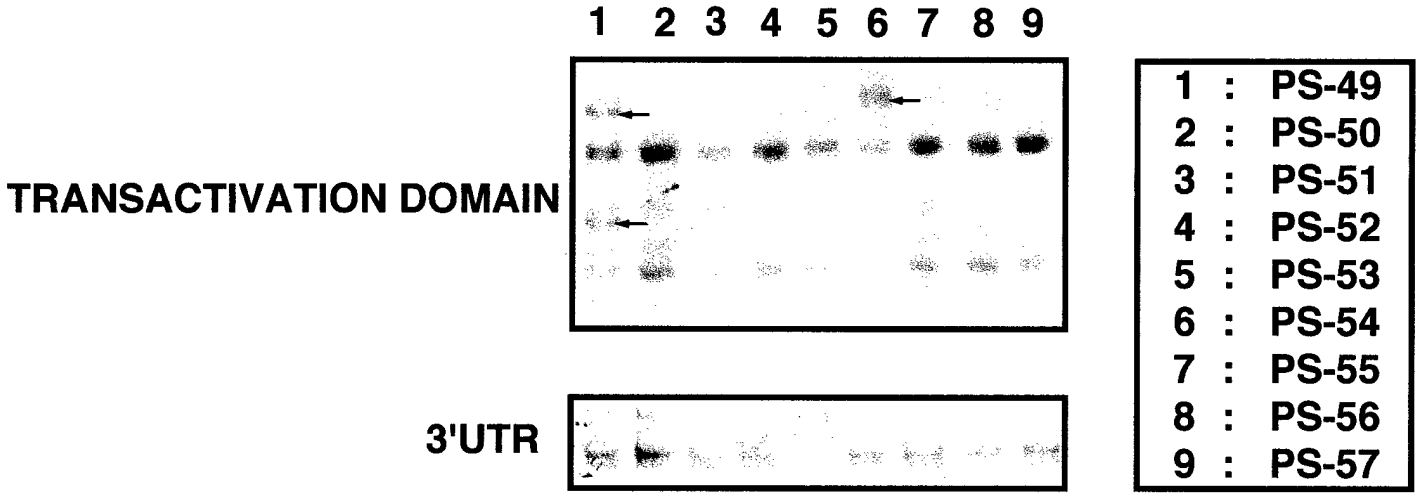


Figure 17. Autoradiograph of PCR-SSCP analysis of the Egr-1 5' (transactivation domain-AGC6/33/6) and 3'(3'UTR-A16) regions using retrospective tumor samples of prostate cancer. Mutations in transactivation domain were detected. Tumors from PS-49 and PS-54 showed mutation in transactivation domain.

Appendix II
(Abstracts presented: AACR'99; NCI grant funding: Critiques and Award letter; and Revised USAMRMC Assistance Agreement)

#948 Ionizing radiation down-regulates *p53* protein in *Egr*-1$^{-/-}$ MEF cells causing enhanced resistance to apoptosis. Ahmed, M.M[1]., Chendil, D[1]., Dey, S[1]., Parekh, S[1]., Mohiuddin, M[1]., Rangnekar, V.M[2] and Milbrandt, J.D[3]. [1]*Departments of Radiation Medicine and* [2]*Urology, University of Kentucky, Lexington, KY and* [3]*Department of Pathology, Washington University, St. Louis, MO.*

