Cytoplasmic condensation is both necessary and sufficient to induce apoptotic cell death

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Summary

Programmed cell death (apoptosis) is important in tissue maintenance. Hallmarks of apoptosis include caspase activation, DNA fragmentation and an overall reduction in cell volume. Whether this apoptotic volume decrease (AVD) is a mere response to initiators of apoptosis or whether it is functionally significant is not clear. In this study, we sought to answer this question using human malignant glioma cells as a model system. In vivo, high grade gliomas demonstrate an increased percentage of apoptotic cells as well as upregulation of death ligand receptors. By dynamically monitoring cell volume, we show that the induction of apoptosis, via activation of either the intrinsic or extrinsic pathways with staurosporine or TRAIL, respectively, resulted in a rapid AVD in D54-MG human glioma cells. This decrease in cell volume could be prevented by inhibiting the efflux of Cl^- through channels. Such suppression of AVD also reduced the activation of caspases 3, 8 and 9 and suppressed DNA fragmentation. Importantly, experimental manipulations that reduce the cell volume to 70% of the original volume for periods of at least 3 hours were sufficient to initiate apoptosis even in the absence of death ligands. Hence, this data suggests that cell condensation is both necessary and sufficient for the induction of apoptosis.

Key words: KCC, Apoptosis, Apoptotic volume decrease (AVD), Cell condensation, Chloride channels, Glioma

Introduction

Cell death is an integral part of normal tissue and organ development and in many organs cell death occurs throughout life. There are many forms of cell death (Nicotera et al., 1999; Van Cruchten and Van Den Broeck, 2002), but programmed cell death or apoptosis is the most common form as it results in minimal inflammation to the surrounding tissue. For this reason, apoptosis has been extensively studied in recent years and both the induction of apoptosis and the underlying signaling cascades are well understood (Thorburn, 2004; Van Cruchten and Van Den Broeck, 2002).

It is generally accepted that apoptosis engages one of two distinct pathways. The intrinsic pathway is activated when an injury occurs inside of the cell, such as DNA or mitochondrial damage. The release of factors from the mitochondria and formation of the apoptosome leads to the activation of caspase 9 (Casp9). This pathway culminates in the activation of the principal effector caspase, caspase 3 (Casp3), DNA fragmentation and the formation of apoptotic bodies. One of the most commonly used drugs to initiate apoptosis via the intrinsic pathway is staurosporine.

The extrinsic pathway is also associated with the activation of caspase 3 and DNA fragmentation but is initiated through the binding of extracellular molecules to cell surface receptors. These receptors include the CD95 and the so-called death-ligand-binding receptors found in many different cell types (Thorburn, 2004). Upon ligand binding, these receptors recruit the Fas-associated death domain (FADD) and initiate an apoptotic cascade beginning with the activation of caspase 8 (Casp8). Cell death ligands include CD95L (also known as TNFL6 or FASLG), TNF-α (TNF) and the TNF-α related apoptosis inducing ligand TRAIL (also known as APO2 or TNFSF10).

One of the hallmarks of apoptosis, regardless of which pathway is activated, is cell condensation (Van Cruchten and Van Den Broeck, 2002). Cell shrinkage in this context is often termed apoptotic volume decrease (AVD) in analogy to regulatory volume decrease (RVD), which occurs in most cells following an osmotic challenge (Hoffmann, 1992; Okada and Maeno, 2001). AVD has been observed as an apoptosis-associated feature in many cell types including neural (Wei et al., 2004), epithelial, lymphoid, neuroblastoma, pheochromacytoma (Maeno et al., 2000) and cardiac muscle cells (Takahashi et al., 2005; d'Anglemont de Tassigny et al., 2004). Although the underlying mechanisms are not entirely understood, cell shrinkage requires the efflux of osmolytes, mainly ions, through channels and transporters followed by the concomitant flux of water (Hoffmann, 1992). Similar to RVD, the flux of Cl^- across the plasma membrane is thought to be one of the primary modalities of osmolyte efflux in AVD (Okada and Maeno, 2001). In support of this hypothesis, cell condensation has been observed to be sensitive to the Cl^- channel blockers, 5-nitro-2(3-phenylpropylamino) benzoic acid (NPPB) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (Maeno et al., 2000; Wei et al., 2004; Takahashi et al., 2005; Okada et al., 2006).

Although previous studies have indicated that AVD is inhibited by the alteration of Cl^- efflux, whether or not this effect alters the final outcome of apoptosis is still debated. In neurons, DIDS inhibition of AVD had no effect on caspase 3 activity or DNA laddering (Wei et al., 2004). By contrast, in HeLa and PC12 cells, both the increase in caspase activity and DNA laddering were

mitigated in response to AVD inhibition (Takahashi et al., 2005; Maeno et al., 2000). Hence, the current view of the literature is that AVD is typically associated with apoptosis, yet whether these volume changes are mechanistically linked to apoptosis is less clear.

This study, therefore, asked the specific question of whether AVD is necessary for apoptosis to occur and, importantly, whether AVD is sufficient to induce apoptosis even in the absence of other apoptosis-promoting stimuli. Using human malignant glioma cells as a model system, we demonstrate that cell condensation or AVD is an integral component of apoptosis that is both necessary and sufficient for cell death to occur. Cell condensation occurs regardless of whether apoptosis is initiated through the intrinsic pathway, via application of staurosporine or by the extrinsic pathway ligand, TRAIL. Importantly, we further demonstrate that sustained cell shrinkage of a defined magnitude, in the absence of other stimuli, is sufficient to induce apoptosis.

Results

Cell condensation occurs following initiation of either the intrinsic or extrinsic apoptotic pathways

To examine the role of cell condensation in apoptosis, we used human glioma cells as a model system. We exposed D54-MG glioblastoma cells to 1 μM staurosporine (STS), which initiates apoptosis through the intrinsic pathway, and to 50 ng/ml TRAIL, which initiates apoptosis through the extrinsic pathway, over a period of 24 hours. As determined by a luminescent assay system and by western blot, treatment with either drug resulted in a significant increase in caspase 3 activity after 7 hours (Fig. 1A,B). In addition, western blots revealed an obvious increase in the cleaved, activated form of caspases 8 and 9. As expected, caspase 8 activity is higher with TRAIL application, highlighting its role as one of the first caspases activated in the extrinsic pathway. In the intrinsic pathway, however, caspase 9 is one of the first caspases cleaved, a fact that is supported by the higher activity in STS-treated cells.

