Inhibition of Hypertonicity-Induced Cation Channels Sensitizes HeLa Cells to Shrinkage-Induced Apoptosis

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Key Words

HeLa cells • Cell shrinkage • Volume regulation • Apoptosis • Cation channel • Gadolinium • Flufenamate

Abstract

Hypertonicity-induced cation channels (HICCs) are an effective mechanism of regulatory volume increase (RVI), which is a restoration process of cell volume after osmotic cell shrinkage, in HeLa cells. Since a reduction of cell size is a hallmark of programmed cell death, we tested whether a blockage of HICCs sensitizes HeLa cells to shrinkage-induced apoptosis by using proliferation assays, apoptosis assays, and patch-clamp recordings. Under control conditions, increasing osmolality up to 600 mosmol/kg-H₂O had no detectable effect on either cell proliferation or apoptosis. With HICCs blocked by flufenamate and Gd³⁺, however, a significant reduction of proliferation and a stimulation of apoptosis were observed. Both effects exhibited virtually identical sensitivity profiles to osmotic stress as well as to flufenamate and Gd³⁺. Moreover, the observed concentration dependency of flufenamate and Gd³⁺ on proliferation and apoptosis was in excellent accordance with that on HICC

inhibition. These results suggest that persistent cell shrinkage may function as a specific signal in the induction of apoptosis. In addition, they provide further evidence for the interplay of proliferation vs. apoptosis and the actual role that mechanisms of cell volume regulation do play in these processes.

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Introduction

The proper regulation of cell volume appears to be one of the most crucial elements in cell homeostasis. Hypertonic cell shrinkage, for instance, is immediately compensated by an import of NaCl (plus osmotically obliged water) accomplishing the process of regulatory volume increase (RVI) [1-3].

We have reported previously that, in the human cervix-carcinoma cell-line HeLa, shrinkage activates a cation channel that is non-selective for Na⁺ and K⁺. This “hypertonicity-induced cation channel (HICC)” is clearly the main mechanism of Na⁺ influx coupled to RVI [4].

Under normosmotic conditions, cell shrinkage is a hallmark of early apoptosis and has been termed apoptotic volume decrease (AVD) [5]. AVD is achieved by the activation of K^+ channels occurring in concert with the stimulation of volume-sensitive, outwardly rectifying Cl^- channels [6, 7]. In a variety of cell types, however, osmotic shrinkage *per se* was reported to induce apoptotic cell death [8-13]. Especially, this appears to hold for cells that are devoid of efficient machinery for RVI [6, 8].

We hypothesised, therefore, that a continuous hypertonicity-induced shrinkage of cells might function as an actual signal for apoptosis induction. It is found that in HeLa cells, originally exhibiting a sizeable RVI, hypertonic stress induces apoptosis only following blockage of the HICC. In a dose-dependent fashion, both flufenamate and Gd^{3+} inhibit HICC currents, they inhibit proliferation, and they induce apoptosis.

Material and Methods

Cell Culture

Human cervix HeLa cells were grown to confluency in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 40 IU/ml penicillin G, and 100 μ g/ml streptomycin under 95% air - 5% CO_2 . For sub-culturing, a phosphate-buffered saline containing 0.25% trypsin and 0.02% EDTA was used to detach cells from their substratum. Changes in osmolality were achieved by adding appropriate amounts of mannitol to the culture medium. In experiments with Gd^{3+} , bicarbonate and sulphate/phosphate were replaced by 25 mmol/l HEPES and chloride, respectively, to avoid any chelation of the lanthanide [14]; in addition, cells were kept in an incubator gassed with 100% air. Cells for patch-clamping were mechanically detached from the bottom of the culture flasks and gently stirred in suspension for 15 to 300 min in a 5%- CO_2 -gassed incubator.

