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Response of *Cyclin B1* to Ionizing Radiation: Regulation by NF- κ B and Mitochondrial Antioxidant Enzyme MnSOD

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Abstract

Background—To understand the molecular response of tumor cells to therapeutic ionizing radiation (IR), we previously reported that human breast cancer cells derived following chronic exposure to fractionated ionizing radiation (MCF+FIR) showed a transient radioresistance. MCF+FIR cells also demonstrated increased activity of NF- κ B, increased expression of the mitochondrial antioxidant enzyme (MnSOD), and increased expression of a cell cycle regulatory protein (Cyclin B1). The present studies were designed to determine the relationship of NF- κ B, MnSOD and Cyclin B1 expression in cellular adaptive responses to ionizing radiation.

Materials and Methods—The first intron of the cyclin B1 gene with a putative NF- κ B element was cloned into the pGL3 luciferase reporter (pGL3CB1E11). pGL3CB1E11 and control NF- κ B luciferase activities were determined in MCF-7 and MCF+FIR cells treated with a single dose of radiation, over expression of the dominant negative mutant I κ B (mI κ B) or over expression of the SOD2 gene.

Results—MCF+FIR cells derived from fractionated IR demonstrated increased transactivation of the pGL3CB1E11 and NF- κ B controlled reporter activities, relative to the parental cell line. Transfection of dominant negative mutant I κ B that inhibits NF- κ B nuclear translocation, inhibited pGL3CB1E11 and NF- κ B activity, indicating the NF- κ B dependence of pGL3CB1E11 mediated transcription. In addition, over expression of the human SOD2 gene (MnSOD) inhibited NF- κ B and pGL3CB1E11 activity, indicating that superoxide or some species derived from superoxide may have participated in the up-regulation of reporter activity in response to chronic exposure to fractionated ionizing radiation. These results provide evidence suggesting that a signaling pathway involving NF- κ B and Cyclin B1 may contribute to adaptive radioresistance induced by chronic exposure to fractionated IR and support the conclusion that MnSOD appears to be a negative regulator of this pathway.

Keywords

Cyclin B1; ionizing radiation; NF- κ B; mitochondrial antioxidant enzyme; MnSOD

Mammalian cells treated with ionizing radiation (IR) induce a stress response associated with an enhanced tolerance to the subsequent cytotoxicity of radiation (1-3). This adaptive cell phenotypic alteration is originally observed in cells pre-exposed to a low or very low dose of IR (4-7). Tumor cells treated *in vitro* with relatively high doses of IR, *e.g.*, fractionated ionizing radiation (FIR) used for clinical radiotherapy, also demonstrate a transient radioresistant phenotype (8-11). The molecular mechanisms responsible for IR induced radioresistance remain to be elucidated. Accumulating gene profiling data have demonstrated that exposure to IR is able to activate the transcription of many stress genes, and a small fraction of IR inducible proteins, *i.e.*, transcription factors and elements signaling antiapoptosis and cell cycle control, are believed to be required for increasing cell survival after a lethal dose of IR (11-13). Using chronic exposure of FIR, we have described a gene expression profile altered in breast cancer MCF-7 cells after radiation (9-11). A group of stress responsive elements, *i.e.*, transcription factor NF- κ B, mitochondrial antioxidant enzyme manganese-containing superoxide dismutase (MnSOD) and cell cycle protein Cyclin B1 are activated in the radiation-adapted radioresistance (11, 14).

Cyclin B1 has been shown to be an essential cell cycle component required for the process of transition going from G2- to M-phases (15-18). Cyclin B1 and the phosphorylated Cdc2 are able to form the complex with 14-3-3 proteins (19) that accelerates Cyclin B1/Cdc2 translocation into nucleus and cell cycle regulation. Cyclin B1 has been reported to increase when G2 delay is extended (20) and G2 delay is decreased when Cyclin B1 is over expressed by gene transfection (17). Clinically, Cyclin B1 is found to be associated with the radioresistance observed in patients with squamous cell carcinoma (21, 22) and regional recurrence of head and neck tumors treated by radiotherapy (23). cDNA microarray profiles and Western blotting have shown that Cyclin B1 is activated in human breast cancer MCF-7 cells exposed to fractionated ionizing radiation (9, 10) and in MCF-7 cells stably transfected with human *SOD2* gene that encode MnSOD (11). Antisense blocking of MnSOD or Cyclin B1 expression has been shown to increase the radiosensitivity of radiation-derived MCF +FIR cells (9). However, the mechanisms of *cyclin B1* activation in radiation-adapted radioresistance have not been identified.

