A global perspective of radiation-induced signal transduction pathways in cancer therapeutics

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Radiation is a well established therapeutic modality for the treatment of solid tumors. By merging molecular biological approaches with radiation biology, a significant number of signaling events elicited by ionizing radiation have been delineated. These signaling pathways include events leading to cell cycle arrest, apoptosis or cell survival. There are two major signaling events that affect radiation response. One is the intrinsic/constitutive pro-survival signaling event that is present in proliferating tumor cells while the other is “induced pro-survival event” in response to radiation, both of these events confer resistance to the killing effects of radiation.

In this review, signaling pathways that lead to either apoptosis or survival of cells following ionizing radiation are discussed in detail. In addition, mechanisms of action for gene/drug based inhibitors that modulate the expression and function of various genes and gene products involved in pro-survival signaling pathways are described. Further, novel strategies to abrogate the “induced radiation resistance” leading to enhanced therapeutic efficacy of ionizing radiation have been proposed. These novel strategies include the use of radio-gene therapy, low dose fractionated radiation therapy as a chemopotentiator and therapeutic utility of high radiation dose induced bystander effect. The complete understanding of the molecular pathways leading to apoptosis/survival of cells following ionizing radiation will help in tailoring more effective novel strategies and treatment modalities for complete eradication of cancer.

Key words: Radiation, Genes, Signal transduction pathways, Apoptosis, Inhibitors and therapy.

Ionizing radiation is one of the most widely used therapeutic modalities for the treatment of tumors. Radiation-induced cell death results from damage to either cell membrane or DNA. Lesions in DNA can be induced either by direct ionization of DNA or indirectly through formation of free radicals. These lesions include base damage, intra- or inter-strand cross-linking and single- or double-strand breaks. The cellular responses to these lesions include arrest in cell cycle progression at cell cycle checkpoints and the induction of DNA repair. However, the residual un-repaired or mis-repaired DNA damage leads to genetic instability, mutations and chromosomal aberrations. This may result in death of the progeny after several mitotic cycles. This type of cell death called “mitotic or clonogenic death” or “mitotic catastrophe” is most common in solid tumors exposed to radiation. Alternatively, radiation induces apoptosis, referred to as interphase death, which avoids the damaged cell to become cancerous. The choice of response depends on the cell type, the location and the extent of the damage.

Thus, apoptosis is regarded as a genetically programmed form of cell death, a physiological event distinct from necrosis or accidental cell death associated with inflammatory responses. Morphologically, apoptosis is characterized by cell shrinkage, active membrane blebbing, chromatin condensation and cellular fragmentation into membrane-enclosed vesicles called apoptotic bodies (Fig. 1). These events are accompanied biochemically with changes both at the cell surface or at various intra-cellular locations. At the cell surface, externalization of phosphatidylserine and other alterations that promote recognition by phagocytes occur. Intracellular changes include the loss of mitochondrial membrane potential, activation of intracellular cystein proteases (caspases), degradation of DNA into high molecular weight and oligonucleosomal fragments and cleavage of specific cellular proteins. Activation of caspases is an evolutionary conserved event and is central to the execution of apoptosis. Activation of caspases can occur by two pathways: extrinsic and intrinsic. Intrinsic pathway involves dysfunction of mitochondria in response to UV irradiation, ionizing radiation, chemotherapeu-
tic drugs, withdrawal of growth factors, loss of cell adhesion (an oikis) \textsuperscript{17-22}. Extrinsic pathway is mediated by death-inducing membrane receptors (such as tumor necrosis factor-\(\alpha\), CD95 and hormone receptors) upon binding with their ligands \textsuperscript{23-25}.

Since one of the modes of cell death induced by treatment strategies such as chemotherapy and radiation involve elimination of malignant cells by the induction of apoptosis, defects in the apoptotic pathway can lead to cancer cell resistance. Therefore, it is to the advantage of tumor cells to down-regulate the genes involved in apoptosis. These defects or alteration in the genes responsible for induction of apoptosis allow cancer cells to survive beyond their normal life spans, provide protection from hypoxia and oxidative stress, promote angiogenesis and enhance invasiveness of the tumor \textsuperscript{15}.

The pathways that govern apoptosis are complex and involve a network of cell death blockers and inducers working against each other to maintain tissue homeostasis \textsuperscript{15,26}. These programs are tissue specific and consist of activation of early, mediator and effector genes that initiate the apoptosis signaling cascade (Fig. 1). The next section describes the role of gene signaling transduction pathways in these early/proximal, mediatory and effector events leading to the induction of apoptosis.

**Gene signaling pathways involved in induction of apoptosis by ionizing radiation**

**Early genes**—Exposure of cells to growth factors, ionizing radiation and other stresses results in the activation of \(c-jun\), \(c-fos\), \(Egr-1\), \(\beta\)-actin, interleukin-1, protein-kinase C, GADD45, NFKB gene families etc. \textsuperscript{27} in the absence of new protein synthesis. The products of these early response genes are mostly transcription factors that regulate downstream target genes such as cytokine and growth factor genes and DNA repair genes that help to adapt to the radiation-induced stress \textsuperscript{27}.

\(c-jun\) gene encodes a protein of AP-1 transcription factor family (\(c-jun\) protein), a DNA binding protein that is present in dimerized form with various jun and

**Gené signaling events**

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<td>ATM activation</td>
<td>P53, Ceramide, Bax, Bid cleavage, Dephosphorylation of Bad, Par-4, PUMA, TRADD/FADD</td>
<td>Cleavage of pro-caspases, Endonucleases, Smac/DIABLO, Release of Cytochrome C, Apaf-1, PARP cleavage, Cleavage of cytoskeleton proteins</td>
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![Morphological changes](image)

**Morphological changes**

Fig. 1—A model of molecular and cellular events induced by ionizing radiation leading to apoptosis.
fos proteins\textsuperscript{27}. Induction of c-fos by ionizing radiation has been reported in fewer cell lines than c-jun. Regulatory domains that regulate the transcriptional activity of c-jun by activating or inhibiting the transcription exist within the jun protein. AP-1 sequences are found in radiation-induced promoters of several genes including c-jun, Egr-1, platelet-derived growth factor (PDGF), basic fibroblast growth factor, tissue plasminogen activator and tumor-necrosis factor-\(\alpha\) (TNF-\(\alpha\)).

