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Mechanism of Radiation Carcinogenesis: Role of the *TGFBI* Gene and the Inflammatory Signaling Cascade

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Abstract

Using an immortalized human bronchial epithelial cell line, we showed previously that the transforming growth factor beta-induced (*TGFBI*) gene was consistently downregulated by six- to sevenfold among radiation-induced tumorigenic human cells when compared with controls. Transfection of *TGFBI* gene into tumor cells resulted in a significant reduction in tumor growth as well as in vitro anchorage independent growth. The observations that *TGFBI* knock-out animals showed increased spontaneous tumor incidence and chemically induced tumors highlight the suppressive nature of the gene. There is evidence that extranuclear/extracellular targets are important in low-dose radiation response and that the cyclo-oxygenase-2 signaling pathway mediates the process. The involvement of NFκB-dependent cytokines and the resultant inflammatory response works in concert with in modulating radiation-induced bronchial carcinogenesis.

13.1 Introduction

Radiation is a two-sided sword; on the one hand, it is a well-established human carcinogen, and, on the other hand, it is an effective therapeutic regimen against a variety of human malignancies. Although ionizing radiation is a well-established human carcinogen and is known to induce tumors on various sites in human, the underlying cellular and molecular mechanism of radiation carcinogenesis remains unknown. The carcinogenic risk for human epithelial cells after exposure to low LET radiation has been estimated to be in the range of 10^{-12} per cell per Gy based on the breast cancer incidence among Japanese A-bomb survivors [1], an incidence too low to be reproduced under any laboratory setting. Immortalized human lung epithelial cell cultures offer the next best alternatives as useful models in assessing the various phenotypic and genotypic changes leading to malignancies after exposure to ionizing radiation. Although these cells are immortalized either by viral transduction [2] or by ectopic expression of telomerase [3], they are phenotypically normal and do not express any transformed characteristics such as anchorage independent growth and tumorigenicity in nude mice. After exposure to either 150 keV/μm α particles or 1 GeV/nucleon ^{56}Fe ions, transformed cells arise through a series of sequential stages including

altered growth pattern, resistance to serum-induced terminal differentiation, agar-positive growth, tumorigenicity, and metastasis [4, 5].

Previous studies have shown that a single 60 cGy dose of these high LET alpha particles or heavy ions irradiations can induce neoplastic transformation of the BEP2D cells in a step-wise fashion at a frequency of $\sim 10^{-6}$ [4]. The immortalization step, therefore, increases the transformation yield of primary human epithelial cells by more than a million fold. It should be noted that, while the majority of agar-positive BEP2D clones are nontumorigenic, they all demonstrated the propensity to resist serum-induced terminal differentiation. In addition, each preceding stage represents a necessary, yet insufficient step toward the later, more malignant phase. Furthermore, tumorigenic BEP2D cells show no mutations in any of the *ras* oncogenes [4], a finding that is consistent with the observation that radiation is clastogenic that induces primarily multilocus deletions and chromosomal loss.

13.1.1 Changes in Gene Profiling in Radiation-Induced Tumorigenic Cells

The differential expression of known genes between tumorigenic and nontumorigenic control cells can be compared using a cDNA expression array. Briefly, total RNAs from both tumorigenic and control BEP2D cells are isolated and then treated with DNase I to remove any contaminating DNA from the RNA preparation. PolyA⁺ RNA is then isolated, and ³²P-labeled complex cDNA probes are generated by reverse transcription in the presence of ³²P-labeled dATP. The cDNA probes can then be hybridized to the nylon membrane and the hybridization patterns can be analyzed by autoradiography. The intensity of the signal, which corresponds to the expression levels of the individual genes, can be easily quantified using phosphorimaging technique.

