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POSITIVE AND NEGATIVE CONSEQUENCES OF FAS/FAS LIGAND INTERACTIONS IN THE ANTITUMOR RESPONSE

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1. ABSTRACT

Understanding the mechanisms by which T lymphocytes mediate antitumor activity *in vivo* may have important implications for the design of active, adoptive and combination immunotherapies against neoplastic progression. The Fas/Fas ligand (FasL) system utilized by antigen (Ag)-specific T cells has been now demonstrated to play important roles in lymphocyte-mediated tumor regression *in vivo*. However, the process of tumor eradication by Fas/FasL interactions *per se* may serve also as an immune-based selective pressure. Indeed, more recent studies have illustrated that this same Fas/FasL system may have negative contributions, perhaps serving as a novel mechanism of tumor escape of Fas-resistant subpopulations. In addition to Fas-resistance, functional FasL expression by certain cancer cell types has been implicated in tumor escape via destruction of infiltrating Fas-bearing lymphocytes. Thus, the acquisition of Fas--resistance by advancing neoplastic subpopulations, possibly in combination with FasL induction may serve as countermeasures against immune attack and contribute favorably toward metastatic development. Further appreciation of the complex nature of this Fas/FasL system, exploited not only by innate or adaptive elements of the immune response, but also by a developing neoplasm may have important implications for the regulation of tumor progression in favor of clinical regression. Thus, this review will focus on both positive and negative consequences of the Fas/FasL system during host/tumor interactions. Emphasis will be on the importance of the Fas/FasL pathway for antitumor activity, as well as a potential selective force influencing the escape of Fas--resistant aggressive tumor variants.

2. INTRODUCTION

The goal of cancer immunotherapy is clear; however, efforts to achieve that goal have been much more elusive. In fact, tumor-specific immune responses often develop or can be induced in cancer-bearing hosts via active or adoptive immunotherapy, yet complete tumor eradication occurs infrequently (1-9). The inability of the immune system to more consistently and effectively eradicate neoplastic disease in immune competent hosts is not fully understood and has remained a fundamental paradox in tumor immunology and immunotherapy. It is now generally well acknowledged, however, that cancerous cells exploit diverse mechanisms to counterattack the immune response (10-14). Preclinical and clinical studies now strongly support the contention that neoplastic cells can evade cell-mediated immunity at multiple levels of the effector/target interaction, including events associated with antigen (Ag) recognition, cell-cell contact, costimulation and, perhaps, induction of cell death.

Therefore, although a variety of molecular alterations have been observed in neoplasms as they become more progressive and better equipped to evade or inhibit host defenses, it still remains to be fully understood how such changes in cancer cells occur initially and whether immunologically driven events also may contribute to the generation of tumor escape variants expressing those more malignantly proficient phenotypes. One interesting hypothesis is that neoplastic subpopulations expressing these more aggressive genetic or epigenetic traits emerge as a result of an endogenous immune-based selection process (9). This phenomenon is conceptually akin to the generation of radioresistant or chemoresistant neoplastic clones.

The Fas/Fas ligand (FasL) system has been characterized as an integral process for the maintenance of immune privilege, and the regulation of immune homeostasis of peripheral lymphoid interactions under both normal and pathologic conditions (15-20). It is a death receptor-initiated pathway mediated by FasL expressed by a number of cell types, including activated T and B cells, natural killer (NK) cells and macrophages, as well as certain tissues and organs that constitutively express it,

namely cells of the eye and testis which have been implicated in the preservation of immune privilege at those sites. So, why is this system of homeostasis also important for the regulation of host/tumor immune interactions *in vivo?* The Fas/FasL system has also been considered one of two major pathways of cell-mediated cytotoxicity that induces apoptotic or programmed cell death of susceptible targets (21-23). In responsive tumor cells, ligation of cell surface Fas by FasL expressed by the effector cell population engages the caspase signaling pathway in those targets, which ultimately contributes to DNA fragmentation and cell death (15-20, 22, 24).

Cellular components of both the innate and adaptive immune responses, namely NK cells and $CD8^+$ T lymphocytes, respectively, engage the perforin/granzyme and Fas pathways as the principal effector mechanisms to mediate cellular cytotoxicity (21-23). Furthermore, the production of interferon-gamma (IFN-gamma) by these activated lymphocytes, for example, has been shown to contribute significantly to antitumor reactivity via a number of mechanisms. Some of these IFN-gamma-mediated effects include phenotypic or functional modification of neoplastic cells rendering them more amenable to immune recognition and attack via Fas-dependent and Fas-independent pathways (2, 25-30). Indeed, earlier studies had pointed toward the perforin pathway as a major force regulating tumor development and progression (21-23, 31-33). Recent studies in mice have demonstrated that the Fas pathway also plays a crucial role against localized tumor growth or tumor progression, including those models reflecting spontaneous or experimental lung metastasis (34-40). Therefore, both the perforin and Fas pathways constitute significant or dominant barriers against tumor growth and spread. In addition to these two pathways, other members of the tumor necrosis factor (TNF) family also may be involved in mediating tumor regression, such as TNF-alpha, TNF-beta (lymphotoxin-alpha) and TRAIL (TNF-related apoptosis-inducing ligand) (41-44).

The fact that multiple cytolytic effector mechanisms exert positive antitumor properties also raises the opposing hypothesis that if neoplastic subpopulations develop resistance to either one or more pathways, this may facilitate tumor escape, which in turn, may influence metastatic formation. Indeed, it is also now known that the downregulation or loss of Fas expression and function is frequently found in the progression of a number of human malignancies, including carcinomas of the colon, breast and lung (45-48). Thus, it is conceivable that an antitumor response, during the process of mediating tumor cell destruction, may unintentionally impose a "selective pressure," which influences the outgrowth of neoplastic clones bearing heightened apoptotic-resistant, malignant characteristics. The goal of this review, therefore, is to focus on positive and negative consequences of an antitumor immune response, with emphasis on the role of the Fas/FasL system.

