Neem Leaf Extract Induces Radiosensitization in Human Neuroblastoma Xenograft Through Modulation of Apoptotic Pathway

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Abstract. Induction of apoptosis is directly correlated with the biological effectiveness of ionizing radiation (IR). Accordingly, we investigated the efficacy of neem leaf extract (NLE) on IR-associated apoptotic transcriptional modulation and cell death in neuroblastoma (NB). Materials and Methods: NB xenografts exposed to single dose (SDR, 10 Gy) or fractionated (FIR, 2 Gy/d×5d) with or without NLE were examined for transcriptional activation of 84 apoptotic pathway genes using quantitative polymerase chain reaction. Apoptosis was measured using TdT nick-end labeling. Results: FIR induced 55 and suppressed 10 genes, while SDR induced 49 and suppressed 5 genes. Of these, 46 and 4 genes were commonly up/down-regulated after FIR and SDR. NLE inhibited IR-induced NAIP, BIRC6, BIRC8, NOL3 and enhanced BAK1, BAX, BCL10, CASP1, CASP10 CARD8 and CRADD. Furthermore, NLE conferred FIR- and SDR-induced cell death. Conclusion: These data imply that NLE may exert radiosensitization by activating pro-apoptotic signaling and negating survival signaling and may thus potentiate radiotherapy in NB.

Neuroblastoma (NB), the most frequent extra cranial solid tumors in children (>90% are seen in patients aged ≤5 years), accounts for 8-10% of all childhood cancers (1) and 15% of childhood cancer fatalities (2). Although a dramatic increase in the survival rate has been achieved during the last two decades (53% to 71%), the risk of relapse and propensity to metastasize pose major challenges in the cure of NB. Recurring tumor may arise from remnant cells of the original neoplasm that have evaded therapeutic intervention and later become visible at the original site. To that end, radiotherapy (RT) is now widely used for high-risk NB patients after chemotherapy and the irradiation dosage depends on the age of the child. Traditionally, RT is delivered in multiple 2 Gy fractions (FIR) for 5 days a week to total ionizing radiation (IR) dose of 50-75 Gy in around 5-7 weeks. IR induces genomic instability (3, 4), adaptive radioresistance (5), and apoptotic response (6). Induced radioresistance in cancer cells might be associated with enhanced survival advantage resulting in tumor regrowth at the treatment site (7). Dose rates ranging from 1-2.5 Gy are enough to increase significantly the risk of a second malignant neoplasm (8). Thus, it is imperative to identify radiosensitizing agents that spare normal cells and induce cell death preferentially in tumor cells. To that end, neem (Azadirachta indica), a versatile medicinal plant, possess a wide spectrum of biological activities (9). The antioxidative properties of neem leaf extract (NLE) have been documented both in vitro and in vivo (9-11). Studies have revealed that NLE inhibits the development of experimental carcinogenesis by modulating multiple molecular targets in key signaling pathways (10-14). Azadirachtin and nimbolide, present in leaves and flowers, are recognized as the most potent neem limonoids that exhibit apoptosis-inducing effects against cancer cell lines in vitro (15, 16). Furthermore, studies have demonstrated the modulatory effects of azadirachtin and nimbolide on xenobiotic metabolizing enzymes, oxidative DNA damage, invasion, and angiogenesis in vivo (17). More importantly, azadirachtin and nimbolide, both of which are antiproliferative, target PCNA, p21, cyclin D1, GST-P, NFkB, p53, FAS, BCL-2, BAX, APAF-1, cytochrome c, Survivin,
caspases 3, 6, 8 and 9, and PARP. Therefore, this study aimed to delineate the radiosensitizing potential of NLE in the NB setting.

Many factors determine tumor resistance in RT, including tumor size, hypoxia, and intrinsic radiosensitivity. The fate of irradiated cells is believed to be controlled by the network of signaling elements that lead to different modes of cell death or survival. Many studies have correlated gene expression and response to RT (18-21) and in vitro radiosensitivity (22). Though many stress-responsive genes are inducible by IR (23), only a fraction of these are believed to play a key role in the stress-tolerance phenotype, including cell cycle checkpoints, apoptosis, and DNA repair (24, 25). More importantly, induction of apoptosis is directly correlated with the biological effectiveness of the IR (6, 26). To that end, the alteration of apoptotic transcriptional response after single-dose radiation (SDR) (27) was recently demonstrated as a function of time (28) and the response after chronic FIR (29) was further delineated in NB cells. Accordingly, herein, using an in vivo human NB xenograft model, this study investigated the apoptosis-specific transcriptional response after SDR (10 Gy) or FIR (2 Gy x5) and further elucidated the effect of NLE in modulating IR-induced apoptotic transcriptional alterations in this setting.