Using cDNA microarrays to compare differentially expressed genes in radiation-induced tumorigenic cell lines relative to parental BEP2D cells, there is evidence that *TGFBI* gene is down-regulated by more than six- to seven fold in the tumorigenic cells [5]. *TGFBI* is a secreted protein induced by the transforming growth factor- β (TGF- β) in human adenocarcinoma cells as well as other human cell types [6]. *TGFBI* was first identified in a human lung adenocarcinoma cell line (A549) treated with TGF- β . This gene encodes a highly conserved 683 amino-acid protein that contains a secretory signal sequence and four internal homologous domains, the last of which contains an RGD (Arg-Gly-Asp) motif which can serve as a ligand recognition site for integrins [6]. *TGFBI* product has been shown to be a component of extracellular matrix (ECM) in lung and mediates cell adhesion and migration through interacting with integrin via integrin receptors: $\alpha 3\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ [7]. It is ubiquitously expressed in human normal tissues; however, its role in cancer appears to be cell type specific. Although downregulation or lost expression of this gene has been found in human tumor cell lines including lung, breast, and leukemia as well as in human primary lung and breast tumor specimens [5, 8], there are several reports that *TGFBI* is over-expressed in human colorectal, colon, esophageal, and pancreatic cancers [9, 10]. Furthermore, there is evidence that *TGFBI* mediates tumor metastasis and cancer cell extravasation [10]. For human lung cancer CpG island hypermethylation in the promoter region correlates with the silencing of *TGFBI* promoter and its subsequent down-expression [11]. In vitro studies have implicated its role in maintaining microtubule stability, and

inhibiting tumorigenicity and tumor angiogenesis, suggesting a tumor suppressor function in vivo.

13.1.2 Ectopic Re-Expression of *Bigh3* Suppress Tumorigenic Phenotype

To ascertain the tumor suppressive effect of *TGFBI* gene in human lung cancer, it is necessary to re-express the lost or downregulated gene in tumor cells and determine the tumor outcome upon inoculation into nude mice. The approach was to ectopically express the gene in a highly malignant cell line (TL1) using pRc/CMV2 *TGFBI* expression vector. Thirty G418-resistant colonies (TL1–TL30) that expressed different levels of *TGFBI* gene were chosen for further studies [12]. From the northern and western blotting results, the parental TL1 cells and TL1-pRc/CMV2 cells expressed a low, detectable, and similar level of *TGFBI* gene. After transfection, the expression of *TGFBI* gene in TL1-clone 18 cells was restored to a level similar to that of control BEP2D cells, whereas in TL1-clone 28 cells it was fourfold higher than control. Upon inoculation into athymic nude mice, no tumor (0/8 mice) was detected in animals injected with the parental BEP2D cells after more than 20 weeks. However, 8/8 mice that were injected with either TL1 or TL1-pRc/CMV2 tumorigenic cells with vector control developed progressively growing tumors at 4 weeks, with average volumes of 1021.8 mm³ and 970.6 mm³, respectively. In contrast, only 9/16 mice injected with TL1-clone 18 formed small tumor nodules at 4 weeks, with an average volume of 86.7 mm³ which was significantly smaller than that of the parental TL1 cells ($P < 0.01$). The results of these studies provide unequivocal evidence of the *TGFBI* gene function in radiation carcinogenesis.

13.1.3 *TGFBI* Knock Out Mice Have Higher Spontaneous Tumor Incidence

To explore the physiological function of *TGFBI* and its role in tumorigenesis, *TGFBI*-deficient mice were generated by homologous recombination [13]. The correct targeting resulted in the replacement of exons 4–6 of *TGFBI* gene in mice with a neomycin-resistant gene and was identified by Southern analysis. *TGFBI*^{−/−} mouse embryo fibroblasts (MEF) still expressed *TGFBI* mRNA, but the level was about sixfold lower than in wild-type MEFs. Moreover, the deletion of exons 4–6 in *TGFBI*^{−/−} MEFs was demonstrated by RT-PCR, and the absence of *TGFBI* protein was revealed by western blot.

To determine the tumor-suppressor function of *TGFBI* in vivo, *TGFBI*^{−/−}, *TGFBI*^{+/-}, and wild-type *TGFBI*^{+/+} animals were generated from back-crossing of heterozygous *TGFBI*^{+/-} mice and the development of spontaneous malignancies for up to 20 months was examined [13]. From ages of 9 to 16 months, over 20% of *TGFBI*^{−/−} mice died of systemic illness, whereas all *TGFBI*^{+/+} mice were alive. To determine the cause of death, the moribund *TGFBI*^{−/−} mice between ages of 9 and 16 months were sacrificed and subjected to detailed histopathological analysis. Four out of 12 mice developed malignancies including one invasive lung adenocarcinoma and three lymphomas, one of which was a highly disseminated lymphoma infiltrating liver and lung tissues. By the end of 20 months, 8.3% of *TGFBI*^{+/+} mice, 13.3% of heterozygotes, and 37.04% (20/54) of *TGFBI*^{−/−} mice had developed tumors. The tumor incidence in heterozygotes is higher than that in wild-type mice, but did not reach statistical significance ($P > 0.05$, χ^2 test). Furthermore, topical treatment of *TGFBI*^{−/−} animals with the chemical carcinogen 7,12-

dimethylbenz(a)anthracene (DMBA) resulted in a tenfold higher skin tumors by 6 months of age when compared with wild-type animals. This data further confirmed the tumor suppressor function of the *TGFBI* gene.

