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Erucylphosphocholine-induced apoptosis in glioma cells: involvement of death receptor signalling and caspase activation

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Abstract

Erucylphosphocholine (ErPC) is a promising anti-neoplastic drug for the treatment of malignant brain tumours. It exerts strong anti-cancer activity *in vivo* and *in vitro* and induces apoptosis even in chemoresistant glioma cell lines. The purpose of this study was to expand on our previous observations on the potential mechanisms of ErPC-mediated apoptosis with a focus on death receptor activation and the caspase network. A172 and T98G glioma cells were treated with ErPC for up to 48 h. ErPC effects on the expression of the tumour necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL) receptor system, and on caspase activation were determined. ErPC had no effect on the expression of TNFα or TRAIL. Inhibition of the TNF or TRAIL signalling pathway with antagonistic antibodies or fusion proteins did not affect apoptosis induced by ErPC, and a dominant-negative FADD construct did not abolish ErPC-induced effects. Western blot analysis indicated that ErPC-triggered apoptosis resulted in a time-dependent processing of caspases-3, -7, -8 and -9 into their respective active subunits. Co-treatment of A172 cells with different caspase inhibitors prevented apoptosis but did not abrogate cell death. These data suggest that A172 cells might have an additional caspase-independent pathway that insures cell death and guarantees killing of those tumour cells whose caspase pathway is incomplete.

Keywords: apoptosis, caspases, death receptor signalling, erucylphosphocholine, glioma.

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Malignant gliomas are the most common human primary brain tumours and remain largely incurable (Hosli *et al*. 1998). The development of malignant tumours results not only from deregulated proliferation but also from an inability of cells to undergo apoptotic cell death. It is now well established that apoptosis is the main response of cells to anti-cancer agents and irradiation, and is mediated by different signalling pathways and cellular targets (Kamesaki 1998; Kaufmann and Earnshaw 2000). Resistance to apoptosis has been implicated as a major mechanism in treatment failure whereby tumour cells avoid drug-induced destruction (Hannun 1997).

Death signals are communicated by two essentially different apoptotic pathways (Reed 2000). The 'mitochondrial pathway' is triggered by intracellular signals originating largely from the mitochondrion (Green and Reed 1998; Ferri and Kroemer 2001) and is regulated by pro- and anti-apoptotic members of the Bcl-2 family (Antonsson and Martinou 2000). In contrast, the 'receptor-dependent pathway' is initiated by extracellular death messengers and the subsequent activation of death receptors belonging to the tumour necrosis factor (TNF)-receptor superfamily (Ashkenazi and Dixit 1998; Locksley *et al*. 2001). However, many apoptotic stimuli do not act accordingly to a single signalling pathway, but rather rely on the interaction of numerous pathways. It has been discovered that both the 'receptor-dependent pathway' and the 'mitochondrial pathway' are interconnected via the activation of the Bcl-2 protein Bid. Cleavage of Bid by caspase-8 results in translocation of the cleaved Bid to the mitochondria where it induces the release of cytochrome c (Li *et al*. 1998; Luo *et al*. 1998). A series of enzymes known

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Abbreviations used: APC, alkylphosphocholine; DED, death effector domain; DMSO, dimethylsulphoxide; ELISA, enzyme-linked immunosorbant assay; ErPC, erucylphosphocholine; FAK, focal adhesion kinase; FBS, fetal bovine serum; GFP, green-fluorescent protein; PARP, poly(ADP-ribose)polymerase; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; zIETD-fmk, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone; zLEHD-fmk, benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

as caspases form a proteolytic network and play a pivotal role in the execution of apoptosis (Bratton *et al*. 2000). Apoptogenic factors such as cytochrome c, caspase-9 and caspase-8 initiate the caspase cascade that ultimately cleaves cellular targets, e.g. poly(ADP-ribose)polymerase (PARP), and results in cell death (Medema *et al*. 1997; Ashkenazi and Dixit 1998).

In a new approach to cancer chemotherapy, the cell membrane was described as a target for cytostatic drugs (Noseda *et al*. 1989). It is known that alkylphosphocholines (APC) are membrane-seeking agents that do not directly target cellular DNA (Hilgard *et al*. 1993). Nevertheless, they exert strong anti-cancer activity against a variety of tumour cells *in vivo* and *in vitro* (Kötting *et al*. 1992; Konstantinov *et al*. 1998; Konstantinov and Berger 1999). Erucylphosphocholine (ErPC) is the first intravenous (i.v.) injectable APC derivative without haemolytic properties (Berger *et al*. 1998) and represents the prototype of a novel class of APC. After repeated i.v. administration it has been shown to cross the blood–brain barrier (Erdlenbruch *et al*. 1999). Interestingly, cytostatic and cytotoxic effects of ErPC have been shown in a multitude of brain tumour cell lines (Erdlenbruch *et al*. 1998; Jendrossek *et al*. 1999). Most important, ErPC has been reported to induce apoptosis in glioma cell lines resistant to treatment with standard chemotherapeutics, e.g. cisplatin and etoposide, suggesting that it follows signalling events different from those of conventional cytotoxic drugs (Jendrossek *et al*. 2001). ErPC is therefore thought to be a promising anti-neoplastic drug in a novel approach for the treatment of malignant brain tumours.

To date, only a few details on the mechanisms of ErPC-mediated apoptosis have been elucidated. In this study we extend our previous observations that ErPC-induced apoptosis was independent of p53 signalling and the Fas/FasL system (Jendrossek *et al*. 2001) with a focus on the TNF and TNF-related apoptosis-inducing ligand (TRAIL) death receptor system and the caspase network.