In addition to caspase activation, a majority of cells treated for 7 hours with either STS or TRAIL contained nuclei that were TUNEL positive (Fig. 1C). During TUNEL procedure, nuclei are exposed to fluorescein-labeled nucleotides in the presence of terminal deoxynucleotidyl transferase, which transfers nucleotides onto the fragmented ends of DNA strands. Increased fluorescein labeling indicates that the DNA in the treated cells was fragmented. These results confirm that both the extrinsic and intrinsic apoptotic pathways are intact in human glioma cells and culminate in the activation of caspase 3 and DNA fragmentation.

We next sought to determine whether apoptosis in human glioma cells was accompanied by an apoptotic volume decrease (AVD), similar to that demonstrated in other cell types (Okada et al., 2006; Maeno et al., 2000; Wei et al., 2004). D54-MG cells were suspended in an isotonic bath solution (normal bath) and cell volumes were measured using a Coulter Counter. After recording baseline cell volumes for 10 minutes, STS or TRAIL was added to the suspension. As demonstrated in Fig. 2, both drugs resulted in a dramatic volume decrease. Treatment with STS caused the cells to shrink to approximately 60% of their original volume within 80 minutes (Fig. 2A). Cell condensation in the presence of TRAIL was less rapid, yet cells did condense to approximately

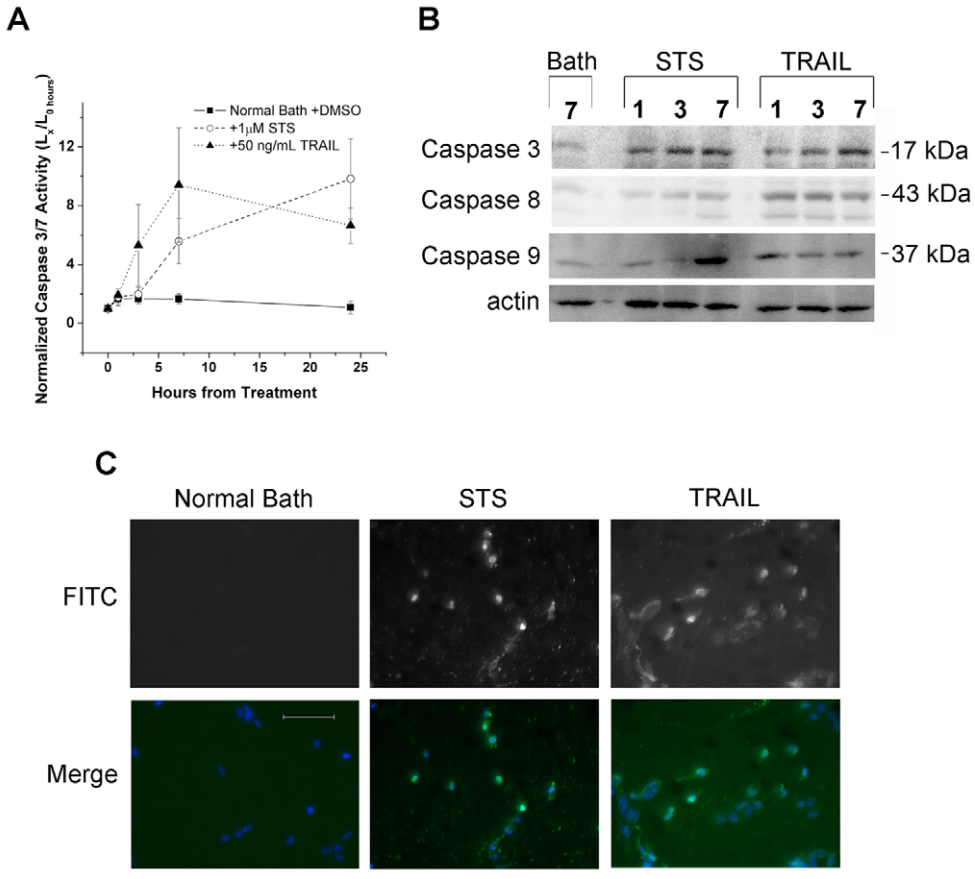

Fig. 1. Apoptosis is initiated in human glioma cells in response to staurosporine (STS) and TRAIL treatment. (A) Caspase activity was measured in D54-MG cells treated with normal bath, 1 μM STS or 50 ng/ml TRAIL by a luminescence assay. Each time point was normalized to the luminescence of cells after 0 hours of treatment with normal bath. Data are from three experiments. Error bars represent s.e.m. and *n*=3 samples per experiment. (B) Western blots of cell lysates from cells treated for 1, 3 or 7 hours were probed with antibodies against the cleaved, activated forms of caspases 3, 8 and 9. (C) Representative images demonstrate DNA fragmentation (fluorescein-labeled nuclei) after treatment with normal bath, STS or TRAIL for 7 hours. Merged images show both fragmented nuclei (green) and total nuclei present by DAPI staining (blue). Bar, 100 μm.

68% of their original volume within 5 hours of drug exposure (Fig. 2B).

