Immune Escape of Tumors In Vivo by Expression of Cellular FLICE-inhibitory Protein

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Summary

The antiapoptotic protein cellular FLICE (Fas-associated death domain–like IL-1β-converting enzyme) inhibitory protein (cFLIP) protects cells from CD95(APO-1/Fas)-induced apoptosis in vitro and was found to be overexpressed in human melanomas. However, cytotoxic T cell-induced apoptosis, which is critically involved in tumor control in vivo, is not inhibited by cFLIP in vitro, as only CD95- and not perforin-dependent lysis is affected. This calls into question whether cFLIP is sufficient to allow escape from T cell–dependent immunity. Using two murine tumors, we directly demonstrate that cFLIP does result in escape from T cell immunity in vivo. Moreover, tumor cells are selected in vivo for elevated cFLIP expression. Therefore, our data indicate that CD95-dependent apoptosis constitutes a more prominent mechanism for tumor clearance than has so far been anticipated and that blockade of this pathway can result in tumor escape even when the perforin pathway is operational.

Key words: CD95 • apoptosis • CTL • perforin • cytotoxicity

Apoptosis is a carefully controlled suicide program that serves to dispose of superfluous or unwanted cells (1). It can be induced by the triggering of specific death receptors, such as CD95(APO-1/Fas) (2). Activation of CD95 can lead to recruitment and activation of caspase-8 (3–6), which then enganges the cell death machinery (7, 8). Several mechanisms have been proposed over the last few years that can interfere with death receptor–induced apoptosis (7, 9). Of these, cFLIP (also called Casper/iFLICE/FLAME-1/CASH/CLARP/MRIT/usurpin) (9) is probably the most receptor proximal. Interestingly, cFLIP expression is increased in human melanomas (references 10 and 11; Medema, J.P., and J. de Jong, unpublished observations), suggesting that these tumor cells become refractory to CD95-induced apoptosis.

CTLs play a critical role in the control of tumor growth (12). They lyse their targets through CD95- as well as perforin-dependent pathways (13–15). Although both pathways can converge at an early step in the signaling cascade that leads to target cell apoptosis (16), cFLIP was shown to only inhibit CD95-dependent and not perforin-dependent apoptosis (17). In effect, cFLIP overexpression fails to protect cells from lysis by an effector population consisting of polyclonal alloreactive CTLs in vitro (17). We obtained comparable results with tumor-specific CTL clones that have a defined peptide specificity and Raji, a human Burkitt B cell lymphoma, loaded with the relevant peptide epitope as target cell (Medema, J.P., and J. de Jong, unpublished observations). Clearly, cFLIP overexpression efficiently blocks the CD95 pathway but does not inhibit CTL-mediated lysis in vitro.

Here, we set out to analyze whether cFLIP overexpression can affect the sensitivity of tumor cells to T cell immunity in vivo and permit tumorigenesis. We employed two murine tumors whose eradication critically depends on the CTL response (18, 19). We demonstrate that overexpression of cFLIP results in their escape from T cell immunity. Moreover, we show that tumor cells are selected in vivo for elevated cFLIP expression.

Materials and Methods

Mice. C57BL/6 perforin−/− (PKO, H-2b; reference 20; a gift from Dr. M. van den Broek) and C57BL/6 Kh (B6, H-2b) mice were bred at TNO-PG (Leiden, The Netherlands). C57BL/6 nu/nu (B6 nude, H-2b) mice were obtained from Bomholtgard (Ry, Denmark).

Cells. A adenovirus type 5 E1A plus mutant EJ-ras–transfected (AR 6; reference 19) and MBL2-Fas (MF; reference 20) (a gift from Dr. M. van den Broek, Zürich, Switzerland) were transfected by electroporation (240 V, 960 μF) with a plasmid encoding murine FLAG-tagged cFLIP under the control of a CMV promoter (cDNA provided by Dr. J. Tschopp, Lausanne, Switzerland) or a control plasmid. Sam-D b is a mouse embryo fibroblast transfected with a plasmid encoding the E1A epitope coupled to a signal sequence and murine H-2D b and therefore presents high levels of the Ad5E1A epitope in the context of
H-2D<sup>b</sup>, CD95L-expressing C57BL/6<sup>imr/lpr</sup> mouse embryo cells (MECs) were obtained by Ca<sub>2+</sub>(PO<sub>4</sub>)<sub>2</sub> transfection with a plasmid encoding murine CD95L. Clone 1 was obtained by immunizing mice with RMA and is specific for an epitope encoded by the FMR-MuLV gagLeader sequence (CCLCLTVFL). The E1A (line 5) and gagLeader (clone 1)–specific CTLs were cultured as previously reported (16, 19).