In our previous studies, we used tumor cell lines such as wild-type *p53* melanoma cells (*J Biol Chem* 271 (46): 29231–29237, 1996) and *p53*-deficient prostate cancer cells (*J. Biol. Chem.* 272(52): 33056–33061, 1997) to understand the regulatory role of *Egr*-1 in radiation-induced apoptotic processes. These studies strongly suggested that *Egr*-1 can mediate it's apoptotic action irrespective of *p53* status. In this study, we sought to investigate this mechanism in normal cell background using mouse embryonic fibroblast (MEF) cells established from homozygous (*Egr*-1$^{-/-}$) and heterozygous (*Egr*-1$^{+/-}$) *Egr*-1 knock-out mice. Radiation-induced apoptosis of MEF cells was determined using TUNEL and flow cytometry assays. Radio-induction of EGR-1 and *p53* protein was determined by Western blot analysis. Ionizing radiation caused signifcantly enhanced apoptosis in *Egr*-1$^{+/-}$ cells (22.8%; $p<0.0001$) when compared to *Egr*-1$^{-/-}$ cells (3.5%). *Egr*-1$^{+/-}$ cells showed radio-induction of EGR-1 expression and this was not evident in *Egr*-1$^{-/-}$ cells. Also, radiation elevated *p53* protein in *Egr*-1$^{+/-}$ cells in 3-6 hours. However, in *Egr*-1$^{-/-}$ cells, the *p53* protein was down-regulated in 1 hour after radiation and was completely absent in the later time points. Since, EGR-1 protein was previously found to upregulate *p53*, the loss of *p53* transactivation in *Egr*-1$^{-/-}$ cells might have contributed to enhanced resistance to apoptosis. More studies are currently being investigated to understand the precise role of wild-type *p53* protein upon DNA-damage in cells lacking EGR-1 function.

#949 Radiosensitivity of thymidylate synthase-deficient human tumor cells is affected by progression through the G_1 restriction point into S-phase: Implications for fluoropyrimidine (FP) radiosensitization (RS). Hwang, H-S., Houghton, J.A., Davis, T.W., and Kinsella, T.J. *Radiation Oncology, Case Western Reserve University and University Hospitals of Cleveland/Ireland Cancer Center, Cleveland, OH 44106; Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN 38101.*

Recently, studies of RS have focused on the molecular mechanisms underlying the control of cell cycle. We hypothesize a mechanism for FP-mediated RS(FP-RS) which is independent of RNA-directed effects. This mechanism is dependent upon progression into S-phase under conditions of an increased dATP:dTTP ratio, which results in DNA fragmentation and cell death. To better understand the mechanism of FP-RS, we characterized the IR response using different synchronization techniques in two thymidylate synthase (TS)-deficient mutants, TS$^-$ and Thy4 clones. Following G_0-synchronization by leucine deprivation, these clones differ under conditions of dThd withdrawal, where Thy4 cells have an intact G_1 arrest and delayed cell death (>5 d). In contrast, TS$^-$ cells progress into early S-phase and undergo acute cell death (<1 d). No difference in the late S and G_2/M populations were noted between the cell lines. The intracellular ratio of dATP:dTTP increased substantially in TS$^-$ cells compared to Thy4 cells. The synchronized TS$^-$ cells showed significantly decreased clonogenic survival and an increase in DNA fragmentation following IR when compared to Thy4 cells. The second trial of synchronization used mimosine to induced a block at close to G_1/S border. TS$^-$ and Thy4 cells now progress into S-phase identically under dThd withdrawal conditions. Also, TS$^-$ and Thy4 cells show similar clonogenic survival following IR. We suggest, based on the RS differences between G_0 and G_1/S synchronized cells that S-phase progression through the G_1 restriction point can result in improved RS.

#950 MAP kinase pathway signaling is required for release/progression of cells through G_2/M after exposure to ionizing radiation. Jong-Sung Park, Rupert Schmidt-Ullrich, and Paul Dent.

In A431 squamous carcinoma cells, ionizing radiation causes transient increases in the activities of the Mitogen Activated Protein kinase (MAPK) and c-Jun NH_2-terminal kinase (JNK) pathways. Radiation (2 Gy) caused both immediate primary (0–10 min) and secondary activations (90–240 min) of the MAPK and JNK pathways. Expression of either antisense EGF receptor or addition of a neutralizing antibody to TGFα blocked the ability of radiation to cause secondary activations of both pathways. Inhibition of the MAPK pathway potentiated the ability of radiation to activate the JNK pathway and to increase apoptosis. However, inhibition of JNK pathway function via antisense oligonucleotides towards JNK1 and JNK2 failed to block the potentiation of apoptosis. These data argue that inhibition of MAPK signaling potentiates radiation-induced cell killing via a JNK-independent pathway. Radiation induced a transient G_2/M growth arrest which was maintained for up to 24 h by inhibition of the MAPK pathway. Irradiation/MAPK inhibition caused prolonged elevation of Cdc2 Y15 and reduction of Histone H3 S10 phosphorylations, respectively. MAPK inhibition abrogated radiation-induced $p21^{Cip-1}$ and Cyclin D1 expression but did not effect expression of Cyclins A, B1, E. Caffeine treatment of irradiated/MAPK inhibited cells permitted release from the prolonged G_2/M arrest at 24 h, and returned Cdc2 Y15 and Histone H3 S10 phosphorylations to control levels. The amount of radiation-induced apoptosis 24 h after irradiation was reduced in caffeine treated cells. Data argue that inhibition of the MAPK pathway radio sensitizes A431 cells is by maintaining radiation-induced G_2/M arrest. Increased signaling by the MAPK pathway may be required to re-energize cells following radiation-induced cell cycle arrest.