Materials and methods

Cell culture

The human malignant glioblastoma cell lines A172 and T98G were obtained from European Collection of Cell Cultures (Salisbury, UK). A172 cells were maintained in RPMI-1640, T98G cells in Eagle modified essential medium (EMEM) supplemented with 1% non-essential amino acids and 1% sodium pyruvate in an atmosphere of 5% CO_2 at 37°C. Both culture media contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. HeLa cells stably transfected with a chimeric construct of a dominant-negative FADD mutant or the vector alone fused to green-fluorescent protein (GFP) were kindly provided by Dr H. Wajant (University of Stuttgart, Stuttgart, Germany; Wajant *et al*. 1998) and cultivated in RPMI-1640 supplemented with 5% FBS, 10 mmol/L HEPES and penicillin/streptomycin. Microscopic analysis of HeLa cells expressing GFP-FADD-DN and vector only was performed with non-fixed cells in an inverse fluorescent microscope and fluorescein isothiocyanate (FITC) filter set.

Drugs and materials

Erucylphosphocholine (ErPC) was synthesized as previously described (Erdlenbruch *et al*. 1999). Agonistic anti-human Fas IgM monoclonal antibody (clone CH-11) was from Upstate Biotechnology (Lake Placid, NY, USA). Neutralizing mouse monoclonal antibodies against human TNFα (clone B-C7), human TNF-R1 (clone H398), or human TRAIL (clone RIK-2) and IgG1 isotype control were from Biosource (Nivelles, Belgium), Bender MedSystems (Vienna, Austria) and eBioscience (San Diego, CA, USA), respectively, recombinant human chimeric TRAIL-R1:Fc and TRAIL-R2:Fc were from Alexis Corp. (Grünberg, Germany), control human IgG:Fc fragment from Chemicon (Hofheim/Ts, Germany). Recombinant human TNFα and TRAIL were from R & D Systems (Wiesbaden, Germany) and PeproTech (Rocky Hill, NJ, USA), respectively. Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was obtained from Bachem (Heidelberg, Germany), benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (zIETD-fmk) and benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone (zLEHD-fmk) from Calbiochem (Schwalbach, Germany).

Measurement of cell viability and apoptosis

Cell viability was determined by the WST-1 test as previously described (Jendrossek *et al*. 1999). Briefly, 5×10^3 cells were seeded in flat-bottom, 96-well microtiter plates (100 μL/well), adhered for 24 h and treated for 24 h as indicated. Absorbance at 450 nm, reference filter 690 nm, was determined 90 min after addition of the WST-1 reagent. Apoptosis was measured using the cell death detection ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany). For induction of apoptosis, cells (10^4 A172 cells, 5×10^3 HeLa cells) were seeded in 96-well microtiter plates (200 μL/well). After 24 h, cells were treated for 24 h with different agents. For apoptosis inhibition assays, cells were pre-incubated for 1 h with the indicated caspase inhibitor before a 23-h treatment with 50 μM ErPC. Control cultures were treated with the appropriate amount of solvent [dimethyl sulphoxide (DMSO), 0.1% final concentration]. The cytosolic fraction of treated or untreated cells was used as an antigen source in a sandwich enzyme-linked immunosorbent assay (ELISA) with a streptavidin-coated microplate and a mixture of anti-histone–biotin and anti-DNA–peroxidase antibody (components of the assay kit), following the manufacturer's instructions. Absorption was measured by a SUNRISE microplate reader (TECAN, Crailsheim, Germany) at 405 nm, reference filter 492 nm. Data of WST-1 test or cell death ELISA are expressed as mean values ± SD and are calculated from triplicate experiments that have been performed at least three times.

RNA isolation and RT–PCR

Total RNA from A172 cells was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase digestion following the manufacturer's recommendations. Single-strand cDNA was synthesized from 500 ng of total RNA using random primers and Superscript RNase H^- reverse transcriptase (Life Technologies, Karlsruhe, Germany). The cDNAs were amplified

by PCR using published primer sequences specific for TRAIL (specific annealing temperature, T_a, 55°C; Dörr *et al*. 2002), TNFα (T_a 62°C), TNF-R1 (T_a 66°C; Yamaguchi *et al*. 2000), TNF-R2 (T_a 61°C; Duan *et al*. 2001) as well as for Actin (T_a 55°C; Raff *et al*. 1997) and HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). The PCR reaction profile, performed in a Biometra TRIO-thermoblock (Biometra, Göttingen, Germany), was 1× (15 min 95°C); 30× [30 s, 94°C, 30 s at T_a, 40 s 72°C]; and 1× (3 min 72°C). PCR products were analysed by agarose gel electrophoresis and visualized by ethidium bromide. Result were repeated in two independent RNA preparations.

Immunoblotting

Western blot analysis with whole-cell detergent extracts was performed as described (Jendrossek *et al*. 2001). Primary antibodies against the following proteins were used at the indicated dilutions: polyclonal rabbit anti-DR4/TRAIL-R1 (Upstate Biotechnology, Lake Placid, NY, USA) and anti-DR5/TRAIL-R2 (StressGen, Victoria, BC, Canada) 1 : 1000 each, 1 : 2000 of monoclonal mouse anti-TNF-R1, 1 : 1000 of monoclonal mouse anti-TNF-R2, 1 : 1000 of monoclonal mouse anti-caspase-3 (all Santa Cruz Biotechnology, Heidelberg, Germany), 1 : 1000 of monoclonal mouse anti-caspase-7 and 1 : 10 000 of polyclonal rabbit anti-DFF (Pharmingen, Heidelberg, Germany), 1 : 2000 each of monoclonal mouse anti-caspase-8 and polyclonal rabbit anti-caspase-9 (Cell Signalling, Frankfurt, Germany), 1 : 5000 of monoclonal mouse anti-PARP (Enzyme Systems Products, Livermore, CA, USA), and 1 : 2000 of monoclonal mouse anti-FAK (Transduction Laboratories, Heidelberg, Germany). The loading and transfer of equal amounts of protein was confirmed by staining the membrane with Ponceau S. Protein content was quantified using the bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Results are representative of three independent experiments. ScanPack 3.0 software (Biometra, Göttingen, Germany) was used for quantification analysis.

