Regulation of Radiation-Induced Apoptosis by Early Growth Response-1 Gene in Solid Tumors

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Abstract: Ionizing radiation exposure is associated with activation of certain immediate-early genes that function as transcription factors. These include members of *jun* or *fos* and early growth response (EGR) gene families. In particular, the functional role of EGR-1 in radiation-induced signaling is pivotal since the promoter of EGR-1 contains radiation inducible CArG DNA sequences. The *Egr*-1 gene belongs to a family of *Egr* genes that includes EGR-1, EGR-2, EGR-3, EGR-4, EGR-α and the tumor suppressor, Wilms' tumor gene product, WT1. The *Egr*-1 gene product, EGR-1, is a nuclear protein that contains three zinc fingers of the C_2H_2 subtype. The EGR-1 GC-rich consensus target sequence, 5'-GCGT/GGGGCG-3' or 5'-TCCT/ACCTCCTCC-3', has been identifiedin the promoter regions of transcription factors, growth factors, receptors, cell cycle regulators and proapoptotic genes. The gene targets mediated by *Egr*-1 in response to ionizing radiation include TNF-α, p53, Rb and Bax, all these are effectors of apoptosis. Based on these targets, *Egr-1* is a pivotal gene that initiates early signal transduction events in response to ionizing radiation leading to either growth arrest or cell death in tumor cells. There are two potential application of *Egr*-1 gene in therapy of cancer. First, the *Egr*-1 promoter contains information for appropriate spatial and temporal expression *in-vivo* that can be regulated by ionizing radiation to control transcription of genes that have pro-apoptotic and suicidal function. Secondly, EGR-1 protein can eliminate 'induced-radiation resistance' by inhibiting the functions of radiation-induced pro-survival genes (NFκB activity and bcl-2 expression) and activate proapoptotic genes (such as bax) to confer a significant radio-sensitizing effect. Together, the review of reported findings demonstrate clearly that EGR-1 is an early central gene that confers radiation sensitivity and its pro-apoptotic functions are synergized by abrogation of induced radiation resistance.

Keywords: EGR-1, apoptosis, TNF-α, bax, p53, bcl-2, bax, NFκB and ionizing radiation

INTRODUCTION

Treatment strategies such as chemotherapy and radiation eliminate malignant cells by the induction of apoptosis as well as by "mitotic death". For example, therapeutic ionizing radiation can cause DNA strand breakage or distortion of the DNA nucleoprotein conformation which may transduce signals that result in activation of early response genes whose gene products may then stimulate later genes. These later genes (such as cross-point regulators of apoptosis) are important in cellular response to radiation injury (such as cell death). Therefore, it is to the advantage of tumor cells to acquire mutation and overexpression of these cellular genes that protect against these processes. The later genes that act as cross-point regulators of apoptosis are involved in the induction of apoptosis programs. These programs are tissue-specific and generally consist of proximal component that initiates the apoptosis signaling cascade. The proximal component includes pro-apoptotic genes such as Bax, Bad, Bak, Bcl-xs, Bid, Bik and Hrk [1,2]. These proximal component events are coupled to downstream effector component that includes caspases, ICE etc, activation of which leads to initiation of death signal causing proteolytic breakdown of the substrates of caspases and thereby changing the cell structure that is characteristic of apoptosis [3-5]. Thus, the phenotypic cell injury caused by ionizing radiation is transduced through early genes that signals proapoptotic genes and thereby activate the caspases leading to cell death. The apoptotic pathways consist of an early component that includes molecular events specific for an inducer or a group of inducers and of downstream effector components common to diverse apoptotic signals [3, 5, 6]. Apoptosis has been extensively reported in variety of experimental tumor systems following exposure to radiation [7-11]. Ionizing radiation alters the expression of specific genes, the products of which may contribute to the events leading to apoptotic cell death [9]. Ionizing radiation exposure is associated with activation of certain immediate-early genes that function as transcription factors [12]. These include members of *jun* or *fos* and early growth response (EGR) gene families [12, 13]. In particular, the functional role of EGR-1 in radiation-induced signaling is crucial since the promoter of EGR-1 contains radiation-inducible DNA sequences [14].

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THE *Egr*-1 GENE

The *Egr*-1 gene [15] belongs to a family of *Egr* genes [16] that includes EGR-2 [17], EGR-3 [18], EGR-4 [19], EGR-α [20] and the tumor suppressor, Wilms' tumor gene product, WT1 [21]. The *Egr* family shows high degree of homology in the amino acids constituting the zinc finger

