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Research Article

Anti-glioma action of aloe emodin: the role of ERK inhibition

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Abstract. The effect of aloe emodin (AE), a herbal anthraquinone derivative, on the rat C6 glioma cell line was investigated. In addition to cell cycle block and caspase-dependent apoptosis, AE led to the formation of intracytoplasmic acidic vesicles indicative for autophagic cell death. Moreover, differentiation of surviving cells toward the astrocytic lineage was confirmed by typical morphological changes and increased expression of glial fibrillary acidic protein (GFAP). AE did not affect the activation of mitogen-activated protein kinase p38, Jun-N-terminal kinase, or transcription factor NF-κB, but markedly inhibited the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in C6 cells. A selective inhibitor of ERK activation, PD98059, mimicked the effects of AE on glioma cell morphology and GFAP expression, but failed to induce either apoptosis or autophagy. Taken together, these results indicate that the anti-glioma action of AE involves ERK-independent induction of both apoptosis and autophagy, as well as ERK inhibition-mediated differentiation of glioma cells.

Key words. Aloe emodin, glioma, apoptosis, autophagy, differentiation, ERK.

Induction of tumor cell death and/or differentiation by certain phytochemicals present in medicinal herbs and dietary plants is one of the most attractive approaches in cancer chemotherapy. Emodin, a natural anthraquinone derivative from herbs used in Chinese traditional medicine, has been shown to exert a potent anti-tumor effect both in vitro [1–6] and in vivo [7]. The anti-cancer action of emodin was shown to involve protein tyrosine kinase (PTK) inhibition-mediated growth arrest in ras-transformed bronchial epithelial cells [1] or breast cancer cells overexpressing the HER-2/neu oncogene [2], as well as caspase-dependent apoptotic death of human lung carcinoma cells [4], promyeloleukemic HL-60 cells [5], or cervical cancer cells [6]. In addition, emodin was reported to senzitize cancer cells to various chemotherapeutic drugs [7, 8] and to suppress HER-2/neu-induced cellular transformation and metastasis-associated properties [9]. Importantly, the data obtained with HER-2/neu-transformed breast cancer cells indicate that induction of differentiation toward more mature cells with reduced growth capacity might be one of the mechanisms for the anti-tumor action of emodin [2]. Aloe emodin (AE), a hydroxyanthraquinone from *Aloe vera* leaves that is structurally very similar to emodin, was also able to induce cell death in several tumor cell lines, including human lung carcinoma and hepatoma cell lines [10–12]. Interestingly, AE expressed a special affinity for neuroectodermal tumor cell lines, resulting in an efficient inhibition of human neuroblastoma growth in mice with severe combined immunodeficiency without any appreciable toxic effects [13].

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According to several authors [10–13], the cytotoxic mechanism of AE mainly involves the induction of caspase-dependent apoptosis, whereas the greater sensitivity of neuroectodermal tumor cell lines could be related to an energy-dependent pathway of drug incorporation [13].

Among neuroectodermal tumors, gliomas are the most aggressive and represent the most common primary malignancy in the human central nervous system [14]. Despite many technologic advances in neuroimaging, neurosurgery, radiation therapy and chemotherapy, gliomas remain incurable in most cases. An increased activity of proliferation-promoting signaling pathways downstream of growth factor receptors, including those controlled by mitogen-activated protein kinases (MAPKs), has been proposed to be at least partly responsible for uncontrolled growth and invasive properties of glioma cells [15, 16].

Our recent study clearly demonstrated the in vitro antiproliferative effect of AE in the rat glioma cell line C6, while primary astrocytes were mainly resistant to AE action [17]. This prompted us to examine whether the observed effect of AE might involve cell death and/or differentiation of glioma cells. In addition, a possible role of the MAPK signaling pathway in the anti-glioma action of AE was examined.

Materials and methods

Cells and reagents

All chemicals used throughout the present study were products of Sigma (St. Louis, Mo.), unless specified otherwise. The rat glioma cell line C6 and human glioma cell line U251 were a kind gift from Dr. P. Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). C6 cells were a late-passage (>70 passages) subclone of the original CCL107 clone (ATCC). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2, in 25-cm^2 tissue culture flasks containing HEPES-buffered RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamine, 0.01% sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and antibiotics (culture medium). The cells were used for experiments after a conventional trypsinization procedure. AE stored at –20 °C at 200 µM in DMSO was diluted with culture medium immediately before use. Control cell cultures contained DMSO corresponding to its content in the solution with the highest concentration of AE used in a particular experiment.

