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REVIEW

Dale B. Chappell · Nicholas P. Restifo

T cell–tumor cell: a fatal interaction?

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Abstract Fas (Apo-1/CD95) is a cell-surface protein that is responsible for initiating a cascade of proteases (caspases) culminating in apoptotic cell death in a variety of cell types. The function of the Fas/FasL system in the dampening of immune responses to infectious agents through the autocrine deletion of activated T cells has been well documented. More recently, it has been proposed that tumor cells express FasL, presumably to avoid immune detection. In this review, we focus on the role of the interaction of Fas and FasL in the modulation of antitumor responses. We critically examine the evidence that FasL is expressed by tumor cells and explore alternative explanations for the observed phenomena in vitro and in vivo. By reviewing data that we have generated in our laboratory as well as reports from the literature, we will argue that the Fas/FasL system is a generalized mechanism used in an autocrine fashion to regulate cell survival and expansion in response to environmental and cellular cues. We propose that FasL expression by tumor cells, when present, is indicative of a perturbed balance in the control of proliferation while "immune privilege" is established by "suicide" of activated antitumor T cells, a form of activation-induced cell death.

Key words FasL · Cancer · Immmunotherapy · Caspase · Apoptosis

Does FasL kill antitumor T cells?

The failure of the natural immune defenses to eliminate tumor is a basic and unresolved question in the field of immunotherapy. Tolerance to "self" antigens has been used as one explanation but this does not account for the over-expression of proteins, translation of alternative open reading frames, and the presentation of mutated epitopes, all of which have been documented in tumor cells [38, 50]. Although much has been learned about the death of tumor cells mediated by cytotoxic T cells, few data exist regarding the fate of antitumor T cells after they encounter tumor. In an effort to investigate mechanisms that subvert immune responses, antitumor T cells were found to be susceptible to programmed cell death mediated by FasL (CD95L). Given that some tumor cells may express FasL and that T cells constitutively express Fas (CD95), there appeared to be a straightforward explanation for these results [12, 15, 33, 34, 39, 42, 44]: the Fas^+ T cells were killed by $FasL^+$ tumor cells. In contrast to this explanation, we and others have found that T cells are capable of producing FasL upon activation through the T cell receptor (TCR) and kill themselves in a classical negative-feedback loop after encountering tumor [5]. Thus, there appear to be two distinct methods that involve FasL that could limit the extent of the immune reaction to tumor cells. This article presents an overview of tumor cell expression of FasL, the in vivo role of FasL expression by tumor cells, and the role of activation-induced cell death in the physiological regulation of the immune system. Potential therapeutic manipulations of the Fas pathway as a mechanism to improve the immune response to tumor will be discussed.

D.B. Chappell
The Howard Hughes Medical Institute-National Institutes of Health Research Scholars Program, Bethesda MD 20814, USA

N.P. Restifo (✉)
Surgery Branch, National Institutes of Health, 10 Center Drive MSC-1502, Bethesda, MD 20892-1502, USA

The interaction of FasL with Fas induces apoptotic cell death

The Fas receptor is widely expressed in cells of the immune system including T cells, B cells, and monocytes [6, 32]. The FasL is a type II transmembrane protein and a member of the tumor necrosis Factor/nerve growth Factor family. FasL is expressed in activated T cells, activated NK cells, some macrophage/monocytes and in

areas of immune privilege such as Sertoli cells and the anterior chamber of the eye [9, 14]. A recent report documents the expression of FasL by vascular endothelial cells where it may act to regulate extravasation of lymphocytes [40]. The membrane-bound FasL is cleaved by metalloproteases resulting in soluble FasL [23]. Binding of the transmembrane form of FasL to Fas results in the trimerization of the Fas receptor and a death signal to susceptible cells [32]. Soluble FasL is less efficient at inducing an apoptotic signal than is the membrane-bound FasL [46]. The downstream signaling from the Fas receptor is initiated by the interaction of FADD (Fas–associating protein with death domain) with the death domain of the trimerized Fas receptor. FADD is then responsible for the cleavage of the first of a series of cysteine proteases (caspases). The first caspase to be activated is FLICE (FADD-like interleukin-1 beta-converting enzyme) which results in a protease cascade that culminates in a defined process of cell death [29] (Fig. 1).