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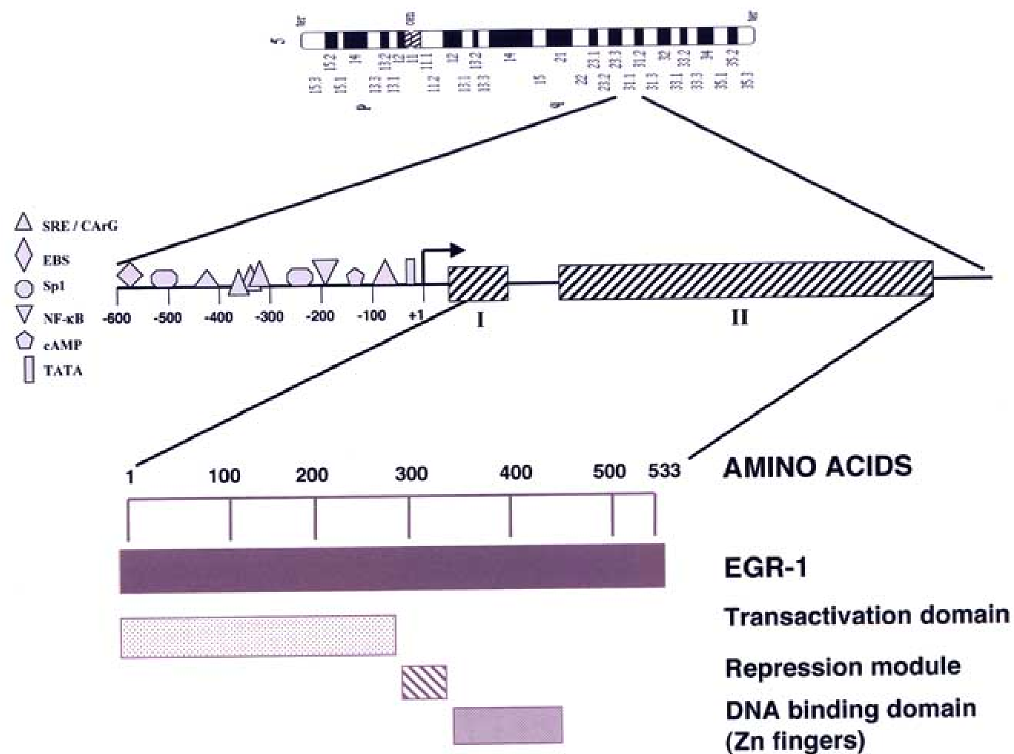

Fig. (1). Localization and gene structure of zinc finger transcription factor *Egr*-1. *Egr*-1 is localized in at 5q31 locus. The 5'-flanking promoter regulatory region consists of 600 bp that contains several serum-response elements (or CArG, radiation inducible sequences), cyclic AMP response element, binding sites for EGR-1, Sp1 and NFκB. The gene has two exons and one 3.6 kb length intron. The EGR-1 protein contains transactivation domain at amino-terminus and a DNA-binding domain consisting of three zinc finger motifs. in between the DNA binding domain and zinc fingers, there is repression domain and this functions as a binding site of transcriptional co-repressor proteins NAB1 and NAB2.

domain and binds to the same GC-rich consensus DNA sequence [22, 23]. The *Egr*-1 gene, as illustrated in Fig. (**1**), is located on 5q31, a region that is commonly deleted in myelodysplastic syndrome and acute myeloid leukemia [24]. The *Egr*-1 gene product, EGR-1, is a nuclear protein that contains three zinc fingers of the C_2H_2 subtype [25, 26]. Structure function mapping studies on EGR-1 protein suggest that the amino acids constituting the zinc finger motif confer DNA binding function, whereas the NH_2-terminus amino acids confer transactivation function [26, 27] [Fig. (**1**)]. Crystallography studies have confirmed the direct interaction between EGR-1 and its consensus target sequence and this occurs at 2.1 Å [28]. More recent studies have found that sequences diverging from the consensus may also bind EGR-1 [29, 30]; thus, having a broader spectrum of potential target genes. It is interesting to note that within this family of transcription factors, EGR-1 was found to be a positive activator of transcription, whereas WT1 is a transcriptional repressor, both acting via binding to the same GC-rich consensus sequence in reporter constructs [21, 31, 32]. Depending on the cell type, EGR-1 may behave as a positive or negative regulator of gene transcription [15, 33, 34]. Similarly, WT1 can also act as a transcriptional activator in cells lacking p53 protein [35].

These findings suggest that the cellular context determines the transcriptional regulatory functions of the EGR-family DNA binding proteins. The EGR-1 GC-rich consensus target sequence, 5'-GCGT/GGGGCG-3' or 5'-TCCT/ACCTCCTCC-3' [36-38], has been identified in the promoter regions of: (a) transcription factors, such as MYC and NUR77 [16]; (b) growth factors or their receptors, such as TGF-β1, TNF-α [39,40], PDGF-A and PDGF-B [41], IGF-II, βFGF, or EGF-receptor [15, 16, 42]; (c) cell cycle regulators such as the retinoblastoma susceptibility gene *Rb* [43], cyclin D1 [44] and c-Ki-ras [15]; and (d) thymidine kinase, an enzyme crucial in DNA biosynthesis [29]. A list of EGR-1 known and putative target genes are listed in previous reviews which include genes that has pro-survival (transformation or proliferation), pro-apoptotic, differentiation and angiogenesis functions [15, 16]. Between the activation and DNA-binding domains, an inhibitory

domain was identified. This domain functions as a binding site for two transcription co-factors known as NGFI-A binding proteins 1 and 2 (NAB 1 and NAB 2) and binding of these factors causes inhibition of *Egr*-1 biological activity [45-47].