MTT, crystal violet, and lactate dehydrogenase release assay

The mitochondrial-dependent reduction of MTT to formazan reflects the mitochondrial activity of cultured cells [18], the intensity of crystal violet staining is directly proportional to the number of adherent cells [19], while the release of cytosolic lactate dehydrogenase (LDH) indicates the loss of membrane integrity that occurs in necrotic cells [20]. The cells were seeded in flat-bottom 96-well plates at 3×10^4 cells/well in a final volume of 200 µl of culture medium containing different agents, and the assays were performed exactly as previously described [18–20]. Mitochondrial-dependent production of formazan and crystal violet absorption by adherent cells were assessed by an automated microplate reader at 570 nm, while the pyruvate-mediated conversion of 2,4-dinitrophenylhydrazine into visible hydrazone precipitate in the LDH assay was measured at 492 nm. The results are presented as percent of control values obtained in untreated cultures.

Cell cycle and apoptosis analysis by propidium iodide staining

For flow cytometric analysis of the cell cycle, the cells (1×10^6 per sample) incubated in 60-mm Petri dishes were scraped in cold PBS-EDTA, washed in PBS and fixed in 70% ethanol for 30 min at 4 °C. After washing with PBS, the cells were incubated with the DNA-binding dye propidium iodide (PI; 40 µg/ml) and RNase (1.0 mg/ml) for 30 min at 37 °C in the dark. Finally, the cells were washed and red fluorescence analyzed by a FACSCalibur flow cytometer (BD, Heidelberg, Germany), using a peak fluorescence gate to discriminate aggregates. Cell distribution among the cell cycle phases was determined by Cell Quest Pro software (BD). To assess apoptosis-associated morphological changes, cells incubated in slide chambers (3×10^4/well) were fixed for 15 min at ambient temperature with 4% paraformaldehyde and stained with 20 µg/ml PI in 0.1% Triton X-100, 0.1 mM EDTA pH 8.0, and 50 µg/ml RNase in PBS. Afterwards, the cells were thoroughly washed with PBS, covered with 50% glycerol in PBS, and the morphology of PI-stained red-fluorescent nuclei was analyzed under the fluorescence microscope.

Acridine orange staining of autophagic vesicles

The acidic autophagic vesicles were visualized by supravital acridine orange staining as previously described [21]. Briefly, cells (3×10^4/well) cultivated on glass chamberslides were washed with PBS after the treatment and stained with acridine orange (1.0 µM; Labo-Moderna, Paris, France) for 15 min at 37 °C. Subsequently, the cells were washed and analyzed under a fluorescence microscope. Depending on their acidity, autophagic lysosomes appeared as yellow-orange to bright-red fluorescent cytoplasmic vesicles, while nuclei were stained green.

Immunocytochemistry analysis of glial fibrillary acidic protein expression

For immunocytochemistry analysis of glial fibrillary acidic protein (GFAP) expression, the cells (3×10^4/well)

cultivated on glass chamber-slides were fixed in 4% paraformaldehyde for 15 min at ambient temperature. After cell membrane permeabilization with 0.5% Triton X-100 for 30 min, the activity of endogenous peroxidase was blocked with 3% H_2O_2 in 10% methanol/PBS for 5 min. Following 1 h incubation with 5% mouse serum, primary mouse anti-GFAP antibody (Bio-Yeda, Rehovot, Israel) was added for an additional hour (1:300 in PBS). The detection was performed with a mouse extravidin-peroxidase staining kit according to the manufacturer's instructions (Sigma). Finally, the slides were incubated for 2–3 min with diaminobenzidine (R&D Systems, Minneapolis, Minn.) as a substrate, counterstained with Mayer's hematoxylin and mounted with 50% glycerol.

Cell-based ELISA

A slightly modified method for cell-based ELISA by Versteeg et al. [22] was used to measure the expression of GFAP and galactocerebroside, as well as the activation of MAPKs [p38 MAPK, extracellular signal-regulated kinase (ERK) and Jun-N-terminal kinase (JNK)] or nuclear factor-κB (NF-κB). Since MAPKs are activated by phosphorylation, and phosphorylation of the inhibitory subunit IκB precedes the NF-κB activation, we used antibodies specific for phosphorylated forms of MAPKs (p-p38, p-ERK and p-JNK) and IκB (p-IκB). After incubation with various agents in 96-well flat-bottom plates (3×10^4 cells/well), the cells were fixed in 4% paraformaldehyde, endogenous peroxidase was quenched with 1% H_2O_2 in PBS containing 0.1% Triton X-100 (PBST) and unspecific binding of antibodies blocked with PBST solution containing 10% FCS. Primary mouse monoclonal antibodies specific for rat/mouse p-ERK, p-p38, p-JNK, p-IκB (1:200; all from Santa Cruz Biotechnology, Santa Cruz, Calif.), GFAP (1:200) and galactocerebroside (O1; 1:100; Boehringer Mannheim, Mannheim, Germany) were introduced in PBST supplemented with 2% bovine serum albumin (PBSTB), followed by secondary peroxidase-conjugated goat anti-mouse IgG (1:2500 in PBSTB; USB Corporation, Cleveland, Ohio) for anti-p-ERK, anti-p-JNK, anti-p-IκB and anti-GFAP, or anti-mouse IgM (1:4000; USB Corporation) for the detection of anti-p-p38 and O1. Both incubations were performed at 37 °C for 1 h. The absorbance at 450 nm was measured in an automated microplate reader 15 min after the incubation with peroxidase substrate TMB and subsequently upon addition of 0.1 M HCl. To enable comparison between different treatments, the obtained absorbances were corrected for the cell number that was determined by crystal violet staining, as described in the original protocol. The results are presented as relative expression in comparison with the control value, which was arbitrarily set to 1.

