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Transformation, Translation and TRAIL: An unexpected intersection

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Abstract

Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is a cytokine with roles in tumor surveillance and tolerance. TRAIL selectively induces apoptosis in many malignant but not normal cells but the underlying cause for spontaneous TRAIL sensitivity remains elusive. We propose a novel hypothesis that links TRAIL sensitivity to translational arrest following stresses that inactivate eukaryotic elongation factor 2 (EF2). Affected cells experience a reduction in apoptotic threshold because, due to their short half-lives, levels of anti-apoptotic proteins quickly drop off once translation elongation is inhibited leaving pro-apoptotic proteins unchallenged. This change in protein profile renders affected cells sensitive to TRAIL-mediated apoptosis and places EF2 into the role of a sensor for cellular damage.

Keywords

Apoptosis; Tumor necrosis factor related apoptosis; inducing ligand (TRAIL); Eukaryotic elongation factor 2 (EF2); Free radicals; Cancer

The tumor necrosis factor (TNF) superfamily includes several members capable of efficiently killing cancer cells *in vitro* and *in vivo*. Their suitability for clinical use, however, has been limited by inflammation and sepsis (for TNF- α) or severe liver toxicity (for FasL) (1). In contrast, TNF related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in cancer cells while sparing non-transformed normal cells (2,3). Recombinant TRAIL is well tolerated and, unlike TNF- α and FasL, is actively being explored as a new anticancer agent in clinical trials. Although TRAIL appears to have clinical potential, its physiological role is less clear. TRAIL knockout mice are not only more susceptible to tumor growth but also more likely to develop various autoimmune diseases (4,5). This suggests that TRAIL may not be cancer-specific but play a larger role in immune surveillance by targeting cells that are harmful, either by reason of tumorigenic potential or self-reactive potential. In this perspective we discuss how translational arrest can alter TRAIL phenotype as well as the potential benefit of free radicals in innate anti-tumor responses mediated by TRAIL.

1. Maintaining TRAIL resistance

Activated immune cells have long been known to express TRAIL but the protein is also expressed in other tissues including liver, colon and the eye (6–8). Co-expression of TRAIL

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receptors and ligand in the same tissue points to the existence of a mechanism that protects cells from autocrine TRAIL signaling under physiologic conditions yet permits TRAIL-induced apoptosis upon cellular transformation. The molecular mechanisms behind the selective activity of TRAIL towards certain transformed cells remain elusive. A number of hypotheses have been advanced and discarded as data has accumulated. For instance, the receptor array for TRAIL is unusually complex, consisting of two active surface receptors, two decoy surface receptors, and a soluble receptor(9). Some researchers have theorized that the ratio of active receptors to decoy receptors could determine the vulnerability of cells to TRAIL. Measurements of receptor expression, however, have generally failed to correlate with TRAIL sensitivity (9). TRAIL signaling downstream of the receptors is similar to FasL: a caspase cascade and mitochondrial involvement have been clearly demonstrated, along with the potential blockage of the signal by anti-apoptotic proteins such as cFLIP, XIAP, survivin and the anti-apoptotic Bcl2 family members (Figure 1).

A 2002 *Oncogene* paper revealed that cycloheximide, a protein synthesis inhibitor, could increase TRAIL sensitivity in non-transformed cells(10). The authors speculated that a short-half life protein may hold apoptotic signaling in check and that inhibition of protein synthesis would clear these protein(s) allowing successful death ligand signaling. Protein life spans are of particular interest in apoptotic signaling, since anti-apoptotic proteins are disproportionately short lived relative to their pro-apoptotic counterparts (Table 1) (11–18). This disproportional allotment of half-lives suggests that any interruption of protein synthesis could quickly render a cell defenseless against pro-apoptotic signals such as TRAIL. The idea that resistance must be actively maintained was recently bolstered by a report by Adams and Cooper which confirmed a 30 minute half life for Mcl-1 and further demonstrated that perpetual replenishment of the Mcl-1 pool was required to inhibit apoptosis(19). In addition, elegant work by Willis and co-workers indicates that apoptosis may be the default mode of cells, which is continually challenged by anti-apoptotic proteins in healthy cells(20). They show that BH3-only proteins, such as the pro-apoptotic Bid and Puma, exert their function by binding and sequestering anti-apoptotic proteins(20). Rather than activating pro-apoptotic proteins such as Bak and Bax, the BH3-only proteins help to induce apoptosis by removing anti-apoptotic challengers of Bak and Bax. Thus, survival requires the presence and activity of anti-apoptotic proteins and, due to their short half-lives, presence of these proteins requires active translational machinery. In this perspective we will discuss how modulating the activity of one critical translational enzyme, eukaryotic elongation factor-2 (eEF2, EF2), could contribute to TRAIL-induced apoptosis. EF2 has been extensively studied in the field of ageing but its potential role in cancer and apoptosis has not been explored.

