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REVIEW

Shared Pathways: Death Receptors and Cytotoxic Drugs in Cancer Therapy*

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Death ligands (TNF, FasL, TRAIL) and their respective death receptor signaling pathways can be used to induce tumor cells to undergo apoptosis. Chemotherapeutic drugs can induce apoptosis and the upregulation of death ligands or their receptors. Downstream events following cytotoxic stress-induced DNA damage and the signaling pathways that lead to the induction of apoptosis may be either dependent or independent of death receptor signaling. The involvement of the Fas signaling pathway in chemotherapy-induced apoptosis has been the most extensively studied, with the current emergence of information on the TRAIL signaling pathway. Fas-mediated and chemotherapy-induced apoptosis can converge at the level of the receptor, FasL, DISC formation, activation of the initiator caspase-8, at the level of the mitochondria, or at the level of downstream effector caspase activation. Convergence is influenced by the specific form of DNA damage, the cellular environment, and the specific pathway(s) by which death receptor-mediated or drug-mediated apoptosis are induced. This review discusses the different levels of interaction between signaling pathways in the different forms of cell death. (Pathology Oncology Research Vol 7, No 2, 95–106, 2001)

Keywords: apoptosis, tumor, death receptor, Fas, drug, p53

Death receptors and their ligands

Cells of higher metazoans activate their self-destructive mechanisms in response not only to enviromental stress, internal damage or lack of survival signals but also by the "instruction" of specific death signals (reviewed in 1,2). Death receptors are cell surface receptors that transmit apoptosis signals initiated by their specific "death ligands". Death receptors belong to the TNF receptor superfamily, and are defined by homologous cysteine-rich extracellular domains. There are six members of this family (DR1-DR6) that contain a cytoplasmic "death domain". These death domains enable the receptors to deliver apoptotic signals in sensitive cells upon binding of the specific ligand. In addition, there are three "decoy" receptors with functional extracellular domains and absent or truncated death domains that bind death ligands (DcR 1-3), but are unable to transmit a cell death signal (*Figure 1*).

Death ligands

Death ligands that bind to specific receptors of the TNF receptor superfamily are type II transmembrane glycoproteins with an extracellular C-terminus, a retained transmembrane region, and a cytoplasmic tail (reviewed in 1,2)

Death ligands form oligomers, most often trimers, that are important for their ability to crosslink their receptors. Proteolytic processing is a common postranslational feature of death ligands. TNF[3] and FasL[4,5] are each processed by metalloproteinases, whereas TRAIL[6] is cleaved by cysteine proteases at the cell surface to yield a soluble

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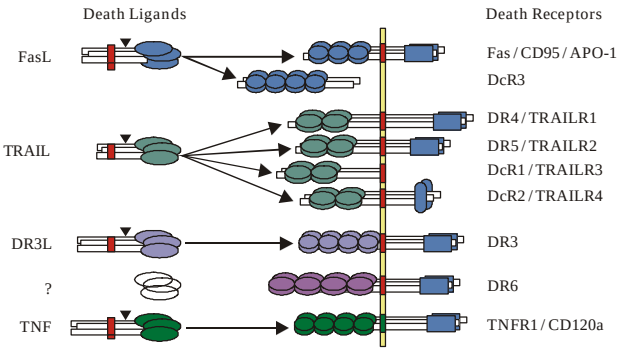

Figure 1. Death receptors belong to the TNF receptor superfamily and share a distinctive intracytoplasmic domain called the "death domain". Their respective death ligands can also bind to "decoy" receptors, where the death domain is truncated or absent.

cytokine. Data indicate tissue-specific differences in death ligand processing determined by different expression of various proteases (e.g. MMPIs) and protease inhibitors (e.g. TIMPs). Therefore, the postranslational modification of death ligands may influence their biological activity in different tissues. The membrane-bound position may confer distinct receptor binding and cellular responses not seen in soluble counterparts. Soluble FasL may induce autocrine signaling in the same cell or paracrine signaling to induce apoptosis in neighboring cells.[7] Others have found that soluble FasL is less potent than the membrane-bound form, and that cell surface "shedding" of the ligand is the mechanism of inactivation of its cytotoxicity.[4] In xenograft models FasL-expressing tumors attracted infiltrating neutrophil granulocytes resulting in decreased tumor growth compared to FasL-negative tumors. Further, adenoviral mediated gene transfer of FasL induced regression of Fas^- CT26 colon carcinoma xenografts, which was mediated by inflammatory cells.[8]

The study of these death receptor/death ligand systems and their signaling mechanisms can influence the search for improved cancer therapy in several ways: 1) Death ligands can be used directly to target cancer cells expressing their specific receptor, 2) DNA damaging agents may induce apoptosis dependent on death receptor signaling, 3) Chemotherapeutic agents and cytokines can modulate the death receptor sensitivity of tumor cells, and 4) Drugs can directly activate or inhibit signal components downstream of death receptors.