Assays for cell proliferation and cell death

Cell proliferation, caspase-3 activity, and DNA fragmentation were determined by using a colorimetric MTT assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan), fluorometric measurements (Apo-ONE Homogeneous Caspase-3/7 Assay; Promega, Madison, WI), and an enzyme-immunoassay for mono- and oligonucleosomes (Cell Death Detection ELISA^{PLUS}; Roche Diagnostics, Mannheim, Germany), respectively. For quantification of necrotic cell death, a double staining with Hoechst 33342 (2 μ g/ml) and propidium iodide (PI; 5 μ g/ml) was performed, the former yielding overall cell numbers. In all assays, HeLa cells were incubated for 2 days under the various experimental conditions tested, because sustained but not transient osmotic cell shrinkage by hypertonicity was needed to affect proliferation and cell death.

Electrophysiology

Membrane currents were measured on single detached HeLa cells in whole-cell patch-clamp recordings. Patch pipettes had resistances of some 2 M Ω ; pipette offsets, series resistance, and capacitive transients were compensated for on the patch-clamp amplifier (Axopatch 200A; Molecular Devices, Union City, CA). Data acquisition and analysis were done with the pCLAMP 8.0 software (Molecular Devices). Currents were filtered at 1 kHz and digitized with an AD converter (DigiData 1200B; Molecular Devices) at 5 kHz. Holding voltage was -30 mV, and voltage ramps from -80 to +20 mV and 1 sec duration were applied every 10 sec.

The pipette solution (pH 7.3) contained (in mmol/l): NaCl, 26; Na-gluconate, 69; $MgCl_2$, 1; TEA-Cl, 2; Na_2 -ATP, 2; Na_2 -GTP, 0.5; HEPES, 10; EGTA, 1. Osmolality was adjusted to 300 mosmol/kg- H_2O by addition of mannitol under osmometric control (OM802; Vogel, Giessen, Germany). The bath solution (pH 7.5) contained (in mmol/l): NaCl, 94; Na-gluconate, 6; $MgCl_2$, 1; $CaCl_2$, 1; TEA-Cl, 2; HEPES, 10. Osmolality was adjusted to 340 (isotonic) and 450 mosmol/kg- H_2O (hypertonic). With the above ion gradients, the equilibrium potential for Na^+ and Cl^- (as the only permeant ions) is set at 0 and -30 mV, respectively.

Statistical Analysis

Data are presented as means $\pm$ S.E.M. with n denoting the number of cells tested. For comparison of data sets, Student's t -tests for unpaired data were employed. A p value < 0.05 is considered significant.

Results

Within a two-day period as experimental time frame, HeLa cells were proliferating to $283.7 \pm 14.5\%$ of basal cell numbers ($n = 8$). As depicted in Figure 1A (left), increasing the osmolality of the culture medium up to 600 mosmol/kg- H_2O had no detectable effect on this proliferation rate. In the presence of 100 μ mol/l flufenamate, however, which is an effective blocker of HICC currents as well as RVI, in HeLa cells [4], cell proliferation was significantly decreased, already under isotonic conditions (Figure 1A, right). Moreover, there was a clear further decrease of proliferation with increasing osmolality. In Figure 1B, the flufenamate-induced decrease in cell proliferation (as flufenamate minus control) is computed.

To test, whether the observed decrease in cell proliferation reflects an actual induction of apoptosis, the activity of caspase-3 was determined. As shown in Figure 2A (left), hypertonic stress *per se* did not induce apoptosis. In the presence of 100 μ mol/l flufenamate, however, there was a significant increase of caspase-3 activity, at 300 mosmol/kg- H_2O already, and a continu-

Fig. 1. Flufenamate sensitizes the proliferation of HeLa cells to hypertonic stress ($n = 29$, for each experimental condition). A, proliferation after 2 days of cell culturing at various osmolarities, both under control conditions and in the presence of $100 \mu\text{mol/l}$ flufenamate, as indicated. Data are normalized to the isotonic control value. In B, the difference of flufenamate minus control is given, i.e. the flufenamate-induced decrease of cell proliferation in HeLa cells,

Fig. 2. Flufenamate-induced apoptosis in HeLa cells is intertwined with hypertonic stress ($n = 9$, for each experimental condition). A, caspase-3 activity after 2 days of cell culturing at various osmolarities, both under control conditions and in the presence of $100 \mu\text{mol/l}$ flufenamate. In B, the difference of flufenamate minus control is given, i.e. the flufenamate-induced increase of caspase-3 activity.

ous further stimulation with increasing osmolarity could be observed (Figure 2A, left). In Figure 2B, the flufenamate-induced stimulation of caspase-3 activity (as flufenamate minus control) is depicted. Of note, the flufenamate-induced decrease in cell proliferation (Figure 1B) and the stimulation of caspase-3 activity (Figure 2B) exhibit osmotic sensitivity profiles that are virtually

identical.

