Radiation-triggered Tumor Necrosis Factor (TNF) α-NFκB Cross-signaling Favors Survival Advantage in Human Neuroblastoma Cells*§▼

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Induced radioresistance in the surviving cancer cells after radiotherapy could be associated with clonal selection leading to tumor regrowth at the treatment site. Previously we reported that post-translational modification of IκBα activates NFκB in response to ionizing radiation (IR) and plays a key role in regulating apoptotic signaling. Herein, we investigated the orchestration of NFκB after IR in human neuroblastoma. Both in vitro (SH-SY5Y, SK-N-MC, and IMR-32) and in vivo (xenograft) studies showed that IR persistently induced NFκB DNA binding activity and NFκB-dependent TNFα transactivation and secretion. Approaches including silencing NFκB transcription, blocking post-translational NFκB nuclear import, muting TNF receptor, overexpression, and physiological induction of either NFκB or TNFα precisely demonstrated the initiation and occurrence of NFκB → TNFα → NFκB positive feedback cycle after IR that leads to and sustains NFκB activation. Selective TNF-dependent NFκB regulation was confirmed with futile inhibition of AP-1 and SP-1 in TNF receptor muted cells. Moreover, IR increased both transactivation and translation of Birc1, Birc2, and Birc5 and induced metabolic activity and clonal expansion. This pathway was further defined to show that IR-induced functional p65 transcription (not NFκB1, NFκB2, or c-Rel) is necessary for activation of these survival molecules and associated survival advantage. Together, these results demonstrate for the first time the functional orchestration of NFκB in response to IR and further imply that p65-dependent survival advantage and initiation of clonal expansion may correlate with an unfavorable prognosis of human neuroblastoma.

Neuroblastoma (NB),2 the most frequent extra cranial solid tumor in children (aged ≤5 years) accounts for 8–10% of all childhood cancers (1) and 15% of childhood cancer fatalities. To that note, NB recurrences remain high (20.2%), and a substantial fraction (46.8%) of those develop metastatic disease. With only 13 months from first diagnosis to recurrence, the survival ratio was 43% for local and 10% for systemic recurrences. Clinical and laboratory evidence suggests that several human cancers contain populations of rapidly proliferating clonogens that can have substantial impact on local control following chemoradiation or radiotherapy (RT) (2). Tumor cell repopulation may arise from remnant cells of the original neoplasm that have escaped therapeutic intervention and later become visible at the original site. RT is now widely used for high risk NB patients after chemotherapy, and the survival rate is significantly improved (76%) by using RT with chemotherapy alone (46%) (3). Overall goal of the RT is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue, a major complication with RT. Conversely, ionizing radiation (IR)-induced neoplasms occur at the edges of the irradiated field, where the IR does not cause cell death but is sufficient to induce malignant transformation (4). Unfortunately, these treatment-induced tumors are often more aggressive than primary tumors and are highly refractory to therapy (5).

IR has been shown to activate various transcription factors including NFκB (6), and studies have suggested their influential role in tumorigenesis (7). NFκB is a member of the c-rel protooncogene family found within the promoter and enhancer region of a wide variety of genes involved in proliferation, apoptosis, inflammation, differentiation, and cell cycle control (8, 9). Unlike other inducible transcription factors, a multitude of conditions/agents can activate NFκB, and elevated NFκB activity has been linked with tumor resistance to chemotherapy and IR (10). Soon after we first reported that clinically relevant doses of IR induces NFκB (11, 12), innumerable studies both in vitro and in vivo demonstrated that IR specifically activates

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2 The abbreviations used are: NB, neuroblastoma; IR, ionizing radiation; RT, radiotherapy; PFC, positive feedback cycle; Gy, gray(s); TNFR, TNF receptor; Ab, antibody; QPCR, quantitative PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide; FIR, fractionated irradiation; ALLN, N-acetyl-leucyl-leucyl-norleucinal-H; SNP, sodium nitroprusside; IAP, Inhibitor of Apoptosis; H, recombinant human.
NFκB. We identified that IR profoundly activates NFκB in human NB cells (13, 14), leading to induced radioprotection, and further that forced inhibition of NFκB enhanced IR-induced cell death. To that end, disruption of aberrantly regulated survival signaling mediated by NFκB has recently become an important task in the therapy of several chemoresistant/radioresistant cancers (15). However, mechanistic orchestration of NFκB after clinical doses of IR and its functional role in induced survival advantage and/or tumor recurrence is poorly understood.

