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Original Paper

Serotoninergic Modulation of Cell Volume Response to Estramustine: an Image-analysis Study on Perifused Individual Glioma Cells

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A technique of microscopy with computerised detection of early morphological changes during continuous perifusion was used to monitor the geometry changes of cultured glioma cells (MG-251) when exposed to 40 mg/L estramustine phosphate (EMP) alone or in combination with granisetron (0.1 μmol/L), ondansetron (0.1 μmol/L), or serotonin (1 μmol/L). When the cells were exposed to EMP, cell volume measured as projected cell area (PCA) rapidly increased. Serotonin and ondansetron, but not granisetron, prevented the acute EMP response (PCA). Serotonin, but none of the 5-HT_3 receptor antagonists, protected against the cytotoxicity of EMP to the glioma cells as measured by a fluorometric microculture assay. Our results demonstrate hitherto unknown differences between selective 5-HT_3 receptor antagonist on the cellular response to EMP and shows the necessity to study the receptor antagonists from viewpoint of interference with the antitumour drug effects on malignant cells. The perifusion technique could be used to study the effects of serotoninergic agonists and antagonists on cell volume regulation of cells exposed to anticancer drugs. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: cell volume, cytotoxicity, estramustine, granisetron, microperifusion, ondansetron, potassium flux, serotonin
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INTRODUCTION

CELL VOLUME regulation is essential in cell growth and proliferation and includes a balanced flow of organic solutes and ions through strictly regulated ion channels [1]. Previously we have shown a concentration-dependent increase in cell size following estramustine phosphate (EMP)-treatment of glioma cells [2] and that inhibitors of transmembrane ion transport could modify these EMP-induced cell volume increases [3]. The estramustine-induced increase in cell volume indicates membrane ion leakage, also shown from a decreased ^{86}Rb influx with a net flow of ions and water into the cell [2]. Cation transport mechanisms can be primary targets for antineoplastic drugs [4–6] as the cytotoxic activity of the drugs is altered by inhibitors of cation transport mechanisms at the level of the plasma membrane [7, 8]. Thus, it is possible that there are interactions between drugs that influence transmembrane fluxes of cations reflected in cell volume changes and the cytotoxicity of antineoplastic drugs.

Some anti-emetics have been shown to modulate cytotoxic chemotherapy [9–11] and we have also previously shown that the anti-emetics, dixyrazin, metoclopramide, chlorpromazine and droperidol, sensitised cultured cells to cytotoxicity of epirubicin [12, 13]. Even the recently introduced effective anti-emetic and 5-HT_3 receptor antagonist, granisetron (but not ondansetron), was demonstrated to potentiate the cytotoxicity of epirubicin to cultured fibroblasts [14]. It is known that serotoninergic and similar drugs are involved in the regulation of several important cellular processes [15]. As such we found it of interest to evaluate the interaction between EMP and the 5-HT_3 receptor antagonists, ondansetron and granisetron, using a new digitised image processing for rapid and precise measurements of cancer cell size and morphology.

MATERIALS AND METHODS

Cell culture

The human malignant glioma cell line, MG-251, was grown as a monolayer in Eagle's minimal essential medium

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(MEM) supplemented with 10% fetal calf serum. The cells were incubated at 37°C in humidified atmosphere containing 5% CO_2. Medium was changed three times a week. Cells were harvested by incubation with 0.2 mL EDTA (5.0 mmol/L) for 5 min followed by trypsin (0.1%). The cells were portioned into plastic tissue culture dishes containing basal medium and then kept under controlled conditions (37°C and 5% CO_2) before usage. Estramustine phosphate (oestradiol-3-N-bis (chloroethyl) carbamate phosphate) was diluted in Eagle's MEM to appropriate concentrations and included in the incubation media.