Results

Induction of apoptosis by ErPC involves neither the TNF nor the TRAIL receptor/ligand system in A172 cells

Involvement of death receptor/ligand systems has been proposed to mediate apoptosis induced by several anti-cancer drugs in a variety of tumour cells (Fulda *et al*. 1997; Herr *et al*. 1997). However, we have recently shown that ErPC-induced apoptosis is independent of the Fas/FasL system (Jendrossek *et al*. 2001). Thus, we examined the putative role of the TNFα/TNF-R or the TRAIL/TRAIL-R system in mediating ErPC-induced apoptosis of A172 cells. First, we considered ErPC-induced changes in TNFα or TRAIL expression. Our data show neither expression of TNFα or TRAIL mRNA in untreated and ErPC-treated A172 cells (Fig. 1a) nor the constitutive release of TNFα as assessed by ELISA (levels slightly above detection threshold; not shown). Second, to explore whether ErPC-induced apoptosis was mediated by up-regulating TNF or TRAIL receptors-1 and -2 we analysed their expression by western blotting. Untreated cells constitutively expressed both TNF

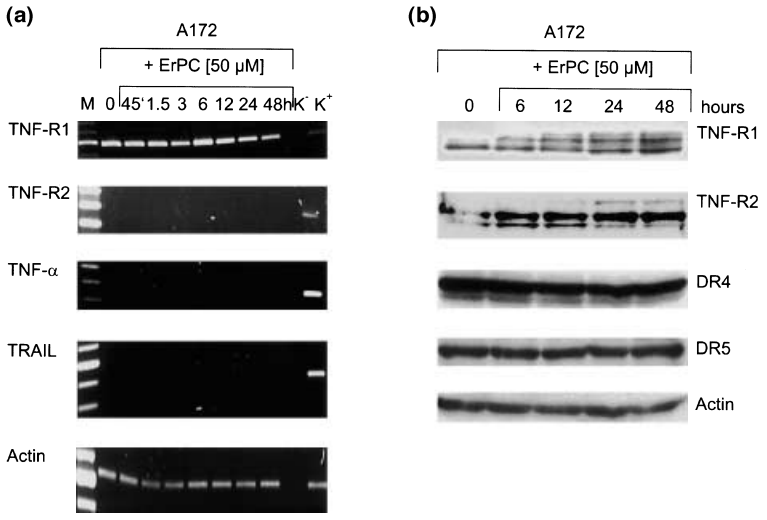

Fig. 1 Expression of TNFα, TRAIL, TNF receptor 1 (TNF-R1), TNF receptor 2 (TNF-R2), TRAIL receptor 1 (DR4) and TRAIL receptor 2 (DR5). (a) A172 cells were incubated with or without 50 μM ErPC for the indicated times. cDNA was synthesized by reverse transcription and PCR was performed to analyse mRNA expression for TNF-R1 (587 bp), TNF-R2 (360 bp), TNFα (325 bp) and TRAIL (406 bp) using specific primers. Expression of β-actin (626 bp) was used as an internal control. M, 100 bp DNA ladder; K^-, negative control, i.e. without template; K^+, positive control, i.e. cDNA prepared from LPS-stimulated peripheral blood monocytes (TNF-R1, TNFα) and from untreated T98G cells (TNF-R2, TRAIL), respectively. (b) A172 cells were incubated with or without 50 μM ErPC for the indicated times and immunoblot analysis was performed for TNF-R1, TNF-R2, DR4 and DR5. Actin was used as a control for protein loading.

and TRAIL receptors and, upon ErPC treatment, minor changes in TNF-R1 or TNF-R2 expression (up to 1.8-fold) were observed. The levels of both apoptosis-mediating TRAIL receptors (TRAIL-R1 and -R2) did not change (Fig. 1b). These data are in agreement with RT–PCR analyses, indicating that ErPC treatment led to a slight increase in TNF-R1 mRNA expression in A172 cells (about 1.7-fold), whereas TNF-R2 mRNA was not expressed at all (Fig. 1a).

To further evaluate the potential implication of TNFα/TNF-R1 or TRAIL/TRAIL-R interaction we analysed whether blocking the receptor pathway would interfere with apoptosis in response to ErPC. We found that neutralization of the endogenous TNFα or TNF-R1 with the respective blocking antibodies had neither a protective effect on ErPC-induced apoptosis (Figs 2a and b) nor blocked cytotoxicity in A172 cells as assessed by the WST-1 test (not shown). The same holds true for experiments blocking the TRAIL receptor pathway. ErPC-mediated apoptosis was not prevented by TRAIL receptor fusion proteins (Figs 2c and d) or a neutralizing anti-TRAIL antibody (Fig. 2e). To document that the amount of neutralizing antibody or fusion protein used was sufficient to block any death receptor-mediated event, A172 cells were exposed to soluble TRAIL and TRAIL-induced apoptosis was blocked by an anti-TRAIL antibody or TRAIL receptor fusion proteins. An isotype-matched control IgG or an IgG:Fc fragment had no effect (Fig. 2f). Together these data suggest that the mechanism underlying ErPC-induced apoptosis is not mediated via TNF or TRAIL ligand/receptor signalling.

ErPC-induced activation of apoptosis is not dependent on death receptor signalling

To extend our investigations into a potential role of death receptors in ErPC-mediated apoptosis, we analysed the effect of ErPC in HeLa cells, which stably expressed a GFP-tagged dominant-negative mutant of the adaptor protein FADD lacking the essential death effector domain (DED) region (Wajant *et al*. 1998). It has been shown that FADD transduces apoptotic signals triggered by Fas, TNF-R1 and the TRAIL receptors (Wajant *et al*. 1998; Locksley *et al*. 2001). Thus, the mutant blocks the death receptor pathway by preventing recruitment of caspase-8 to the receptor (Medema *et al*. 1997). In agreement, $HeLa_{FADD\text{-}DN}$ cells are resistant to anti-Fas and TRAIL and have an intrinsic resistance to TNF-mediated apoptosis (Wajant *et al*. 1998).