TNFα has been demonstrated to induce NFκB via receptor activation (16). Details of the NFκB pathways responding to TNFα have been well established (17). Mutual activation of NFκB and TNFα required for the inflammatory response induced by IR has also been suggested (18). TNFα can activate NFκB through TNF receptor associated factors that in turn interact with NFκB-interacting kinase, which plays a key role in cytokine-induced NFκB activation in irradiated cells. Furthermore, ERK activated by TNFα regulates NFκB activation (19, 20) through IκB kinase phosphorylation. To that extent, blocking NFκB has been demonstrated to sensitize cancer cells to TNFα-induced killing (21). Recent evidence suggests that endogenous production of TNFα is a potent trigger of NFκB activation by IR. In addition, molecular cloning analysis has disclosed the presence of one or more putative binding sites for NFκB in the promoter/enhancer region of TNFα (17, 22). Accordingly, we investigated whether the cells of the original neoplasms that have escaped IR insult result in the development of concurrent radioadaptation and survival advantage mediated by persistent activation of NFκB through positive feedback (NFκB → TNFα → NFκB) cycle (PFC). Our data suggests that at least in human NB cells, clinical doses of IR results in the (i) occurrence of NFκB → TNFα → NFκB PFC; (ii) feedback cycle-supported sustained activation of NFκB; (iii) NFκB-dependent regulation of prosurvival IAP1, IAP2, and Survivin; and (iv) NFκB-mediated radioprotection and survival advantage.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human SK-N-MC, IMR-32, and SH-SY5Y cells were obtained from ATCC (Manassas, VA). Culture and maintenance of SK-N-MC cells were performed as described earlier (23). SH-SY5Y cells were maintained as monolayer cultures in DMEM/F-12 50/50 (Mediatech Inc., Herndon, VA) supplemented with 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 1% Nonessential amino acids, 1% minimal essential medium vitamins, 5000 IU/ml penicillin, 5000 μg/ml streptomycin, 1% sodium pyruvate, and 10% FBS (Invitrogen). Likewise, IMR-32 cells were maintained in minimum essential medium supplemented with 1.5 g/L sodium bicarbonate, 5000 IU/ml penicillin, 5000 μg/ml streptomycin, 0.011% sodium pyruvate, and 10% FBS. For passage and for all of the experiments, the cells were detached using trypsin (0.25%) and EDTA (1%), resuspended in complete medium, counted (Countess; Invitrogen), and incubated in a 95% air, 5% CO2 humidified incubator.

**Inhibition/Overexpression and Irradiation Experiments**—For IR-induced sustained activation of NFκB and initiation of NFκB-TNFα PFC, the cells were exposed to 2 Gy using Gamma Cell 40 Exactor (Nordion International Inc., Ottawa, Canada) at a dose rate of 0.81 Gy/min. Mock irradiated cells were treated identical except that the cells were not subjected to IR. Irradiated cells were incubated at 37 °C for an additional 1, 3, 6, 24, 48, and 72 h. All of the experiments were repeated at least three times in each group. For NFκB inhibition studies, the cells were treated for 3 h prior to IR or 1 and 24 h after IR with 50 nm of SN50 NFκB cell-permeable peptide (Calbiochem, La Jolla, CA). Likewise for TNFα inhibition, 100 ng/ml TNFR1 antibody (Santa Cruz Biotech, Santa Cruz, CA) was used. Conversely, for physiological induction of NFκB, the cells were treated with 1 mM SNP (Sigma-Aldrich) or 20 ng/ml of endotoxin-free exogenous human recombinant TNFα (ProSpec-Tany Ltd., Ness-Ziona, Israel).

**Plasmid Preparation, DNA Transfection, and Luciferase Reporter Assay**—Transient transfection of NFκB p65 and p50 subunits was carried out by the lipofection method using Effectene™ reagent (Qiagen) as described in our earlier studies (24). NFκB inhibition was achieved using 150 ng of siRNAs (Qiagen) targeting RelA, NFκB1, NFκB2, and Rel. siRNA mixed with 12 μl of HiPerfect transfection reagent (Qiagen) was incubated for 15 min and slowly added to 80% confluent cells grown in 30-mm plates. After 18 h, transfection medium was replaced with growth medium before IR. Moreover, NFκB inhibition was also accomplished using transient transfection of S32A/S36A double mutant IκBα (ΔIκBα; Upstate Biotechnology, Lake Placid, NY). The mutated form of IκBα with a serine-to-alanine mutation at residues 32 and 36 does not undergo signal-induced phosphorylation and thus remains bound to NFκB, subsequently preventing nuclear translocation and DNA binding. In addition, alterations in IR-induced NFκB promoter activation were investigated in SH-SY5Y and IMR-32 cells treated with TNFR1 Ab or transfected with RelA siRNA. The pNFκB-Luc plasmid construct was amplified and purified as described earlier (22). Cell lysates were assayed for luciferase activity as per the manufacturer’s protocol (Biovision Research Products, Mountain View, CA).

**Electrophoretic Mobility Shift Assay**—Nuclear protein extraction and electrophoretic mobility shift assay were performed as described in our earlier studies (13, 14). For the competition assay, the nuclear extract was preincubated with unlabeled homologous NFκB oligonucleotide followed by the addition of [γ-32P]ATP-labeled NFκB probe. Supershift analysis was performed as described earlier (25).

**Immunoblotting**—Total protein extraction and immunoblotting were performed as described in our earlier studies (26). For this study, the protein-transferred membranes were incubated with either mouse monoclonal anti-pIκBα antibody; rabbit polyclonal anti-IκBα, -cIAP1, -cIAP2, or -TNFα antibody; or Survivin antibody (Santa Cruz). The blots were stripped and rebotted with mouse monoclonal anti-α-tubulin antibody (Santa Cruz) to determine equal loading of samples.