AVD requires a DIDS-sensitive Cl^- efflux

Rapid cell volume changes are the result of the movement of osmolytes, especially ions, across the plasma membrane followed by the obligatory movement of water (Hoffmann, 1992). In human glioma cells, shrinkage has been studied in the context of volume regulation following a hyposmotic challenge, and has been shown to involve the NPPB-, Cd^{2+}- and DIDS-sensitive flux of Cl^- through channels and the [(dihydroindenyl)oxy]acetic acid (DIOA)-sensitive flux of Cl^- through K^+-Cl^--coupled co-transporters (KCCs) (Ernest et al., 2005). To determine whether these same mechanisms were involved in the AVD of human glioma cells, D54-MG cells were exposed to STS or TRAIL in the presence of 40 μM NPPB, 125 μM Cd^{2+}, 200 μM DIDS or 40 μM DIOA. Unlike regulatory volume decrease, AVD in human glioma cells was insensitive to the Cl^--channel inhibitors NPPB and Cd^{2+}, and to the KCC inhibitor DIOA (Cd^{2+} and DIOA data not shown for TRAIL treatments). In the presence of either STS or TRAIL, however, cell condensation was sensitive to the Cl^--channel inhibitor DIDS (Fig. 2). We hypothesized that the mechanism of DIDS inhibition of AVD was through the inhibition of an apoptosis-enhanced Cl^- efflux. To confirm this hypothesis, we visualized changes in Cl^- flux using the fluorescent Cl^- indicator, SPQ. SPQ is quenched in the presence of Cl^- and intensifies when the Cl^- concentration is reduced. However, like other fluorescent dyes, its signal will also intensify as the cell shrinks and the dye becomes more concentrated. To account for changes in cell size, SPQ was loaded into cells that expressed the enhanced green fluorescent protein (eGFP), and the change in SPQ intensity was normalized to the change in eGFP intensity. To validate this method, we exposed these cells to a hyperosmotic challenge by the addition of 100 mOsm mannitol to the bath solution, resulting in a final osmolarity of approximately 430 mOsm. This challenge should result in rapid cell shrinkage, with no decrease in Cl^- concentration. As expected, both the intensity of SPQ and the intensity of GFP increased because of cell shrinkage, resulting in a slight decrease in the ratio of SPQ:GFP (Fig. 3B). By contrast, when cells were exposed to 1 μM STS, the ratio of SPQ:GFP increased dramatically, indicating that there was an increase in SPQ intensity that could not be accounted for simply by a decrease in cell volume (Fig. 3A,B). This increase in SPQ fluorescence is indicative of an enhanced Cl^- movement across the plasma membrane in the presence of STS. However, when the cells were treated with 200 μM DIDS prior to the addition of STS, the increase in SPQ intensity did not occur (Fig. 3B). These data confirm our hypothesis that initiation of apoptosis results in an early enhancement of Cl^- efflux and an ensuing DIDS-sensitive volume decrease.

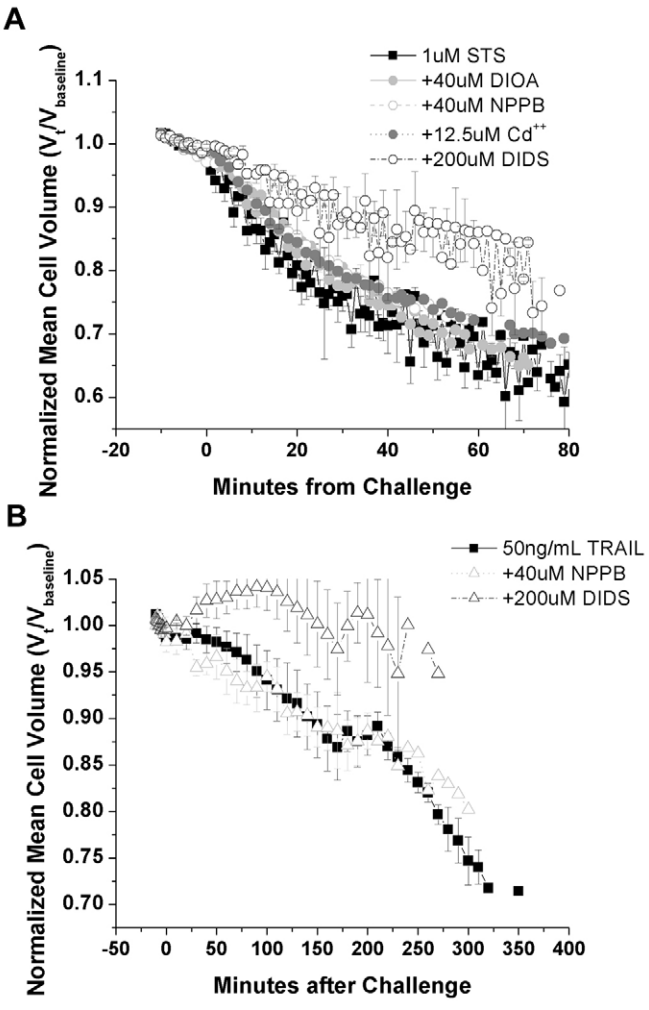

Fig. 2. Apoptotic cell condensation is sensitive to the Cl^--channel inhibitor DIDS. (A,B) Normalized mean cell volumes (MCVs) of D54-MG were recorded following addition of STS (A) or TRAIL (B) in the presence of the Cl^--channel inhibitors NPPB, DIDS or Cd^{2+} or the KCC inhibitor DIOA. Data are the mean results from three independent experiments. Error bars represent s.e.m. with n=10,000-20,000 cells.

AVD is necessary for apoptosis, but not cell death

In order to determine whether the observed Cl^--mediated volume decrease was necessary for apoptosis, we treated confluent monolayers of D54-MG cells for 7 hours with STS or TRAIL in the presence or absence of DIDS. As shown in Fig. 4, both caspase 3 activation and DNA fragmentation, late points in the apoptotic pathway, were effectively inhibited by DIDS application (Fig. 4A,B). Surprisingly, the activation of the initiation caspases 8 and 9 also appeared reduced by DIDS application to either the STS- or TRAIL-treated cells, suggesting that at least some of the cleavage occurs downstream of the necessary volume decrease (Fig. 4B).

Furthermore, we sought to determine whether inhibition of cell condensation would also lead to an increase in cell viability. As demonstrated in Fig. 5, application of STS for 7 hours resulted in a decrease in cell viability to 82%, whereas only 67% of cells were viable in the presence of TRAIL. DIDS application had no effect on cell viability with STS treatment. Interestingly, however, DIDS application significantly increased viability of TRAIL-treated cells.