13.1.4 *TGFBI*^{-/-} MEFs Exhibit an Increased Frequency of Chromosomal Aberrations

To clarify whether disruption of *TGFBI* resulted in an increased frequency of chromosomal aberrations, *TGFBI*^{-/-} and wild-type MEFs at passage 2 were treated with 0.05 µg/ml colcemid for 3–6 h, and chromosomal metaphases were prepared using previously published procedures. Various types of chromosomal aberrations in *TGFBI*^{-/-} MEFs were identified. Overall, 43.75% (7/17) of metaphases prepared from *TGFBI*^{-/-} MEFs contained chromatid breaks, centric fragments, or chromosomal breaks, whereas only 13.3% (2/15) metaphases from wild-type MEFs contained only centric fragments [13]. In addition, frequency of micronuclei was also examined in the early passage of MEFs (P2). Twenty-four hours post-plating, cells were fixed with acid alcohol and stained with acridine orange in dark for 10 min. A total of 3,000 cells were counted for each experiment, and three independent assays were performed. Micronuclei frequency in *TGFBI*^{-/-} MEFs was found to be 4.7-fold higher than that in wild-type MEFs.

13.1.5 Nontargeted Effects of Ionizing Radiation

Radiation is a well-established human carcinogen. Based principally on the cancer incidence found in survivors of the atomic bombs in Japan, the International Commission on Radiological Protection (ICRP) and the US National Council on Radiation Protection and Measurements (NCRP) have recommended that estimates of cancer risk for low-dose exposure be extrapolated from higher doses where data are available using a linear, no-threshold model. This recommendation is based on the dogma that the DNA of the nucleus is the main target for radiation-induced genotoxicity and, since fewer cells are directly damaged at lower doses, the deleterious effects of radiation decline proportionally. However, there is increasing evidence from a number of laboratories indicating that extranuclear targets/extracellular events may also play an important role in determining the biological responses of ionizing radiation, particularly, at low doses (reviewed in 14, 15). A major conceptual shift in our understanding on the target theory of ionizing radiation in the last decade has resulted from the discovery of the bystander effect.

The radiation-induced bystander effect is defined as the induction of biological effects in the cells that are NOT directly traversed by a charged particle, but are in close proximity to cells that are. The additional responding cells that received no radiation exposure were “bystanders” of either directly hit cells or resulted from agents released from the irradiated medium. Although circumstantial evidence in support of a bystander effect appears to be consistent, direct proof of such extranuclear/extracellular effects are most convincingly demonstrated using charged particle microbeams [16, 17].

Using microbeam technology, a variety of biological endpoints in both human and other mammalian cell lines have firmly established the presence of a bystander effect under either confluent or sparsely populated culture conditions. In general, as few as one cell in a population that is targeted by a single particle has been shown to be sufficient in eliciting a

bystander response [17]. Furthermore, increasing the number of particle traversals per cell or the total dose delivered to the irradiated fraction does not increase the intensity of the bystander response. Thus, there is no evidence of a dose response in any of the biological endpoints examined thus far.

The apparent lack of a dose response in bystander effects observed in many in vitro studies conducted using either a microbeam or through medium-mediated approach could have significant, broader implication in radiation risk assessment. The dose-response concept and the linear no threshold model form the cornerstone in our current management of radiation risk, particularly at the low-dose region. However, at the low-dose range, there are other radiobiological effects come into play including adaptive response, genomic instability, and genetic susceptibility. Although the role of bystander effects in human health is not clear at the moment, there is evidence that it may modulate tumor incidence after heavy ion irradiation in mice [18].