Receptors for FasL (Fas [DR2], DcR3)

The cell surface receptor Fas (APO-1, CD95) and its ligand (FasL) regulate apoptosis in cells of the immune system and in neoplastic cells derived from other tissues. However analyses of tissues from mice have demonstrated expression of Fas in tissues largely characterized by high rates of cell turnover and apoptotic cell death, including epithelial tissues.[9,10] The decoy receptor DcR3 can also bind FasL and is frequently expressed in malignancies[11] but does not necessarily correlate with resistance to Fas-mediated apoptosis. Following trimerization of Fas after ligation, apoptosis is initiated.[12] Several proteins have been identified that bind to the intracellular death domain of Fas to form the death-inducing signaling complex (DISC). The adaptor protein FADD[13,14] binds to Fas via the death domain, and subsequently recruits procaspase-8 to the DISC.[15-17] Following release of active caspase-8 from the DISC, a cascade of ICE-like proteases is activated. An anti-apoptotic factor, c-FLIP, has been shown to exist in two isoforms, $c\text{-FLIP}_S$ and $c\text{-FLIP}_L$, which appears to be the predominant form expressed in mammalian cells.[18,19] C-FLIP can be recruited to the DISC and can block further recruitment of procaspase-8 into the complex, thereby inhibiting the activation of caspase-8.[18-20]

In Fas-mediated apoptosis, two signaling pathways have been demonstrated (*Figure 2*).[20-21] In type I cells, ligation of Fas leads to strong caspase-8 activation at the DISC thereby directly activating other caspases including caspase-3 in the absence of mitochondrial involvement. In type II Fas-mediated cell death, only a small amount of DISC is formed leading to the activation of a small amount caspase-8, which cleaves the cytosolic substrate Bid. The proteolytically modified Bid induces conformational changes in Bax that lead to pore formation in the mitochondrial membrane. This process can be blocked by anti-apoptotic members of the Bcl-2 family.[22] Release of mitochondrial cytochrome c and dATP triggers the formation of the "apoptosome" with caspase-9 and the adaptor molecule Apaf-1. In the apoptosome caspase-9 undergoes autoactivation and further activates the effector caspases-3, -6, and –7.[23] The active caspase-6 can activate additional caspase-8 molecules such that the apoptotic signal of Fas can be further amplified.[21,22]

Receptors for TRAIL (DR4, DR5, DcR1, DcR2)

In contrast to the Fas signaling pathway, considerably less is known about the biologic function of TRAIL in the regulation of apoptosis in cells and tissues. Also, in contrast to the Fas system, the only primary cells identified as susceptible to apoptosis induction by TRAIL are activated T lymphocytes, suggesting a role in limiting the immune response,[24,25] hence normal cells are in general TRAIL-resistant. In contrast, tumor cell lines of diverse origin are sensitive to TRAIL.[6,26-29] Like FasL, TRAIL is a type II transmembrane protein with an intracellular amino terminal portion and extracellular carboxyl terminus, and functions optimally in the trimeric form.[28,29] In contrast to other members of the TNF family whose expression is tightly

regulated and only transiently expressed on activated cells, mRNA for TRAIL is detected in a wide range of tissues including peripheral blood lymphocytes, spleen, thymus, prostate, ovary, colon and placenta, but not brain, liver or testis.[30] There are four receptors for TRAIL. DR4[31] and DR5,[32,33] like Fas, are type I transmembrane proteins with extracellular cysteine-rich residues and a cytoplasmic death domain. There are two non-death-domain-containing TRAIL receptors, DcR1 (lacking a cytoplasmic domain;[34]) and DcR2 (containing a truncated death domain;[35,36]), that bind the ligand with comparable affinity to DR4 and DR5 but cannot transmit a cell death signal, and may protect normal tissues from the cytotoxic action of TRAIL, providing a rationale for the use of TRAIL as a cytotoxic. In neoplastic diseases, DR4 and DR5 are expressed in primary human brain tumors,[37] melanomas[38] including those resistant to FasL,[39] and colon carcinomas.[28,40] Further, TRAIL has induced apoptosis in malignant glioma cell lines,[41] melanoma cell lines,[38] breast and colon carcinoma cell lines.[28,40]