Statistical analysis

The results are presented as means ± SD of triplicate observations from one representative of at least three experiments with similar results, except if indicated otherwise. The significance of the differences between various treatments was assessed by analysis of variance (ANOVA), followed by a Student-Newman-Keuls test. A p value less than 0.05 was considered significant.

Results

AE induces apoptosis and autophagy in the C6 glioma cell line

The results of the MTT test presented in figure 1A confirmed our recent observation that AE treatment significantly reduced the mitochondrial respiration in cultured C6 cells [17]. This anti-glioma action of AE coincided with a reduction in tumor cell number, as revealed by crystal violet staining of adherent tumor cells in the present study (fig. 1A), as well as in the previous report [17]. We therefore assumed that AE could induce glioma cell death and/or suppress proliferation. The LDH release assay has frequently been used to distinguish between the two major types of cell death, necrosis or apoptosis (type I programmed cell death. Namely, a significant damage of the cellular membrane and consequent release of intracellular LDH occurs mainly during necrosis, but not apoptosis [23, 24]. However, the observed effect of AE was apparently not due to cell necrosis, as the LDH release assay did not show a significant increase in cell membrane permeability of AE-treated cells (fig. 1A). On the other hand, the cell cycle analysis revealed the accumulation of AE-treated cells in the S phase and sub-G (hypodiploid) compartment, suggesting a cell cycle block accompanied by induction of apoptotic cell death (fig. 1B). Chromatin condensation, a typical morphologic feature of apoptosis, was readily apparent in AE-treated cells stained with PI (fig. 1C). However, the toxicity of AE was only partly blocked in the presence of the pan-caspase inhibitor VAD-fmk (fig. 1D), suggesting that apoptosis was not solely responsible for the anti-glioma action of AE. This led us to search for type II programmed cell death – autophagy – which is characterized by the formation of acidic vesicular organelles that can be visualized by acridine orange supravital staining [21]. Indeed, numerous bright-red fluorescent acidic vesicles were observed in 50–60% of C6 cells exposed to AE for only 6 h (fig. 1E). Thus, the anti-glioma action of AE apparently involved a cell cycle block accompanied by both apoptosis and autophagy.

AE induces differentiation of C6 cells

Microscopic observation of the glioma cells that survived AE treatment revealed a major change in their morphol-