NF- κ B regulates a great variety of genes involved in signaling different stress responses (24) and NF- κ B itself has been shown to be sensitive to IR-induced cell damage (25, 26). IR induces IKK, the protein kinase that phosphorylates I κ B- α at Ser-32 and Ser-36, which accelerates activation and nuclear translocation of NF- κ B (25). After activation, a major function of NF- κ B appears to be signaling the antiapoptotic responses in cells exposed to IR (13, 26-28). Inhibition of NF- κ B activation is able to increase apoptosis and radiosensitivity (9, 10, 26, 29-31), and activation of NF- κ B is found causally related to the adaptive resistance to IR in human breast carcinoma MCF-7 cells (9, 11) and human keratinocytes transformed by papilloma virus (10). Blocking NF- κ B by over expressing the mutant form of I κ B that reduces NF- κ B translocation to the nucleus decreases cell survival and Cyclin

B1 expression (11). These results suggest that determining mechanisms of radiation-induced Cyclin B1 expression may be important to understanding adaptive responses to IR.

Genotoxic stress often requires the co-operation of several signaling pathways. This study was designed to answer the question of whether radiation-induced Cyclin B1 expression is regulated at the level of transcription by NF- κ B and MnSOD as well as determining the relevance of this pathway to IR-induced adaptive responses. An intronic NF- κ B element but not the NF- κ B elements in the 5' and 3' flanking regions has been identified for an active induction of the human *SOD2* gene (32). Therefore, NF- κ B of the intronic region of IR inducible genes may play an important role in gene expression. The present study addresses the possibility that the first intron of *cyclin B1* with a NF- κ B element is regulated by radiation *via* NF- κ B and mitochondrial antioxidant MnSOD that is also induced by radiation. Using a specific luciferase reporter controlled by the first intron of *cyclin B1* isolated from human breast cancer MCF-7 cells and NF- κ B reporters, we demonstrate here that radiation-induced NF- κ B is responsible for the transactivation controlled by the first intron of *cyclin B1* and mitochondria MnSOD appears to function as a negative control for NF- κ B-mediated gene regulation, suggesting a role for superoxide in this process.

Materials and Methods

Plasmid construction

Cyclin B1 1st intron was amplified from genomic DNA from MCF-7 cells by two-step PCR using the following primers: 5'-TGCGGGGTTTAAATCTGAGGCTAGG-3' and 5'-TAGGCATTTTGGCCTGCAGTTG-3' for 1st step, 5'-TGTTGGTTTCTGCTGGTTGTAGGTC-3' and 5'-CTTTGCGCC TGCCATGTTGATCTT-3' for 2nd step. PCR products were separated by agarose electrophoresis, and the DNA fragment was recovered from a gel to construct the Cyclin B1 first intron controlled luciferase reporter pGL3CB1E11. The enzyme site attached DNA fragment by PCR was cloned into pGL3E luciferase assay vector (Clontech Laboratories Co., Palo Alto, CA, USA). The NF- κ B luciferase reporters were the same as described before (11).

Cell culture and gene transfection

MCF-7 human breast cancer cell line was obtained originally from the American Type Culture Collection (Manassas, VA, USA). MCF+FIR cells were established as described before (11). All the wild-type and transfected cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-bovine serum. For gene transfection, cells were plated at a density of 2×10^5 cells in a 12-well plate for overnight culture, and the cells were transfected with 1 mg of experimental plasmids by LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were exposed to a single-dose IR and further incubated for 24 h. The transfected cells were washed with ice-cold PBS and lysed in a passive lysis buffer (Promega, Madison, WI, USA) for measuring luciferase activity by Luciferase Reporter Assay System (Promega) or in protein extraction buffer for Western blotting. The samples of each well were analyzed for

protein concentration using the BCA protein assay (PIERCE, Rockford, IL, USA) for equal loading controls.

