Radiation-Induced Apoptosis

The Ceramide-SAPK Signaling Pathway and Clinical Aspects

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Apoptosis, or programmed cell death is an important regulatory mechanism that is involved in a variety of homeostatic processes. Decreased cellular sensitivity or inappropriate responses to apoptotic stimuli may be important factors in tumorigenesis and resistance to anticancer treatments. It is generally accepted that all mammalian cells constitutively express the biochemical machinery to execute apoptosis. It is, however, not clear which signal transduction pathways are involved, or to which extent various stimuli activate independent or partially overlapping pathways. In this paper we discuss the involvement of a ceramide-mediated stress-activated protein kinase (SAPK) signaling cascade in radiation-induced apoptosis. Furthermore, examples are presented of pharmacological intervention in specific signal transduction pathways that lead to modulation of the apoptotic response. Finally, data are presented to illustrate the potential clinical relevance of apoptosis.

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Apoptosis, or programmed cell death, is a distinct mode of cell death and considered to be a major regulatory mechanism in eliminating abundant cells during embryonal development, growth, differentiation and normal cell turnover (1). It is fundamentally different from necrotic cell death based on specific morphologic and biochemical features (2) (Table 1). Apoptosis can be induced by a wide variety of stimuli, including ionizing radiation (Table 2).

The cellular target for radiation-induced apoptosis may be the plasma membrane, cytosol, nuclear DNA or a combination. Classically, radiation-induced DNA damage and subsequent failure to maintain structural and metabolic reproductive integrity is considered as the main mechanism that leads to (postmitotic) cell death. The recent (re-)emergence of apoptotic or interphase cell death in response to a variety of stress factors (1, 3), constitutes an alternative model of cell kill. Its contribution to the clinical effects of radiation, however, remains unclear.

THE SPHINGOMYELIN SIGNAL TRANSDUCTION PATHWAY

Apoptotic cell death occurs rapidly and may be triggered by membrane-derived signals, independently of nuclear DNA damage (4–6). One of these signaling systems is the sphingomyelin pathway, a ubiquitous and well-preserved signaling system which couples specific cell surface receptors to the nucleus (7–10). Activation of this pathway involves hydrolysis of plasma membrane-localized sphingomyelin (SM) by the enzyme sphingomyelinase (SMase), resulting in rapid generation of the second messenger ceramide (Fig. 1A). In some cell systems ceramide was found to mediate mitogenesis and cell differentiation, while in others it was shown to be a potent inducer of apoptosis (4, 11–14). A number of different extracellular stimuli have now been reported to activate the SM pathway, including TNFα, CD95/Fas, CD28, IL-1, corticosteroids, interferon-γ, vitamin D_3 and ionizing radiation (4, 7, 9, 14–17).

The first convincing evidence suggesting that ionizing radiation employs the SM pathway to generate ceramide and initiate apoptosis was provided by Haimovitz-Friedman et al. (4) who showed that postnuclear supernatants of irradiated endothelial cells were signaling-competent and that addition of synthetic ceramide analogs mimicked radiation to induce apoptosis. Conclusive evidence for a critical role of the SM signal transduction pathway in radiation-induced apoptosis originates from observations by Santana et al. (18). This group found that lymphoblasts

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from patients with Niemann-Pick disease (NPD), a severe neurological disorder characterized by an inherited deficiency of acidic SMase activity, did not generate ceramide and were resistant to radiation-induced apoptosis. Retroviral transduction of the human acidic SMase gene in these NPD cells restored the cellular capacity to generate ceramide and undergo apoptosis after irradiation. Similar results were found in vivo using an acidic SMase deficient knockout mouse model (18).

Recently, we reported on an alternative mechanism for ceramide generation which appeared to be essential in signaling apoptosis for the chemotherapeutic agent daunorubicin (Dx) (19). This anthracyclin drug induced a progressive elevation of ceramide in U937 cells and murine P388 leukemia cells. In contrast to the acute rise in ceramide levels as observed after radiation, Dx-induced elevation of ceramide was delayed and detected after only 4–6 h of stimulation (19). Dx did not activate sphingomyelinase and the increase in ceramide levels was not associated with a concomitant decrease in SM levels (as observed immediately after irradiation). In fact, an increase in cellular SM content was observed. Dx-induced ceramide generation occurred via activation of the enzyme ceramide synthase, which catalyzes de novo ceramide synthesis from sphingoid bases and fatty acids (20) (Fig. 1B). Ceramide elevation appeared to be obligatory in Dx-induced cell death since fumonisin B1, a natural specific inhibitor of ceramide synthase, blocked Dx-induced ceramide synthesis and apoptosis (19). Collectively, these studies define a pivotal role for ceramide as a mediator of various types of stress-induced apoptosis.

