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Acquired Tumor Cell Radiation Resistance at the Treatment Site Is Mediated Through Radiation-Orchestrated Intercellular Communication

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Abstract

Purpose—Radiation resistance induced in cancer cells that survive after radiation therapy (RT) could be associated with increased radiation protection, limiting the therapeutic benefit of radiation. Herein we investigated the sequential mechanistic molecular orchestration involved in radiation-induced radiation protection in tumor cells.

Results—Radiation, both in the low-dose irradiation (LDIR) range (10, 50, or 100 cGy) or at a higher, challenge dose IR (CDIR), 4 Gy, induced dose-dependent and sustained NFκB-DNA binding activity. However, a robust and consistent increase was seen in CDIR-induced NFκB activity, decreased DNA fragmentation, apoptosis, and cytotoxicity and attenuation of CDIR-inhibited clonal expansion when the cells were primed with LDIR prior to challenge dose. Furthermore, *NFκB* manipulation studies with small interfering RNA (siRNA) silencing or *p50/p65* overexpression unveiled the influence of LDIR-activated NFκB in regulating CDIR-induced DNA fragmentation and apoptosis. LDIR significantly increased the transactivation/translation of the radiation-responsive factors tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α), cMYC, and SOD2. Coculture experiments exhibit LDIR-influenced radiation protection and increases in cellular expression, secretion, and activation of radiation-responsive molecules in bystander cells. Individual gene-silencing approach with siRNAs coupled with coculture studies showed the influence of LDIR-modulated TNF-α, IL-1α, cMYC, and SOD2 in induced radiation protection in bystander cells. NFκB inhibition/overexpression studies coupled with coculture experiments demonstrated that TNF-α, IL-1α, cMYC, and SOD2 are selectively regulated by LDIR-induced NFκB.

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Conclusions—Together, these data strongly suggest that scattered LDIR-induced NF κ B-dependent TNF- α , IL-1 α , cMYC, and SOD2 mediate radiation protection to the subsequent challenge dose in tumor cells.

Introduction

American Cancer Society estimates a total of 1,638,910 new cancer cases will develop in the United States for 2012 (1), and nearly two-thirds of all cancer patients will receive radiation therapy (RT) as part of their treatment plan. RT is used in curative, palliative, and prophylactic treatment plans and is delivered through external beam, internal placement, or systemic administration, depending on the type of cancer and treatment goals (2). The overall goal of RT is to damage as many cancer cells as possible while limiting harm to nearby healthy tissue. Conversely, radiation-induced tumor radiation resistance stands as a fundamental barrier limiting the effectiveness of RT (3). Recent data strongly imply that pre-exposure to low-dose irradiation (LDIR) is able to activate specific proteins that may increase cellular tolerance to subsequent IR injuries (Supplementary Table 1) (4). We have reported a relative adaptive radiation resistance in human breast adenocarcinoma (5) and neuroblastoma (6) cells after fractionated IR (FIR; as opposed to single-dose radiation) and have identified several potential targets that may effect radiation resistance. All of the information suggests that a specific prosurvival signaling network is required for the development of an adaptive response. Studies have demonstrated the activation of transcription factors in cells exposed to IR (7), including NF κ B. We demonstrated that clinically relevant IR upregulates NF κ B-DNA binding activity in many tumor models including neuroblastoma, breast-cancer, pancreatic-cancer, and Ewing sarcoma (5, 6, 8, 9). Recently, we determined the functional orchestration of NF κ B in surviving tumor cells after radiation and validated its influence in tumor relapse (10). Accordingly, in this study, we investigated the influence of sublethal (scattered) radiation in the NF κ B-dependent onset and mechanistic inflow of tumor cell radiation protection. To that end, we elucidated the indispensable role of NF κ B-dependent radiation-responsive tumor necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α), cMYC, and SOD2 in intercellular communication and their sequential orchestration in endorsing radiation protection in surviving tumor cells.