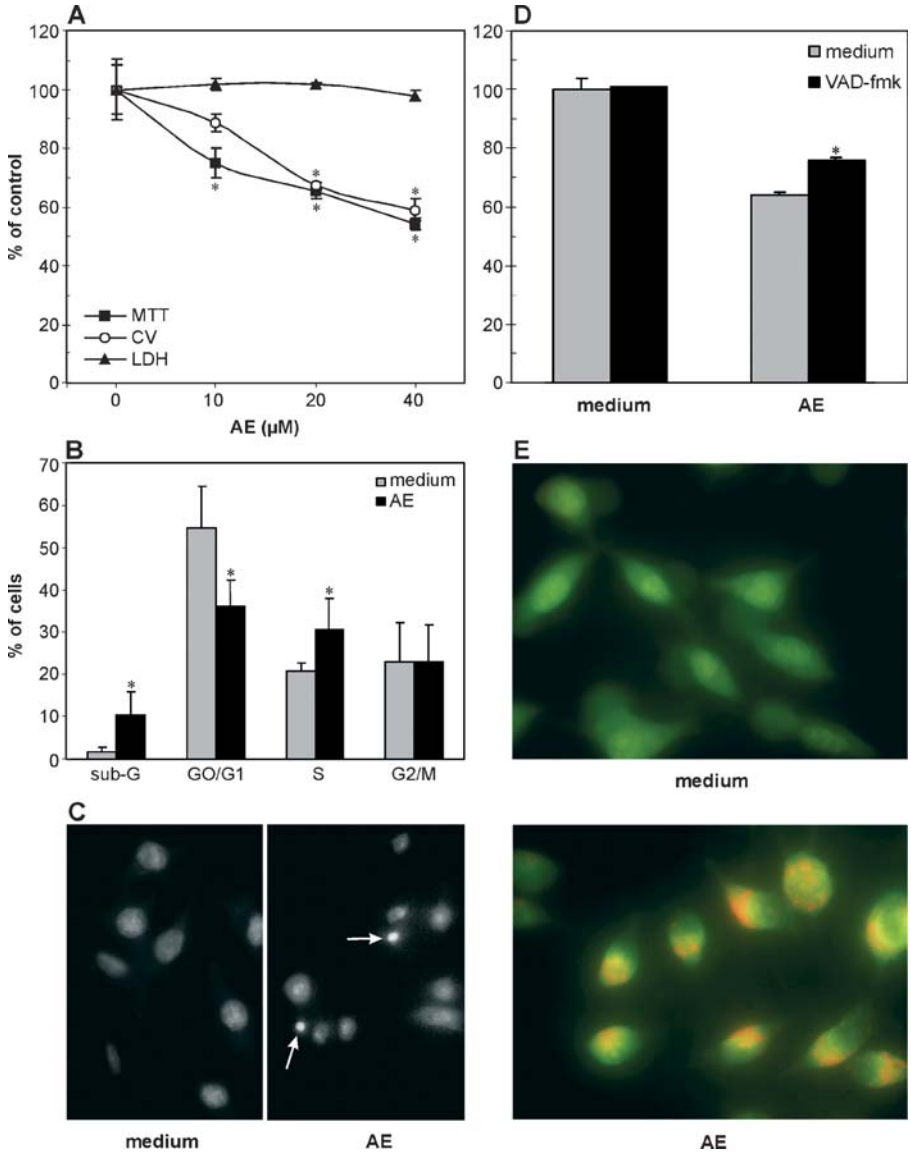

Figure 1. Cytotoxicity of AE toward C6 cells. (*A*) C6 cells were incubated with different doses of AE for 24 h, and an MTT, crystal violet or LDH release assay were performed (*p < 0.05). (*B*) After 24 h incubation with 20 μM AE, C6 cells were stained with PI and the cell cycle analyzed by flow cytometry. The values are means ± SD from five separate experiments (*p < 0.05). (*C*) C6 cells were treated with 20 μM AE for 24 h and nuclear morphology characteristic of apoptosis (arrows) was observed by fluorescence microscopy after PI staining. (*D*) C6 cells were incubated for 24 h with 20 μM AE, in the absence or presence of a pan-caspase inhibitor VAD-fmk (0.5 μM), and the MTT test was performed (*p < 0.05). (*E*) After 6 h incubation with AE (20 μM), C6 cells were stained with acridine orange for the presence of orange-red acidic autophagic vesicles.

ogy. Unlike the mainly polygonal morphology of control, untreated glioma cells, the shape of AE-treated cells was similar to that of mature astrocytes, with smaller cell bodies and much longer, fine, tapering processes (fig. 2A). As this indicated that a surviving fraction of AE-treated C6 cells entered the maturation process toward the astrocytic lineage, we further examined whether morphological changes were accompanied by the expression of GFAP, a well-established marker of mature astrocytes. Indeed, both immunocytochemical analysis and cell-based ELISA confirmed a significant up-regulation of GFAP expression in AE-treated compared to control C6 cells (fig. 2B, C). At the same time, the reactivity of C6 cells with the oligodendrocyte-specific O1 antibody was markedly reduced upon AE treatment (fig. 2C), thus further indicating the commitment of C6 cells toward the astrocytic lineage.

The role of ERK inhibition in anti-glioma action of AE

As both the MAPK pathway and the transcription factor NF-κB participate in a wide range of cellular programs controling proliferation, differentiation and survival [25, 26], we examined the influence of AE on the activation of