Ionizing radiation

The wild-type MCF-7 and gene-transfected cell lines were maintained in a humidified incubator (5% CO₂) at 37°C. Ionizing radiation was performed at room temperature by using Cs-137 mark I irradiator (Dose rate 436 cGy/min; J.L. Shepherd & Associates).

Western blotting

MCF-7, MCF+FIR, and mutant IκB transfected cells were collected from T-25 flasks, washed with cold PBS, and lysed in protein extraction buffer. Protein concentrations were determined using the BCA protein assay kit (PIERCE) and equal amount of proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Millipore, Rockford, IL, USA). Proteins were detected using antibody against each proteins and visualized by ECL Western blotting detection system (Amersham Biotech, Piscataway, NJ, USA).

Results and Discussion

Activation of transcription factor NF- κ B, cell cycle regulating protein Cyclin B1, and the mitochondrial antioxidant enzyme MnSOD, as well as other factors appear to be required for signaling a prosurvival pathway in irradiated cells (9-11, 23, 33, 34). Our prior results demonstrate that Cyclin B1 is strongly inducible in breast cancer MCF-7 cells that showed adaptive radioresistance after chronic exposure to fractionated IR (9, 11). Furthermore, blocking Cyclin B1 by antisense transfection increases MCF-7 radiosensitivity (9, 11). Interestingly, Cyclin B1 has been found to be casually related to the radioresistance in head and neck cancers that show a persistent radioresistance following clinical radiotherapy (23). The present study provides evidence that radioresistant MCF+FIR cells derived following exposure to fractionated IR show increases in Cyclin B1 expression. Comparing to NF- κ B controlled reporters, luciferase reporters pGL3CB1EI1 controlled by the first intron of *cyclin B1* that contains a potential NF- κ B element is found sensitive to radiation in wild-type MCF-7 and MCF+FIR cells. Transactivation of pGL3CB1EI1 and NF- κ B reporters are inhibited by overexpression of mutant IκB, suggesting NF- κ B dependence of *cyclin B1* induction. Co-transfection of pGL3CB1EI1 with MnSOD expression vectors show a negative control on NF- κ B and pGL3CB1EI1-mediated transcription. Overall, these results suggest that a specific signaling network involving NF- κ B, Cyclin B1 and MnSOD contribute to radioresistance in breast cancer cells treated with fractionated ionizing radiation.

Construction of cyclin B1 1st intron reporters

The promoter and intron regions of human *cyclin B1* gene (GenBank No. M25753) was analyzed and computational search for transcription factor-binding sites revealed that, except the NF- κ B binding sites located in the promoter region, a putative NF- κ B site was identified in the first intron as shown in Figure 1. Published work that intronic but not promoter NF- κ B elements are responsible for MnSOD induction encourage us to focus on the function of the first intron of *cyclin B1* that may play a potential role in radiation-

induced *cyclin B1* expression. In order to elucidate the mechanism underlying NF- κ B-mediated regulation, we generated the luciferase reporter pGLCB1E11 with PCR fragment of the whole first intron that contains the NF- κ B site, as shown in Figure 1. Co-transfection experiments were performed in parental MCF-7 and radiation-adapted MCF+FIR cells to determine the pGLCB1E11 luciferase response with the wild-type NF- κ B controlled reporters.

