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Chemotherapy primes malignant glioma cells for CD95 ligand-induced apoptosis up-stream of caspase 3 activation

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Abstract

The cytotoxic cytokine, CD95 ligand, is an experimental anti-cancer agent. Here, we describe a novel pathway of drug-mediated augmentation of CD95 ligand-induced apoptosis. We report that prolonged pre-exposure of human malignant glioma cells to different cytotoxic agents, VM26, cytarabine and cisplatin, induces strong sensitization to CD95 ligand-induced apoptosis. *CD95* gene transfer does not prevent sensitization, suggesting that sensitization is not mediated by drug-induced CD95 expression. Priming with cytotoxic drugs greatly increases CD95 ligand-induced caspase 3 activity, indicating that the cytotoxic drugs positively modulate the CD95-dependent signalling cascade up-stream of caspase 3 activation.

Keywords: CD95; Chemotherapy; Apoptosis; Glioma; Caspase

1. Introduction

CD95 ligand is a cytotoxic cytokine that signals apoptosis to susceptible target cells via trimerization of the intracellular domains of CD95, a receptor of the nerve growth factor/tumor necrosis factor receptor superfamily. Signalling involves the formation of a death-inducing signalling complex (DISC) that involves the recruitment of caspase 8 and down-stream activation of caspase 3 (Medema et al., 1997). The physiological functions of the CD95/CD95 ligand system involve the negative control of peripheral immune effector cell populations and possibly maintaining immune privilege in distinct sites of the body, including eye, testis and brain. More recently, CD95/CD95 ligand interactions have also been suggested to mediate cytotoxic drug toxicity (Friesen et al., 1996; Müller et al., 1997) even though presumably not in glioma cells (Winter et al., 1998).

Many tumor cells including glioma cell lines are resistant to CD95 ligand-induced apoptosis despite expression of CD95 at the cell surface. Since such cells are sensitized to CD95-mediated apoptosis when RNA or protein synthesis are inhibited, potent short-lived inhibitors of apoptosis are likely to be expressed by these cells (Weller et al., 1994). We have had a specific interest in overcoming glioma cell resistance to apoptosis by combined immunochemotherapy involving CD95 ligand and cytotoxic drugs (Roth et al., 1997; Winter et al., 1998). Here, we report on a novel pathway of tumor cell sensitization to CD95-mediated apoptosis that involves pre-exposure to cytotoxic drugs and seems to target the death signalling pathway down-stream of CD95 expression but up-stream of caspase 3 activation.

2. Materials and methods

T98G cells were obtained from the American Type Culture Collection (Rockville, MD). LN-229 and LN-308 cells were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland) (Van Meir et al., 1994). The cell culture conditions have been described (Weller et al., 1994, 1995a). Non-clonal sublines of LN-229 and LN-308 cells transfected with BCMGS-CD95 were generated as outlined before (Weller et al., 1995b; Winter et al., 1998). CD95

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expression was analysed by flow cytometry (Weller et al., 1995b). Caspase 3 activity was measured by conversion of the fluorescent substrate, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) (Schulz et al., 1997). The preferential caspase 3 inhibitor, DEVD-CHO, was purchased from Biomol (Hamburg, Germany). Cisplatin and cytosine arabinoside (cytarabine) were purchased from Sigma (St. Louis, MO). Teniposide (VM26) was obtained from Bristol (Syracuse, NY).

3. Results

3.1. Chemotherapy primes malignant glioma cells for CD95 ligand-induced apoptosis

We have previously reported that co-exposure of human malignant glioma cells to CD95 ligand and cytotoxic drugs