3. T CELL SUBSETS AND THEIR EFFECTOR MECHANISMS

$CD4^+$ and $CD8^+$ T cells have been classified as the two major subpopulations of peripheral T lymphocytes. Subset dichotomy, in part, reflects intrinsic differences in major histocompatibility complex (MHC)/peptide recognition requirements and the nature of the resulting cellular immune responses (49, 50). $CD4^+$ lymphocytes have been shown to play an important and central role in immunoregulation through the production and action of lymphokines, while $CD8^+$ lymphocytes have been described as cytotoxic T lymphocytes (CTL) that mediate the destruction of Ag-bearing targets. Although both $CD4^+$ and $CD8^+$ T cells independently recognize antigenic determinants expressed by an antigen-presenting cell (APC), optimal development and regulation of the cellular immune response typically require the cellular cooperation between these two subpopulations (49, 51-53). It is becoming clearer, however, that the seemingly simple division of T cells into $CD4^+$ and $CD8^+$ T cell subsets is actually more complex. In fact, multiple functional subtypes of $CD4^+$ and $CD8^+$ T cells have been described. These subtypes have been termed type 1 (i.e., $CD4^+$ Th1; $CD8^+$ Tc1) and type 2 (i.e., $CD4^+$ Th2; $CD8^+$ Tc2), which predominantly reflect differences in their cytokine secretion patterns following T cell receptor (TCR) stimulation (42, 54-56).

The TCR of the $CD8^+$ CTL recognizes antigenic peptides (epitopes) displayed on the cell surface of the APC/target cell in the context of self-MHC class I molecules. The resulting effector cell response, whether it reflects a Tc1 or Tc2 phenotype, is the death of the Ag-bearing target cell via Fas-dependent and/or Fas-independent pathways, as described in detail below (Figure 1). The TCR of $CD4^+$ T cells recognize antigenic peptides displayed on the cell surface of the APC/target cell in the context of self-MHC class II molecules. $CD4^+$ T cells, chiefly the Th1 subtype, have been shown to exert antitumor effects *in vivo* in various models of active or adoptive immunotherapy (57, 58). Antitumor reactivity may result from cytokines that modify tumor-cell viability directly (TNF-alpha or TNF-beta) or indirectly (interleukins, IFN-gamma, granulocyte/macrophage-colony stimulating factor; GM-CSF) by recruitment and further activation of other cytotoxic effector cells, such as $CD8^+$ T cells, macrophages, neutrophils or NK cells.

In general, the precise mechanisms leading to CTL-mediated tumor regression *in vivo* may reflect both cell contact-dependent and cell contact-independent, lymphokine-based (e.g., IFN-gamma, TNF-alpha or TNF-beta) pathways (21, 22, 27, 29, 33, 41, 42, 59). If CTL do mediate tumor regression *in vivo* by direct cell contact, then this may occur via two major effector mechanisms involving the secretion of perforin/granzymes and/or ligation of Fas by FasL expressed by the Ag-activated CTL (Figure 1). The extent of contribution of each effector mechanism to the overall lytic response likely depends upon intrinsic characteristics of the given target cell population. Although the prevailing view has been that perforin/granzyme-mediated lysis is a dominant pathway (21-23, 31-33, 59), it recently has been shown in preclinical models that FasL-mediated cytotoxicity is additionally required for optimal tumor regression *in vivo* (30). Interestingly, some studies even have challenged the