Studies using free radical scavengers have suggested that c-jun and Egr-1 are induced by radiation secondary to the generation of radical oxygen intermediates\textsuperscript{28}. Induction of Egr-1 has been shown in several cell lines following mitogenic or differentiation signals\textsuperscript{29}. Egr-1 gene promoter contains six serum response elements and three of them bind to transcription factors called as serum responsive factors (SRF) when exposed to X-rays\textsuperscript{30}.

Nuclear factor \(\kappa B\) (NF-\(\kappa B\)) mediate cellular response to a variety of stimuli, including TNF-\(\alpha\), Interleukin-\(\beta\), reactive oxygen species and radiation\textsuperscript{30-35}. This dimeric transcription factor is composed of different members of the Rel family, p65 (RelA), p50, p52, c-Rel and RelB\textsuperscript{36} and activates genes involved in inflammation, cell growth and apoptosis. In un-stimulated cells, it is present in the cytoplasm and sequestered by inhibitor of kappa B (IkB) proteins. Following stimulus, it can be rapidly released to enter the nucleus without a requirement for protein synthesis. Anti-apoptotic proteins such as Bcl-X\(_1\), Bcl-2 and Bfl-1 are up-regulated by NF-\(\kappa B\)\textsuperscript{37-40}. Thus, it can regulate apoptosis by modulating the expression of anti-apoptotic proteins.

Since, Egr-1 and c-jun genes may initiate apoptosis depending on the environmental factors to eliminate cells carrying the DNA damage, it is to the advantage of tumors to down-regulate their expression. Further, reduced expression of these early genes\textsuperscript{41} with constitutive activation of NF-\(\kappa B\)\textsuperscript{40,42,46} in tumors compared to normal tissues has been observed, suggesting their role in radiation-induced apoptosis.

Mediators—The process of growth inhibition and apoptosis require coordinate expression of specific genes. Gene function studies in diverse experimental models have identified several gene products as mediators of growth inhibition and apoptosis which include p53\textsuperscript{47-49}, TGF-\(\beta\)\textsuperscript{50,52}, c-myc\textsuperscript{53}, Bcl-2 related proteins\textsuperscript{54} in various mammalian cell systems.

The tumor suppressor protein, p53, contains distinct domains that mediate transcription activation, sequence specific DNA binding, recognition of DNA damage, activation of the p53 molecule by phosphorylation/acetylation and promotion of protein-protein interactions\textsuperscript{55}. p53 up-regulates the expression of many genes involved in cell cycle checkpoint, repair and apoptosis. These include \textit{CIP1/WAF1} coding for p21, \textit{GADD45} coding for the “growth arrest and DNA damage responsive response” protein, \textit{MDM2} coding for the “murine double minute-2 protein” and \textit{PCNA} coding for the “proliferating cell nuclear antigen protein”\textsuperscript{46}. Various biochemical responses generated by p53 activation determine whether the cell will survive or undergo apoptosis. The factors that determine the decision may include the type and extent of damage, genetic make-up and cellular context.

Transforming growth factor beta (TGF-\(\beta\)) isoforms (TGF-\(\beta\)\textsubscript{1}, TGF-\(\beta\)\textsubscript{2}, TGF-\(\beta\)\textsubscript{3}) are 25kDa homodimer polypeptides that regulate cell proliferation, differentiation, deposition of the extracellular matrix, immunosuppression and apoptosis\textsuperscript{56,58}. Radiation induces TGF-\(\beta\) isoform in various cell types\textsuperscript{59,60}, downregulates TGF-\(\beta\) isoform and does not alter the expression of TGF-\(\beta\)\textsuperscript{241}. TGF-\(\beta\) is involved in apoptotic pathways as it has been shown to cooperate with Fas\textsuperscript{62} and TGF-\(\alpha\)\textsuperscript{63}. Further, it downregulates the antiapoptotic proteins Bcl-x\(_1\) and Bcl-2-1 and activates caspase-1, 3, 8 and 6\textsuperscript{44,65}. TGF-\(\beta\) also downregulates NF-\(\kappa B\) implicating it in the induction of apoptosis\textsuperscript{56}. TGF-\(\beta\) signaling process is elicited through complex downstream effector gene components that include TGF-\(\beta\) receptors, SMAD genes and the distal effector genes that are usually CDK inhibitors\textsuperscript{66,67}. Recent studies have shown that several tumors escape from TGF-\(\beta\) induced apoptosis that provide them with selective growth advantage accelerating tumor progression\textsuperscript{68,71}.

Recently, we have reported in a pancreatic cancer cell line that TGF-\(\beta\) signaling through receptors and SMADs is pivotal for radiation-induced apoptosis\textsuperscript{72}.

Apoptosis is mediated mainly by members of tumor necrosis factor alpha (TNF-\(\alpha\)) death receptor superfamily proteins. These include TNF-\(\alpha\), FasL/CD95L/Apo1L, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L and TWEAK/DR3/Apo3L\textsuperscript{73,74}. Receptors of these protein ligands contain a conserved cytosolic domain, known as death domain that recruits adapter proteins such as FADD/TRADD (Fas-associated death domain pro-
tein/TNF-α associated death domain protein). Through interaction of another domain referred to as death effector domain (DED), recruitment of initiator caspases occur resulting in activation of executioner caspases and cell death. TNF-α also has been shown to have anti-apoptotic activity. This could be due to its ability to induce cytokines like IL-1 and IL-6 and activation of NF-κB through type II TNF receptor. Deregulation of these pathways may contribute to abnormal tumor growth.