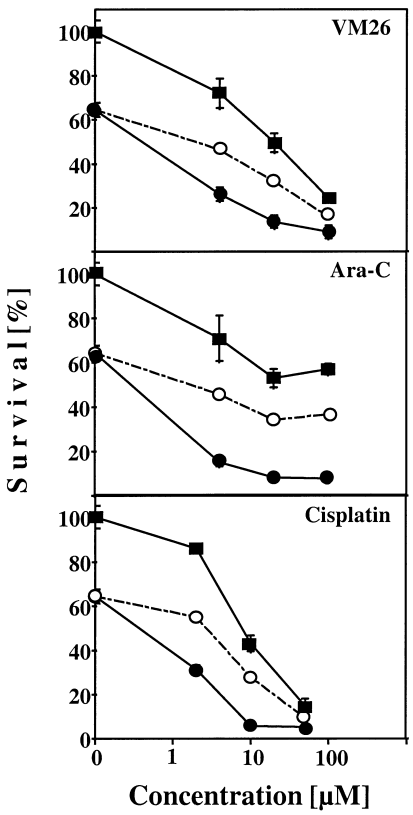

Fig. 1. Chemotherapy primes T98G glioma cells for CD95 ligand-induced apoptosis. The cells were pre-exposed to VM26 (A), cytarabine (Ara-C) (B) or cisplatin (C) for 36 h and then co-exposed to these drugs and CD95 ligand (20 U/ml) for 12 h. Survival was assessed by crystal violet staining. Data are expressed as mean percentages and S.E.M. ($n = 3$). Filled squares correspond to drug effect alone, filled circles correspond to the effects of co-exposure of CD95 ligand and drugs, open circles with dashed line correspond to the predicted independent (additive) effect of co-exposure (Roth et al., 1997). A comparison of open circles and filled circles illustrates the degree of synergistic killing.

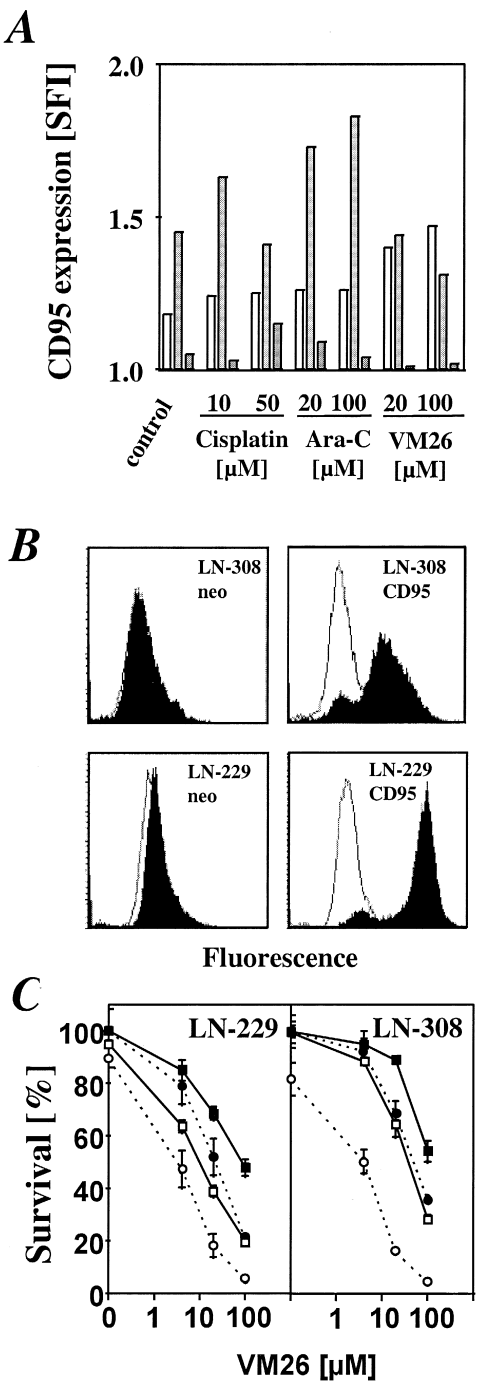

Fig. 2. Drug-induced sensitization to CD95 ligand-induced apoptosis is independent of CD95 expression. (A) T98G (open bars), LN-229 (grey bars) or LN-308 (black bars) cells were exposed to the drugs for 36 h. CD95 expression was monitored by flow cytometry (Weller et al., 1995b). (B) LN-229 or LN-308 cells transfected with the control vector (neo) or BCMGS-CD95 were assessed for CD95 expression by flow cytometry. The SFI values were 1.45 for LN-229 neo, 21.1 for LN-229 CD95, 1.05 for LN-308 neo, and 7.45 for LN-308 CD95. (C) Neo control cells (squares) or CD95-transfected (circles) LN-229 (left) or LN-308 (right) cells were pre-treated with VM26 for 36 h and then further treated for 12 h with VM26 alone (filled symbols) or with VM26 and CD95 ligand at 20 U/ml (filled symbols) as in Fig. 1.