The role of Fas/FasL in the regulation of cell proliferation and cell death is not confined to the immune system. A number of tissues have been shown to express Fas, such as colonic epithelium, hepatocytes, spleen, thymus, pancreas, prostate, and other areas where cellular turnover is present [9, 44, 49]. In these tissues, the Fas/FasL system may operate downstream of other cellular controls such as c-Myc or p53 and function as a generalized mechanism of cell death in response to lack of survival signals from the surrounding micro-environment [9, 13, 18]. The Fas/FasL system has also been demonstrated to regulate the proliferation of tumor cells. Trasfecting colon carcinoma cells with wild-type p53 results in Fas-mediated apoptosis [45]. Cytotoxic drugs and γ irradiation act, at least in part, through the p53 system to activate the Fas pathway [10, 31, 35]. Thus, the Fas/FasL system may have a more generalized homeostatic role, acting as an intermediary between pro-life signals, such as Bcl-2, and inducers of cell death, such as p53 or Bax [17, 45] (Fig. 1).

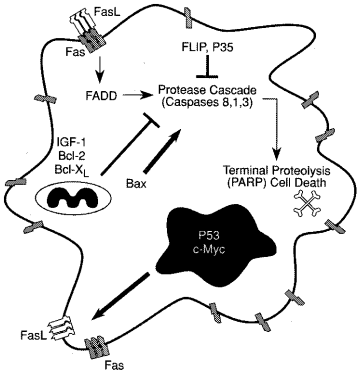

Fig. 1 The apoptotic cascade. Maintaining the balance between pro-life signals from Bcl-2 and Bcl-X_L with pro-death signals from Bax, p53, c-Myc, and FasL. The two pathways converge at the level of caspase activation. The activation of caspases can be abrogated by FLIP (FLICE inhibitory protein) and baculovirus P35 protein. The apoptotic cascade is highly conserved and operates in T cells as well as in cells of various histologies

The interaction of Bax with Bcl-2 and Bcl-X_L sets up a balance that will determine the fate the cell. The proteins Bcl-2 and Bcl-X_L (CED-9 *Caenorhabitis elegans* functional homologues) have been demonstrated to block the activation of caspases (CED-3 functional homologue) and inhibit cell death [36]. Bcl-2 and Bcl-X_L are localized to the outer membrane of the mitochondria, the endoplasmic reticulum, and the nuclear envelope. These proteins have structural similarity to the bacterial pore-forming colicins and can function as ion and protein channels [30]. Not all Bcl-2 family members are anti-apoptotic as Bax has been shown to promote cell death. New details are emerging about the exact mechanism used by Bcl-2 and Bcl-X_L to promote cell survival. In the *C. elegans* model, CED-9 (Bcl-2) forms a trimolecular complex with CED-3 (caspase) and another protein CED-4. Although CED-4 is a caspase activator, the interaction with CED-9 may interfere with caspase activation. The human homologue of CED-4, apoptosis protease-activating factor (Apaf-1), has been recently cloned [54]. Apaf-1 has been shown to bind to cytochrome *c*, which is released from the mitochondria during apoptotic stress. The complex of Apaf-1 and cytochrome *c* participates in the activation of caspase-3 [54]. Bcl-2 may act to block the release of cytochrome *c* from the mitochrondria during apoptosis or may interact directly with the Apaf-1/cytochrome *c*/caspase complex to regulate the activation of the protease cascade. Bax, on the otherhand, may block the interaction of Bcl-2 with the Apaf-1/cytochrome *c*/caspase complex or may promote the release of cytochrome *c* from the mitochondria during apoptotic stress.