2. Eukaryotic Elongation Factor 2 (EF2)

2.1 EF2 in the role of “sensor”

Elongation Factor 2 (EF2) is an essential component of the cell's translation machinery, functioning as the critical facilitator for movement of the mRNA codons through the ribosomes. EF2 has proved to be surprisingly sensitive to inhibition via phosphorylation or ADP-ribosylation, both of which lead to an immediate translational arrest (21),(22). We found that the commonly used chemotherapeutic agent doxorubicin enhances sensitivity to TRAIL, unfortunately though without selectivity towards malignant cells(23). The drug also significantly inhibits cellular protein synthesis and in investigating the cause of this inhibition, we found that doxorubicin induced the production of free radicals which in turn elicited phosphorylation of EF2 (24). Phosphorylation of EF2 was accompanied by a decrease in short half-life anti-apoptotic proteins and enhanced TRAIL sensitivity which helped to form our hypothesis that TRAIL sensitization is linked to the loss of anti-apoptotic proteins via inactivation of EF2 following an increase in free radical load (and possibly other mechanisms of inactivation). According to this hypothesis, EF2 functions as a “sensor” for potential cellular

damage with the consequence of lowering the apoptotic threshold of an affected cell (Figure 2).

2.2 Carcinogenesis and EF2

Free radicals are, of course, both damaging and ubiquitous. Basal metabolism and respiration routinely generate free radicals and activated immune cells generate oxidative bursts to defend against pathogens. Indeed, the body is awash in free radicals but spikes in free radical concentrations have well-documented adverse affects (25). Free radicals are one of the most common culprits in carcinogenesis, an observation that has fueled much research (and product marketing) around anti-oxidants, the scavengers of free radicals. We suggest that cells, in addition to endogenous anti-oxidants, possess a fundamental defense against free radical damage, which has so far gone unexplored. Envisioning EF2 as a cellular sensor for free radical exposure, excessive exposure to free radicals results in phosphorylation of EF2, inhibition of protein synthesis, and subsequent disappearance of anti-apoptotic proteins (Figure 2). This causal relationship between the potentially carcinogenic action of free radicals and TRAIL-sensitization may address how TRAIL manages to induce apoptosis in specific target cells without damaging healthy cells. Such a pathway would constitute a simple and efficient innate and pre-emptive cancer surveillance system, removing cells that would otherwise be predisposed to tumorigenesis.

The source of TRAIL in this system would not necessarily be limited to endogenous stores of the affected cells. For instance, neutrophils are prolific producers of TRAIL and are certainly important participants in immune surveillance. Always in circulation, neutrophils are quite sensitive to the surrounding levels of free radicals such as reactive oxygen species (ROS) and are primed proportionally to the concentration of ROS so that their activity is appropriate to the level of damage encountered (26). A robust housekeeping system such as suggested here could be easily envisioned as a critical player in tissues which are routinely exposed to free radicals, such as the lungs (especially in smokers) or in the colon during metabolism. In tissues that co-express, TRAIL receptors and ligand, such as the liver, generation of ROS could lead to fratricide. Liver NK cells, which constitutively express TRAIL, may exacerbate the effect (27).

The link between carcinogenic exposure and EF2 inactivation is not limited to free radicals. UV radiation also inhibits EF2, although it does so through ADP-ribosylation rather than phosphorylation (28). The net effect is the same however, placing EF2 in the role of “sensor” and removing the activity of anti-apoptotic proteins upon carcinogenic exposure (Figure 2). In addition to potential autocrine signaling by damaged cells, it has been reported that neutrophils are the first responders to UV damage in the dermis(29). Their infiltrates are likely to play a role in the death of damaged cells, providing (1) a method for continued sensitization by production of oxidative bursts and (2) a source of exogenous TRAIL.