results in synergistic growth inhibition in clonogenic cell death assays but induces little synergistic killing in acute cytotoxicity assays (Roth et al., 1997). Here, we show that strong sensitization to induction of apoptosis can be achieved if the glioma cells are pre-exposed to cytotoxic drugs for 36 h and subsequently co-treated with these drugs and CD95 ligand for 12 h only. Sensitization is achieved with different drugs (VM26, cytarabine, cisplatin) in different cell lines (LN-229, LN-308, T98G) and over a broad range of drug concentrations from subtoxic to toxic. Representative data are shown for T98G cells (Fig. 1). The same dramatic augmentation of killing is observed in LN-229 and LN-308 cells (data not shown, but see control transfectants in Fig. 2C).

3.2. Chemotherapy-induced priming for CD95 ligand-induced apoptosis does not depend on the induction of CD95 expression

A drug-induced increase in CD95 expression is one conceivable mechanism of how prolonged pre-exposure to cytotoxic drugs could enhance tumor cell sensitivity to a subsequent CD95 ligand challenge. This hypothesis predicts that cells, whose level of CD95 expression can be no further enhanced by cytotoxic drugs, are not synergistically killed by CD95 ligand after drug pre-treatment. Using stable CD95 transfectants of LN-229 and LN-308 cells, we show that this is not the case. We find that drug exposure indeed results in increased CD95 expression. However, the patterns of induced changes in CD95 expression are both drug-specific and cell line-specific and do not correlate with the sensitization to a subsequent CD95 ligand challenge (Figs. 1 and 2A). Thus, at 36 h, VM26 has prominent effects on CD95 expression in T98G cells only whereas cytarabine is most active in LN-229 cells and the induced changes are negligible in LN-308 cells, except for cisplatin. Of note, these differential changes are also not accounted for by the p53 status of the cell lines (LN-229, wild-type; T98G, mutant; LN-308, deleted). Although the highly elevated cell surface expression of CD95-transfected LN-229 and LN-308 cells (Fig. 2B) precludes further drug-induced increases (data not shown), these cells are nevertheless subject to the same strong priming effect of chemotherapy prior to exposure to CD95 ligand (Fig. 2C). Note that the *CD95* gene transfer alone induces moderate sensitization to CD95 ligand-induced apoptosis both without and with pre-exposure to the drugs.

3.3. Chemotherapy-induced priming for CD95 ligand-induced apoptosis operates up-stream of caspase 3 activation

Caspase 3 activation has been established as an essential step during CD95-mediated apoptosis. Therefore, we asked next whether the facilitation of apoptosis mediated by pre-exposure to cytotoxic drugs operates up-stream or down-stream of caspase 3 activation. To this end, we measured caspase 3-like enzymatic activity 4 h after stimulation with CD95 ligand. Fig. 3A shows that CD95 ligand