One of the major apoptosis-regulatory gene families is Bcl-2 family of proteins that play an important role in cell’s decision to live or die. This family can be divided into two categories: pro-apoptotic (Bax, Bad, Bak, Bcl-xs and Hrk) and anti-apoptotic (Bcl-xL, Bcl-2 and mcl-1). In general, Bcl-2 family proteins can alter cell death pathways through multiple mechanisms such as caspase-independent effects on mitochondria, regulation of CED-4 family proteins and homo- or hetero-dimerization with other members to block their function. Two types of actions have been described for these proteins: binding to other proteins and ion-channel activity. Expression of Bcl-2 family of proteins is regulated by p53 and NF-κB, both of which have opposite roles; p53 activates expression of pro-apoptotic proteins like Bax, while NF-κB activates anti-apoptotic proteins like Bcl-2 and Bcl-xL. Alteration in the expression of these proteins such as up-regulation of anti-apoptotic proteins and down-regulation of pro-apoptotic members has been shown in several tumors. Thus, assessment of levels of Bcl-2 members of proteins will be of significant prognostic value in cancer therapy.

**Effectors**—The final steps of apoptosis are the downstream effectors that act as executioners involving proteases and endonucleases.

Proteases: The proteolytic activities of caspases, granzyme B, cathepsin B, Ca²⁺-regulated serine protease and histone-associated protease have been implicated in eliciting characteristic nuclear changes that occur during apoptosis. However, caspases appear to be the most important proteases responsible for the induction of apoptosis. Caspases are the highly conserved proteases that cleave the substrate after aspartic acid (Asp). All caspases are synthesized as proenzymes and get activated by cleavage at Asp residue thereby generating large and small subunits to form the active hetero-tetramer. Caspases initiate and execute cell death by inactivating anti-apoptotic proteins, shutting down DNA replication and repair, reorganization of the cytoskeleton and disruption of the nuclear lamina. Initiator caspases such as 8, 9 and 10 activate effector caspases 3, 6, 7 that are the executioners of apoptosis. Caspases can be activated in a mitochondria-dependent and independent fashion. While, death receptors activate caspases in mitochondria-independent manner, most other stimuli require release of cytochrome c from mitochondria. Examples of loss of expression or mutational inactivation of specific caspase genes have been observed in human tumor cell lines.

Endonucleases: One of the well conserved biochemical hallmarks of apoptosis is genomic DNA fragmentation. From the numerous studies trying to find out the nucleases responsible for this fragmentation, it appears that this process is mediated in vivo by two systems, one operating cell autonomously in the dying cells and the other in phagocytes after the dying cells are engulfed. In cell autonomous DNA degradation, first the genome is cleaved into 50-300 kb DNA fragments at the nuclear scaffold region and this is followed by internucleosomal cleavage that generates mono- and oligo-nucleosomal DNA fragments termed DNA laddering.

Several enzymes were earlier proposed including DNase I, DNase II, cyclophilins and DNase γ but none appeared to fulfill the criteria for the apoptotic DNase. Subsequently, major nucleases responsible for internucleosomal degradation of DNA has been elucidated by several groups in 1997-1998 and were called DNA fragmentation factor (DFF), caspase-activated DNase (CAD) or caspase-activated nuclease (CPAN) although endonuclease G, unidentified AIF-activated nuclease and others may degrade DNA in certain circumstances. Subsequently, it was found that DFF is a complex of CAD (DFF40) and ICAD (DFF 45). ICAD inhibits CAD by blocking its binding to DNA. Caspases 3 and 7 cleave ICAD and thereby inhibit its CAD-inhibiting activity. However, this system is dispensable for cell death.

It has been shown that the DNA of apoptotic cells can be degraded in phagocytes after the cells are engulfed. In vivo, macrophages engulf apoptotic cells by recognizing phosphatidylserine that is exposed on the cell surface in a caspase-dependent fashion and DNase II digests DNA. The accumulation of undigested DNA in various tissues in DNase II null mice but not CAD-deficient mice suggests that...
DNA degradation in macrophages may play a more vital role for proper development and homeostasis\(^\text{16}\).

**Ionizing radiation induced pro-survival signal transduction pathways as therapeutic targets**

Functional links between cellular signal transduction responses and DNA damage recognition, repair and cell death have been well recognized. Ionizing radiation (IR) is known to induce signal transduction pathways that lead to apoptosis. However, most tumors respond to the effects of IR oppositely by inducing pro-survival signal transduction pathways. This is due to specific gene aberrations involving overexpression or underexpression or mutant forms or homozygous deletions, together helping in evasion of the killing effects of IR. Thus, selective suppression of a specific gene represents an important approach to understand the functional relevance of radiation induced cascade leading to the ultimate phenotypic change as well as of anticancer therapy. Suppression of gene expression can be achieved either by gene disruption or by introduction of a genetic element that inhibits the function of the target gene. Use of protein mutants that interfere with the function of the wild-type protein in a dominant fashion can be used to suppress the function of a specific gene\(^\text{17}\). The antisense RNA approach involves the production of RNA sequences complementary to mRNA of the target gene\(^\text{18}\). Double-stranded RNA-mediated interference (RNAi) is a simple and rapid method of silencing specific gene expression. The silencing of a gene is a consequence of degradation of RNA into short RNAs that activate ribonucleases to target homologous mRNA. The resulting phenotypes either are identical to those of genetic null mutants or resemble an allelic series of mutants\(^\text{19,20}\). In addition, several inhibitors specific to a particular molecule important in radiation-induced signal transduction pathway can be used as drugs for cancer therapy. The next section will review the radiation-induced signal transduction pathways that lead to apoptosis or pro-survival events and their modulation by either chemical inhibitors or by suppression of target genes with an overall intent to enhance the killing effects of radiation (Table 1).