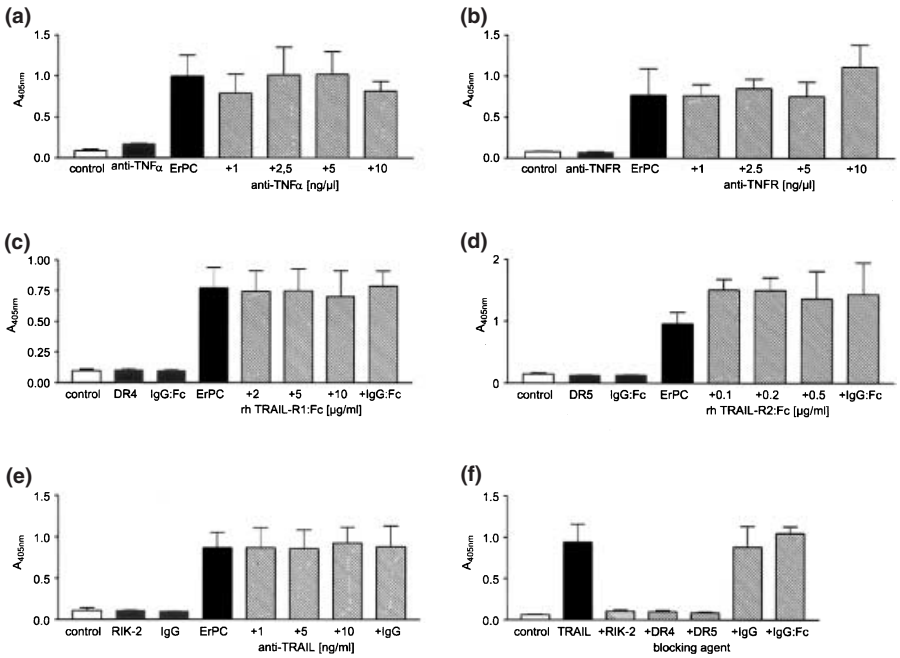

Fig. 2 Effect of blocking the TNF or TRAIL receptor pathway on ErPC-induced apoptosis. A172 cells were treated for 24 h with 50 µM ErPC in the absence or in the presence of increasing concentrations of antagonistic anti-TNFα (a), anti-TNFR (b) and anti-TRAIL (e) antibody, respectively, or of recombinant human (rh) TRAIL-R1:Fc (c) or TRAIL-R2:Fc (d) fusion protein. Untreated control cells and cells treated with antibody or fusion protein alone (highest concentration used in neutralization experiments) were run in parallel, IgG1 isotype control (10 ng/mL) or IgG:Fc fragment (10 µg/mL) were used as negative controls. As a positive control (f), TRAIL-mediated apoptosis (100 ng/mL) was blocked by an anti-TRAIL antibody (RIK-2; 10 ng/mL) or TRAIL receptor fusion proteins (DR4 at 10 µg/mL or DR5 at 0.5 µg/mL). Apoptosis was determined as outlined under Materials and methods. Results are mean values ± SD of at least three independent experiments.

Microscopic analysis revealed that stimulation with agonistic anti-Fas antibody did not induce any apoptotic alterations in $HeLa_{FADD\text{-}DN}$ cells, thus confirming the protective effect of the dominant-negative FADD-mutant (Fig. 3a). In contrast, treatment with ErPC caused the appearence of typical apoptotic features such as cell condensation and membrane blebbing (Fig. 3a). The parallel assessment of apoptosis induction revealed that ErPC and mitomycin C (used as positive control; Wesselborg *et al*. 1999) induced apoptosis in both cell types, whereas cytotoxic anti-Fas induced apoptosis only in HeLa cells transfected with the vector alone (Fig. 3b). Accordingly, ErPC activated caspase-8 in both $HeLa_{vector}$ and $HeLa_{FADD\text{-}DN}$ cells, whereas anti-Fas induced cleavage of procaspase-8 only in HeLa-control cells (Fig. 3c). These data indicate that, at least in HeLa cells, apoptosis by ErPC does not require death receptor signalling and that caspase-8 can be activated in the absence of a FADD-containing receptor signalling complex.

Processing of multiple caspases and caspase substrates during ErPC-induced apoptosis

We have analysed by western blotting the time-dependent proteolytic processing of caspases-3, -7, -8 and -9 during ErPC-induced apoptosis in two human glioblastoma cell lines. In untreated A172 cells, caspase-8 was expressed primarily as two isoforms of 55 and 53 kDa (Fig. 4a, lane 1). Exposure to ErPC resulted in a time-dependent processing of caspase-8 initially to two fragments of 43 and 41 kDa (p43/41), corresponding to cleavage of both isoforms between the large and small subunits. This was followed by the appearance of a p18 subunit resulting from removal of the death effector domains from the 43- and 41 kDa fragments (Fig. 4a, lane 4). An increase in the processing of caspase-8 was first observed 6 h after ErPC treatment, the appearance of the active p18 subunit as a faint band after 24 h. The control lane in A172 cells showed a very small amount of the intermediate cleavage products, likely due to background