**QPCR**—IR-induced NFκB-dependent regulation of TNFα mRNA expression and persistent activation of NFκB-dependent transcriptional response of cIAP1, cIAP2, and survivin were analyzed by real time QPCR as described earlier (13). Likewise, inhibition of IR-induced p65 transcriptional levels in RelA siRNA transfected SH-SY5Y and IMR-32 cells was validated.
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using QPCR. We used β-actin as a positive control, and a negative control without template RNA was also included. Each experiment was carried out four times, and the ΔΔCT values were calculated by normalizing the gene expression levels to β-actin, and the relative expression level was expressed as a fold change. Group-wise comparisons were made using analysis of variance with Tukey’s post-hoc correction.

ELISA—ELISA was performed as described in our earlier studies (27). In this study, conditioned medium from cells either mock irradiated or exposed to IR was recovered after 10 min through 72 h and concentrated using 9KD ICON concentrators (Thermo Scientific, Rockford, IL). Samples and standards coated on high binding microwell plates were blocked and labeled with human TNFα antibody (Santa Cruz) and tagged with anti-rabbit IgG HRP conjugate (Alpha Diagnostics, San Antonio, TX). TMB substrate was used as a detection system, and the reaction was stopped using 1 N HCl. The absorbance at 450 nm was read on a Synergy II multi-detection microplate reader (Biotek). Cell survival response was compared using analysis of variance with Tukey’s post-hoc correction.

RESULTS

IR-induced Persistent Activation of NFκB—To delineate whether IR persistently induces NFκB activity in NB cells, SK-N-MC, SH-SY-5Y, and IMR-32 cells were both exposed to mock IR or 2 Gy and harvested after 15 min through 72 h (Fig. 1A). IR profoundly induced NFκB DNA binding activity as early as 15 min (253.6 ± 7.4% in SK-N-MC cells) and reached the maximum of 757.7 ± 44, 279.5 ± 44, and 381 ± 41.7 at 1 h in SK-N-MC, IMR-32, and SH-SY5Y cells, respectively. We observed a consistent and persistent induction of the DNA binding activity in response to single dose of IR after 3, 6, 12, and 24 h in NB cells investigated. More importantly, this robust activity was evident even after 48 (349.7 ± 14, 253.8 ± 52.7, and 309.7 ± 53) and 72 h (203.5 ± 3.8, 298 ± 73.14, and 383.1 ± 24.23) in SK-N-MC, IMR-32, and SH-SY5Y cells, respectively (supplemental Fig. S1). Specific binding of NFκB to its sequence-specific oligonucleotide was confirmed with competition binding assay (supplemental Fig. S2A). Moreover, supershift assay revealed that the major subunits in all three cell types are p50 and p65 (supplemental Fig. S2, B–F). The differences seen between the cell types are the magnitude of p50/p65 heterodimers and p50/p50 homodimers. The distance between the heterodimer and homodimer band is varied between different cell types because the electrophoretic runs were performed separately at different time points and because the levels of NFκB in a given amount of total protein varied and changed the exposure time to properly show both bands. Furthermore, immunoblotting revealed relatively reduced levels of constitutive IκBα levels after 1, 3, 24, 48, and 72 h in irradiated SK-N-MC, IMR-32, and SH-SY5Y cells (Fig. 1B). The expression of IκBα decreased at 1 h and continues to be at a reduced level until 24 h after exposure. The IκBα expression again started increasing at 48 and 72 h. This correlates well with the reduced activation of NFκB at that later time points, i.e. 48 and 72 h (Fig. 1A, left panel). Increasing IκBα at a later time point and the corresponding decrease in NFκB activity indicate the feedback cycling of NFκα. Conversely, we observed a consistent induction of IκBα phosphorylation in these cells after IR. To that note, we observed an induction in IκBα phosphorylation immediately (1 h) after IR, and this induced phosphorylation remained consistent at least up to 72 h in all three NB cell lines investigated (Fig. 1B). Once IκBα is phosphorylated, phospho-IκBα undergoes ubiquitination and subsequent degradation. It was not apparent in neuroblastoma cells. These findings
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prompt us to determine in future studies the possibility of deregulated ubiquitination pathway in these cell types. Because the occurrence of phosphorylation of IkBα results in an immediate release of active NFκB and the active NFκB translocates into the nucleus (confirmed by the DNA binding activity of NFκB in nuclear extracts), its downstream function is not affected by the sustained levels of phospho-IκBα in the cytosol.