Sustained cell condensation is sufficient to induce apoptosis

Although Cl^- efflux and cell shrinkage were demonstrated to be tightly related events during apoptosis, we sought to determine whether a volume decrease alone can initiate apoptosis. We previously demonstrated that human glioma cells shrink rapidly in response to a hyperosmotic challenge. Following cell shrinkage, cells are able to recover their cell volume, but only in the presence of glutamine; cells in the absence of glutamine remain small (Ernest and Sontheimer, 2007). As shown in Fig. 6A, the addition of 150 mOsm mannitol to the normal bath solution, in the absence

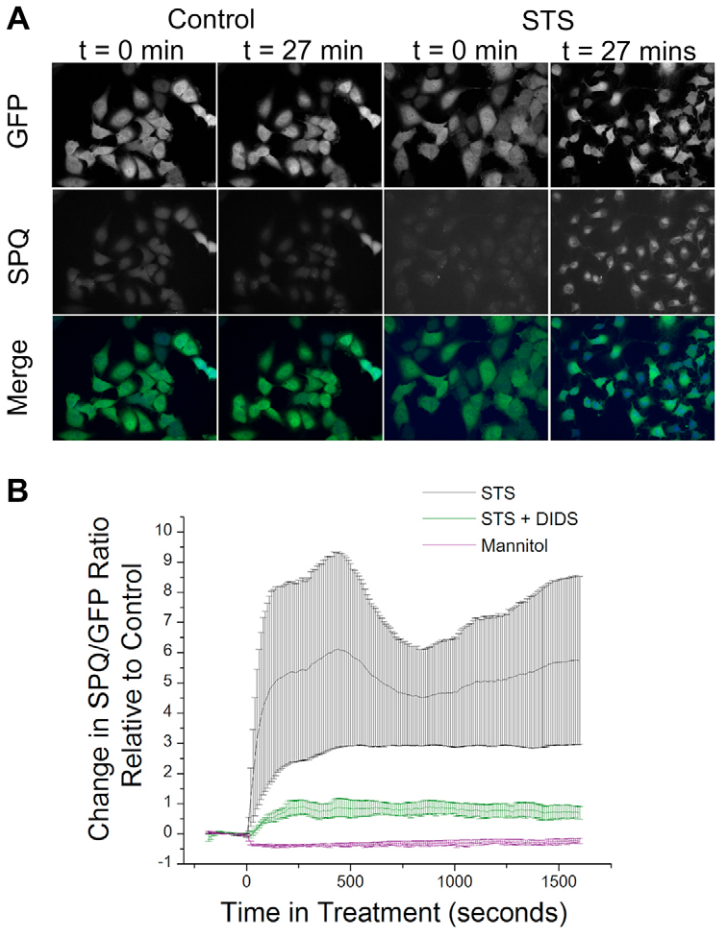

Fig. 3. Apoptosis initiation results in DIDS-sensitive Cl^- movement across the plasma membrane. (A) Representative images depict cell size as well as SPQ and GFP intensity just prior to (t=0) and 27 minutes after the addition of control or STS media. (B) Mean time normalized intensity curves quantify the changes depicted in A. Data are the mean results from three independent experiments. Error bars represent s.e.m. with n=10 samples in each experiment.

of glutamine, resulted in a volume decrease to approximately 68% of the original size. In the presence of 2 mM glutamine, however, the cells were able to recover to approximately 75% of their original volume. As expected, the extent of cell condensation was dependent on the degree of osmotic challenge. Exposure to 300 mOsm mannitol resulted in a more dramatic shrinkage to 52% in the absence of glutamine. Similarly, however, these cells were also able to undergo a volume recovery in the presence of glutamine to approximately 60% of their original volume (Fig. 6A). Note that this cell shrinkage induced through the addition of mannitol was not accompanied by a decrease in the intracellular Cl^- content as demonstrated in Fig. 3.

To determine whether cell shrinkage was, indeed, sufficient to induce apoptosis, we measured the caspase 3 activity of cells treated with a hyperosmotic challenge by the addition of mannitol or NaCl (data not shown) for 24 hours. Although any increase in osmolarity would result in cell shrinkage, a significant increase in caspase 3 activity was only observed when 150 mOsm, or more, was added to the normal bath solution (final osmolarity 450 mOsm). However, in the presence of glutamine, the caspase 3 activity was significantly reduced. Under these conditions, an addition of 300 mOsm was necessary to enhance caspase 3 activity (Fig. 6B). These data suggest that there is a volume threshold that cells must reach to induce apoptosis. Only hyperosmotic challenges eliciting a sustained cell shrinkage below 70% of the original volume of the cell (Fig. 6A, shaded area) resulted in the initiation of apoptosis.

Notably, cells that underwent an initial shrinkage to below 70% of their original volume, but were able to recover within 1 hour to above 70%, were prevented from undergoing apoptosis (Fig. 6A,B, 150 mOsm addition of mannitol plus glutamine). This result indicates that cell shrinkage must be sustained for a certain period of time before apoptosis is initiated. To determine what the 'time threshold' is for the activation of apoptosis via cell shrinkage, we exposed the D54-MG cells to the same hyperosmotic conditions by the addition of NaCl to the normal bath solution. After a specified time, however, we 'rescued' the cells by the addition of water to the bath solution such that the final osmolarity of the solution was returned to 300 mOsm. A representative experiment in Fig. 7A demonstrates how this 'rescue' results in a rapid volume increase to the cell's original volume. After 24 hours, D54-MG cells exposed to an increase in osmolarity by 150 mOsm, resulting in a shrinkage to 68%, showed an increase in caspase 3 activity. This increase also occurred when the cells were rescued at 8 hours, but did not occur if the reduced cell size was maintained for only 4 hours or less. By contrast, cells forced to a more reduced cell size (approximately 54%) by the addition of 300 mOsm NaCl showed a significant increase in caspase 3 activity even if the cells were rescued after 4 hours (Fig. 7B). These data indicate that cell shrinkage must be maintained for a period of time to induce apoptosis, but the amount of time that the cells must remain condensed before apoptosis is initiated is dependent on the degree of cell condensation.

Discussion

In this study, we have demonstrated that cell condensation, or AVD, occurs when apoptosis is initiated either through the extrinsic pathway in response to TRAIL or through the intrinsic pathway in response to STS. Our examination of AVD clearly shows that the process requires Cl^- efflux via DIDS-sensitive ion channels. We further show that the resulting cell condensation is a necessary step in the apoptotic pathway. Most importantly, however, our data indicate that cell shrinkage, even in the absence of Cl^- efflux, is sufficient to initiate apoptosis.

