Lack of P-Glycoprotein Expression by Low-Dose Fractionated Radiation Results from Loss of Nuclear Factor-κB and NF-Y Activation in Oral Carcinoma Cells

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Abstract
Multidrug resistance (MDR) is associated with the overproduction of the 170-kDa transmembrane protein P-glycoprotein (MDR1) caused by transcriptional activation. However, the activity of the MDR1 promoter in response to different doses of ionizing radiation has not been investigated. In this study, two squamous cell carcinoma oral cavity cell lines, T-167 and T-409, were exposed to either a standard clinical dose of 2 Gy or low-dose fractionated radiation therapy (LDFRT), delivered as 0.5 Gy in four fractions. MDR1 gene expression and degree of cell death were assessed. Clinically relevant 2-Gy dose of radiation resulted in increased expression of MDR1 by reverse transcription-PCR and luciferase reporter assays in both cell lines (T-167 and T-409), whereas LDFRT did not. LDFRT caused enhanced apoptosis when compared with the 2-Gy dose in T-167 and T-409 cells as assessed by terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assays. Transcription of the MDR1 gene is regulated by numerous transcription factors, which include nuclear factor-κB (NF-κB), NF-Y, SP1, YB1, MEF1 (MDR1 promoter-enhancing factor 1), p53, and NF-R1. Interestingly, 2 Gy robustly induced both NF-κB and NF-Y in T-167 and T-409 cells, but did not show induction when exposed to LDFRT. Silencing the expression of the DNA binding subunit of NF-κB, p50, by small interfering RNA vector resulted in a decrease of MDR1 function by rhodamine 123 efflux assay in T167 cells exposed to 2 Gy. Together, these results provide evidence for the lack of induction of P-glycoprotein expression by LDFRT, which has important implications in combinatorial cancer therapy, including the use of LDFRT as an adjuvant for chemotherapy. (Mol Cancer Res 2008;6(1):89–98)

Introduction
Squamous cell carcinoma of the oral cavity (SCCOC) is the most prevalent cancer of the upper aerodigestive tract and is the most common form of cancer on the Indian subcontinent (1, 2). The primary modality for treatment is surgery with postoperative, adjuvant, external beam radiation therapy for advanced stages. Despite aggressive treatment of advanced SCCOC, patients at high risk based on clinicopathologic criteria frequently develop locoregional recurrence leading to 5-year survival rates that have remained flat at 30% to 50% (3). Chemotherapy, as combined adjuvant therapy with radiation, has not been efficacious against SCCOC. Intrinsic drug resistance of oral cavity cancers to chemotherapy is believed to be a primary reason for its ineffectiveness. Due to their complex, heterogeneous make-up, many tumors tend to be resistant to therapy inherently or develop resistance during initial therapy, which is termed “acquired” resistance.

Evidence implicates MDR proteins as major factors in intrinsic and acquired drug resistance. P-glycoprotein (encoded by the MDR1 gene) was the first multidrug-resistant protein (4). P-glycoprotein and multidrug resistance protein 1 (MRP1), the most studied MDR proteins (5), have <15% amino acid identity (6). Although only few studies have been done that correlate oral cancer and multidrug resistance expression, Hirata et al. (7) did not find significant differences in the levels of MDR1 mRNA expression in head and neck squamous cell carcinoma and the mucosa. Ng et al. (8) proposed that the induction of P-glycoprotein expression by standard radiation might affect the efficacy of subsequent or concurrent chemotherapy. Thus, P-glycoprotein may confer chemoresistance and be responsible for the poor treatment outcomes realized with nonsurgical modalities in oral cancer. It has been postulated that P-glycoprotein, besides acting as an efflux pump of xenobiotics,
may also confer resistance to apoptosis from direct stimuli such as chemotherapy agents, Fas ligation, serum starvation, and irradiation through mechanisms that affect caspase-3 activation (9-12).