IR-induced TNFα Transactivation and Sustained Secretion of TNFα—To delineate whether IR-induced alterations in TNFα actively contribute to the induced persistent NFκB activity and subsequent survival advantage, we elucidated whether IR modulates TNFα. The cells exposed either to mock IR or 2 Gy and harvested after 10 min through 72 h were analyzed for alterations in TNFα mRNA. Compared with mock IR, 2 Gy significantly induced TNFα transactivation after 10, 15, and 30 min and 1, 3, 6, 12, and 24 h (Fig. 1C). Furthermore, we observed a sustained and significant (p < 0.001) TNFα mRNA induction after 48 and 72 h in SK-N-MC cells. Secondly, concentrated medium recovered after 10 min through 72 h from SK-N-MC, IMR-32, and SH-SY5Y cells exposed to either mock IR or 2 Gy were examined for secreted levels of TNFα. IR significantly increased secreted levels of TNFα after 10, 15, 30, or 45 min and 1, 3, and 12 h post-IR in SK-N-MC cells. More importantly, significant and sustained induction of secreted TNFα was evident after 24, 48, and 72 h post-IR in all three NB cell lines (Fig. 1D).

IR-induced NFκB-TNFα-NFκB PFC—To precisely identify and validate the occurrence of IR-induced NFκB-TNFα-NFκB PFC, we investigated whether (i) inhibition of TNFα or NFκB disrupts IR-induced PFC and (ii) simulating the IR response by activating NFκB or TNFα initiates PFC. First, to accomplish the disruption of IR induced PFC, IR-induced TNFα was studied after inhibiting NFκB. Inhibition of NFκB in SK-N-MC cells was accomplished using SN50 cell-permeable peptide (50 nM for 3 h), whereas SH-SY5Y and IMR-32 cells were transfected with RelA siRNA. The cells were then exposed to mock IR or 2 Gy and harvested after 15 min through 72 h. Treating SK-N-MC cells with SN50 markedly inhibited IR-induced NFκB DNA binding activity as early as 15 min, and this induced inhibition remained significant consistently at all time points at least up to 72 h (Fig. 2A). More importantly, induced inhibition of NFκB concordantly inhibited secreted TNFα (Fig. 2B). To that end, inhibiting NFκB significantly (p < 0.001) inhibited sustained elevation of NFκB-dependent secreted TNFα in SK-N-MC cells. Coherently, parallel cultures of RelA siRNA transfected SH-SY5Y and IMR-32 cells showed significant inhibition of IR-induced NFκB activity (Fig. 2A). Autorads were overexposed to capture the reduced activities that were lower than mock IR controls. QPCR analysis revealed a significant inhibition of IR-induced NFκB transcriptional activation as early as 1 h and remained consistent at least up to 72 h in RelA siRNA transfected SH-SY5Y and IMR-32 cells (Fig. 3A). Consequently, when IR-induced persistent NFκB activation is muted, we observed a significant (p < 0.001) decrease in secreted TNFα (Fig. 2B). These results clearly elucidate that inhibition of IR-induced NFκB resulted in the sustained suppression of TNFα and thereby demonstrates the initiation of NFκB-TNFα link in NB cells after clinical doses of IR.

Furthermore, to delineate the occurrence of a TNFα-NFκB feedback, we adopted two approaches. First, we investigated the
effect of post-IR-induced NFκB in TNFα transactivation and secretion. To achieve this, human SK-N-MC, SH-SY5Y, and IMR-32 cells were exposed to mock IR or IR (2 Gy) and were incubated at 37 °C for additional 1 or 24 h. The cells were then treated with SN50 and examined for TNFα transactivation (at 1 and 3 h) and secretion (at 24, 48 and 72 h). EMSA analysis showed complete suppression of IR-induced NFκB after both 1 h and 24 h post-IR SN50 treatment (Fig. 3B). Further, this post-IR SN50-induced inhibition of NFκB was sustained at least up to 72 h (Fig. 3B). Coherently, in all three cell lines, we observed a significant inhibition of IR-induced TNFα transactivation after both 1 and 24 h post-IR SN50 treatment (Fig. 3B). Consistently, ELISA analysis revealed a significant (p < 0.001) and sustained (at least up to 72 h) inhibition of TNFα intercellular secretion in all three of the cell lines investigated (Fig. 3D).

Next, we investigated the alterations in IR-induced NFκB after blocking TNFα. Blocking TNFα was achieved by treating the cells with TNFR1 Ab and validated with ELISA. Treatment with TNFR1 Ab completely (p < 0.001) suppressed the IR-induced secreted TNFα in SK-N-MC cells as early as 15 min after IR, and this induced inhibition remained consistent up to 72 h after IR (Fig. 2C). More importantly, blocking TNFα inhibited IR-induced NFκB activity at all time points investigated (Fig. 2C). Autorads were overexposed to capture the reduced activities that were lower than mock IR. Similarly, compared with IR-exposed cells, both SH-SY5Y and IMR-32 cells treated with TNFR1 Ab and exposed to IR showed complete (p < 0.001) inhibition of secreted TNFα after 24, 48, and 72 h (Fig. 2C). Concordantly, silencing TNFα significantly inhibited IR-induced NFκB activity after 1 h through 72 h in both cell lines.
investigated (Fig. 2D). The specificity of NF\(\kappa\)B inhibition with blocking secreted TNF\(\alpha\) (with TNFR1 antibody) was examined by analyzing the alterations in AP1 and SP1-DNA binding activity. SH-SY5Y, IMR-32, and SK-N-MC cells exposed to mock IR or IR or transfected with RelA siRNA and exposed to 2 Gy were analyzed for changes in p65 mRNA levels after 1, 3, 24, 48, and 72 h. \(\beta\)-actin was used as the positive control. The histogram shows the fold change in relation to mock irradiated controls. IR significantly induced p65 transcription in these cells, and these induced p65 levels were significantly and consistently inhibited with RelA siRNA transfection in both cell lines investigated. 