We used human glioma cells as model system because of their propensity to undergo apoptosis in vivo and their sensitivity to the extrinsic pathway ligand, TRAIL. To our knowledge, this is the first study demonstrating a role for AVD in the apoptosis of human glioma cells. The here described requirement for AVD to occur as cells undergo apoptosis is not unique to glioma cells. Indeed, initiation of the intrinsic pathway of apoptosis by STS, doxorubicin and ceramide has been demonstrated to engage an apoptotic volume decrease in many cell types including neurons, lymphoid cells, PC12 cells, rat neuroblastoma cells and cardiomyocytes (Okada et al., 2006; Takahashi et al., 2005; Maeno et al., 2000). Furthermore, similar to what we have observed for glioma cells, AVD in these cell types can be inhibited by application of Cl^--channel inhibitors. However, different cell types appear to engage different Cl^- channels as release pathways are sensitive to different

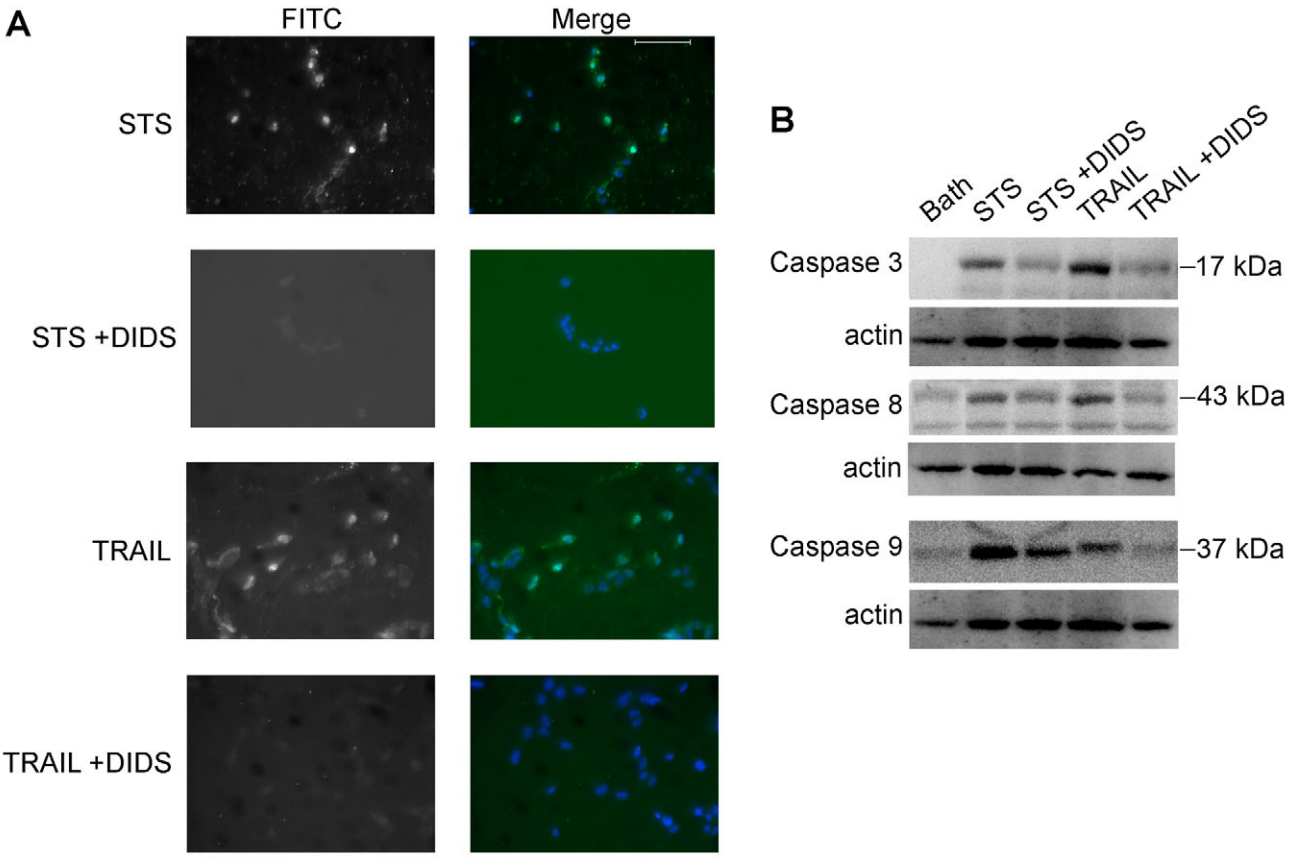

Fig. 4. DNA fragmentation and caspase activation are also sensitive to DIDS inhibition of AVD. (A) Representative images demonstrate the presence or absence of DNA fragmentation (FITC-labeled nuclei) in cells treated for 7 hours with STS or TRAIL in the presence or absence of DIDS. Merged images show fragmented nuclei (green) as well as DAPI staining of all nuclei present (blue). (B) Western blots of lysates from similarly treated cells were probed with antibodies to activated, cleaved caspases 3, 8 and 9. Actin was used as a loading control.

pharmacological reagents. For example, AVD in PC12 cells can be strongly inhibited by the relatively non-specific Cl^- channel inhibitor, NPPB, a drug that was ineffective in human glioma cells in our studies. The most effective drug in preventing AVD in human glioma cells was DIDS which is frequently used to inhibit Cl^- channels including ClC-3 and the volume activated Cl^- channel ($I_{Cl-,\,swell}$). Although DIDS also inhibits the Cl^-/HCO_3^- exchanger (Taylor et al., 2006), all critical experiments were performed in the absence of HCO_3^- in the bath solution. As the exchanger absolutely requires the presence of HCO_3^- in both the intracellular and extracellular fluids to function, any contribution from this transport system to the observed volume decrease is unlikely. Taken together with the very rapid efflux of Cl^- demonstrated by fluorescence imaging, this strongly suggests that the observed effects of DIDS were due to inhibition of Cl^- efflux through channels.

Importantly, we also found that the mechanisms engaged in AVD differ from those that operate when cells regulate their volume in response to a hyposmotic challenge. The latter response can be inhibited by Cd^{2+}, an inhibitor of the voltage-gated and volume-sensitive channel ClC-2 and by DIOA, an inhibitor of the K^+Cl^--coupled co-transporter family (KCCs) (Ernest et al., 2005). AVD in glioma cells was not sensitive to either of these drugs. Therefore, although other studies suggest that AVD and RVD are related processes (Okada et al., 2006), our data indicate that the underlying ion channels are, indeed, not identical and must be studied separately.