Joiner et al. (13) revolutionized beliefs about the effects of low doses of radiation (<100 cGy) by demonstrating that the initial phase of hyper-radiosensitivity (HRS) at doses ranging from 1 to 80 cGy. The hyper-radiosensitivity observed with low-dose radiation is a unique radiobiological phenomenon (14). The finding that hyper-radiosensitivity does not initiate cellular repair mechanisms such as those observed at higher doses provides a plausible explanation as to why there is no induction of radiation resistance with hyper-radiosensitivity, as measured in vitro. In clinical and preclinical trials, we employed an original strategy that combines low-dose fractionated radiation therapy (LDFRT) and chemotherapy. In this scheme, LDFRT acts as an enhancer or chemo-potentiator of taxanes and seems to circumvent the development of resistance found with higher, conventional doses of chemoradiation. Our recent studies showed that exposure of p53-mutant tumor cells to paclitaxel alone or in combination of LDFRT and paclitaxel, or the combination of the two in vitro (15). Subsequent studies using SCCOC xenografts in nude mice upheld the in vitro findings in preclinical testing (16).

Recently, Kuo et al. (17) reported the up-regulation of P-glycoprotein expression by the multiple effectors of the phosphoinositide-3-kinase (PI3K) signaling pathway in human hepatoma and embryonic fibroblast 293 cells. The induction of MDR1 was mediated through PI3K activation of the nuclear factor-κB (NF-κB) binding site located at −6092 from the transcription start site of the MDR1 promoter. Interestingly, Ogretmen and Safa (18) showed that the NF-κB/p65-cFos complex is involved in the negative regulation of the MDR1 promoter. Kato et al. (19) showed sensitization of head and neck cancer cell line UM-SCC-9 to radiation using a mutant IκB (inhibits NF-κB). These results suggest that NF-κB and its heterodimeric components may play multiple roles in the regulation of the human MDR1 gene, which may be cell type specific. MDR1 promoters also contain putative CCAAT elements (Y-boxes). The Y-box especially is absolutely required for the basal and inducible expression of human MDR1 as shown by mutational analysis of the MDR1 promoter (20, 21).

The nuclear protein NF-Y complex, which consists of A, B, and C subunits, recognizes the Y-box sequences (22, 23). NF-Y plays a pivotal role in the regulation of MDR1 gene expression under genotoxic stress conditions, and it is thought to be a good molecular target for manipulating the MDR phenotype (24).

In the present study, we have elucidated the roles of the transcription factor NF-κB and NF-Y in the modulation of MDR1 expression in response to conventional dose (2 Gy) and LDFRT (0.5 Gy delivered in four fractions).

**Results**

**Status of p53 in SCCOC Cells**

Cancer cells are frequently considered radiation resistant when they possess a nonfunctional p53 protein (25). Whether this occurs through mutation or nullity, it is believed that p53 mutations act in a dominant negative manner to suppress the activity of wild-type p53 (26). We previously reported the influence of functional p53 status of two SCCOC cell lines SQ-20B (p53mut) and SCC-61 (p53wt) in resistance to 2 Gy radiation (15). To show the functional status of p53 in SCCOC cell lines used in the present studies, T167 and T-409, we used ionizing radiation to induce p53 activation with 7 Gy dose of X-ray irradiation (Fig. 1; ref. 27). Reporter assay for p53 function was used. p53 transcriptional activity was not detected in both cell lines T167 and T409.

**Low-Dose Hyper-Radiosensitivity of SCCOC Cells**

Before initiating our experiments, we examined whether the SCCOC cell lines T167 and T409 showed low-dose hyper-radiosensitivity. T167 cells were exposed to doses ranging from 0.1 to 6.0 Gy. In the low-dose range (between 0.1 and 1 Gy), 0.1-Gy increments were measured, and the degree of surviving fraction was determined by colony-forming assay. Figure 2A clearly shows the presence of increased hyper-radiation sensitivity in both lines; single 0.7- and 0.8-Gy doses produced the greatest amount of cell killing in T167 and T409 cells, respectively. At a point immediately after the 1.0-Gy dose, the cell killing resumed a linear-quadratic response (Fig. 2B). Based on the single-hit multi-target model, T167 was found to be more radiosensitive (D0 = 189 cGy, n = 1.1) than T409 cells (D0 = 355 cGy, n = 1.1; Fig. 2B). These results confirm the presence of hyper-radiosensitivity in T167 and T409 cells despite the lack of functional p53.
LDFRT Induces Robust Apoptosis