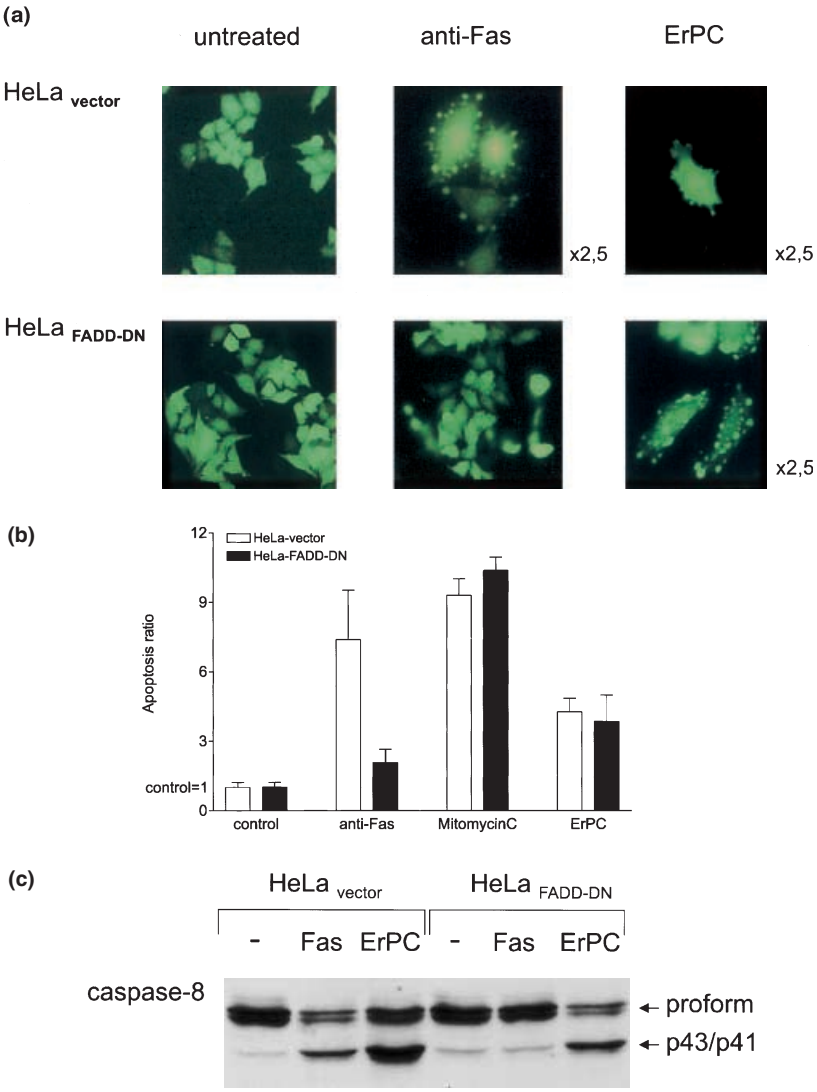

Fig. 3 ErPC-induced apoptosis and activation of caspase-8 is independent of FADD signalling. (a) HeLa cells stably expressing a GFP-tagged dominant-negative mutant of FADD (■, $HeLa_{FADD\text{-}DN}$) or the GFP-tagged vector alone (□, $HeLa_{vector}$) were treated with agonistic anti-Fas antibody (1 μg/mL), ErPC (30 μM) or left untreated. After 24 h, the cells were analysed for the induction of apoptosis under a fluorescent microscope using a 16× or a 40× oil immersion objective and FITC filter set. Photos from an 8-s exposure are shown. (b) Subsequently, the induction of apoptosis in $HeLa_{FADD\text{-}DN}$ and $HeLa_{vector}$ cells was analysed by incubating the cells with medium (control), agonistic anti-Fas antibody (1 μg/mL), mitomycin C (5 μg/mL) or ErPC (30 μM) for 24 h. The enrichment of mono- and oligonucleosomes in the cytoplasm was determined by ELISA. Data obtained are presented as percentage of untreated control cells (untreated control=1). (c) $HeLa_{FADD\text{-}DN}$ or $HeLa_{vector}$ cells were incubated with medium, agonistic anti-Fas antibody (1 μg/mL), or ErPC (30 μM) for 24 h. Cellular proteins were immunoblotted with anti-caspase-8. Caspase-8 processing is reflected by the occurrence of the intermediate cleavage forms p43/41.

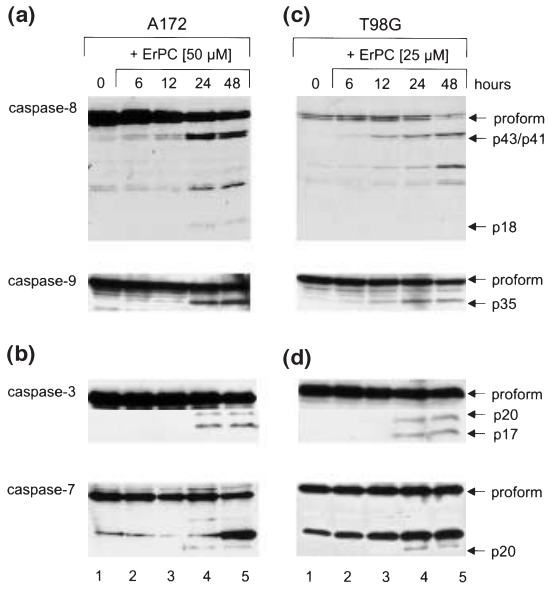

Fig. 4 Time-dependent caspases processing induced by ErPC treatment. Extracts from A172 (a and b) and T98G cells (c and d) treated with 50 μM and 25 μM ErPC, respectively, for the indicated times were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and analysed for the processing of initiator caspases-8, -9 (a and c), and effector caspases-3, -7 (b and d). The migration position of full-length procaspases and the corresponding cleavage intermediates and active subunits are indicated.

caspase activity. In T98G cells, similar processing of caspase-8 was detected (Fig. 4c).

Recent studies have shown that caspase-9 is activated by cytochrome c due to clustering of caspase-9 by Apaf-1, leading to activation of caspases-3 and -7. Because ErPC induced cytochrome c release (Jendrossek *et al*. 2001) and caspases-3 and -7 activation (see below), we then examined whether caspase-9 was also activated by ErPC. Untreated A172 and T98G cells contained the 46 kDa proform of caspase-9 (Figs 4a and c, lane1), which on ErPC treatment was processed in a time-dependent manner to yield a fragment of 35 kDa (p35). The first detectable processing of caspase-9 was evident at 6 h in both cell lines (Figs 4a and c, lane 2).

In A172 cells, the effector caspase-3 was present as its intact 32 kDa proform (Fig. 4b, lane 1). Induction of ErPC-mediated apoptosis resulted in partial loss of the proform of caspase-3 and appearance of the intermediary 20 kDa (p20) subunit and the mature 17 kDa (p17) form. Processing was first detected after 24 h (Fig. 4b, lane 4). In T98G cells, caspase-3 was also cleaved with the same kinetics in response to ErPC (Fig. 4d).