Methods and Materials

Cell culture and irradiation

Human Ewing sarcoma (SK-N-MC), neuroblastoma (SH-SY5Y), and breast (MCF-7, MDA-MB-435, MDA-MB-468), bladder (TCC-SUP, J82), colon/gastric (Colo-205, AGS), prostate (DU-145) and lung (A549) cancer cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured and maintained as described previously (5, 6, 8-10). Exponentially growing cells were exposed to LDIR (2, 10, 50, 100 cGy) or challenge-dose IR (CDIR, 4 Gy) using Gamma Cell 40 Exactor at a dose-rate of 0.81 Gy/min. Irradiated cells were incubated for an additional 1 hour through 72 hours. For LDIR-induced radiation protection, cells were exposed to 10, 50, or 100 cGy, allowed to respond for 24 hours and then exposed to CDIR.

Coculture

Cells cultured in 24-well plates were incubated for 24 hours with LDIR-exposed cells in 0.4- μ m cell culture inserts. For NF κ B-silencing studies, small interfering RNA (siRNA)-transfected cells (after 12 hours) were seeded (on the inserts) and allowed to settle (12 hours) before exposure to LDIR. For TNF- α , IL-1 α , cMYC, and SOD2 silencing studies, LDIR-exposed cells on inserts were incubated with TNF- α , IL-1 α , cMYC, or SOD2 muted (with gene specific siRNA) cells for 24 hours that were then exposed to CDIR.

Electrophoretic mobility shift assay

Nuclear protein extraction, electrophoretic mobility shift assay (EMSA), and specificity assays were performed as described in our previous studies (11).

QPCR

TNF- α , *IL-1 α* , *cMYC*, and *SOD2* transcriptional alterations after LDIR, CDIR with/without LDIR-priming, and gene-specific siRNA silencing and after manipulating LDIR-induced NF κ B were analyzed by real-time quantitative polymerase chain reaction (QPCR) as described previously (11). A positive control β -actin and a negative control without template RNA were also included.

Immunoblotting

Total protein extraction and immunoblotting were performed as described in our previous studies (12). For this study, the protein-transferred membranes were incubated with rabbit anti-IAP1, anti-IAP2, anti-survivin, anti-SOD2, and anti-TNF- α or cMYC.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed as described in our previous studies (13). In this study, ELISA was performed in conditioned medium from cells exposed to CDIR with/without LDIR priming and were concentrated using 9KD-ICON (Thermo Scientific, Rockford, IL) concentrators.

Cell viability

Trypan blue dye exclusion coupled with Countess automated cell counting was used to identify the cell viability after LDIR and CDIR with/without LDIR priming as described previously (9).

Cell survival by MTT and clonogenic assay

Cell survival was analyzed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and clonogenic assays as described in our previous studies (11).

DNA fragmentation

DNA fragmentation analysis was performed using a fluorescein Fragel DNA fragmentation detection kit as previously described (14).

Apoptosis detection

Alterations in apoptosis was analyzed on a single-cell basis by flow cytometry using Aposcreen annexin V-fluorescein isothiocyanate (FITC) conjugate as described in our previous studies (9).

Caspase activity

To determine the modulations in the activity levels of caspases-3/-7, we used SensoLyte homogenous AMC caspase-3/-7 assay system as described in our previous studies (9).

SOD2 activity

SOD2 activity analysis was performed using a superoxide dismutase assay kit as described in our previous studies (15).

Plasmid preparation and DNA transfection

Transient transfection of NF κ B p65/p50 subunits was carried out as described previously (12, 16). Inhibition of NF κ B, SOD2, TNF- α , IL-1 α and cMYC was achieved using gene-specific siRNAs (150 ng) as described in our previous studies (12). For ecotopic inhibition of NF κ B, cells were transiently transfected with the dominant negative mutant (S32A/S36A double mutant) I κ B α (I κ B α).