#951 Caffeine does not override the G2/M block induced by UVc-radiation in normal human skin fibroblasts (NHFs). Deplanque[2] G., Goldblum[2] S., Vincent[2] F., Cazenave[1] JP., Bergerat[2] JP., Klein-Soyer[1] C. *INSERM U311[1], ETSS, 10 rue Spielmann, 67065 Strasbourg Cedex, France and Laboratoire d'Oncologie Moléculaire[2], IRCAD, HUS, BP426, 67091 Strasbourg-Cedex, France. E-Mail: Claudine.Soyer@etss.u-strasbg.fr.*

Caffeine (Caff) has for many years been known to be involved in the sensitization of DNA to damage, presumably in particular through its capacity to promote overriding of the G2/M block induced by irradiation. However, the variety of responses displayed by different cell types does not yet allow the establishment of a clear cut mechanism, especially as little is known about the capacity of this agent to enhance DNA damage in normal, untransformed cells. Hence we examined the effects of caffeine on NHFs. Continuous exposure to caffeine (1 to 5 mM) inhibited cell proliferation in a dose-dependent manner attaining 80 % at 5 mM and this phenomenon was reversible within 24h of removal of caffeine. Exposure of exponentially growing NHFs to UVc radiation (20 J/m2) in the absence of caffeine led to a 45 % inhibition of proliferation and resulted in a protracted accumulation of cells in the G2/M phase.

	% G2/M at 16h	Cells/cm2 at 16h	Cells/cm2 at 72h
Control	1	12.000	54.000
Control + Caff	1	10.000	21.000
UV	**50**	9.100	**29.000**
UV + Caff	**27**	8.400	**11.000**

Addition of 2 mM caffeine after UV-irradiation induced slowing of the S phase traverse, with a resultant delay in G2/M accumulation mimicking a G2/M block override. In fact, after the first 24h, G2/M accumulation was greater in the presence than in the absence of caffeine. As it has been shown that caffeine directly interferes with DNA repair synthesis, these results correlate with more unrepaired cells arriving and accumulating in G2/M.

#952 Proapoptotic gene, *par*-4, abrogates radiation-induced G_2 block and bcl-2 delays the occurrence of radiation-induced G_2 block in prostate cancer cell line PC-3. Chendil, D[1]., Dey, S[1]., Parekh, S[1]., A1-Jumaily, W[2]., Mohiuddin, M[1]., Rangnekar, V.M[3] and Ahmed, M.M[1]. [1]*Departments of Radiation Medicine,* [2]*Surgery and* [3]*Urology, University of Kentucky, Lexington, KY.*

par-4 encodes a 38-kd protein that is required for enhanced apoptosis. Recent studies have indicated that the induction of this gene is exclusively associated with the induction of apoptosis. Recently, we had reported that *par*-4 overexpression caused reduction in the endogenous bcl-2 protein leading to sensitization of PC-3 cells to apoptosis upon serum deprivation. In this study, we sought to investigate the role of *par*-4 and bcl-2 in radiation-induced clonogenic inhibition and cell cycle changes in PC-3 cells. Radiation-induced clonogenic inhibition and cell cycle analysis of PC-3/Vector, PC-3/CMV-*par*-4 and PC-3/CMV-BCL2 cells were determined using colony-forming and flow cytometry assays respectively. Radiation caused a delay in the occurrence of G_2 block in PC-3/CMV-BCL2 cells (this block was evident only 24 hours after radiation) when compared to PC-3/Vector cells (evident in 12–24 hours after radiation) and these cells were highly radio-resistant. Whereas, in PC-3/CMV-*par*-4 cells, no G_2 block was observed and the cells progressed to G_0/G_1 after 24 hours of radiation. In these cells, clonogenic inhibition was similar to that of PC-3/Vector cells. Currently, we are investigating the mechanism through which *par*-4 abrogates the radiation-induced G_2 block by analysing the G_2 checkpoint genes.