Experimental set-up for cell microperifusion

The microperifusion device (ECZ-250, Carl Zeiss, Svenska AB, Stockholm, Sweden) consisted of a plastic frame, 75×25×4.5 mm with a thin bottom glass slide. At one end and at a 250 μm distance from the bottom, there is a smaller glass slide dividing two compartments; one cell compartment for perifusion (approximately 85 μL) and a larger medium reservoir (approximately 3.5 mL). The cell compartment is open towards the medium reservoir for medium to enter during perifusion. Evaporation from the reservoir was prevented by a glass cover. During a pause in perifusion, the medium reservoir is drained of untreated medium and a drug-containing medium is added. When the pump is restarted, the test medium is aspirated into the cell compartment with a sharply defined interface between the two media. By knowing the pump speed, the geometry of the cell compartment and the location of the cell (approximately 8 mm from the compartment entrance) the timing of cell exposure can be predicted, here being of the order of 10 sec. Note, in this study and for best experimental reproducibility, this 10 sec delay was incorporated into the first minute of perfusion and thus the first 60 sec recording rather represents 50 sec of true drug exposure. A peristaltic pump (LKB 12000 Varioperpex, LKB AB, Bromma, Sweden) was used and connected in series with a flow vibration damper to prevent vibrations from the pump dislodging the cells in the chamber. The inverted phase contrast microscope, Zeiss Axiovert™ (with oil objective lens 100/1.25), on which the microperifusion device was mounted, was equipped with a thermostatically controlled box and inlet media were preheated before entering the chamber reservoir in which the temperature was further stabilised prior to its entrance into the cell space. The technique has been used in previous reports on EMP effects on cultured cells [2, 3].

Cells were recorded continuously on the computer screen and with automatic, intermittent computer inputs. The morphology of individual cells was automatically measured by a computerised image analyser IBAS 25 (KONTRON Image Analysis Division, Zeiss, Oberkochen, Germany). The computer was programmed for automatic timing of the experimental phases, with one input per minute and separating untreated and test medium perifusions. One cell was measured during each perifusion experiment. The original images were stored on disc followed by a re-run after the experiment during which background subtraction, contour enhancement and object identification was performed. The image processing was semi-interactive in that the cell images were digitally identified but interactively edited for grey-scale and possible artefact of the cell contour. The microscope image produces