Materials and Methods

**NB Xenograft development and irradiation experiments.** All experiments conformed to American Physiological Society standards for animal care and were carried out in accordance with guidelines laid down by the National Research Council and were approved by the host Institutional Animal Care and Use Committee. Seven-week-old athymic NCr-nu/nu nude mice (NCI, Frederick, MD, USA) received subcutaneous injections of SK-N-MC (5×10^6) cells suspended in 100 μl of culture media into their right flank. Tumor growth was periodically monitored, tumor volume was calculated using the formula volume= [(π/6) × length × width^2] (30) and tumors were allowed to grow to a volume of approximately 500 mm^3.

**DNA fragmentation.** The Fluorescein-FragEL kit (Oncogene Research Products, Boston, MA, USA) was used to detect DNA fragmentation in NB xenograft from mice treated with NLE (10, 20, 50 mg/kg), exposed to 10 Gy, 2 Gy x5 days with or without NLE as described earlier (33). Relative fluorescence intensity levels were quantified and the groups were compared using ANOVA with Tukey’s post-hoc correction. A p-value less than 0.05 was considered to be statistically significant.

**Results**

IR modulated apoptosis-related genes in human NB xenograft. Overall, compared to the mock-IR group, FIR induced 60 genes and down-regulated another 23 genes. One gene, *RIPK2*, did not show any modulations after FIR. On the other hand, SDR induced 65 genes and suppressed 19 genes. Interestingly, in this setting, FIR and SDR commonly
up-regulated 60 genes (Figure 1A) and down-regulated another 19 genes (Figure 1B). BCL2A1, BNIP2, CASP4, RIPK2 and TRAF4 were selectively induced after SDR, while BCL2A1, BNIP2, CASP4, TRAF4 were selectively suppressed after FIR. ANOVA with Tukey's post-hoc correction revealed that FIR significantly induced 55 out of 60 genes including TNF ligands (CD40LG, FASLG, LTA, TNF, CD70, TNFSF8) (Figure 2A), TNF receptors (CD40, FAS, TNFRSF 10A, 10B, 1A, 21, 25) (Figure 2B), BCL-2 family (BAG1, BAG3, BAG4, BAK1, BAX, BCL10, BCL2L1, BCL2L10, BCL2L11, BIK, BNIP1, BNIP3, BNIP3L, MCL1) (Figure 2C and 2D), caspases (CASP1, 10, 2, 3, 5, 6) (Figure 2E), anti-apoptotic molecules (BFA1, NAIP, BIRC6, BIRC8, BIRC9, CFLAR, IGF1R) (Figure 2F), TRAF and CARD family (NOD1, CARD6, 8, CARDD, NOL3, PYCARD, TRAF3) (Figure 2G) and death/CIDE domain, p53 and DNA damage-response molecules (CIDEA, CIDEB, DAPK1, FADD, GADD45, TP53BP2, TP73, TRADD) (Figure 2H). Conversely, 59 SDR down-regulated genes were completely suppressed (Figures 2C, 2F and 2H). Interestingly, 46 genes were significantly up-regulated and another four genes were completely suppressed commonly after FIR or SDR exposure (Figure 1C and 1D).