Caspase-7 was present in control A172 cells primarily as a 35-kDa protein (Fig. 4b, lane 1). Treatment with ErPC resulted in a time-dependent activation of caspase-7 accompanied by the formation of a 20-kDa (p20) fragment, which corresponds to the catalytically active large subunit (Fig. 4b, lane 4). Processing of caspase-7 was first observed as a faint band 24 h after treatment. Similarly, p20 was detected in T98G cells (Fig. 4d). We did not further analyse the identity of the prominent protein band of about 24 kDa, which is already present in untreated cells and increased in a time-dependent manner. Thus, ErPC-induced apoptosis was accompanied by the activation of both the initiator caspases-8 and-9 and the effector caspases-3 and -7. Interestingly, the precursor molecules of the caspases studied were not depleted during apoptosis.

We further investigated the activation of caspases in ErPC-mediated cell death by monitoring cleavage of the known caspase substrates PARP (D'Amours *et al*. 2001), focal adhesion kinase (FAK; Schaller 2001), and inhibitor of caspase-activated DNase/DNA fragmentation factor (ICAD/DFF; Gu *et al.* 1999; Zhang and Xu 2000) in immunoblot analyses. Upon treatment of A172 cells with ErPC, substrate cleavage products include the 85 kDa fragment of PARP, the 90 kDa proteolytic species of $p125^{FAK}$ and the 45 and 30 kDa bands of DFF (Fig. 5a). Consistent with the results in A172 cells, treatment of T98G cells with ErPC displayed a time-dependent cleavage of specific caspase substrates (Fig. 5b).

Effect of peptide inhibitors on ErPC-induced apoptosis

To substantiate the essential role of caspases in mediating ErPC-induced apoptosis, we analysed the effect of different peptide inhibitors on apoptosis by determination of cytosolic nucleosomes as a result of apoptotic DNA damage (Jarvis *et al*. 1994). A172 cells were stimulated with 50 μM ErPC for 24 h in the presence or absence of various concentrations of different peptide inhibitors (10–200 μM). Cells treated with 0.1% DMSO or peptide inhibitor alone were used as controls. The highest inhibitor concentration used in these experiments had no effect on nucleosome enrichment or cell viability *per se* (Figs 6a–f). In the presence of Ac-DEVD-CHO, which primarily inhibits caspases-3, -7 and -8 (García-Calvo *et al*. 1998), the amount of apoptotic cells was reduced to about 40% of ErPC-treated cells (Jendrossek *et al*. 2001). Similarly, the peptide z-IETD-fmk, which preferentially inhibits caspases-6, -8, -9 and -10 (García-Calvo *et al.* 1998), dose-dependently blocked the enrichment of mono- and oligonucleosomes in the cytoplasm after ErPC-induced apoptosis (Fig. 6a). Treatment with the optimal sequence inhibitor of caspase-9, z-LEHD-fmk, reduced the enrichment of cytoplasmic histone-associated-DNA-fragments with maximal effect achieved with 100 μM z-LEHD-fmk (Fig. 6b). zVAD-fmk, a general caspase inhibitor, showed a dose-dependent inhibition of nucleosome enrichment, with maximal protection observed at concentrations above 40 μM (Fig. 6c). Further, the selective inhibitors z-IETD-fmk and z-LEHD-fmk failed to prevent toxicity of ErPC in A172 cells

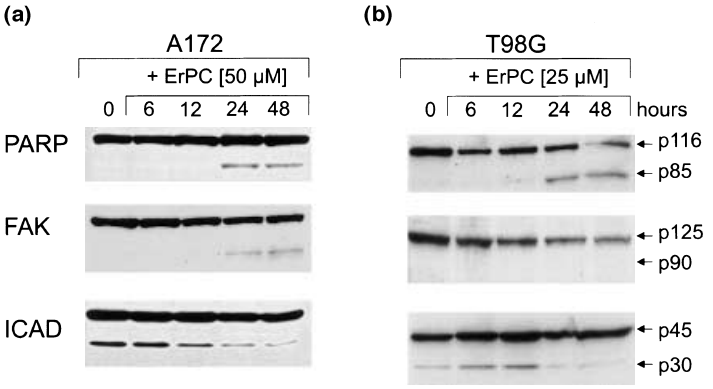

Fig. 5 Time-dependent processing of caspases substrates induced by ErPC treatment. Extracts from A172 (a) and T98G cells (b) treated with 50 μM and 25 μM ErPC, respectively, for the indicated times were resolved by SDS–PAGE and analysed for the processing of poly(ADP-ribose) polymerase (PARP), focal adhesion kinase (FAK), and inhibitor of caspase-activated DNase (ICAD). The migration position of full-length proteins and the corresponding cleavage products are indicated.

