Diva/Boo is a negative regulator of cell death in human glioma cells

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Abstract Diva is a novel proapoptotic member of the Bcl-2 protein family which binds apoptosis activating factor-1 (APAF-1). Diva is identical with Boo which was identified as a novel antiapoptotic Bcl-2 family protein. Here, we report that Diva promotes the cell cycle exit of human glioma cells in response to serum deprivation and inhibits apoptosis of these cells induced by CD95 ligand or chemotherapeutic drugs. In glioma cells, Diva interferes with apoptotic signaling downstream of cytochrome *c* release, but upstream of caspase activation, consistent with an inhibitory effect on the mitochondrial amplification step involving the apoptosome and APAF-1.

Key words: Glioma; Apoptosis; Diva; Caspase; Boo

1. Introduction

Gene transfer-mediated antisense inhibition of the expression of antiapoptotic Bcl-2 family members, or overexpression of proapoptotic Bcl-2 family members, are promising therapeutic strategies for cancers which are resistance to radiochemotherapy-induced apoptosis. Of the four Bcl-2 homology domains BH1–BH4, BH3 has been identified as the essential domain for proapoptotic Bcl-2 homologs to interact with the principal antiapoptotic family members, Bcl-2 and Bcl-X_L, and to induce apoptosis [1]. Diva (death inducer binding to vBcl-2 and apoptosis-activating factor, APAF-1) was identified as a proapoptotic member of the Bcl-2 family that appeared not to require the BH3 domain for the induction of apoptosis [2]. Instead, Diva was hypothesized to inhibit the association of Bcl-X_L and APAF-1 via direct interactions with APAF-1, thereby antagonizing the antiapoptotic activity of Bcl-X_L. Diva mRNA was abundantly expressed in embryonal tissues including brain, but was restricted to ovary and testis in adult mice. Diva is identical with Boo (Bcl-2 homolog of ovary) which was characterized as an antiapoptotic Bcl-2 homolog interacting with APAF-1 [3].

2. Materials and methods

2.1. Materials and cell lines

Lomustine (CCNU) was obtained from Medac (Hamburg, Germany), DEVD-amc from Bachem (Heidelberg, Germany). CD95L-containing supernatant was harvested from CD95L-transfected N2A neuroblastoma cells [4]. The human malignant glioma cell lines LN-18, U138MG, U87MG, LN-428, D247MG, T98G, LN-319, LN-229, A172, U251MG, U373MG and LN-308 were kindly provided by N. de Tribolet (Lausanne, Switzerland). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Eggenstein, Germany) containing 10% fetal calf serum, glutamine (2 mM) and penicillin (100 IU/ml)/streptomycin (100 μg/ml). Transfections were done by lipofection with pcDNA3 as a control (mock) or pcDNA3-myc-Diva, kindly provided by G. Nunez (Ann Arbor, MI, USA). Pooled stably transfected sublines were generated by selection with G418 (50 μg/ml). Transient transfections were done by lipofection with pcDNA3neo or pcDNA3-myc-Diva in combination with pEGFP encoding enhanced green fluorescent protein to assess the efficacy of transfection.

2.2. Immunoblot analysis

The general procedure has been described [5]. The following antibodies were used: anti-myc (9E10, Santa Cruz SC40, Santa Cruz, CA, USA); anti-actin (1-19, Santa Cruz SC1616); anti-cytochrome *c* (65981A, Pharmingen-Becton Dickinson, Heidelberg, Germany); anti-caspase 3 (#C31720, Transduction Laboratories, Lexington, KY, USA); anti-caspase 8 (kindly provided by P.H. Krammer, Heidelberg, Germany).

2.3. Proliferation and viability assays

For acute drug cytotoxicity assays, the cells were seeded at 10^4 cells per well in microtiter plates, allowed to attach for 24 h and exposed to CD95L for 24 h or cancer chemotherapy drugs for 72 h in serum-free medium. Viable cells were stained by crystal violet. For transient transfections, cell death was measured by flow cytometry, counting sub-G0/1 double-transfected neo/EGFP- or Diva-EGFP-positive cells.