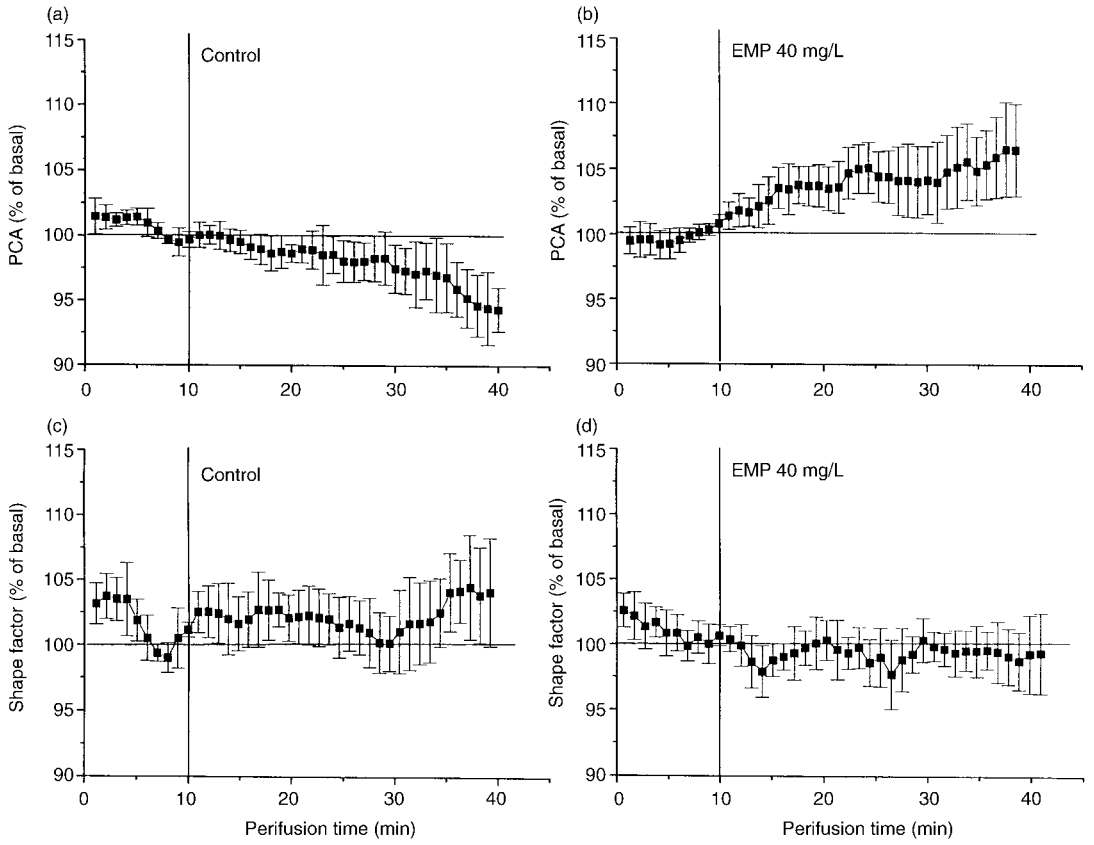

Figure 1. Dose–response curves for control cells (untreated medium only a,c), or EMP (b,d) on PCA (a,b) and shape factor (c,d) in human malignant glioma cells. After 10 min of basal medium perifusion the medium was abruptly changed to perifusion without/with EMP 40 mg/L for 30 min. Mean values ± SEM. Number of cells analysed were 5 for control and 9 for EMP.

Table 1. *The effect of estramustine on glioma cell geometry.*

Parameters	(unit)	Control	EMP
Untreated mean 5–10 min	(%)	100	100
PCA			
Mean 10–40 min	(%)	97.90 ± 1.41	104.05 ± 1.93
Mean 20–40 min	(%)	97.25 ± 1.85	104.65 ± 2.31
Mean 30–40 min	(%)	96.13 ± 2.22	105.13 ± 2.76
Slope 10–20 min	(%/min)	− 0.15 ± 0.08	0.33 ± 0.13
Slope 10–40 min	(%/min)	− 0.17 ± 0.09	0.14 ± 0.10
Shape factor			
Mean 10–40 min	(%)	102.17 ± 2.16	99.32 ± 1.03
Mean 20–40 min	(%)	102.14 ± 2.31	99.27 ± 1.19
Mean 30–40 min	(%)	102.67 ± 2.94	99.35 ± 1.73
Slope 10–20 min	(%/min)	− 0.00 ± 0.17	0.02 ± 0.21
Slope 10–40 min	(% min)	0.03 ± 0.09	− 0.00 ± 0.07

Glioma cells were harvested and introduced into a perifusion chamber to measure the geometry of individual cells by computerised image processing. The geometric parameters in the table refer to the mean values and slopes of all tested cells during perifusion with or without estramustine phosphate (40 mg/L). The PCA and perimeter were measured by the computer to calculate the shape factor. Number of cells analysed were 5 for control and 9 for EMP.

an optical halo around the cell due to differences in refractive indices (cell/medium) and in which the true cell membrane is found. Instead of tracing the outer boarder of the halo, which would be technically easier, the digital image was inverted to measure the inner contour of the halo. The inner halo contour better follows the membrane irregularities and thus gives a better estimate of the true cell morphology.

The cell geometry was measured in terms of projected cell area (PCA) and perimeter shape factor (PSF). The PCA was the two-dimensional area of the cell whereas PSF was calculated by the computer from the PCA and the perimeter;

$$\text{Perimeter shape factor} = 4\pi\text{PCA}/(\text{perimeter})^2 \quad (1)$$

The PSF represents the roundness of the cell and for which the value 1.0 represents a perfect circular shape.

Experimental operation of perifusion

A small volume of cell suspension, approximately 50 μL, was injected into the slit entrance of the cell compartment and the cells were allowed to settle for 1 min before the perifusion with untreated medium (Eagle's MEM without fetal calf serum) was started. A cell was selected at random and perifused for 10 min after which time the pumping was stopped. The untreated medium in the reservoir was then exchanged for a test medium (containing 40 mg/L estramustine phosphate, 0.1 μmol/L granisetron, 0.1 μmol/L ondansetron, or/and 1 μmol/L serotonin) and the perifusion continued for another 30 min. During medium replacement and with the pump stopped, the cell remained in untreated medium to be exposed to the test medium only after the pump was restarted. This exposure becomes very distinct due to the sharp medium interface between basal and test medium that enters the cell compartment.