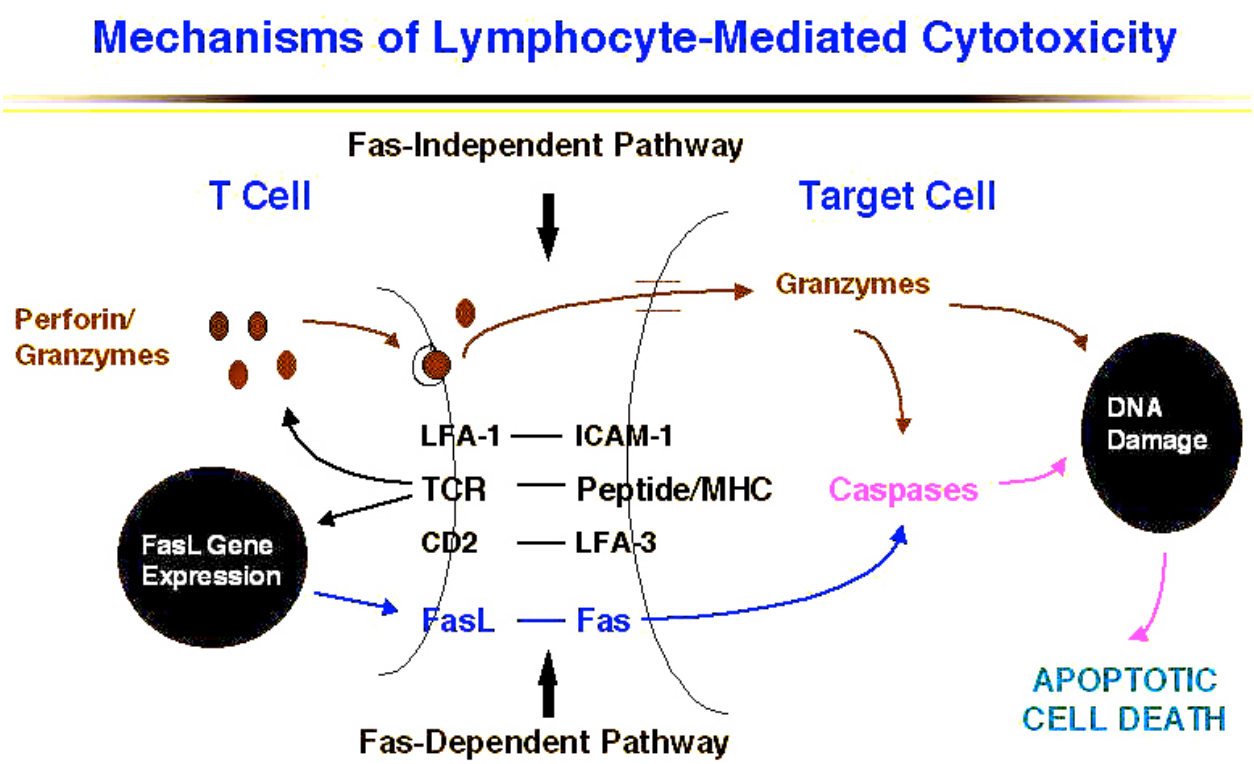

Figure 1. Mechanisms of Lymphocyte-Mediated Cytotoxicity. Lysis of Ag-bearing tumor cells by cytotoxic lymphocytes may occur principally via two major effector mechanisms, generally referred to as the Fas-independent or the perforin/granzyme pathway, and the Fas-dependent pathway. Execution of both pathways requires TCR recognition of MHC/peptide complexes. Ag-independent interactions involving LFA-1-ICAM-1 and/or CD2-LFA-3 serve to enhance conjugate formation between effectors and targets and, consequently, the strength of signaling through the TCR. The degranulation of perforin/granzyme contents results in target cell death via caspase-dependent and caspase-independent events. The Fas-dependent pathway involves ligation of cell surface Fas on the target cell surface by membrane-bound FasL (or soluble FasL) expressed by the TCR-activated lymphocyte. Productive Fas/FasL interactions then initiate caspase signaling, which ultimately leads to DNA fragmentation and cell death (see Figure 2 for further details). The extent of contribution of each effector mechanism to the overall lytic response likely depends upon intrinsic characteristics of the given target cell population. Because of that biologic caveat, tumor cell subpopulations that acquire Fas-resistance, for example, may escape immune attack (see Figure 3). Reprinted with permission from Landes Bioscience. Figure 2, Page 144. Abrams SI. Regulation of Tumor Progression by Anti-Neoplastic T Cell Responses. Cancer Biology & Therapy **3**:140-146 (2004).

importance of the perforin pathway in CTL-mediated tumor rejection *in vivo* (29, 41, 42, 59). Studies in mice also sustain the idea that the host Fas/FasL system may be important for the regulation of local tumor growth. For example, transfection of the cFLIP (cellular-derived FLICE-inhibitory protein) gene, an inhibitor of Fas-mediated signaling (60), into syngeneic tumor cells enhances the frequency and decreases the latency of subcutaneous (sc) tumor growth (35, 36).

Several studies in humans (25, 26, 45-47) also support the notion that loss of Fas expression or function by diverse neoplasms associates with a more malignant phenotype. Furthermore, the notion that loss of sensitivity to Fas-mediated apoptosis may play an important role in the progression of malignant behavior in human cancer is supported by the observations that as hematopoietic or non-hematopoietic malignancies: (a) acquire a more malignant phenotype, they downregulate Fas expression or function (25, 45-47, 6165) and (b) develop resistance to gamma-irradiation or certain chemotherapeutic agents, such as 5-fluorouracil (5-FU), doxorubicin, cisplatin or anthracenes, they may exhibit cross-resistance to Fas-dependent lysis (66-69). Furthermore, other work has demonstrated an important role for Fas-dependent interactions in human $CD8^+$ CTL-mediated lysis of human colon carcinoma cells *in vitro* (25, 26, 28). Thus, the relative participation of Fas/FasL interactions in tumor immunity *in vivo,* whether in mouse or human systems, is intimately linked to the functional status of Fas on the neoplastic cell, which in some instances can be further modulated by pro-inflammatory cytokines such as IFN-gamma and/or TNF-alpha (26, 29, 61, 62, 70).

4. FAS/FAS LIGAND SYSTEM AND APOPTOTIC SIGNALING

Fas also known as CD95 or APO-1, is a member of the TNF/NGF receptor superfamily (15, 17, 18, 47, 71, 72). The Fas gene encodes a 45-kDa type 1 transmembrane

