The Granzyme B-binding serpin PI-9 interferes with Fas-induced apoptosis: implications for interpretation of prognostic factors in relation to therapy of cancer

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Patients with leukemia and other hematological malignancies can be successfully treated with chemotherapy followed by allogeneic hematopoietic stem cell transplantation. Chemotherapy mainly kills malignant cells via activation of the mitochondrial pathway of apoptosis, but has also been described to activate the death receptor pathway of apoptosis. After allogeneic stem cell transplantation, natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) can mediate target cell death using two different effector mechanisms: perforin (PFN) and granzyme B (GrB) dependent granule-mediated cell death and death receptor-mediated apoptosis. Malignant cells protect themselves from chemotherapy- and CTL-induced apoptosis using several escape mechanisms including upregulation of anti-apoptotic proteins such as B cell lymphoma-2 (Bcl-2), FLICE (caspase-8) Inhibitory Protein (FLIP) and serine protease inhibitor-9 (PI-9). Bcl-2 is a specific inhibitor of the mitochondrial pathway of apoptosis, and overexpression has been shown to be an adverse prognostic factor for outcome of chemotherapeutic interventions. FLIP, an enzymatically inactive homologue to caspase-8, has been shown to efficiently block the death receptor pathway of apoptosis, and enhanced FLIP expression has been associated with poor clinical outcome after treatment with chemotherapy and immunotherapy. PI-9, a protein that specifically binds to GrB, was shown to efficiently inhibit PFN/GrB-mediated apoptosis in both in vitro and in vivo studies, and is considered to be a specific prognostic marker for outcome of cellular immunotherapy. However, due to the similarity between PI-9 and the viral serpin cytokine response modifier A (CrmA), an efficient inhibitor of Fas-mediated and to a lesser extent of GrB-induced cell death, we hypothesized that PI-9 may also protect cells against Fas-mediated apoptosis, and hence may be less specific for certain immunological processes than expected. In this study, we therefore examined the effect of enhanced PI-9 expression on Fas-induced apoptosis.

Both EBV-transformed B cells (EBV-JTO) and Jurkat J16 target cells were transduced with retroviral constructs encoding FLIP or PI-9. Transduced cells were purified (>95%) on basis of NGFR or GFP marker gene expression using cell sorting. Wildtype (WT) and enhanced FLIP- or PI-9 expressing EBV-JTO and Jurkat cells were exposed to various concentrations of Fas agonistic antibody (Fas Ab, 7C11; Beckman Coulter Inc., Fullerton, CA, USA), and cell death was determined after 5 and 24 hrs of exposure using a CFSE-based cytotoxicity assay. Fas Ab titration curves for both EBV-JTO and Jurkat cells are shown in Figure 5.1A. EBV-JTO cells were less sensitive to Fas Ab treatment than Jurkat cells, since maximal lysis of EBV-JTO cells after 5 hrs of exposure was 30% using 500 ng/mL of Fas Ab, whereas 10 ng/mL of Fas Ab was sufficient to kill 30% of Jurkat cells. The IC$_{50}$ value for EBV-JTO cells after 5 and 24 hrs of exposure was approximately 100 ng/mL of Fas Ab, while IC$_{50}$ values for Jurkat...
Figure 5.1. Effect of FLIP and PI-9 expression on Fas- and camptothecin (Camp)-induced apoptosis.

(A) Fas Ab titration curves for EBV-JTO (left panel) and Jurkat (right panel) cells after 5 and 24 hrs of exposure. The IC$_{50}$ value for EBV-JTO was 100 ng/mL of Fas Ab; IC$_{50}$ values for Jurkat were 10 and 1 ng/mL of Fas Ab after 5 and 24 hrs of exposure, respectively. (B) Percentages of inhibition of Fas-induced apoptosis by FLIP and PI-9 after 5 hrs (left panel) and 24 hrs (right panel) of exposure of EBV-JTO and Jurkat cells. Percentages of lysis were determined with CFSE-based cytotoxicity assays using IC$_{50}$ concentrations and concentrations of Fas Ab causing maximal lysis of EBV-JTO and Jurkat cells. Data shown are mean values (+SD) of 9 (EBV-JTO) or 4 (Jurkat) independent experiments. NA; not applicable, lysis in WT < 10%. (C) Percentages of inhibition of Camp-induced apoptosis by FLIP and PI-9 after 24 hrs of exposure of EBV-JTO and Jurkat cells. Lysis was 83% and 88% in wild type EBV-JTO and Jurkat cells, respectively, using 10$^{-5}$ M Camp. Data shown are mean values (+SD) of 2 (EBV-JTO) or 4 (Jurkat) independent experiments.