Results

LDIR instigates NF κ B-dependent survival advantage

In order to delineate whether LDIR instigated NF κ B-dependent survival advantage, we sequentially determined whether LDIR (1) activates/maintains NF κ B activity, (2) activate NF κ B-dependent downstream survival molecules, and (3) promotes tumor cell survival/clonal expansion. LDIR-induced a significant and dose-dependent NF κ B DNA-binding activity (Fig. 1A). Competitor-binding assay recognizes sequence-specific binding of NF κ B (Fig. 1B). Immunoblotting displayed a dose-dependent I κ B α phosphorylation in LDIR-exposed cells (Fig. 1C), and this correlated well with NF κ B-activity results. Furthermore, EMSH revealed consistently high levels of NF κ B activity from 3 hours through 24 hours (Fig. 1D), demonstrating that LDIR-induced NF κ B was persistent and not a rapidly reversible process. LDIR significantly transactivated (QPCR) *IAP1*, *IAP2*, *Survivin*, and *Bcl2* at 24 hours post-IR (Fig. 1E). Coherently, immunoblotting demonstrated a significant increase of IAP1, IAP2, and surviving after all doses of LDIR (Fig. 1F). MTT analysis revealed a robust ($P<.001$) increase in cell survival after LDIR was examined (Fig. 1G). Furthermore, clonogenic assay displayed an increase in clonal expansion in LDIR-exposed cells (Fig. 1H). Together, these results demonstrate the flow-through of LDIR-induced NF κ B-dependent IAP-mediated survival advantage.

LDIR-induced NF κ B activates radiation protection

Armed with the evidence that LDIR activates NF κ B-dependent survival advantage, we investigated its role in radiation protection for subsequent CDIR. To validate any measurable radiation protection involved, we used 4 Gy (double the clinical radiation dose)

as CDIR. To define the NF κ B role in LDIR-induced radiation protection, cells exposed to CDIR with/without LDIR priming were examined for alterations in NF κ B 1 hour post-CDIR. CDIR induced NF κ B-DNA binding activity and served as the positive controls for the study (Fig. 2A). However, LDIR-priming studies showed LDIR dose-dependent increases in CDIR-induced NF κ B (Fig. 2A). Furthermore, LDIR-primed cells (10 cGy, to validate the possibility of very low scattered-dose in promoting radiation protection) exposed to CDIR showed robust and sustained (3 hours through 3 days) increase in NF κ B activation (Fig. 2B). CDIR induced significant ($P<.001$) DNA fragmentation at as early as 1 hour (Fig. 2C) and remained consistent through 48 hours. Conversely, LDIR priming completely ($P<.001$) and consistently hampered the CDIR-induced DNA fragmentation (Fig. 2C and D). Similarly, CDIR resulted in a robust increase in apoptosis. However, LDIR priming prior to CDIR significantly muted the induced apoptosis (Fig. 2E). Moreover, CDIR significantly induced caspase activity, and priming with LDIR extensively inhibited CDIR caspase-3/-7 activity (Fig. 2F). Caspase activity, apoptosis, and DNA fragmentation results correlated well with increased NF κ B, suggesting LDIR induced NF κ B-dependent inhibition of apoptosis. Substantiating this NF κ B cause—effect, NF κ B-overexpressed cells exposed to CDIR showed complete ($P<.001$) and consistent (up to 48 hours) inhibition of CDIR-induced DNA fragmentation (Fig. 2D). Coherently, we observed a significant inhibition of CDIR-induced apoptosis in NF κ B-overexpressed cells (Fig. 2E). Next, NF κ B muted (with siRNA) cells primed with LDIR followed by CDIR significantly increased the LDIR-priming associated inhibition of CDIR-induced DNA fragmentation (Fig. 2D) and apoptosis (Fig. 2E). Furthermore, LDIR priming reverted the CDIR-inhibited cell survival both after 3 hours and 24 hours post-CDIR (Fig. 2G). Likewise, LDIR-priming resulted in an increased clonal expansion as opposed to CDIR alone (Fig. 1H).

LDIR induces radiation-responsive SOD2, cMYC, IL-1 α and TNF- α

To determine the NF κ B-dependent functional orchestration of signal transduction in induced radiation protection, LDIR-exposed cells were examined for activation of effector molecules (SOD2, cMYC, IL-1 α , TNF- α) at 24 hours post-IR. Conversely, LDIR profoundly increased *TNF- α* , *IL-1 α* , *SOD2*, and *cMYC* (Fig. 3A). LDIR resulted in robust (>5-fold) *SOD2* and *TNF- α* transactivation. Immunoblotting revealed that LDIR resulted in significant induction of SOD2, TNF- α , IL-1 α , and cMYC and remained consistent up to at least 48 hours (Fig. 3B, Supplementary Fig. 1A).