To determine the means by which LDFRT causes enhanced cell killing, an apoptosis assay was done using terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) staining. The SCCOC cell lines T167 and T409 were treated with LDFRT, 2 and 7 Gy. The temporal expression of radiation-induced apoptosis was assessed at time points of 3, 6, and 24 h (Fig. 3).

In both cell lines, all the treatment groups showed TUNEL-positive apoptosis significantly greater than the untreated control groups. However, the proportion of cells undergoing apoptosis was twice as large in the LDFRT-treated group as compared with cells treated with conventional 2 Gy radiation. Interestingly, the SCCOC cell lines T167 and T409 are p53 mutants, and still, a significantly greater proportion of the cells underwent apoptotic cell death (Fig. 3). This observation is consistent with our previously reported studies that have established that at the very least, LDFRT causes cell death by a p53-independent mechanism and may in fact activate an alternative apoptotic pathway that uses mediators of apoptosis downstream of p53 (15, 16).

Clonogenic Inhibition in SCCOC Cells in Response to LDFRT

The effects of different radiation doses on the reproductive death of SCCOC cells were assessed by colony-forming assay. No significant differences were observed in the fraction of surviving cells exposed to 2 Gy alone or LDFRT in T-167 cells (Fig. 4). Furthermore, the cell survival was marginally higher in cells exposed to LDFRT when compared with that of 2 Gy in T-409 cells (Fig. 4). When compared with the TUNEL results from Fig. 3, this suggests that LDFRT-mediated cell killing is primarily through apoptosis rather than reproductive cell death (Fig. 4).

LDFRT Does Not Induce MDR1 Expression

Ruth and Roninson reported that cells that overexpress P-glycoprotein cause inhibition of radiation-induced apoptosis

![FIGURE 3. LDFRT induces maximal apoptosis as compared with conventional and high-dose radiation. T-167 and T-409 cells were treated with either 2 or 7 Gy or LDFRT and were scored for TUNEL-positive cells at 6 and 24 h posttreatment. Apoptosis is assessed by TUNEL analysis, and the results are represented as histograms. Increased apoptosis was seen in cells treated with LDFRT as compared with conventional 2-Gy or high-dose 7-Gy exposure.](image-url)
Role of Transcription Factors NF-κB and NF-Y in Radiation-Induced MDR1 Expression

Our previous studies with SCC-61 and SQ-20B cell lines established a role for NF-κB in radiation-induced Bcl-2 expression (15). We wanted to know whether NF-Y and NF-κB, which are critical transcription factors that regulate MDR1 expression, have a role in radiation-induced MDR1 expression. To test this, appropriate MDR1 promoter reporter constructs were used. The observation that LDFRT does not induce MDR1 expression was confirmed by the luciferase assay using the full-length MDR1 promoter construct that contained both NF-κB and NF-Y binding sites (6228, Figs. 5A and 6) or with a truncated promoter containing only the NF-Y binding site (2930, Figs. 5A and 6). Expression of the hu-MDR1 promoter luciferase reporter assay was analyzed 24 h after the cells were exposed to 2 Gy or LDFRT. In all treatment regimens, the luciferase activity was reduced particularly in the reporter construct lacking the NF-κB binding site as compared with reporter construct containing both NF-κB and NF-Y binding sites. This indicates the cooperative contributions of both NF-κB and NF-Y transcription factors in the expression of MDR1 gene. MDR1 expression as reflected in the luciferase activity was significantly induced in both the T-167 and T-409 cells exposed to the 2-Gy radiation as compared with the cells that did not receive any treatment. However, there was no significant difference between the LDFRT-exposed cells as compared with that of control untreated cells. Thus, conventional dose but not LDFRT causes the activation of MDR1 gene expression (Fig. 6).

