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 engages 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/FLICE/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 Interestingly, cFLIP expression is increased in human melanomas (references 10 and 11; Medema, J.P., and J. de Jong, unpublished observations), suggesting that these tumor cells become refractory to CD95-induced apoptosis.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/6nu/nu (B6 nude, H-2b) mice were obtained from Bomholtgard (Ry, Denmark).

Cells. A adenovirus type 5 E1A plus mutant EJ-ras-transfected (AR6; 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-Db is a mouse embryo fibroblast transfected with a plasmid encoding the E1A epitope coupled to a signal sequence and murine H-2Db and therefore presents high levels of the Ad5E1A epitope in the context of...
vivo present throughout the assay (22). E1A-specific CTL response in concanamycin A (CMA; Sigma Chemical Co.), which was

Cells and counting in the presence of scintillation fluid. To de-

body Jo2 in vitro to apoptosis induced by the murine CD95–specific anti-

MECs) were obtained by Ca

killed when tumors reached a size

were injected intraperitoneally into 6-wk-old female mice and

Both lines express identical amounts of MHC class I and

growth.

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 murine leukemia virus–induced lymphoma that can be

high levels (MF-FLIP

low) of FLAG-tagged cFLIP (Fig. 1 A, inset). Transfectants of MF were generated either expressing

whereas injection of the same dose of MF-FLIP

low cells into syngeneic PKO (filled symbols) or wild-type mice (open symbols) at 10^6 and 10^5 cells respectively. (D) 10^6 (filled symbols) or 10^5 (open symbols) MF cells were injected into nude mice. In both C and D, squares represent MF-FLIP

low and circles represent MF-FLIP

high. Figure 1. Increased tumorigenicity of MF cells by expression of cFLIP. (A) MF cells (20) were tested for their sensitivity to CD95–induced apoptosis with the murine CD95–specific antibody Jo2 at increasing concentrations. After 16 h, apoptotic nuclei were determined using the Nucleoli assay (21). Inset shows expression of FLAG–tagged cFLIP, which was immuno-precipitated using a rabbit polyclonal anti-FLAG mAb and subsequently detected on Western blot with an mAb against FLAG. (B) CTL–induced DNA fragmentation of MF-FLIP

low (circles) or MF-FLIP

high (squares) in a 6-h DNA fragmentation assay using the Moloney virus gagLeader–specific CTL clone 1, either left untreated (open symbols) or preincubated for 2 h with CMA (filled symbols). Experiments shown are representative of at least three performed with similar results. (C) MF cells were injected intraperitoneally into PKO (filled symbols) or wild-type mice (open symbols) at 10^6 and 10^5 cells respectively. (D) 10^6 (filled symbols) or 10^5 (open symbols) MF cells were injected into nude mice. In both C and D, squares represent MF-FLIP

low and circles represent MF-FLIP

high.

Next we tested whether cFLIP expression also allowed

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

high is completely lost, whereas MF-FLIP

low 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 10^2 MF-FLIP

low 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

high 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 pathway is functional. Wild-type mice are far more resistant to MF-FLIPlow. Injection of up to $10^6$ of these cells does not efficiently induce tumors (Fig. 1 C), whereas such doses give rise to progressively growing tumors in PKO mice (data not shown). This indicates that perforin-dependent killing is an essential aspect of the immune defense against this tumor, an observation that is in line with previous observations (20) and our in vitro data (Fig. 1 B). Nevertheless, we found that injection of $10^6$ M-FLIPhigh cells leads to much higher tumor-injection in wild-type mice than injection of the same number of M-FLIPlow cells (Fig. 1 C). Importantly, M-FLIPlow and M-FLIPhigh are equally efficient in inducing tumor growth in T cell-deficient nude mice (Fig. 1 D). This indicates that overexpression of cFLIP enables M F to escape from T cell-dependent immunity in immunocompetent mice. Even in the presence of perforin-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-FLIPhigh 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 AR 6 has been generated by transfection of B6 MECs with the adenovirus type 5 E1A and mutant EJ ras oncogenes. Subcutaneous injection of AR 6 results in initial tumor growth, after which the tumor regresses due to an inefficient CTL response directed against an epitope encoded by E1A (19). AR 6 expresses low but detectable levels of CD95 (Fig. 2 A). We transfected AR 6 with FLAG-tagged cFLIP (AR 6-FLIP) or as control with vector alone (AR 6-vector) (Fig. 2 B, inset). Two subclones were selected on the basis of comparable MHC class I expression, growth kinetics, and morphological features in vitro. AR 6-vector 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 AR 6-vector is induced but AR 6-FLIP is fully resistant (Fig. 2 B). Even though CD95-induced apoptosis is completely blocked, we find that cFLIP does not affect lysis of AR 6-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 AR 6-FLIP (Fig. 2 C, inset), which has been shown to correlate with resistance (10, 24).

When injected into nude mice, the AR 6 lines display equal and uncontrolled tumor growth (data not shown). In contrast, both PKO and wild-type mice can control the AR 6-vector tumor (Fig. 2, D and E). However, comparable to our findings with M-FLIPhigh, neither of these mouse strains is capable of rejecting the cFLIP-overexpressing tumor AR 6-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 AR 6-FLIP tumor grows almost as efficiently as do 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 dependence is
Figure 3. cFLIP-induced immune evasion is due to CTL resistance. (A) Tumor growth was analyzed in wild-type mice injected simultaneously with AR6-vector (2 × 10^7) into the left flanks and AR6-FLIP (2 × 10^7) into the right flanks of mice (△; n = 10). For comparison, mice injected in the right flanks with AR6-FLIP (2 × 10^7) alone are added (●; 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 processing 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 AR6-FLIP may involve prevention of T cell priming rather than escape from the T cell response, we injected B6 mice simultaneously with AR6-FLIP and AR6-vector in either flank. As expected, most mice were again capable of rejecting the AR6-vector cells, pointing to the induction of an efficient immune response. Despite this response, AR6-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 AR6-vector, AR6-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-FLIP^high 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-FLIP^high (data not shown). As challenge of nude mice with MF-FLIP^low 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 subphysiological 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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