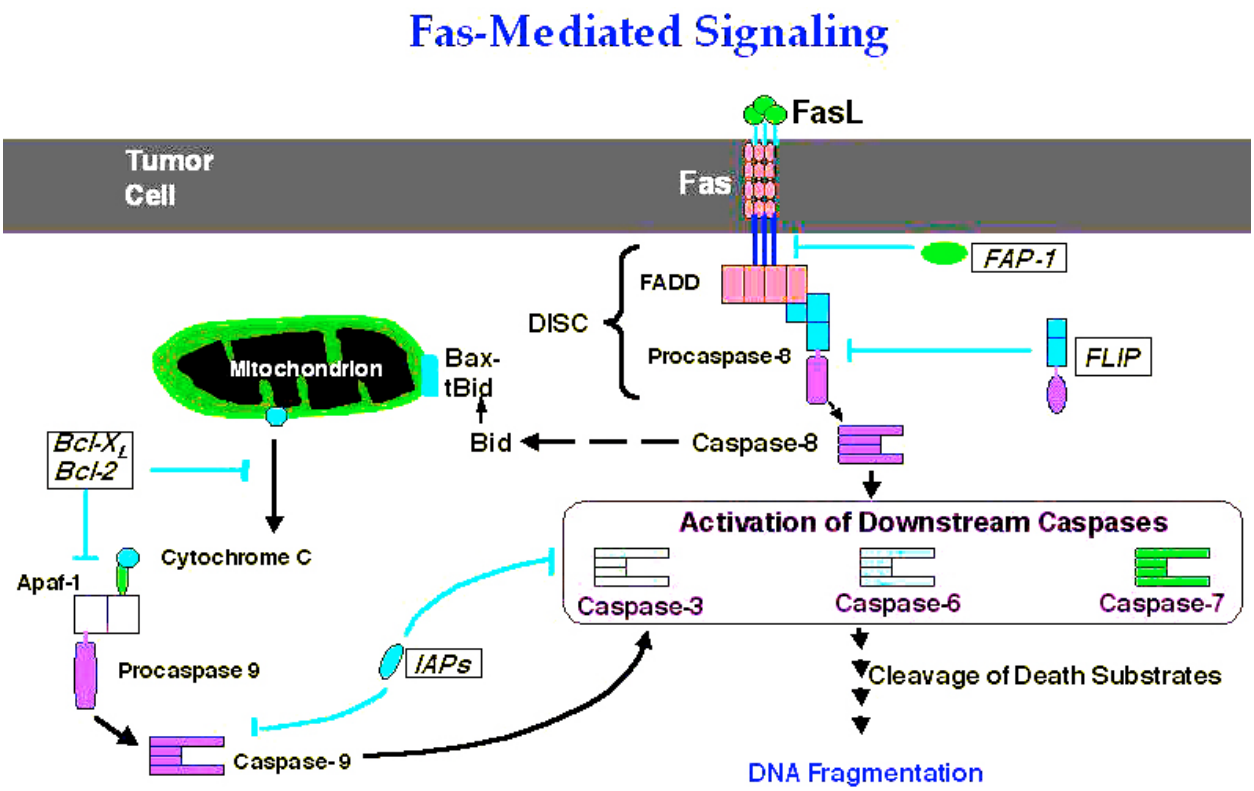

Figure 2. Fas-Mediated Signaling. The process of Fas-mediated apoptosis is triggered by cross-linking (trimerization) cell surface Fas by its cognate ligand (FasL) or a surrogate agonistic anti-Fas stimulus. Once engaged, Fas recruits FADD, which, in turn, interacts with procaspase-8 molecules forming the DISC. In a cascade-type reaction, activation of caspase-8 leads to the activation of downstream effector caspases, such as caspases-3, -6 or -7, resulting in DNA fragmentation and cell death. A second major pathway involves damage to the mitochondria induced by other types of stimuli, such as gamma-irradation, anti-neoplastic agents and the perforin/granzyme pathway of cytotoxic lymphocytes. Bcl-2 family members have either pro-apoptotic (Bax, Bak, Bid) or anti-apoptotic (Bcl-2, Bcl-X_L) functions. Pro-apoptotic Bcl-2 members stimulate the mitochondria to release cytochrome C, which then forms a complex with Apaf-1 and procaspase-9. Activation of caspase-9 then activates caspase-3, ultimately leading to nuclear degradation. Endogenous inhibitors of the Fas pathway (which are illustrated in boxes alongside the flat-head arrows), downregulate the amount of signaling and resulting cell death. Aberrant expression of some of these inhibitory mechanisms in neoplastic cells may contribute to increased Fas-resistance and, consequently, tumor escape from Fas-dependent cytotoxicity.

protein that is constitutively expressed on the surface of a broad range of cells or tissues, including those of lymphoid and non-lymphoid lineages. FasL, also known as CD95L, is a member of the TNF/NGF superfamily (15, 17, 18, 71, 73). FasL is a 40-kDa type II transmembrane protein that is expressed by lymphocytes, mainly by type 1 $CD4^+$ and $CD8^+$ T cells, and B cells, after engagement of the Ag-specific T or B cell receptor. NK cells and macrophages also express FasL. FasL is constitutively expressed by cells of the eye, reproductive organs (testis, uterus), lung and small intestine in addition to cells of the immune system. FasL is functionally active in a membrane-bound form and, as reported for activated human T cells, a secreted, soluble form as well (74-76). Soluble FasL (sFasL) is generated as a 26-kDa protein fragment lacking the transmembrane and cytosolic domains by matrix metalloproteinases (74).

Briefly, the apoptotic pathway is initiated by cross-linking (trimerization) cell surface Fas by FasL or an anti-Fas stimulus (Figure 2). Engagement of Fas leads to the aggregation of the receptor, which enables the adapter molecule, Fas-associated death domain (FADD) protein, to bind to the cytosolic domain of Fas (15, 17, 18, 47, 71, 77). FADD, a multimeric protein, then recruits and interacts with multiple procaspase-8 molecules forming the death-inducing signaling complex (DISC). The oligomerization of procaspase-8 within the DISC leads to its proteolytic activation, which initiates activation of the caspase pathway. Caspase-8 then cleaves procaspases-3, which is thought to be an integral downstream effector element ultimately contributing to the disintegration of the cellular genome. Effector caspases, such as caspases-3, -6 or -7, cleave numerous target substrates, including structural proteins and those involved in cellular signaling, cell cycle and DNA repair.

A second major pathway is independent of Fas engagement, but still caspase-dependent, and it involves damage to the mitochondria induced by other types of stimuli, such as gamma-irradation, anti-neoplastic agents

and the perforin/granzyme pathway of cytotoxic lymphocytes (46, 66, 78-80). Members of the Bcl-2 family are crucial regulators of this process, and either have pro-apoptotic (Bax, Bak, Bid) or anti-apoptotic (Bcl-2, Bcl-X_L) functions (81). Pro-apoptotic Bcl-2 members stimulate the mitochondria to release cytochrome C, which then forms a complex with Apaf-1 (apoptotic protease activating factor-1). The ultimate effectors of apoptosis, as with the Fas pathway, are the caspases. In this case, procaspase-9, instead of procaspase-8, is thought to be the principal initiator, which is activated by the cytochrome C/Apaf-1 apoptosome complex. Caspase-9 then activates procaspase-3, ultimately leading to nuclear degradation. Thus, both Fas-dependent and -independent pathways converge at the level of caspase-3, suggesting a potentially crucial role of that component in the regulation of apoptosis by multiple types of death-inducing stimuli.