LDFRT Does Not Induce Activation of NF-κB in SCCOC Cells

In unstimulated cells, the transcription factor NF-κB is stabilized in the cytoplasm in an inactive state by the inhibitor protein IκBα. Stimulation of cells results in rapid phosphorylation and degradation of IκBα, thus releasing NF-κB, which translocates to the nucleus and activates transcription of responsive genes. Localization of the NF-κB subunits p50 and p65 in the nucleus is indicative of its activation. In both the cell lines studied, T167 and T409, 2 and 7 Gy radiation induced the translocation of NF-κB subunits into the nucleus as shown by the co-localization of these subunits in the nucleus (yellow staining) using double immunofluorescence microscopy. In the untreated and the cells exposed to LDFRT, the majority of the signal was present in the cytoplasm (Fig. 7).

LDFRT Does Not Cause Activation of NF-κB and NF-Y in MDR1 Gene Promoter

We investigated the contribution of these NFκB and NF-Y in the modulation of MDR1 gene expression (Fig. 6). Next, we analyzed the response of NF-κB activity in different treatment doses of radiation using a 3X-κB-CAT reporter plasmid transfected into T167 cells. Figure 8A shows that the CAT activity of the cells treated with 2 Gy was equivalent to that of the cells transfected with the positive control p65. On the other hand, the activity of NF-κB in response to LDFRT was almost identical to that of the untreated cells. This result shows that LDFRT does not up-regulate NF-κB. This reporter assay characterizes the response of NF-κB to ionizing radiation as either direct activation or indirect activation by involvement of intermediary factors.

To better understand the way in which LDFRT impacts MDR1 gene expression relative to standard 2 Gy radiation through NF-Y, electrophoretic mobility shift assay (EMSA) studies were done on nuclear extracts from T167 cell lines treated with LDFRT, 2- and 7-Gy fractions of ionizing radiation. The gel shifts used NF-Y double-stranded oligo-DNA probes with specificity for hu-MDR1. Nuclear extracts from T167 SCCOC cells showed lower levels of NF-Y binding after LDFRT treatment compared with 2- and 7-Gy doses of radiation at different time points (Fig. 8B). A super-shift was
done using anti–NF-Y antibody to establish specificity of the NF-Y DNA binding complexes.

Thus, NF-Y–specific oligonucleotides failed to show meaningful binding activity in T167 SCCOC cells treated with LDFRT. This result directly illustrates that the two critical transcription factors NF-κB and NF-Y in MDR1 gene regulation are not activated by low doses of fractionated radiation therapy, and that this is a putative mechanism for overcoming induced chemoradiation resistance in SCCOC. In fact, Ng et al. (8) have shown that patients who previously received external beam radiation to oral cavity cancers overexpressed P-glycoprotein, which may confer multidrug resistance to these characteristically chemoresistant tissues.

MDR1-Mediated Efflux Is Up-Regulated in Response to the Conventional Radiation Exposure but not LDFRT

All our earlier results of RT-PCR and reporter assays clearly indicate a role of NF-κB, and the NF-YA subunit is involved in the up-regulation of MDR1 expression in response to conventional clinical dose of 2 Gy. To further characterize the role of NF-κB in MDR1-mediated transport of rhodamine 123, T-167 cells were stably silenced for p50 expression with p50 small interfering RNA (siRNA) and analyzed for the efflux mediated by MDR-1. Down-regulation of the expression of the p50 subunit was assessed by Western immunoblot (Fig. 9A). The rhodamine efflux increased in response to conventional 2-Gy and high-dose 7-Gy radiation, but not in the LDFRT treatment of T-167 cells. The increase in MDR1 efflux activity in response to 2 and 7 Gy was abrogated in T-167 cells stably transfected with p50 subunit of NF-κB, but not in T-167 cells transfected with control vector. No significant differences were observed in efflux activity in response to LDFRT exposure of T167 cells irrespective of the p50 silencing, demonstrating the role of NF-κB in the functional MDR1 expression in SCCOC cells exposed to conventional 2-Gy and high 7-Gy doses but not in LDFRT (Fig. 9B). Thus, the up-regulation of the transport activity of the MDR1 pump may lead to radio- and chemoresistance during the conventional and high-dose ionizing radiation treatments, but not during LDFRT.