Another cellular protein that blocks apoptosis is FLIP (FLICE inhibitory protein). FLIP blocks the interaction of FADD with FLICE (caspase-8), thereby preventing downstream signaling from the Fas receptor [20]. FLIP has been shown to be expressed in the early stage of T cell activation but the levels of FLIP fall as the T cell becomes susceptible to FasL-mediated death. Melanoma cells have been shown to express FLIP, which may block apoptotic signals leading to dysregulated cellular proliferation [20].

Do tumor cells express FasL?

A number of reports have asserted that tumor cells express FasL as a mechanism of immune evasion [12, 15, 33, 34, 39, 42, 44]. Tumors that have been reported to express FasL include melanoma, colon carcinoma, hepatocellular carcinoma, astrocytoma, and lung carcinoma (Table 1). In these models, lymphocytes infiltrating into the tumor bed would come into contact with FasL expressed by the tumor cells and undergo death via apoptosis. The result would be a continuously renewing population of lymphocytes at the tumor site but little tumor destruction and inevitably tumor outgrowth

Table 1 Tumor cell expression of FasL. Melanoma, colon carcinoma, hepatocellular carcinoma, astrocytoma, and lung carcinoma have all been reported to express FasL by various methods of detection and by functional assays

Histological type	mRNA (FasL)	Protein detection	Functional assay
Melanoma [15]	Data not shown	Western blot, immunoblot of serum, immunohistochemistry	Targets *not* controlled for Fas expression. No FasL$^-$ melanoma lines shown
Melanoma [2]	Not done	Not detected by FACS	No killing of Jurkat cells (6 lines)
Melanoma[a]	Not detected in 23 lines	Not detected by FACS	No killing of Jurkat, A20, or L1210-Fas in assays controlled for Fas expression
Colon carcinoma [34]	RT-PCR (SW620)	Immunofluorescence, immunohistochemistry	Anti-sense oligonucleotides used to control target Fas expression
Colon carcinoma [42]	RT-PCR (SW480 + other lines)	Immunofluorescence, immunohistochemistry	Blocking antibody to FasL but no FasL$^-$ effector cell control.
Colon carcinoma[a]	RT-PCR (SW480)	Not done	Not functional on Jurkat and L1210-Fas
Hepatocellular carcinoma [44]	in situ hybridization RT-PCR	Immunohistochemistry	Target *not* controlled for Fas expression
Astrocytoma [39]	Not shown	Western blot, FACS, immunohistochemistry	Targets controlled for Fas expression FasL$^-$ effector cell not included
Lung carcinoma [33]	Nested RT-PCR	Western blot, immunofluorescence, immunohistochemistry	Targets not controlled for Fas expression FasL$^-$ effector cell not included

[a] Data obtained in the Surgery Branch, NCI

(Fig. 2A). One problem with this model is that T cells are not uniformly susceptible to FasL. TCR engagement has been demonstrated to sensitize T cells to FasL-mediated death and it is known that TCR signaling results in FasL production by T cells [16]. Therefore, the T cells that are the most susceptible to FasL expressed by tumor cells will also be producing FasL in an autocrine fashion.

Other confounding variables need to be considered when analyzing the literature concerning FasL expression by tumors. First, any fresh tumor samples will have lymphocyte contamination and any FasL detected can not be attributed to the tumor cells unless precautions are taken to insure the removal of lymphocytes and other non-transformed cells that may express FasL, such as vascular endothelial cells [40]. Functional assays are also difficult to interpret as the target cells are usually not controlled for Fas expression but are often differentially susceptible to apoptosis in a mechanism that may involve caspases, FLIP, or possibly Bcl-2 expression. Since caspases can be activated through mechanisms independent of Fas/FasL, the differential killing can not be attributed to the Fas/FasL system and must be viewed more cautiously [29]. Another potential confounding variable is the regulation of autocrine cell death by c-Myc and p53 [18, 45]. A crowding effect or lack of growth factor in a microtiter well could send an apoptotic signal via c-Myc to the apoptosis-sensitive target cell, which may die according to its susceptibility to apoptosis in a Fas/FasL-dependent manner without regard to the FasL status of the effector cell. Therefore, functional assays comparing apoptosis-resistant cells and an apoptosis-sensitive cells as well as assays that do not control for effects of cell crowding by the addition of a FasL$^-$ effector cell line are difficult to interpret.