REGULATION OF *Egr*-1 GENE

The promoter of *Egr*-1 is 600 bp in length [Fig. (**1**)] that contains 5 serum response elements (SREs), 2 Sp1 site, one NFκB site, one cyclic AMP response element and one EGR-1 binding site (EBS) [48-50]. Datta *et al.* reported that radiation activates the CArG boxes in the promoter, and thereby activates the transcription of EGR-1 [51]. Activation of CArG elements is mediated through reactive oxygen intermediates [14]. These CArG elements in the *Egr*-1 promoter are similar, but not identical, to SREs present in c-*fos* reporter that bind a ternary complex of transcription factors that include serum response factor, SRF accessory proteins (Sap1 and Sap2) and Elk 1. Based on the structural elements present in the promoter of *Egr*-1, synthesis of *Egr*-1 mRNA is induced by serum, platelet-derived growth factor or phorbol or phorbol 12-myristate 13-acetate and this induction was depended on the activation of the Ras-Raf-MEK-ERK signaling pathway [52]. Two types of signal does not stimulate *Egr*-1 promoter activity. The cyclic AMP response element (CRE) in the *Egr*-1 promoter does not function as an enhancing element as observed in other genes, since an activator of adenylate cyclase, forskolin, does not stimulate the *Egr*-1 promoter activity [53, 54]. From this, it is evident that CRE may regulate *Egr*-1 transcription via activation of p38/stressactivated protein kinase-2 mediated signaling [55]. The other repressor of *Egr*-1 transcription is EGR-1 protein that bind to its own promoter via the EBS sequence [56]. This action might constitute a negative feedback loop that allows only a transient but not a sustained induction of *Egr*-1.

To further understand the mechanisms by which the Egr-1 gene is regulated *in-vivo*, a recent study generated transgenic mice with a construct containing 1200 bp of the mouse Egr-1 promoter coupled to nuclear localized LacZ. The reporter gene expression was detected in subsets of endothelial cells, vascular smooth-muscle cells, cardiomyocytes, neurons, and hepatocytes. In addition, partial hepatectomy significantly induced reporter gene activity in regenerating livers. These observations strongly suggest that the *Egr*-1 promoter contains information for appropriate spatial and temporal expression *in-vivo* [57].

UTILIZATION OF *Egr*-1 PROMOTER ELEMENTS IN SPATIAL AND TEMPORAL CONTROL OF GENE EXPRESSION TO TARGET APOPTOSIS IN TUMOR CELLS

Control of gene transcription by ionizing radiation *in-vivo* represents a novel method of spatial and temporal regulation of gene-based medical treatments. Activation of transcription of the *Egr*-1 gene by X-rays is regulated through the CArG elements in the promoter region. Weichselbaum *et al.* [58] linked the radiation-inducible promoter region of the *Egr*-1 gene to the gene encoding the radiosensitizing and tumoricidal cytokine, TNF-α and a replication-deficient adenovirus was used to deliver the Egr-TNF construct to human tumors grown in nude mice. Combined treatment with Ad5.Egr-TNF and 5 Gy resulted in increased intratumoral TNF-α production and increased tumor control compared with treatment with Ad5.Egr-TNF alone or with radiation alone. The increase in tumor control was achieved without an increase in normal tissue damage when compared to tissue injury from radiation alone. In addition, combined Ad.Egr-TNF and radiation produced occlusion of tumor microvessels without significant normal tissue damage [59]. Based on pre-clinical studies [59-65], a phase I study is currently open to define the tolerability and biologic effects of TNFerade (a replication defective adenoviral vector containing TNF-α gene, regulated by the radiation sensitive promoter of Egr-1) in patients with advanced malignancies [66]. Two patients with chest wall masses were enrolled in the protocol comprising twice-weekly injections on weeks 1 and 2 followed by weekly injections thereafter till 6 weeks. Radiation therapy was given on week 2 and was continued maximum of 5 weeks. One patient completed full course of radiation therapy to two lesions (one injected with TNFerade and the other uninjected). Serial CAT scans revealed extensive necrosis in the injected lesion, however, the uninjected lesion shows minimal change from the baseline at the end of radiotherapy. PET scan post-therapy confirmed differential necrosis in the injected lesion. TNFerade was well tolerated in the first two patients. This study is currently open and is conducted at The Albert Einstein College of Medicine, New York, University of Kentucky, University of Chicago and University of South Florida [66].