Figure 10 represents schematically our current understanding of the differential response of P-glycoprotein to ionizing radiation. NF-Y and NF-κB are depicted as positive feedback regulators of MDR1 and are activated by conventional dose radiation, whereas LDFRT does not seem to activate these two transcription factors. In all likelihood, the interplay between MDR1 gene regulators is more complicated than this schema predicts. However, it is clear clinically that chemoresistance and radiation resistance is closely intertwined; and this approach is an attempt to link these therapeutic phenomena.

Discussion

The hyper-radiosensitivity phenomenon seems to be most prominent in the G2 phase of the cell cycle, which suggests that actively proliferating cell populations may have increased radiosensitivity to very low radiation doses (28). Recently, Dey et al. (15) showed that low doses of fractionated radiation cause enhanced cell killing in a p53-independent manner in vitro. The concept of low-dose radiation delivered in fractions has been applied in preclinical experiments in which SQ20B cells xenografted into nude mice and treated with LDFRT combined with docetaxel showed complete tumor eradication and durable response in the nude mice xenografts (16). Arnold et al. (29) enrolled 40 patients in a single-arm neo-adjuvant clinical trial in which patients with advanced head and neck cancer were treated successfully (i.e., excellent response rates) with low-dose fractionated radiation combined with carboplatin and paclitaxel before definitive therapy.

Induced radiation resistance (IRR), a radiobiological phenomenon observed when treating cells with clinical doses of radiation, is often associated with point mutations and deletions of p53, commonly described in head and neck carcinomas (30). The use of LDFRT seems to bypass the classic p53-dependent induction of apoptosis. Marples et al. (31) have described a three-response phase mechanistic process by which
hyper-radiosensitivity operates involving (a) a sensor signal; (b) a transducer signal; and (c) a damage repair step that implicates the protein kinase ATM (ataxia telangiectasia mutated) as a central control protein, but which is independent of p53 status. They also show that existence of low-dose hyper-radiosensitivity in clonogenic survival experiments was found to be associated with an elevated level of apoptosis after low-dose exposures corroborating their earlier observations (32, 33). Therefore, the disassociation between the clonogenic cell survival and apoptosis observed in our studies is not surprising. These data are consistent with current hypothesis to explain low-dose hyper-radiosensitivity, namely, that the enhanced sensitivity of cells to low doses of ionizing radiation reflects the failure of ATM-dependent repair processes to fully arrest the progression of damaged G2-phase cells harboring unrepaired DNA breaks entering mitosis (33).

Medical, radiation, and surgical oncologists have long recognized that cancers that exhibit resistance to chemotherapy drugs are frequently resistant to radiation. Very few studies have investigated whether radiation and multidrug resistance have a common regulator of expression. Ruth and Roninson used a HeLa-derived cell line with inducible MDR1 expression and NIH 3T3 cells transduced with an MDR1-expressing retroviral vector to show the inhibition of radiation-induced apoptosis by P-glycoprotein overexpression (10). Hill et al. irradiated human ovarian tumor cells exposed to 2-Gy doses twice daily for 5 days on 2 consecutive weeks, similar to the clinical setting, and were able to detect overexpression of P-glycoprotein and MRP1 (34). Clinically, Ng et al. (8) showed induction of P-glycoprotein in three different groups of patients irradiated for oral squamous cell carcinoma. The results helped to explain the poor response rate of previously irradiated SCCOC cells to chemotherapy.

