Alkylphosphocholine-induced glioma cell death is BCL-X_L-sensitive, caspase-independent and characterized by massive cytoplasmic vacuole formation

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Abstract

Alkylphosphocholines (APC) are candidate anticancer agents. We here report that APC induce the formation of large vacuoles and typical features of apoptosis in human glioma cell lines, but not in immortalized astrocytes. APC promote caspase activation, poly(ADP-ribose)-polymerase (PARP) processing and cytochrome *c* release from mitochondria. Adenoviral X-linked inhibitor of apoptosis (XIAP) gene transfer, or exposure to the caspase inhibitor, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoro-methylketone zVAD-fmk, blocks caspase-7 and PARP processing, but not cell death, whereas BCL-X_L blocks not only caspase-7 and PARP processing but also cell death. APC induce changes in $\Delta\Psi_m$ in sensitive glioma cells, but not in resistant astrocytes. The changes in $\Delta\Psi_m$ are unaffected by crm-A (cowpox serpin – cytokine response modifier protein A), XIAP or zVAD-fmk, but blocked by BCL-X_L, and are thus a strong predictor of cell death in response to APC. Free radicals are induced, but not responsible for cell death. APC thus induce a characteristic morphological, BCL-X_L-sensitive, apparently caspase-independent cell death involving mitochondrial alterations selectively in neoplastic astrocytic cells.

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Abbreviations: APC, alkylphosphocholine; AnxV, annexin V; SOC-2, (S)-1-O-phosphocholin-2-O-acetyl-octadecane; rac-OMe, (rac)-1-O-phosphocholin-2-O-methyl-octadecane; PARP,

poly(ADP-ribose)-polymerase; XIAP, X-linked inhibitor of apoptosis protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DEVD-amc, acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; COX, cyclo-oxygenase; PI, propidium iodide; zVAD-fmk, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoro-methylketone; zFA-fmk, benzyloxycarbonyl-Phe-Ala-fluoromethylketone; H₂DCFDA, dichlorodihydrofluorescein diacetate; tiron, 1, 2-dihydroxybenzene-3,5-disulfonate; JC-1, 5,5', 6,6'-tetraethyl-benzimidazolylcarbocyanine iodide; crm-A, cowpox serpin – cytokine response modifier protein A; PS, phosphatidylserine; AIF, apoptosis-inducing factor; Ca-074Me, (L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl)-L-isoleucyl-L-proline methylester; ALLN, N-acetyl-Leu-Leu-Norleucinal; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; BAPTA, 1, 2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethylether)N,N',N',N'-tetraacetic acid; MDC, monodansylcadaverine

Introduction

Glioblastoma, the most common malignant intrinsic brain tumor, is rather resistant to current approaches of therapy. The median survival after aggressive multimodality treatment consisting of surgical resection, radiotherapy and chemotherapy has remained in the range of 12 months for decades. According to the meta-analysis of the major clinical chemotherapy trials in malignant glioma, chemotherapy provides a small, but reproducible, increase in the median survival.¹ The failure of current chemotherapy regimens in malignant glioma involves, among others, poor drug penetration of the blood–brain barrier, multidrug resistance proteins expressed by brain endothelial and tumor cells, hypoxia and acidosis, and an intrinsic tumor cell resistance to the induction of apoptosis.²

Alkylphosphocholines (APC), a novel type of antitumor agent targeting cellular membranes, are lipophilic ether lipids derived from alkyllysophospholipids and related to natural lysophospholipids and ceramides. APC exhibit cytotoxic activity against a variety of tumor cell lines *in vitro* and antineoplastic activity *in vivo*.^{3–6} Since an increase of free cytosolic calcium ions is an early effect after APC treatment, calcium-dependent lysosomal enzymes like calcium-activated calpains or cathepsins may be involved in APC-induced cell death.^{7,8} Investigations of the structure-activity relationship among synthetic APC indicated that a long alkyl chain and a phosphocholine moiety are essential for their antineoplastic effects.⁹ A clinical application of APC appears to be feasible in view of the fact that APC are cytotoxic preferentially to leukemia cells, but spare normal blood progenitors, a feature attributed to differences in the lipid metabolism of tumor and non-neoplastic cells.¹⁰ Also, APC have no intense

substrate affinity for phospholipid-metabolizing enzymes, which increases their half-life and may allow accumulation in tumor cells.¹¹ Finally, the accumulation of APC in brain¹² provides an additional rationale to assess such agents in the experimental treatment of gliomas.

Here, we characterize the cytotoxic effects of two APC compounds ((*S*)-1-*O*-phosphocholin-2-*O*-acetyl-octadecane (SOC-2))¹³ and (rac)-1-*O*-phosphocholin-2-*O*-methyl-octadecane (racOMe)), patent pending) in a panel of human malignant glioma cell lines. We find that both agents induce a novel variant of a BCL-X_L-sensitive, mitochondrially mediated cell death in human malignant glioma cells, but not in immortalized human non-neoplastic astrocytes.