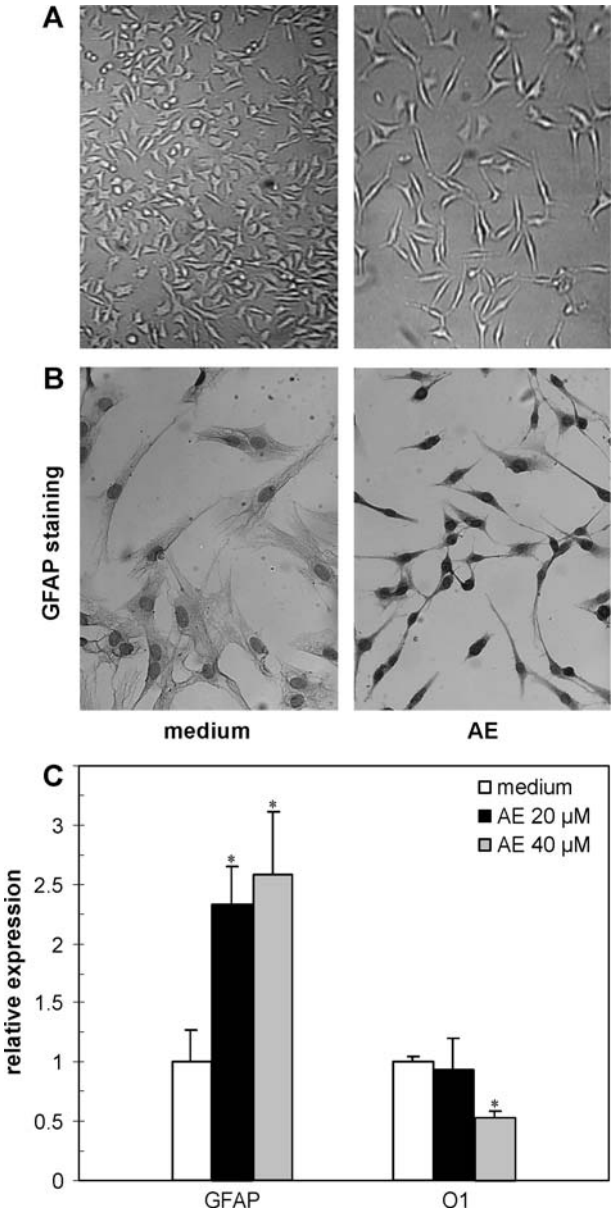

Figure 2. AE induces differentiation of C6 cells. (*A–C*) C6 cells were incubated with 20 μM AE for 24 h and their morphology was analyzed by inverted microscopy (*A*). GFAP expression was analyzed by immunocytochemistry as described in Materials and methods (*B*). The expression of GFAP and galactocerebroside was assessed by cell-based ELISA with anti-GFAP and O1 antibodies, respectively, as described in Materials and Methods (*p < 0.05) (*C*).

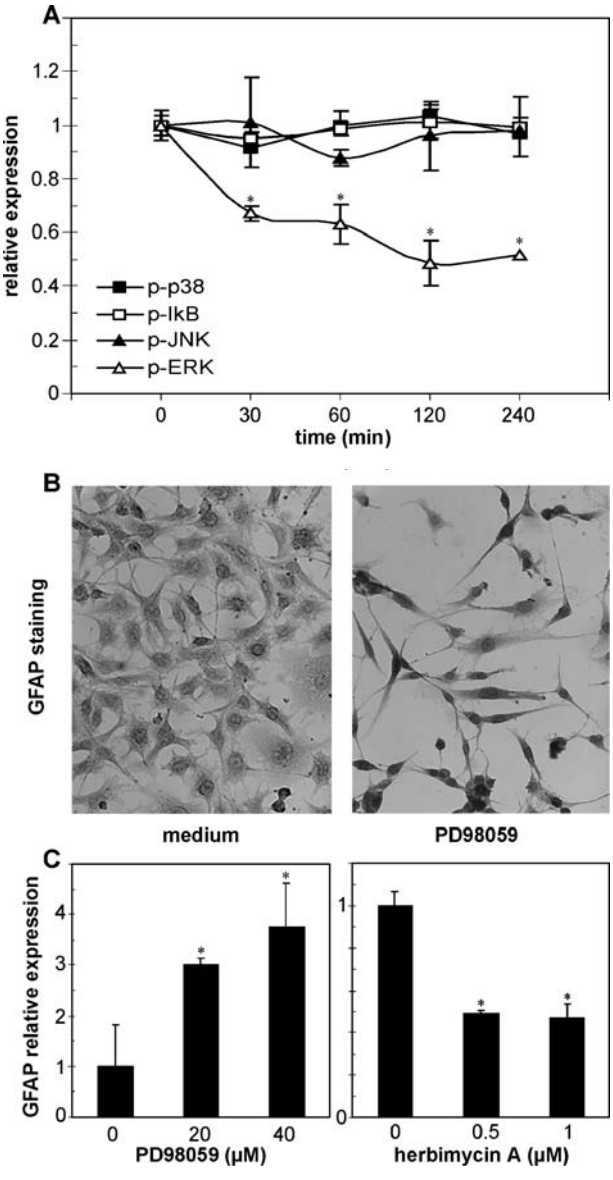

Figure 3. ERK inhibition is involved in AE-induced differentiation of C6 cells. (*A*) C6 cells were incubated with 20 μM AE and activation of ERK, p38 MAPK, JNK or NF-κB was assessed by cell-based ELISA at the indicated time points (*p < 0.05). (*B*) Immunocytochemistry staining for GFAP expression was performed after 24 h incubation with the ERK activation inhibitor PD98059 (40 μM). (*C*) GFAP expression was analyzed by cell-based ELISA in C6 cells treated with PD98059 or the PTK inhibitor herbimycin A for 24 h (*p < 0.05).