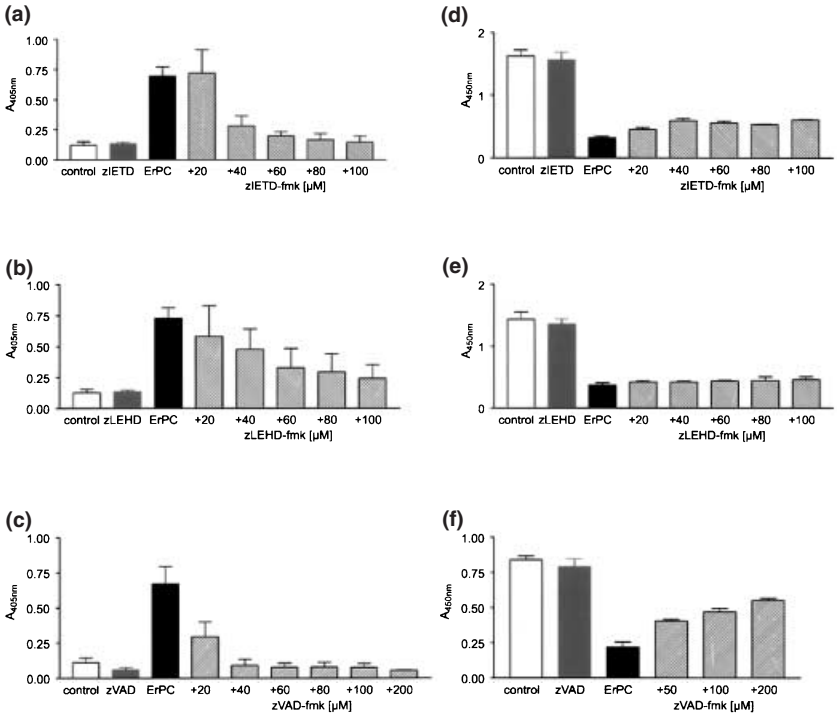

Fig. 6 Effect of peptide inhibitors on ErPC-induced apoptosis and cell viability in A172 cells. Cells were pre-incubated with the indicated doses of caspase-8 (zIETD-fmk), caspase-9 (zLEHD-fmk) or the pan-caspase inhibitor (zVAD-fmk) for 1 h prior to the addition of ErPC (50 μM) and further incubated for 23 h. Untreated control cells and cells treated with the different inhibitors alone (100 μM or 200 μM for zVAD-fmk) were run in parallel. Apoptosis (a–c) and cell viability (d–f) were determined as outlined under Materials and methods. Results are the mean values ± SD of at least three independent experiments.

at any of the concentrations tested (0.1–100 μM), although we observed a slight increase in viability of A172 cells treated with ErPC in the presence of z-IETD-fmk (Fig. 6d). The cytoprotective effect was most pronounced with the broad-specificity caspase inhibitor zVAD-fmk (2.5-fold increase at 200 μM zVAD-fmk; Fig. 6f). Higher concentrations of zVAD-fmk were cytotoxic to A172 cells as assessed by the WST-1 test (not shown). This suggests that the different peptide inhibitors tested were sufficient to block ErPC-induced apoptosis but only partially cell death in A172 cells.

zVAD-fmk inhibits the activation of caspases-3, -7, -8 and -9 in ErPC-treated A172 cells

To evaluate the relative contribution of the different caspase subfamilies to ErPC-induced apoptosis, we analysed by western blotting the proteolytic processing of initiator and effector caspases in cells treated with ErPC in the presence of increasing concentrations of zVAD-fmk. As illustrated in Figs 7(a–d, lane 2), A172 cells incubated with 50 μM ErPC alone for 48 h, resulted in the activation of all caspases examined. Caspase processing is reflected by the occurrence of p43/41/caspase-8, p35/caspase-9, p20/17 caspase-3 and p20/caspase-7. Strikingly, very different inhibitory effects were observed in ErPC-induced apoptosis. The presence of zVAD-fmk only partially inhibited the processing of caspases-9 and -3, with caspase-9 being less sensitive to inhibition than caspase-3. It is known that procaspase-3 is processed by an upstream caspase to yield two subunits of 20 and 12 kDa. The 20 kDa subunit is then autoproteolysed to 19- and 17-kDa fragments. Accordingly, the major subunits detected in A172 cells treated with ErPC alone were p17 and

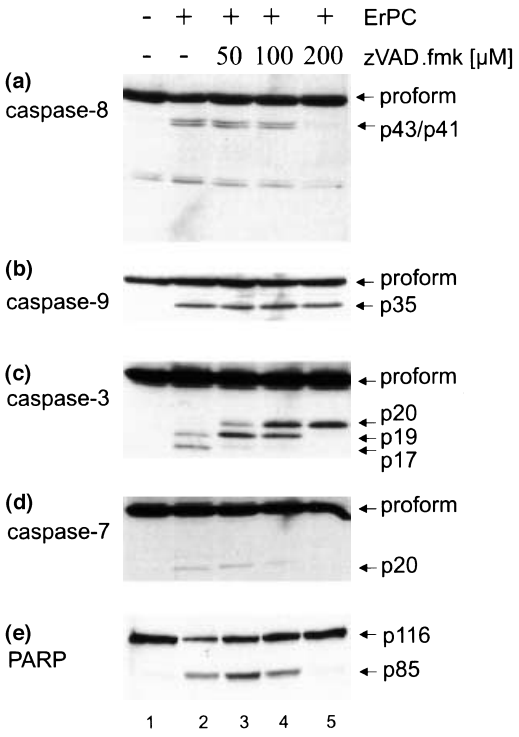

Fig. 7 ErPC-induced caspase processing: Effects of zVAD-fmk. A172 cells were treated with ErPC (50 μM) for 48 h in the absence (control) or in the presence of zVAD-fmk at 50 μM, or 100 μM, or 200 μM. Equal amounts of cell lysates were subjected to electrophoresis and immunoblot analysis was performed for caspases-8, -9, -3, -7 (a–d) and PARP (e).

p19 (Fig. 7c, lane 2). Interestingly, in the presence of zVAD-fmk (200 μM), the p20 subunit of caspase-3 appeared (Fig. 7c, lane 5), whereas further processing to its 19- and 17-kDa fragments was completely inhibited. These results are compatible with increasing concentrations of zVAD-fmk being unable to inhibit the processing of caspase-9. They imply that caspase-9 was activated and able to process caspase-3 to its 20 kDa fragment but that zVAD-fmk had inhibited the autocatalytic activation of caspase-3 and its ability to feedback and further process caspases. However, zVAD-fmk completely inhibited the processing of caspases-8 and -7 (Figs 7a and d, lanes 5) and the cleavage of PARP, a marker for caspase-3-like enzymatic activity (Fig. 7e). Thus, zVAD-fmk was more effective in blocking the enzymatic activity rather than the processing of caspase-3.