LDIR activates radiation-responsive molecules and promotes a survival advantage in bystander cells against CDIR

To determine whether LDIR-induced radiation protection indeed expands to neighboring bystander cells, we examined the expression/secretion and activity of radiation-responsive molecules and associated radiation protection in CDIR-exposed cells cocultured with LDIR-exposed cells. Although CDIR-exposure showed marginal variations in SOD2 activity at early stages (1-6 hours), we observed a profound inhibition from 12 hours through 48 hours (Fig. 3C). However, under coculture conditions, the CDIR-regulated SOD2 activity was greatly induced and remained consistent (through 48 hours) (Fig. 3C). CDIR exposure exerted notable reduction in TNF- α secretion after 48 and 72 hours (Fig. 3D). However, CDIR-exposed cells cocultured with LDIR-treated cells significantly induced soluble TNF-

α secretion (Fig. 3D). Consistently, immunoblotting showed a marked increase in intracellular TNF- α in CDIR-exposed cells cocultured with LDIR-exposed cells (Fig. 3E, Supplementary Fig. 1B). Also, we observed similar increase in cellular expression levels of SOD2, cMYC, and IL-1 α in CDIR-exposed cells cocultured with LDIR-exposed cells (Fig. 3E). Evidently, cells cocultured with LDIR-exposed cells and then challenged with 4 Gy showed increased cell viability (Fig. 3F). Cells cocultured with LDIR-exposed cells and then exposed to CDIR showed robust increase in cell survival as opposed to cells exposed to CDIR alone (Fig. 3G). This LDIR-induced paracrine activation of cell survival remained consistent from 3 hours through 72 hours post-CDIR. Consistently, we observed this LDIR-activated IL1- α , RelA, and TNF- α transcription in nontargeted bystander cells across other tumor models including neuroblastoma (SH-SY5Y), breast (MCF-7, MDA-MB-435, MDA-MB-468), bladder (TCC-SUP, J82), colon/gastric (Colo-205, AGS), prostate (DU-145), and lung-cancer (A549) cells (Fig. 3).

LDIR-induced NF κ B mediates TNF- α , SOD2, cMYC, IL-1 α activation in bystander cells

Equipped with the datum that LDIR induces radiation protection in bystander cells, we further investigated whether LDIR-induced NF κ B mediates the decisive effect. To achieve this, cells cocultured with NF κ B-silenced LDIR-exposed cells were then subjected to CDIR and analyzed after 24 hours for *TNF- α* , *SOD2*, *cMYC*, and *IL-1 α* . For radiation protection controls, we used cells cocultured with LDIR-exposed cells and subjected them to CDIR. QPCR revealed robust *TNF- α* , *SOD2*, *cMYC*, and *IL-1 α* transactivation in bystander cells cocultured with LDIR-exposed cells and subjected to CDIR (Fig. 4A). Conversely, muting NF κ B significantly inhibited LDIR-induced *TNF- α* , *SOD2*, *cMYC*, and *IL-1 α* transactivation in bystander cells challenged with CDIR (Fig. 4A), demonstrating that LDIR-induced NF κ B mediates *TNF- α* , *SOD2*, *cMYC*, and *IL-1 α* signaling in the bystander cells. Furthermore, bystander cells cocultured with NF κ B-overexpressed cells, which were then exposed to CDIR, resulted in profound induction of CDIR regulated SOD2 activity (Fig. 4B), signifying the role of NF κ B in this setting.

LDIR-induced NF κ B-dependent TNF- α , SOD2, cMYC, and IL-1 α in bystander cells mediates radiation protection

Silenced *TNF- α* , *SOD2*, *cMYC*, and *IL-1 α* cells cocultured with LDIR-exposed cells and subjected to CDIR were analyzed for altered DNA fragmentation, apoptosis, and cell survival. Mock-transfected cells cocultured with LDIR-exposed cells and subjected to CDIR were used as positive control. Muting TNF- α in bystander cells significantly recovered LDIR-hampered CDIR-induced DNA fragmentation (Fig. 4C). Consistently, gene-specific silencing of IL-1 α , SOD2, and cMYC considerably recuperated LDIR-blocked CDIR-induced DNA fragmentation (Fig. 4C). Likewise, silencing TNF- α , IL-1 α , SOD2, and cMYC effectively convalesced LDIR-impaired CDIR-associated apoptosis (Fig. 4D). MTT analysis revealed a significant inhibition of LDIR-induced cell survival in *TNF- α* , *SOD2*, *cMYC*, or *IL-1 α* muted cells exposed to CDIR (Fig. 4E).