The baseline in each experiment denotes the PCA and shape factor, respectively, during the last 5 min basal perifusion (5–10 min). Data were normalised (100%) against the baseline.

Fluorometric microculture cytotoxicity assay

To quantify the effects of cytotoxic drugs on the cells, fluorescein diacetate (FDA) was used in a semi-automated fluorometric method [16]. FDA is membrane-permeable and is cleaved to fluorescent fluorescein by unspecific esterases in the cytoplasm of viable cells. Fluorescein is retained intracellularly. The amount of fluorescence correlates to the number of living cells.

Cells were harvested and plated in a volume of 100 μL at 2×10^4 cells/well in 96-well microtitre plates using a multichannel pipette. The plates were first incubated at 37°C for 24 h with culture medium only. Then medium was changed, drugs (0.1 μmol/L granisetron, 0.1 μmol/L ondansetron, 1 μmol/L serotonin, or/and 40 mg/L estramustine phosphate)

Table 2. *The effects of serotonin, granisetron and ondansetron on glioma cell geometry. Glioma cells were harvested and introduced into a perifusion chamber to measure the geometry of individual cells by computerised image processing. The geometric parameters in the table refer to the mean values and slopes of all tested cells during perifusion with or without* 40 *mg/L estramustine phosphate (EMP), combined with serotonin (*1 *μmol/L), granisetron (*0.1 *μmol/L), or ondansetron (*0.1 *μmol/L). The PCA and perimeter were measured by the computer to calculate the shape factor. Number of cells analysed were* 5 *for serotoninergic drug alone and* 5–11 *with drug combined with EMP*

Parameters	(unit)	Granisetron	Granisetron + EMP	Ondansetron	Ondansetron + EMP	Serotonin	Serotonin + EMP
Basal mean 5–10 min	(%)	100	100	100	100	100	100
PCA							
Mean 10–40 min	(%)	99.79 ± 1.29	104.04 ± 1.97	99.58 ± 1.19	99.70 ± 2.34	100.74 ± 1.80	99.93 ± 0.84
Mean 20–40 min	(%)	99.65 ± 1.61	105.17 ± 2.38	99.81 ± 1.50	99.72 ± 2.74	100.99 ± 2.07	100.19 ± 0.95
Mean 30–40 min	(%)	100.35 ± 2.23	105.90 ± 2.54	99.81 ± 1.65	100.18 ± 2.99	101.21 ± 2.14	99.77 ± 0.85
Slope 10–20 min	(%/min)	− 0.21 ± 0.11	0.21 ± 0.31	0.22 ± 0.05	− 0.03 ± 0.27	0.07 ± 0.21	0.14 ± 0.10
Slope 10–40 min	(%/min)	0.03 ± 0.10	0.22 ± 0.10	0.03 ± 0.06	− 0.05 ± 0.10	0.05 ± 0.08	0.02 ± 0.04
Shape factor							
Mean 10–40 min	(%)	100.72 ± 0.55	97.96 ± 1.42	100.24 ± 0.98	95.06 ± 2.26	98.78 ± 0.75	100.81 ± 0.67
Mean 20–40 min	(%)	101.01 ± 0.53	97.64 ± 1.79	99.74 ± 1.14	94.69 ± 2.56	97.99 ± 0.99	100.86 ± 0.77
Mean 30–40 min	(%)	101.11 ± 0.46	97.48 ± 2.06	99.66 ± 1.08	94.61 ± 2.66	98.04 ± 1.39	101.49 ± 0.79
Slope 10–20 min	(%/min)	0.27 ± 0.19	− 0.35 ± 0.31	0.091 ± 0.17	− 0.49 ± 0.19	− 0.17 ± 0.14	− 0.01 ± 0.12
Slope 10–40 min	(%/min)	0.05 ± 0.01	− 0.13 ± 0.10	− 0.077 ± 0.05	− 0.04 ± 0.07	− 0.12 ± 0.07	0.03 ± 0.04

diluted in culture medium were added in triplicate and the cells were then cultured for another 48 h. Plates were then centrifuged (300–400 g, 5 min), medium removed by flicking the plate and wells washed once with 200 μL PBS. To each well was then added 100 μL of PBS containing 10 mg/L FDA, the plates incubated for 60 min at 37°C, followed by fluorescence determination using 485 and 538 nm for excitation and emission, respectively.