To elucidate the mechanism by which LDFRT acts to enhance tumor cell killing, we investigated whether NF-κB and/or NF-Y is induced by this novel fractionation approach. Their consensus sequences significantly contribute to overall promoter strength and may be strictly required for gene activity (35). Kuo et al. (17) have shown that the NF-κB consensus sequence located upstream of the TATA-less MDR1 gene promoter is in part responsible for the expression of MDR1. Additionally, Jin and Scotto (36) have shown that the ubiquitous heteromeric protein NF-Y transcription factor was responsible for the activation of the MDR1 promoter by UV radiation. In the present study, we showed that treatment with a clinical 2-Gy dose can modulate the expression of MDR1 in head and neck carcinoma cell lines through differential activation of NF-κB and NF-Y. In most cases, low-dose radiation does not elevate expression of either transcription factor to a level above that of basal expression. The relative contribution of each of the transcription factor, i.e., NF-κB and NF-Y, in the induction of the MDR1 gene expression is not clear. Our reporter assays results suggest that both factors are required for the expression of MDR1 gene during conventional and high-dose radiation exposure. It is plausible that the major effect of LDFRT is cell killing mediated by apoptosis because the expression of MDR1 is associated with the caspase-mediated apoptosis (9-11). However, it is clear from our studies that LDFRT represents a discrete mode of tumor cell killing through pathways, which interconnect radiation and chemotherapy resistance.

Previous reports show that higher doses (>100 cGy) of radiation lead to induced radiation resistance. In the present study, we have shown by way of molecular analysis that 2 Gy radiation causes an increase in NF-κB activity in SCCOC lines. Furthermore, the transcription factor NF-κB targets the induction of Bel-2 protein, which ultimately contributes to the development of radio-resistance among tumor cells (37). It may also be that at higher doses of radiation, there is sufficient cellular damage to trigger repair systems or other radio-protective mechanisms. This molecular signaling may be the basis of induced radiation resistance. On the other hand, low doses of radiation evince the hyper-radiosensitivity phenomenon and cause a significant increase in the proapoptotic Bax protein without induction of either NF-κB or NF-Y activity, suggesting that the low doses of radiation have the capacity to selectively induce proapoptotic pathways and inhibit the activation of pro-survival pathways. In such a situation, induced radiation resistance is avoided, which results in enhanced cell killing.

Although there are previous studies that have shown the decrease in the level of MDR1 gene expression in multidrug-resistant KB cells in response to fractionated radiation, this phenomenon was thought to be mediated by the radiation-induced loss of extrachromosomally amplified genes from...
tumor cells via their entrapment in micronuclei (38). This is the first report that shows that no regulation of MDR1 occurs at the transcriptional level in LDFRT exposure. This lack of MDR1 response at the transcriptional level seems to be mediated by the fact that transcription factors NF-κB and NF-Y are not induced by LDFRT. Treatment outcomes from our previous studies and clinical trials using adjuvant low-dose radiotherapy with paclitaxel and carboplatin may be explained by the observations in the current study in that multidrug resistance elements are not activated by LDFRT (15, 16, 29).

In this study, we have attempted to connect the physically distinct but clinically complementary modalities of radiation therapy and chemotherapy in the context of modern cancer therapy. The activators and inhibitors of expression of other multidrug resistance proteins need to be investigated with an eye toward defining a common relation among these clinically disparate, but genetically linked proteins.

**Materials and Methods**

**Cell Culture and Treatment**

Two SCCOC lines T167 and T409 were kindly provided by Dr. Gary Clayman (University of Texas M. D. Anderson Cancer Center, Houston, TX). These cells were grown in DMEM/F12 medium containing 10% fetal bovine serum (FBS) with 1% penicillin-streptomycin. They were grown at 37°C and in 5% CO₂. A 100-kV industrial X-ray machine (Phillips) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm Al plus 1-mm Be filter was ~2.64 Gy/min at a focus-surface distance of 10.5 cm.