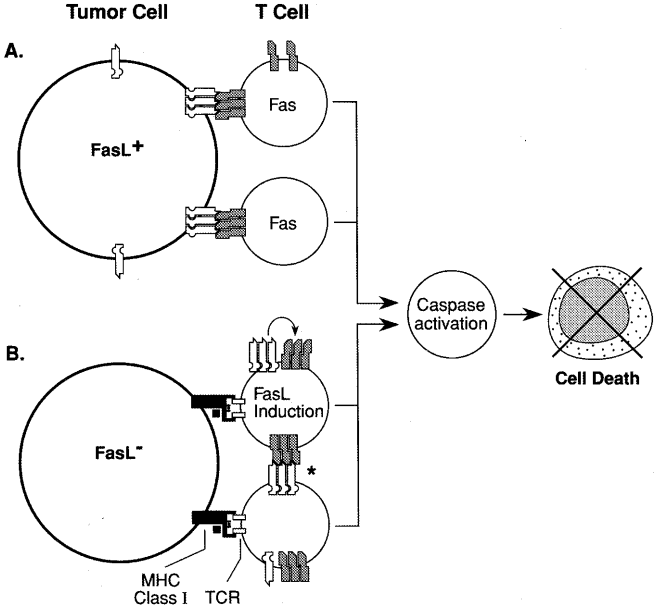

Fig. 2A, B Two models of T cell death after Encounter with Tumor. **A** Tumor cells express FasL and kill Fas-expressing T cells as these lymphocytes travel to the tumor bed. In this model tumor recognition is not mentioned but may be required to sensitize T cells to FasL. **B** T cells recognize antigens presented by the tumor and become activated. The activated T cells express FasL and begin to die in a suicidal (↩) or fratricidal (*) manner. Fratricide would occur if both T cells recognize the tumor since T cell receptor (*TCR*) engagement sensitizes T cells to FasL-mediated death [16]

As an example of the confusion that envelops functional assays, Hahne et. al found low levels of killing of A20 compared A20R (apoptosis-resistant) by melanoma lines [15]. This functional assay used effector cells that were plated out 48 h in advance, which would lead to overgrowth of the well and did not include a FasL$^-$

melanoma cell line. In contrast, Arei et al., did not see any killing of Jurkat cells by six melanoma lines. In their functional assay, the melanoma lines were not allowed to overgrow the well since they were added concurrently with the target cells [2]. In agreement with the data of Arai et al., we have been unable to detect any killing by melanoma lines using A20, L1210/L1210-Fas, and Jurkat cells as targets (manuscript in preparation). We have not been able to detect FasL-mediated killing despite letting the effector melanoma lines adhere for 24 h prior to the addition of target cells. Of these assays, only the L1210/L1210-Fas system controls for Fas expression of the target cell. Since both A20, L1210-Fas, and Jurkat cells express high levels of the Fas receptor, the difference in these assays could involve mechanisms unrelated to FasL expression by the melanoma cell lines.

Despite the ambiguity of the functional assays, some tumor cell lines appear to express FasL at the mRNA level and some tumor samples appear to stain for FasL protein by immunohistochemical techniques [12, 15, 33, 34, 39, 42, 44]. Again cautious interpretation is warranted as the Fas/FasL system can be used as a suicidal mechanism for cells in response to a vast number of conditions. Colon carcinoma lines deficient in thymidylate synthase will up-regulate FasL in response to thymineless stress [17]. Fibroblast cell lines have been shown to undergo Fas/FasL-mediated death in a c-Myc-dependent manner after serum deprivation [13, 18]. Thus, finding FasL protein on a tumor cell after an extended period of overgrowth in vitro does not necessary mean that this tumor would express FasL in vivo. It is likely that FasL is regulated by survival factors within a cell's environment and will not be a constitutive trait that will characterize most cell lines during altered culture conditions. Constitutive functional FasL expression by tumor lines implies a failure in the autocrine signaling for cell death.