Chemicals

Eagle's MEM and L-glutamine was from Gibco Ltd, Paisley, U.K. Fetal calf serum was purchased from Biochrom KG, Berlin, Germany. Ondansetron was from Glaxo Group Research, Greenford, U.K. and granisetron was from Smith-Kline Beecham Pharmaceuticals, Crawley, U.K. Serotonin (5-hydroxytryptamine, 5-HT) and fluorescein diacetate (FDA) was from Sigma Chemical Company, St Louis, Missouri, U.S.A. Estramustine from Pharmacia & Upjohn Sverige AB, Lund, Sweden. Cell culture 24 well cluster plates were from Costar, Cambridge, Massachusetts, U.S.A.

Statistics

Results are given as mean and standard errors (SEM). For the evaluation of microperifusion data, a linear regression was calculated for each individual cell during test perifusion. The mean values and linear slopes were compared by using an unpaired Student's *t*-test with correction for unequal variance and numbers between groups. Statistical significance for the fluorometric cytotoxicity assay was tested with one-way Anova. The level of significance for rejecting the null hypothesis of zero treatment effect was taken to be $P = 0.05$.

RESULTS

Control and estramustine

During control perifusion, there was a continuous decrease in PCA with time (Figure 1a); there was a significant difference between the starting PCA (5–10 min) and that after the following 30 min perifusion ($P < 0.05$, Table 1). This produced a negative slope that did not reach significance ($P < 0.1$). When EMP (40 mg/L) was added, the PCA increased (Figure 1b). The slope during the initial 10 min test perifusion gave a significant positive increase ($P < 0.05$). Furthermore, there was a significant difference in PCA versus control, calculated for the last 10 min perifusion ($P < 0.05$). This EMP-induced difference was also confirmed by comparing the slopes to that of the control curve, for the initial 10 min ($P < 0.01$) and for the entire 30 min test perifusion ($P < 0.05$).

The shape factor showed fluctuations during basal medium perifusion but without significance (Figure 1c, Table 1). When EMP (40 mg/L) was added, the shape factor decreased, suggesting a more irregular cell contour. This occurred during the initial 5 min perifusion but did not reach high significance as it returned towards baseline (Figure 1d).