Table 1

Morphologic and biochemical characteristics of apoptosis versus necrosis

Characteristic	Apoptosis	Necrosis
Morphology		
Distribution	Isolated, single cells	Groups of cells
Cell size	Shrunken	Swollen
Cell bounderies	Distinct	Indistinct
Membrane	Blebs, initially intact	Leakage
Mitochondria	Intact	Swollen
Nucleus	Chromatin condensation	Karyorhexis
Cell fragmentation	Apoptotic bodies	No bodies
Inflammation	No	Common
Biochemistry		
Stimuli	Stress/physiological factors	Irreversible injury
Requires energy	Yes	No
Gene transcription	Yes	No
DNA fragments	Multiples of 180–200 bp	Random

DOWNSTREAM TARGETS OF CERAMIDE ACTION

At present it is not known how ceramide, once generated, links upstream signaling events to downstream effectors of apoptosis. A number of direct targets for ceramide action, however, have been reported. These include: 1) a membrane-bound serine/threonine kinase termed ceramide-activated protein kinase (CAPK), which phosphorylates Raf-1 (21) and was recently identified as a kinase suppressor of Ras (KSR) (22); 2) a cytosolic serine/threonine protein phosphatase, designated ceramide-activated protein phosphatase (CAPP) (23), involved in the regulation of apoptosis and c-myc down-regulation; 3) PKC-ζ, an atypical PKC isoform, which has been associated with the regulation of NF-κB (24, 25); 4) the guanine nucleotide exchange activity of the protooncogene Vav, a putative activator of Ras and related proteins (26). We have recently shown (27) that ceramide couples to a stress-activated protein kinase (SAPK or JNK) cascade (Fig. 2). SAPK belongs to the stress kinases of the mitogen-activated protein kinase (MAPK) family. Activation of the SAPK pathway appeared to be essential in transducing death signals, since disruption of the pathway by dominant-negative transfectants, abrogated both ceramide- and stress-induced apoptosis (27–33). Another MAPK kinase homolog involved in cellular stress responses is p38, the mammalian counterpart of yeast Hog1. Whereas p38 is shown to be involved in various forms of stress-induced apoptosis, it does not seem to play a major role in radiation-induced apoptotic cell death (28, 31–33).

THE ROLE OF THE STRESS-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY IN RADIATION-INDUCED APOPTOSIS

Since cellular stress, and more recently SMase and C2-ceramide were reported to activate the SAPK cascade (34–

Table 2

Triggers of apoptosis

Extracellular stress
Ionizing radiation
UV radiation
Heat shock
Oxidative stress
Osmotic stress
Protein synthesis inhibition
Loss of cell adhesion (*anoikis*)
Chemotherapeutic agents
Receptor-mediated processes
CD95/Fas/APO-1
TNFα
Hormone/growth factor addition/withdrawal
Cytotoxic T-lymphocytes

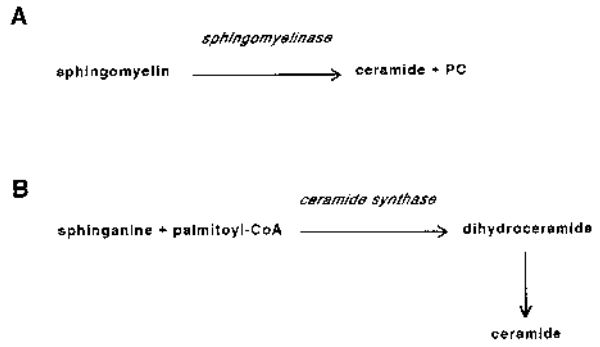

Fig. 1. Mechanisms of ceramide generation. A. The enzyme sphingomyelinase mediates the hydrolysis of sphingomyelin to form ceramide and phosphatidylcholine (PC). B. De novo ceramide synthesis is mediated by the enzyme ceramide synthase, which catalyzes the condensation of sphinganine and fatty acyl-coenzyme A to form dihydroceramide, which is rapidly oxidized to form ceramide.