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<thead>
<tr>
<th>Target</th>
<th>Inhibitors</th>
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<tr>
<td>MAPK</td>
<td>PD98059, SP600125, SB203580, CEP-1347, CI-1040 (PD188352), peptide inhibitors</td>
<td>128, 129, 137-141</td>
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<tr>
<td>EGFR</td>
<td>Iressa, Genistein, GW572016, PKI166, antibody (CC225/ cetuximab), dominant negative mutants</td>
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<td>Onco-RAS</td>
<td>Farnesyltransferase inhibitors (FTI), GGTI</td>
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<td>AR</td>
<td>Casodex (bicalutamide), flutamide (Eulexin), receptor gene inhibition</td>
<td>181-186</td>
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<td>ER</td>
<td>Tamoxifen and analogs, fulvestrant</td>
<td>187</td>
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<td>PI3K, PKB/Akt</td>
<td>Carboxy-terminal modulator protein (CTMP), staurosperine and derivatives, integrin-linked kinase inhibitors, Wortmannin, LY294002 and celecoxib</td>
<td>162, 165-180</td>
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<td>HDAC</td>
<td>Trichostatin A, SAHA, Short chain fatty acids, depsipeptide, cyclic tetrapeptides, benzamide derivatives, hybrid polar compounds</td>
<td>229-244</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Proteasome inhibitor (MG 132, lactacystin, PSI), PS-341, human immunodeficiency virus protease inhibitor (saquinavir), Ad. IxB, Ad. PAr, Antioxidants (N-acetylcysteine, Dithiocarbazates, Thiamidol), Anti-inflammatory drugs (Glucocorticoids, salicylates), Synthetic peptides (SN-50), Viral proteins (Ad. E1A protein), Nucleotides (antisense oligonucleotides, transcription factor decoys), Plasmids (IxB expression plasmids)</td>
<td>246, 248-251, 272</td>
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<tr>
<td>STATs</td>
<td>Antisense oligonucleotides, SiRNA, peptide inhibitors, Gene therapy vector, dominant negative mutant</td>
<td>247, 252-265</td>
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<td>Survivin</td>
<td>Ribozyme, antisense oligonucleotides, cancer vaccine, dominant negative mutants</td>
<td>266-271</td>
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<tr>
<td>BCL-2, BclXL</td>
<td>DNA methyltransferase inhibitors, Gene transfer, antisense oligonucleotides, protease inhibitors</td>
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The exposure of cells to ionizing radiation leads to activation of several messengers generated either at cell membrane, in cytosol or in nucleus and finally results in modulation of expression of several genes that decides the fate of the cell (Fig. 2). Primary messengers are generated at cell membrane following the binding of the ligands to the specific receptors. Activation of ionizing radiation-induced receptors like TNF receptor family TNF-R1, TRAIL receptors DR4 and DR5, TRAMP (DR3) and CD95 and epidermal growth factor receptor (EGFR) leads to activation of several kinases (MAPK, PI3 kinase and MAPK8) in the cytoplasm that finally modulate the proliferation and survival of cells\textsuperscript{121-127}. Activation of EGFR following radiation leads to cytoprotective response through MAPK and MAPK8 pathways\textsuperscript{123,128,129}. Activation of Ras following phosphorylation of EGFR at specific Tyrosine residues as well as PLC\gamma has been observed\textsuperscript{123,130}. Further, activation of RAF1 and protein kinase C (PRKC) leads to enhanced transcription, protein synthesis, mitogenesis, proliferation and survival\textsuperscript{130-134}. Activation of PI3 kinase that plays an important role in protecting cells from growth-factor deprivation-induced apoptosis through stimulation of AKT has also been observed following activation of ERBB receptor tyrosine kinases\textsuperscript{127,135,136}.

Since EGFR pathway is linked to cell proliferation, cellular radiosensitivity can be enhanced by blocking the components of this pathway. Indeed, inhibition of MAPK by PD98059 and other inhibitors\textsuperscript{128,129,137-141}, EGFR by tyrosine kinase inhibitors such as IRESSA or Genistein, dominant negative mutants and anti-EGFR antibodies\textsuperscript{142-157}, RAS by farnesyltransferase inhibitors\textsuperscript{158,159} and RAF1 by antisense-RAF oligonucleotides\textsuperscript{160} has been shown to enhance the radiosensitivity of human tumor cells (Table 1). A review on inhibitors of EGFR for cancer treatment has been published recently\textsuperscript{161}. Upstream growth factors can signal through RAS to PI3K or directly to PI3K and there is a cross talk between estrogen and androgen receptors (ER and AR) and PI3K in tumors refractory to growth hormone treatment. Thus, inhibiting PI3K and the growth hormone receptors should abrogate the resistance from EGFR.

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**Fig. 2**—Activation of signaling pathways depicting a series of changes at cell membrane, cytosol and nucleus levels. These radiation-induced primary and secondary messengers modulate gene expression to elicit specific cellular responses.
RAS, AR and ER signaling. Inhibitors of PI3 kinase and protein kinase B (PKB)/AKT (a serine/threonine kinase) pathways have been shown to increase radiosensitivity both in vitro and in vivo. Carboxy-terminal modulator protein (CTMP), staurosporine, and its derivatives, PKC412, and UCN-01, and integrin-linked kinase inhibitors of PKB/Akt pathway are being examined as anticancer drugs. New selective Akt inhibitors as well as inhibitors of PI3 kinase like Wortmannin and L680,002 and celecoxib are being developed and have shown enhanced radiosensitivity.