Endogenous inhibitors of the Fas pathway regulate the extent of signaling, including caspase activation, and resultant cell death. These include molecules that affect signaling, namely FLIP (FLICE/caspase-8 inhibitory protein) (60), FAP-1 (Fasassociated phosphatase-1) (82), Bcl-2-related proteins (81, 83) or inhibitors of apoptosis proteins (IAP-1, IAP-2, XIAP; survivin) (71, 84, 85). FLIP structurally resembles caspase-8, but lacks proteolytic activity and competes with it for binding to the DISC via FADD, thus blocking its activation. High levels of FLIP have been linked to resistance to Fas-mediated apoptosis in naïve peripheral T cells (86) and in human melanoma cell lines (87, 88), as well as in experimentally engineered murine tumors (36). FAP-1, which associates with a negative regulatory domain of the C-terminus of Fas, similarly has been found to inhibit Fas-mediated signaling in T cells (82, 89). IAPs inactivate the function of caspases-3, -7 or -9 via direct interactions, while Bcl-2 or Bcl-X_L inhibits the redistribution of cytochrome C from the mitochondria into the cytosol or the binding of cytochrome C to Apaf-1 (71, 81, 84). The expression of the IAP is regulated by NF-kB, a nuclear transcription factor (71, 90). Although under normal conditions the Fas/FasL system is a tightly regulated pathway, aberrant expression of one or more inhibitory mechanisms in neoplastic cells can contribute to apoptotic resistance in response to a wide range of death-inducing stimuli, including ionizing radiation, chemotherapeutic agents and even cytotoxic lymphocytes.

5. MECHANISMS OF TUMOR ESCAPE

Although the fundamental basis for the failure to initiate effective T cell responses in unimmunized tumor-bearing hosts may be linked to poor tumor immunogenicity (14, 91), a variety of mechanisms have been presented to account for tumor escape, reflecting multiple levels of the effector/target interaction, including: TCR-MHC/peptide ligand recognition, cellular adhesion (conjugate formation) or the cytotoxic mechanism. (Reviewed in refs. 10-13). For example, it has been proposed that tumor cells may escape as a consequence of (a) reduced expression of MHC alleles, beta-2-microglobulin protein or adhesion (e.g., ICAM-1) molecules; (b) suboptimal expression of the relevant rejection epitope(s), which may reflect intracellular defects in endogenous Ag processing and presentation pathways; (c) production of tumor-derived inhibitory factors, such as interleukin-10 (IL-10), vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-beta) and prostaglandins, which directly or indirectly downregulate cell-mediated immune reactions; (d) failure to activate Ag-specific T cell responses because of tumor-induced alterations affecting TCR structure, signaling or function (92-94); (e) resistance to cell-mediated cytotoxicity, due to the inability of effector T cells to efficiently mediate Fas-dependent apoptosis (25, 35, 36); (f) cell surface expression of FasL, which has been suggested to eliminate infiltrating Fas-bearing $CD4^+$ or $CD8^+$ T lymphocytes, although this latter possibility remains unsettled (13, 95, 96); (g) tumor--induced alterations in T cell subtype cytokine profiles (97, 98) or "suppressor" T cell subpopulations that bear a unique $CD4^+CD25^+$ phenotype (99), which may exist in lymphoid compartments and at sites of tumor growth (100-102), that might downregulate productive antitumor immune responses; and (h) inefficient tumor penetrance of Ag-specific T cells, particularly in the treatment of large tumor masses (103).

Although a variety of molecular or biologic alterations have been observed in tumors as they become more progressive, such as those described above, it remains to be fully understood how such changes are thought to occur initially and whether immunologically driven events, in addition to genetic and epigenetic determinants, contribute to the generation of tumor escape variants expressing those aggressive phenotypes. Studies by Schreiber and colleagues (2, 4, 9) have now provided key insights into a mechanistic basis for tumor progression, and a critical role of host T cells in an immune-based selective process. Consequently, the concept of "cancer immunoediting" was advanced (9), which constituted a refinement of the original concept of "cancer immunosurveillance" proposed by Burnet and Thomas almost five decades ago (104). The concept of cancer immunoediting encompasses the process of immune surveillance, but goes beyond to predict that the antitumor immune reaction during the act of surveillance concurrently imposes a selective pressure toward developing neoplasms, resulting in the potential outgrowth of immune-resistant malignant variants. The production of IFN-gamma turns out to be a critical determinant of this selective pressure. Ordinarily, IFN-gamma, secreted primarily by activated T cells and NK cells after receptor engagement, is thought to modulate antigenic properties of the tumor rendering it more susceptible to Ag-specific immune attack. In so doing, those tumor cells that are most responsive to IFN-gamma-mediated effects are eliminated by such effector cells, whereas those tumor cells that are least responsive are potentially less immunogenic. Consequently, this immune/IFN-gamma selective pressure, at least in certain models, has led to an antigenic or immunogenic reshaping of the reemerging tumor population possessing enhanced malignant properties.

6. REGULATION OF FAS EXPRESSION IN NEOPLASIA

Thus, it is now generally well established that a functional innate and adaptive immune system is crucial for the control of tumor development and growth (2, 5, 9, 14,

105). This introduces the opposing notion that if the immune system becomes functionally impaired or if neoplastic cells develop resistance to host defense mechanisms, then the outcome is progressive tumor development and growth. Therefore, understanding the nature and molecular bases of tumor escape in the face of intact immune defense mechanisms is critical to the control of the neoplastic process.