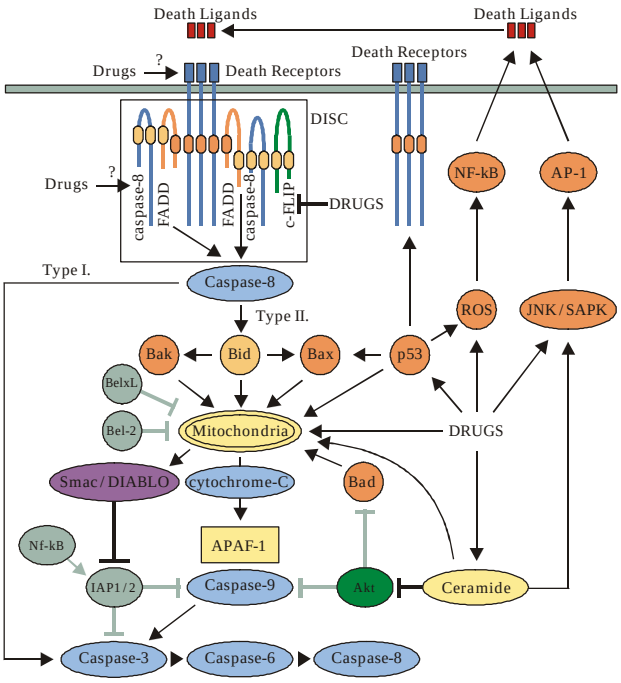

Figure 2. Induction of apoptosis by death ligands and chemotherapeutic drugs: Upon activation of death receptors by their ligands a death-inducing signaling complex (DISC) is formed at the receptor. Activated caspase-8 is released and can directly activate caspase-3 in type I cells. Alternatively, further amplification of the apoptotic signal is required with the involvement of the mitochondria in type II cells. Cleavage of Bid by caspase-8 triggers the release of cytochrome C and Smac/DIABLO from the mitochondria. Cytochrome C activates formation of the apoptosome and subsequently the activation of caspase-9. Smac/DIABLO facilitates the activation of caspase-3 and caspase-9 by blocking the caspase inhibitory proteins (IAP1/2). The activation of caspase-6 can lead to the further activation of caspase-8. Cytotoxic drugs can directly target the mitochondria or can activate various signaling pathways. P53 can induce apoptosis by translocating to the mitochondria, by increasing the expression of the death receptors or pro-apoptotic Bax protein, or by production of reactive oxidative species (ROS). ROS activates the transcription factor NF-kB, which increases the expression of several death ligands including FasL. Stress response signals to drugs include the jun kinase/stress activated protein kinase (JNK/SAPK) system, leading to the activation of AP-1 which transcriptionally regulates the expression of death ligands. Increased production of ceramide can indirectly induce the mitochondrial release of pro-apoptotic molecules and also the inhibition of the anti-apoptotic Akt/PKB kinase. Drugs can also inhibit the expression of the anti-apoptotic c-FLIP molecule and may induce direct activation of receptor aggregation, DISC formation, and caspase-8 activation by unknown mechanisms.

C-FLIP can also inhibit TRAIL-induced apoptosis.[38,42] To date, little information is available concerning the receptor complexes for the TRAIL receptors DR4 and DR5. Studies have reported direct binding of the adaptor proteins FADD and TRADD to these receptors and inhibition of TRAIL-induced apoptosis in the presence of dominant negative (DN) mutant forms of FADD and TRADD.[38] Further, TRAIL-induced DISC formation has been demonstrated with recruitment of FADD and procaspase-8, similar to the Fas-DISC.[42-45] Hence TRAIL signals through formation of a DISC downstream of DR4 or DR5, which explains an inhibitory role for c-FLIP in TRAIL-induced apoptosis.

Apoptosis induced by chemotherapy

It is known that tumor cells can die in response to sublethal stimuli, such that reversible damage can trigger apoptosis. However, when irreversible lethal damage occurs, cell death is not necessarily dependent on the caspase-mediated apoptotic machinery. Irreversible damage can cause cell death without initiating apoptosis, or if triggered, cell death is still not necessarily dependent on caspase activation. Thus, discrepancies can exist between apoptosis, growth inhibition and clonogenic assays.