Moreover, to substantiate the occurrence of PFC, IR simulation experiments were performed. First, IR-induced molecular response was simulated with increased NF\(\kappa\)B by overexpressing NF\(\kappa\)B or by treating the cells with physiological inducer, SNP (50 \(\mu\)g/kg). Overexpressing NF\(\kappa\)B subunit significantly induced NF\(\kappa\)B DNA binding activity (Fig. 4A) after 24 h and remained at elevated levels after 48 and 72 h. Immunoblotting revealed an increased p65 and p50 expression in these transfected cells (supplemental Fig. S2F). Consistently, overexpressing NF\(\kappa\)B profoundly induced secreted TNF\(\alpha\) levels in these cells and remained high up to 72 h (Fig. 4B). Likewise, cells exposed to SNP and harvested after 15 min through 72 h...
showed robust NFκB DNA binding activity in SK-N-MC cells (Fig. 4C). Concordantly, SNP-induced NFκB resulted in a marked and significant (p < 0.001) up-regulation in secreted TNFα levels (Fig. 4D) as early as 15 min and remained consistent up to 72 h. These results validate that persistent activation of NFκB in response to a stimuli (in our case clinical doses of IR) initiate a NFκB-TNFα link and subsequent feedback cycle.

Conversely, occurrence of TNFα-dependent feedback was investigated by exposing the cells to exogenous human recombinant TNFα. SK-N-MC cells treated with rH-TNFα and harvested after 15 min through 72 h were first validated for significant (p < 0.001) induction of secreted TNFα (Fig. 4E). Interestingly, TNFα induction significantly activated NFκB in NB cells as early as 30 min (Fig. 4F) and remained persistent up to 72 h. Taken together, these results clearly delineate that IR-induced NFκB initiates the activation of TNFα, and the secreted TNFα in turn activates NFκB and thereby promotes a NFκB-TNFα-NFκB PFC. Moreover, as an end product of this IR-induced PFC, elevated NFκB activity remains persistent and mediates the IR-induced downstream survival response.

NFκB Regulates IR-induced cIAP1, cIAP2, and survivin—SK-N-MC cells exposed to 2 Gy and harvested after 15 min through 24 h were analyzed for the IR-induced transcriptional alterations in cIAP1, cIAP2, and survivin. Compared with mock IR, QPCR analysis revealed a robust and significant induction of cIAP1 mRNA at all time points investigated (Fig. 5A). Furthermore, we observed an elevated level of cIAP2 and survivin in SK-N-MC cells. Consistently, immunoblotting analysis showed an induced level of cIAP1, cIAP2, and Survivin as early as 30 min (Fig. 5B) and reached maximum at 90 min. We observed a unrelenting induction of these proteins at least up to 72 h. Likewise, SH-SY5Y cells exposed to IR showed a significant (p <
0.001) increase in cIAP1 mRNA after 3 h post IR and remained high even after 24, 48, and 72 h (Fig. 5C). Compared with the mock irradiated controls, we observed a significant increase in cIAP2 as early as 1 h, and cIAP2 remained at elevated levels up to 24 h. Only a marginal difference in cIAP2 mRNA was observed after 48 h in these cells. However, after 72 h post IR, the cIAP2 levels were significantly (p < 0.001) induced in SH-SY5Y cells. Similarly, a significant (p < 0.001) and profound increase in survivin mRNA levels was observed after 1, 3, 24, 48, and 72 h (Fig. 5C). Coherent with the mRNA expression data, immunoblotting analysis showed a robust induction of Survivin, cIAP1, and cIAP2 proteins after 24, 48, and 72 h (Fig. 5D). Consistent with expression patterns observed in SK-N-MC and SH-SY5Y cells, QPCR analysis showed a significant induction of cIAP1, cIAP2, and survivin mRNA levels in IMR-32 cells at all time points investigated (Fig. 5E). Interestingly, both cIAP2 and survivin reached almost baseline levels after 48 h while reaching the maximal levels after 72 h. Conversely, compared with the mock irradiated controls, Western blot analysis showed a robust induction of cIAP1 after 24, 48, and 72 h and of cIAP2 and Survivin after 48 and 72 h post IR in IMR-32 cells (Fig. 5D).

Taken together, these results precisely demonstrate the up-regulation of pro-survival cIAP1, cIAP2, and Survivin after clinical doses of IR in human NB cells.

