CD95-mediated apoptosis: no variation in cellular sensitivity during cell cycle progression

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Abstract Sensitivity of CD95-mediated apoptosis has been reported to vary during cell cycle progression (FEBS Lett. (1997) 412, 91–93). Here, we report that three human glioma cell lines with different p53 status (i) undergo growth arrest and synchronous cell cycle re-entry after prolonged serum deprivation, (ii) do not exhibit cell cycle-related changes in CD95 expression at the cell surface, and (iii) do not exhibit cell cycle-related changes in susceptibility to DC95 ligand-induced apoptosis. In contrast, cell cycle-specific activity was demonstrated for various cancer chemotherapy drugs. Further, CD95 expression and susceptibility to CD95 ligand-induced apoptosis does not vary during cell cycle progression of Jurkat T cells, HeLa cervical carcinoma and HepG2 hepatocellular carcinoma cells. These results do not support a role for the cell cycle phase as an important predictor of vulnerability to CD95-mediated apoptosis.

Key words: Apoptosis; CD95; Cell cycle

1. Introduction

CD95 ligand (CD95L) is a cytokine of the nerve growth factor/tumor necrosis factor family that induces apoptosis in susceptible target cells. The death signalling pathway triggered by CD95 involves activation of caspases 8 and 3 and does not require new mRNA or protein synthesis. Although the cell cycle-associated $p34^{cdc2}$ kinase may be required for CD95-mediated apoptosis [1,2], no direct links between cell cycle progression and CD95-mediated apoptosis have been noted. However, a recent report in this Journal suggested that agonistic CD95 antibodies killed WIL-2 B lymphoma cells in a cell cycle-specific fashion with preferential killing in G0/1 [3]. A related observation had previously been made by other authors [4]. Moreover, CD95-mediated apoptosis was reported to involve translocation of p53 from the cytoplasma to the nucleus [3]. These unexpected findings prompted us to reevaluate the role of cell cycle progression in CD95-mediated apoptosis of various glioma and non-glial tumor cell lines.

2. Materials and methods

All chemical reagents, vincristine, doxorubicin, taxol, cisplatin and camptothecin were from Sigma (St. Louis, MO), 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)-3-nitrosourea (ACNU) and teniposide (VM26) from Bristol (Munich, Germany). Soluble CD95L was obtained from CD95L cDNA-transfected N2A neuroblastoma cells. T98G human glioma, HeLa human cervical carcinoma, HepG2 human hepatocellular and Jurkat human T cells were from ATCC (Rockville, MD). LN-18 and LN-229 human glioma cells were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland) [5]. The glioma cells were cultured as described [6]. Cell cycle analysis was performed using ethidium bromide staining of ethanol-permeabilized cells. CD95 expression was monitored by flow cytometry [7]. Survival was measured by crystal violet assay [6].

3. Results and discussion

We compared serum deprivation and several pharmacological approaches for their ability to induce a stable, but reversible, cell cycle arrest in human malignant glioma cell lines. Aphidicolin (10 μM) and hydroxyurea (650 μM) induced a G1 arrest within 24 h but most cells did not survive a recovery period for 24 h after drug withdrawal. Nocodazole (0.1 μM) induced a G2 arrest within 24 h but, again, most cells died after a recovery period of 24 h. Deferoxamin (500 μM) and mimosine (300 μM) did not lead to a cell cycle arrest at 40 h but were toxic. In contrast, serum deprivation of LN-18, LN-229 and T98G cells for 60 h reliably induced a cell cycle arrest in G0/1 without significant cell loss. Reexposure to serum-containing medium induced synchronous cell cycle re-entry without cytotoxicity, unlike the release from cell cycle block, e.g. induced by nocodazole (Fig. 1A, B).

We then asked whether the glioma cells exhibited changes in CD95 expression at the cell surface during cell cycle progression which might account for a preferential vulnerability to CD95-mediated cytotoxicity at specific phases of the cell cycle. This was not the case. There was no significant modulation of the SFI value, which indicates the signal ratio of the specific antibody versus an isotype control antibody upon flow cytometric analysis of CD95 expression [7] in either cell line (Fig. 1C). Next, we examined whether the exposure to CD95L for 4 or 8 h resulted in differential effects either in acute cytotoxicity assays or in clonogenic cell death studies, depending on the cell cycle phase. Again, there was no prominent difference in CD95L-induced cytotoxic or clonogenic cell death in either cell line (Fig. 1D). Since the negative data obtained for CD95 expression and sensitivity to CD95L-induced apoptosis might have been cell type-specific, we performed experiments corresponding to those of Fig. 1B–D in three more human cancer cell lines of different histogenetic origin, Jurkat T cells, HepG2 hepatocellular carcinoma and HeLa cervical carcinoma cells. To induce S phase exit and loss of [^{3}H]thymidine incorporation, Jurkat cells were serum-deprived for 48 h, and HepG2 and HeLa cells for 60 h. Cell cycle arrest in these cell lines was less complete than in the glioma cell lines, and cell cycle re-entry most synchronous in HepG2 cells. Similar to the glioma cells, there was no significant change in either CD95 expression or susceptibility to