Discussion

The results show that sublethal LDIR activates NF κ B activation in surviving and in nonirradiated bystander tumor cells through collaborative actions of two mechanisms: increased activation of NF κ B with subsequent radiation, which leads to stimulation of de novo synthesis of IAPs in surviving tumor cells; and stimulation of SOD2, cMYC, IL-1 α , or TNF- α function, which leads to bystander cell survival stabilization. Our previous work has suggested that IR-induced NF κ B in surviving tumor cells is a critical determinant of overall response to RT (5, 6, 14) and further demonstrated that selectively targeting IR-induced NF κ B alleviated radiation resistance in many tumor models (8, 9, 11, 17-19). Therefore, targeting the NF κ B pathway involved in the secretion of radiation-responsive factors such as SOD2, cMYC, IL-1 α or TNF- α may prove a promising strategy to mitigate IR-induced radiation protection. Recently, we showed that IR-triggered NF κ B orchestrated a TNF- α -dependent feedback that maintains NF κ B in the system which, in turn, mediates IAPs-dependent survival advantage (10). To that end, a plethora of evidence in multifarious tumor models implicated NF κ B in initiation, flow-through and/or functional orchestration of radiation-protective response (Supplementary Table 1) (20). However, these studies are focused on understanding (1) the mechanisms involved in intrinsic radiation resistance of select tumor systems, (2) the molecular signal transduction in targeted radiation sensitization and, very few studies (including from our group) dissecting out (3) the induced radiation protection in response to clinical doses of IR. Outcomes of these investigations portrayed the signal transduction(s) and associated functional responses pertaining only to surviving tumor cells and to clinical dose of IR. To our knowledge, this is the first study that demonstrates the influence of scattered, sublethal IR-induced radiation protection and, furthermore, delineates the mechanistic molecular orchestration involved in both surviving and bystander tumor cells.

Focusing on the mechanisms for radiation protection in cancer cells, we observed that treatment with clinical mimicking of scattered (sublethal) doses of IR (2, 10, 50, or 100 cGy) markedly induced post-translational modification of I κ B α -mediated NF κ B activation, which increased the NF κ B-dependent transactivation/translation of IAPs and subsequent cell survival and clonal expansion. We further showed that priming cells with LDIR significantly increased subsequent CDIR-induced NF κ B. CDIR (4 Gy) did not induce robust NF κ B activation but rather provoked extensive cell death. However, concomitant scattered dose priming reverted this CDIR-induced DNA fragmentation cell death but rather stabilized cell survival and provoked clonal expansion. Furthermore, NF κ B overexpression studies with CDIR exposure produced cell death, survival, and clonal expansion effects comparable to that of LDIR priming. Likewise NF κ B silencing studies with LDIR priming followed by CDIR produced cell death, survival, and clonal expansion effects comparatively equivalent to that of cell exposed only to CDIR. Therefore, it is likely that scattered dose radiation-induced increase in NF κ B expression could contribute to induce radiation protection to the subsequent radiation exposure.

It has been known that NF κ B-dependent gene expression in directly irradiated cells resulted in producing the cytokines and their receptors, with autocrine/paracrine functions (21, 22). As a result of paracrine functions of effector molecules, which were produced by directly