Furthermore to throw light on the mechanism of IR-induced survival signaling, we investigated whether IR-induced persis-
tent activation of NFκB mediates the expression of cIAP1, cIAP2, and Survivin. First, human SH-SY5Y and IMR-32 cells were transfected with pNFκB-Luc construct that expresses the luciferase reporter gene in an NFκB-dependent manner. It contains four tandem copies of the NFκB consensus sequence fused to a TATA-like promoter region of the herpes simplex virus thymidine kinase promoter. Binding of NFκB to the promoter activates transcription, allowing the Luc reporter gene to be expressed. Transfected cells were then mock irradiated, exposed to 2 Gy, treated with TNFR1 Ab, and exposed to 2 Gy or co-transfected with RelA siRNA and exposed to 2 Gy. The luciferase assay was performed in extracts obtained from cells harvested after 24 and 72 h. Compared with 2 Gy, silencing IR-induced NFκB completely suppressed clonal expansion. Moreover, inhibition of IR-induced NFκB-dependent TNFα revealed robust inhibition of clonogenic activity in SK-N-MC cells.

![Figure 6](http://www.jbc.org/)

**Figure 6.** IR induced NFκB dependent survival advantage. A and B, MTT analysis showing survival response in NB cells exposed to 2 Gy after 24, 48, and 72 h (A) or after forced inhibition of IR-induced NFκB or TNFα (B). C, clonogenic analysis of SK-N-MC cells either mock irradiated or exposed to 2 Gy. D, histograms showing colony forming capacity of SK-N-MC cells exposed to IR, transfected with ΔIkBα, and exposed to IR or treated with TNFR1 Ab and exposed to IR. Compared with 2 Gy, silencing IR-induced NFκB completely suppressed clonal expansion. Moreover, inhibition of IR-induced NFκB-dependent TNFα revealed robust inhibition of clonogenic activity in SK-N-MC cells.
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To assess the IR-induced survival advantage and to delineate the influence of NFκB activity in mediating the induced survival response, we examined the induced modulations in both metabolic and clonogenic activity of the cells. To determine the changes in cell survival, SK-N-MC, SH-SY5Y, and IMR-32 cells were exposed to 2 Gy and examined using MTT analysis after 24, 48, and 72 h. Compared with the mock irradiated cells, IR significantly reduced cell survival after 24, 48, and 72 h post IR in all three cell lines investigated (Fig. 6A). However, compared with the 2 Gy exposed cells, forced inhibition of NFκB (through RelA siRNA transfection) completely (p < 0.001) inhibited the cell survival after 24, 48, and 72 h in all three cell lines investigated, demonstrating that inhibition of IR-induced NFκB significantly enhances IR-induced cell killing (Fig. 6B). Further, we investigated whether inhibition of other subunits of NFκB has similar effect in cell killing. To achieve this, SH-SY5Y, IMR-32, and SK-N-MC cells transfected with NFκB1, NFκB2, or Rel siRNA were exposed to 2 Gy and examined for cell survival after 24, 48, and 72 h. Interestingly, we did not observe any significant difference in cell survival in these transfected cells as opposed to 2 Gy exposure (Fig. 7). Furthermore, inhibition of IR-induced NFκB-dependent TNFα (with TNFR1 Ab) profoundly enhanced the IR-induced cell killing after 24, 48, and 72 h in SK-N-MC, SH-SY5Y, and IMR-32 cells, suggesting that IR-induced NFκB-initiated NFκB-TNFα-dependent persistent activation of NFκB mediates survival advantage. Abrogation of the induced feedback cycle inhibits the survival advantage and promotes radiosensitivity. To substantiate these findings, human SK-N-MC cells either mock irradiated or exposed to 2 Gy were seeded after 24, 48, and 72 h to assess the colony forming capacity (Fig. 6C). Compared with the mock irradiated cells, IR significantly (p < 0.001) induced clonogenic activity after 24, 48, and 72 h (Fig. 6D). Conversely, compared with the 2 Gy exposed cells, forced inhibition of IR-induced NFκB completely suppressed (p < 0.001) the colony forming capacity, demonstrating that NFκB regulates IR-induced survival advantage (Fig. 6D). Moreover, inhibition of IR-induced NFκB-dependent TNFα revealed robust inhibition of clonogenic activity in human SK-N-MC cells, flaunting that disruption of IR-induced feedback cycle inhibits induced survival advantage.

IR-induced NFκB-TNFα-dependent Persistent Activation of NFκB Mediates Survival Advantage in Xenograft—To substantiate our in vitro findings, human SK-N-MC xenografts developed in athymic nude mice (Fig. 8A) that were either mock irradiated, exposed to FIR (2 Gy five times), treated with SNP and exogenous rH-TNFα, treated with ALLN or TNFR1 Ab, and exposed to FIR were examined for modulation in NFκB DNA binding activity, TNFα expression, and alterations in pro-survival cIAP1, cIAP2, and Survivin. EMSA analysis revealed that compared with mock irradiated control, FIR significantly induced NFκB DNA binding activity in FIR-exposed xenografts after 3 days (Fig. 8B). Conversely, FIR-induced NFκB activity was significantly suppressed in ALLN-treated animals. Similarly, forced inhibition of IR-induced NFκB-dependent TNFα with TNFR1 Ab profoundly inhibited IR-induced NFκB DNA binding activity in the tumors. Consistently, QPCR analysis revealed a marked increase in cIAP1, cIAP2, and survivin mRNA in the xenografts exposed to FIR (Fig. 8C). Similarly we observed a significant induction of cIAP1, cIAP2, survivin, and TNFα mRNA levels in xenografts exposed to exogenous rH-TNFα or NFκB physiological inducer SNP, suggesting that NFκB induction in tumor initiates a link with downstream TNFα and subsequently induces survival advantage. Conformingly, when we inhibited the FIR-induced NFκB with ALLN or TNFα with TNFR1 antibody, FIR-induced cIAP1, cIAP2, and survivin were significantly inhibited in the xenografts (Fig. 8C). Furthermore, immunoblotting analysis revealed a consistent increase in TNFα expression in xenografts exposed to FIR, SNP, or exogenous rH-TNFα (Fig. 8D). To that end, compared with FIR-exposed xenografts, inhibition of FIR-induced NFκB with ALLN significantly inhibited TNFα.