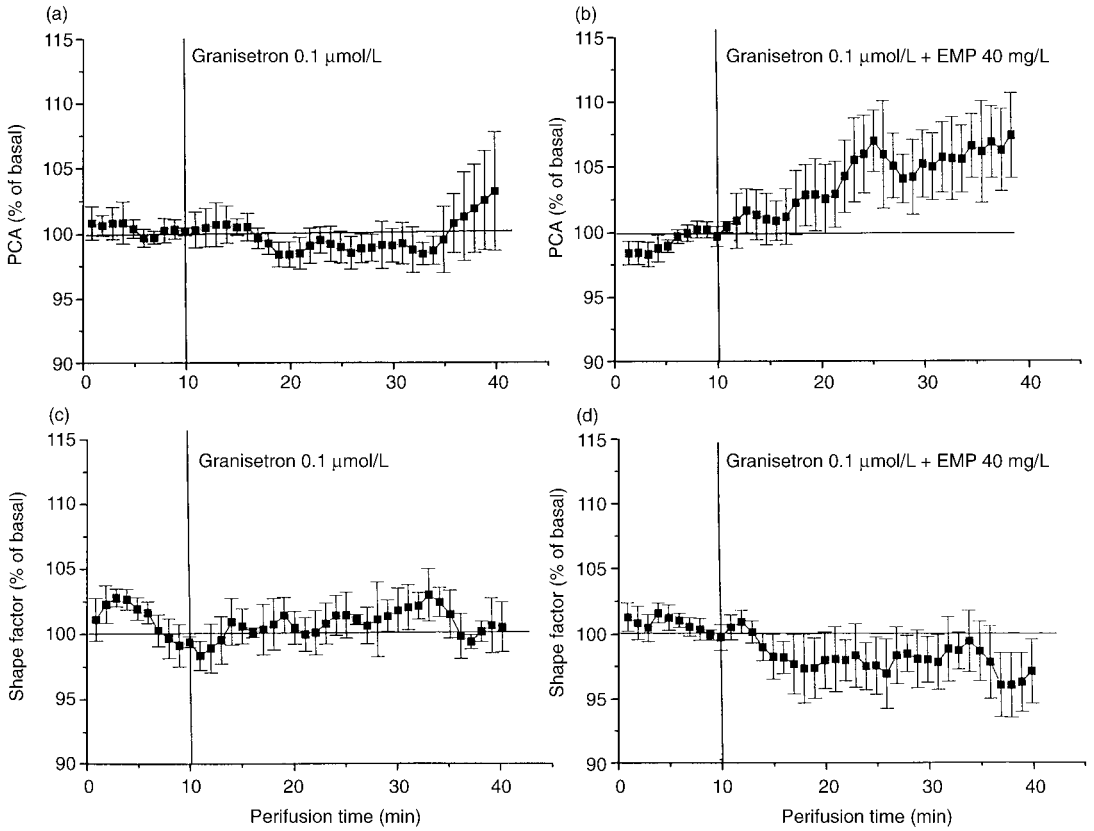

Figure 2. Effects of granisetron on PCA and shape factor and EMP-induced cell geometry change. Glioma cells were harvested and introduced into a perifusion chamber and the geometry of an individual cell was monitored by computerised image processing. After 10 min of basal medium perifusion, the medium was abruptly changed to either (a) granisetron 0.1 μmol/L for PCA, (b) granisetron 0.1 μmol/L plus EMP 40 mg/L for PCA, (c) granisetron 0.1 μmol/L for shape factor, or (d) granisetron 0.1 μmol/L plus EMP 40 mg/L for shape factor within the test-perifusion medium for 30 min perifusion. Mean values ± SEM. Number of cells analysed were 5 for granisetron alone and 9 for granisetron +EMP.

Granisetron and estramustine

When granisetron alone (0.1 μmol/L) was added to the untreated medium, no change in PCA was recorded, although, at the end of the perifusion (last 5 min) one of the tested cells increased in PCA to produce an apparent change (Table 2, Figure 2a). Except for the last 5 min perifusion, a modest decrease in PCA was seen with time, similar to that seen for the control curve. When EMP (40 mg/L) was added to the granisetron-containing medium an increase in PCA was recorded, close to identical with that for EMP alone (Figure 2b). This gave a positive PCA slope that nearly reached significance ($P < 0.06$) and the mean PCA calculated for the last 10 min perifusion showed a significant increase ($P < 0.05$).

Granisetron (0.1 μmol/L) had no effects on the shape-factor response, in fact, all characteristics of the control and EMP curve was seen also when granisetron was added (Figure 2c, d). In terms of PCA and shape factor changes, granisetron showed no differences to that of EMP alone and appeared without effect on its own.

Ondansetron and estramustine

When ondansetron (0.1 μmol/L) was added to the untreated medium the PCA showed an early decrease during the initial 3 min perifusion ($P < 0.01$, not indicated in table) and then increased so that the net slope for the initial 10 min became positive ($P < 0.05$, Table 1, Figure 3a). The PCA then remained fairly stable during the remaining perifusion period. Because of fluctuations in PCA during the control perifusion, with a gradual decrease during the 10 min perifusion, the exact significance of the ondansetron-response remains unsure. However, the last recordings of PCA prior to medium shift seemed fairly stable and may, therefore, justify the above interpretations.