ROLE OF RADIATION-INDUCED EGR-1 PROTEIN TRANSACTIVATION FUNCTION TO TARGET APOPTOSIS IN TUMOR CELLS

Egr-1 is a pivotal gene that initiates early signal transduction events in response to ionizing radiation leading to either growth arrest or cell death in tumor cells. This is because in most type of cells, ionizing radiation causes increased EGR-1 expression and the induced EGR-1 protein is a strong transcription activator of key genes involved in cell death pathway. To support some of the above facts, it has been documented that cell injury caused by cellular response to radiation leads to transient induction of *Egr-1* within 0.5 to 3 hours of exposure to X-rays in the absence of new protein synthesis [12]. When melanoma cells, A375-C6, were exposed to different doses of radiation, a dose-dependent increase in EGR-1 expression was observed as assessed by Western blot analysis, and this increase correlated with dose-dependent growth inhibition as judged by [^{3}H] thymidine-incorporation assay. Transfected A375-C6 cells stably expressing the dominant-negative mutant protein of EGR-1 showed significantly reduced (<50%) sensitivity to radiation-inducible growth inhibition and this resistance was found to be dose-dependent [67]. Functional studies in A375-C6 cells expressing the *Egr*-1 dominant-negative mutant, whose product competes functionally with EGR-1 [22], or an antisense *Egr*-1 oligomer, which blocks the expression of *Egr*-1 [67], suggested that EGR-1 function is required for the radiation-inducible growth inhibition. The inhibition of EGR-1 expression with the AS oligomer or

function with the dominant-negative mutant leads to partial, but not complete, radioresistance. Because in A375-C6 cells the dominant-negative mutant abrogates EGR-1-dependent transactivation substantially, but not completely [67], and because treatment with the AS oligomer does not result in complete ablation of endogenous EGR-1 levels [67], it is possible that incomplete radioresistance may, in part, be due to incomplete interference with endogenous EGR-1. In addition, however, there exists the possibility that the induction of other redundant growth inhibitory pathways by ionizing radiation may prevent complete radioresistance despite ablation of EGR-1. Thus, EGR-1 may control only a specific component of the radiosensitive pathways. These melanoma cells A375-C6 have an extra copy of the p53 gene with trisomy for chromosome 17 and that none of the three alleles of p53 contains mutations in exons 2 through 11.

Exposure to ionizing radiation led to an induction of p53 protein in the nucleus of A375-C6 vector and A375-C6 EGR-1 dominant-negative transfected cells. It is possible that induction of p53 protein may have rendered the parental cells radiosensitive, leading to growth inhibition and apoptosis. However, despite the induction of wild-type nuclear p53 protein, the transfected cells expressing the dominant-negative mutant of *Egr*-1 were resistant to ionizing radiation. These findings suggest that *Egr*-1 plays a key functional role in the growth inhibitory response to ionizing radiation.

Egr-1 AND TNF-α IN RADIATION-INDUCED APOPTOSIS

In p53 null prostate cancer cell line, PC-3, the genes encoding EGR-1 and TNF-α that induced apoptosis were up-regulated by ionizing radiation in the PC-3 cells, and inhibition of EGR-1 transactivation function by the dominant-negative WT1-EGR-1 chimera abrogated ionizing radiation-inducible TNF-α induction and apoptosis. Consistent with these observations, ectopically expressed EGR-1 enhanced ionizing radiation-inducible TNF-α expression and apoptosis [40]. These findings suggest that EGR-1 is an upstream modulator of TNF-α induction and apoptosis in the pathway evoked by ionizing radiation. Moreover, EGR-1 causes transcriptional activation of the TNF-α promoter via a consensus EGR-1- binding site providing a mechanism for EGR-1-inducible expression of the TNF-α gene [40]. Thus, EGR-1 is an important mediator of radiation responsiveness in prostate cancer cells that lack functional p53 protein. Both EGR-1 and TNF-α are induced by ionizing radiation in diverse tumor types, and future studies may design approaches to further exploit this novel pathway for the containment of radio-resistant tumors [40].

Wild-type *p53* has been shown to be functionally necessary for growth inhibition and apoptosis following exposure to ionizing radiation, and *p53* mutations have been reported to increase resistance to apoptosis [8]. On the other hand, EGR-1 is an important mediator of radiation responsiveness irrespective of p53 functional status [40,67]. It was found that EGR-1 protein transactivates the promoter of p53 gene and up-regulates p53 mRNA and protein levels in response to apoptotic stimuli [68]. In this study, the apoptotic stimulus was not ionizing radiation. Skepticisms arise if p53 is regulated by EGR-1 in response to radiation. In wild-type p53 A375-C6 melanoma cells expressing the dominant-negative mutant of EGR-1, exposure to ionizing radiation led to induction of p53 protein in the nucleus [67]. This observation is in contrast to the fact that EGR-1 directly regulates p53 gene, since induction of p53 protein was observed in response to radiation inspite of these cells expressing the dominant-negative mutant of EGR-1 protein. The other issue that might be of great concern is that EGR-1 protein might potentially drive the mutant p53 gene and this might contribute enhanced resistance to ionizing radiation-induced cell killing. These skepticisms underscore further studies to resolve the role of p53 regulation by EGR-1 in response to ionizing radiation.

