Chapter 7

APOPTOSIS INDUCTION BY TUMOR-TARGETED TOXINS

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Abstract: Tumor cell-targeted toxins are recombinant proteins that consist of a targeting domain that preferentially recognizes tumor cells and facilitates entry of the protein into the target cells with a bacterial or plant toxin that kills the tumor cells. A large number of toxins targeted against different kinds of tumor cell have been developed in recent years and the first such toxins are approved for use against specific cancers. In most cases, targeted toxins kill their target cells by inducing caspase-dependent apoptosis. However the mechanism by which the apoptotic machinery is activated may differ with different toxins and in different cell types. Moreover, recent work shows that the same toxin can kill different kinds of tumor cells through different molecular mechanisms.

Key words: Diphtheria toxin, tumor cell targeting, caspases, caspase-independent.

1. INTRODUCTION

Despite improvements in cancer treatment in recent years some tumors remain stubbornly resistant to available therapies. Even where useful treatments currently exist many patients respond poorly creating a continuing need to develop and understand the mode of action of new therapies that are targeted against particular tumor types. Tumor cell-targeted toxins represent one such kind of therapy and have been developed to treat a variety of tumors including various kinds of leukemia and lymphomas, indeed the first FDA approved targeted toxin, ONTAK, which consists of a diphtheria toxin fused to IL-2 has been used successfully to treat cutaneous T-cell lymphoma [1]. Promising results have also been

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obtained treating solid tumors especially brain tumors where the toxin is delivered directly to the tumor tissue. Targeted toxins that have been developed for brain cancers using targeting domains that react with receptors that recognize Interleukin13, transferrin, urokinase and IL4 [2-5]. Intratumoral administration of these agents can result in tumor regression and clinical remissions in glioblastoma multiforme patients that can last for years [2, 3]. This finding is encouraging because glioblastoma multiforme patients have a dismal prognosis even with aggressive radiation, chemotherapy and surgical treatments with median survival of only about 9 months and a five year survival rate of ~1% [6].

Targeted toxins are fusion proteins that combine a targeting molecule, which selectively binds to and enters tumor cells, with a toxin that kills the target cells. Tumor cells selectively take up the fusion protein through receptor-mediated endocytosis, the toxin portion is released from the endosome into the cytoplasm and the toxin kills the cell. Different kinds of targeting protein can be used including antibodies that recognize tumor cell-specific epitopes or growth factors that bind to cell surface receptors [7]. Toxins are derived from bacterial pathogens (e.g. diphtheria toxin, DT, or Pseudomonas exotoxin A, PE) or plants (e.g. ricin). These toxins block protein synthesis by different mechanisms. Ricin cleaves ribosomal RNA to disrupt the ribosome whereas DT and PE ADP-ribosylate the translation elongation factor 2 to prevent protein synthesis. The molecular mechanisms through which targeted toxins kill are incompletely understood [8]. In this chapter, we will review how these agents work and discuss how the differences in mechanism between tumor cell types, differences between different kinds of toxin and combinations of toxins and other agents may allow us to design tailored cancer treatment strategies.

2. TARGETED TOXINS CAN INDUCE CASPASE-DEPENDENT APOPTOSIS

Because the toxin molecules (DT, PE or ricin) that have been used to make targeted toxins inhibit protein synthesis and protein synthesis is an essential cellular activity, it seems obvious that the target cells should die in response to the toxin. However mechanistic studies on the way that tumor cells die when treated with these agents has provided several surprises. For example, toxins that inhibit protein synthesis by different mechanism can kill cells by distinct mechanisms suggesting that the way toxin-treated cancer cells die is determined not by the lack of protein synthesis but rather by the way that protein synthesis is inhibited. The best evidence for this arose from a non-biased screen to identify inhibitors of toxin-induced apoptosis.