Results

APC are cytotoxic to human malignant glioma cells, but not to non-neoplastic human astrocytes

LN-18, LNT-229 or LN-308 human malignant glioma cells, or SV40-immortalized human astrocytes (SV-FHAS), were incubated with increasing concentrations of APC (Figure 1a) for up to 72 h (Figure 1b) or with APC at 10 μ M for increasing periods of time (data not shown). The first cytotoxic effects were detected at 8 h of incubation with 30 μ M APC. Figure 1b shows the growth inhibitory effects of SOC-2 and racOMe at 24 h. The three glioma cell lines were sensitive to both APC, whereas SV-FHAS cells were resistant to APC concentrations of up to 30 μ M for more than 48 h. Since maximal cytotoxic effects were detected after 18–24 h of APC incubation, this time period was used for all further experiments. The EC₅₀ values for SOC-2 and racOMe were 23 and 18 μ M in LN-18, 27.5 and 20.5 μ M in LNT-229 and >30 μ M in LN-308 cells. APC not only induced growth inhibition but also actual cell death (Figure 1c). The exposure of phosphatidylserine (PS), which is physiologically confined to the inner plasma membrane, on the outer plasma membrane is characteristic of apoptosis and should precede the loss of membrane integrity detected by propidium iodide (PI) uptake, which is suggestive of (secondary) necrosis. An externalization of PS in response to APC was detected at 16–24 h. In parallel, APC induced the formation of a second population of cells, negative for annexin V (anxV), but positive for PI indicating necrotic cell death. Cells treated with lomustine served as a positive control for apoptosis¹⁴ (Figure 1d).

Morphological features of APC-induced cell death

We next characterized the morphology of cell death induced by APC. LN-18 and LNT-229 glioma cells were treated with racOMe at 20 μ M and analyzed by electron microscopy at different time points. Untreated LN-18 cells were flat cells with an elliptic nucleus and cytoplasmic organelles and commonly formed some superficial protrusions like short microvilli, which appeared as small blebs surrounding the cells (Figure 2a). After 12 h treatment with APC, the cells were structurally largely intact, but the nuclei became altered and assumed a globular shape. The overall volume of the cells increased, and the organelles were still intact. However, small vacuoles

appeared in some cells (Figure 2b). After 16 h, approximately 50% of the LN-18 cells developed numerous vacuoles, some of which fused to form larger vacuoles. The size of the vacuoles varied between 0.5 and 2 μ m. Morphologically, the origin of vacuoles was indistinct (Figure 2i), since organelles such as mitochondria and Golgi cisterns were still intact (Figure 2c). At 24 h, most cells were strongly vacuolated, and the size of the vacuoles increased to 3–4 μ m (Figure 2d). At this time point, nearly all cells also revealed an apoptotic or secondary necrotic phenotype with a characteristic chromatin redistribution, although apoptotic bodies were not formed (Figure 2d and h, insets). The alterations induced in LNT-229 cells were similar to those observed in LN-18 cells (Figure 2e–h).

Biochemical features of APC-induced cell death: calcium, lysosomal enzymes and free radicals

An early effect of APC treatment of glioma cells was the increase of intracellular free Ca²⁺, which was detected as early as 2 h and peaked at 6 h (Figure 3a). Since Ca²⁺ activates the lysosomal enzymes calpain-1 and -2,^{15,16} the ability of lysosomal enzyme inhibitors to prevent APC-induced cell death was tested. Cell death was unaffected by different lysosomal enzyme inhibitors. Neither *N*-acetyl-Leu-Leu-Norleucinal (ALLN) (inhibitor of calcium-activated enzymes calpain-1 and -2, cathepsin B and L, cysteine proteases and proteasome) nor Ca074Me (selective inhibitor of cathepsin B) and zFA-fmk (inhibitor of cathepsins B, H, L and S, papain and cruzain), nor E-64d (inhibitor of cathepsin B, C, H, L and S¹⁷), Ac-RRRXXX-NH₂ (specific inhibitor of cathepsin C¹⁸) nor iodoacetate (broad spectrum peptidase inhibitor) (Figure 3b and data not shown), nor Ca²⁺ chelators-like ethyleneglycol-*bis*(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) or 1,2-*bis*(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) (Figure 3c) were protective. Further, the specific staining of lysosomes, golgi vesicles or endoplasmic reticulum indicated that APC-induced vacuoles appeared not to originate from lysosomes or endoplasmic reticulum (Figure 4). Macroautophagy is a widespread phenomenon in the degradation of cellular components of the lysosomal compartment. Coexposure of glioma cells to APC and the autophagy inhibitor 3-methyladenine modulated neither racOMe-induced cell death nor vacuole formation (Figure 5a and b). Further, the staining of autophagosomes with monodansylcadaverine did not show any accumulation of this dye in APC-induced vacuoles (Figure 5c).

Exposure to racOMe induced the formation of free radicals in a time- and concentration-dependent manner (Figure 6a and b). The radicals were first detected at 2–4 h in both cell lines. The levels of free radicals further increased and remained elevated until the cells died approximately 24 h later. Although antioxidants such as uric acid, a scavenger of peroxynitrite, 1,2-dihydroxybenzene-3,5-disulfonate (tiron), a scavenger of superoxide radicals, or catalase, which degrades hydrogen peroxide, decreased the levels of free radicals (Figure 6c), these agents had no effect on cell death (Figure 6d).

Figure 1 APC-induced cell death in human malignant glioma cells. **(a)** Structure of the synthetic APC. **(b)** The cells were treated with vehicle (open circles), SOC-2 (filled squares) or racOMe (filled triangles) for 24 h. Data are expressed as the mean percentages of cell density relative to nontreated cells ($n=3$). **(c)** The cells were treated as in **(b)** for 18 h and cell integrity was assessed by CASY measurement (open bars: vehicle; filled bars: SOC-2; shaded bars: racOMe). Data are expressed as the mean percentages of living cells ($n=3$, $^*P<0.05$, t -test). **(d)** LNT-229 cells were treated with racOMe ($20\ \mu\text{M}$) for increasing time periods, or with lomustine ($200\ \mu\text{M}$) as a control,¹⁴ and assessed for the exposure of PS by anV staining and for viability by PI exclusion by flow cytometry (lower left: living cells, anV/PI-negative; lower right: early apoptotic cells, anV positive/PI negative; upper left: nonapoptotic dead cells, anV negative/PI positive; upper right: late stages of apoptosis and secondary necrosis, anV/PI positive) (one representative of three similar experiments)