Discussion

The present study together with previously reported data (Jendrossek *et al*. 2001) excludes a role of endogenous death ligand/receptor interactions, including the FasL/Fas, the TNFα/TNF-R1 and the TRAIL/TRAIL-R system, in apoptosis associated with ErPC. This conclusion is based on the observations that: (i) ErPC induces a potent apoptotic response in glioma cells, whereas FasL/Fas or TNFα/TNF-R1 interactions fail to promote a significant cell death, suggesting that ErPC does not induce the same signalling events triggered by FasL or TNFα; (ii) ErPC does not induce FasL, TNFα, or TRAIL expression at the mRNA level in human glioma cells; (iii) co-exposure to an anti-FasL, anti-TNFα, or anti-TRAIL antibody, which prevent death ligand-dependent signalling, has no effect on ErPC-induced apoptosis, (iv) addition of an anti-TNF-R1 blocking antibody or neutralizing TRAIL receptor fusion proteins, that prevent cell killing elicited by death receptor/ligand interactions, do not block ErPC-induced apoptosis in A172 cells; (v) ErPC-induced induction of apoptosis was independent of protein synthesis as it could not be inhibited by cycloheximide indicating that activation of apoptosis was not mediated by death-inducing ligands; and (vi) a dominant-negative caspase-8 construct fails to block ErPC-induced cytotoxicity, suggesting that the observed minor changes in CD95 and TNF-R1 expression were epiphenomenal to the death process. However, it has to be taken into account that the receptor activation experiments were done in different cell lines than the FADD–/– experiments. Therefore, the findings in HeLa cells cannot be directly related to the discussed pathways in glioma cells. Furthermore, in contrast to Fas and TNF signalling, the role of FADD in TRAIL receptors is highly controversial. Complexes of FADD and caspase-8 have been implicated in DR4- and DR5-mediated apoptosis (Wajant *et al*. 1998; Suliman *et al*. 2001). However, there is also evidence suggesting they might not be required for DR4- and DR5-induced apoptosis (Marsters *et al*. 1996; Yeh *et al*. 1998). Our results are in contrast to recent observations with ET-18-OCH3 and several alkylphosphocholines. It was shown that these substances strongly induce TNFα production by U-937 cells (Eue *et al*. 1995; Pushkareva *et al*. 2000). Furthermore, ET-18-OCH3 induces apoptosis in mitogen-activated peripheral blood human T lymphocytes via the Fas/FasL system (Cabaner *et al*. 1999). Liposomal ET-18-OCH3, however, leads to apoptosis independently of CD95 signalling in Jurkat, H9 and U-937 cells (Cuvillier *et al*. 1999). These differences are possibly due to the different structure of the drugs and cell lines used.

We report that in A172 and T98G cells ErPC triggers apoptosis via proteolytic processing of caspases-8, -9, -3 and -7 that was blocked by caspase inhibitors, confirming the requirement of caspase activation for apoptosis to occur. Activation of caspases may be triggered by at least two different pathways: the death receptor pathway or the mitochondrial pathway (Kaufmann and Earnshaw 2000). Stimulation of the Fas, the TNF, or the TRAIL ligand/receptor system did not appear to account for activation of caspases induced by ErPC inasmuch as blocking the receptor/ligand interaction had no effect on ErPC-induced cell death in A172 cells (see above). Recent evidence suggests that the mitochondrion is an essential component of

apoptosis mediated by cellular stress (Kroemer and Reed 2000; Fulda *et al*. 2001). This is in line with our data showing that ErPC induces cytochrome c release, alterations of the mitochondrial membrane potential (Jendrossek *et al*. 2001), and that cleavage of caspase-9 precedes cleavage of caspase-8, indicating that caspase-9 is the most apical caspase of the cascade. Although it was initially assumed that caspase-8 acts as the proximal initiator caspase in the death receptor pathway, our experiments with ErPC in HeLa cells and several other reports now show that caspase-8 can be activated by anti-cancer drugs downstream in a receptor-independent manner (Slee *et al*. 1999, 2000; Wesselborg *et al*. 1999). This suggests that caspase-8 can also function as an effector caspase in the mitochondrial pathway (Engels *et al*. 2000). Treatment of A172 or T98G cells with ErPC results further in cleavage of effector caspases-3 and -7 which correlates with the onset of apoptosis (Jendrossek *et al*. 1999) and leads to cleavage of specific substrates, e.g. PARP. Activation of caspases-3, -7, -8 and -9 with subsequent cleavage of PARP was also observed in liposomal ET-18-OCH3-induced apoptosis in Jurkat, H9 and U937 cells (Cuvillier *et al*. 1999).

We were surprised by the mode of action of the pan-caspase inhibitor zVAD-fmk which prevents DNA fragmentation but does not abolish cell death after ErPC exposure. Nevertheless, treatment with zVAD-fmk completely inhibits the processing of PARP and of caspases-7 and -8 (Figs 7a and d, lane 5), suggesting that caspases-7 and -8 are activated downstream of caspases-9 and -3. The fact that zVAD-fmk attenuates, but does not abrogate, cell death suggests that, in addition to a caspase-dependent pathway, A172 cells might have a caspase-independent pathway that insures cell death. Caspase-activity itself may be required to amplify the killing cascade. This is also supported by the failure of all caspase inhibitors tested to fully prevent cell death and by the observation that none of the procaspase molecules under study are fully depleted during ErPC-induced apoptosis. Indeed, an increasing number of cases have been reported recently where the inhibition of caspase activities in mammalian apoptotic systems failed to prevent cell death (Xiang *et al*. 1996; Hirsch *et al*. 1997; McCarthy *et al*. 1997; Miller *et al*. 1997). Xiang *et al*. (1996) demonstrated that, although Bax does induce caspase activation and apoptotic cell death in Jurkat cells, zVAD-fmk only blocks DNA fragmentation, cleavage of caspases and substrates for caspases. These cells, however, still die. A similar observation was made in other cell types, e.g. cerebellar granule cells (Miller *et al*. 1997). In all cases, cells exhibited a more necrotic morphology. This could also be shown by electron microscopy in preliminary experiments exposing A172 cells to 50 μM ErPC for 48 h (unpublished results). We conclude that in ErPC-induced cell death a caspase-independent cell death programme operates in concert with a caspase-dependent apoptosis programme, either in parallel or sequential. Caspase-independent pathways may signal to the nucleus the initiation of the death programme and reinforce mitochondrial and/or cytoplasmic signals as suggested by Ferrando-May *et al*. (2001). In addition, the ability of ErPC to bypass the requirement for caspases in the death pathway may guarantee killing of those tumour cells whose caspase pathway is incomplete, e.g. with reduced levels of caspase expression or activation.

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