Genetic selection for cDNAs that confer resistance to PE led to the isolation of specific cDNAs that could prevent PE-induced apoptosis [9]. These cDNAs also confer resistance to DT, but not ricin. One cDNA encoded an antisense fragment of cellular apoptosis susceptibility gene (CAS). Down regulation of endogenous CAS was responsible for resistance to toxin-induced apoptosis. However, CAS antisense had no effect on ADP-ribosylation or inhibition of protein synthesis [10]. Because CAS antisense blocks apoptosis by only some protein synthesis inhibitors and has no effect on protein synthesis inhibition itself, these data suggest that a toxin's ability to induce apoptosis depends on its mechanism of action rather than its ability to inhibit protein synthesis per se. As discussed below this theme, whereby different toxins seem to kill cells through different mechanisms even when all the drugs inhibit protein synthesis is becoming a common refrain in this field. These complexities provide scientific interest from a purely basic perspective– why would cells respond differently when two agents inhibit protein synthesis by different methods? Why do two different cells respond differently when the same agent inhibits protein synthesis? Moreover, they may also provide opportunities to maximize the anti-tumor effect when used clinically.

Targeted toxins can induce apoptosis as shown by caspase activation in the dying cells and classical apoptotic morphology [11]. This raises the question of which apoptosis signaling pathways are activated in the tumor cells. We possess a relatively sophisticated understanding of the apoptotic machinery [12] at least as regards caspase activation. Apoptotic caspases include "initiator caspases" (caspase-2, -8, -9 and -10) that start an apoptotic cascade and "effector caspases" (caspase-3, -6 and –7) that disassemble the cell. Caspases cleave specific substrates at a few sites [13] to alter the activity of the target protein resulting in the apoptotic phenotypes. Two main pathways leading to caspase activation have been characterized [14].

The extrinsic or death receptor pathway is activated by receptors of the Tumor Necrosis Factor Receptor superfamily [15]. These receptors contain an intracellular protein interaction domain called a death domain (DD) and induce apoptosis by forming a multiprotein complex called the Death-Inducing Signaling Complex (DISC). Upon ligand binding, activated death receptors recruit an adapter protein called Fas Associated Death Domain protein (FADD) [16]. FADD consists of two protein interaction domains: a DD and a death effector domain (DED). The DED interacts with a DED on the initiator procaspase-8. FADD binds to the Fas and TRAIL receptors (DR4 and DR5) receptor through interactions between the two death domains and activities that are regulated by the DED [17, 18]. This complex recruits the inactive pro-form of caspase-8. Aggregation of caspase-8 leads to dimerization, which activates protease activity [19-21]. For a recent review,

see [22]. Initiator caspases activate effector caspases such as caspase-3 causing the cell to undergo apoptosis by cleaving specific substrates [13].

Diverse stress pathways cause release of mitochondrial proteins to activate the other well known apoptosis pathway– the "intrinsic" pathway [23]. A defining characteristic of this pathway is that anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-xL inhibit caspase activation and apoptosis induced by stimuli that work through this pathway. Protein release occurs through mechanisms that are still unclear [24]. Released cytochrome c (cyt c) interacts with Apaf-1, pro-caspase 9 and dATP to form a complex called the apoptosome [25]. This complex dimerizes and activates caspase 9, which then activates effector caspases to induce apoptosis. Other released proapoptotic mitochondrial proteins include Apoptosis Inducing Factor (AIF) [26], Smac/Diablo [27, 28] and Endonuclease G [29] and Omi/HtrA2 [30-33]. Death receptors can activate the intrinsic pathway through cleavage of Bid, which translocates to mitochondria [34] providing a link between the extrinsic and intrinsic apoptosis pathways.

3. TARGETED TOXINS ACTIVATE DISTINCT APOPTOTIC PATHWAYS

Targeted toxins can activate caspase 3-like activities that cleave known caspase substrates such as Poly(ADP) ribose polymerase (PARP) [35, 36]. Furthermore, PE-immunotoxin-induced apoptosis was inhibited and cell viability increased by treatment of T cell leukemia cells with a caspase inhibitor, z-VAD.fmk [35]. Diphtheria toxin fusions with GMCSF can activate caspases in myeloid target cells [11, 37], even when these cells display multi-drug resistance to other chemotherapeutic agents [37]. These data suggest that caspase activation is an important aspect of targeted toxin-induced cell death however they do not determine which caspase activation pathway (intrinsic, extrinsic or another pathway) starts the process. A clue comes from experiments where Bcl-2 was overexpressed in MCF7 breast cancer cells and inhibited a PE-fused immunotoxin from inducing apoptosis [36]. However, while this result suggests that mitochondrial dysfunction is important in the response, these data do not discriminate between direct activation of the intrinsic pathway and activation after activation of the extrinsic death receptor pathway. Indeed, other data suggest that components of the extrinsic pathway may be important in toxin-induced tumor cell death. In myeloid leukemia cells, treated with a GMCSF-targeted diphtheria toxin protein both caspase-8 and caspase-9 were activated by the toxin. However, caspase-8, not caspase-9, was the apical caspase responsible for initiating the apoptosis pathway because while caspase-9 inhibition did not affect cell