Figure 2 Ultrastructural features of racOME-induced cell death. LN-18 (a–d) or LNT-229 (e–h) cells were untreated (a,e) or treated with racOME (20 μ M) for 12 (b,f), 16 (c,g,i) or 24 h (d,h), fixed and analyzed by electron microscopy. The insets in (d) and (h) confirm that some cells exhibited typical features of apoptosis (for details, see text)

Biochemical features of APC-induced cell death: the role of caspases

A possible involvement of caspase activity in APC-induced cell death was assessed by caspase activity assays using the preferential caspase-3/7 substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-amc) and by immunoblot analysis for specific caspases or the caspase substrate poly(ADP-ribose)-polymerase (PARP). As a control for caspase activation, the cells were treated with CD95L^{19,20}. DEVD-amc-cleaving caspase activity was induced in a concentration-dependent manner in both cell lines (Figure 7a), roughly paralleling the induction of cell death (Figure 1b). In contrast, no caspase activity was detected in APC-treated SV-FHAS cells (data not shown). Caspases-8, -9, -3 and -7 were processed in response to both APC in LN-18 cells, whereas these concentrations were insufficient to promote detectable caspase processing in LNT-229 cells, with the exception of caspase-7 in SOC-2-treated cells. This minor caspase-7 processing might correspond to the DEVD-amc cleavage in response to SOC-2 shown in Figure 7a. Altogether, the striking difference in caspase processing in response to APC among the two cell lines did not translate into corresponding differences in sensitivity to cell death induction (Figure 1). APC-induced PARP cleavage was observed in both cell lines, but the levels of processed PARP were much lower in LNT-229 than in LN-18 cells, and in LNT-229 cells lower with racOME than with SOC-2. In contrast, there appeared to be a comparable release of cytochrome *c* from mitochondria in both cell lines, despite the different degree in caspase activation (Figure 7b). BID was also cleaved, but BID cleavage was a rather late event, detected at 12 h after racOME treatment, a time point where 50% of the cells had died (Figure 7c). In contrast to cytochrome *c*, apoptosis-inducing factor (AIF) was not released from mitochondria after racOME treatment in either cell line (Figure 7d). SV-FHAS-immortalized astrocytes showed neither caspase nor PARP processing nor mitochondrial cytochrome *c* release under these conditions (data not shown).

We next examined the modulation of APC-induced caspase-7 and PARP processing and cell death by the broad-spectrum caspase inhibitor, benzylloxycarbonyl-Val-Ala-DL-Asp-fluoro-methylketone (zVAD-fmk), the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP), the viral preferential caspase-1/8 inhibitor, cowpox serpin – cytokine response modifier protein A (crm-A), or a dominant-negative FADD mutant, DN-FADD, all of which interfere with death receptor-mediated apoptosis. Further, a death ligand-resistant subline LN-18^R,²¹ was examined. Caspase-7 and PARP processing were strongly attenuated by zVAD-fmk and XIAP, moderately attenuated by crm-A and FADD-DN, but unaffected in LN-18^R cells (Figure 8a). Moreover, DEVD-amc-cleaving activity induced by APC exposure was blocked by zVAD-fmk and XIAP, and attenuated not only by crm-A and FADD-DN but also in LN-18^R cells (Figure 8b). However, neither zVAD-fmk, Ad-XIAP, crm-A nor FADD-DN inhibited cell death induced by either APC, suggesting that the inhibition of APC-induced caspase activity is not sufficient to inhibit cell death. Similarly, LN-18^R cells were not resistant to APC-induced cell death (Figure 8c). Interestingly, zVAD-fmk

Figure 3 APC increases free intracellular Ca^{2+} levels, but lysosomal enzyme inhibitors do not block APC-induced cell death. **(a and b)** The cells were treated with racOME (20 μM) for increasing time periods (left panel) or at increasing concentrations for 4 h (right panel). Intracellular free Ca^{2+} was measured by flow cytometry using Fluo-3AM. Data are representative of three experiments with similar results. **(b)** The cells were treated with increasing concentrations of racOME in the absence or presence of ALLN, Ca074Me, zFA-fmk, E-64d or Ac-RRRXXX-NH₂ for 18 h. Cell density was assessed by crystal violet staining (mean and s.e.m., $n = 3$). **(c)** LN-18 or LNT-229 cells were exposed to racOME (20 μM) in the absence or presence of 1.45 mM EGTA or 12 μM BAPTA for 16 h. Cell density was assessed by crystal violet staining (mean and s.e.m., $n = 3$)

and Ad-XIAP (Figure 8d and e), although not crmA, FADD-DN or the LN-18^R phenotype (data not shown), blocked the evolution of apoptosis defined as PI-negative/anxV-positive cells. In contrast, there was no such effect on the necrotic

population defined as PI-positive/anxV-negative. Also, zVAD-fmk neither altered the levels of free radicals nor of free cytoplasmic calcium nor suppressed the formation of vacuoles after APC treatment (data not shown).

Figure 4 racOMe-induced vacuoles are of unidentified origin. LNT-229 cells were untreated or treated with 30 μ M racOMe for 21 h. (a) Lysosome distribution was assumed using Lysotracker-Red. (b) The formation of golgi vesicles was monitored by staining the cells with BODIPY-C₅-ceramide. (c) The endoplasmatic reticulum was stained using BODIPY-brefeldin (a)

Further, p53 appeared to play no role in APC-induced cell death. This is because the highly APC-sensitive LN-18 cell line harbors the transcriptionally inactive mutant p53^{C238S}, whereas the least sensitive cell line, LN-308, is deficient for p53 and the intermediate sensitivity cell line, LNT-229, retains p53 wild-type function. Moreover, transfection of LNT-229 cells with the dominant-negative murine p53^{V135A} mutant, which abrogates p53 function in these cells,²⁰ has no effect on APC sensitivity (data not shown).