39), we investigated the role of the SM pathway on this signaling system (27). Signaling through the SAPK cascade may be initiated via MEKK1 and involves sequential phosphorylation and activation of SEK1, SAPK and c-Jun (34, 36–38, 40) (Fig. 2). In order to study SAPK activation by ceramide, we stimulated U937 cells and bovine aortic

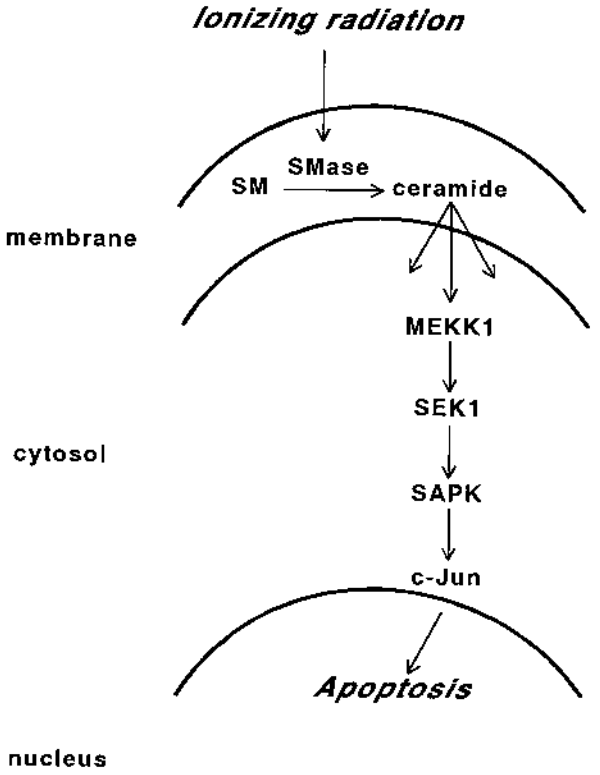

Fig. 2. Radiation induces a ceramide-mediated SAPK activated signal transduction pathway leading to apoptosis.

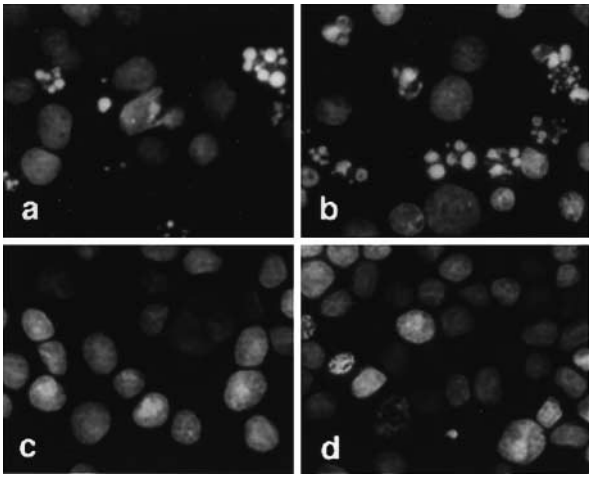

Fig. 3. Radiation- and ceramide-induced apoptosis is blocked in U937 cells, overexpressing a dominant-negative c-Jun mutant (TAM-67). Apoptotic nuclear changes at 12 h in U937/wild type (a, b) and U937/TAM-67 cells (c, d). Cells were irradiated to 5 Gy (a, c) or treated with 100 μM C2-ceramide (b, d).

endothelial cells (BAEC) with synthetic ceramide analogs or *S. aureus* sphingomyelinase. Both C2-ceramide and bacterial sphingomyelinase induced concentration-dependent activation of SAPK up to 20–40-fold of control (27). Other potential lipid second messengers, including C2-dihydroceramide, diacylglycerol, phosphatidic acid and arachidonic acid, failed to activate SAPK, indicating that ceramide is a specific and efficient activator of the SAPK cascade. Subjecting the cells to stress treatment, including ionizing radiation, significantly activated SAPK within 10–20 min (27).