death, caspase-8 inhibition prevented subsequent caspase-9 activation, effector caspase activation and cell death [11]. The involvement of caspase-8 in toxin-induced death suggests that other components of the extrinsic pathway may be involved and this was confirmed by the finding that the adaptor protein FADD was required for GMCSF-DT-induced apoptosis. However, inhibition of death receptor signaling had no effect on toxin-induced death suggesting that this effect does not involve the death receptors themselves [11]. These data suggest that the targeted toxin may be able to activate the death receptor machinery but through a mechanism that is independent of receptor activation.

4. THE SAME TARGETED TOXIN CAN KILL DIFFERENT TUMOR CELLS BY DIFFERENT MECHANISMS

Different types of cancer sometimes overexpress the same receptor. For example the Epidermal Growth Factor (EGF) Receptor is expressed in many epithelial tumors such as breast cancer and is also frequently overexpressed in other tumor types such as glioblastoma. This allows one to study the mechanism of killing by the same targeted toxin in different tumor cell types. Recent unpublished work from our group shows that an EGF-diphtheria toxin protein kills epithelial tumor cells by activating caspases that lead to classical apoptosis with its associated hallmarks such as membrane blebbing and fragmentation into apoptotic bodies. The same toxin also kills glioma cells [38, 39]. However we do not detect caspase activation in glioma cells, which die without showing the hallmarks of caspase-dependent apoptosis. Moreover, unlike epithelial cells, caspase inhibitors do not affect EGF-diphtheria toxin-induced glioma cell death. These data indicate that different tumor cell types can activate different cell death pathways when treated with a targeted toxin. This result is interesting from a basic mechanistic perspective because it again implies that it is not the fact that protein synthesis is inhibited but rather the way that it is inhibited that regulates how the cells die. More importantly, these data also suggest that different tumor types may respond optimally to combinations of toxins with other agents and could develop different resistance mechanisms. For example, tumor cells that undergo classical apoptosis may develop resistance by inactivating caspases or other components of the extrinsic or intrinsic pathways while this would have no effect in glioma cells.

5. TARGETED TOXINS SYNERGIZE WITH OTHER ANTI-CANCER DRUGS

Targeted diphtheria toxins have been shown to synergize with standard chemotherapeutic agents such as AraC [40]. Other protein synthesis inhibitors such as ricin did not synergize with AraC in these studies providing yet more evidence that the way that the toxin works is more important than the fact that it inhibits protein synthesis in determining how it kills cells. Targeted toxins can also synergize with other targeted toxins. For example, EGF-targeted diphtheria toxin can synergize with IL13-targted pseudomonas exotoxin to kill glioma cells that possess both receptors [39].

Recent unpublished work from our group also demonstrates synergy with other "targeted" anti-cancer therapeutics. In this case we showed that combining EGF-targeted diphtheria toxin with an antibody that activates the TRAIL receptor DR5 led to synergistic cell killing. Interestingly, when the combination was used to treat glioma cells, which as mentioned above do not activate caspases in response to the toxin on its own, robust caspase activation occurred. Thus at least in the case of some tumor cell types, combining a targeted toxin with another anti-cancer agent can not only increase the amount of tumor cell death but can also change the way that the cells die. This could have practically important consequences for cancer therapy. One difference between classical, caspase-dependent apoptosis and other forms of cell death is that caspase-dependent apoptosis is associated with reduced inflammation. This might be good or bad depending on the circumstances. Tumor cell killing with increased inflammation might be more effective at reducing tumor burden because it stimulates host immune-mediated anti-tumor responses, which may work better when the tumor cells die by caspase-independent mechanisms [41]. In other situations, effects associated with increased inflammation such as tissue swelling might cause serious problems. This problem may be more important in specific tissues. For example, inflammation in subcutaneous tissues might be less problematic than inflammation and swelling in the brain.