Although alterations in the genetic or epigenetic program of the evolving neoplastic population are undoubtedly crucial for tumor progression and metastatic development (78, 106), immune-mediated selective pressures may help drive the emergence of such aggressive neoplastic subpopulations possessing those tumorigenic characteristics (2, 4, 9, 39, 40). If that were the case, then a T cell response directed against a parental or primary tumor mass will have not only positive, but also negative consequences affecting neoplastic formation. One would predict, therefore, that an antitumor T cell response would mediate meaningful levels of tumor regression initially. However, if tumor regression is incomplete, this may reflect the survival of neoplastic subpopulations resistant to T cell attack. Thus, an anti-neoplastic T cell response may alter the composition of the resulting mass or lesion such that it may be enriched for a more malignantly proficient or aggressive phenotype.

The possibility that aggressive tumor variants emerge in response to NK cell or Ag-specific CTL interactions *in vivo* is supported by several studies, which reveal the outgrowth of Ag loss variants (107-110) or Fas-resistant subpopulations expressing heightened malignant or metastatic properties (34-40). For example, in a mouse model of experimental lung metastasis, the relationship between Fas expression and metastasis was examined in three groups of cells: the parental line (CMS4), the *in vivo*-selected metastatic subline (CMS4-met) and a CMS4 subline biologically selected *in vitro* from the parental population by serial culture with agonistic anti-Fas stimuli (CMS4.sel) (39). These studies revealed an inverse correlation between Fas expression and metastatic phenotype. Moreover, in a transgenic mouse model of spontaneously arising primary and metastatic mammary carcinoma, tissue sections from both primary (mammary) and metastatic (lungs) sites of tumor growth were analyzed for Fas expression by immunohistochemistry. It was found that Fas was highly expressed in mammary gland tumors. In contrast, Fas expression was considerably less in metastatic foci in the lung when compared with primary mammary gland carcinoma. As with the experimental lung metastasis model, these observations revealed an inverse correlation between Fas expression and metastatic phenotype *in vivo*. In other work, it was reported that Fas-sensitive, non- or poorly metastatic murine melanoma clones spontaneously form lung metastases in *gld* mice as efficiently as their Fas-insensitive metastatic clonal counterparts in wild-type mice (34). These data suggested that endogenous Fas/FasL interactions in the host played a direct role in the regulation of metastatic formation. Loss of Fas function alone, therefore, was characterized as both necessary and sufficient for tumor progression in that model.

Similarly, in human colon tumorigenesis, immunohistochemical evidence suggests that diminished Fas expression is a common occurrence of an advancing neoplastic phenotype (46, 61). However, the biologic significance of loss of Fas expression and the mechanisms underlying these phenomena have remained unclear. To better explore the link between functional Fas status and malignant phenotype in human colon carcinoma, several studies took advantage of two naturally occurring primary and metastatic cell lines, termed SW480 and SW620 (25, 40, 111). The SW480 and SW620 tumor cell lines have been previously characterized as primary and metastatic colon adenocarcinoma cell lines, respectively, established from the same patient (112). The SW620 cell line was derived as a lymph node-metastasis identified six months later during disease recurrence. Furthermore, both cell lines were isolated from the patient without any prior chemotherapy (113).

It was first demonstrated that SW480 cells displayed an IFN-gamma-inducible Fas-responsive phenotype, whereas SW620 cells remained Fas-resistant under these experimental conditions (25, 26, 40, 111). Next, it was examined whether such differences in functional Fas status influenced tumor progression toward a more metastatic phenotype (40). The approach taken was to produce sublines from the primary tumor *in vitro* for Fas resistance using an agonistic anti-Fas stimulus to deplete the Fassensitive subpopulations. Conversely, sublines were produced from the primary tumor *in vivo* (in nude mice) from sites of spontaneous distal splenic metastases (following a sc tumor transplant). In so doing, several SW480 sublines were established by these two approaches. Thus, any functional and/or molecular differences observed with such SW480-derived sublines were compared with the naturally occurring primary and metastatic tumor cell lines. Overall, those findings revealed that such SW480-derived sublines were Fas-resistant, and that they morphologically, functionally and molecularly resembled the naturally occurring metastatic SW620 cell line. These data supported the hypothesis that metastatic subpopulations possessing a Fas-resistant "SW620-like" phenotype already preexisted within the primary tumor population (Figure 3).

Taken collectively from both mouse and human studies (34-40), these findings revealed a novel contribution of the Fas pathway in tumor progression, and suggested that Fas-based interactions mechanistically imposed an immunologic or biologic selective pressure favoring the emergence of such preexistent metastatic subpopulations (Figure 3). These observations are consistent with the "cancer immunoediting" hypothesis (9), but extend it further to implicate the Fas/FasL system as a potentially important element of the selective process. Thus, in addition to the diversity of tumor escape mechanisms already reported (see Section 5), these data lend strong support to the identification of Fas-resistance as a novel tumor escape mechanism from immune attack. Further molecular characterization of such tumor escape variants will improve not only an understanding of the neoplastic process, but perhaps may also aid in the design of more effective therapies.