BCL-X_L inhibits APC-induced cell death

Since BCL-X_L inhibits glioma cell death induced by death ligands and chemotherapeutic agents,^{14,22} we also examined whether BCL-X_L modulates APC-induced cell death. BCL-X_L strongly inhibited APC-induced cell death, whereas neo control cells were as susceptible to APC as the parental cells. BCL-X_L also abrogated DEVD-amc cleavage induced by APC (Figure 9a). The initial BID cleavage was unaltered in BCL-X_L-expressing LN-18 cells but BID levels were recovered at 16 h in BCL-X_L-expressing cells, although not in neo control cells (Figure 9b). In contrast, caspase-7 and PARP cleavage were abolished in LN-18 and LNT-229 cells expressing BCL-X_L (Figure 9c). To assess whether BCL-X_L protects the cells by rescuing mitochondria from the lysolipidic activity of APC, the ATP content of APC-treated glioma cells was determined. LN-18 and LNT-229 cells were treated with APC for 8 h, an early time point in the killing cascade, or for 16 h, a time point where approximately 50% of the cells were dead. Neither at early nor at late stages of APC-induced cell death was there a reduction in the ATP content, hypoxia being used as a positive control for ATP loss (data not shown). BCL-X_L overexpression did not alter the levels of free cytoplasmic calcium either (data not shown), but diminished the increase in free radical levels, even though there was no difference at later time points in LNT-229 (Figure 9d), despite the different fates of control- and BCL-X_L-transfected glioma cells (Figure 9a). AnxV/PI staining revealed that BCL-X_L blocked both the evolution of the apoptotic as well as the necrotic cell populations (Figure 9e), resulting in a corresponding increase in surviving (double-negative) cells. Electron microscopic analysis of racOMe-treated BCL-X_L-transfected LN-18 cells showed that most cells were protected from cell death (Figure 9f, right panel), but those cells which died after APC treatment showed the same morphologic alterations like vacuolization as APC-treated LN-18 neo control cells. In contrast to the protection afforded by the antiapoptotic BCL-2 family member BCL-X_L (Figure 9), ectopic expression of the proapoptotic proteins BAK or BAX had no effect on APC-induced cell death (Figure 10). The same BAX-overexpressing cells have previously been shown to be more sensitive to irradiation.²³

APC-induced loss of the mitochondrial membrane potential in sensitive glioma cells

To further delineate a role for mitochondria in the cell death process, the mitochondrial membrane potential ($\Delta\Psi_m$) was measured in APC-treated LN-18, LNT-229 or SV-FHAS cells. $\Delta\Psi_m$ alterations were monitored using 5,5',6,6'-tetraethylbenzimidazolylcarbocyanine iodide JC-1, a mitochondrial-specific dye, which changes its fluorescence from red to green upon breakdown of the mitochondrial membrane potential. The proportion of green cells increased during APC treatment in LN-18 and LNT-229 cells, but not in SV-FHAS astrocytes. As a control for $\Delta\Psi_m$ breakdown, LN-18 and LNT-229 cells were treated with CD95L and CD95L-resistant SV-FHAS cells were treated with lomustine (Figure 11a and b, left panel). Figure 11b shows that the breakdown in $\Delta\Psi_m$ was associated with the development of cell death since it was blocked by BCL-X_L, which inhibited cell death (Figure 9), but was

Figure 5 APC does not induce autophagy. **(a)** LNT-229 cells were treated with increasing amount of racOMe in the absence or presence of increasing concentrations of 3-Me-Ad for 18 h. Cell density was assessed by crystal violet staining (mean and s.e.m., $n = 3$). **(b)** LNT-229 cells were treated with 20 μ M racOMe in the absence or presence of 3-methyladenine (5 mM) and vacuole formation was assessed by electron microscopy. **(c)** LNT-229 cells were untreated or treated with 30 μ M racOMe for 21 h. Autophagosomes were monitored using MDC

unaffected by zVAD-fmk, crmA or XIAP, which were not protective (Figure 8). Yet, overexpression of BAX enhanced the breakdown of $\Delta\Psi_m$ after racOMe treatment (Figure 11c), but did not translate into enhanced or accelerated cell death (Figure 10a and b), suggesting that BAX is involved in the APC-induced alterations of $\Delta\Psi_m$, but that the extent of such alterations does not always translate into corresponding changes in cell death.

Discussion

We report here that two APCs, SOC-2 and racOMe, are cytotoxic to human malignant glioma cell lines, but not to immortalized SV-FHAS astrocytes, and induce a mixed type of cell death involving features of apoptosis and necrosis (Figure 1). Electron microscopic studies revealed that APC induced an atypical form of cell death essentially featuring

Figure 6 APC induces free radical formation. (a and b) The cells were untreated (open bars) or treated with racOME (20 μ M) (black bars) for different time periods (a) or at increasing concentrations for 4 h (b) and assessed for free radical formation ($n = 3$, $*P < 0.05$). (c and d) The cells were treated with racOME (20 μ M) for 5 h in the absence or presence of uric acid (U, 500 μ M), tiron (T, 25 μ M), catalase (C, 500 U/ml) or uric acid plus catalase. Free radical formation was assessed at 5 h (c), cell density at 24 h (d) (mean and s.e.m., $n = 3$)

vacuole formation as well as some hallmarks of classical apoptosis like chromatin condensation (Figure 2). The origin of APC-induced vacuole formation was not clarified, since neither staining of lysosomes, endoplasmic reticulum, golgi vesicles nor autophagosomes showed any enrichment of the

specific dyes in the lumen of vacuoles or of vacuole membranes (Figures 4 and 5c). Early responses to APC treatment induced an increase in free intracellular Ca^{2+} (Figure 3a), suggesting a cell death pathway dependent on the activity of lysosomal enzymes or cytoplasmic proteases