**Western Blot Analysis**

Total protein extracts from stable NF-κB p50 siRNA T-167 cells and its control lacking the insert for siRNA in T-167 cells were made and separated on SDS-PAGE and blotted. The NF-κB p50 protein was detected using a polyclonal antibody (Santa Cruz Biotechnology). Anti–β-actin antibody (Sigma Chemical Co.) was used as an internal loading control. These proteins were detected using the chemiluminescent method.

**Clonogenic Assay**

Cell lines were left untreated or exposed to a clinical dose (2 Gy) or LDFRT (0.5 Gy in four fractions) radiation. Hyper-radiosensitivity was assessed using doses from 0.1 to 6 Gy in both T-167 and T-409 cell lines by colony-forming assay to assess clonogenic inhibition as described earlier (30, 39).

**Quantification of Apoptosis**

Apoptosis was quantified by the TUNEL assay. The ApopTag in situ apoptosis detection kit (Oncor) that detects DNA strand breaks by TUNEL was used as described earlier (40). Briefly, T-167 and T-409 cells were seeded in double-chambered slides in duplicates and were exposed to single-dose radiation (2 or 7 Gy) or four fractions of 0.5-Gy radiation doses, and apoptosis was assessed at 3, 6, and 24 h post-irradiation.

**Isolation of RNA and 32P-RT-PCR**

Total RNA was isolated from cultured cells, and tumor tissues using TRIzol reagent (Life Technologies-BRL) was reverse transcribed into cDNA. The PCR conditions were 5 min at 94°C, followed by 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C for 30 cycles, followed by 72°C for 7 min. The PCR primer sequences of MDR1 and GAPDH (used as internal control) were as follows: MDR1, sense primer 5’-CCCATCATGGCAATAGCAGG-3’ and antisense primer 5’-GTTCAACCTTCTGCTCCTGA-3’ corresponding to 167 bp (residues 2733-2752); GAPDH, sense primer 5’-CCCCTGGCAGTTGTCAAACTTCTGCCCTCTGA-3’ and antisense primer 5’-GGCCATGAGGTCCACCACCCTGTTGCTGTA-3’ corresponding to 513 bp (residues 515-1027). The PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide, photographed, and scanned. We did densitometric analysis to determine the relative mRNA expression levels of MDR1.

**FIGURE 7.** NF-κB is activated by 2 and 7 Gy, but not by LDFR. Co-localization of the p50 and p65 subunits (yellow) of NF-κB was largely seen in the nucleus (blue) of T-167 and T-409 cells treated with either 2 or 7 Gy ionizing radiation. On the other hand, co-localization of these subunits was seen primarily in the cytoplasm of cells that were either untreated or exposed to four fractions of 0.5-Gy dose of X-ray radiation.
Relative mRNA expression was normalized after dividing the absorbance values of each band by the absorbance values obtained for the respective β-actin band of each sample.

**Transient Transfection and Luciferase Assay**

MDR1 promoter reporter activity was measured using the pMDR1-luciferase (−6228-1-MDR1-LUC harboring both NF-κB and NF-Y sites; and −2930-1-MDR1 lacking NF-κB site but has NF-Y site) assay. p53 reporter activity was also assessed in T167 and T409 using luciferase reporter (Panomics Inc.). The pMDR1-luciferase or p53 reporter and pRL-TK control vector (Promega) were transiently co-transfected by LipofectAMINE PLUS (Life Technologies-BRL), according to manufacturer’s directions. Twenty-four hours post-transfection, the cells were either left untreated or treated with radiation. Six hours (for p53 reporter) or 24 h (for MDR1 reporter) after treatment, the cells were lysed in reporter lysis buffer and subjected to three freeze-thaw cycles. The activity of luciferase was measured using a Dual-Luciferase Reporter assay (Promega) and expressed as a ratio of pMDR1 or p53/pRL-TK for normalization.