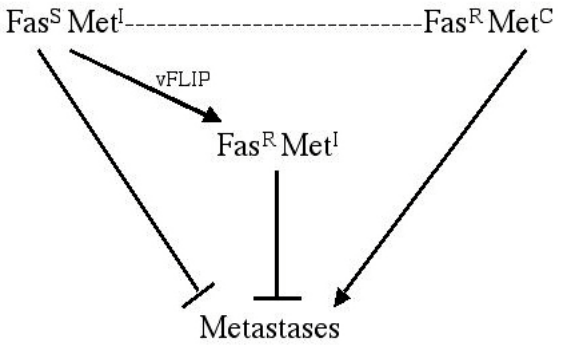

Figure 3. Model of Fas-based interactions in tumor progression in a human colon carcinoma system. The SW480 primary tumor population appears to comprise multiple subpopulations, embracing the gamut of both Fas-sensitive (Fas^S) and Fas-resistant (Fas^R) cells, including those that are metastatic-incompetent (Met^I) and metastatic-competent (Met^C), illustrated by the dashed line. In contrast, the SW620 metastatic tumor population appears to be more homogeneous in terms of expressing a Fas^RMet^C phenotype. Fas^RMet^C cells were identified within the parental SW480 population, based on the isolation of both *in vitro*- and *in vivo*-derived SW480 sublines. The observation that such SW480 sublines harboring a Fas^RMet^C phenotype morphologically and molecularly resembled the naturally occurring SW620 population suggests that such metastatic subsets already pre-existed within the primary tumor and that anti-Fas interactions served as a biologic selective pressure for their outgrowth. However, the idea that Fas status alone was sufficient for this biologic outcome is unlikely, since parental SW480 cells engineered to express a Fas^RMet^I phenotype (via FLIP transfection) did not display detectable metastatic behavior under these experimental conditions. Thus, these data supported the idea that a Fas^RMet^C phenotype likely reflected Fas-resistant neoplastic subpopulations that also co-possessed additional malignant/metastatic-associated genes and properties. Reprinted with permission from The Journal of Immunology (Copyright 2003, The American Association of Immunologists, Inc.) Figure 8, Page 4173. Liu K, McDuffie E, and Abrams SI. Exposure of Human Primary Colon Carcinoma Cells to Anti-Fas Interactions Influences the Emergence of Pre-existing Fas-resistant Metastatic Subpopulations. J Immunol171:4164-4174 (2003). Similar conclusions were derived in a mouse model of experimental metastasis (39).

Although an antitumor immune reaction may influence the outgrowth of developing or preexisting Fas-resistant clones, the precise mechanisms underlying Fas--loss-of function initially in malignant cells remain to be fully understood. Loss of Fas function has been reported to occur at three major levels: (a) downregulation of *fas* gene transcription, perhaps mediated by activated or oncogenic *ras* or a loss of wild-type p53 function causing repression (47, 114-116); (b) production of soluble forms of the Fas receptor (117) which may compete with membrane-bound Fas for binding to functional FasL or expression of a Fas decoy receptor that lacks bioactivity (118); and (c) inhibition of Fas-mediated signaling as a consequence of aberrant expression of anti-apoptotic mechanisms, such as FLIP, FAP-1, IAP or Bcl-2, as described in Section 4.

In addition to the acquisition of Fas-resistance, appreciable interest has surrounded the "tumor counterattack model", which theorizes that FasL expression by malignant cells contributes to tumor escape as a consequence of inducing apoptosis of infiltrating Fas-bearing effector cells (10, 119, 120). Despite the molecular basis for FasL expression or production (i.e., cell surface or secreted), this model has been recently challenged given the findings that FasL-bearing tumor cells are more easily rejected as compared to Fas-negative parental cells via a pro-inflammatory-based, neutrophil-mediated antitumor mechanism (48, 96). Because considerable debate still surrounds the soundness of the FasL counterattack model, it remains unresolved whether this phenomenon indeed serves as a viable mechanism driving tumor escape and progression *in vivo.* Therefore, additional investigations are necessary to elucidate the molecular basis by which FasL-bearing cancer cells promote an anti-inflammatory (immune cell destruction) versus a pro-inflammatory (tumor cell destruction) outcome *in vivo.*

7. PARADIGM FOR T CELL-TUMOR CELL INTERACTIONS *IN VIVO*

The following paradigm can be envisioned for how an immune response may have both positive and negative consequences during host-tumor interactions *in vivo.* Active immunization of the tumor-bearing host with tumor-derived antigenic materials reflecting $CD4^+$ and/or $CD8^+$ T cell epitopes leads to the *in vivo* priming and expansion of the Ag-specific T cell precursor pools. *In vivo* sensitized $CD4^+$ and/or $CD8^+$ T cell subpopulations may also be then isolated *ex vivo* from immunized hosts and expanded *in vitro* to achieve larger quantities of immune effector cells for adoptive transfer. Thus, the combination of both active and passive immunotherapies may have a more comprehensive impact on the control of metastatic development. This notion is supported by recent preclinical studies in a mouse model of melanoma which demonstrated that the combination of both active and adoptive immunotherapies, under conditions of extensive disease, led to dramatic antitumor responses significantly more so than either modality administered separately (121).

The Ag-specific $CD4^+$ T lymphocyte may be a central player important for the optimal induction and development of adaptive and antitumor immunity (57, 122). At the tumor site, or more likely within lymph nodes draining the tumor site, Ag-specific $CD4^+$ T cells may be stimulated by specialized APC populations, such as dendritic cells, macrophages or activated B cells that have infiltrated these metastatic lesions. Such APC populations, expressing a spectrum of adhesion and costimulatory molecules, may exogenously process tumor-associated Ag (supplied via the vaccine strategy or tumor cell themselves) and present them as antigenic peptides in association with self-MHC class II or class I alleles via cross-priming pathways for initiation of the cellular immune response by both $CD4^+$ and $CD8^+$ subpopulations, respectively.

Lymphokines produced by the Ag-primed $CD4^+$ T cell response, such as IL-2, may further intensify clonal expansion of both Ag-sensitized $CD4^+$ and $CD8^+$ T cell populations.