DISCUSSION

NB remains a major therapeutic challenge in pediatric oncology despite the high response rates. The radiobiological consid-
erations predict that the combination of radio and chemotherapy administered as initial treatment for NB would be the optimum clinical strategy. Convincingly, IR delivered to the local NB sites has several well recognized applications including respiratory distress relief for stage IV patients apart from its direct tumoricidal effect. To that note, functional links between cellular signal transduction responses and DNA damage recognition, repair, and cell death have been well recognized. IR is known to induce signal transduction pathways that lead to apoptosis. However, most tumors including NB (31) respond to the effects of IR oppositely by inducing pro-survival signal transduction pathways. Furthermore, we (14, 23, 32) and others (31) have reported an elevated constitutive level of NFκB in NB cells and enhanced NFκB DNA binding activity as an IR response. More importantly, studies have causally linked the induced NFκB activity to the responsiveness to therapy and survival of NB cells (33–35). Together, these observations tie NFκB responsiveness to the tumorigenic behavior of NB, particularly with treatment resistance and relapse. Here, we provide insight into the mechanistic regulation of NFκB in response to IR and its direct role in subsequent survival advantage. The results of the present study clearly elucidated an induced level of NFκB activity as a response to IR, and further we have shown that the induced levels were persistent with no recovery from the altered activation at least for 3 days. However, sustained elevation of NFκB activity has been reported in normal lung tissue in response to IR (36); to our knowledge, for the first time, this study provides evidence of persistent activation of NFκB in tumor cells, in particular, NB cells exposed to single clinically relevant dose of IR.

Moreover, our results implicate IR-induced NFκB mediated initiation of a TNFα-dependent positive feedback mechanism. Consistent with our NFκB activity data, sustained activation of TNFα transcription and intercellular TNFα levels were evident in all three NB cell lines investigated. TNFα, produced originally by activated T cells and macrophages, has been demonstrated to induce NFκB via receptor activation (16). Details of the NFκB pathways responding to TNFα have been well estab-
NFκB Regulation in Induced Radioresistance

A mutual activation of NFκB and TNFα is required for the inflammatory response induced by IR has also been suggested (18). TNFα can activate NFκB through TNF receptor associated factors (38) that in turn interact with the downstream NFκB-interacting kinase (39), which plays a key role in cytokine-induced NFκB activation in irradiated cells. Furthermore, ERK activated by TNFα regulates NFκB activation (19, 20, 40) through IκB kinase phosphorylation. To that extent, blocking NFκB has been demonstrated to sensitize cancer cells to TNFα-induced killing (21, 41). Similarly, studies, both in vitro and in vivo, have demonstrated that pretreatment of cells with TNFα resulted in increased NFκB activation (42, 43). Recently, it has been demonstrated that endogenous production of TNFα is a potent trigger of NFκB activation by IR. In addition, molecular cloning analysis has disclosed the presence of one or more putative binding site for NFκB in the promoter/enhancer region of TNFα. Using five independent strategies, our results show that IR-induced NFκB-dependent TNFα is required for the persistent activation of NFκB in NB cells and clearly portrayed the initiation and occurrence of NFκB-TNFα-NFκB feedback mechanism. Regulation of expression of TNFα is a complex process. TNFα is transcriptionally active within minutes and protein production begins within few hours after being initially stimulated with an inducer (in this case radiation exposure). The secreted TNFα lasts as a soluble factor in the culture supernatant for less than an hour. The second synthesis involves receptor binding, activation of NFκB, transcriptional initiation of its promoter, regulation of message splicing, regulation of message turnover, and regulation of translational product as a mature protein (44), which accounts for a lapse in the availability of TNFα in the culture supernatant examined. This pattern of initial induction, lapse in the availability of TNFα, and again secretion of the second synthesis is shown as two-phase induction. Although this type of time-dependent bi-phasic induction is not significantly demonstrated in NFκB activation, findings of TNFα inhibition and NFκB blocking studies clearly demonstrated that the activation of NFκB and the production of TNFα are interdependent regulation that maintains the feedback loop. In addition, NFκB measure by EMSA may decept a clear bi-phasic induction because of the intranuclear availability of pre-existing and newly synthesized NFκB after initiating the cycle. Conformingly, results of in vivo xenograft studies provide evidence that TNFα-dependent feedback mechanism plays a definite role in the sustained induction of NFκB in NB. As shown in supplemental Fig. S3, we outline a pathway in which NFκB activation leads to TNFα-dependent MAPK activity, resulting in NFκB activation through the IκB kinase phosphorylation of IκBα.