irradiated cells through activation of receptor-mediated pathways, bystander cells also express signal transduction molecules followed, again, by autocrine/paracrine stimulation of NF κ B and other pathways (23). Recently, we demonstrated that radiation-induced NF κ B-dependent TNF- α initiates an autocrine/paracrine second-signaling feedback that maintains NF κ B and promotes survival advantage and clonal expansion (10). Furthermore, we and others have shown that blocking TNF- α functions with TNF- α /TNF-R1 antibodies decreased NF κ B activation both in irradiated and bystander cells, confirming the existence of autocrine/paracrine feedback loop and NF κ B-dependent gene expression in both irradiated and nontargeted cells (10). Our ongoing efforts to determine the basics of radiation protection in cancer cells have provided evidence that scattered sublethal doses of radiation plays a major and definite role in induced radiation protection. We observed that (1) LDIR-increased NF κ B activation translates downstream IAPs and promotes survival advantage and clonal expansion; (2) treatment with LDIR resulted in robust and sustained increase in CDIR-activated NF κ B, leading to effectively reduced radiation-induced tumor cell death and increased survival/clonal expansion; (3) LDIR treatment induced the radiation-responsive second-signaling effector molecules TNF- α , IL-1 α , SOD2, and cMYC; (4) translation of TNF- α , IL-1 α , SOD2, and cMYC in nontargeted cells after LDIR coculture caused increased cell survival; (5) LDIR-induced NF κ B mediated the translation of TNF- α , IL-1 α , SOD2, and cMYC in nontargeted cells; and (6) the translation of TNF- α , IL-1 α , SOD2, and cMYC in bystander cells serves as the effector molecules and are absolutely needed for the induced radiation protection.

These findings provide a significant step toward the basic understanding of tumor cell radiation protection and also throw light on the molecular blueprint that underlies induced radiation protection. However, we acknowledge the limitations in the current study: (1) observations only in in vitro settings, (2) observations of bystander signal transduction only from and to cancer cells. To that end, we will extend our findings to dissect out bystander signaling from nontumor cells such as endothelial and/or fibroblasts by using appropriate, clinically translatable animal models.

Conclusions

In conclusion, our findings provide a strong rationale for establishing therapeutic strategies for the use of inhibitors of NF κ B or IKK in combination with RT. In this perspective, the benefit of the tumor-targeted IKK β inhibitor in potentiating RT with appropriate preclinical in vivo models are warranted and are currently in progress in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Summary

Clinical radiation therapy-associated bystander response in surviving tumor cells plays a definite role in tumor relapse and dissemination. This study identified the molecular blueprint that underlies low-dose (scattered) radiation that drives the radiation resistance. By exploiting complex bystander approach coupled with clinically mimicking step-wise radiation schemes and selective gene(s) manipulations, this study demonstrated the low-dose radiation-induced NF κ B-driven intercellular communication and intricate mechanistic inflow that endorses radiation protection in bystander tumor cells.

Fig. 1.

(A) NFκB DNA-binding activity in cells exposed to mock IR or LDIR and analyzed after 1 hour. Densitometry shows LDIR-induced dose-dependent increase in NFκB activity. (B) Histograms show specificity of DNA-binding activity. (C) Western blot shows levels of phosphorylated IκBα after 1 hour in cells exposed to LDIR. Densitometry shows dose-dependent induction of pIκBα in LDIR-exposed cells. (D) Kinetics of NFκB DNA-binding activity (EMSA) in cells exposed to LDIR at 3 to 24 hours post-IR. Densitometry shows LDIR-induced consistent increases in NFκB activity. (E) *cIAP1*, *cIAP2*, survivin, and *Bcl2* transactivation (QPCR) in cells exposed to LDIR at 24 hours post-IR. (F) Increased expression levels are shown of IAP1, IAP2, and survivin in cells exposed to LDIR after 24 hours. Densitometry shows induction of IAP1, IAP2, and survivin in cells exposed to LDIR. (G) MTT analysis shows significant increase in survival in cells exposed to 10, 50, or 100 cGy in contrast to mock irradiation. (H) Computed colony counting (Image Quant) shows clonogenic activity of cells either mock irradiated or exposed to LDIR. EMSA = electrophoretic mobility shift assay; IR = ionizing radiation; LDIR = low-dose irradiation; MTT = tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; QPCR = quantitative realtime polymerase chain reaction.

Fig. 2.