It should also be pointed out that constitutive FasL expression has only been suggested for a few tumor lines. Two separate reports describe FasL expression by colon carcinoma [34, 42]. The lines studied in detail include SW480 and SW620. Inspection of these two lines revealed that they arose from the same patient. The paper by Shiraki et al. demonstrates FasL mRNA for 3/10 colon carcinoma lines but only SW480 was studied in further detail for protein expression and ability to kill Fas^+ targets [42]. For the sake of argument, the assumption will be made that some small percentage of tumors constitutively express functional FasL protein and we will next examine the animal model data for the role of FasL in immune evasion.

Contrary to the predicted outcome, tumor cells transfected with FasL are eliminated while the wild-type tumor cells or neomycin-transfected controls grow progressively. This has been reported for B16, CT26, and RENCA [2, 41]. In addition, injecting a subcutaneous tumor nodule with adenovirus expressing FasL leads to the elimination of the tumor in vivo, while adenovirus alone had no effect on the growth of the tumor cells [2]. This observation can be explained in the RENCA model, since RENCA is known to express the Fas receptor. Introduction of FasL into RENCA cells would result in fratricidal and suicidal death of the tumor cells. It is more difficult to explain why the production of FasL by a Fas-deficient tumor cell would lead to its elimination. The histology suggests that neutrophils are attracted to the tumor bed and these may play a role in the antitumor response. This suggestion is further supported by the fact that treatment with an anti-Ly-6G (Gr-1) antibody inhibits the inflammatory response. A similar finding of inflammation as opposed to immune suppression has been reported when FasL is expressed in pancreatic β cell transplants and syngeneic myoblast transplants [21, 22]. One explanation for the pro-inflammatory effect of FasL may be the activity of caspases in Fas-expressing bystander macrophages. The Fas/FasL pathway results in the activation of caspase-1 (interleukin-1β-converting enzyme), which is known to cleave pro-IL-1β into active IL-1β [29]. The production of IL-1β by macrophages at the tumor bed may generate a pro-inflammatory cytokine to recruit other non-specific immune effectors such as neutrophils. Thus, even if tumor cells are found to express functional FasL convincingly in a constitutive fashion, the effect on the immune system will not be straightforward. Inflammation may predominate over immunosuppression.

T cells commit suicide

It is now well established that T cells express FasL after activation with antigen, TCR crosslinking by antibody, or calcium ionophores [27]. T cells are capable of using FasL to kill Fas-expressing target cells but this protein also acts to regulate the expansion of lymphocytes by acting in an autocrine fashion to delete recently activated T cells in a process termed activation-induced cell death (AICD) [25]. Bystander killing of T cells that do not recognize the target is minimal since TCR engagement sensitizes T cells to FasL-mediated killing [16]. Since the Fas/FasL pathway requires new protein synthesis, perforin-mediated pathways can eliminate target cells before lymphocytes begin to die via apoptosis [11]. In addition, FLIP expression is high during the early stages of T cell activation, which would inhibit Fas-mediated signaling [20]. This accounts for the ability of a normal immune response to eliminate antigen without continually expanding the immune response at the site of infection. The perforin and granzyme pathways act quickly to eliminate the target, followed shortly by the elimination of the effector lymphocytes. The evidence that supports the role of the Fas pathway in the deletion of activated T cells in vivo can be found in patients with mutations in the Fas receptor who suffer from autoimmune lymphoproliferative syndrome [7, 8, 43]. These patients are born with a normal appearance but develop disfiguring lymphadenopathy in childhoood as activated T cells continually expand. This mechanism of deleting

recently activated T cells upon re-encounter with antigen has been used to explain the phenomenon of high zone tolerance and peripheral tolerance to "self" [25]. As it relates to peripheral self-tolerance, it may depend more upon the amount of antigen and the continual exposure to antigen than upon the nature of the peptides presented [1, 19, 28]. The level of antigen determines whether the immune system will be able to eliminate the challenge fully before propriocidal mechanisms dampen the immune response. Thus, even viral, bacterial, and, mutated tumor antigens could lead to tolerance if the immune system is continually confronted with an overabundance of these proteins.