Constitutive activation of cell survival signaling pathways is a general mechanism underlying tumor development and resistance to therapy and constitutes a major clinical problem in cancer. Disruption of aberrantly regulated survival signaling mediated by NFκB has recently become an important task in the therapy of several chemoresistant and radioresistant cancers (15). IAPs are expressed at high levels in many tumors and have been reported to contribute to the resistance of cancers to therapy including resistance to radiotherapy (45). In the current study, we provide evidence that clinical doses of IR significantly induced IAPs and Survivin in NB cells. Therefore, our results showed a good correlation in the pattern of increased IAPs and Survivin to that of induced NFκB activity in response to IR. More importantly, our results obtained from two independent strategies demonstrated that IR-induced NFκB mediates the expression of these pro-survival proteins. Because inhibition of caspases by IAPs occurs at the core of the apoptotic machinery (45), therapeutic modulation of IAPs could target a key control point in deciding cell fate. To that end, there is mounting evidence that IAPs determine sensitivity to radiotherapy in human cancers (31, 46–48). Inhibition of Survivin or IAP using antisense oligonucleotides was shown to enhance the efficacy of radiotherapy by reducing survival and increasing apoptosis of lung cancer cells (46, 47). NFκB enhances cell survival by switch on cIAP-1, cIAP-2, XIAP, the FLICE inhibitory protein cFLIP, and members of the Bcl-2 family (Bcl-XL and A1/Bfl-1), as well as TNF receptor associated factors 1 and 2, which dampen pro-apoptotic signals and attenuate the apoptotic response to anticancer drugs and IR (49, 50). Conformingly, we showed that IR-induced NFκB is both necessary and sufficient to activate prosurvival proteins and to limit IR-induced cell death in NB cells. It is tempting to speculate that its sustained and pro-survival activity in NB explains the direct correlation between NFκB expression and unfavorable outcomes in patients receiving radiochemotherapy. It is intriguing that TNFα is generally understood to promote tumor cell death but to possess pro-survival role in NB. These results support the need to define the function of these signaling proteins (in this case TNFα) according to disease and cell type before applying therapeutic strategies that target these proteins.

With respect to NB, for the first time, our data suggest that clinical doses of IR induce NFκB, which initiates a TNFα-dependent positive feedback effect that in turn maintains the elevated levels of NFκB activity for an extended period. This persistent activation of NFκB mediates the IR-induced expression of the cell death inhibitors that enhance the cellular proliferation and survival advantage, thereby limiting the therapeutic potential of IR. Therefore, therapeutic measures that selectively target NFκB-TNFα link and disrupt the IR-induced FDC may mitigate local failure of NB control after radiotherapy.

REFERENCES

1. Bernstein, M. L., Leclerc, J. M., Bunin, G., Brisson, L., Robison, L., Shuster, J., Byrne, T., Gregory, D., Hill, G., Dougherty, G., Scriber, C., Lemieux, B., Tuchman, M., and Woods, W. G. (1992). J. Clin. Oncol. 10, 323–329
2. McGinn, C. J., and Kinsella, T. J. (1992). Semin Oncol. 19 (4 suppl II), 21–28
3. Castellbery, R. P., Kun, L. E., Shuster, J. J., Alshuler, G., Smith, I. E., Nitschke, R., Wharam, M., McWilliams, N., Joshi, V., and Hayes, F. A. (1991). J. Clin. Oncol. 9, 789–795
4. Parisi, M. T., Fahmy, J. L., Kaminsky, C. K., and Malogolowkin, M. H. (1999). Radiographics 19, 283–297
5. Vázquez, E., Castellote, A., Piqueras, I., Ortuño, P., Sánchez-Toledo, J., Nogués, P., and Lucaya, J. (2003). Radiographics 23, 1155–1172
6. Aggarwal, B. B. (2004). Cancer Cell 6, 203–208
7. Abal, M., Planaguma, J., Gil-Moreno, A., Monge, M., Gonzalez, M., Baro, T., García, A., Castelví, J., Ramon, Y., Cajaí, S., Xercavins, J., Alameda, F., and Reventos, J. (2006). Histol. Histopathol. 21, 197–204
8. Baueuler, P. A., and Baltimore, D. (1991) in Molecular Aspects of Cellular Regulation (Cohen, P., and Foulkes, J. G., eds) pp. 409–432, Elsevier/ North Holland Biomedical Press, Amsterdam
9. Lenardo, M. J., and Baltimore, D. (1989) Cell 58, 227–229
Radiation-triggered Tumor Necrosis Factor (TNF) α-NFκB Cross-signaling Favors Survival Advantage in Human Neuroblastoma Cells

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