One of the major goals in cancer therapy is to trigger tumor selective cell death. The discovery of distinct, highly efficient molecular pathways that link cellular stress to cell death led to an explosion of apoptosis research, and its relevance to cancer chemotherapy. It is now widely accepted that in tumor cells many anticancer agents act as apoptosis inducers, and an important determinant of drug resistance is the inability of drugs to trigger apoptosis.[46,47] Although the primary intracellular targets of action of chemotherapeutic agents are distinct, it has become evident that induced cytotoxicity ultimately converges on a common pathway that induces apopto-

sis.[48] Cells treated with cytotoxic agents can show the typical characteristics of apoptosis analogous to apoptosis induced by "physiologic" stimuli such as growth factor withdrawal or death receptor activation. Anti-apoptosis genes including Bcl-2 and Bcl-xL can inhibit apoptosis induced by a wide variety of stimuli including chemotherapeutic drugs.[49] Further, the p53 tumor suppressor gene plays an important role in the regulation of apoptosis induced by DNA damage, which can influence tumor sensitivity to cancer therapy.[50] Most importantly, drug-induced cell death is frequently mediated by caspase-dependent apoptosis.[51]

For hematologic malignances and certain drug-sensitive solid tumors there is rapid induction of apoptosis following drug treatment, where apoptosis has proven to be a prominent mechanism associated with the induction of tumor remission. In contrast, in other solid malignances, the association is less clear (Reviewed in 48). Hematopoetic cells can undergo rapid apoptosis within a few hours,[52] while epithelial cells require at least 24 hours,[53] hence the form and kinetics of cell death are clearly dependent upon the cell type. Further, the drug concentration not only influences the cytotoxic response in a quantitative manner, but can also trigger qualitatively different response mechanisms.[54-57] For doxorubicin and daunomycin, EC_{90} concentrations led to G2/M arrest and cytostasis in HeLa cells in the absence of inhibition of DNA synthesis, ultimately resulting in delayed apoptosis and cell death. In contrast at 10 x EC_{90} concentrations, apoptosis was detected as early as 3 hours after drug treatment.[55] The delay or inhibition of an apoptotic response does not always correlate with long-term cell survival. In HeLa cells rapid apoptosis induced by VP-16 was prevented by Bcl-2, while the loss in clonogenic survival and the formation of giant, multinucleated cells characteristic of mitotic catastrophe, was not affected by Bcl-2.[57] It has been demonstrated that caspase inhibitors can inhibit apoptosis induced by VP-16 in colon carcinoma cells without affecting loss in clonogenic survival.[53] In contrast, DNA damage induced by *5-fluorouracil/leucovorin* (FUra/LV) triggered apoptosis and loss in clonogenic potential, and both were prevented by inhibition of caspase activation.[53] Chemotherapeutic agents can induce loss in clonogenic survival at therapeutically relevant concentrations without acute induction, but with delayed induction, of apoptosis.[53]

Although many different anticancer drugs ultimately mediate cell death by activating the caspase signaling cascade, the molecular mechanisms linking the primary drug target and diverse cellular responses to apoptotic effectors are unclear. The present discussion will examine how drug-induced DNA damage activates apoptosis, and how this may be involved or converge on death receptor-mediated apoptosis pathways.

Sharing pathways between drug – and death receptor – induced apoptosis

Some reports indicate that certain DNA damaging agents may induce apoptosis via Fas signaling,[58-61] while others suggest that stress-induced apoptosis caused by drug treatment may be independent of Fas[62,63] (*Figure 2*). Fas dependency of apoptosis can require interaction between Fas and FasL. However other possibilities exist for crosstalk between pathways of drug-induced cell death and death receptor-induced apoptosis. It is clear that drugs and death receptor ligands induce apoptosis that can converge at the level of the death receptor itself, the initiation phase of caspase activation, or via activation of the same effector caspases resulting in similar morphologic and biochemical features of cell death. Evidence is also emerging that chemotherapeutic agent-induced signaling and signaling via the TRAIL receptors can be convergent in the induction of apoptosis.[64-66]

Sharing the death receptors

In Jurkat T leukemia cells, apoptosis induced by doxorubicin appeared to proceed via activation of FasL,[58] analogous to the dependence of activation-induced apoptosis in T lymphocytes upon transcription and expression of FasL.[67,68] In addition, competitive binding of either FasL by using a Fas-Fc chimeric fusion protein,[58] or of Fas by using a neutralizing anti-Fas Ab, effectively inhibited death induced by VP-16 and VM-26,[60,61] and the early stages of doxorubicin-induced apoptosis,[69] suggesting that apoptosis induced by these agents was mediated via Fas signaling. In Type I cells (BJAB), both the receptor and the mitochondrial pathway were activated upon treatment with doxorubicin or VP-16, since blockade of the receptor pathway by overexpression of DN-FADD or of the mitochondrial pathway by Bcl-xL only partially inhibited apoptosis.[70] Also in these cells, drug treatment induced formation of a FADD- and caspase-8-containing Fas-DISC. It is also well documented that thymineless stress-induced apoptosis induced in thymidylate synthase-deficient (TS^-) human colon carcinoma cells,[71] wt colon carcinoma cells[72] and other cell types[73,74] treated with FUra/LV, that apoptosis signals via Fas both in vitro[71-73] and in vivo.[74] Sensitization of colon carcinoma cells to FUra/LV has been enhanced following upregulated expression of Fas by the cytokine IFN-γ.[72] 5-Fluorouracil and other cytotoxic agents upregulate the expression of FasL,[58,71-73,75] which can be transcriptionally regulated by NF-κB[58,75] or AP-1,[58,73,75] and also upregulate Fas, dependent upon p53.[76,77] In some systems however from data derived using blocking Abs against Fas,[63,78-80] FasL,[53] or DN-FADD,[80,81] drug-induced apoptosis clearly proceeds via a Fas-independent pathway. This has also been demonstrated in cells