Figure 8 Inhibition of death receptor signaling and caspases fails to protect glioma cells from APC-induced cell death. LN-18 cells were treated with zVAD-fmk (100 μ M), infected with 100 MOI of Ad-LacZ or Ad-XIAP for 24 h, or stably transfected with crm-A or DN-FADD; an LN-18 cell subline resistant to death ligand-induced cell death (LN-18^R) was also examined. (a) The cells were treated with racOMe (20 μ M) for 18 h and analyzed by immunoblot. Caspase activation was visualized by the detection of p20/caspase 7(*) and p85/PARP(*). (b and c) DEVD-amc-cleaving caspase activity was assessed at 14 h after exposure to racOMe ($n=3$, s.e.m. <10%; small insets: transgene expression was assessed by immunoblot) (b). Survival was assessed by crystal violet staining, and the data are expressed as mean percentages of cell density relative to untreated control cells (c). In (b) and (c), exposure to CD95L (50 U/ml) for 6 h (b) or 12 h (c) was used as a control (* $P<0.05$, t -test). (d and e) LN-18 cells were treated with racOMe (20 μ M) in the absence or presence of 100 μ M zVAD-fmk for 24 h (d) or infected with 300 MOI Ad-LacZ or Ad-XIAP and 24 h after infection treated with racOMe (20 μ M) for further 24 h (e). The cells were analyzed by anxnV/PI flow cytometry as in Figure 1d

Figure 7 Caspase activation in APC-treated glioma cells. (a) DEVD-amc-cleaving caspase activity was assessed at 18 h after exposure to SOC-2 or racOMe or at 4 h after exposure to CD95L (50 U/ml for LN-18, 250 U/ml + 10 μ g/ml cycloheximide for LNT-229) (* $P<0.05$, ** $P<0.01$). (b) The processing of caspase-8, -9, -3, and -7 and the caspase substrate, PARP, was assessed by immunoblot at 18 h after exposure to SOC-2 or racOMe (20 μ M) or 4 h exposure to CD95L (as in a). Caspase activation is reflected by the formation of p18/caspase-8, p37/35/caspase-9, p12/caspase-3 or p20/caspase-7 (*active caspase fragment). The release of cytochrome c into the cytosol was assessed in parallel. Note that the intermediate protein band in the caspase-9 immunoblot in LNT-229 cells is not the active caspase-9 fragment, but an unspecific staining pattern produced by the caspase-9 antibody. (c) LN-18 cells were treated with racOMe (20 μ M) for increasing time periods. BID cleavage was assessed by immunoblot and cell death was assessed by crystal violet staining in parallel. (d) LN-18 cells were treated with 20 μ M racOMe for increasing periods of time or, as a positive control, with 7.5 μ M staurosporine for 4 h. Cytosolic lysates were prepared as described and mitochondrial AIF release was assessed by immunoblot. As a control for mitochondrial integrity, and equal loading, the levels of the mitochondrial proteins COX1/2 and the cytoplasmic protein GAPDH were assessed

like cathepsins or calpains. Yet, activation of protease-dependent apoptotic pathways seemed not to mediate APC-induced cell death, since neither calpain, cathepsin nor autophagy inhibitors nor calcium chelators prevented cell death (Figures 3b, c and 5a). Macroautophagy, a widespread phenomenon in the degradation of cellular components of the lysosomal compartment,^{24,25} was excluded as the origin of APC-induced vacuole formation and cell death, since inhibitors of autophagy neither rescued glioma cell death nor vacuole formation, and since staining APC-treated cells with monodansylcadaverine (MDC) did not show any enrichment of the dye in APC-induced vacuoles (Figure 5c).

A second phenomenon of APC treatment was the formation of free radicals (Figure 6a and b). However, free radical formation was epiphenomenal to cell death, since antioxi-

Figure 9 Bcl-X_L inhibits APC-induced glioma cell death. (a) LN-18 or LNT-229 cells stably transfected with BCL-X_L (black bars) or mock-transfected neo cells (open bars) were treated with SOC-2 for 18 h, or as a control, with CD95L for 12 h (upper panel) or 6 h (lower panel). Cell density was assessed by crystal violet staining (upper panel) and caspase activity was assessed by DEVD-amc cleavage (lower panel). Data are expressed as means and s.e.m. ($n = 3$, s.e.m. < 10%, * $P < 0.05$, ** $P < 0.01$). The small insets show transgene expression assessed by immunoblot. (b) LN-18 neo control or BCL-X_L-overexpressing LN-18 cells were treated with 20 μ M racOme for increasing periods of time. BID cleavage was assessed by immunoblot. (c) Neo- or BCL-X_L-transfected cells were treated with 20 μ M SOC-2 for 18 h. Caspase-7 processing is visualized by the detection of p20/caspase-7 (*). PARP processing involves the formation of p85/PARP (*). (d) Neo- (open bars) or BCL-X_L-transfected (black bars) LN-18 or LNT-229 cells were treated with racOme (20 μ M). Free radical formation was assessed by flow cytometry. (e) LN-18 neo (open bars) or BCL-X_L (black bars) cells were treated with racOme (20 μ M) for 16 h and examined as in Figure 8d. (f) Electron microscopic analysis of LN-18 neo or BCL-X_L cells was performed at 16 h after racOme treatment (20 μ M). Note vacuolization and condensed chromatin in neo control cells (left panel), whereas most BCL-X_L-expressing cells were unaffected (right panel)

dants did not protect glioma cells from APC-induced cell death (Figure 6d).