Induction of Cyclin B1 expression by radiation and TNF- α

Currently, specific genes causing radiation adaptive resistance have not been fully identified. Accumulating results, especially gene microarray profiles, suggest a group of IR-induced cell cycle elements may decide the fate of irradiated cells (35, 36). We have shown that Cyclin B1 expression was induced following multiple doses of IR in human breast cancer MCF-7 cells that demonstrated radioresistant phenotype (9). The function of Cyclin B1 in the signaling radioresistant phenotype is further evidenced by the facts that radiation-induced adaptive radioresistance is reduced by antisense blocking Cyclin B1 expression (9) and that Cyclin B1 is activated in radioresistant tumors (23). We hypothesized that IR-induced adaptive radioresistance is mediated by induction of Cyclin B1 that is due to activation of NF- κ B. Following this reasoning, we analyzed the induction of Cyclin B1 in wild-type MCF-7 cells treated with TNF- α that induces NF- κ B activation as a positive control. Figure 2 shows that a single dose of 5 Gy increases Cyclin B1 expression, and the basal Cyclin B1 protein level is elevated in the multiple dose-adapted radioresistant MCF+FIR cells that showed a further induction of Cyclin B1 after a single dose 5 Gy IR (Figure 2 A). In agreement with IR induction, Cyclin B1 is also induced by exposure to TNF- α (Figure 2B). Since TNF- α has been well documented to be an inducer of NF- κ B, these results suggest, but do not prove, that IR-induced Cyclin B1 expression is a consequence of NF- κ B induction.

Transcription controlled by the first intron of Cyclin B1 was paralleled by NF- κ B-mediated transactivation by IR

To get insights of transcriptional activation of *cyclin B1*, Figure 3 further demonstrates that luciferase expression controlled by the first intron of Cyclin B1 (pGLCB1E11) or a wild-type NF- κ B is similarly induced by radiation in parental MCF-7 as well as the IR-adapted MCF+FIR cells (Figure 3A). In addition, MCF+FIR cells demonstrated an increased basal level of NF- κ B and pGKCB1E11-mediated luciferase activity that was increased by 30% and 90%, respectively, for pGLCB1E11 and NF- κ B-controlled luciferase activities compared to the level of IR-treated wild-type MCF-7 cells. Exposure to a single dose of 5 Gy IR further enhanced NF- κ B and pGLCB1E11 activity in MCF+FIR cells (~35%; Figure 3). The present results provide the first evidence suggesting that IR-induced NF- κ B activation is responsible for Cyclin B1 expression and this signaling pathway may play an important role in long-term radiation induced radioresistance.

Blocking NF- κ B inhibited pGLCB1E11 transcription

Next, we determined if over expression of the dominant negative mutant I κ B (mI κ B) that inhibits NF- κ B nuclear translocation inhibits pGLCB1E11-controlled luciferase

transcription. Experiments in Figure 4 indicate that over expression of mI κ B that was co-transfected with luciferase NF- $\hat{\kappa}$ B or pGL3ECCNB1, inhibited the basal and IR-induced NF- $\hat{\kappa}$ B as well as pGLCB1E11-controlled luciferase activity (Figure 4A and 4B). mI κ B-mediated inhibition of luciferase activity by pGLCB1E11 was found to be consistent with that of NF- $\hat{\kappa}$ B inhibition by mI κ B. As a control, over expression of mI κ B also inhibited TNF- α induced NF- $\hat{\kappa}$ B and pGLCB1E11 luciferase activity. These results clearly support the hypothesis that NF- $\hat{\kappa}$ B is involved in radiation-induced increases in Cyclin B1 expression.

Over expression of human SOD2 genes inhibited NF- $\hat{\kappa}$ B and pGLCB1E11-controlled transcription