these important signaling molecules in C6 cells. While AE did not affect the expression of either phosphorylated, active forms of MAPKs, p38 and JNK, or the phosphorylated NF-κB inhibitory subunit IκB, it significantly reduced the level of phospho-ERK in C6 cells, as determined by cell-based ELISA (fig. 3A). The inhibition of ERK activity was evident already after 30 min exposure to AE, and persisted for at least 4 h (fig. 3A). To assess a possible contribution of ERK inhibition to the observed effects of AE on C6 glioma cells, we used PD98059, a fairly specific inhibitor of ERK1/2 that acts by inhibiting the activity of its upstream activator MEK [27]. Preliminary experiments showed that 1-h treatment with 20 or 40 μM PD98059 reduced the intracellular level of activated ERK to 35 or 25% of control values, respectively, as assessed by cell-based ELISA (not shown). While the crystal violet assay revealed that 24-h incubation with PD98059 only slightly, but significantly, reduced the

number of C6 cells (100 ± 3% vs 79.5 ± 4.5% in control vs PD98059-treated cultures, $p < 0.01$), this inhibitor of ERK activation caused elongation of cellular processes (fig. 3B) similar to that previously observed upon AE treatment. Moreover, the expression of GFAP in C6 cells was markedly up-regulated in the presence of PD98059, as judged by both immunocytochemistry analysis (fig. 3B) and cell-based ELISA (fig. 3C). In contrast, the PTK inhibitor herbimycin A not only failed to mimic the AE-mediated increase in GFAP expression, but even significantly reduced GFAP levels in C6 cells (fig. 3C). While herbimycin A had a slight anti-proliferative effect on C6 cells similar to that observed with PD98059 (data not shown), neither herbimycin nor PD98059, applied separately or in combination, were able to induce either apoptosis or autophagy in C6 cells, as revealed by PI or