In contrast to BJAB lymphoma cells,⁶ APC cytotoxicity of glioma cells did not depend on death receptor activation since neither CD95L-resistant LN-18^R cells nor cells transfected with dominant-negative FADD were resistant (Figure 8c). Moreover, APC cytotoxicity also did not involve the classical

mitochondrial death pathway mediated by cytochrome *c*-dependent caspase activation in glioma cells, contrasting with similar studies in lymphoid cells.²⁶

Caspase-7 was activated, and its candidate substrate PARP was cleaved in response to APC (Figure 7), but prevention of caspase-7 and PARP processing did not block cell death, as indicated by the differential effects of adenoviral

Figure 10 BAX or BAK do not sensitize for APC-induced cell death. (a and b) LN-18 (a) or LNT-229 cells (b) were transiently transfected with the empty pcDNA3 vector (open symbols) or pcDNA-BAK (black symbols). At 36 h after transfection, the cells were treated with racOME for 18 h. The insets show BAK expression in the transiently transfected cells. (c) LN-18 puro control cells (open symbols) or BAX-expressing cells (black symbols) were treated with 20 μM racOME for increasing time periods. The insets show BAX expression in LN-18 BAX cells. (d) The same cells were treated with increasing concentrations of racOME for 12 h. Cell density was assessed by crystal violet staining (mean and s.e.m., $n = 3$)

XIAP gene transfer and zVAD-fmk cotreatment on caspase and PARP processing and survival. Interestingly, caspase inhibitors prevented some aspects of apoptotic cell death such as annexin V labeling, even though without a significant effect on overall survival (Figure 8), again indicating an involvement of cell death pathways besides caspases. Aside, p53 appeared to play no role in APC-mediated cell death since p53 mutant LN-18 cells, p53 wild-type LNT-229 cells and p53-null LN-308 cells were all sensitive to APC. Further, ectopic expression of dominant-negative mutant p53 in LNT-229 glioma cells had no effect on APC sensitivity.

Interestingly, high-level expression of BCL-X_L provided strong protection from APC-induced cell death. BCL-X_L may directly inhibit the activation of caspase-7,²⁷ and APC-induced caspase-7 processing and PARP cleavage were indeed inhibited in BCL-X_L-expressing glioma cells (Figure 9). However, as deducible from the observations made in XIAP-infected or zVAD-fmk-treated cells (Figure 8), prevention of caspase-7 and PARP cleavage alone cannot be responsible for the cytoprotection mediated by BCL-X_L.

The minor differences in sensitivity to APC among the three glioma cell lines cannot be attributed to endogenous expression patterns of the antiapoptotic proteins BCL-X_L or XIAP, since these cell lines express similar levels of these proteins.^{19,28} In contrast to the sensitive glioma cells, resistant SV-FHAS astrocytes express only very low levels of caspase-9 and PARP, and similar levels of caspase-7, but are deficient in caspase-3 (data not shown).

Prevention of mitochondrial changes induced by APC may be involved in the cytoprotective effects of BCL-X_L, since the alteration in $\Delta\Psi_m$ in response to APC (Figure 10a) was blocked in cells overexpressing BCL-X_L (Figure 11b), and not by the different strategies of caspase inhibition (XIAP, zVAD-fmk, crmA) that had no effect on survival either (Figure 8). Since tBID activates the BAX- and BAK-dependent mitochondrial apoptotic pathway²⁹ and since tBid activity is blocked by BCL-2,³⁰ BCL-X_L might also block tBid activity in APC-treated glioma cells. However, overexpression of BCL-X_L in LN-18 cells did not prevent p22 BID cleavage at early time points after APC exposure (Figure 9). Further, the proapoptotic proteins BAX or BAK seemed not to be involved in APC-induced cell death, since overexpression of BAK or BAX did not alter glioma cell sensitivity to APC (Figure 10). The cells dying in response to racOME under conditions of caspase inhibition (Figure 8d) showed the same morphological alterations as unprotected control cells (Figure 9f, data not shown). In contrast, BCL-X_L inhibited both the apoptotic and necrotic cell death pathways activated by APC, possibly by preventing the breakdown of the mitochondrial membrane potential. Thus, there was generally a close correlation between the loss of $\Delta\Psi_m$ and ensuing cell death, and their modulation occurred in parallel except for the differential effects of BAX on $\Delta\Psi_m$ and cell death (Figure 10c, d and 11c).

The present study thus defines APC as antitumor agents inducing cell death in neoplastic, but not in non-neoplastic immortalized cells of astrocytic origin. Induction of APC-induced cell death seemed to induce a variety of apoptotic and/or necrotic processes and morphological alterations, latterly termed necroptosis.³¹ Induction of cell death is BCL-X_L sensitive, independent of p53, caspases, lysosomal enzymes or autophagy, but involves mitochondrial alterations

Figure 11 APC induce a BCL-X_L-sensitive loss of the mitochondrial membrane potential. (a) LN-18 or SV-FHAS cells were treated with racOMe (20 μM) and ΔΨ_m was assessed by flow cytometry. As a positive control, the cells were treated with CD95L (100 U/ml) for 4 h or 200 μM lomustine for 24 h. (b) The modulation of APC-induced alterations in ΔΨ_m in LNT-229 parental cells (left panel) and by stable expression of BCL-X_L, zVAD-fmk (100 μM), infection with Ad-ΔE1 or Ad-XIAP (300 MOI) or stable expression of crm-A was examined in LNT-229 cells treated with racOMe (20 μM) for 24 h or CD95L (100 U/ml) for 4 h (*P < 0.05, t-test). (c) LN-18 puro control or BAX cells were treated with racOMe (20 μM) or CD95L (100 U/ml, 4 h) and ΔΨ_m was assessed by JC-1 staining