#953 Mitochondrial DNA is important for cell death induced by adriamycin. Barbara Sigala[1], James Russell[1], Yonggong Zhang[1], Jerry Williams[1], Kylie F. Keshav[2], Keshav K. Singh[1]. [1]*Johns Hopkins Oncology Center, 600 North Wolfe Street, Baltimore, MD 21287,* [2]*Department of Bioscience and Biotechnology, Drexel University, 32nd and Chestnut Streets, Philadelphia, PA 19104.*

While nuclear DNA is an established cellular target for cancer therapy it is unclear whether mitochondrial DNA (mitDNA) is also targeted by therapeutic agents. To examine the importance of mitDNA in cancer therapy, we have determined the clonogenic survival of HSL2, (Rho$^+$, HeLa subline) and its derivative cell line lacking mitDNA (Rho^0) after exposure to different anticancer agents. We found that isogenic Rho^0 cells lacking mitDNA were extremely resistant to adriamycin induced cell death, whereas the Rho$^+$ cell line was sensitive. However, there was no measurable difference in the responses of these cell lines to either akylating agent or γ-radiation. We show that the development of resistance to adriamycin-induced cell death was not due to alterations in cell cycle response or to the uptake of adriamycin in isogenic Rho^0 cells. We also demonstrate that exposure of HSL2 cells to adriamycin leads to deletions in mitDNA. These studies provide direct evidence that mitDNA play an important role in cell death induced by adriamycin. Since most cancer cells are deficient in mitochondrial function, our studies indicate that Rho^0 cancer cells may serve as an excellent system for screening anticancer drugs for potential clinical efficacy.

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

National Institutes of Health
National Cancer Institute
6130 Executive Blvd., Suite 800
Rockville, MD 20852-7440
Phone (301) 496-9360
FAX (301) 480-5785

March 26, 1999

Mansoor M. Ahmed, Ph.D.
University of Kentucky Medical Center
Department of Radiation Medicine
800 Rose Street
Lexington, KY 40536-0084

Re: 1 R01 CA78471-01A1

Dear Dr. Ahmed:

Enclosed is a copy of the summary statement prepared by the scientific review administrator of the initial review group (study section) that evaluated your application. The evaluation of an application for its scientific merit is the first step in a two-step process of peer review. The second step will be carried out by the National Cancer Advisory Board (NCAB) at its next meeting.

In deciding which research grant applications to fund, the single most important factor is the study section's assessment of the scientific and technical merit of the application. However, the Institute also considers other factors such as programmatic needs and balance; therefore, no final conclusions should be made concerning the probability of funding based on priority score or percentile ranking alone.

I would like to call your attention to one problem identified on your Summary Statement which precludes possible funding until it is resolved. On page 5 under GENDER AND MINORITY SUBJECTS you will find the comment: " The applicant, however, does not provide the minority composition of this study population."

If we are able to fund this project, we will contact you concerning the details. However you could eliminate one problem by providing to me, at your earliest convenience, the projected minority composition of your study. Before you do this, it is advisable to acquaint yourself with NIH policies on this subject. The following website should be of help to you:

< http://www.nih.gov/grants/funding/phs398/section_3.html#gender>

If you have any questions or need elaboration, please do not hesitate to call me.