13.1.6 Nature of the Signaling Molecules and Pathways Involved in Radiation-Induced Nontargeted Response

In our quest to identify the signaling pathways involved in radiation-induced bystander effect, we first focused on the genes that are differentially expressed among the bystander vs. control cells. Since the microbeam can only irradiate one cell at a time and a large number of cells are needed for gene array analyses, we employed a novel double mylar dish approach to define the bystander response. Briefly, two concentric stainless steel rings were fitted with mylar bottoms with the outer and inner rings covered by a 6- and 38- μ m-thick mylar sheet, respectively. The thicker mylar sheet of the inner ring was sliced into strips. After sterilizing with 70% ethanol and air-dried, exponentially growing normal human lung fibroblasts (NHLF) were plated in the concentric strip dishes 3 days before irradiation to ensure a confluent state. Since the fibro-blasts seeded on the 38- μ m-thick mylar strips would not be irradiated due to the short penetrating distance of the α particles, these cells would effectively become the bystander cells, being seeded right next to the cells plated on the 6- μ m mylar dishes that were directly irradiated.

Using a signal transduction pathway specific SuperArray, we compared the differentially expressed genes among the nonirradiated control NHLF cells and the bystander cells. Among the 96 genes represented on the platform, transcription level of one gene, cyclooxygenase-2 (*COX-2*), was found to be consistently upregulated by more than threefold, while the RNA level of insulin growth factor binding protein-3 (*IGFBP3*) was found to be consistently lower by more than sevenfold in multiple analyses of multiple bystander samples [19]. Semiquantitative reverse transcription (RT)-PCR was used to confirm the expression levels of these two genes using expression level of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene as an internal control. The expression of the COX-2 protein in the nonirradiated bystander cells was further confirmed by western blotting. Addition of the COX-2 inhibitor NS-398 (50 μ M) suppressed COX-2 activity in NHLF cells and finally, after 24 h, reduced the COX-2 protein level in bystander cells to a nondetectable level [19]. These results indicated that expression of COX-2 is associated with the bystander effect.

13.1.7 Effects of COX-2 Inhibitor on the Nontargeted Effect

If the *COX-2* gene is causally linked to the bystander-signaling pathways, it should be possible to modulate the bystander response using a specific inhibitor of the *COX-2* enzymatic activity. Experiments were conducted to show the effect of a noncytotoxic and nonmutagenic dose of NS-398, a specific inhibitor of *COX-2* activity, on bystander mutagenesis at the hypoxanthine guanine phosphoribosyltransferase (*HPRT*) locus in NHLF cells irradiated with a 0.5-Gy dose of particles using the track segment beam [19]. NHLF cells showed a bystander mutagenic yield of $\sim 4.2 \pm 1.2$ mutants per 10^6 survivors. By contrast, in cultures co-treated with NS-398 (50 μ M) that did not increase the spontaneous mutant yield by itself, the bystander mutant fraction was reduced by more than six fold to a level of $\sim 0.7 \pm 0.2$ mutants per 10^6 survivors. Although NS-398 treatment was able to reduce the *HPRT* mutant fraction in the directly irradiated population as well, the magnitude of suppression, from 9.2 ± 3.5 to 5.9 ± 2.2 mutants per 10^6 survivors was only 36%.

13.1.8 Activation of MAPK Signaling Pathways in Bystander Cells

Insulin growth factor and other cytokines activate mitogen-activated protein kinase (MAPK) signaling cascade; and activation of extracellular signal-related kinase (ERK) by phosphorylation is a critical upstream event preceding *COX-2* expression. Our previous findings based on western blot analysis demonstrated a strong upregulation of phospho-ERK levels in both α -irradiated and bystander NHLF 4 h after treatment [19]. To further confirm the activation of ERK in bystander cells, PD98059 (50 μ M), a specific inhibitor of MAP kinases (MEK) – extracellular signal-regulated kinase (ERK) – was added to cell cultures immediately after irradiation for a period of 4 h. In the presence of PD98059, the phosphorylated form of ERK and its activation were suppressed in both a particle irradiated and bystander cells. If activation of the MAPK signaling cascade and ERK phosphorylation are essential in mediating the bystander effect, it should be possible to mitigate the later response by using a specific inhibitor of the MEK–ERK signaling cascade. In fact, treatment of cells with a noncytotoxic dose of PD98059 (50 μ M) completely suppressed bystander toxicity observed in NHLF cultures [19].