selected for resistance to Fas-mediated apoptosis.[53,63,82] Interaction of anticancer drugs with the TRAIL receptors is due to upregulated expression of DR5 which enhances the induction of apoptosis.[64,65]

Sharing the caspase signaling cascade

Following initial caspase activation, these molecules subsequently activate each other in the signaling cascade leading to apoptosis, and form positive feedback loops. There may be a critical threshold level of caspase activation for the "chain-reaction" to be initiated (e.g. Type I vs. Type II cells in Fas-mediated apoptosis). It is possible that independent mechanisms can synergistically activate the same caspases, whether at the level of initiator caspases (e.g. caspase-8) or effector caspases (e.g. caspase-3). This mechanism is a general way of positive interaction between death receptor-induced and drug-induced cell death. Drug-induced and death receptor-induced apoptosis pathways can converge upstream of the mitochondria at the level of caspase-8, or downstream of the mitochondria at the level of effector caspases. Further, drug-induced downregulation of inhibitory factors (c-FLIP, Bcl-2) also enhance caspase activation.

A. Upstream of the mitochondria

It has been demonstrated that anticancer drugs can activate caspase-8 dependent upon or independent of signaling via Fas. Doxorubicin or VP-16 activated caspase-8 as the most apical caspase in Type I cells (BJAB) downstream of DISC formation.[70] Bid was cleaved prior to changes at the level of the mitochondria demonstrating activation of caspase-8 upstream of these organelles. This has also been reported for staurosporine-mediated apoptosis.[83] In addition doxorubicin-induced apoptosis involved FasL-mediated aggregation of Fas followed by DISC formation and activation of caspase-8 in H9 T leukemia cells.[69] However other reports have demonstrated initial caspase-8 activation independent of Fas.[82,84] In the NSCLC cell line H460, overexpression of DN-FADD did not prevent procaspase-8 cleavage and subsequent apoptosis induced by cisplatin, topotecan or gemcitabine.[84] Further overexpression of caspase-9S or XIAP, both inhibitors of caspase-9 activation, failed to block drug-induced apoptosis, indicating lack of mitochondrial involvement in the apoptotic response. In the Jurkat T leukemia model, cells resistant to Fas-mediated apoptosis were equally susceptible to anticancer drugs (doxorubicin, daunorubicin, VP-16, mitomycin-C) and activated caspase-8 with similar kinetics and dose response as Fas-sensitive cells.[82] In addition a neutralizing Fas decoy or DN-FADD selectively abrogated Fas but not drug-induced effects.

Another level upstream of the mitochondria at which Fas-induced and drug-induced apoptosis may converge is at the level of c-FLIP expression, which may determine caspase-8 activation, dependent upon death receptor signaling. In human osteosarcoma MG-63 cells cisplatin downregulated expression of c-FLIP$_L$ in a time-dependent manner thereby sensitizing the cells to Fas-mediated apoptosis, but did not influence the expression of other anti-apoptotic proteins including XIAP, c-IAP-1 or c-IAP-2.[85] Down regulated expression of c-FLIP$_L$ using antisense oligonucleotides sensitizied MG-63 cells to Fas-mediated apoptosis thereby confirming the importance of c-FLIP in the mechanism of sensitization. Inhibitors of protein (cycloheximide) or RNA (actinomycin-D) synthesis have also downregulated c-FLIP expression and sensitized cancer cells to Fas-mediated apoptosis[86,87] and TRAIL-induced apoptosis.[88,89] However this is not a universal mechanism in all cell types, since Jurkat cells transfected with c-FLIP remained sensitive to doxorubicin, VP-16 and vincristine.[90]