Sincerely,

Francis J. Mahoney, Ph.D.
Program Director
Radiation Research Program
Division of Cancer Treatment
and Diagnosis

Enclosure
cc: Business Official

DR. FRANCIS J. MAHONEY
301 496-9360
mahoneyf@dtpepn.nci.nih.gov

SUMMARY STATEMENT
(Privileged Communication)

Application Number: 1 R01 CA78471-01A1

DUAL PROGRAM CLASS CODE: 1BPHYFB DUAL: AG
REN

Review Group: REPRODUCTIVE ENDOCRINOLOGY STUDY SECTION

Meeting Dates: IRG: FEB/MARCH 1999 COUNCIL: MAY 1999 11 2753
Requested Start Date: 07/01/1999

AHMED, MANSOOR M, PHD
UNIV OF KENTUCKY MEDICAL CENTER
DEPT OF RADIATION MEDICINE
800 ROSE STREET
LEXINGTON, KY 40536-0084

Project Title: ERG-1 AND APOPTOSIS IN PROSTATE CANCER

IRG Action: Priority Score: 180 Percentile: 9.1
Human Subjects: 30-HS INV-CERTIFIED NO IRG CONCERNS/COMMENTS
Animal Subjects: 10-NO LIVE VERTEBRATE ANIMALS INVOLVED
Gender: G3A-ONLY MEN, SCIENTIFICALLY ACCEPTABLE
Minority: M4U-MINORITY MAKE-UP UNKNOWN, SCIENTIFICALLY UNACCEPTABLE
CLINICAL RESEARCH - NOT NIH-DEFINED PHASE III TRIAL

NOTE TO APPLICANT FOLLOWS SUMMARY STATEMENT.

NEW INVESTIGATOR

RESUME AND SUMMARY OF DISCUSSION: This revised application proposes to study the role of Egr-1 in radiation-induced apoptosis in prostate cancer. The relationship of Egr-1 and expression of TGF-alpha and p53 in outcome of radiation therapy in prostate cancer was proposed. The applicant has addressed the majority of comments from the Summary previous critique. This is an improved application to ask an important question regarding the molecular mechanisms of radiation resistance in prostate cancer. The application overall is well-written with appropriate controls and design. The addition of substantial, new preliminary studies to support the hypothesis have increased the enthusiasm for the application.

DESCRIPTION: Egr-1 (early growth response) gene is up-regulated by radiation in prostate cancer cells. The PI has postulated that Egr-1 is a key upstream regulatory gene in the control of apoptosis process, which is mediated through wild-type p53. Four specific aims were proposed.

Specific Aim 1 will determine effect of radiation on the induction of Egr-1 expression in DU-145 (androgen independent and p53 mutated cell line) and LNCaP

has been strengthened by clarification of the anticipated results and potential problems and alternative methods. Aim #2 is further strengthened by the demonstration in the Preliminary Studies that EGR-1 regulates p53. Here, Dr. Ahmed will determine if a dominant negative mutation protects radiation-induced growth inhibition and whether the cDNA for Egr-1 enhances radiation-induced growth inhibition. This aim is relatively straightforward and problems should not be significant. This aim is strong.

Aim #3 is to elucidate the pathway of Egr-1 mediated up regulation of the target genes (TNF-alpha and p53) so that the mechanism of radiation-inducible apoptosis in prostate cancer cells can be understood. Again, this aim has been strengthened by the additional Preliminary Studies. This aim is strong. Aim #4 will determine the clinical relevance of EGR-1 mutations in human prostate cancer using PCR-SSCP, immunohistochemical and RT-PCR analyses. Again, this approach (SSCP/sequencing) will provide no information regarding the significance of any observed variants (i.e. are the changes benign polymorphisms or do they affect the function of the protein?). Unless a large deletion or large insertion or stop codon is affected, it may not be immediately obvious whether the mutations have any functional consequence.

3. INNOVATION: The application is moderately innovative. It uses standard molecular biology techniques that are appropriate for the questions to be asked. The focus on the molecular aspects of radiation resistance of prostate cancer and this focus is important and somewhat novel as well.

4. INVESTIGATOR: Dr. Ahmed has been moderately productive over the past 5 years, although it appears that the last few years have been particularly productive with some publications in strong journals. He appears to have expertise that is appropriate for the proposed studies. Other investigators have expertise in clinical radiology, urology, pathology and this expertise is needed for the project.

5. ENVIRONMENT: The environment is good and Dr. Ahmed will have the facilities needed for this project.

OVERALL EVALUATION: This is an improved application to ask an important question regarding the molecular mechanisms of radiation resistance in prostate cancer. The application overall is well-written with appropriate controls and design. The addition of substantial, new preliminary studies to support the hypothesis have increased the enthusiasm for the application.