Next, the concentration dependency of the effects of flufenamate on hypertonicity-induced proliferation-decrease and caspase-3 activation were examined. All experiments of this series were performed under constant hypertonic stress, i.e. by elevating osmolality from 300 to 600 mosmol/kg- H_2O . As shown in Figure 3A & B,

Fig. 3. Effects of flufenamate (FFA) on proliferation (A), caspase-3 activity (B), and DNA fragmentation (C) in HeLa cells under hypertonic stress. Data are presented as the difference of the hypertonic (600 mosmol/kg-H₂O) minus the isotonic (300 mosmol/kg-H₂O) value obtained (n = 16, for A & B; n = 12, for C).

	Control	100 μM Flufenamate	Hypertonicity	Hypertonicity + Flufenamate
PI-positive cells (%)	2.2 ± 0.7	3.0 ± 0.8	2.8 ± 0.8	4.3 ± 0.8
	Control	100 μM Gd ³⁺	Hypertonicity	Hypertonicity + Gd ³⁺
PI-positive cells (%)	0.6 ± 0.3	0.7 ± 0.3	4.0 ± 1.7	1.2 ± 0.6

Table 1. Screen for necrotic cell death. To test for necrotic cell death, HeLa cells were double-stained with Hoechst 33342 and PI (see Methods). There is no significant difference in the amount of PI-positive cells among the various experimental groups tested (n = 6 for each data set).

both parameters exhibit sensitivity profiles that are undistinguishable. In addition, flufenamate added to hypertonic solution was found to increase DNA fragmentation. Enrichment of mono- and oligonucleosomes released into the cytoplasm was enhanced by the presence of flufenamate under hypertonic conditions (Figure 3C). Furthermore, there was no detectable induction of cell necrosis under any of the above experimental conditions, as was determined by a double staining of HeLa cells with Hoechst 33342 and PI (Table 1).

To further test for the putative correlation between HICC currents and proliferation/apoptosis in HeLa cells, whole-cell patch-clamp recordings were performed. As depicted in Figure 4A, hypertonic cell shrinkage significantly increased membrane currents (at -30 mV, which is the equilibrium potential for Cl⁻) from -0.6 ± 0.14 to -3.2 ± 0.46 pA (n = 26, p < 0.05). As expected for HICC activation, the reversal potentials of the shrinkage-activated current was -2.5 ± 1.8 mV (n = 26), suggesting that the current is cation selective. Of note, HICC currents

Fig. 4. Inhibition of HICC currents by flufenamate. A, a representative experiment illustrating the currents under isotonic and hypertonic (450 mosmol/kg-H₂O) conditions, as well as the hypertonic current in the presence of 30 and 100 μmol/l flufenamate. B, summary of currents at -30 mV (= E_{Cl}) with reference to the hypertonic control (n = 4 to 9).

Fig. 5. Concentration-dependence of the effects of Gd³⁺ on proliferation (A) and caspase-3 activity (B), in HeLa cells under hypertonic stress. Data are presented as the difference of the hypertonic (600 mosmol/kg-H₂O) minus the isotonic (300 mosmol/kg-H₂O) value obtained (n = 22, for each experimental condition).

were dose-dependently inhibited by flufenamate (Figure 4B) and the overall profile of this effect was very similar

to those observed for proliferation-decrease and caspase-3 activation (Figure 3). These results strongly suggest

Fig. 6. Inhibition of HICC currents by Gd³⁺. A, a representative experiment illustrating the currents under isotonic and hypertonic (450 mosmol/kg-H₂O) conditions, as well as the hypertonic current in the presence of 100 μmol/l Gd³⁺. B, summary of currents at -30 mV (= E_{Cl}) with reference to the hypertonic control (n = 3 to 17).

that hypertonicity-triggered apoptosis-induction in HeLa cells is mediated via the actual inhibition of HICC channels.