13.1.9 NF- κ B Signaling Pathway in Modulating Radiation-Induced Nontargeted Response

Exposure of mammalian cells to ionizing radiation induces two completely different “information flows” that regulate cell response: from the nucleus to the cytoplasm and from the plasma membrane receptors via the cytoplasm to the nucleus. One of the universal and well-documented effects of ionizing radiation is the production of double-stranded breaks (DSB) in genomic DNA and DSB-induced signaling that targets and activates Ataxia telangiectasia mutated (ATM) kinase in the nucleus followed the initiation of the downstream ATM-mediated signaling pathways [20]. ATM-mediated phosphorylation and stabilization of p53 is one of the critical events in directly irradiated cells, which determines the fate of a cell whether to undergo growth arrest or to cell death via the mitochondrial apoptotic pathway. A general role of ATM/ATR in the regulation of radiation-induced bystander response was described several years ago and was confirmed by the subsequent investigation [21, 22]. However, ATM-p53 signaling pathway was not directly involved in

the initiation of bystander response [19, 22]; furthermore, bystander response was observed for p53-null cells [23].

There is clear evidence that alpha particle irradiation upregulates NF κ B binding activity in both directly irradiated and bystander cells, while Bay 11-7082, a pharmacological inhibitor of IKK/NF κ B, efficiently suppresses this up-regulation and also reduces levels below the basal amount [24]. This inhibitor of NF κ B activity also efficiently downregulates COX-2 and iNOS-expression levels in both directly irradiated and bystander fibroblasts. The NF- κ B-dependent gene expression of *Interleukin 1 beta (IL1B)*, *IL6*, *IL8*, *IL33*, *tumor necrosis factor (TNF)*, and *PTGS2/COX2*, in concert with other NF- κ B-target genes, in directly irradiated human skin fibroblasts resulted in the production of other cytokines and their receptors, as well as COX-2-dependent prostaglandin E2 (PGE2), with autocrine/paracrine functions. These signaling molecules might further activate signaling pathways and target NF- κ B-dependent gene expression in both directly irradiated and nontarget cells, using the plasma membrane receptor-initiated pathways through the cytoplasm into the nucleus.

13.1.10 Relevance of the Nontargeted Response in Radiation Carcinogenesis

Using the radiosensitive *Patched-1^{+/-}* (*Ptch1^{+/-}*) mouse model system that has a defect in radiation-induced activation of the ATR-Chk1 checkpoint signaling pathway, there is evidence that irradiated of the lower half of the animal with a 3-Gy dose of X-rays resulted in the induction of medulloblastoma in the nonirradiated brain tissues [25]. A significant increase in medulloblastoma (39%) occurred in the partial body irradiated heterozygous mice compared to the sham-treated group.

On the basis of human serum analyses, there is clear evidence that plasma clastogenic factors are present many years after radiation exposure from the Japanese atomic bomb survivors, Chernobyl liquidators, and from patients who underwent radiotherapy [26]. To provide a better estimate of the frequency distribution of second primary tumor sites in relation to previous irradiation volumes, a cohort of 115 pediatric patients who developed such cancers was studied [27]. It was estimated that ~22% of these secondarily derived tumors arose from a distance of at least 5 cm from the irradiated site and ~6% arose from a distance that was > 10 cm away, i.e., from out-of-field locations. Although these findings are suggestive, nonetheless, the data highlight the potential of second tumor development outside the treatment field and at much lower dose level.

13.2 Conclusion

In vitro oncogenic transformation assays based on immortalized human bronchial epithelial cells are useful surrogate models in dissecting molecular mechanisms associated with each of the multiple steps involved in radiation carcinogenesis. In an attempt to identify the genes involved in the neoplastic process, cDNA arrays have been used to screen differentially expressed genes between control and radiation-induced tumorigenic cells. The identification of *TGFBI* as a tumor suppressor gene in tumorigenic human bronchial epithelial cells and human lung cancer samples is further confirmed by the availability of the *TGFBI* knock-out mouse model. In the mean time, advances in microbeam technology have resulted in the unequivocal demonstration of an extra-nuclear and an extracellular target in modulating

radiobiological responses. The presence of COX-2 and various inflammatory cytokines in the nontargeted cells/tissues signified the critical role of the stress response in the process. The demonstration of a radiation-induced nontargeted effect in 3D human tissues in whole organism and that the progeny of nontargeted cells shows an increase in genomic instability ([28] for review) highlights the contributions of COX-2 and the inflammatory cascade in radiation carcinogenesis.

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