REGULATION OF p53 AND pRB BY Egr-1 IN RADIATION-INDUCED APOPTOSIS

The requirement of EGR-1 protein function to confer radiation sensitivity was extensively studied using tumor cells† [40, 67]. Further, this mechanism was investigated in a normal cell background using isogenic normal primary culture cells derived from mouse embryonic fibroblasts (MEF) with varied genomic status for *Egr*-1 gene (cells with both intact *Egr*-1 alleles: *Egr*-$1^{+/+}$, cells with homozygous deletion of *Egr*-1 alleles: *Egr*-$1^{-/-}$ and heterozygous deletion of one *Egr*-1 allele: *Egr*-$1^{+/-}$). In this study [69], in contrast to *Egr*-$1^{+/+}$ MEF cells, *Egr*-$1^{-/-}$ MEF cells were significantly resistant to radiation-inducible apoptosis and showed no elevation of p53 protein after radiation. These findings indicate that radiation-induced EGR-1-mediated transactivation of downstream genes is essential for radiation sensitivity. Thus, in support of previous reports, this study demonstrated that EGR-1 is the upstream mediator for the initiation of the radiation-induced signaling cascade leading to cell death. The tumor suppressor gene *p53* is a central mediator of apoptotic pathways in diverse model systems [70-73]. The p53 protein can cause transcriptional up-regulation of a number of downstream genes, such as *mdm-2*, p21$^{waf1/cip1}$, *bax, fas/apo1*, insulin-like growth factor-binding protein-3, which are implicated in growth inhibition and apoptotic cell death [70-73]. Interestingly, the mRNA levels of *p53*, p21$^{waf1/cip1}$, *mdm-2,* and *bax* were elevated after irradiation in *Egr*-$1^{+/+}$ cells but not in *Egr*-$1^{-/-}$ cells [69]. In addition, the basal levels of p53 and its target genes were high in *Egr*-$1^{-/-}$ MEF cells when compared with *Egr*-$1^{+/+}$ cells. Loss of radiation-induced elevation of p53 may be attributed to the loss of *Egr*-1 mediated transregulation of p53 in *Egr*-$1^{-/-}$ MEF cells, and this may have led to the loss of up-regulation of *p53* target genes, p21$^{waf1/cip1}$, *mdm-2*, and *bax*. The most important observation in this study was that radiation caused degradation of p53 protein in *Egr*-$1^{-/-}$ cells, and this led to enhanced resistance to radiation-inducible apoptosis. Re-expression of EGR-1 protein by transient overexpression of EGR-1 protein in *Egr*-$1^{-/-}$ cells restored radiation sensitivity and stabilized the p53 protein

† Das, A.; Chendil, D.; Dey, S.; Mohiuddin, M.; Rangnekar, V.; Ahmed, M. M. in *91st Annual Meeting of American Association for Cancer Res. earch*; AACR: San Francisco, 2000; Vol. 41; Pp 546-546

levels. Thus, this observation suggests that EGR-1 protein is necessary for the up-regulation and the stability of p53 protein and radiation sensitivity. Moreover, radiation elevated the p53-CAT reporter activity in *Egr*-$1^{+/+}$ cells but not in *Egr*-$1^{-/-}$ cells. This finding supports a reported study that EGR-1 can directly bind with the *p53* promoter at two consensus EGR-1-binding sites and induce the p53 mRNA and protein [68]. Thus, *Egr*-1 is an important transregulator of p53. However, at this point we cannot rule out the possibility that other genes that are also regulated by *Egr*-1 may play a role in the stability of p53 protein. A marginal induction of radiation-induced apoptosis observed in $p53^{-/-}$/ CMV-EGR-1 MEF transfectants when compared with $p53^{+/+}$/CMV-EGR-1 MEF cells suggests that p53 played an important downstream role in regulation of *Egr*-1-mediated radiation-induced apoptosis. It also suggests that the absence of p53 may not contribute toward complete abrogation of EGR-1-mediated radiation-induced apoptosis. This is further supported by previous data that in p53 null prostate cancer cell line PC3, EGR-1 overexpression caused super induction of radiosensitivity [40]. However, the degree of induction of apoptosis was much higher in p53 null PC3/CMV-EGR-1 cells when compared with $p53^{-/-}$/CMV-EGR-1 MEF transfectant cells [69]. The difference may be due to the tumor cell background *versus* the normal cell background. Thus, in the absence of p53, EGR-1 may mediate the proapoptotic action of radiation via TNF-α [40] or other downstream cell-death effector genes. p53 is a known transactivator of MDM2, forming an autoregulatory loop between the expression and function of p53 and MDM2 [74]. It is also known that MDM2-p53 interaction can target p53 for degradation [75]. Rb can regulate the apoptotic function of p53 through binding to MDM2, thus preventing MDM2- mediated degradation of p53 [76]. Rb can also prevent MDM2 from inhibiting p53-mediated apoptosis. In addition, Rb can protect p53 from MDM2-mediated degradation by forming a trimeric complex with p53 via binding to MDM2 [76]. Since Rb regulates the apoptotic function of p53 by mitigating MDM2 mediated degradation [76] and the Rb gene promoter contains EGR-1-binding sites