B. Downstream of the mitochondria

Depolarization of the mithochondrial membrane and downstream activation of effector caspases is part of the apoptotic machinery in both drug- and death receptor-induced apoptosis. Deficient activation of caspases leads to cross resistance between these two types of induced cell death,[91] and hence the effector caspases form an additional level at which these two signaling pathways can converge. In Type II Fas-mediated apoptosis caspase-8 cleavage occurs predominantly downstream of the mitochondria in contrast to Type I.[20] In Jurkat cells (Type II), variant clones selected for resistance to Fas-mediated apoptosis were cross resistant to VP-16.[92] The pathways did not converge at the level of Fas ligation or caspase-8 signaling, since both effector caspase processing and cytochrome c release were inhibited in the Fas-resistant variants as well as in Bcl-2 transfectants, suggesting the two apoptosis signaling pathways are under common mitochondrial control. The time dependence of activation of caspases-3, -7, -8 and -9 were also similar.[93] In B lymphoma cells epirubicin-induced caspase-8 activation occurred independent of death receptor (Fas) signaling and was mediated downstream of caspase-3 activation.[81] Caspase-3 was also activated after treatment with doxorubicin alone or combined with anti-Fas.[94] These data indicate that drug-induced apoptosis can also act solely in the execution phase of apoptosis, thereby converging downstream of the mitochondria with death receptor signaling pathways.

Convergence at the level of p53

Chemotherapeutic agents including cisplatin, mitomycin, methotrexate, mitoxantrone, doxorubicin and bleomycin can induce Fas expression in a variety of different cell lines, dependent on the presence of a wtp53

gene.[76,77] The upregulation of Fas by chemotherapeutic agents can also sensitize tumor cells to Fas-mediated apoptosis. This has been substantiated in studies in which the wtp53 gene has been transfected, followed by the induction of Fas and enchancement of apoptosis.[95-98] Overexpression of Fas has sensitized cells to p53-mediated apoptosis.[97] Further, FUra combined with LV upregulated expression of Fas in human colon carcinoma cells, dependent on the presence of a wtp53 gene, and HCT116 cells became sensitive to anti-Fas after FUra/LV treatment.[77] This in turn may make cancer cells more vulnerable to cytotoxic lymphocytes expressing FasL. Theoretically, a combination of drugs and recombinant FasL or agonistic anti-Fas mAB could have synergistic antitumor activity in vivo. However FasL is toxic, primarily due to the induction of apoptosis in the liver.[99] The death receptor for TRAIL, DR5, is also upregulated by cytotoxic drugs in a p53-dependent manner.[100] In glioma cell lines, cisplatin and VP-16 upregulated DR5 expression, and demonstrated synergistic activity with TRAIL.[101] TRAIL has not demonstrated toxicity in vivo and clinical evalutaion is currently being initiated using TRAIL as a cytotoxic ligand alone and in combination with chemotherapeutic agents.

Sharing JNK/SAPK and NF-kB signaling pathways

The most controversial interaction between drugs and death receptor signaling is at the level of upregulation of death ligands. It has been shown in several cell types that FasL,[58,59] TRAIL[102] and TNF[102] are upregulated in cytotoxic stress induced by anticancer drugs, independent of p53. Upregulation of FasL in colon carcinoma cell lines has been demonstrated in response to thymineless stress, and after treatment with FUra, doxorubicin, VP-16 or topotecan.[53,71] The transcriptional regulation of FasL is not fully understood. However, there are reports indicating that drugs may activate JNK/SAPK (JNK: c-Jun N-terminal kinases, SAPK: stress-activated protein kinases) and NF-κB pathways by generating ROS[103] or via ceramide[104] signaling.