selectively in neoplastic astrocytic cells. It is increasingly recognized that the morphological changes of apoptosis vary between cell types and death stimuli. This heterogeneity reflects the wide range of cellular proteins and enzymes involved in apoptotic pathways: APC might induce a more widespread kind of cell death, involving caspase dependent and independent as well as yet unknown pathways. Some atypical apoptotic morphological changes in APC-treated cells like the formation of large vacuoles again argue against a classical apoptotic cell death. Interestingly, Cardenas-Aguayo Mdel *et al.* postulated an alternative nonapoptotic death mechanism, which is inhibited by BCL-2, in cells derived from neural precursor cells. This type of cell death was linked to the formation of vacuoles. In contrast to APC-induced vacuole formation, the formation of those vacuoles was abrogated by autophagy inhibitors like 3-methyladenine.³² Thus, the precise molecular pathways mediating APC-induced cell death await identification.

Materials and Methods

Materials and cell lines

SOC-2 and racOMe were synthesized and purified by one of the authors (UM). The APC were micellarily dissolved in water and stored at -80°C

until used. Lomustine was obtained from Medac (Hamburg, Germany). zVAD-fmk and DEVD-amc were obtained from Bachem (Heidelberg, Germany). ALLN was purchased from Calbiochem (Bad Soden, Germany), CA-074Me was from Peptide Institute (Osaka, Japan) and benzyloxycarbonyl-Phe-Ala-fluoromethylketone (zFA-fmk) from Enzyme System Products (Livermore, CA, USA). Ac-RRRXXX-NH₂ was synthesized and purified by S Stefanovic (Institute of Immunology, Tübingen, Germany). Dichlorodihydrofluorescein diacetate (H₂DCFDA), Fluo-3AM, LysoTracker-Red, BODIPY-C5-ceramide and BODIPY-brefeldin A were from Molecular Probes (Leiden, The Netherlands), monodansylcadaverin, iodoacetate, E-64d and all other reagents were obtained from Sigma (Deisenhofen, Germany). pcDNA3-FADD-DN plasmid was a kind gift from VM Dixit (Ann Arbor, MI, USA), and Ad-XIAP was kindly provided by P Liston (Ottawa, Ontario, Canada).¹⁹ The generation of Ad-LacZ has been described.³³ CD95L-containing supernatant was harvested from CD95L-transfected N2A neuroblastoma cells.³⁴ LN-18, LNT-229 and LN-308 human malignant glioma cells were kindly provided by N de Tribolet (Lausanne, Switzerland). SV-FHAS SV40-transformed human astrocytes were provided by A Muruganandam (Ottawa, Canada). All cell lines were maintained in Dulbecco's modified eagle's medium (DMEM) (GIBCO Life Technologies, Eggenstein, Germany) containing 10% fetal calf serum, glutamine (2 mM) and penicillin (100 IU/ml)/streptomycin (100 μg/ml).²⁸ Stably transfected, pooled cells expressing crm-A or BCL-X_L were generated as described.²² BAX-overexpressing LN-18 cells were generated by retroviral infection.³⁵

The respective control cell lines contain neo (FADD-DN, BCL-X_L, BAX) or puro (crm-A) resistance genes only. Stably transfected, pooled cells expressing mutant murine p53^{V135A} were generated as described.²⁰ To overexpress BAK, LN-18 and LNT-229 cells were transiently transfected with pcDNA-BAK using FuGene⁶ (Roche, Mannheim, Germany). The death ligand-resistant cell line, LN-18^R, has previously been characterized.²¹

Cell proliferation and viability assays

The malignant glioma cell lines, LN-18, LNT-229 and LN-308, which differ in their genetic and functional p53 background as well as in their sensitivity to cytotoxic drugs and death ligands,^{28,36} and SV-FHAS cells were seeded at 10⁵ cells/well in 12-well plates or at 10⁴ cells/well in 96-well plates and incubated for up to 72 h with increasing concentrations of APC. Cell density was measured by crystal violet staining. Viability was estimated by counting viable cells via measuring cell size and cell integrity using the photoptical system of a CASY cell counter (CASY, Schärfe Systeme, Reutlingen, Germany).³⁷ For cathepsin inhibitor assays, the cells were seeded at 2 × 10⁴ cells/well in 96-well plates and treated with APC and the inhibitors for 18 h. To overcome poor membrane permeability of Ac-RRRXXX-NH₂, the cells were pretreated for 10 min with hypoosmolar buffer (Eppendorf, Hamburg, Germany) followed by treatment with the cathepsin c inhibitor in hypoosmolar buffer at increasing concentration for 20 min.

Immunoblot analysis

The general procedure has been described, including the preparation of cytoplasmic lysates for the detection of cytochrome *c* and AIF release from mitochondria.³³ The following antibodies were used: anti-actin (I-19, SC1616, Santa Cruz, CA, USA); anti-AIF (E-1, SC13116, Santa Cruz); anti-BAX (N-20, SC-493, Santa Cruz); anti-BAK (G-23, SC-832, Santa Cruz); anti-BCL-X_L (S18, SC694, Santa Cruz); anti-BID (AB1730, Chemicon, Hofheim, Germany); anti-caspase-3 (#C31720, Transduction Laboratories, Lexington, KY, USA); anti-caspase-8 (PH Krammer, Heidelberg, Germany); anti-caspase-7 and -9 (Y Lazebnik, Cold Spring Harbor Laboratory, New York, NY, USA); anti-crm-A (clone A71-1, #556427, Pharmingen-Becton Dickinson, Heidelberg, Germany); anti-cyclo-oxygenase 1(COX1) (C-20, SC1752, Santa Cruz); anti-COX2 (C-20, SC-1745, Santa Cruz); anti-cytochrome *c* (clone 7H8.2C12, #65981A, Pharmingen-Becton Dickinson); anti-FADD (#F36620, Transduction Laboratories); anti-glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) (MAB374, Chemicon); anti-PARP (clone 4C10-5, #556494, Pharmingen-Becton Dickinson) and anti-XIAP (P Liston, Ottawa, Ontario, Canada).