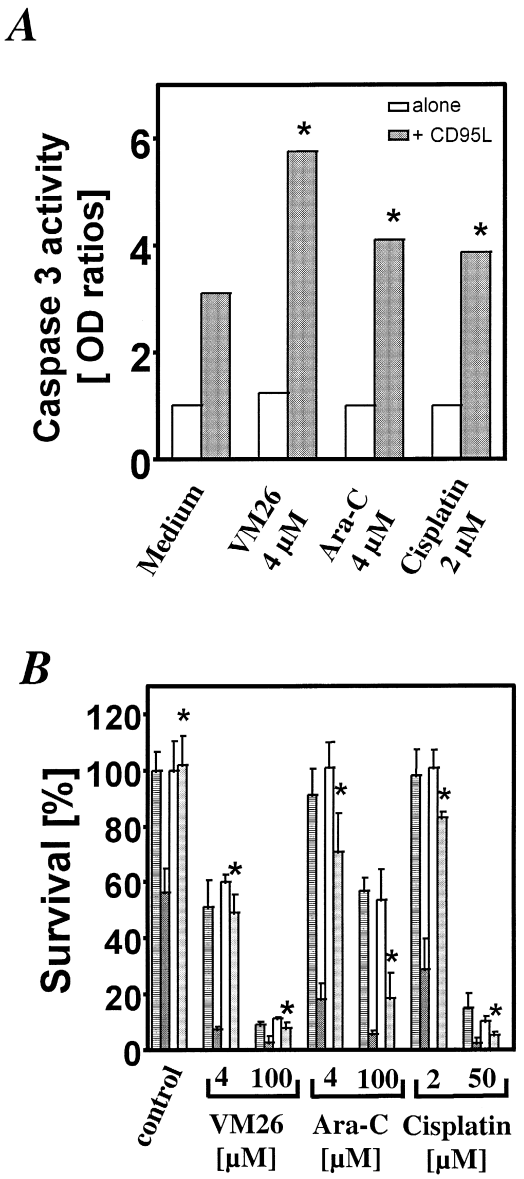

Fig. 3. Drug-mediated sensitization to CD95 ligand-induced apoptosis operates up-stream of caspase 3 activation. (A) T98G cells were treated with the drugs for 36 h as in Fig. 1. The cells were then further treated with drug alone (open bars) or drugs plus CD95 ligand (20 U/ml, grey bars). Caspase 3-like enzymatic activity was determined by DEVD-AMC (12.5 μM) cleavage 4 h later. Data are expressed as mean fluorescence units and S.E.M. normalized as ratios to untreated controls ($* P < 0.05$, *t*-test, compared with CD95 ligand alone). (B) The cells were treated as in Fig. 1 in the absence or presence of the caspase 3 inhibitor DEVD-CHO (horizontally striped bars, drug alone; dark grey bars, drug plus CD95 ligand; open bars, drug plus DEVD-CHO; light gray, drug plus CD95 ligand plus DEVD-CHO). DEVD-CHO (400 μM) was added 1 h prior to CD95 ligand exposure, that is, 35 h after drug exposure. Survival was assessed by crystal violet staining. Data are expressed as mean percentages and S.E.M. ($n = 3$, $* P < 0.05$, *t*-test, protection afforded by DEVD-CHO).

evokes much higher caspase 3 activity in drug-pre-treated cells than in drug-naive cells whereas drug treatment alone does not induce significant enzymatic activity at this time point, suggesting that the drugs modulate the CD95-dependent killing cascade up-stream of caspase 3 activation. The preferential caspase 3 inhibitor, DEVD-AMC, provides good protection from CD95 ligand-induced apoptosis even after drug-mediated sensitization (Fig. 3B), consistent with the hypothesis that activation of caspase 3 is a central step in the CD95-dependent killing cascade and that drug-mediated sensitization feeds into that pathway as well.

4. Discussion

Targeting the CD95-dependent killing cascade is a promising therapeutic approach to chemoresistant cancers such as malignant glioma (Weller et al., 1994) even though hepatic and other organ toxicity may preclude its systemic application (Ogasawara et al., 1993). Here, we report a hitherto unknown pathway of tumor cell sensitization to CD95 ligand-induced apoptosis that is activated by prolonged pre-exposure to cytotoxic drugs with different modes of action, such as VM26, a topoisomerase II inhibitor, cytarabine, an antimetabolite, or cisplatin, a DNA-damaging agent (Fig. 1).

Classically, exposure of cancer cells to subtoxic concentrations of cancer chemotherapy drugs has been associated with induction of resistance, e.g., the emergence of the multidrug resistance phenotype (Chin et al., 1993) rather than sensitization to apoptosis. However, the role of multidrug resistance in the intrinsic resistance of malignant glioma cells to chemotherapy is not well-defined since multidrug resistance expression is predominantly found on the endothelial cells but not on the tumor cells in human malignant gliomas in vivo (Toth et al., 1996; Takamiya et al., 1997).

CD95 gene transfer studies confirm that drug-induced changes of CD95 expression are dispensable for sensitization to apoptosis (Fig. 2), suggesting that signalling by CD95 is facilitated down-stream of CD95 expression. Further, drug-induced sensitization to CD95 ligand-induced apoptosis does not require p53 since LN-229, but not T98G or LN-308, cells have retained wild-type p53 activity (Van Meir et al., 1994). The site of action of the drugs can be located up-stream of caspase 3 activation since drug-pre-treated cells develop much more caspase 3 activity than naive cells. Consistent with this model, a caspase 3 inhibitor protects even drug-primed cells from CD95 ligand-induced apoptosis (Fig. 3). Possible targets of drug priming may include the negative regulation of recently identified gene products, e.g., of the inhibitor-of-apoptosis-protein (Liston et al., 1996) or caspase 8-inhibitory-protein families (Irmler et al., 1997) which interfere with up-stream elements of cytotoxic cytokine-dependent signalling.

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