**Chloramphenicol Acetyl Transferase Assays**

3x-NF-κB-CAT constructs were as described previously (41, 42). As a control, MIA PaCa-2 cells were transfected with a positive control vector (pCAT-control, Promega) to monitor the general transcriptional activity. Transfections were done by the calcium phosphate coprecipitation method, and 20-h post-transfection, cells were treated conventional 2 Gy radiation or LDFR. Cells were harvested after 24 h of treatment and normalized by the pCAT-control activity, and standard chloramphenicol acetyl transferase (CAT) assays were done using by TLC as described previously (43).

**EMSA**

Nuclear extracts from untreated and treated cells were isolated and prepared, and EMSA was done. Analysis of DNA-binding was done using 2 μg of poly(deoxyinosinic-deoxyctydidyric acid) (Sigma Chemical Co.) as nonspecific competitor DNA. The binding reactions contained 10,000 cpm of 32P-labeled double-stranded oligonucleotide probe (with a high affinity for NF-Y binding). Binding reactions were electrophoresed on a 4% PAGE in 0.5× Tris-borate EDTA buffer to separate the bound and unbound probe. The following oligonucleotide primers and their complements were used: wild-type NF-Y binding site (−82 to −55), 5′-TGTTGTTAG-GCTGGATTGGCTGGGCAGGA-3′.

**Localization of p65/p50 Using Double Immunofluorescence**

Cells were cultured on Lab-Tek chamber slides (Nunc Inc.) and exposed to radiation at 80% confluency. Slides were blocked with 3% bovine serum albumin in PBST [PBS (pH 7.4), with 0.25% Tween 20] for 30 min. Slides were then incubated overnight at 4°C in primary antibodies, mouse anti-p65, and/or...
rabbit anti-p50 (Santa Cruz Biotechnology) that were diluted in 1:100 with the blocking buffer. Following washes in PBST (3 × 15 min), the cells were overlaid with secondary antibodies, Cy2-conjugated anti-mouse immunoglobulin M (IgM) and Cy3-conjugated anti-rabbit IgG, diluted 1:1,000 in blocking buffer. After three washes in PBST (3 × 15 min) and one wash in PBS (1 × 15 min), the slides were mounted with aqueous mounting media with antifade and 4',6-diamidino-2-phenylindole (DAPI, VectaShield, Vector) and visualized using a Zeiss epi-fluorescence microscope.

**Construction of Stable NF-κB p50 Silencing Lines**

The siRNA for p50 subunit of the NF-κB under the regulation of human U6 promoter and the control vector lacking the siRNA (Panomics) encoding sequence were stably transfected into T-167 cells using the LipofectAMINE reagent (Invitrogen). The stable clones selected with 100 μg neomycin/mL were maintained in medium containing 50 μg of neomycin/mL. Suppression of the p50 gene subunit of NF-κB was assessed by Western immunoblot.

**Cellular Efflux Assay**

To study the transport function of P-glycoprotein in T167 cells exposed to different irradiation treatment regimens, rhodamine 123 was used in an efflux assay buffer (44). After 12 h of incubations following the treatment, cells were resuspended in cold rhodamine 123 loading buffer (1.0 × 10⁶ cells/mL) and incubated on ice for 2 h. After incubation and washing with cold efflux buffer, cells were either resuspended in cold efflux buffer or warmed (37°C) efflux buffer with DMSO or incubated for 1 h on ice or at 37°C. Cells were then washed twice in cold efflux buffer to remove excess rhodamine 123 in the medium and incubated in propidium iodide containing cold efflux assay buffer on ice and analyzed by flow cytometry using a FACScan by measuring events in the FL1 (rhodamine 123) channel. To quantify the effect of radiation on the rhodamine 123 efflux from T167 cells, we measured the shift of the histogram to the right as compared with that of the control (rhodamine 123 alone in cold efflux assay buffer incubated on ice).

**Statistical Analysis**

Student’s t test was used to test the statistical significance of the data obtained from the clonogenic, TUNEL, and efflux assays.

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**References**


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