Similarly, previous studies have concluded, based on the pharmacological inhibition of AVD, that this cell shrinkage is primarily mediated through the putative volume sensitive outwardly rectifying Cl^- channel (VSOR), believed to be responsible for the majority of Cl^- efflux in RVD (Okada et al., 2006; Okada and Maeno, 2001). Although we demonstrated that

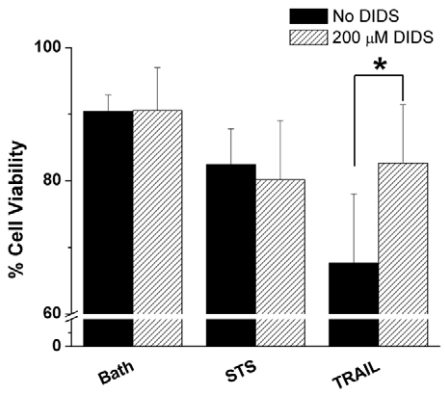

Fig. 5. DIDS inhibition of AVD has variable effects on cell viability. Percentage cell viability as measured by Trypan Blue exclusion after application of STS or TRAIL for 7 hours. *P<0.05 compared with untreated control cells.

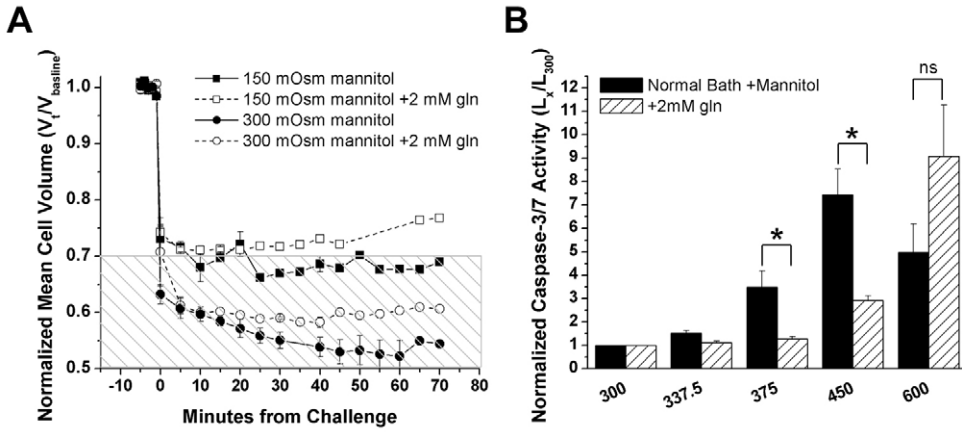

Fig. 6. Hyperosmotic cell shrinkage results in activation of caspase 3. (A) Normalized MCVs of D54-MG cells were recorded following the addition of 150 or 300 mOsm mannitol in the presence or absence of 2 mM glutamine. Data are the mean results from three independent experiments. Error bars represent s.e.m. with n=10,000-20,000 cells. (B) Caspase 3 activity, as measured by luminescence, in cells exposed to isosmotic (300 mOsm) or hyperosmotic (337.5-600 mOsm) bath solutions for 24 hours. Activity normalized to 300 mOsm exposure. Data are from three experiments. Error bars represent s.e.m. and n=3 samples per experiment. *P<0.05.

application of DIDS inhibited AVD, this does not confirm the involvement of the VSOR. In fact, the lack of inhibition by NPPB strongly questions the involvement of this channel, which has been shown to be blocked by NPPB in all preparations studied to date.

Irrespective of the identity of the Cl^- efflux pathway, there appears to be general agreement that cell condensation is a morphological feature of apoptosis, yet the necessity for volume decrease in apoptosis has been debated. In this regard, an important finding of our study was that DIDS not only inhibited apoptosis-associated cell shrinkage but also prevented the activation of caspase 3 and DNA fragmentation in response to either intrinsic or extrinsic apoptotic pathway activation. In contrast, an important observation was that the inhibition of apoptosis did not always result in an increase in cell viability. In cells treated with STS, cell viability was already reduced to 80% by 7 hours. Although only 20% of the cells were confirmed to be 'not viable', we believe that this is an early estimate of viability and, according to the DNA fragmentation data many more cells were committed to cell death along the apoptotic pathway. Although apoptosis was aborted in the presence of DIDS, cells programmed to die by initiation of the intrinsic pathway were still found to undergo cell death by other means. By contrast, when apoptosis was initiated through the extrinsic pathway, inhibition of the cell condensation resulted in an increase in cell survival. These differences are most likely due to the distinguishing features of the extrinsic versus the intrinsic pathways. Specifically, the intrinsic pathway is initiated when the mitochondria, nucleus or other vital organelles are damaged directly. If irreparable, this damage may lead to eventual cell death even when the apoptotic pathway is unavailable. In contrast, the extrinsic pathway is initiated through ligand binding to the cell surface. If the downstream apoptotic pathways are unavailable, then the cell may remain undamaged and survive the insult. Taken together these data suggest that regardless of the pathways of activation, AVD is indeed a necessary step in apoptosis. Further, the data implicate AVD as an early effector of the process in human glioma cells.

The most surprising, and arguably most important contribution of this paper to the apoptotic literature pertains to our finding that the volume decrease alone was sufficient to cause apoptosis even in the absence of a death ligand. Although other studies have examined the interdependence of cell volume changes and apoptosis, none was able to establish such a clear causal relationship. Here we demonstrate the existence of a volume threshold, below which cells activate their apoptotic machinery. We have discovered that human glioma cells will undergo apoptosis if, and only if, they are unable to recover from cell shrinkage below approximately 70%. If cells remain shrunken to just below 70% of their original volume for 8 hours, then apoptosis will occur within 24 hours. However, if they are rescued from their condensed state prior to this critical time, apoptosis is prevented. Finally, in the presence of a more substantial cell volume decrease to 52% of their original volume, apoptotic machinery is activated even if the cells are osmotically rescued within 4 hours. Our findings explain one