2.4. Cell cycle analysis

The cells were seeded in 75 cm^2 flasks and allowed to attach. The cells were washed twice with phosphate-buffered saline (PBS) and then serum-deprived for up to 96 h. Detached cells were harvested from the supernatant by centrifugation and added to the non-detached cells harvested by trypsinization. The cells were washed with PBS, fixed in 70% ice-cold ethanol, centrifuged, washed with PBS, and 10^6 cells per condition were stained with propidium iodide (50 μg/ml) diluted in PBS containing RNase A (100 μg/ml). The cells were subjected to flow cytometric analysis of DNA content using a Becton Dickinson FACS-calibur cytometer. Percentages of cells in the different cell cycle phases were calculated by CellQuest software (Becton Dickinson).

2.5. DEVD-amc cleaving caspase activity assay

The cells (10^4) were seeded on microtiter plates, allowed to attach for 24 h and then treated with CD95L. At 0, 3 and 6 h after CD95L exposure, the cells were lysed in 25 mM Tris–HCl, pH 8.0, 60 mM NaCl, 2.5 mM EDTA, 0.25% Nonidet-P40 for 10 min, and DEVD-amc was added at 12.5 μM. Caspase activity was assessed by fluorescence using a Millipore fluorimeter at 360 nm excitation and 480 nm emission wave lengths [6].

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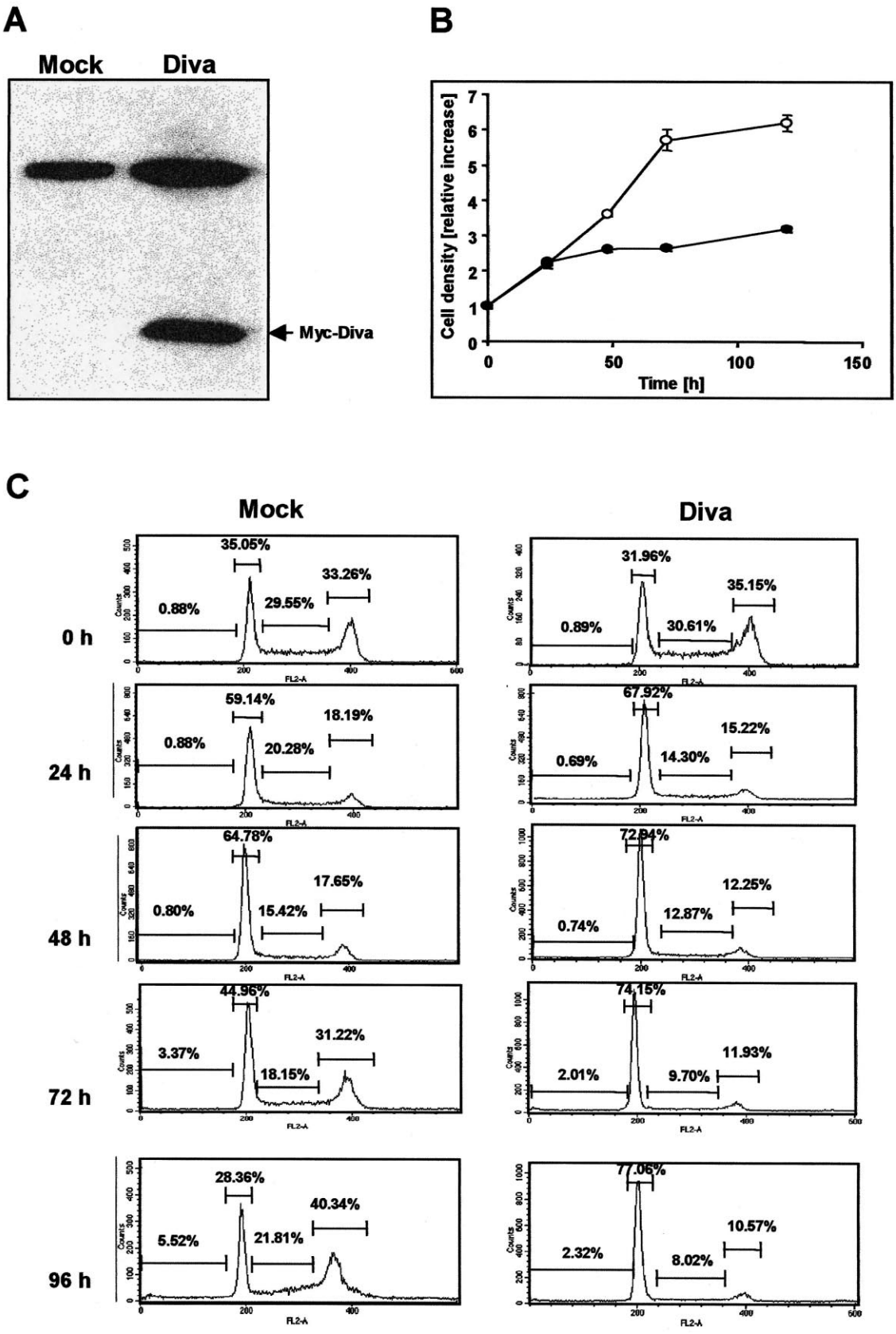