NLE modulates cell death associated gene transcription. Altogether, NLE activated 65 genes and suppressed another 18 genes in NB xenografts. Of the 65, activated genes, 49 including TNF ligands (CD40LG, FASLG, LTA, TNF, CD70, TNFSF8) (Figure 2A), CD40, FAS, TNFRSF 10A, 10B, 1A, 21, 25 (Figure 2B), BAG1, BAG4, BAK1, BAX, BCL10, BCL2L11, BIK, BNIP1, BNIP3, BNIP3L, MCL1 (Figures 2C and 2D), CASP1, CASP10, CASP5, CASP6 (Figure 2E), BFA1, NAIP, BIRC8, CFLAR, IGF1R (Figure 2F), APAF1, NOD1, CARD6, 8, CARDD, NOL3, PYCARD, TRAF3, 4 (Figure 2G), CIDEA, CIDEB, DAPK1, FADD, GADD45, TP53BP2 and TRADD (Figure 2H) were significantly induced. Conversely, BCL2, BIRC2, ABL1, AKT1, DFFA of 19 SDR down-regulated genes were completely suppressed (Figures 2C, 2F and 2H). Interestingly, 46 genes were significantly up-regulated and another four genes were completely suppressed commonly after FIR or SDR exposure (Figure 1C and 1D).
Figure 2. continued
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Figure 2. Histograms showing the levels of (A) TNF ligands, (B) TNF receptors, (C&D) BCL2 family, (E) caspases, (F) IAPs and other anti-apoptotic molecules, (G) TRAF and CARD family, (H) death/CIDE domain, p53 and DNA damage-response molecules and (I) in-house controls in NB xenograft exposed to either mock-IR, SDR, FIR, NLE or treated with NLE and exposed to SDR/FIR.
Figure 3. Photomicrographs showing DNA fragmentation in NB xenograft exposed to SDR/FIR, treated with NLE (10, 20 or 50 mg/kg) with or without SDR/FIR. (B) Histogram showing significant and profound conferring effect of NLE on SDR- and FIR-induced DNA fragmentation in NB xenograft.
NLE significantly enhanced FIR-induced xenografts. Conversely, NLE treatment reverted the induction genes was observed in NLE pre-treated and FIR exposed $BCL2A1$, $BIRC3$, $TRAF2$. Furthermore, NLE significantly induced FIR-inhibited $BAX$, $BCL10$, $CASP1$, $CARD8$. To that note, except for $TRAF4$, these IR-inhibited molecules were only brought back to the basal level with NLE. Conversely, NLE significantly inhibited FIR-induced $TNRFSF10A$, $BCL2L1$, $BCL2L10$, $BCL2L11$, $BCLAF1$, $BNIP3$, $MCL1$, $NAIP$, $BIRC6$, $BIRC8$, $APAF1$, $NOL3$, $DAPK1$ and $TP73$. Moreover, NLE completely suppressed FIR-inhibited XIAP, ABL1 and AKT1 in the xenografts (Figure 2). Like wise, compared to SDR exposed xenografts, NLE up-regulated 40 and suppressed another 44 genes. ANOVA revealed that NLE significantly suppressed SDR-induced $BIK$, $BNIP3$, $BFA$, $NAIP$, $BIRC6$, $BIRC8$, $BRAF$, $IGF1R$, $APAF1$, $NOD1$, $CARD6$, $CARD8$, $CRADD$, $NOL3$ and PYCARD and further inhibited SDR-inhibited ABL-1 (Figure 2). Conversely, NLE significantly enhanced SDR-induced $TNRFSF25$, $BAG1$, $BAX$, $BCL1$ and $CASP10$ (Figure 2). The functional importance of these NLE regulated molecules in SDR and/or FIR exposed NB xenografts is discussed below.

NLE regulates IR-induced DNA fragmentation. Compared to mock-IR, FIR ($p<0.01$) and SDR ($p<0.001$) significantly increased DNA fragmentation in the NB xenografts. Likewise, NLE (50 mg/kg)-treated xenograft demonstrated a significant ($p<0.05$) induction of DNA fragmentation as opposed to mock-IR. Furthermore, it was observed that NLE markedly and significantly ($p<0.001$) induced DNA fragmentation after FIR and SDR exposure of xenograft respectively (Figures 3A and 3B).

Discussion

Drug development from natural products is currently emerging as a highly promising strategy to identify novel anticancer agents. Recently, limonoids, modified triterpenes formed as secondary metabolites by plants, have attracted considerable attention as promising anticancer candidates (34). Neem contains a vast array of bioactive phytochemicals, one-third of which are limonoids. Azadirachtin and nimbolide, present in neem leaves, are recognized as the most potent limonoids that exhibit cytotoxic-effects against various cancer cell lines (15, 16). Also, studies have shown their modulatory effect on xenobiotic metabolizing enzymes, oxidative DNA damage, invasion, and angiogenesis (17). Thus, azadirachtin and nimbolide have been shown to exert apoptosis by selectively targeting PCNA, p21, cyclin D1, GST-P, NFkB, Jnk, p53, FAS, BCL2, BAX, APAF-1, cytochrome c, survivin, caspase 3, 6, 8, 9, and PARP (35). In the present study, for the first time, it was shown that NLE significantly inhibits clinically relevant radiation-induced $BNIP3$, $NAIP$, $BIRC6$, $BIRC8$, APAF1, NOL3 in human NB xenograft.