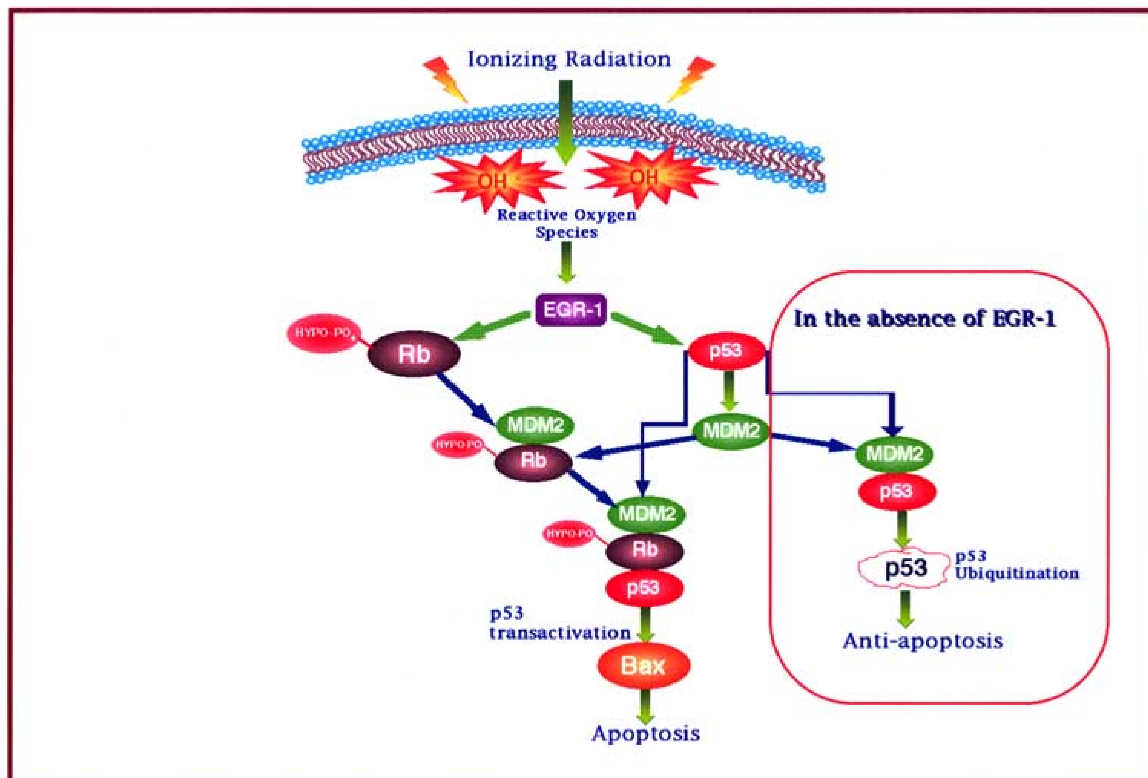

Fig. (2). A model for apoptosis by ionizing radiation. Ionizing radiation requires the induction of EGR-1 protein, that then elevates the expression of p53 protein and regulates the stability of p53 via transactivation of RB. In the absence of EGR-1, lack of Rb transactivation causes diminished RB binding to MDM2 and p53 is directly degraded by MDM2 leading to inactivation of the p53-mediated apoptotic pathway.

that conform to the GC-rich consensus [43], it might be speculated that there must be a pivotal role of Rb in regulating p53 degradation through *Egr*-1 transactivation function in response to radiation. Das *et al.* [69] found low expression levels of hypophosphorylated forms of Rb and decreased Rb-CAT reporter activity in *Egr*-$1^{-/-}$ MEF cells before and after irradiation when compared with *Egr*-$1^{+/+}$ MEF cells. Relatively higher levels of Rb-MDM2-bound complex and lower levels of p53-MDM2-bound complex were observed in irradiated *Egr*-$1^{+/+}$ MEF cells. In contrast, higher amounts of p53-MDM2 complex and low bound forms of the Rb-MDM2 complex were observed in *Egr*-$1^{-/-}$ cells. Lower amounts of the Rb-MDM2 complex along with higher amounts of p53-MDM2 in *Egr*-$1^{-/-}$ MEF cells might have contributed to p53 degradation after radiation [Fig. (**2**)]. Thus, apoptosis caused by ionizing radiation requires the induction of EGR-1 protein, which then transregulates the expression of p53 protein and also indirectly regulates the stability of p53 via Rb [69].

REGULATION OF BAX BY EGR-1 PROTEIN IN RADIATION-INDUCED APOPTOSIS

On further investigation of EGR-1 function in radiation-induced apoptosis, it was found that EGR-1 protein directly transactivates the bax promoter and enhances the radiation sensitivity. This was tested in two prostate cancer cell lines, p53 mutant DU-145[†] and wild-type p53 22Rv1[*]. Enhanced radiation sensitivity of DU145 or 22Rv1 cells expressing EGR-1 protein was associated with up-regulation of Bax at the RNA and protein level. Simultaneously, radiation caused down regulation of Bcl-2 in DU145/CMV-EGR-1 or 22Rv1/Ad-GFP-EGR1 cells when compared to unaltered levels of Bcl-2 in DU145/Vector or 22Rv1/Ad-GFP and DU145/CMV-WT1-EGR1 or 22Rv1/Ad-GFP-NAB1 cells. In addition, significant activation of Caspase-3 and Caspase-9 with increased PARP cleavage was observed in DU145/CMV-EGR-1 or 22Rv1/Ad-GFP-EGR1 cells when compared to DU145/Vector or 22Rv1/Ad-GFP and DU145/CMV-WT1-EGR1 or 22Rv1/Ad-GFP-NAB1 cells. Gel shift analysis and CAT reporter assay indicated that EGR-1 transactivates the promoter of the Bax gene that contains two overlapping GC-rich EGR-1 binding sites.