Inhibition of AR function using antisense oligodeoxynucleotides, ribozymes or small interference RNAs (RNAi) have been successful as approaches to the treatment of hormone-refractory, apoptosis-resistant prostate tumors. Tamoxifen and analogs and fulvestrant have been used for abrogation of ER signaling as well. Radiation-induced cytotoxic pathway at the membrane level is mediated mainly by TNF receptor family. Two forms of TNF-α receptor, CD4 and TNFRSF1B have been characterized, and CD4 directly signals the caspase cascade leading to apoptosis. TNF receptors can activate acid sphingomyelinase (ASMase) that cleave lipids to form ceramide. Agonist-induced elevation of ceramide, the second messenger, has been shown to precede biochemical and morphological manifestations of apoptosis. Also, elevation of endogenous ceramide using ceramide analogs, sphingolipid analogs or pharmacological agents that interfere with enzymes of ceramide metabolism, mimics the effects of stress on apoptosis. Ceramide through the family of GTP binding molecules RHO/RAC and RAS, activates MEKK1/2 that phosphorylate and activate MAPK4/7 and MAP2K3/6. MAPK2K4/7 signals MAPK8 that phosphorylate transcription factor Jun leading to apoptosis in some cell types. The role of MAPK8 activation following radiation in inducing either proliferation or death vary substantially with the cell type.

Agnostic antibodies to TNF receptor can induce apoptosis in some tumor cells. Alternatively, the overexpression of TNF-α protein by gene therapy or treatment with TRAIL has been shown to result in enhanced radiosensitivity of tumor cells in vitro and in vivo. Expression of TNF-α in response to radiation is tightly regulated by transcription factor Egr-1. Thus, we have reported that adenoviral expression of Egr-1 in PC-3 tumor bearing mice in combination with radiation showed significant regression of tumors and also inhibition of NF-κB and upregulation of TNF-α and Bax suggesting that Egr-1 is a pro-apoptotic sensitizer of radiation.

Since ionizing radiation leads to DNA damage, the response to this damage requires sensors. ATM and p53 have been identified as sensors of radiation damage. In a wild type p53 background, cells either repair the damage or undergo apoptosis leading to radiation sensitivity. However, mutations in p53 are frequently associated with reduced chemosensitivity, reduced radio-sensitivity and "induced radiation resistance". Loss of function of the tumor suppressor gene p53 also has been associated with an increase in both radiation resistance and radiation sensitivity, suggesting that clinical radio-response may be dependent on cell type or yet unknown genetic factors. ATM, phospho-protein (localized primarily in the nucleus) appears to act upstream of p53. ATM can bind and phosphorylate non-receptor tyrosine kinase ABL in response to DNA damage. Interactions between ATM and ABL may facilitate interactions with signal transduction and cell cycle regulatory pathways. ABL can phosphorylate RNA polymerase II which may modulate transcription from specific promoters. ATM can also regulate S- and G2/M phase cell cycle progression after irradiation that involve Chk protein kinases, CDK1 and the phosphatase CDC25. ATM also plays a role in double-strand break repair and recombination through its binding to ABL which in turn binds RAD51.

ATM protein binds directly to p53 and stabilizes it by phosphorylation. Cells expressing wild type p53 protein arrest at the G1 phase checkpoint with possible additional effects on G2 phase following irradiation. Several proteins as described before (MDM2, GADD45, PCNA etc.) are involved in the p53-dependent apoptosis and DNA repair. Although, use of p53 wild-type and p53-knock out mice has shown the advantages of anti-p53 therapy, its applicability depends on the isolation and characterization of p53 inhibitors.

Gene expression is also influenced by chromatin remodeling by acetylation, deacetylation, methylation etc. Several enzymes like histone acetylases (HATs), histone deacetylases (HDACs) and DNA methyltransferases are responsible for chromatin modulation which in turn regulates transcriptional activation of specific genes through the relaxation/compaction of...
chromatin conformation. Inhibition of HDACs by short chain fatty acids\textsuperscript{229,230}, benzamide derivatives\textsuperscript{231}, trichostatin and analogues\textsuperscript{232,233}, hybrid polar compounds\textsuperscript{234}, cyclic tetrapeptides\textsuperscript{235,236}, depsipeptides\textsuperscript{237} leads to hyperacetylation of histones that have anti-tumor activities in both tumor cells and xenografted models (Table 1)\textsuperscript{238-240}. HDAC inhibitors can be used in combination with certain established anti-tumor agents to augment clinical efficacy and/or to reduce toxicity. Indeed, HDAC inhibitors have shown radiosensitizing potential in colon and prostate cancer cell lines\textsuperscript{241-244}.

Since a number of pro-survival signaling pathways converge on a limited set of nuclear transcription factors (TFs) and these TFs alter the gene expression patterns leading to proliferation, they are the choice targets for anticancer drugs\textsuperscript{245}. Novel cancer treatment strategies have been developed, for example to inhibit these factors like NF-κB, survivin and STATs, since these are involved in the transcriptional regulation of genes important in anti-apoptosis, cell cycle, cellular adhesion and malignant progression\textsuperscript{246,247}.

A number of agents that inhibit NF-κB and have variable specificities have been reviewed by Schwartz et al.\textsuperscript{246}. Though many tumors are likely to be resistant to NF-κB inhibition alone, the sensitization of these tumors by either radiation or other agents make NF-κB inhibition a promising, novel therapy in the treatment of solid tumors\textsuperscript{248-251} (Table 1). However, implication of NF-κB inhibition on normal tissues and in vivo efficacy is yet to be determined. STATs (STAT3) have been shown as valid targets for cancer