‡ p > 0.05; inhibition was considered significant if p ≤ 0.05.
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cells after 5 and 24 hrs of exposure were 10 and 1 ng/mL of Fas Ab, respectively. Percentages of inhibition of Fas-induced apoptosis by FLIP and PI-9 after 5 hrs (left panel) and 24 hrs (right panel) of exposure are shown in Figure 5.1B. FLIP expression resulted in efficient and significant ($p<0.05$) inhibition of Fas-mediated apoptosis in EBV-JTO cells (75-90%), while the inhibitory effect of the same construct was less pronounced in Jurkat cells (30-50%). Unexpectedly, PI-9 also caused 40-70% and 20-30% inhibition of Fas-induced apoptosis in EBV-JTO and Jurkat cells, respectively. The lower the concentration of Fas Ab used, the higher the inhibitory effect of both FLIP and PI-9 we observed.

To ensure that introduction of PI-9 did not render EBV-JTO and Jurkat cells into less responsive cells in general, we exposed the WT, FLIP- or PI-9-transduced cell lines to various concentrations of the cytostatic agent camptothecin (Alexis Corp., Lausanne, Switzerland). Camptothecin-induced cell death was not inhibited by FLIP or PI-9 in both cell lines (Figure 5.1C). Moreover, upregulation of PI-9 in EBV-JTO cells did not influence lysis caused by other chemotherapeutic agents such as Ara-C or daunorubicin (data not shown).

We demonstrated that in both EBV-JTO and Jurkat cells, PI-9 significantly inhibited Fas-induced apoptosis. This is in contrast with results from other groups showing that PI-9 selectively inhibits GrB-mediated apoptosis without affecting Fas-induced cell death in Jurkat cells. 8;9 We showed that the inhibition of Fas-induced cell death by PI-9 was much more pronounced in EBV-JTO cells than in Jurkat cells, suggesting that the inhibitory effect of PI-9 on Fas-induced apoptosis is dependent on the cell line studied. Jurkat cells are so-called type II cells, in which Fas-induced apoptosis is partially executed via the mitochondria through caspase-8-mediated cleavage of BH3-interacting-domain death agonist (Bid), a member of the pro-apoptotic B-cell lymphoma 2 (Bcl-2) family. 1 This explains the partial inhibition of Fas-induced apoptosis we observed in Jurkat cells by FLIP and also by Bcl-2 (data not shown). In EBV-JTO cells, Fas-induced apoptosis was completely mediated via the death receptor pathway, since FLIP expression almost completely inhibited Fas-induced apoptosis (Figure 5.1B).

It is not known at what level PI-9 interferes with the death receptor pathway. We speculate that PI-9 perturbs the death receptor pathway downstream from caspase-8 activation. Fas-induced apoptosis in Jurkat cells will be less affected by this intervention, since apoptosis can still be executed via the mitochondrial bypass. Bird et al. demonstrated that PI-9 poorly inhibited caspase-8 activation in vitro (10%), but efficiently blocked activation of caspase-4 (70%), and to a less extent (35%) of caspase-10. 8 Caspase-4 has a thus far unknown function, but intervention of Fas-induced apoptosis by PI-9 may occur at the level of caspase-10, since this protein has been reported to play a role in the death receptor pathway of apoptosis. 10
In conclusion, here we demonstrate that PI-9 interferes with the Fas cell death pathway, and hence is less specific for the PFN/GrB pathway than previously reported. Consequently, poor clinical outcomes associated with elevated PI-9 levels may not automatically implicate disruption of T-cell-induced PFN/GrB-mediated apoptosis, but can also be the result of blockade of the death receptor pathway which may affect chemotherapy treatment.

REFERENCES