By using dominant-interfering mutants of members of the SAPK pathway, we were able to evaluate the crucial involvement of this signaling system in radiation-induced apoptosis. In the kinase-inactive SEK1 mutant (SEK 1[K-R]) (36) the critical lysine (Lys-129) of the ATP-binding site is replaced by arginine. In the dominant-negative construct TAM-67 (Ref. (41)) the amino-terminal transactivation domain of c-Jun (amino acids 3–122) is deleted, including Ser-63 and Ser-73, the sites of phosphorylation and activation via the SAPK pathway (42, 43). Cells overexpressing these dominant-negative mutants were resistant to radiation- and C2-ceramide-induced apoptosis as compared to wild type and vector-only transfected cells (27) (Fig. 3). Transfection with these inhibitory constructs did not interfere with the cellular capacity to generate ceramide after exposure to radiation. Furthermore, we could not demonstrate significant activation of p38 in response to irradiation (unpublished data). These studies clearly show that expression of radiation-induced apoptosis in U937 cells and BAEC is mediated via ceramide and requires activation of the SAPK cascade.

CLINICAL RELEVANCE OF APOPTOSIS IN RADIOTHERAPY

Although important progress has been made in understanding the biochemical pathways involved in apoptosis, the role of this mode of cell death in radiotherapy is still uncertain (44). Two areas of apoptosis research with potential relevance for clinical radiation therapy are discussed here.

The first relates to the possibility of intervening in specific signal transduction pathways to modulate the apoptotic response. It has been suggested that cell death and cell survival are tightly regulated by opposing actions of pro-apoptotic and anti-apoptotic signals (32, 45). Whereas activation of the SAPK pathway leads to apoptosis in many cell systems (27–33), signaling through the mitogen-activated protein kinase (MAPK) pathway is associated with cell survival and differentiation (46). The balance between both signaling systems might determine whether a cell survives or undergoes apoptosis. Accordingly, protein kinase C (PKC) being a promoter of the MAPK pathway, might counteract ceramide/SAPK-mediated apoptosis. In this respect, it has been shown that in cultured endothelial cells, PKC activation by basic fibroblast growth factor (bFGF) or phorbol ester protects against radiation-induced apoptosis (47). This radioprotective effect was abolished by pretreatment with the PKC inhibitor H-7. To explore whether bFGF would also protect endothelial cells against radiation-induced apoptosis in vivo, Fuks et al. (48) injected mice intravenously with bFGF prior to and after whole-lung irradiation. Histopathological analyses of irradiated lung tissue of bFGF-treated animals showed an inhibition of the apoptotic response in the endothelium, but not in other pulmonary cell types. This resulted in a significant protection from lethal radiation pneumonitis as compared to irradiated control animals (48). This example of a signaling-based apoptosis therapy provides an important basis for further (clinical) studies.

We have recently pursued a different approach to modulate the apoptotic response following irradiation. By using synthetic membrane-permeable alkylphospholipids (or ether-phospholipids), we aim at inhibiting the anti-apoptotic MAPK pathway, thereby favoring the SAPK pathway leading to apoptosis. Unlike the classical chemotherapeutic drugs which target the DNA, these alkylphospholipids exert their action at the cell membrane level where they interfere with mitogenic signal transduction pathways. Classical examples of such alkylphospholipids are 1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine (ET-18-OCH_3; Edelfosine) and hexadecylphosphocholine (HePC; Miltefosine). These and related compounds have been extensively tested as anticancer agents for more than a decade, also in clinical trials (49, 50). Although the mode of action of alkylphospholipids has not yet been fully established, both in vitro and in vivo studies indicate that these compounds interfere with mitogenic signaling at different levels. These antiproliferative mechanisms include: modulation of the biosynthesis and turnover of phospholipids (51, 52), reduction of inositol 1,4,5-triphosphate (IP_3) formation and calcium release (53), inhibition of phospholipase C (PLC) and PKC activity (53) and induction of c-Jun expression (54). Recent studies have suggested that apoptosis is an important mechanism by which alkylphospholipids exert their cytotoxic effect on tumor cells (55). Indeed, ET-18-OCH_3 and other alkylphospholipids were reported to induce apoptosis in a variety of human tumor cell lines (52, 55–57) and in primary tumor cell cultures from cancer patients (55). More importantly, whereas human leukemic cells were found highly sensitive to the lethal action of ET-18-OCH_3, normal cells remained unaffected (55), illustrating the selective antitumor properties of this class of drugs. Preliminary data from our laboratory indicate that HePC may potentiate the apoptotic effect by radiation. In the murine T-lymphoma cell line BW5147, which does not undergo significant apoptosis after exposure to radiation and displays only minimal apoptotic changes after HePC, we demonstrated that the combination of both treatments resulted in high levels of apoptosis.