Fig. 1. Diva promotes growth arrest in response to serum deprivation. A: LN-18 glioma cells were transfected with the empty vector (mock) or pcDNA-myc-Diva. Transgene expression was assessed by immunoblot using the anti-myc antibody. The upper unspecific band serves as a loading control. B: LN-18 mock (open circles) or Diva-transfected (filled circles) LN-18 cells were seeded on microtiter plates, allowed to attach, and serum-deprived for up to 120 h. Growth was assessed by cresyl violet staining. C: Cell cycle analysis of LN-18 mock and Diva-expressing LN-18 cells was performed at various time points after serum deprivation.

3. Results

The present study was performed to assess whether Diva acted as a proapoptotic or an antiapoptotic gene in human malignant glioma cells. Northern blot analysis revealed that Diva/Boo mRNA was not expressed constitutively in any of 12 human malignant glioma cell lines (data not shown). LN-18 glioma cells were transfected by lipofection with a pcDNA3-myc-tagged Diva plasmid. Pooled polyclonal sublines stably expressing Diva were selected in medium containing G418 (50 μg/ml) for 6–8 weeks. To avoid artifacts from clonal selection, all experiments were performed with pooled

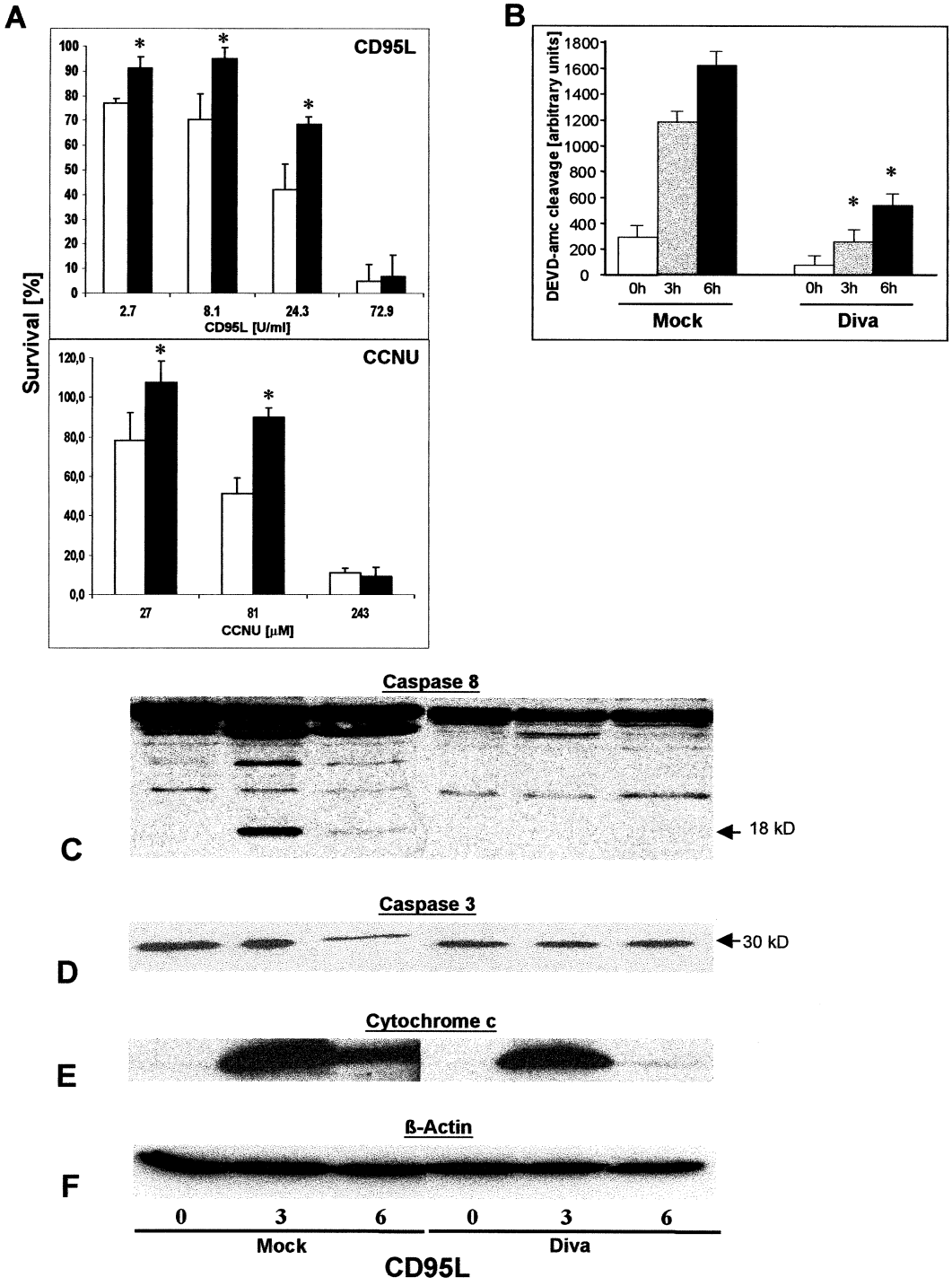

Fig. 2. Diva inhibits CD95L- and CCNU-induced apoptosis in glioma cells. A: Mock (open bars) or Diva-transfected (black bars) LN-18 cells were treated with increasing concentrations of CD95L (upper panel) or CCNU (lower panel) for 72 h. Survival was measured by cresyl violet staining. Note the logarithmic increase in concentration to assess the degree of protection mediated by