Another critical feature of adaptive radioresistance in tumor cells is the finding that mitochondrial antioxidant MnSOD, that is low or absent in many human cancer cells (37), appears to be sensitive to radiation and involved in signaling pro-survival (11). MnSOD has been shown to regulate several redox sensitive transcription factors including NF- $\hat{\kappa}$ B (38) and *Cyclin B1* is found increased in MCF-7 cells transfected with SOD2 gene (MCF+SOD) (11). Therefore, the question of whether MnSOD up- or down-regulates *cyclin B1* is to be answered. To clarify the relationship between MnSOD expression and NF- $\hat{\kappa}$ B-mediated cyclin B1 induction, we analyzed the luciferase reporters controlled by pGLCB1E11 or NF- $\hat{\kappa}$ B under the condition of over expression of human *SOD2* gene. As shown in Figure 5A, by transient transfection of SOD2 plasmids, MnSOD protein levels showed a dose-dependently increase with the amount of *SOD2* DNA transfected (Figure 5A). However, a significant inhibition was induced in cells cotransfected with NF- $\hat{\kappa}$ B (Figure 5B) or pGLCB1E11 (Figure 5C) luciferase reporters with MnSOD expression vectors compared to the control of empty vectors. Together, these results suggest that although NF- $\hat{\kappa}$ B, Cyclin B1 and MnSOD are induced by IR, overexpression of MnSOD functions as a negative regulator NF- $\hat{\kappa}$ B-Cyclin B1 pathway. Since MnSOD is only active after translocation to mitochondria, these results suggest that alterations in the NF- $\hat{\kappa}$ B-Cyclin B1 pathway may be mediated by mitochondrial superoxide in cells exposed to fractionated ionizing radiation.

Conclusion

NF- $\hat{\kappa}$ B is sensitive to redox alterations (39, 40) and both Cyclin B1 and the mitochondrial antioxidant enzyme MnSOD have been shown to be induced by radiation *via* NF- $\hat{\kappa}$ B regulation (11). Our present data demonstrate that Cyclin B1 expression is increased *via* NF- $\hat{\kappa}$ B activation in MCF-7 cells treated with fractionated ionizing radiation. Over expression of MnSOD demonstrated a negative control on NF- $\hat{\kappa}$ B and Cyclin B1 expression. Overall, these results suggest that regulation of the NF- $\hat{\kappa}$ B-Cyclin B1 pathway in cells exposed to fractionated IR may be involved with adaptive radioresistance and mediated, at least in part, by alterations in mitochondrial superoxide production.

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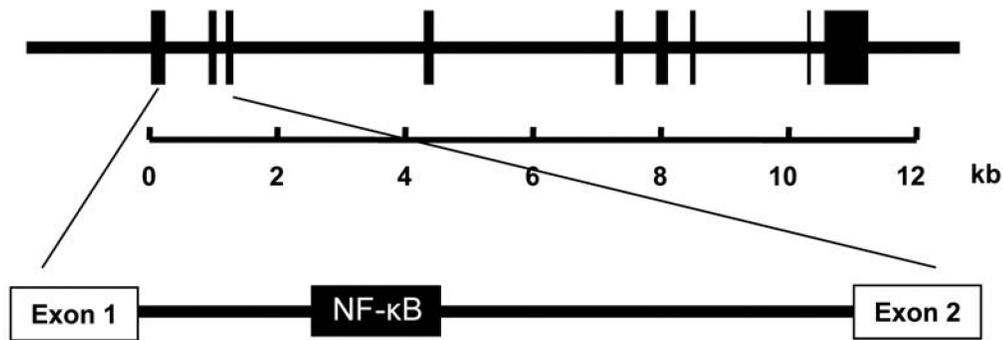
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Cyclin B1