Cancer cells are able to acquire resistance to apoptosis by up-regulating multiple survival factors. Inhibitors of apoptosis (IAPs) are a pivotal class of intrinsic cellular inhibitors of apoptosis (36). IAPs widely and potently suppress apoptosis against a large variety of apoptotic stimuli, including radiation, in cancer cells. Induced expression of IAPs (NAIP, BIRC6, BIRC8) observed in the current study, both after SDR or FIR in NB xenograft, confirms earlier findings and also serves as the positive control for the current study. Since IAPs function at the convergence of mitochondrial and death-receptor pathway, they can be described as an apoptosis ‘brake’ and IAP antagonists/inhibitors function to release this ‘brake’ (37). Inside a live cell, upon irradiation, multiple apoptosis pathway proteins are involved in shifting the balance of life and death signals. In the context of SDR/FIR, induction of NAIP, BIRC6 and BIRC8 observed in this study throws light on their roles in regulating IR-induced apoptosis and further dictates the outcome of the cell’s response to therapy. Furthermore, NLE significantly inhibiting these molecules demonstrates IAP-inhibition-mediated radiosensitization and provides critical information as to how NLE works in the context of IR, and how NB cells respond better to the therapy. The latter has clear clinical relevance in that the information will be useful to predict or select the patients who will benefit the most from the molecular therapy targeting IAPs (37). Similarly, NOL3 (ARC), an endogenous inhibitor of apoptosis antagonizes both central death (extrinsic death receptor or intrinsic mitochondrial/ER) cascades (38). While ARC binds to FAS, FADD, and procaspase-8 precluding the formation of death-inducing signaling complex, disabling the extrinsic pathway, it interacts with BAX preventing conformational activation and translocation to the mitochondria, thereby antagonizing the intrinsic pathway (38). To the Authors’ knowledge, this study is the first report of clinically relevant doses of IR (SDR/FIR) robustly inducing the transcription of NOL3, at
least in NB cells. More importantly, the ARC inhibitory effect of NLE delineates its potential in conferring radiosensitization by inducing cell death. However, it is interesting to note that NLE also inhibits IR-induced pro-apoptotic BNIP3 and APAF1 in this setting. Although NLE inhibition of these molecules has been realized in earlier studies in other settings (17), the mechanism underlying this response and its functional significance, if any, in NLE-induced cell death in NB needs further investigation.

Many in vitro studies have demonstrated that the antiproliferative activities of NLE are mediated through induction of apoptosis. Here, it was shown that NLE radiosensitizes NB xenografts by blocking anti-apoptotic machinery and inducing apoptosis. Comprehensively, NLE either inhibits IR-induced anti-apoptotic molecules, completely confers IR-inhibited XIAP, AKT1 or profoundly enhances proapoptotic genes such as BAX. Mitochondria, which play a pivotal role in apoptosis, are a major site of reactive oxygen species (ROS) generation. Excessive ROS generation can lead to the opening of the mitochondrial permeability transition pore with consequent release of cytochrome c from the inter membrane space into the cytosol, culminating in activation of the caspase cascade and apoptotic cell death. BCL-2 inhibits ROS production, cytochrome c release and caspase-3 activation, whereas BAX facilitates cytochrome c release, triggering caspase-mediated apoptotic cell death. BCL-2 and BAX have become attractive targets for designing new anticancer drugs, and agents that lower the BCL-2/BAX ratio are regarded as promising chemopreventive and chemotherapeutic agents. The decrease in the BCL-2/BAX ratio seen after exposure of NB xenograft to NLE that was observed in this study, together with decreased IAPs, ARC and others throw light on the initial molecular events that directs NLE associated radiosensitization in NB cells. The present study provides compelling evidence showing that NLE transduces apoptosis by both the mitochondrial and death receptor pathways.

In summary, the results demonstrate for the first time that NLE exerts radiosensitization in NB cells by inducing apoptosis. The data indicate that NLE potentiates IR-induced cell death by targeting both extrinsic and intrinsic pathways, in particular, by inhibiting the anti-apoptotic signaling cascade. Furthermore, owing to the limitations of in vitro studies in delineating molecular orchestration in response to a stimuli or selective targeting, a more relevant pre-clinical human NB xenograft coupled with quantitative PCR profiling of more direct, yet comprehensive and functionally (apoptosis) characterized molecules were utilized to elucidate the molecular blueprint that underlies NLE associated radiosensitization. This study allowed the identification of a potential ‘deliverable’ that targets apoptosis transcriptional response. However, further studies are warranted to delineate its efficacy in mitigating NB progression and radiotherapy-associated NB relapse and metastasis.

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References


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