Promoter sequence analysis of Bax gene showed two potential overlapping Egr-1 binding site flanking from –21 to –13 (GCGGCGGCG) and –15 to –7 (GCGGGAGCG). When compared to normal consensus sequence of Egr-1 binding site, GCGGGGGCG, both the overlapping Egr-1 binding sequence in the Bax promoter has one base variation in the center of the consensus sequence. These sites are present just after CACGTG motifs which are potential binding sites for transcription factors that includes Myc and its homologs [77-79]. These findings establish that radiation-induced proapoptotic action of EGR-1, in a mutant p53 background, directly transactivates Bax that alters the Bcl-2: Bax ratio resulting in significant activation of caspases and induction of cell death pathway[†*]. Thus, these observations strongly indicate that Bax is target gene for Egr-1 in radiation-inducible apoptosis (Chendil and Ahmed, unpublished results).

ADENOVIRAL Egr-1 GENE THERAPY IS A POTENT SENSITIZER OF IONIZING RADIATION

Ectopic expression of EGR-1 significantly suppressed the cell growth and tumorigenicity in tumor cells that express low levels of EGR-1 [80, 81]. In-vitro studies demonstrated that ectopic expression of EGR-1 sensitized to the effects of radiation. To further evaluate these findings in in-vivo, the combined effect of radiation therapy and EGR-1 adenovirus therapy were tested on the growth of prostate xenograft tumors in nude mouse model using PC-3, a p53 null prostate tumor cell line. Five groups were designed for the following treatment regimen: Group I: Ad/GFP-EGR-1 injected xenografts treated with total dose of 20 Gy irradiation; Group II: Ad/GFP injected xenografts treated with total dose of 20 Gy irradiation; Group III: Ad/GFP-EGR-1 injected xenografts only; Group IV: Ad/GFP injected xenografts only and; Group V: parental xenografts only with no treatments. Ad/GFP or Ad/GFP-EGR-1 constructs were injected into the tumor at a dose of 100 MOI (3×10^8 PFU) at the first day of treatment, followed by 2 Gy radiation dose per day to the tumor for 5 days. On 7th and 8th days, the animals received two more injections of 100 MOI of Ad/GFP or Ad/GFP-EGR-1 constructs into the tumor, and they were subsequently treated with 2 Gy dose of radiation per day for 5 days. On the 14th day of treatment, the animals were injected with last intra-tumoral dose of 100 MOI of Ad/GFP or Ad/GFP-EGR-1 adenovirus and were observed for the following two weeks. No differences were observed between Group V and IV, suggesting that Ad/GFP alone did not exert any tumor regression effect. However, when compared to Group IV and V, Group II and Group III showed modest regression but was not significant suggesting that radiation or Ad/GFP-EGR-1 alone conferred modest tumor regression. Interestingly, when Ad/GFP-EGR-1 was combined with radiation, a significant enhancement in tumor regression was observed till 26th day ($p<0.0001$). Thus, the results of this study demonstrated that EGR-1 in combination with radiation significantly regressed the PC-3 xenografts. These findings imply similar results as observed in our reported *in-vitro* studies, strongly suggesting that EGR-1 protein is a pro-apoptotic sensitizer of radiation in prostate cancer (Dimova and Ahmed, unpublished results).

LACK OF Egr-1 FUNCTION CORRELATES RADIATION RESISTANCE IN SOLID TUMORS

Expression of EGR-1 in tumors is heterogeneous. Gliomas [82], lung [83-85], hepatocellular and esophageal carcinomas [81, 86] show reduced or lack of EGR-1 expression. On the other hand, prostate tumors show high incidence of EGR-1 overexpression at the mRNA and

[*] Chendil, D.; Sathiskumar, S.; Mohiuddin, M.; Ahmed, M. M. in *93rd Annual Conference of American Association for Cancer Research*: San Francisco, CA, 2002.

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protein level [87, 88]. Our recent study in androgen independent prostate cancer cell line DU-145, showed three alleles of *Egr*-1 gene with elevated basal levels when compared to other prostate cancer cell lines PC-3 and LNCaP†. No evidence of mutation was detected in the coding region of *Egr*-1 gene in DU-145 cells and interestingly the endogenous EGR-1 protein showed low transactivation ability in response to radiation†. DU-145 cells were highly resistant to radiation-induced apoptosis. In prostate tumor samples, we found a similar situation in which most tumors showed elevated levels of EGR-1 expression and this expression correlated with treatment failure or recurrence as demonstrated in post-irradiated biopsies. Thus, the presence of EGR-1 overexpression 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 is 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. Or, if *Egr*-1 gene is amplified and lacks its transcriptional activity (as seen in DU-145) and this will eventually shut the *Egr*-1 mediated pro-apoptotic signaling leading to enhanced resistance to apoptosis. In conclusion, based on the small number of samples studied, EGR-1 overexpression (potentially in mutant form or in amplified form or loss of *Egr*- 1 transcriptional activity) may provide an indication of clinical failure [89].