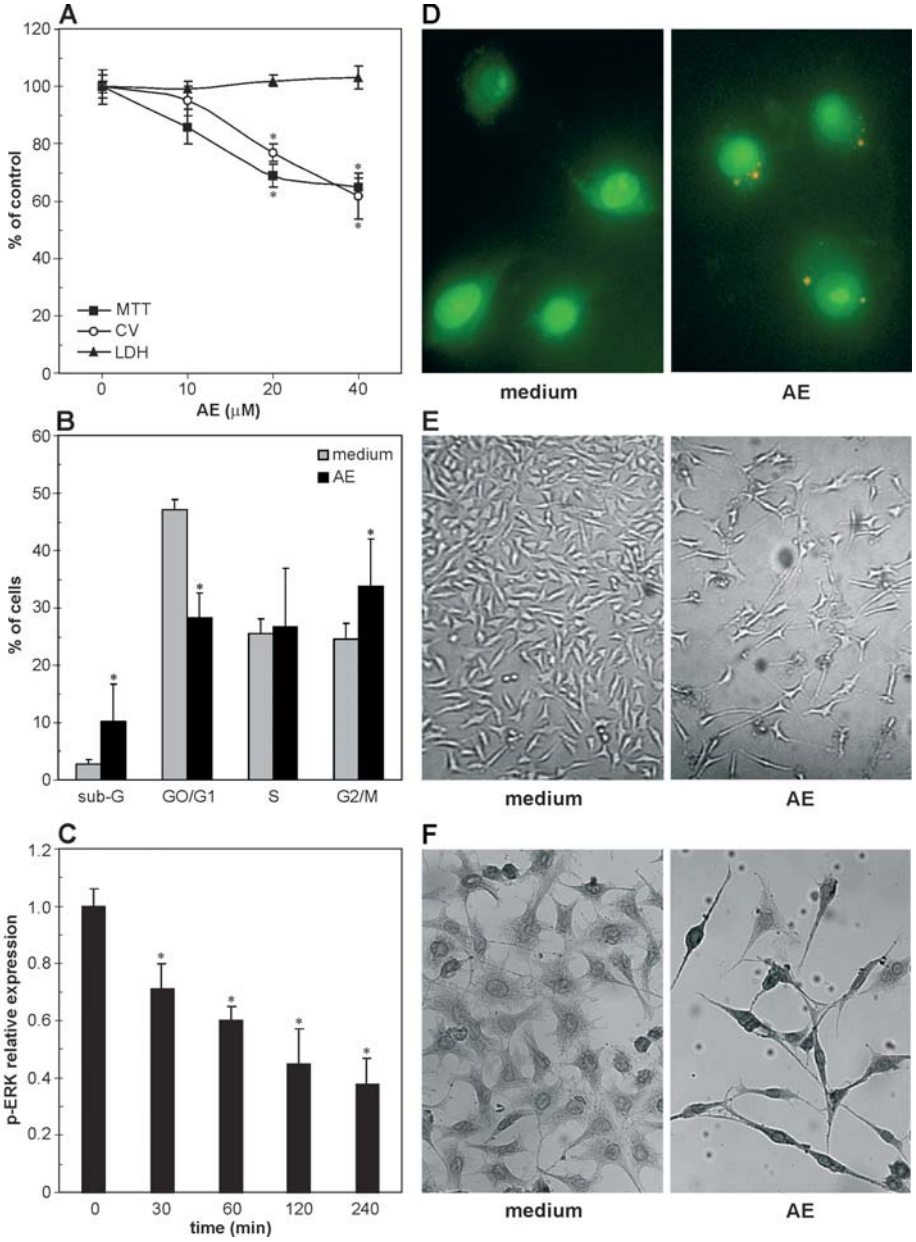

Figure 4. The effects of AE on U251 glioma cells. (*A*) U251 cells were incubated with different AE doses and an MTT, crystal violet or LDH assay was performed after 24 h. (*B*) U251 cells were incubated with or without AE (20 μM) for 24 h and the cell cycle was analyzed by flow cytometry after PI staining. The values are means ± SD from five separate experiments (*$p < 0.05$). (*C*) U251 cells were incubated with or without AE (20 μM) and the amount of activated ERK was analyzed by cell-based ELISA (*$p < 0.05$). (*D*) U251 cells were incubated with or without AE (20 μM) for 6 h and the autophagy was assessed by supravital acridine orange staining followed by fluorescence microscopy. (*E, F*) After 24 h incubation of U251 cells with AE (20 μM), their morphology was examined (*E*), or immunocytochemistry staining for GFAP expression performed (*F*).

acridine orange staining, respectively (not shown). Taken together, these data indicate that ERK inhibition probably participated in AE-induced differentiation of C6 cells, while the induction of apoptosis and autophagy was independent of the drug interference with either ERK or PTK activity.

The effect of AE on U251 glioma cells

Finally, to exclude the possibility that AE-induced changes were restricted to C6 cells, we examined the effects of AE treatment on the human glioma cell line U251. Similar to the results obtained with C6 cells, the incubation of U251 cells with AE led to a significant decrease in cell number, as assessed by the MTT and crystal violet test, without affecting cell membrane integrity measured by LDH release (fig. 4A). This was accompanied by an increase in the number of hypodiploid apoptotic cells (sub-G) and the cell cycle block in G2/M phase (fig. 4B). The ability of AE to interfere with ERK activation in U251 cells was confirmed by cell-based ELISA, which showed that the intracellular level of the phosphorylated, active form of ERK1/2 was markedly reduced upon incubation of U251 cells with AE (fig. 4C). Furthermore, the appearance of acidic autophagic vesicles was readily observed in the cytoplasm of more than 50% of U251 cells treated with AE (fig. 4D). Finally, AE treatment triggered differentiation of U251 cells toward the astrocytic lineage, as judged by pronounced elongation of cellular processes (fig. 4E) and more intensive staining for the astrocyte marker GFAP (fig. 4F).

Discussion

The results of the present study demonstrated for the first time the ability of AE, a herbal anthraquinone derivative with potential anti-cancer therapeutic activity, to induce apoptosis, autophagy and differentiation of glioma cells. Maturation toward the astrocytic lineage, but not cell death, was at least partly mediated by the interference of the drug with the constitutive activity of the ERK1/2 pathway in glioma cells.

Apoptosis (programmed cell death type I), typically characterized by caspase-dependent DNA and cell disintegration into small fragments, is the best-defined cell death program induced by various anti-cancer treatments [28]. However, some authors have indicated recently that certain drugs or therapeutic procedures, including arsenic trioxide [29], tamoxifen [30] and ionizing radiation [21], could also lead to programmed cell death type II, autophagy, in which the engulfment of cytoplasm and/or cytoplasmic organelles by multiple-membrane cytoplasmic vesicles leads to their destruction by the lysosomal system of the same cell [31]. The anti-tumor potential of emodin and AE has usually been associated with their ability to induce a cell cycle block accompanied by caspase-mediated tumor cell apoptosis [4–6, 10–13]. Although similar, the results obtained throughout the present study strongly indicate that AE is also capable of triggering autophagy in glioma cells, as demonstrated by the presence of numerous acidic vesicles in more than 50% of AE-treated C6 and U251 cells. However, since we did not follow the fate of autophagic cells, how and to what extent could autophagy contribute to the AE-mediated reduction in glioma cell number is still unknown. Since in some settings, autophagy might actually serve as a protective mechanism against ongoing apoptosis [29], a similar scenario for autophagy involvement in the anti-glioma effect of AE deserves further exploration. Regarding the putative inability of AE to induce necrosis, one might argue that spontaneous LDH release should decrease with lowering the cell number and that unchanged LDH release coupled with reduced cell number, as in our study, would actually mean an increased LDH release *per cell*. However, since spontaneous LDH release in our experiments was marginal, due to the very low spontaneous necrosis of glioma cells (data not shown), our data indeed seem to reflect a genuine absence of necrotic cell death in AE-treated gliomas.