CRITIQUE 2:

1. SIGNIFICANCE: This is a first revision of an application in which the PI will study the role of Egr-1 in radiation-induced apoptosis in prostate cancer cells. The PI has addressed reviewer's comments and has made a significant amount of changes in the revised proposal. Specifically, the PI has included in the revised proposal comments on expected results and potential problems. The PI also addressed the impact of this study in clinical relevance. However, due to circumstance beyond the PI's control, the PI was unable to satisfy some of the comments. For example, the PI was unable to obtain the pre-treatment cancer specimens for determination of EGR-1 staining. Nevertheless, the revised proposal has improved the merit of this project. The PI has also added more

worthwhile information. There are no major concerns.

OVERALL EVALUATION: The major strengths of this well-written proposal are 1) a very important problem addressing a focused hypothesis 2) sound rationale for testing this hypothesis on the basis of preliminary data, and 3) a well qualified young investigator. There are no perceived major weaknesses.

GENDER AND MINORITY SUBJECTS: This study involes the use of prostate tissue, hence only male subjects will be used. The applicant, however, did not provide the minority composition of this study population.

BUDGET: The salaries were not properly calculated based upon NIH guidelines. Otherwise, the request is appropriate.

NOTICE: The NIH has modified its policy regarding the receipt of amended applications. Detailed information can be found by accessing the following URL address: http://www.nih.gov/grants/policy/amendedapps.htm

++Beginning with the previous round of reviews (i.e., for October 1998 Councils), NIH peer review groups are being asked to recalibrate their scoring using 3.0 as the target median score and to spread scores over a wider range of the priority score scale. (This measure is being taken for two reasons: 1) inconsistent scoring practices among different review groups, and 2) in many review groups, scores have become so compressed that the ability to discriminate among applications has been compromised.) As a result of this recalibration, priority scores for applications reviewed this review cycle may not be comparable to those received in the past. Furthermore, in order to prevent influence from priority scores given in prior rounds, percentiles for applications reviewed this round are based on scores assigned during the previous round and this round.

REPRODUCTIVE ENDOCRINOLOGY STUDY SECTION
ENDOCRINOLOGY AND REPRODUCTIVE SCIENCES INITIAL REVIEW GROUP
CENTER FOR SCIENTIFIC REVIEW
REN
February 21, 1999 - February 23, 1999

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DEPARTMENT OF INTERNAL MEDICINE AND ENDOCRINOLOGY
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SCIENTIFIC REVIEW ADMINISTRATOR
SHAIKH, ABUBAKAR A., DVM, PHD
SCIENTIFIC REVIEW ADMINISTRATOR
CENTER FOR SCIENTIFIC REVIEW
NATIONAL INSTITUTES OF HEALTH
BETHESDA, MD 20892

X-Sender: ddavis@pop.uky.edu
Date: Fri, 30 Jul 1999 10:14:59 -0400
To: ahmm
From: Deborah Davis <ddavis@pop.uky.edu>
Subject: Fwd: NGA: 1 R01 CA78471-01A1 PI: AHMED, MANSOOR M
Mime-Version: 1.0

Dr. Ahmed: Here is your NIH award. I normally forward these to the Principal Investigator when they arrive, but I could not find an e-mail address for you.