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10      20      30      40      50      60      70      80      90      100
ACGAACAGGCCAAT AAGGAGGGAGCAGT CGGGGTTTAAATCTGAGGCTAGGCTGCTCTTCTCGGCGTGCTGCGGCGAAOCGCTGTTGGTTTTCTGCTG
                                CB1F1*
110     120     130     140     150     160     170     180     190     200
GGTGTAGGTCCTTGCTGGT CGGGCTCGGGTGTCTGCTTCTCCCGCTGAGCTGCTGCCTGGTGAAGAGGAAGCCATGGCGCTCCGAGTCAOCAGGGT
                                M A L R V T R
210     220     230     240     250     260     270     280     290     300
GAGCGCTTCGGACTGCGAACTAACGGGCTTCTTAGCTGCTGCTCTCCCTGCCTCGGCTGCGGAGCCTCCGAGCGGGAGGGCCGACAGGAG
310     320     330     340     350     360     370     380     390     300
CGATTTGGGGAGGAAGGTGGGAGGGACTCACCAAGAGAGCGCCGAGGTGGGCCAGGCGCTGGTGAAGAGTGTGGGGACGATGGATGGAGGGGAAG
                                NF-κB
410     420     430     440     450     460     470     480     490     500
GTGAGAAAGAGAACTGGACGGATATTGGATAAATGTTTTGGGAGGTGGAGAGTGGACTGGGAACCTTTTGAAAAAGTGATAGAGGGTCCCTGAGTGGCC
510     520     530     540     550     560     570     580     590     600
CCGCCAGCAACTCTGTAAACCCCTTCCAGAGAGAGGTGTCTGCAATTGGAGGCTTTTTCGGTTTCTTTCAAATGTAATCTCGGTATTTTAGGCT
610     620     630     640     650     660     670     680     690     700
GGCCAGGACTAATCAGGAATCCCTCAATTGGTAAATGTAGAGGTGGGCGAAACTGACTTGTCAAGCCCGCAGAGTAGACTCTGGACCCATGTTTTCC
710     720     730     740     750     760     770     780     790     800
CTCGGAACCCATTTTAGTCGGCTTTCTTTCTGGGAATTCTCCTTGTGCCCCACCTTAATTAACCCCTTGACTTACTCGAGCCCTCGTGATCAGCTCTT
810     820     830     840     850     860     870     880     890     900
AAAGTGGTCTTGCTTCTTT CAGAACTCGAAAATTAAATGCTGAAAATAAGCCGAAGATCAACATGGCAGGCCAAAAGCGGTTCTACGGCCCTGCTGCA
                                N S K I N A E N K A K I N M A G A K R V P T A P A A
                                • CB1R2
910     920     930     940     950     960     970     980     990     1000
ACCTCAAGCCCGGACTGAGGCCAAGAACAGCTCTTGGGACATTGGTAAACAAAGTCAGTGAACAACCTGCAGGCCAAAATGCCTATGAAGAAG
T S K P G L R P R T A L G D I G N K V S E Q L Q A K M P M K K
                                • CB1R1

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Figure 1. A putative binding site of NF- κ B of cyclin B1 first intron. A putative NF- κ B binding site was located in the first intron of human cyclin B1 gene (GenBank #M25753). The marked regions (CB1F2 and CB1R2) were designed to produce fragment of cDNA from human breast cancer MCF-7 cells used for construction of cyclin B1-first-intron luciferase reporters (pGL3CB1E1).