In addition to cell cycle block, apoptosis and autophagy, AE-treated glioma cells displayed dramatic morphological changes indicative of their transition to a more mature state. This was confirmed by an increased expression of GFAP, a 50-kDa type III intermediate filament protein considered to be a reliable differentiation marker of normal astrocytes and tumors of astrocytic lineage [32]. A recent study reported that C6 glioma cells, under the influence of saikosaponin A, could also acquire an oligodendrocyte phenotype characterized by an increase in oligodendrocyte-specific enzyme 2′,3′-cyclic nucleotide 3′-phosphohydrolase [33]. However, as in our experiments the GFAP increase was accompanied by a concomitant reduction in the reactivity with the oligodendrocyte-specific anti-galactocerebroside antibody O1 [34], AE-exposed C6 cells appear to have been committed to the astrocytic, rather than oligodendrocyte lineage. This might be due to the fact that, in contrast to the cells in early passages (<30), the late-passage (>70) C6 cells used in the present study displayed a mainly astrocytic phenotype [35] and lost their ability to differentiate into oligodendrocytes [36]. As the C6 cell line has also been shown to contain progenitors of radial [37] and neuronal stem cells [38], of interest would be to examine whether these progenitor cells could survive the AE treatment and differentiate into GFAP-positive astrocytes.

There is a question of mechanism(s) underlying the anti-glioma effects of AE observed in the present study. Emodin, which is structurally very similar to AE, is well known for its ability to inhibit the activity of various ty-

rosine kinases [1, 39, 40]. The inhibition of HER-2/neu tyrosine kinase seems particularly important for the anti-cancer effect of emodin, as it was associated with suppressed transformation and differentiation of breast cancer cells [2], as well as sensitization of breast and lung cancer cells to chemotherapeutic drugs [7, 8]. However, a suppression of PTK activity with herbimycin A did not mimic AE-induced differentiation of C6 glioma cells in our study, and even caused a significant down-regulation of the astrocyte marker GFAP in these cells. Similarly, Roymans et al. [41] described herbimycin-mediated reduction of GFAP mRNA and protein expression in C6 cells differentiated with cAMP analogs or β-adrenergic receptor agonists. Our findings, together with those of Roymans et al. [41], indicate a positive role for PTK in controlling GFAP expression, and argue against the PTK inhibition as a mechanism for the AE-induced GFAP increase in C6 cells. On the other hand, emodin has been recently shown to inhibit the activation of ERK, p38 MAPK, JNK and NF-κB in cancer cell lines [42]. In our experiments, however, AE potently inhibited the activation of ERK, but did not significantly affect the activation of other MAPKs or NF-κB in C6 glioma cells. Importantly, a selective blockade of ERK activation with PD98059 mimicked the effects of AE on glioma cell morphology and GFAP expression, suggesting that suppression of ERK activation might at least partly account for AE-induced differentiation of glioma cells. Accordingly, PD98059 treatment has recently been reported to enhance GFAP expression and to synergize with oncostatin M for the induction of differentiation in the 86HG39 glioblastoma cell line [43]. Furthermore, the finding that an increase in intracellular cAMP is one of the most potent signals directing C6 cell differentiation toward the astrocytic lineage [44–46] and is also a strong inhibitor of ERK activity in these cells [47–49], indicates a possible role for ERK in the negative regulation of GFAP expression and glioma cell differentiation. Finally, in our experiments, both herbimycin A and PD98059 reduced the number of C6 cells to some extent, which is consistent with previous findings that increased PTK/ERK activity in response to various growth factors might participate in uncontrolled proliferation of glioma cells [15, 16]. However, neither herbimycin A or PD98059, nor their combination, was able to induce apoptotic or autophagic cell death, indicating that PTK and ERK inhibition, while possibly contributing to AE-mediated growth arrest, were completely dispensable for its ability to trigger apoptosis and autophagy in glioma cells. On the other hand, emodin has been recently reported to inhibit the activity of PI3-kinase [50], which provides a universal cell survival signal by inhibiting both apoptosis and autophagy [51, 52]. Thus, further studies on a possible involvement of PI3-kinase inhibition in the anti-glioma action of AE seem to be justified.

In conclusion, we have described multiple effects of the anthraquinone derivative AE on glioma cells which might be of therapeutic interest, and which include the induction of cell growth arrest accompanied by apoptosis, autophagy and astrocytic differentiation. While AE-triggered apoptosis and autophagy were independent of either PTK or ERK activity, inhibition of the ERK pathway was probably responsible for the differentiation of glioma cells. The ability of AE to trigger both caspase-dependent apoptosis and caspase-independent autophagy deserves special attention, because drugs possessing back-up mechanisms for inducing tumor cell death are considered as potential 'magic bullets' in anti-cancer therapy [31]. Future studies, however, must extend these findings to in vivo tumor models and examine a possible contribution of autophagic cell death or tumor cell differentiation to the therapeutic efficacy of AE in glioma treatment.

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