Diva ($^*P < 0.05$, *t*-test). B: LN-18 mock- or Diva-transfected cells were treated with CD95L (30 U/ml) for 0 (open bars), 3 (gray bars) or 6 h (black bars). Caspase activity was assessed by fluorescence ($^*P < 0.05$, *t*-test). C–F: LN-18 mock- or Diva-transfected cells were treated with CD95L (25 U/ml). Protein levels and caspase cleavage were assessed by immunoblot using (C) anti-caspase 8; (D) anti-caspase 3; (E) anti-cytochrome *c* or, as a loading control, (F) β-actin antibody.

transfected LN-18 sublines. Stable transgene expression was confirmed by immunoblot using an antibody to the myc tag of transgenic Diva protein (Fig. 1A). There was no effect of ectopic Diva expression on the doubling times (Diva-transfected LN-18 cells, 16.5 ± 1 h; mock-transfected LN-18 cells, 18 ± 0.5 h) or on the clonogenicity (Diva-transfected LN-18 cells, 13.2%; mock-transfected LN-18 cells, 12.8%), as assessed by colony formation assays. However, Diva-transfected cells were more sensitive to serum deprivation-induced growth arrest. In contrast to parental LN-18 cells (not shown) and mock-transfected LN-18 cells which continued to grow in serum-free medium, Diva-transfected LN-18 cells no longer proliferated under these conditions (Fig. 1B). Flow cytometric cell cycle analysis revealed that neither mock- nor Diva-transfected cells died in the time frame of the experiments, but that Diva-transfected cells, in contrast to mock-transfected cells, arrested in G0/1 in response to serum deprivation (Fig. 1C). Thus, 77% of Diva-expressing cells were found in G0/1, compared with 28% in the control transfectants, at 96 h of serum deprivation.