Another intriguing observation with potential clinical applicability, relates to the predictive value of pretreatment levels of apoptosis for treatment outcome. Radiation is known to enhance apoptosis in both normal tissues and tumors, and it has been suggested that the acute apoptotic response following irradiation may be a feature of radiosensitivity (3). In some normal tissues this phenomenon has already been illustrated convincingly (6, 58–60). For tumor cells, however, the data are less consistent. Several investigators have quantified apoptosis in a variety of murine tumor types. Although these studies showed a heterogeneity in the apoptotic response after radiation, a correlation was established between the level of apoptosis prior to, and the tumor response following irradiation (61, 62). These findings raise the possibility that pretreatment levels of apoptosis may predict for tissue responsiveness to radiation. A number of clinical studies have been carried out correlating spontaneous apoptosis with treatment outcome. A review of the data from the literature (63–73) shows conflicting results; whereas half of the studies show a correlation between a high baseline apoptotic index (AI) and good prognosis, the other half show the opposite (Table 3). For example, a retrospective analysis on human rectal tumors showed a trend for higher apoptosis scores in those responding well to radiotherapy (63). Recently, Wheeler et al. (64) reported that elevated levels of pretreatment apoptosis in cervical cancer were correlated with improved survival. These and other studies support the hypothesis that tissues with a relatively high baseline AI

Table 3

Apoptosis as a predictor for treatment outcome

Tumor	Stage	n	Treatment	Correlation	Reference
Lymphoma	I–IV	50	Chemo	High AI = poor prognosis	66
Rectal carcinoma	T3	30	Surgery + XRT	High AI = good prognosis	63
Prostate adenocarcinoma	All	279	Surgery; hormonal; XRT	High AI = poor prognosis	67
Prostate adenocarcinoma	T2	28	Surgery	High AI = poor prognosis	68
Cervix carcinoma	T1–3	66	XRT	High AI = poor prognosis	65
NSCLC		47	Surgery	High AI = poor prognosis	69
SQC	T1–3N0-2				
Cervix adenocarcinoma	Ib	44	XRT	High AI = good prognosis	64
Bladder carcinoma	T3b	51	XRT	High AI = good prognosis	70
Lymphoma	Low grade	60	Chemo + XRT; chemo	High AI = good prognosis	71
NSCLC	N1		surgery; surgery +	SQC:high AI = good prognosis;	72
SQC		86	XRT; chemosurgery +	AC/LC:high AI = poor prognosis	
AC/LC		76	XRT + chemo		
Follicular lymphoma	I–II	144	XRT; chemo; chemo + XRT	High AI = good prognosis	73

Chemo = chemotherapy; AI = apoptotic index; XRT = radiation therapy; NSCLC = non-small cell lung cancer; SQC = squamous cell carcinoma; AC = adenocarcinoma; LC = large cell carcinoma.

are more sensitive to ionizing radiation than those with a low AI. On the other hand, Levine and co-workers (65) found, also in cervical cancer, that high baseline apoptosis was associated with poor tumor response and patient survival. This group also observed a correlation between apoptosis and mitotic indices. This may be consistent with the observation that in normal developmental tissues high incidences of apoptosis and mitosis often coexist (3). It would furthermore imply that fast-proliferating tumors with high mitotic (and apoptotic) indices respond less well to conventional radiotherapy than more slowly growing tumors, due to clonogenic repopulation during treatment. Most recently, a correlation was established between radiation-induced apoptosis, measured in vitro and early response to in vivo radiotherapy in 26 patients with low-grade non-Hodgkin's lymphoma (74). These data illustrate the need for further evaluation of apoptosis as a predictor for tumor radio responsiveness.

CONCLUDING REMARKS

Several signal transduction pathways have been identified as being critically involved in the induction of apoptosis following exposure to ionizing radiation. One of these pathways is the sphingomyelin pathway which is initiated by hydrolysis of sphingomyelin leading to the generation of ceramide, a potent inducer of apoptosis. Ceramide was recently found to couple to the stress-activated protein kinase (SAPK) cascade, transducing death signals from the cell membrane to the nucleus. Accumulating data suggest the coexistence of both anti- and pro-apoptotic signaling pathways, and indicate that the balance between these opposing signals may determine whether a cell survives or undergoes apoptosis. This provides a potential target for pharmacological intervention in specific signaling pathways, which may lead to the development of new therapeutic strategies. Finally, several groups of investigators have evaluated whether pretreatment levels of apoptosis can be used to predict tumor radio responsiveness. Thus far, the data are inconclusive, illustrating the need for more, prospective studies.

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