**Results and Discussion**

The inability of cFLIP to prevent CTL killing in vitro makes it questionable whether increased cFLIP expression, as observed in human melanomas (references 10 and 11; Medema, J.P., and J. de Jong, unpublished observations), can protect tumor cells from CTL-mediated cytotoxicity in vivo and thereby provide a mechanism of tumor escape. To analyze this directly, we used the murine tumor line MF. MF is a CD95 transfectant of MBL2 (20), a Moloney leukemia virus–induced lymphoma that can be controlled in vivo by virus-specific CTLs (18). Due to the high sensitivity of MF to CD95-induced apoptosis, it is well suited to testing the potential of cFLIP to modulate tumorigenicity.

Transfectants of MF were generated either expressing high levels (MF-FLIP<sup>high</sup>) or, as a control, very low levels (MF-FLIP<sup>low</sup>) of FLAG-tagged cFLIP (Fig. 1 A, inset). Both lines express identical amounts of MHC class I and CD95 (data not shown), but only MF-FLIP<sup>low</sup> is sensitive to apoptosis induced by the murine CD95–specific antibody Jo2 in vitro (Fig. 1 A). Overexpression of cFLIP does not affect tumor-specific CTL–induced cytotoxicity (Fig. 1 B). However, when the perforin pathway of this CTL is blocked by preincubation with CMA, a substance that results in specific degradation of perforin (22), the sensitivity of MF-FLIP<sup>high</sup> is completely lost, whereas MF-FLIP<sup>low</sup> killing is only slightly reduced (Fig. 1 B). These data also indicate that for the MF tumor, cFLIP expression only affects CD95- and not perforin-dependent killing and that both pathways need to be inhibited for MF to escape from CTL-induced apoptosis in vitro.

We subsequently set out to analyze the role of perforin- and CD95-dependent cytotoxicity in vivo. Injection of 10<sup>2</sup> MF-FLIP<sup>low</sup> cells into syngeneic PKO mice results in clearance of the tumor cells in the majority of these mice, whereas injection of the same dose of MF-FLIP<sup>high</sup> cells results in progressive tumor growth (Fig. 1 C). To our knowledge, this outcome represents the first direct proof that overexpression of cFLIP enables tumor cells to escape CD95-dependent cytotoxicity not only in vitro but also in vivo.

Next we tested whether cFLIP expression also allowed...
these tumor cells to escape destruction by the immune response in wild-type mice, in which not only the CD95-dependent cytotoxicity, which increases the resistance of mice to these tumors and is sufficient to obtain complete lysis in vitro, the T cell–dependent immune response in vivo is not adequately equipped to eradicate MF-FLIP<sup>high</sup> tumors. These data underscore the crucial role of CD95-induced apoptosis in tumor clearance by a physiological T cell response and indicate that blockade of the CD95 pathway can tip the balance in favor of the tumor cells.

Due to the high levels of CD95 on MF, CTL activity against this line may be skewed toward this pathway. Therefore, we analyzed the effect of cFLIP on a tumor that expresses only modest levels of endogenous CD95. Tumor line AR6 has been generated by transfection of B6 MECs with the adenovirus type 5 E1A and mutant EJ-ras oncogenes. Subcutaneous injection of AR6 results in initial tumor growth, after which the tumor regresses due to an efficient CTL response directed against an epitope encoded by E1A (19). AR6 expresses low but detectable levels of CD95 (Fig. 1 A). We transplanted AR6 with FLAG-tagged cFLIP (AR6–FLIP) or as control with vector alone (AR6–vector) (Fig. 2 B). Two subclones were selected on the basis of comparable MHC class I expression, growth kinetics, and morphological features in vitro. AR6–vector tumor is resistant to Jo2 (anti–murine CD95)–induced apoptosis, probably due to the low cytotoxic potential of this antibody (Medema, J.P., and J. de Jong, unpublished observation). However, in cocultures with CD95L-expressing MECs, apoptosis of AR6–vector is induced but AR6–FLIP is fully resistant (Fig. 2 B). Even though CD95–induced apoptosis is completely blocked, we find that cFLIP does not affect lysis of AR6–FLIP by E1A-specific CTLs (Fig. 2 C). Inhibition of the CD95 pathway during CTL–induced apoptosis by cFLIP is, however, suggested by the partial cleavage of FLAG-tagged cFLIP in AR6–FLIP (Fig. 2 C, inset), which has been shown to correlate with resistance (10, 24).

When injected into nude mice, the AR6 lines display equal and uncontrolled tumor growth (data not shown). In contrast, both PKO and wild-type mice can control the AR6–vector tumor (Fig. 2, D and E). However, comparable to our findings with MF-FLIP<sup>high</sup>, neither of these mouse strains is capable of rejecting the cFLIP-overexpressing tumor AR6–FLIP (Fig. 2, D and E). Thus, escape from T cell–mediated immunity in vivo by cFLIP overexpression is not limited to tumors with very high CD95 surface expression.