CONCLUSION

Activation of transcription of the *Egr*-1 gene is regulated through the CArG elements in the promoter region. In most type of cells, the induced EGR-1 protein is a strong transcription activator of key genes involved in cell death pathway. Thus, *Egr-1* is a pivotal gene that initiates early signal transduction events in response to ionizing radiation leading to either growth arrest or cell death in tumor cells. Signaling gene targets mediated by *Egr*-1 in response to ionizing radiation include TNF-α, p53, Rb and Bax, all these are effectors of apoptosis. It is known that EGR-1 protein phosphorylates to confer DNA binding function [25]. However, it is not clear whether the phosphorylation sites in EGR-1 protein differ in response to ionizing radiation and if it is true, whether specific phosphorylation site dictate specific target gene. There are two potential application of *Egr*-1 gene in therapy of cancer. First, the *Egr*-1 promoter contains information for appropriate spatial and temporal expression *in-vivo* that can be regulated by ionizing radiation to control transcription of genes that have pro-apoptotic and suicidal function and this represents a novel method of gene-based medical treatments through *Egr*-1. Secondly, EGR-1 protein can eliminate 'induced-radiation resistance' by inhibiting the functions of radiation-induced pro-survival genes and confer a significant radio-sensitizing effect. For example, more often in p53 mutant background, radiation-induces TNF-α which then upregulates the NF-κB-dependent bcl-2 expression. Induction of these pro-survival and anti-apoptotic genes indicate that tumor cells harbor a tight regulatory loop that operates to prevent the cell killing effects of ionizing radiation [90]. Together, this signaling constitutes 'induced-radiation resistance'. Ectopic or wild-type EGR-1 function will effectively eliminate the 'induced-radiation resistance' signaling by (i) inhibiting the NF-κB activity through direct EGR-1 protein binding to p65 sub-unit protein [91]; (ii) indirectly inhibit Bcl-2 expression [92]; (iii) by activating pro-apoptotic genes such as bax†, par-4† and TNF-α [40]; and (iv) further down-regulating TNF-α induced NF-κB activity through EGR-1 induced par-

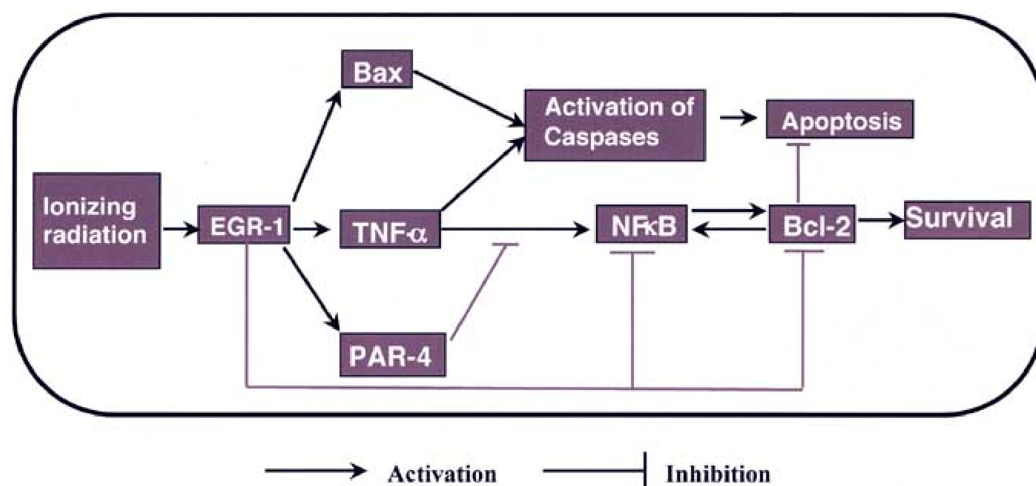

Fig. (3). Abrogation of induced radiation resistance by EGR-1. To evade from the killing effects of radiation, tumor cells that lack p53 function show induction of pro-survival factors such as NFκB and bcl-2 expression in response to ionizing radiation. Radiation induced EGR-1 directly inhibits NFκB or indirectly inhibits TNF-α induced NFκB via pro-apoptotic gene Par-4 resulting in diminished bcl-2 expression. Simultaneously, EGR-1 induces bax and TNF-α, both of these signaling direct towards the execution of downstream effector cell death signaling.

4 [90] [Fig. (**3**)]. Together, these studies demonstrate clearly that EGR-1 is a pivotal gene that confers radiation sensitivity and its pro-apoptotic functions are synergized by abrogation of induced radiation resistance.

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