Diva-transfected cells were also assessed for a modulation of sensitivity to apoptosis. Fig. 2A shows that Diva-transfected cells were more resistant to the induction of apoptosis mediated by CD95L and the nitrosourea CCNU than mock-transfected cells. Similar data were obtained for the topoisomerase II inhibitor, teniposide (VM26) (data not shown). Protection from apoptosis mediated by Diva was consistently found in different sublines of Diva-transfected LN-18 cells.

The Diva-mediated protection from CD95L-induced apoptosis was associated with reduced DEVD-amc cleaving caspase activity (Fig. 2B). Further, the CD95L-evoked processing of caspases 8 and 3 was reduced in the Diva-transfected LN-18 cells at 3 and 6 h (Fig. 2C,D). Reduced caspase 8 processing is reflected by the prevention of caspase 8/p18 formation. Reduced caspase 3 processing becomes apparent from the prevention of loss of the 30 kDa full length caspase 3. The protection mediated by Diva in the DEVD-amc cleavage assay at 3 h (Fig. 2B), a time point where no major caspase 3 processing became apparent by immunoblot analysis (Fig. 2D), indicates that the enzyme assay is more sensitive than immunoblot analysis and probably reflects the early activation of caspases other than caspase 3. Interestingly, the release of cytochrome *c* was not prevented by Diva at 3 h, but was instead less sustained since almost no more cytochrome *c* release was detected at 6 h in Diva-transfected LN-18 cells (Fig. 2E).

4. Discussion

The previous studies yielded conflicting results regarding the modulation of apoptosis by Diva. Transient Diva transfection induced cell death in 293T kidney cells, Ramsey melanoma cells and T47D breast cancer cells [2]. Conversely, stable expression of Boo inhibited cyclosporine-, irradiation- and interleukin 3 deprivation-induced apoptosis of the FL5.12 prolymphocytic cell line and the bone marrow-derived Baf-3 cell line [3].

The present study defines an antiapoptotic effect of Diva/Boo in stably transfected human glioma cells treated with CD95L or CCNU (Fig. 1). In contrast to previous observations in other cell types [2], there was no proapoptotic effect of Diva upon treatment with CD95L or CCNU in transient transfection assays assessed up to 48 h after transfection (data not shown).

The failure of Diva to prevent the initial cytochrome *c* release evoked by CD95L is consistent with the hypothesis that Diva acts at the level of the apoptosome to modulate apoptosis [2,3]. At least in the paradigms examined here, Diva appeared to prevent the efficient triggering of the caspase cascade by the apoptosome. That fact that caspase 8 processing was inhibited by Diva in the glioma cells is consistent with our previous observation of insufficient processing of caspase 8 at the death-induced signaling complex during CD95-mediated apoptosis [7]: although caspase 8 is thought to be the most apical caspase activated by CD95L, a mitochondrial amplification step is required to promote efficient caspase 8 processing. Accordingly, Bcl-2 and Bcl-X_L also inhibit CD95L-induced caspase 8 processing, but, in contrast to Diva, Bcl-2 and Bcl-X_L also block mitochondrial cytochrome *c* release, and thus act at a level upstream of the action of Diva.

The present study identified a second, presumably independent effect of Diva expressed in glioma cells. Diva promoted the exit from the cell cycle in glioma cells in response to serum deprivation. Similar results have been obtained in Bcl-2-transfected FDC-P1 cells, an interleukin 3-dependent mouse promyelomonocytic cell line [8], and Bcl-2 transgenic mouse thymocytes [9]. The physiological role of antiapoptotic Bcl-2 family proteins mediating cell cycle exit under unfavorable growth conditions remains to be identified.

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