Fluorescence microscopy

To detect autophagosomes, the cells were grown on poly-(L)-lysine covered microslides and stained with 0.1 mM monodansylcadaverine at 37°C for 60 min. The cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Fluorescence was detected with an Axiovert 135 microscope (Zeiss, Cologne, Germany). Excitation illumination was from an argon laser (488 nm) and fluorescence was detected using a 515 nm filter. For the staining of lysosomes (LysoTracker-Red), endoplasmic reticulum (BODIPY-Brefeldin A) and golgi vesicles (BODIPY-C₅-ceramide), living cells were stained with the appropriate fluorescence dyes and analyzed according to the manufacturer's instruc-

tions. Fluorescence was detected with a Nikon Eclipse TS-100 microscope (Düsseldorf, Germany).

AnxV staining

Alterations in membrane structure were assessed by double staining cells with anxV (Becton Dickinson) and PI. Briefly, the cells were trypsinized, harvested into ice-cold complete medium containing 10% fetal calf serum and centrifuged for 10 min at 4°C. The cells were washed twice with anxV binding buffer (10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), stained with FITC-labelled anxV (1 µg/ml in binding buffer) for 20 min at 20°C, washed twice, counterstained with PI and analyzed in a Becton Dickinson FACScalibur cytometer using the CellQuest analysis program.

Electron microscopy

Cells treated as indicated were fixed in 2.5% glutaraldehyde in Hank's modified salt solution, postfixed in 1% OsO₄ in 0.1 M cacodylate buffer, scraped off and dehydrated in a series of ethanol. The 70% ethanol step was saturated with uranyl acetate for contrast enhancement. Dehydration was completed in propylene oxide and the specimens were embedded in Araldite (Serva, Heidelberg, Germany). Ultrathin sections were produced on an FCR Reichert Ultracut ultramicrotome (Leica, Bensheim, Germany), mounted on pioloform-coated copper grids and contrasted with lead citrate. Specimens were analyzed and documented with an EM 10A electron microscope (Zeiss, Oberkochen, Germany).

DEVD-amc-cleaving caspase activity assay

The cells (2 × 10⁴/well) were seeded on microtiter plates, allowed to attach, treated with the APC for 12–24 h and lysed in 25 mM Tris-HCl, pH 8.0, 60 mM NaCl, 2.5 mM EDTA and 0.25% Nonidet-P40 for 10 min. DEVD-amc was added at 12.5 µM and fluorescence was measured at 1–6 h after lysis on a cytofluor II fluorimeter (Perkin-Elmer, Weiterstadt, Germany) at 360 nm excitation and 480 nm emission wavelengths.²¹

ATP assay

The cells (2.5 × 10⁴/well) were seeded in microtiter plates, exposed to the APC and lysed in an ATP releasing agent (Sigma) at defined time points. ATP was determined by luciferase assay with the CLS II kit (Boehringer Mannheim, Mannheim, Germany).³⁸ Hypoxia was induced by incubating the cells in Becton Dickinson anaerobic system gaspack pouches for 12 h.³⁹ The ATP content was normalized to percentages of viable cells determined by crystal violet staining.

Mitochondrial membrane potential (ΔΨ_m)

ΔΨ_m was assessed using the mitochondrial membrane potential detection kit (Biocarta, Hamburg, Germany), which uses the cationic dye JC-1 (5,5',6,6'-tetraethyl-benzimidazolylcarbocyanine iodide) to signal changes in the mitochondrial membrane potential. In healthy cells, the dye stains the mitochondria bright red. The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye to enter the mitochondrial matrix where it accumulates. After exceeding a critical concentration, JC-1 forms aggregates which exhibit red fluorescence. Under conditions where ΔΨ_m collapses, JC-1 cannot accumulate within the mitochondria, but remains in the cytoplasm in a green fluorescent monomeric form.⁴⁰ In these assays, the cells were analyzed by flow

cytometry gated to viable cells (FSC/SSC) and relative counts of green fluorescent cells were obtained. As a control for $\Delta\Psi_m$ breakdown, the cells were treated for 4 h with 100 U/ml CD95L for CD95L-sensitive cells or for 24 h with 200 μ M lomustine for CD95L-resistant cells.

Detection of intracellular free radicals

The cells were treated with APC at increasing concentrations or for different time periods. As a control, the cells were treated with ethacrinic acid.⁴¹ To assess the generation of free radicals, the cells were washed with PBS and incubated with H₂-DCFDA (5 μ M) for 15 min. The cells were washed with PBS again, trypsinized and harvested by centrifugation. Cell pellets were resuspended in PBS, counterstained with PI to detect necrotic cells and viable cells defined by PI exclusion were analyzed by flow cytometry.

Detection of free intracellular calcium levels

For the detection of free intracellular calcium ions, the cells were treated with APC or vehicle for increasing time periods. Fluo-3AM was added for 30 min. The cells were scraped off, washed with PBS and resuspended in PBS containing PI (50 μ g/ml). The signal from Fluo-3 bound to Ca²⁺ was analyzed by flow cytometry using channel FI-1 H. Dead (PI-positive) cells were eliminated from analysis.

Statistical analysis

The figures show representative data from three separate experiments with similar results. Data were assessed for significant differences using *t*-test (**P* < 0.05, ***P* < 0.01).

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