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**Fig. 3—A model of radiation-induced gene signaling events in wild-type p53 background.** Ionizing radiation (IR) causes activation of p53 through ATM kinase leading to upregulation of CDK inhibitor p21 causing a cell cycle arrest. During the cell cycle arrest, the damage caused by ionizing radiation is repaired by repair proteins such as Brca1 and Nbs1. Concomitantly, functions of pro-survival genes such as Ras, Akt/PI3 kinase or NF-κB are inhibited by p53 function. If the damage caused by IR is beyond the capacity of internal repair proteins, Bax is activated directly by p53 leading to downstream apoptotic effector events.
therapy with the use of a gene-therapy vector in a mouse model of melanoma. Also, the blocking of STAT3 signaling using dominant negative mutant forms or other inhibitors in normal cells does not generally lead to apoptosis and thus makes it specific for tumor cells. Small-molecule inhibitors of upstream tyrosine-kinases (JAK, SRC, BCR-ABL, FLT-3 and EGFR) that activate STATs have been shown to block STAT3 or STAT5 signaling and induce tumor cell apoptosis. Various approaches to directly target STATs have been developed that include antisense oligonucleotides, decoy oligonucleotides, dominant negative expression vectors, small interfering RNA molecules and specially designed peptide inhibitors of STATs. However, use of these may be limited in terms of their clinical development as therapeutic agents.

The transcription factor NF-κB is proposed to act as a radio-protector and is downstream of ErbB and TNF-α receptors. It has been reported that NF-κB signaling is regulated by the PI3K pathway. Others have suggested regulation by MAPK signaling through autocrine mechanisms. PAR-4, a protein inhibitor of PKCζ and NF-κB function, and downstream of mutant Ras molecules, may be inhibited by MAPK signaling. Recently, we demonstrated that ectopic expression of PAR-4 radio-sensitizes prostate tumor cells and this is mediated through inhibition of NF-κB activity and Bcl-2 expression. Thus, PAR-4 may be a link between MAPK signaling, NF-κB function and radio-sensitivity. Hence, depending on cell type, PI3K, NF-κB or MAPK signaling downstream of receptors and Ras molecules or their combined signals, may play a radio-protective role.

Fig. 4—A model of radiation-induced gene signaling events in mutant p53 background. Lack of p53 function leads to ineffective cell cycle arrest. This causes increased function of pro-survival genes leading to "induced radiation resistance" or chemo-resistance.
Survivin is a smallest member of the mammalian inhibitor of apoptosis family and is also required for cell division. Because survivin is selectively expressed in tumor and its neovasculature and is important to maintain their viability, it is an attractive target for cancer therapy. Strategies of targeting survivin include antisense oligonucleotides, ribozymes, dominant negative mutants and cancer vaccine (Table 1)\(^{266-271}\). The inhibition of survivin by these approaches has been shown to significantly enhance the cytotoxic effects of radiation in melanoma, pancreatic and lung cancer cells\(^{266-268}\).

The radiation-induced signals generated either at cell membrane or at the level of DNA are sensed and relayed by specific kinases leading to the activation/degradation of various transcription factors. These TFs modulate the transcription of the various genes that regulate functions such as cell cycle, DNA repair or apoptosis. Blocking of any of these pathways may modulate the response of the cell to the damage. Events that occur further downstream may also be used to enhance the effects of radiation. Biological manipulation of Bcl-2 family of proteins as well as caspases may synergize with radiation to enhance tumor cell killing. Modulation of c-FLIP, Bcl-2, Bcl\(_{xL}\) or caspase activity by specific gene transfer, demethylating agents or DNA methyltransferase inhibitors, antisense oligonucleotides and protease inhibitors may form novel approaches to improve outcome in cancer treatment (Table 1)\(^{281-287}\).

**Novel radiation therapy strategies to enhance tumor cell killing**

In addition to the abovementioned strategies for cancer treatment that are based on the well studied mechanisms of radiation-induced damage and response, some other phenomena induced by radiation may be utilized to improve response of cancer cells to radiation. These novel strategies are: radio-gene therapy, low dose fractionated radiation (LDFRT) as a chemo-potentiator and therapeutic utility of high dose radiation-induced bystander effects and will be reviewed in the following sections.

**Radio-gene therapy**—Control of gene transcription by ionizing radiation in vivo represents a novel method of spatial and temporal regulation of gene-based medical treatments. Activation of transcription of Egr-1 gene by X-rays is regulated through the CArG elements in the promoter region. Weichselbaum et al.\(^{27}\) linked the radiation-inducible promoter region of Egr-1 gene to the gene encoding the radiosensitizing and tumoricidal cytokine, TNF-\(\alpha\) and a replication-deficient adenovirus was used to deliver the Egr-TNF construct to human tumors grown in nude mice. Combined treatment with Ad/Egr-TNF and 5 Gy resulted in increased intratumoral TNF-\(\alpha\) production and increased tumor control compared to treatment with Ad/Egr-TNF alone or with radiation alone. The increase in tumor control was achieved without an increase in normal tissue damage when compared to tissue injury from radiation alone. In addition, combined Ad/Egr-TNF and radiation produced occlusion of tumor microvessels without significant normal tissue damage\(^{288}\).

Based on pre-clinical studies\(^{288-294}\), a phase I study is currently in progress to define the tolerability and biologic effects of TNFerade (a replication defective adenoviral vector containing TNF-\(\alpha\) gene, regulated by the radiation sensitive promoter of Egr-1) in patients with advanced malignancies\(^{295}\). Two patients with chest wall masses were enrolled in the protocol comprising twice-weekly injections on weeks 1 and 2 followed by weekly injections thereafter till 6 weeks. Radiation therapy was given on week 2 and was continued for a maximum of 5 weeks. One patient completed full course of radiation therapy to two lesions (one injected with TNFerade and the other un.injected). Serial CAT scans revealed extensive necrosis in the injected lesion, however, the un.injected lesion shows minimal change from the baseline at the end of radiotherapy. Post-therapy PET scan confirmed differential necrosis in the injected lesion. TNFerade was well tolerated in the first two patients. This study is currently open and is conducted at The Albert Einstein College of Medicine, New York, University of Kentucky, University of Chicago and University of South Florida\(^{295}\).