During the effector phase of the immune response, *de novo* activated or adoptively transferred $CD8^+$ CTL may lyse susceptible target populations *directly,* if displaying the relevant MHC/peptide ligand complexes via Fas-dependent and/or independent (i.e., perforin/granzymes) mechanisms. IFN-gamma, perhaps in concert with other pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha) may be important components for improving the overall efficacy of cancer immunotherapy. IFN-gamma-induced augmentation of MHC and adhesion molecules may thus serve to facilitate and strengthen both specific (i.e., TCR-MHC/peptide) and nonspecific (i.e., LFA-1-ICAM-1) aspects of the effector/target interaction, leading to enhanced T cell activation and triggering of the cytolytic pathways. In the context of the metastatic tumor microenvironment, IFN-gamma and other potentially relevant cytokines may be provided endogenously by immune system interactions, most notably by a subclass of Ag-activated $CD4^+$ T cells, termed Th1, following interaction with MHC class II^+ Ag-bearing APC. In addition to IFN-gamma, $CD4^+$ Th1-derived cytokines such as IL-2 and GM-CSF may further influence the recruitment, activation and expansion of various other cytotoxic effector cells of the innate immune response, including granulocyte subpopulations, macrophages and NK (or lymphokine-activated killer/LAK) cells. Furthermore, $CD8^+$ CTL or $CD4^+$ Th1 cells may lyse susceptible tumor cells *indirectly* through the release of cytotoxic lymphokines, such as TNF-alpha or soluble FasL (39, 75, 111) following Ag-specific immune stimulation. In this context, the initiation of the immune response remains Ag-specific, while the effector mechanisms become Ag-independent and bystander in nature. These cell contact-independent mechanisms as well as any additional or alternative innate immune effector elements may represent biologically significant pathways for the elimination of low MHC- and/or low Ag-expressing tumor cells thus circumventing, at least in part, tumor antigenic heterogeneity associated with the loss or downregulation of MHC/peptide ligand expression.

Although this paradigm clearly has positive implications for tumor immunotherapy, it also may have potential negative consequences, as discussed earlier in Sections 5 & 6. Persistent exposure to an immunotherapeutic procedure or agent over time may unintentionally impose a selective pressure favoring tumor escape of immune-resistant neoplastic subclones and may account, at least in some way, for the failure of a given immunotherapy to sustain long-term antitumor effects. Of course, it remains to be fully understood whether this immunologic phenomenon naturally occurs in cancer patients, and whether T cell-based immunotherapies can circumvent effectively such barriers of tumor self-protection and escape or, for that matter, unintentionally influence the process of neoplastic progression.

8. PERSPECTIVES

An understanding of tumor rejection mechanisms *in vivo* may help design or improve immunotherapies against metastatic disease in clinical settings. Indeed, a number of studies have now demonstrated that the Fas/FasL system utilized by both innate and adaptive forces of the immune system plays an important role in the regulation of tumor growth and spread. The Fas/FasL system, however, constitutes one of several host defense mechanisms, and collectively with perforin and other TNF family members optimally control neoplastic development in an immune competent host. The contributions of FasL-dependent interactions to tumor immunity, or other cytotoxic moieties, depend upon the intrinsic susceptibility of the aberrant target population to a given effector mechanism. Many variables likely determine tumor-cell responsiveness to immune attack, and may be related, at least in part, to tumor heterogeneity and the composition of neoplastic clones reflecting diverse stages of malignant proficiency. In both mouse tumor models and human neoplasia, Fas expression or function has been inversely related to metastatic phenotype. Furthermore, in some studies, loss of Fas function has been causally linked to tumor progression. Thus, the Fas/FasL system may serve as a novel biologic selective pressure against a progressing mass or lesion, causing an elimination of the Fas-bearing clones with a reciprocal enrichment of potentially lower frequencies of the Fas-resistant ones. Consequently, this phenomenon is consistent with the concepts of "immunoselection" and "cancer immunoediting" (9), but extends it to implicate the Fas/FasL system as an underlying component of the selective process. Although preclinical data support this "Fas selection hypothesis", it remains to be formally demonstrated in human neoplasia.

So, how can the Fas/FasL system be exploited to favor tumor regression over tumor progression? One possibility is to integrate or implement combination therapies, attacking the neoplastic process from multiple vantage points. For example, combination therapies involving vaccination or adoptive transfer with other oncological treatments, such as radiation (123, 124), chemotherapy (5, 125), cytokines (e.g., IL-2, IL-12, IL-15) (126-128), passive administration of tumor-specific monoclonal antibodies (129), angiogenic inhibitors (130), or non-steroidal anti-inflammatory drugs (e.g., cyclooxygenase-2 inhibitors) (131) may prove even more beneficial to facilitate long-term clinical regressions concomitant with a lower risk toward the generation of potential aggressive tumor escape variants. The intention is that such combinatorial therapies may sufficiently enhance or restore the capacity of tumor cells to undergo cell death through Fas-dependent, as well as Fas-independent mechanisms. Clearly, these and other factors represent potentially significant challenges confronting effective cancer immunotherapy. Continued investigations likely will not only shed light into a potential explanation of tumor development in the face of a competent immune system or tumor recurrence in the face of immunotherapy, but also eventually will aid improvement into the nature, breadth

and effectiveness of combination immunotherapy strategies.

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Abbreviations: Ag Apaf-1 APC cFLIP CTL DISC FADD FAP-1 FasL GM-CSF IAP IFN-gamma IL-2 MHC NK sc TCR TIL TNF TRAIL APC cFLIP CTL DISC FADD FAP-1 FasL GM-CSF IAP IFN-gamma IL-2 MHC NK sc TCR TIL TNF TRAIL Antigen, apoptotic protease activating factor-1, antigen-presenting cell, cellular-derived FLICE inhibitory protein, cytotoxic T lymphocyte, death-inducing signaling complex, Fas-associated death domain, Fas-associated phosphatase-1, Fas ligand, granulocyte/macrophage-colony stimulating factor, inhibitors of apoptosis proteins, interferon-gamma, interleukin-2, major histocompatibility complex, natural killer (cells) subcutaneous, T cell receptor, tumor-infiltrating lymphocytes, tumor necrosis factor, TNF-related apoptosis-inducing ligand

Key Words: Cytotoxic T lymphocytes, Fas, Fas ligand, Immunotherapy, Neoplasia, Tumor escape, Immune System, Cancer, Gene, Review

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