>From: OD OER NGA Mailer <ODOERNGAMAILER@OD.NIH.GOV>
>To: "'ddavis@pop.uky.edu'" <ddavis@pop.uky.edu>,
> NCI GAB IMPAC2
> <ncigabimpac2-l@exchange.nih.gov>
>Subject: NGA: 1 R01 CA78471-01A1 PI: AHMED, MANSOOR M
>Date: Tue, 20 Jul 1999 00:05:37 -0400
>X-Mailer: Internet Mail Service (5.5.2448.0)
>
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>
>********** NOTICE OF GRANT AWARD
>**********
>RESEARCH Issue Date:07/20/1999
>Department of Health and Human Services
>National Institutes Of Health
>NATIONAL CANCER INSTITUTE
>**********
>****
>
>Grant Number: 1 R01 CA78471-01A1
>Principal Investigator: AHMED, MANSOOR M PHD
>Project Title: ERG-1 AND APOPTOSIS IN PROSTATE CANCER
>
>
>ASSOC DIRECTOR
>UNIVERSITY OF KENTUCKY
>214 KINKEAD HALL
>LEXINGTON, KY 40506-0057
>
>
>
>
>Budget Period: 08/01/1999 - 05/31/2000
>Project Period: 08/01/1999 - 05/31/2003
>
>
>Dear Business Official:
>
>The National Institutes of Health hereby awards a grant in the amount of
>$158,832(see "Award Calculation" in Section I) to University of
>Kentucky Research Foundation in support of the above referenced
>project. This award is pursuant to the authority of 42 USC 241 42 CFR
>52 and is subject to attached terms and conditions.

>Acceptance of this award including attached Terms and Conditions is
>acknowledged by the grantee when funds are drawn down or otherwise
>obtained from the grant payment system.
>
>Award recipients are responsible for appropriate acknowledgment of NIH
>support when preparing publications, or issuing statements, press
>releases, request for proposals, bid solicitations, and other documents
>describing projects or programs funded in whole or in part with NIH
>support.
>
>If you have any questions about this award, please contact the
>individual(s) referenced in the attachments.
>
>Sincerely yours,
>
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>
>Kelli Oster
>Grants Management Officer
>NATIONAL CANCER INSTITUTE
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>Attachments
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>SECTION I - AWARD DATA - 1 R01 CA78471-01A1
>
>AWARD CALCULATION (U.S. Dollars):
>
>Salaries and Wages $74,125
>
>Fringe Benefits $14,338
>

>Personnel Costs $88,463
>
>Supplies $18,900
>
>Travel Costs $900
>
>Other Costs $900
>
>Direct Costs $109,163
>F&A Costs $49,669
>
>APPROVED BUDGET $158,832
>TOTAL $158,832
>
>
>
>Recommended future year total cost support, subject to the availability
>of funds and satisfactory progress of the project, is as follows.
>
>02 $189,521
>03 $194,618
>04 $199,866
>
>
>
>FISCAL INFORMATION:
>CFDA Number 93.395
>
>EIN: 1616033693A1
>Document Number: R1CA78471A
>IC/ CAN / FY1999 / FY2000 / FY2001 / FY2002
>
>CA/8422753/ 158,832/ 189,521/ 194,618/ 199,866
>
>NIH ADMINISTRATIVE DATA:
>
>PCC: 11AV2753 / OC: 41.4A /Processed: OSTERK 990714 0429
>
>Award e-mailed to: ddavis@pop.uky.edu
>
>
>SECTION II - PAYMENT/HOTLINE INFORMATION - 1 R01 CA78471-01A1
>
>For Payment and HHS Office of Inspector General Hotline Information,
>see the NIH Home Page at
>http://www.nih.gov/grants/policy/awardconditions.htm
>
>SECTION III - TERMS AND CONDITIONS - 1 R01 CA78471-01A1
>
>This award is based on the application submitted to, and as approved by,
>the NIH on the above-titled project and is subject to the terms and
>conditions incorporated either directly or by reference in the
>following:
>
>a. The grant program legislation and program regulation cited in this
>Notice of Grant Award.