Low dose fractionated radiation therapy as a chemo-potentiator—Until recently, in the field of radiation biology, the initial slope of the cell survival curve following irradiation (doses of < 1 Gy) was presumed to be an ineffective dose range for human tumor therapy. However, Joiner et al.\(^{286,287}\) revolutionized the thinking regarding low doses of radiation (< 1 Gy) by demonstrating an initial phase of hypersensitivity to radiation (HRS). Increased resistance to radiation was found from doses > 1 Gy, a phenomenon termed induced/increased radiation resistance (IRR).
Low-dose radiation effects have been studied extensively \textit{in vitro}. At doses <1 Gy, several cell lines from various cancer types have demonstrated the presence of HRS region in the initial slope of cell survival curve\textsuperscript{29,30}. Although this has been studied in murine models as well\textsuperscript{30}, it has not been adequately explored in humans. Interestingly, the phenomenon of HRS at low doses of radiation is most pronounced in radio-resistant cells, defined as those with mutant p53 expression\textsuperscript{30,30}. The discovery that HRS does not stimulate cellular repair mechanisms seen at higher doses, provides a plausible explanation of why there is no induction of radio-resistance with HRS, as measured \textit{in vitro}\textsuperscript{30}. Evidences for the role of other mechanisms like apoptosis, cell cycle delay, induction of different type of lesions and involvement of specific cellular repair pathways in the low dose radiation induced effects are not consistent\textsuperscript{30,30}

However, as suggested by Short et al.\textsuperscript{30} to take advantage of the benefits of HRS in the clinical setting, therapy would have to be extended over 7-12 weeks, allowing tumor growth that would abolish the gain attributable to enhanced cell killing. A novel strategy combining low dose radiation as an "enhancer" of full dose chemotherapy may provide a way to avoid the development of resistance found with standard clinical doses of radiation and chemotherapy. Our recently published \textit{in vitro} studies have demonstrated that low-dose fractionated radiation therapy (LDFRT) is a chemo-potentiator of Taxol in two tumor types \textit{viz.}, head and neck and colorectal cancer\textsuperscript{30,30}

Extensive data has emerged on the HRS/IRR phenomenon observed in more than 40 tumor cell lines in response to single low dose radiation. Studies have also shown that HRS occurs after fractionated low doses \textit{in vitro}\textsuperscript{30,30}. Previously, we reported that Paclitaxel failed to radio-sensitize mutant p53 colorectal tumor cell line, HT-29. However, when radiation was delivered in four fractions of 0.5 Gy, a significant sensitization was observed in this mutant p53 cell line\textsuperscript{30}. It appears that there is a different mechanism of synergy between chemotherapy and radiation when LDFRT is used: the induction of DNA repair and pro-survival mechanisms seen with higher doses of radiation does not develop at lower doses. This led us to hypothesize that LDFRT in combination with chemotherapy will render enhanced chemopotentiation and eliminate the "IRR" irrespective of complex genetic alterations that may be found in tumors. Also the toxicities associated with chemotherapy and radiotherapy in cancer treatment can adversely affect short- and long-term patient quality of life and may be life threatening. The ideal sequencing of chemotherapy and radiotherapy in cancer treatment is not known. Optimizing the way radiotherapy and chemotherapy are combined is important in reducing long-term adverse effects\textsuperscript{30}. Concurrent chemotherapy and reduced dose radiotherapy (such as LDFRT) is a novel approach that requires additional evaluation.

In a recent report, the effect of low dose ultra-fractionation schedule (0.4 Gy per fraction-3 fraction per day for 21 days, an approach to exploit the HRS phenomenon) was compared with conventional fraction schedule (1.68 Gy per fraction, 1 fraction per day and 5 fractions per week) in glioma xenografts\textsuperscript{30}. It was found that the low-dose ultra-fractionation caused a significant decrease in tumor growth delay but increased the top-up TCD\textsubscript{50}, dose and further failed to prove the existence of HRS in \textit{in vivo}\textsuperscript{30}. It was not clear from the results of this report that whether there existed any potential utility of low-dose ultrafractionation in translating to clinical practice\textsuperscript{30}. However, in another recent report, repeated irradiation with low dose (0.8 Gy 3 times/day for 4 days/week to a total of 2 consecutive weeks) was markedly more effective than irradiation with single conventional dose (2 Gy/day, for 4 days/week to a total of 2 consecutive weeks) in inhibiting the growth of tumor in mice\textsuperscript{30}. The authors implied that the ultra-fractionation as opposed to continuous hyperfractionation accelerated radiotherapy, reduced long-term injuries and prevented tumor re-population in radiosensitive tumors\textsuperscript{30}. These reported findings are in concordance with results observed in our preliminary study where the LDFRT group showed a significant inhibitory effect and a prolonged tumor re-growth delay when compared with 2 Gy group\textsuperscript{30}. Thus, a thorough evaluation of novel treatment options in animal models remains an essential requirement for clinical translation of the HRS phenomenon. The unique significance of the data generated in our study was that LDFRT in combination with Taxotere significantly controlled tumors of SQ20B xenografts with no tumor re-growth; and remarkably, 30% of animals showed complete tumor cure\textsuperscript{30}. This observation is in agreement with our previous findings reported using tumor cell lines that demonstrated the chemo-potentiating effect of low-dose fractionated radiation\textsuperscript{30,30}. Also, LDFRT at 0.5 Gy in four fractions potentiated the effect of cisplatin and gemci-
tabine, the two non-G2 M arrest inducing drugs, as demonstrated by *in vitro* experiments. The chemopotentiation was achieved by abrogating NF-κB/ERE activity and by direct induction of Bax, translocation of Bax into mitochondria with increased cytochrome C release. LDFRT mediated chemopotentiating effects were independent of p53 functional status. In addition, we recently found that 2 Gy or 7 Gy single fraction induced MDR-1 expression (multi-drug resistant-1 or P-Glycoprotein), whereas, LDFRT failed to induce MDR-1 gene expression. Recently, it has been shown that activators of NF-κB cause induction of MDR-1 gene expression. Thus, we have proposed that lack of induction of MDR-1 gene expression in LDFRT treatment might be due to absence of NF-κB induction, whereas, the presence of MDR-1 gene induction in 2 Gy single fraction is due to induction of NF-κB activity. Thus, the lack of MDR-1 gene expression in LDFRT group may be an important step in providing the chemo-potentiating effect for Taxotere (Fig. 5).