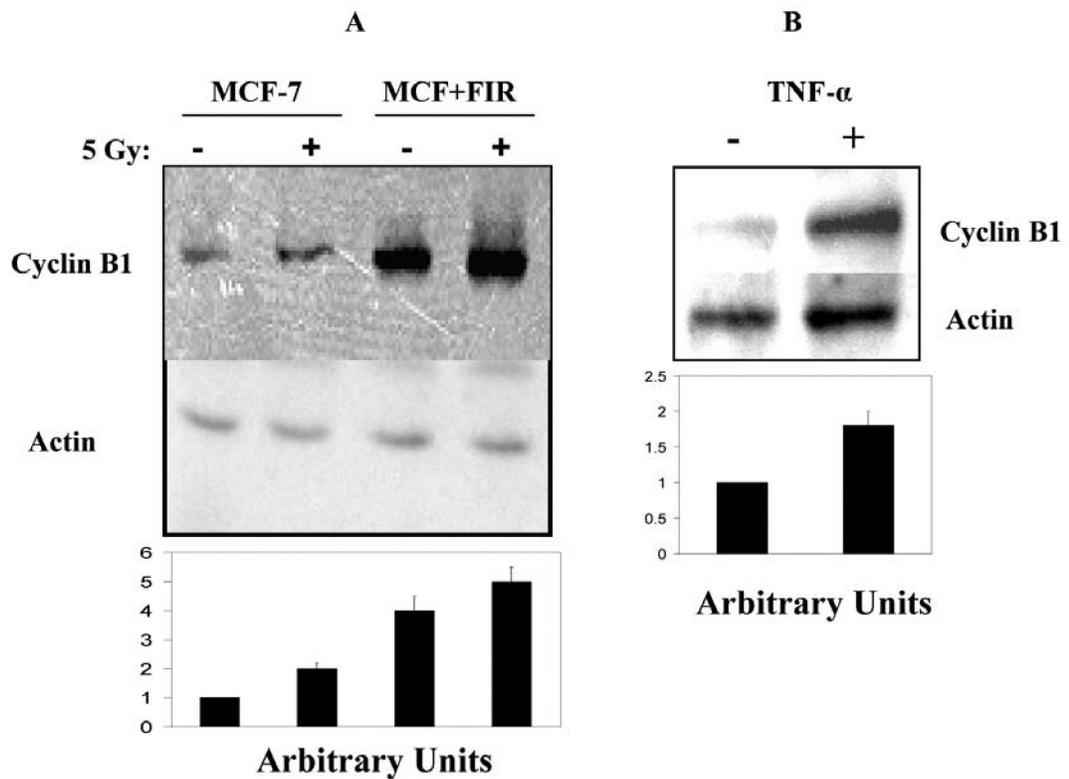


Figure 2.

A. Cyclin B1 expression was induced by IR and TNF- α in wild-type MCF-7 and radiation-treated MCF-7 cells MCF+FIR. Wild-type MCF-7 and radiation-treated radioresistant MCF+FIR cell populations were exposed to a single dose 5 Gy IR and cyclin B1 expression levels were measured with Western blot 24 h after radiation. B. Cyclin B1 expression was induced in MCF-7 cells by TNF- α . Wild-type MCF-7 and radiation-treated radioresistant MCF+FIR cells were exposed to TNF- α (1 ng/ml for 24 h) and cyclin B1 expression levels were measured with Western blot 24 h after exposure to TNF- α .

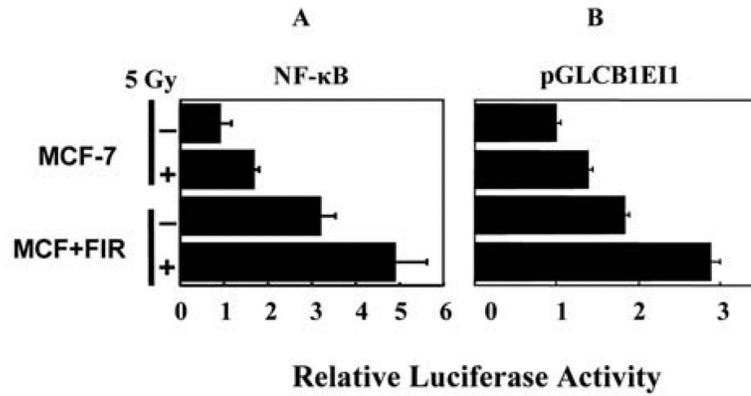


Figure 3.

NF- κ B and pGL3CB1EI1 controlled luciferase reporters were highly activated in MCF+FIR cells. Wild-type MCF-7 and radiation-treated radioresistant MCF+FIR cells were transfected with NF- κ B or pGL3CB1EI1 reporters for 6 h and cultured for 18 h before irradiation with a single dose of 5 Gy. Luciferase activities were measured 24 h after radiation and normalized to the value of basal MCF-7 cells without radiation (mean \pm S.D., n =3).