>b. The restrictions on the expenditure of federal funds in
>appropriations acts, to the extent those restrictions are pertinent to
>the award.
>c. 45 CFR Part 74 or 45 CFR Part 92 as applicable.
>
>d. The NIH Grants Policy Statement, including addenda in effect as of
>the beginning date of the budget period.
>
>e. This award notice, INCLUDING THE TERMS AND CONDITIONS CITED BELOW.
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>(see NIH Home Page at
>http://www.nih.gov/grants/policy/awardconditions.htm for certain
>references cited above.)
>
>This grant is included under Expanded Authorities.
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>
>This grant is subject to Streamlined Noncompeting Award Procedures
>(SNAP).
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>
>Treatment of Program Income:
>Additional Costs
>
>INFORMATION This award reflects the budget and the budget period
>negotiated between Jill Rogers of the National Cancer Institute and
>Suzanne Leibee on July 14, 1999.
>
>INFORMATION In accordance with the National Cancer Institute's (NCI's)
>implementation of the National Institutes of Health core principles for
>FY 1999 funding decisions, NCI staff have determined that critical
>program objectives can be met with the funding of this grant at 90% of
>the corrected recommended level*. Future year committed levels** have
>been adjusted accordingly.
>
>* corrected recommended level: Summary Statement recommended level of
>support with arithmetic errors corrected, with adjustments made in
>accordance with applicable grant policies as appropriate, direct
>salaries and associated fringe benefits adjusted to comply with the
>$125,900 salary cap, and no more than a 3% cost of living factor used to
>calculate the level of support recommended for each future budget
>period.
>
>** committed level: The level of support calculated by applying the NCI
>funding plan to the corrected recommended level for each budget category
>for all years of the project period.
>
>INFORMATION Future year total cost commitments appearing on the award

>notice under "Recommended Future Year Total Cost Support" have been
>calculated by applying the negotiated facilities and administrative cost
>rate(s) in effect at the time of this FY 1999 award to the committed
>total direct cost level for each future year.
>
>INFORMATION In order to redistribute awards more evenly throughout the
>year, budget periods are being adjusted. In accordance with the
>discussion between Jill Rogers of the National Cancer Institute and
>Suzanne Leibee on July 14, 1999, this award is issued with a 10-month
>budget period with support reduced in accordance with the NCI FY99
>recycling plan. Continuation awards will cycle each year on June 1st.
>Noncompeting continuation applications are due two months prior to this
>date.
>
>INFORMATION The recycling of this award has changed the receipt date
>for the next competing continuation (Type 2) application. Consult
>"Application Receipt Dates, Review and Award Schedule" in the Grant
>Application Form PHS 398 for the established deadline date.
>
>INFORMATION None of the funds in this award shall be used to pay the
>salary of an individual at a rate in excess of 125,900 per year.
>Therefore this award and future years are adjusted accordingly.
>
>INFORMATION Included in the Notice of Grant Award is a spreadsheet
>showing the committed level of funding for each year of this competitive
>segment. Spreadsheets showing the "Corrected Recommended levels" of
>funding and spreadsheets for individual projects and/or consortia (if
>applicable) are available upon request from the Grants Management
>Specialist referenced in the terms of award.
>
>INFORMATION For administrative and management concerns, contact the
>Grants Management Specialist, Jill Rogers, at (301) 496-8699. For
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>programmatic and scientific concerns, contact the Program Director, Dr.
>Francis J. Mahoney, at (301)496-9360.
>
>INFORMATION In a continuing effort to provide exceptional customer
>service, the NCI Grants Administration Branch has set up a Feedback
>address on its web site (http://www.nci.nih.gov/admin/gab/index.htm).
>General concerns and issues related to NCI grants policies, procedures,
>and practices can be sent to the Customer Liaison using this feature.
>Specific questions or concerns related to this grant should be addressed
>to the Grants Management Specialist listed in the Terms of Award.
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>
>Jill Rogers, Grants Specialist
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>SPREADSHEET

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>GRANT NUMBER: 1 R01 CA78471-01A1
>P.I.: AHMED, MANSOOR M
>
>INSTITUTION: University of Kentucky Research Foundation
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	YEAR 01	YEAR 02	YEAR 03	YEAR 04
>Salaries and Wages	74,125	91,278	93,677	96,147
>Fringe Benefits	14,338	17,656	18,120	18,598
>Personnel Costs	88,463	108,934	111,797	114,745
>Supplies	18,900	19,467	20,051	20,652
>Travel Costs	900	927	955	984
>Other Costs	900	927	955	984
>TOTAL DC	109,163	130,255	133,758	137,365
>TOTAL F&A	49,669	59,266	60,860	62,501
>TOTAL COST	158,832	189,521	194,618	199,866

>

	YEAR 01	YEAR 02	YEAR 03	YEAR 04
>F&A Cost Rate 1	45.50%	45.50%	45.50%	45.50%
>F&A Cost Base 1	109,163	130,255	133,758	137,365
>F&A Costs 1	49,669	59,266	60,860	62,501

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