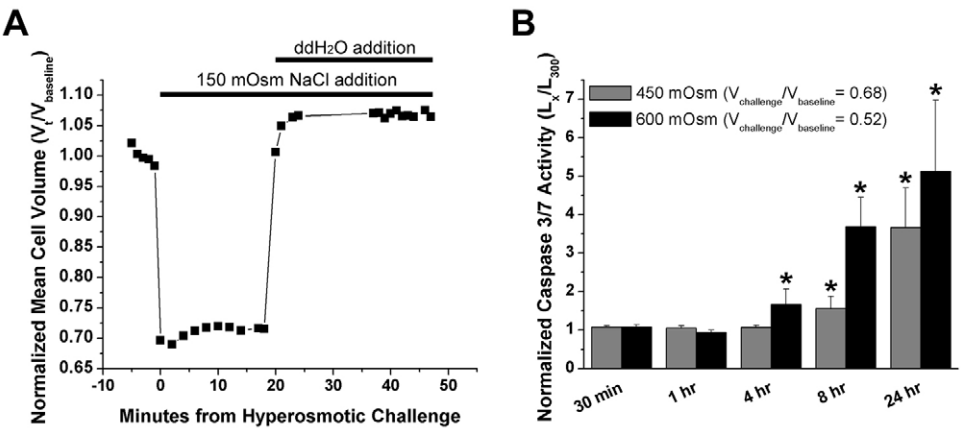

Fig. 7. The 'time threshold' to activate is reduced with a greater cell shrinkage. Cells were exposed to bath solution made hyperosmotic by the addition of NaCl (final osmolarity 450 or 600) and were then 'rescued' from shrinkage by the addition of ddH_2O after 30 minutes, 1, 4 or 8 hours. (A) Normalized MCVs of D54-MG cells during exposure to and rescue from hypertonic challenge in a representative experiment. (B) Caspase 3 activity as measured by luminescence. Activity is normalized to 300 mOsm (no challenge) exposure. Data are from three independent experiments. Error bars represent s.e.m. and n=3 samples per experiment. *P<0.05 compared to unchallenged control cells (not shown).

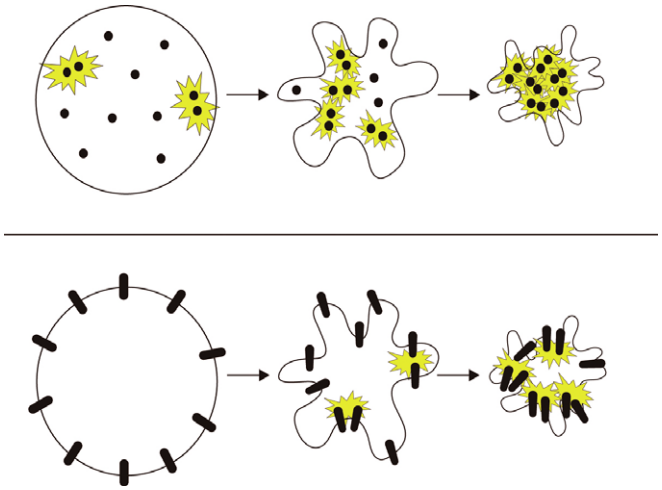

Fig. 8. Proposed model for the initiation of apoptosis by cell shrinkage. Cell shrinkage may result in the increased concentration of auto-activating enzymes (top panel) or the increase in proximity of cell surface receptors (bottom panel). The activation of such enzymes/receptors may result in the initiation of apoptosis in the absence of other drugs or the acceleration of the pathway in the presence of other apoptosis initiators.

previous study where S49 Neo lymphocytes, cells that cannot regulate their cell volume in response to hypertonicity, could be induced to undergo apoptosis if chronically exposed to hypertonic medium (Bortner and Cidlowski, 1996).

Our data also provided a lead into mechanisms that may initiate apoptosis. As illustrated in Fig. 8, we hypothesize, based on this data, that cellular enzymes or cell surface receptors become auto-activated in response to an increase in concentration or molecular crowding. In this model, apoptosis would be expected to occur, naturally, at a very slow rate because of the low concentration of these enzymes and/or receptors. However, as the cell shrinks, it would increase the rate at which the enzymes/receptors were activated and, hence, increase the rate of apoptosis. The feasibility of this model is supported by the observation that caspases, principal enzymes involved in apoptosis, can auto-activate (Thorburn, 2004) and the observation that cell shrinkage increases the pro-apoptotic activity of divalent anti-CD95 antibodies in leukemia-derived cells (Fumarola et al., 2001). In further support of this hypothesis, unpublished observations in our lab indicate that even a small hyperosmotic challenge, induced by the addition of 30 mOsm mannitol, can greatly enhance the rate of STS-induced apoptosis. Although the mechanisms of osmolyte efflux may vary from cell type to cell type, the basic principles of this model has the potential to apply to many cell types. Importantly, we suggest that the fact that cell shrinkage is both a necessary and sufficient mediator of cell apoptosis applies much more broadly to other cell systems.

Materials and Methods

Cell culture

All experiments were performed on the glioma cell line D54-MG (glioblastoma multiforme, WHO grade IV; a gift from D. Bigner, Duke University, Durham, NC). Cells used for 2D ratiometric time-lapse experiments were stably transfected with peGFP-N1 (Clontech, Mountain View, CA). All cells were maintained in 1:1 DMEM-F12 (Mediatech, Hendon, VA) supplemented with 2 mM L-glutamine (CellGro, Herndon, VA) and 7% bovine growth serum (HyClone, Logan, UT). The cells were incubated at 37°C in a 10% CO_2 humidified atmosphere.

Solutions

The control NaCl bath solutions (normal bath) contained the following (in mM): 130 NaCl, 5.0 KCl, 10.5 glucose, 32.5 Hepes and 1 $CaCl_2$. The pH of each solution was adjusted to 7.4 with 10 M NaOH and the osmolarity of each solution was confirmed with a freezing point micro-osmometer (Fiske Model 210; Fiske Associates; Norwood, MA). The osmolarities of all bath solutions were 310±10 mOsm.

Drugs were added directly to bath solutions from stock solutions. Stock solutions of NPPB, DIOA, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and staurosporine (STS) were dissolved at ×1000 final concentration in DMSO whereas TRAIL and $CdCl_2$ were dissolved at 1000× final concentration in double-distilled H_2O. DMSO at its final concentration (0.1%) did not perturb cell volume or affect volume regulation (data not shown). TRAIL was obtained from Chemicon International (Temecula, CA). All other drugs were purchased from Sigma-Aldrich (St Louis, MO).

Cell volume measurements

Cell volumes were measured as previously described (Ernest et al., 2005) by electronic sizing with a Coulter counter Multisizer 3 (Beckman-Coulter, Miami, FL). The counter determines cell volume by measuring the voltage step that occurs when a cell displaces its volume in electrolyte solution as it passes through a small aperture. The aperture size used for these experiments was 100 μm.