Transcription factors including NF-κB and AP-1 have been implicated in the induction of apoptosis in response to the cytotoxic agents VP-16 and VM-26,[58] daunorubicin and mitoxantrone,[105] and to ionizing radiation.[58] NF-κB is a dimer of two subunits, NFKB1 (p50) and RelA (p65).[106,107] Activation of NF-κB is controlled by phosporylation and proteolysis of an inhibitory subunit, IκB, which retains the NFKB1/RelA dimer in the cytoplasm. Upon stimulation, IκB is phosphorylated by an as yet unknown kinase and subsequently degraded.[108] The free NF-κB dimer then migrates to the nucleus and binds to specific sequences. Such κB elements are found in promoter regions of genes that are crucial for immune or acute phase responses, including FasL.[58] AP-1 is a sequence-specific transcriptional activator composed of members of the Jun and Fos families (reviewed in 109). These proteins associate to form a variety of homo- and hetero- dimers that bind to a common site. A putative AP-1 binding domain has also been identified in the promoter region of FasL.[58,73] Further implication for the involvement of AP-1 in the induction of apoptosis comes from studies of the involvement of the JNK/SAPK in apoptosis induction. The JNK, members of the MAPK family, phosphorylate the stimulatory sites of c-Jun,[109] and it is well established that AP-1 is induced via activation of the JNK/SAPK pathway.[110,111] Recent studies have identified JNK to be involved in cellular responses to environmental stresses including uv light,[112,113] ionizing radiation,[113,114] DNA-damaging drugs,[59,115-118] and Fas-mediated[119] apoptosis. However, in individual systems, the role of JNK in mediating apoptosis appears dependent upon the agent used to induce DNA damage.[59,120] Thus, activation of JNK by cytotoxic drugs[59,118] has led to apoptosis, and dominant negative mutants of some of the elements of this pathway have delayed or significantly inhibited apoptosis induced by certain cytotoxic agents.[59,121] Ectopic expression of JNK has also induced apoptosis.[113] Additionally, introduction of a mutation into the putative binding site for AP-1 in the promoter region of a FasL reporter construct, resulted in the inhibition of transcriptional activation following VP-16 or VM26 treatment in Jurkat cells.[58] Similar results were obtained with a FasL reporter construct following mutation of the NF-κB binding site.[58] The pathways responsible for NF-κB activation are considerably less clear, and may or may not involve the JNK/SAPK pathway.[58,59,122] However, anticancer drugs including VP-16,[58,123,124] VM-26,[58] actinomycin-D,[124] anthracyclines,[105] topoisomerase I inhibitors,[124] and ara-C[123,125] have been reported to activate NF-κB, and inhibition of this activation can inhibit apoptosis.[58,123]

Sharing the ceramide pathway

Ceramide produced by sphingomyelinases (Smases) has been recognized as an important evolutionary conserved intracellular second messenger.[126] Sphingomyelin breakdown products have been implicated in both cytotoxic stress (chemotherapy, ionizing radiation, UV, heat, oxidative stress)[127-131] and death receptor induced apoptosis.[132] The Smases are sphingomyelin-specific C phospholipases and are identified by their activation requirements such as pH optimum and cation dependence.[126] A synthetic production of ceramide de novo also occurs via the enzyme ceramide synthase, which in mammalian cells catalyzes the reaction between the sphingoid base sphinganine and fatty acyl-CoA to produce dihydroceramide.[133] Ceramide synthase activity has been detected in the endoplasmic reticulum and in the mitochondria.[134, 135]

Ceramide generation in response to TNFR and Fas activation has been associated with the adaptor molecules TRADD and FADD. Overexpression of these molecules enchanced TNF-induced stimulation of the acidic form of Smase. Ceramide production was shown to be initiator caspase dependent but upstream of effector caspase activation. However, this initiator caspase has yet to be identified distinct of caspase-8 or caspase-10.[136-138] In type II Jurkat cells activation of Fas induced elevation in sphingosine and ceramide concentrations.[139] Both ceramide- and sphingosine- induced apoptosis was dependent on mitochondrial membrane depolarization and the release of cytochrome c.[139] Apaf-1-deficient fibroblasts were resistant to ceramide-induced apoptosis,[140] and exogenous ceramide-c2-induced apoptosis was inhibited by overexpression of Bcl-2 and Bcl-xL.[139,141] Ceramide directly induced mitochondrial depolarization and cytochrome c release in isolated mitochondria in some experiments but failed in others. Others found that ceramide was metabolized to disialoganglioside (GD3) in the Golgi apparatus, and caspase dependent accumulation of GD3 was involved in Fas-induced mitochondrial membrane depolarization and apoptosis.[142] Further, ceramide may inhibit protein kinase Akt responsible for the phosphorylation of Bad, which may in turn inhibit Bcl-2.[143]

Since the cytotoxic action of many chemotherapeutic agents is dependent on mitochondrial membrane depolarization and inhibited by Bcl-2, this molecular pathway activated by death receptors could sensitize cells to chemotherapy.

Stress signals induced by chemotherapeutic drugs and DNA damage can induce ceramide synthase activation in the mitochondria and endoplasmic reticulum, probably by mechanisms distinct from Smase activation by death receptors. Activation of ceramide synthase was demonstrated in daunorubicin-induced apoptosis in p388 and U937 cells[127] and also in HL60 cells.[144] The exact mechanism of how DNA damage may induce ceramide synthesis is not known. However, the role of p53 activation has been indicated.[145]

Upregulation of FasL in response to chemotherapeutic agents can be dependent on ceramide production and impaired in cells from patients with Niemann-Pick disease deficient in Smase.[104] However ceramide can induce the activation of JNK that leads to activation of AP-1, which in turn can upregulate several death ligands including FasL.[75] In fact, ceramide-induced apoptosis in HL-60 cells was dependent on AP-1 activation and c-jun expression.[146]