6. SUMMARY

Targeted toxins have been shown to be effective anti-tumor agents in many preclinical models and are displaying efficacy in clinical trials and making their way into the clinic as approved drugs. These agents often but not always work by inducing caspase-dependent apoptosis, which at least in some cases appears to be achieved through activation of the death receptor signaling pathway but is also affected by Bcl-2 proteins. However, different

toxins activate different cell death pathways and even the same toxin can activate different signaling pathways that lead to cell death in different cells. These complexities make it difficult for us to work out how any given toxin works and suggests that it may not be feasible to extrapolate based on one kind of toxin or one tumor type to determine how any particular toxin works against any particular tumor type. Added complications come from the finding that some toxins synergize with standard chemotherapy agents and with other targeted therapies such as TRAIL receptor agonists and that different targeted toxins can even synergize with each other in some but not all tumor cells. Further understanding of how these agents work should allow us to improve their use as anti-cancer treatments.

REFERENCES

1. Frankel AE, Fleming DR, Powell BL, Gartenhaus R. DAB(389)IL2 (ONTAK((R))) fusion protein therapy of chronic lymphocytic leukaemia. *Expert Opin Biol Ther* 2003;3:179-86.
2. Laske DW, Youle RJ, Oldfield EH. Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumors. *Nat Med* 1997;3:1362-8.
3. Rand RW, Kreitman RJ, Patronas N, Varricchio F, Pastan I, Puri RK. Intratumoral administration of recombinant circularly permuted interleukin-4-Pseudomonas exotoxin in patients with high-grade glioma. *Clin Cancer Res* 2000;6:2157-65.
4. Vallera DA, Li C, Jin N, Panoskaltsis-Mortari A, Hall WA. Targeting urokinase-type plasminogen activator receptor on human glioblastoma tumors with diphtheria toxin fusion protein DTAT. *J Natl Cancer Inst* 2002;94:597-606.
5. Li C, Hall WA, Jin N, Todhunter DA, Panoskaltsis-Mortari A, Vallera DA. Targeting glioblastoma multiforme with an IL-13/diphtheria toxin fusion protein in vitro and in vivo in nude mice. *Protein Eng* 2002;15:419-27.
6. Davis FG, Freels S, Grutsch J, Barlas S, Brem S. Survival rates in patients with primary malignant brain tumors stratified by patient age and tumor histological type: an analysis based on Surveillance, Epidemiology, and End Results (SEER) data, 1973-1991. *J Neurosurg* 1998;88:1-10.
7. Frankel AE, Kreitman RJ, Sausville EA. Targeted toxins. *Clin Cancer Res* 2000;6:326-34.
8. Thorburn A, Thorburn J, Frankel AE. Induction of apoptosis by tumor cell-targeted toxins. *Apoptosis* 2004.
9. Brinkmann U, Brinkmann E, Pastan I. Expression cloning of cDNAs that render cancer cells resistant to Pseudomonas and diphtheria toxin and immunotoxins. *Mol Med* 1995;1:206-16.
10. Brinkmann U, Brinkmann E, Gallo M, Scherf U, Pastan I. Role of CAS, a human homologue to the yeast chromosome segregation gene CSE1, in toxin and tumor necrosis factor mediated apoptosis. *Biochemistry* 1996;35:6891-9.
11. Thorburn J, Frankel AE, Thorburn A. Apoptosis by leukemia cell-targeted diphtheria toxin occurs via receptor-independent activation of Fas-associated death domain protein. *Clin Cancer Res* 2003;9:861-5.

12. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000;407:770-6.
13. Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* 2003;10:76-100.
14. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999;15:269-90.
15. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998;281:1305-8.
16. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995;81:505-12.
17. Thomas LR, Henson A, Reed JC, Salsbury FR, Thorburn A. Direct binding of FADD to the TRAIL receptor DR5 is regulated by the death effector domain of FADD. *J Biol Chem* 2004;279:32780-32785.
18. Thomas L, Stillman D, Thorburn A. Regulation of Fas-associated death domain interactions by the death effector domain identified by a modified reverse two hybrid screen. *J. Biol. Chem.* 2002;277:34343-34348.
19. Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS. A unified model for apical caspase activation. *Mol Cell* 2003;11:529-41.
20. Donepudi M, Sweeney AM, Briand C, Grutter MG. Insights into the Regulatory Mechanism for Caspase-8 Activation. *Mol Cell* 2003;11:543-549.
21. Chang DW, Ditsworth D, Liu H, Srinivasula SM, Alnemri ES, Yang X. Oligomerization is a general mechanism for the activation of apoptosis initiator and inflammatory procaspases. *J Biol Chem* 2003;278:16466-9.
22. Thorburn A. Death Receptor-induced cell killing. *Cellular Signalling* 2004;16:139-144.
23. Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev* 2001;15:2922-33.
24. Newmeyer DD, Ferguson-Miller S. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* 2003;112:481-90.
25. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997;91:479-489.
26. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999;397:441-6.
27. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000;102:33-42.
28. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000;102:43-53.
29. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 2001;412:95-9.
30. Verhagen AM, Silke J, Ekert PG, Pakusch M, Kaufmann H, Connolly LM, Day CL, Tikoo A, Burke R, Wrobel C, Moritz RL, Simpson RJ, Vaux DL. HtrA2 promotes cell death through its serine protease activity and its ability to antagonise inhibitor of apoptosis proteins. *J Biol Chem* 2002;277:445-454.
31. Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, DuBois G, Lazebnik Y, Zervos AS, Fernandes-Alnemri T, Alnemri ES. Identification of

Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts IAP-caspase interaction. *J Biol Chem* 2002;277:432-438.

32. Martins LM, Iaccarino I, Tenev T, Gschmeissner S, Totty NF, Lemoine NR, Savopoulos J, Gray CW, Creasy CL, Dingwall C, Downward J. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a Reaper-like motif. *J Biol Chem* 2002;277:439-444.
33. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, htra2, is released from the mitochondria and interacts with xiap, inducing cell death. *Mol Cell* 2001;8:613-21.
34. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998;94:481-90.
35. Keppler-Hafkemeyer A, Kreitman RJ, Pastan I. Apoptosis induced by immunotoxins used in the treatment of hematologic malignancies. *Int J Cancer* 2000;87:86-94.
36. Keppler-Hafkemeyer A, Brinkmann U, Pastan I. Role of caspases in immunotoxin-induced apoptosis of cancer cells. *Biochemistry* 1998;37:16934-42.
37. Perentesis JP, Waddick KG, Bendel AE, Shao Y, Warman BE, Chandan-Langlie M, Uckun FM. Induction of apoptosis in multidrug-resistant and radiation-resistant acute myeloid leukemia cells by a recombinant fusion toxin directed against the human granulocyte macrophage colony-stimulating factor receptor. *Clin Cancer Res* 1997;3:347-55.
38. Liu TF, Cohen KA, Ramage JG, Willingham MC, Thorburn AM, Frankel AE. A diphtheria toxin-epidermal growth factor fusion protein is cytotoxic to human glioblastoma multiforme cells. *Cancer Res* 2003;63:1834-7.
39. Liu TF, Willingham MC, Tatter SB, Cohen KA, Lowe AC, Thorburn A, Frankel AE. Diphtheria Toxin-Epidermal Growth Factor Fusion Protein and Pseudomonas Exotoxin-Interleukin 13 Fusion Protein Exert Synergistic Toxicity against Human Glioblastoma Multiforme Cells. *Bioconjug Chem* 2003;14:1107-1114.
40. Kim CN, Bhalla K, Kreitman RJ, Willingham MC, Hall P, Tagge EP, Jia T, Frankel AE. Diphtheria toxin fused to granulocyte-macrophage colony-stimulating factor and Ara-C exert synergistic toxicity against human AML HL-60 cells. *Leuk Res* 1999;23:527-38.
41. Reiter I, Krammer B, Schwamberger G. Cutting edge: differential effect of apoptotic versus necrotic tumor cells on macrophage antitumor activities. *J Immunol* 1999;163:1730-2.