(A) NFκB DNA binding activity in cells exposed to CDIR (4Gy) or primed with LDIR (10, 50, or 100 cGy) followed by CDIR is shown. Densitometry showed increased NFκB DNA binding activity after CDIR. However, LDIR priming robustly increased CDIR-induced NFκB. (B) Kinetics of LDIR priming-associated increase in CDIR-induced NFκB DNA-binding activity after 3 hours through 72 hours post-CDIR are shown. Densitometry shows LDIR-associated robust increase in CDIR-induced NFκB activity remained consistent at least up to 72 hours. (C) Flow cytometry shows complete inhibition of CDIR-induced DNA fragmentation in cells primed with LDIR. (D) Flow cytometry shows the influence of LDIR priming-induced NFκB in inhibiting CDIR-induced DNA fragmentation. NFκB overexpression prevented CDIR-induced DNA fragmentation. Likewise, muting LDIR-induced NFκB brought back CDIR-induced DNA fragmentation consistently up to 48 hours post-CDIR. (E) Flow cytometry of annexin V-FITC staining shows modulation in apoptosis in cells exposed to CDIR with/without LDIR priming or NFκB overexpression and in NFκB-mutated LDIR-primed cells exposed to CDIR. (F) Alterations in caspase-3 and -7 activity in cells exposed to CDIR with/without LDIR priming. (G) MTT analysis shows cell survival after CDIR with/without LDIR priming. (H) Clonogenic activity of cells exposed to CDIR with/without LDIR priming is shown. CDIR = challenge-dose irradiation; FITC = fluorescein isothiocyanate; LDIR = low-dose irradiation; MTT = tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Fig. 3.

(A) *TNF-α*, *IL-1α*, *SOD2*, and *cMYC* transactivation (QPCR) in LDIR-exposed cells at 24 hours post-LDIR. (B) Immunoblots show expression levels of *TNF-α*, *IL-1α*, *SOD2*, and *cMYC* in cells exposed to LDIR. Densitometry shows significant induction of *TNF-α*, *IL-1α*, *SOD2*, and *cMYC* in cells after LDIR. (C-G) Coculture experiments show LDIR-induced translation of *TNF-α*, *IL-1α*, *SOD2*, and *cMYC* in bystander cells and associated radiation protection. (C) SOD2 activity in cells exposed to CDIR with or without LDIR priming is shown. Coculturing CDIR and LDIR-exposed cells significantly induced CDIR-regulated SOD2 activity in bystander cells. (D) ELISA shows secreted *TNF-α* in cells exposed to CDIR with/without coculturing with LDIR-treated cells. Coculturing significantly increased *TNF-α* secretion in bystander cells. (E) Immunoblots show expression levels of *TNF-α*, *IL-1α*, *SOD2*, *pIκBα*, and *cMYC* in cells exposed to CDIR with/without coculturing with LDIR-treated cells. (F) Cell viability in cells exposed to CDIR with/without coculturing with LDIR-treated cells is shown. (G) Cell survival is shown in cells exposed to CDIR with/without coculturing with LDIR-treated cells. (H) LDIR-induced *TNF-α*, *IL-1α*, and *RelA* transactivation (QPCR) in nontargeted bystander cells across tumor models, including neuroblastoma (SH-SY5Y), breast (MCF-7, MDA-MB-435, MDA-MB-468), bladder (TCC-SUP, J82), colon/gastric (Colo-205, AGS), prostate (DU-145), and lung (A549) cancer cell lines. LDIR = low-dose irradiation; QPCR = quantitative realtime polymerase chain reaction.

Fig. 4.

(A) LDIR-induced NF κ B mediates *TNF- α* , *SOD2*, *cMYC*, and *IL-1 α* transactivation in bystander cells. Cells cocultured with NF κ B-silenced, LDIR-exposed cells were then exposed to CDIR and analyzed 24 hours post-LDIR. Silencing LDIR-induced NF κ B inhibited LDIR priming-associated increases in *TNF- α* , *SOD2*, *cMYC*, and *IL-1 α* in bystander cells. (B) LDIR-induced NF κ B mediates increased SOD2 activity in cocultured CDIR-exposed bystander cells. (C-E) LDIR-translated TNF- α , SOD2, cMYC, and IL-1 α mediated radiation protection in bystander cells. Gene-specific knockout cells cocultured with/without LDIR were then exposed to CDIR and analyzed for (C) DNA fragmentation, (D) apoptosis, and (E) cell survival. Knocking out radiation-responsive TNF- α , SOD2, cMYC, and IL-1 α in bystander cells profoundly reverted LDIR priming-inhibited DNA fragmentation and apoptosis and inhibited LDIR priming-induced cell survival. CDIR = challenge-dose irradiation; LDIR = low-dose irradiation.