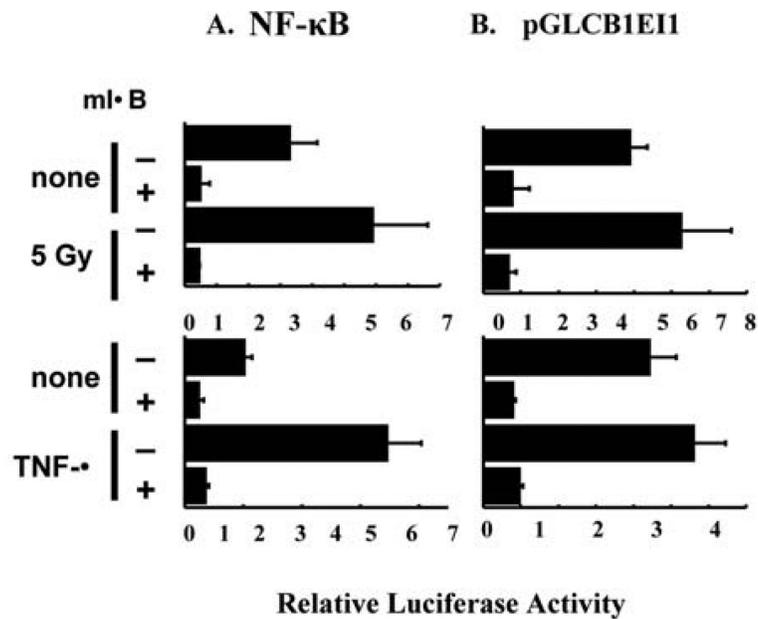


Figure 4. Over expression of dominant negative mutant IκB inhibited basal and IR-induced NF- κ B and pGLCB1EI1 luciferase activities. Luciferase reporters of NF- κ B or pGL3CB1EI1 were co-transfected respectively with 0.5 mg of dominant negative mutant IκB expression vector in MCF-7 cells, and luciferase activities were measured 24 h after 5 Gy IR or exposure to 1 ng/ml TNF- α (mean \pm S.D., n=4).

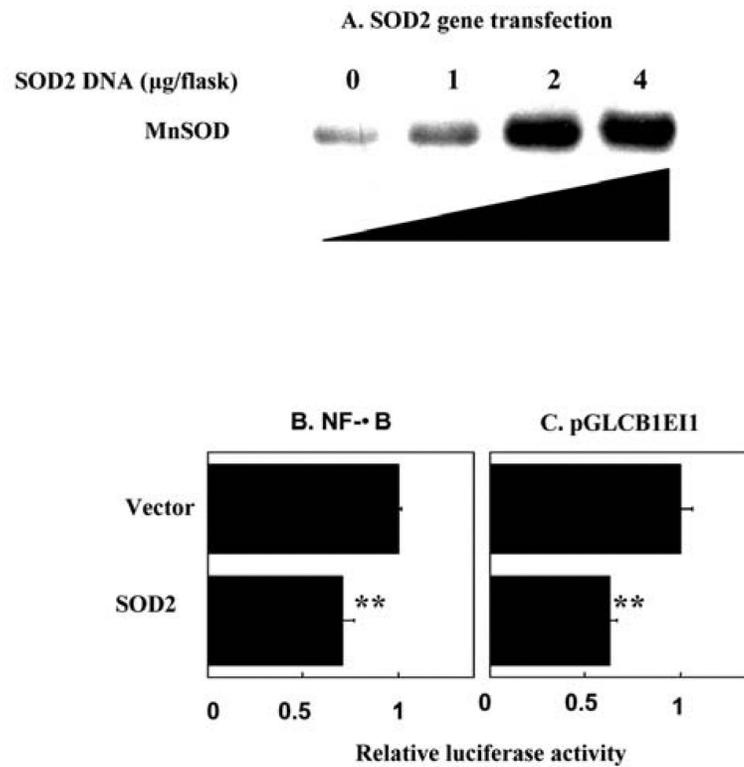


Figure 5.

Over expression of human SOD2 genes inhibited luciferase activity of pGLCB1E11 and NF- κ B. MnSOD proteins were dose-dependently increased with SOD2 gene transfection (A). Gene expression vectors containing human SOD2 (11) gene were transiently transfected into MCF-7 cells and MnSOD protein levels were estimated using Western blotting. Luciferase reporters controlled by NF- κ B (B) or pGL3CB1E11 (C) were co-transfected with (1-4 mg/flask) human MnSOD gene expression vector (SOD2) and luciferase activities were measured 24 h after exposure to 5 Gy IR. Luciferase activity was normalized to protein concentration (mean \pm S. D., n=4; **p<0.01).