In an effort to understand whether or not the Fas/FasL pathway is functional when a T cell encounters a tumor cell, we co-cultured radioactively labeled antitumor T cells with melanoma cells. We found that T cells underwent cell death in a Fas-dependent manner that was completely dependent upon tumor cell recognition (i.e. AICD; manuscript in preparation). There was no T cell apoptosis when the T cell failed to recognize the tumor cell (either because of a lack of appropriate antigen expression or because of lack of a restricting HLA molecule by the melanoma cell). As already mentioned in Table 1, we were unable to find FasL mRNA in 23 melanoma lines by reverse transcriptase/polymerase chain reaction (RT-PCR). The FasL message was detected in both IL-2-stimulated and TCR-activated T cells and Jurkat cells. Similar findings have been reported in a breast cancer model. Although FasL mRNA was not detected in a panel of breast cancer lines, antitumor T cells underwent AICD and produced FasL upon contact with tumor [5]. Given these results, it appears that Fas-mediated AICD of T cells may limit the immune reaction to tumor cells whenever there exist T cells capable of recognizing antigens expressed by tumor cells (Fig. 2B).

Therapeutic modulation of the Fas/FasL pathway

There are multiple mechanisms in addition to activation-induced cell death that are operative in dampening the immune response to tumor cells. The lack of co-stimulatory molecules such as B7.1, tumor secretion of suppressive cytokines such as IL-10, down-regulation of antigen and HLA molecules, and immune suppressor cells may all contribute to tumor immune evasion [4, 37, 51, 52]. Until each of these negative signals is sufficiently blocked in vivo, we will not know to what extent each contributes to immune down-modulation. One particular approach to blocking Fas-mediated killing of T cells would be to transduce cytotoxic T lymphocytes generated in vitro with genes that interfere with the downstream signaling molecules and then adoptively transfer these T cells back to patients. Potential genes for such transduction include apoptosis-linked gene 3 (ALG-3), the baculovirus caspase inhibitor P35 (*Autographa californica* nuclear polyhedrosis virus), or various viral or cellular FLIPs [3, 20, 47, 48]. Such approaches suffer from the difficulty in efficiently transducing human T cells and the risk of inducing a T cell lymphoma. Thus far, gene transduction has not been feasible. Blocking antibodies to the Fas receptor or the use of a Fas receptor Ig construct offers the ability to modulate the apoptotic pathway without transducing T cells. We have had success with anti-Fas blocking antibodies in vitro and are currently developing animal models to test the ability of these antibodies to improve T cell immune responses in vivo.

Conclusion

The initial discovery that FasL regulates the expansion of activated T cells has shaped the way in which we view FasL expression by cells of other histologies. FasL expression has been used to explain the existence of immune-privileged sites. More recent evidence indicates that the Fas/FasL system is a more generalized autocrine regulator of cell proliferation and cell death in tissues throughout the body. In fact, disruption of the Fas signaling pathway may contribute to carcinogenesis [20, 24, 26, 53]. Therefore, one would expect to find variable FasL expression on any number of cell types depending upon the cell's micro-environment. Constitutive FasL expression by proliferating cells suggests an imbalance in the cellular controls governing proliferation and apoptosis, such as a defect in the Fas signaling pathway or over-expression of Bcl-2. Any role that constitutive FasL expression may have in immune evasion by tumor cells would be a serendipitous side-effect and is not supported by the animal data.

Although constitutive FasL expression by tumor cells does not appear to limit the antitumor immune response, is seems likely that the regulated FasL expression by T cells plays some role in limiting the immune response to tumor. Just as antigen dose determines activation or tolerance in a number of experimental models, the tumor burden may play a critical role in determining whether AICD is limiting or whether the immune response will eliminate tumor [1, 19, 28]. Thus, abrogation of Fas-mediated AICD may be a promising strategy in the enhancement of T-cell-based immunotherapies.

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