Of course, many other influences can result in cell transformation, including inherited genetic lesions. In such cases, perhaps no carcinogen is conspicuously involved. However, the early physiology of transformed cells may result in EF2 inactivation as well. For instance, small tumors rapidly outgrow their blood supply resulting in oxidative stress, hypoxia, and nutrient starvation. All of these stresses could lead to inactivation of EF2. Oxidative stress may cause EF2 to be ADP-ribosylated through non-enzymatic binding of free ADP-ribose (30). Alternatively, EF2 kinase is activated when pH is lowered by ischemia and the hypoxia-specific kinase AMP-activated protein kinase phosphorylates EF2 when oxygen levels drop (31,32) (Figure 2). In vitro work has confirmed that hypoxia sensitizes cells to TRAIL(33). The TRAIL system has been implicated to play a role in vivo as well, although concrete evidence so far exists only for TNF(34). However, downregulation of anti-apoptotic proteins following hypoxia would also sensitize cells to other death ligands and thus an involvement of TNF

following ischemic injury does not contradict our hypothesis. These redundant systems of control over EF2 suggest that early transformation itself may be a TRAIL-sensitizing process.

One obvious way for cancer cells to short-circuit the hypothesized EF2 sensor is to upregulate EF2 itself. With more substrate to absorb phosphorylating and/or ribosylating influences, some EF2 would likely remain active. In fact, EF2 upregulation has been observed in tumor tissue compared to normal tissue, although the mechanism by which this may benefit the cancer has not been suggested(35). Overexpression of EF2 has also been demonstrated to protect cells from HIV-1 viral protein R-induced apoptosis, a finding that may be transferable to other apoptosis studies (36).

3. Free radicals – friend or foe?

The damage caused by free radicals is certainly well documented and well studied. However, general consensus has leapt to the conclusion that all free radicals are unilaterally harmful. This may not be the case if, as we propose, cells are able to use free radicals to lower their apoptotic threshold towards innate tumor surveillance mechanisms. Cells exposed to free radicals may contain damaged proteins and/or DNA and thus have the potential to become carcinogenic. Removal of these cells may be best remedied by free radical-induced phosphorylation of EF2 and sensitization to TRAIL. The idea that free radicals are beneficial has found some support in unexpected outcomes of anti-oxidant clinical trials.

A strong link has been established between a diet rich in cruciferous vegetables and lower cancer rates(37). These vegetables, such as broccoli, were shown to have very high levels of the anti-oxidant beta-carotene(37). Since free radicals have long been known to contribute to cancer through covalent damage to both proteins and DNA, it was hypothesized that the anti-oxidant activity in the vegetables effectively scavenges free radicals thereby protecting cells from transformation. Subsequently, several clinical trials investigating mega-doses of anti-oxidants were undertaken in order to examine the correlation between anti-oxidants and cancer rates. Surprisingly, one study using beta-carotene had to be cut short after finding increased rates of cancer in the group of patients taking the anti-oxidants, particularly among smokers (38). Similarly, a recently released meta-study of anti-oxidant clinical trials over the last several decades revealed that mega-doses of anti-oxidants are not protective and may contribute to mortality (39). Researchers of lycopene, the antioxidant in tomatoes, have come to a similar conclusion: lycopene does not appear to be protective against prostate, lung, colorectal, or ovarian cancers(40,41). In addition, a report in September's *Journal of Nutrition* warns that large doses of anti-oxidants may predispose women towards developing skin cancer (42). At the same time, a study published in *Cancer Cell* reports a new insight into cruciferous vegetables: in addition to beta-carotene they also contain beta-phenylethyl isothiocyanate, which the authors of the study report kills cancer cells through a free-radical mediated mechanism (43). It appears that the assumption made decades ago about the protective component in vegetables may have been based on a limited mechanistic understanding regarding the role of free radicals.

4. Clinical relevance

Perhaps the most dramatic and long-standing unexplained observation in the history of cancer investigations is the phenomenon of spontaneous regression. Long before current chemotherapeutic approaches were invented, doctors observed that occasionally a patient's cancer would go into remission and that this unusual course of events was typically associated with a concurrent major infection. The correlation was strong enough to prompt the design of cocktails containing infectious agents as a novel cancer treatment in the hopes of stimulating patients' immune systems (44). One holdover, which is still in clinical use, is BCG (Bacille Calmette-Guerin) therapy against superficial bladder cancer. Of particular relevance is the

recent finding that neutrophil-derived TRAIL positively correlates with response to BCG therapy (45). BCG therapy may therefore activate the same combination of ROS and TRAIL that we suggest functions as a cancer surveillance system in the body.