Finally, a series of experiments with Gd³⁺, which is an additional blocker of HICCs in HeLa cells [4], was performed. As shown in Figure 5, both hypertonicity-induced proliferation-decrease and caspase-3 activation exhibited profiles of sensitivity to the lanthanide that are very similar. Furthermore, the sensitivity of HICC currents totally matched these profiles (Figure 6) providing strong further evidence for the paramount significance of HICCs for RVI, proliferation and apoptosis in HeLa cells. There was no necrosis detectable in any of the experiments performed with Gd³⁺ (Table 1).

Discussion

Apoptotic volume decrease (AVD), under isotonic conditions, is a hallmark of early apoptosis and inhibition of the AVD process has been shown to rescue cells from programmed cell death [5-7]. On the other hand, hypertonic cell shrinkage initiates apoptosis in some cells [8-13], but only if the actual machinery of RVI is

functionally impaired [8]. We have previously reported that flufenamate, which is a blocker of HICCs, completely blocks RVI in HeLa cells [4]. We were wondering, therefore, if continuous cell shrinkage induced by hypertonic stress in conjunction with a block of HICCs may act as a sufficient signal for apoptosis induction. Such a mechanism would be of considerable physiological interest concerning the actual interplay of proliferation vs. apoptosis and the role that mechanisms of cell volume regulation do play in these processes.

In the present study, hypertonic stress up to plus 300 mosmol/kg-H₂O did *per se* neither change the proliferation rate of HeLa cells nor did it stimulate the activity of caspase-3. These results are readily explained in terms of the high potency of HeLa cells to accomplish an effective RVI, even under a sustained hypertonic exposure [4].

In sharp contrast, flufenamate as well as Gd³⁺, as the most effective blockers of the HICC [4], both significantly sensitized HeLa cells to hypertonic stress: As a function of hypertonicity, proliferation was decreasing whereas the activity of caspase-3 was increasing. Furthermore, under constant (maximal) hypertonic stress, proliferation-inhibition and caspase-3

activation exhibited pharmacological profiles that were virtually identical and that, finally, precisely matched those of the inhibition of the HICC. In addition to caspase-3 activation, DNA fragmentation induced by hypertonicity was enhanced by the presence of 100 μ M flufenamate. Furthermore, PI staining revealed that membrane integrity was maintained under these conditions. Considering these results, it appears that inhibition of HICC resulted in early apoptosis but neither late apoptosis nor necrosis within the time scale in the present study.

Flufenamate is a non-specific ion channel blocker and has been reported to inhibit Ca^{2+} -activated [15] and volume-sensitive Cl^- channels [16]. In none of our recordings, however, there was any hypertonic activation of Cl^- channels detectable (data not shown). On the other hand, although several transient receptor potential (TRP) channels such as TRPC3 and/or TRPC6 [17] and TRPC5 [18] are reported to be sensitive to flufenamate, the sensitivity of Gd^{3+} on these channels is not known yet. In addition, it should be noted that the molecular identification of HICCs is missing. In the present study, therefore, judging from the pronounced similarities in the observed sensitivity profiles of flufenamate for proliferation-inhibition, apoptosis-stimulation as well as the obvious parallelism to the effects of Gd^{3+} , HICCs appear to be

the most likely candidate actually involved in the regulation of proliferation and apoptosis in HeLa cells.

In conclusion, our results clearly show that flufenamate and Gd^{3+} , both effective inhibitors of HICC currents, elicit a sensitization of HeLa cells to hypertonic stress. With the RVI blocked, proliferation is blunted whereas apoptosis is stimulated. First of all, these results suggest that persistent cell shrinkage may function as a specific signal in the induction of apoptosis. In addition, they provide further evidence for the interplay of proliferation vs. apoptosis and the actual role that mechanisms of cell volume regulation do play in these processes.

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