Together, our earlier reported *in vivo* and *in vitro* studies demonstrate that LDFRT can be exploited to enhance the effect of chemotherapy for achieving maximum killing of tumor cells. Thus, the approach of combining low-dose fractionated radiation with chemotherapy is novel in setting a platform for clinical translation of the HRS phenomenon.

**High radiation dose induced bystander effects as therapeutic target**—Bystander effect refers to the response in unirradiated cells that occurs as a result of exposure of other cells to radiation. Experiments using irradiated conditioned medium (ICM) show clear evidence of the production of ceratin factors that do not require gap junction-mediated transfer from cell to cell. Bystander effects have been reported at very low doses of radiation and the effects are not proportional with dose and do not contribute significantly to total damage at higher doses. Features of this effect depend on several factors—the way in which the radiation is delivered; the cell type and cell cycle phase; degree of cell to cell attachment;
the number of irradiated cells; the medium constituents; and the end point of the study. Response of cells to bystander signal may include induction of apoptosis, induction of genomic instability or delayed death, induction of enhanced cell growth or induction of mutations. Alterations in the levels of proteins associated with these effects and with a generalized stress response have also been detected. Both p53 dependent and independent pathways to induce signal has been implicated in bystander effects. A calcium signal has been shown to occur within minutes when recipient cells are exposed to ICM. Similarly, changes in mitochondrial membrane permeability and in mitochondrial biochemistry are observed in unirradiated cells. Experiments allowing the identification of specific targets for this phenomenon have been performed using microbeam irradiation of either cytoplasm or nucleus. Relationship of the bystander effect to low dose hypersensitivity has also been studied and it has been shown that only one phenomenon or the other occurred in the cell lines.

While, low dose-induced bystander effect has been implicated in radiation-induced carcinogenesis, high dose radiation induced bystander effect is implicated in the treatment of metastatic tumors. Egr-1 deficient lung cancer cells (H-460) were sensitized by the bystander effect originated from the indirect high dose radiation exposure (10 Gy) and this sensitization was achieved by mitigating the pro-survival effects of NF-kB and Bcl-2 with concomitant induction of TRAIL secretion. Thus, bystander-response can be exploited in clinical situation as shown by the work carried out in our laboratory.

High dose (15-20 Gy) spatially fractionated radiation was delivered to the patients having large and bulky tumors which were unlikely to benefit from conventional radiation therapy using GRID. Significant tumor responses have been observed without side effects. This effect on tumor regression appears to be a combination of both direct physical effect of the radiation and a biological effect (bystander) that may be indirect but more global. Since the bystander effects may be mediated by production of cytokines, the levels of TNF-α and TGF-β in patients’ sera obtained before and after GRID therapy were correlated with patients’ response. The GRID therapy resulted in induction of TNF-α levels (resulting in increased tumor cell killing) associated with reduced TGF-β levels (conferring a protective effect on normal tissues) and thus provided a therapeutic gain factor. GRID radiation also resulted in induction of ceramide and sphingomyelinase activity in serum obtained from patients following treatment and these levels correlated with the clinical response. Further, uptake of LDL (from patients treated with GRID) sensitized the endothelial cells to undergo apoptosis in response to 5 Gy radiation, that by itself had no effect on cell death suggesting that LDL-associated ceramide may be involved in tumor reduction. Thus, spatially fractionated GRID radiation therapy is a novel strategy to treat bulky radio-resistant tumors that is achieved by delivery of high radio-inductive dose (causing bystander signaling) followed by conventional radiation fractions leading to autoradio-sensitization in presence of bystander factors.

Conclusion
Balance of survival and death signals determines the homeostasis of the normal cell systems. Due to the advances in understanding of molecular pathways of apoptosis induction and its regulation in the recent past, several new treatment approaches for cancer have been developed. Many survival signals transduced via cell surface receptors and kinases increase the apoptotic threshold. Active modulation of apoptotic and survival signaling pathways may affect the radiation response. Response to ionizing radiation can be increased by using different drugs as sensitizers or radiation can itself be used to sensitize cells to certain pro-apoptotic stimuli.

Novel apoptosis-inducing drugs that increase the response of cells to radiation have been previously described as drugs that induce apoptosis directly or drugs that alter the threshold for the induction of radiation response in tumor cells. However, the mechanism of action of the combined modality still needs to be understood completely. In addition, since most of the targets control several pathways and there is a cross-talk between these cascades, it is difficult to determine the cause and effect relationship. Also, the genetic background of the cell represents an important criterion for selection of particular molecule as the target.

Another important issue is normal tissue toxicity. Generally, high doses of radiation cannot be delivered due to potential fatal toxicity of the surrounding normal tissue. With novel strategies like low dose fractionated radiation that act as a chemo-potentiator and the delivery of high dose spatially fractionated GRID
radiation that exploit bystander effect, the toxicity to the normal tissue can be reduced significantly and better therapeutic gain can be achieved. However, more in vivo studies are required to completely understand and assess the potential toxicity of any combination treatment. Thus, further understanding of the apoptotic pathways and treatment-induced pro-survival pathways will shed light for the development of novel strategies in the treatment of cancer with the intent for complete local tumor response and elimination of metastatic disease.

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