It is important to note that the AR6–FLIP tumor grows almost as efficiently in PKO and wild-type mice (Fig. 2, D and E), which suggests that eradication of this tumor depends almost exclusively on CD95. This observation is not without precedent; as other murine tumor lines, such as the T cell lymphoma RMA and the melanoma B16, were shown to be equally tumorigenic in PKO and wild-type mice (20). Apparently, clearance of certain tumors does not critically depend on perforin-based cytotoxicity but can be achieved by other mechanisms, which obviously include CD95-induced apoptosis. Importantly, this dependance is
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Figure 3. cFLIP-induced immune evasion is due to CTL resistance. (A) Tumor growth was analyzed in wild-type mice injected simultaneously with AR 6-vector (2 x 10^7) into the left flank and AR 6-FLIP (2 x 10^7) into the right flank of mice (n = 10). Percentage survival of the mice is shown. Death of these mice is not determined by a high sensitivity to CD95-induced apoptosis, as is clear from the two tumor lines tested here. Further evaluation is required to determine the relative importance of perforin-dependent cytotoxicity among different tumor systems, but these data clearly indicate that CD95-mediated apoptosis constitutes a highly important mechanism for tumor clearance even in situations where the perforin pathway is operational.

Recent findings have suggested that apoptosis of tumor cells may be required for efficient uptake and cross-presentation of tumor antigens by dendritic cells and therefore may be essential for priming of effective T cell immunity (25). To test whether immune escape by AR 6-FLIP may involve prevention of T cell priming rather than escape from the T cell response, we injected 86 mice simultaneously with AR 6-FLIP and AR 6-vector in either flank. As expected, most mice were again capable of rejecting the AR 6-vector cells, pointing to the induction of an efficient immune response. Despite this response, AR 6-FLIP tumors in the other flanks of these mice developed progressively, indicating that these cFLIP-expressing tumors are capable of growing out in the face of an effective antitumor response (Fig. 3 A). To directly examine whether antitumor CTL immunity is induced in mice challenged with AR 6-vector, AR 6-FLIP, or both, we analyzed the CTL response against the E1A epitope in splenocytes from these mice. Importantly, comparable E1A-specific CTL immunity was detected in all mice (Fig. 3 B).

Also in the MF model, we obtained evidence that cFLIP overexpression enables escape from antitumor immunity rather than preventing the induction of this response. As shown in Fig. 1 C, a minority of the PKO and wild-type mice challenged with MF-FLIPlow developed progressively growing tumors. Intriguingly, isolation of these tumors and analysis in vitro showed that the cells had acquired resistance to CD95-mediated apoptosis, which correlates well with the increased expression of cFLIP in these cells (Fig. 3 C). Moreover, reinjection of such tumor cells revealed that they are as tumorigenic as MF-FLIPhigh (data not shown). As a challenge of nude mice with MF-FLIPlow does not result in tumors with elevated cFLIP expression (data not shown), this process requires the selective pressure of the T cell immune system.

In conclusion, our data show that cFLIP-overexpressing tumors escape from T cell immunity in vivo despite the fact that they are efficiently killed in vitro. This apparent discrepancy is most likely due to limitations of in vitro assays, which do not accurately reflect the microenvironment in the tumor. For instance, in vitro CTL assays are generally performed at aphysiological E/T ratios and under conditions that allow prolonged CTL–target interactions that may give rise to a different killing potential of the CTL. Alternatively, the experimental generation of CTL clones often favors clones with high affinity, which does not necessarily reflect the response against a tumor in vivo. In addition, in vivo assays target cells are often pretreated with IFN-γ to increase their MHC class I expression and thereby the avidity of the CTL–target interaction. Together, this may change the specificity of the response. Indeed, it has been suggested that decreasing the affinity/avidity of the CTL–target interaction shifts the balance of CTL-mediated cytotoxicity toward the CD95-dependent pathway (26–28). Although the exact reason for this disparity remains to be determined, our data do provide direct evidence that tumor clearance in vivo critically depends on the CD95 pathway. In view of this finding, it is interesting to note that a plethora of mechanisms has been reported by which tumors seem to block CD95-induced apoptosis. For instance, mutation (29) or simply downmodulation of CD95 (30–33) is found in several tumors. Alternatively, secretion of CD95 decoy receptors (34) could provide a separate route to escape from CD95-dependent cytotoxicity. Our findings demonstrate that blockade of the CD95 pathway, for instance through overexpression of cFLIP as was found in human melanomas (10, 11), can indeed serve as an efficient mechanism of immune escape by tumors.
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