To prepare the cells for volume measurements, cells were incubated for 3 minutes with 0.05% trypsin and 0.53 mM EDTA (Invitrogen Corporation, Carlsbad, CA). Trypsin was inactivated by the addition of an equal volume of serum-containing medium. After pelleting the cells by brief centrifugation, the cells were resuspended in normal bath solution in the presence or absence of pharmacological inhibitors and incubated for 10 minutes before the beginning of the first baseline measurement. Cell volume measurements were obtained every minute and each measurement was an average of 10,000-20,000 cells. Ten baseline readings, during which mean cell volume varied less than 100 fl, were collected before the osmotic challenge was applied or apoptosis was induced.

Data analysis for volume regulation experiments

Coulter counter data were collected with Multisizer 3 software and exported to Excel. Time points were rounded to whole minutes and mean cell volumes were normalized to the average baseline value for that experiment. All data were plotted in Origin 7.0 (MicroCal, Northampton, MA) as means ± s.e.m., where V_t is the volume measured at time t and $V_{baseline}$ is the average of the baseline volumes for that experiment. Significance (P) was determined by Student's t-test of relative volumes at 60 minutes after STS addition or hyperosmotic challenge or 300 minutes after TRAIL addition.

Caspase 3 activity

Cells were plated in Nunc F96 white MicroWell plates (Fisher Scientific; Hampton, NH) and allowed to grow for 3 days in culture. Adherent monolayers of cells were exposed to normal bath in the presence or absence of apoptotic inducing agents, pharmacological inhibitors or osmotic challenges. After a specified time, an equal volume of CaspaseGlo 3/7 Reagent (Promega; Madison, WI) was added to the bath. This reagent contains a DEVD-aminoluciferin substrate that luminesces when cleaved, producing a signal that is proportional to the caspase 3 activity. After 30 minutes, the signal was measured using a LUMIstar luminescence plate reader (BMG Labtech; Durham, NC).

Western blots

Cells in 100 mm culture dishes were rinsed with ice-cold PBS, then scraped and collected in 0.5 ml 2% SDS with 1:100 protease inhibitor cocktail (Sigma). The sample was immediately boiled for 10 minutes. Lysates were stored at –20°C until used. Lysate proteins were separated by electrophoresis using a 10-20% SDS-polyacrylamide Ready gel (Bio-Rad). Proteins were transferred onto PVDF membranes (Millipore), probed with antibodies obtained from Chemicon International (Temecula, CA) and visualized using HRP-conjugated anti-rabbit antibodies (Bio-Rad) and SuperSignal West Femto maximum sensitivity substrate (Pierce; Rockford, IL). Images were obtained using a KODAK Image Station 4000MM (Kodak; New Haven, CT).

TUNEL assay for the detection of DNA fragmentation

D54-MG cells were plated in eight-well chamber slides and cultured for 3 days. Confluent monolayers of cells were treated with normal bath plus or minus apoptosis inducers and Cl^--channel inhibitors. After 7 hours, adherent cells were fixed by a 10-minute exposure to 4% paraformaldehyde. After fixation, cells were exposed to a solution containing terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotides, as provided in Roche Diagnostic's In Situ Death Detection Kit (Roche; Mannheim, Germany). Cells were incubated in this solution at 38°C for 1 hour. After mounting coverslips to the slides, five fields were selected at random from each condition and images of the cells was acquired with a Zeiss Axioscope with Axiocam.

Time-lapse fluorescent measurements

Cells were plated on 0.170 mm glass-bottom 35 mm dishes (Mattek, Ashland, MA) to reach 50% confluence after 24 to 36 hours. Twenty-four to 48 hours after plating, cells were loaded with the Cl^--indicator dye, 6-methoxy-*N*-(3-sulfopropyl)quinolinium

(SPQ; Molecular Probes, Eugene, OR) for 6 minutes in room-temperature hypotonic phosphate-buffered saline (PBS) (30% water challenge) at a concentration of 2 mg/ml SPQ. This challenge was followed by a 30 minute incubation in normosmotic PBS (280 mOsm) containing the same concentration SPQ. After loading, cells were washed with medium and allowed to recover in incubation for ⩾30 minutes.

Confocal time-lapse images were acquired with a Hamatsu IEEE1394 Digital CCD camera (325-6, Sunayama-Cho, Hamamatsu City, Japan) mounted on an Olympus IX81 motorized inverted microscope equipped with an Olympus Disk Scanning Unit (Olympus, 2 Corporate Center Drive, Melville, NY 11747-3157) and controlled by Slidebook software (Intelligent Imaging Innovations Inc., Denver Colorado). The microscope was housed in a temperature- and CO_2-controlled humidified incubator maintained at 37°C and 5% CO_2. GFP (excitation=480±15 nm, emission=535±20 nm) and SPQ (excitation=350±25 nm, emission=460±25 nm) images were taken at 10-second intervals through a 40× oil objective (numerical aperture=1.3) and the camera was binned to prevent photobleaching. After 190 seconds of baseline imaging in 1.5 ml of normal maintenance medium or 200 μM DIDS-containing medium, 500 μl of control, drug or 400 mOsm mannitol-containing medium was transferred into the dish without interrupting acquisition. For all experiments, final STS and DIDS concentrations were 1 μM and 200 μM, respectively.

Analysis of confocal images

Background corrected mean GFP and SPQ intensity values were measured within a specified region of interest for each cell analyzed using Slidebook and ImageJ (NIH Image) software. At each time point a SPQ/GFP intensity ratio was generated and normalized to the mean of the baseline values to generate a time normalized SPQ/GFP curve for each cell. For each experiment at least ten cells were analyzed under each condition and the mean curves were control subtracted.

Cell viability (Trypan Blue exclusion assay)

In order to assess cell viability, non-adherent cells were collected by brief centrifugation of the treated medium. Adherent cells were briefly exposed to trypsin, followed by an equal volume of serum-containing medium and centrifugation. Collected cells were resuspended in 2 ml PBS. A 20 μl aliquot of the cell suspension was gently mixes with an equal volume of 0.1% Trypan Blue in PBS and the cells were counted using a hemacytometer. Percentage cell viability was calculated as the number of non-stained cells / total number of cells ×100.

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