Turning from history to recent breakthroughs, we would be remiss not to mention Ontak, a drug recently approved by the FDA (46). This drug is composed of two fused molecules, interleukin-2 and diphtheria toxin. The macromolecule is endocytosed by cells expressing IL-2 receptors, such as the cells involved in several types of lymphoma. Once in the endosome, proteolytic cleavage frees the enzymatically active portion of the diphtheria toxin, which is released into the cytosol where it inactivates EF2 by ADP-ribosylation. Naturally, permanent inhibition of protein synthesis is fatal in time. We suggest that this approach has the added benefit of sensitizing the targeted cells to TRAIL within a short time frame after EF2 inactivation due to decreased levels of anti-apoptotic proteins. Perhaps future studies with Ontak will evaluate whether TRAIL plays a role in therapeutic outcome and patient recovery.

5. Open questions and future challenges

The puzzle of TRAIL specificity for transformed cells has been a hallmark of the death ligand since its discovery. Certainly, the property has proven to be remarkably robust. Both recombinant human TRAIL and TRAIL receptor-agonistic human mAbs are currently under clinical evaluation and recent safety trials have confirmed that systemic TRAIL treatment is not toxic, even at high doses(47,48). The questions, then remain: Why does TRAIL induce the apoptotic pathway in a special population of cells? What cellular circumstances would render cells TRAIL sensitive and how do these circumstances relate to transformation? Is there a causal relationship between transformation and TRAIL sensitivity and if so, how would we pinpoint it? These questions are difficult to address under physiological conditions because the question refers to an extraordinarily small population (possibly a single cell) that has recently transformed and is targeted and destroyed by TRAIL.

In conclusion, we propose that free radicals or other cellular stresses lower a cell's apoptotic threshold via inhibition of EF2, which serves as a damage sensor. Interruption of translation elongation quickly leads to a decline of short-half-life anti-apoptotic proteins leaving potentially harmful cells sensitive to TRAIL, which functions as part of an innate immune surveillance system. Cells may escape this system, if insufficient TRAIL is available, resulting in lesions that could be amenable to exogenous TRAIL therapy. However, selective pressure of TRAIL therapy (or an increase in endogenous TRAIL) may give rise to a population of cells with acquired TRAIL resistance as has been suggested for FasL(49). Thus overcoming acquired resistance in malignant cells without compromising the integrity of innate resistance in normal cells remains the challenge of TRAIL combination therapy.

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Figure 1.

TRAIL-induced apoptosis. Upon ligand binding to TRAIL-R1 or R2, the adapter molecule FADD and initiator caspases are recruited resulting in formation of a death-inducing signaling complex (DISC) and activation of caspase-8 (and/or -10). Activated caspase-8 cleaves Bid, which facilitates oligomerization of the Bax and/or Bak. Bax/Bak-mediated pore formation allows the release of apoptogenic factors, such as cytochrome c, from the mitochondria resulting in apoptosome formation, activation of caspases-9 and -3, and execution of the apoptotic program. This process is inhibited at the DISC by cFLIPs, which interferes with caspase-8 homodimer formation via competitive binding, at the mitochondria by anti-apoptotic members of the Bcl-2 family, and downstream of the mitochondria by inhibitor of apoptosis proteins (IAP). XIAP binds to and interferes with activation of caspases-9 and -3. Survivin has been proposed to bind and inhibit these caspases as well but this has not been uniformly confirmed.

Figure 2. Eukaryotic elongation factor 2 (EF2) in the role of a sensor. The details by which carcinogen exposure or transformation affects TRAIL phenotype are described in the text.

Table 1

Half lives of representative apoptotic/anti-apoptotic proteins

Apoptotic proteins	Half life (hrs)	Anti-apoptotic protein	Half life (hrs)
Bax ¹³	24+	FLIPshort ¹⁴	0.6
Bad/Bik/Bak ¹⁵	22+	Mcl-1 ¹⁶	0.5
Caspase 3 ¹⁷	6	XIAP ¹⁸	2
Caspase 8 ¹⁹	9	survivin ²⁰	0.5