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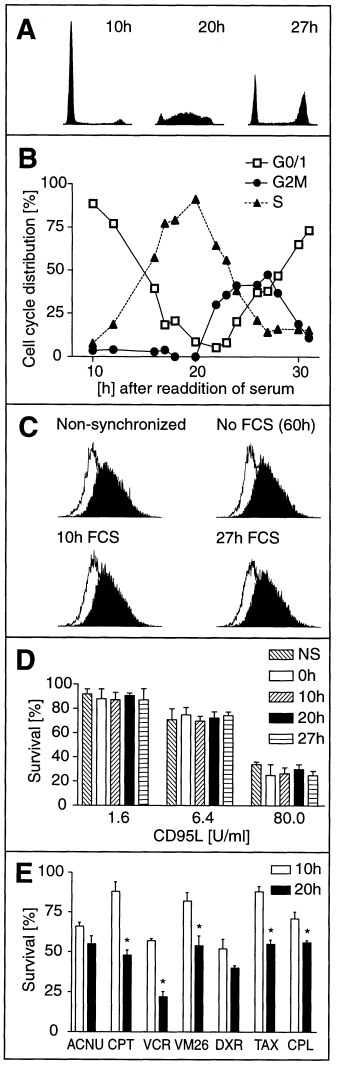

Fig. 1. The sensitivity of human glioma cells to CD95-mediated apoptosis is not modulated during cell cycle progression. A: LN-229 cells were maintained in serum-free medium for 60 h and then reexposed to serum-containing medium. Cell cycle analysis was performed at 10 h, 20 h or 27 h. B: Percentages of LN-229 cells in G0/1 (open squares), G2/M (filled circles) or S (filled triangles, dashed lines) were measured using ModFit LT 2.0 (Verity Software, Topsham, MN, USA). Similar data were obtained in LN-18 and T98G cells (data not shown). C: LN-18 cells were either not synchronized, or serum-deprived for 60 h, or serum-deprived and subsequently restimulated with serum-containing medium for 10 h or 27 h. CD95 expression was monitored by flow cytometry as described [7]. Open profiles correspond to the signal obtained with an isotype control antibody, black profiles to those obtained with the specific CD95 antibody. D: LN-229 cells were not synchronized, or serum-deprived for 60 h (0 h), or serum-deprived and restimulated with serum-containing medium, and exposed to CD95L, for 4 h, at 10 h, 20 h, or 27 h after restimulation, and further cultured for 48 h (two generation times) in fresh medium. Data are expressed as mean percentages and S.E.M. of survival ($n=3$, $P>0.05$, t-test). E: LN-229 cells were serum-deprived for 60 h, restimulated with serum-containing medium for 10 h (open bars) or 20 h (filled bars), then exposed to the drugs (50 μM ACNU, 0.8 μM camptothecin (CPT), 0.4 μM vincristine (VCR), 5 μM VM26, 0.4 μM doxorubicin (DXR), 0.4 μM taxol (TAX), 20 μM cisplatin (CPL)) for 4 h, and further cultured for 48 h (two generation times) in fresh medium. Data are expressed as in D (*$P<0.05$).

Table 1
CD95 expression and susceptibility to CD95L-induced apoptosis in non-synchronized non-glial cancer cell lines

	CD95 expression (SFI)	Survival (%) CD95L (20 U/ml)	Survival (%) CD95L (80 U/ml)
Jurkat	3.0 ± 0.3	34 ± 2	26 ± 3
HepG2	2.8 ± 0.1	86 ± 3	60 ± 3
HeLa	2.0 ± 0.2	76 ± 4	43 ± 3

CD95 expression was assessed by flow cytometry, survival by crystal violet staining after a 4 h pulse exposure to CD95L (20 or 80 U/ml) and recovery of 48 h (for details, see Section 2).

CD95L-induced apoptosis in either of these cell lines after cell cycle re-entry, as assessed at 10 h, 20 h, or 27 h after reexposure to serum-containing medium, compared with non-synchronized or serum-deprived cells (data not shown). We therefore only summarize the baseline data for the cell lines studied here (Table 1).

To confirm that differential sensitivity during cell cycle progression can be detected using our experimental set-up, we also examined possible cell cycle-specific actions of various cancer chemotherapy drugs in the three glioma cell lines (Fig. 1E). Camptothecin, vincristine, VM26, taxol and cisplatin were more active when administered during S phase. Small, but insignificant, differences between sensitivity in G0/1 and S phase were detected for ACNU or doxorubicin. G0/1 cells did not differ significantly in sensitivity from non-synchronized cells but were more resistant to chemotherapy than serum-deprived cells (data not shown).

Since changes in p53 function were hypothesized to play a role in cell cycle-dependent modulation of CD95-mediated apoptosis, we included cell lines which are wild-type (LN-229) or mutant (LN-18, T98G) for p53 [5]. Thus, the p53 status had no impact on the lack of cell cycle dependency of CD95-mediated apoptosis in our cell lines. This is also consistent with the observation that CD95-mediated apoptosis, e.g. during activation-induced T cell death, is not blocked or enhanced in p53 knockout mice [8]. We also note that in the preceding study [3], the cells were most resistant to CD95-mediated apoptosis, immediately after the thymidine block (G1/S) and most sensitive 9 h later (G1), even though most cells are in G1 in both populations. Therefore, we hypothesized that thymidine might have had a direct impact on CD95-mediated apoptosis that was unrelated to cell cycle progression. However, we were not able to delineate any effect of thymidine on CD95-mediated apoptosis of glioma cells (data not shown). Taken together, cell cycle progression had no impact on CD95 expression or sensitivity to CD95L-induced apoptosis in three human malignant glioma cell lines and three other cancer cell lines of differing histogenetic origin (Jurkat, HepG2, HeLa) in this study.

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