Of interest was the report in which exogenous ceramide-C2 induced apoptosis only in type II cells (Fas-mediated apopotosis), such as Jurkat and CEM, but type I cells (H9, SKW6) were not sensitive. Moreover, protein kinase C activation inhibited both Fas- and ceramide- induced apoptosis only in type II cells.[147] Protein kinase C can induce the activation of sphingosine kinase, the enzyme responsible for the conversion of sphingosine into sphingosine-1-phosphate, a metabolite known to inhibit ceramide-induced apoptosis.[148] Based on these observations we can hypothesize that in type II cells induction of ceramide synthesis by chemotherapeutic agents upregulates FasL and other death ligands leading to activation of death receptors, but at the same time ceramide can directly induce apoptosis via mitochondrial membrane depolarization. In contrast in type I cells, drug-induced apoptosis may be more dependent on the activation of death receptor-induced apoptosis via direct activation of effector caspases independently from the mitochondria. Thus the same chemotherapeutic drug can induce apoptosis dependent on DR-DL interactions in type I cells while being „independent“ in type II cells[70]

Conclusions

Death ligands upregulated during cytotoxic stress in turn can activate an autocrine or paracrine cell death in constitutively sensitive cells. Further, cancer therapy can sensitize resistant tumors to death ligands by upregulating death receptors or via other mechanisms that converge on the pathway leading to apoptosis. Therefore death ligands can be considered as “death genes” since their de novo expression is required for apoptosis induction. It is evident that drug-induced apoptosis can require functional death receptors for the apoptotic signal to be transmitted, however this apoptosis can also proceed independent of Fas. The cell type, the molecular target and the drug concentration determine whether the upregulation of FasL and activation of the Fas pathway is involved in drug-induced apoptosis. Since common pathways between drug-induced and death receptor-induced apoptosis involve direct activation of initiator caspase-8 or of the effector caspases downstream of the mitochondria, aberrant expression of a specific caspase would lead to cross resistance to the two forms of cell death. However cytotoxic stress can directly target the mitochondria, thereby simply bypassing the Fas signal. Drugs that generate ROS or induce ceramide production (e.g. doxorubicin, bleomycin) can rapidly activate the JNK/SAPK pathway and upregulate FasL therefore can induce a Fas-FasL dependent early apoptosis. However, in these model systems Fas dependence is only transient and cell death occurs later induced by alternative signaling mechanisms.

The concept of employing therapeutic strategies to modulate death receptor signaling pathways in combination with anticancer agents is only in its infancy. One approach has involved modulation of the Fas death receptor to sensitize human colon carcinomas to FUra/LV-induced cytotoxicity. Apoptosis downstream of DNA damage induced by FUra/LV is dependent on Fas/FasL interactions and on a

functional Fas signaling pathway. Cells are sensitized to FUra/LV by IFN-γ-induced upregulation of Fas expression.[72] In human colon carcinoma cells the dependency of FUra/LV-induced thymineless death on Fas signaling is unique to this cytotoxic agent and has not been demonstrated for other DNA damaging agents. Hence the combination of FUra/LV and IFN-γ is completing Phase I clinical trial in patients with colon carcinoma and will subsequently be evaluated as a therapeutic modality in previously untreated patients. This is an excellent example of how therapeutic strategies can be developed to exploit the dependency and crosstalk between death receptor signaling and drug signaling pathways.

Further, even if drug induced cytotoxicity can induce "Fas-independent" apoptosis, the reactivation of death receptor pathways may open alternative cell death mechanisms. Also, the role of death receptors and ligands in the anti-tumor activity of chemotherapeutic agents is even more complex in vivo. Death ligands may kill bystander drug-resistant cells and attract immune competent cells, and upregulation of death receptors can sensitize tumor cells to the immune response.

For the cytotoxic ligand TRAIL, anticancer agents with diverse mechanisms of action including VP-16, doxorubicin, cisplatin, FUra and Ara-C, have demonstrated the ability to enhance TRAIL-induced apoptosis.[100,101] These agents can upregulate the expression of TRAIL receptors. While the signaling pathway(s) for TRAIL-induced apoptosis remain to be clearly defined, it is probable that the TRAIL signaling and drug signaling pathways converge at various levels in their respective pathways. Therefore the approaches to combine cytokines or ligands that engage death receptor signaling pathways with cytotoxic agents are yielding considerable potential in the exploration of new targets for therapeutic exploitation in the treatment of human cancers.

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