With ondansetron (0.1 μmol/L) added to the medium, the EMP response with its significant increase in PCA, was abolished, in fact, the significant positive slope recorded during the initial 10 min of EMP perifusion became negative with ondansetron present (Table 1, Figure 3b). This possible difference caused by ondansetron was further strengthened by the shape-factor pattern (Figure 3d); when EMP was as added to the ondansetron-containing medium a steep negative slope was produced ($P < 0.05$). When a mean shape factor value was calculated for the entire test perifusion, the difference from baseline was near significance ($P < 0.06$). Further, with ondansetron present, a decrease in shape factor was seen with EMP versus with ondansetron alone (mean of entire test perifusion, $P < 0.06$). This suggests an additive or potentiating effect of ondansetron on the cell geometric response to EMP.

Serotonin and estramustine

With serotonin (1 μmol/L) added, the PCA remained unchanged compared with control during the 30 min obser-

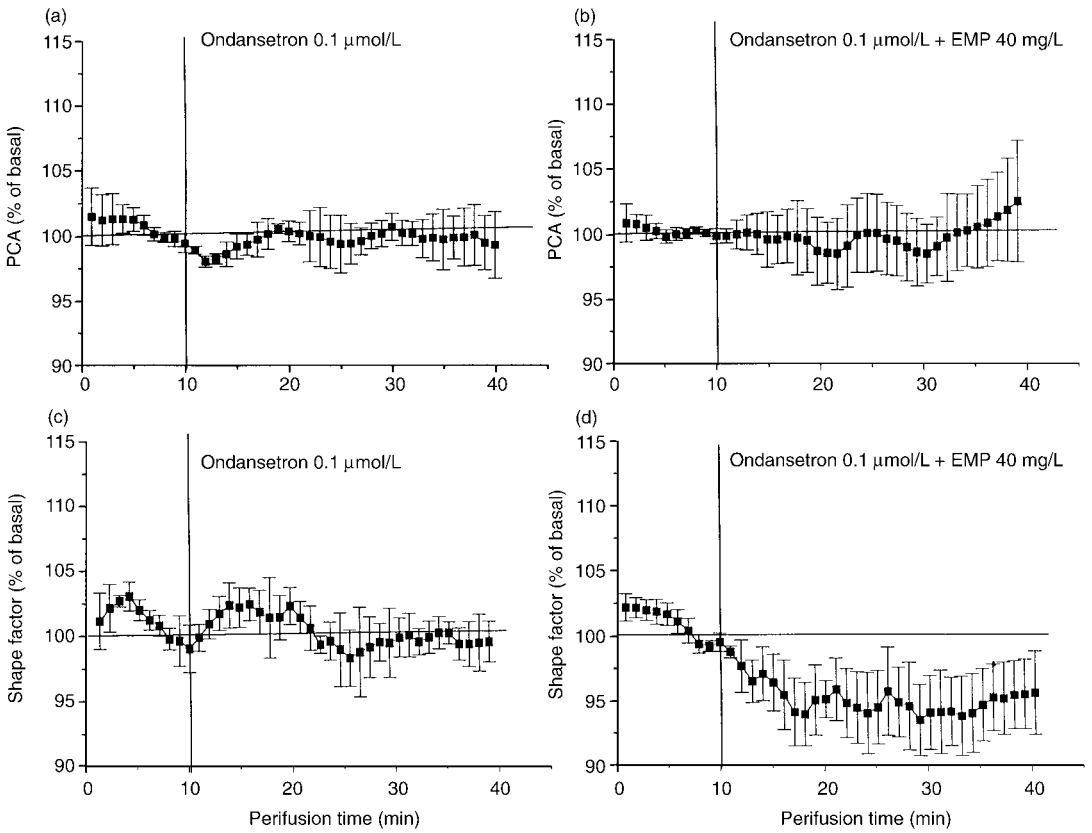

Figure 3. Effects of ondansetron on PCA and shape factor and EMP-induced cell geometry change. Glioma cells were harvested and introduced into a perifusion chamber and the geometry of an individual cell was monitored by computerised image processing. After 10 min of basal medium perifusion, the medium was abruptly changed to either (a) ondansetron 0.1 μmol/L for PCA, (b) ondansetron 0.1 μmol/L plus EMP 40 mg/L for PCA, (c) ondansetron 0.1 μmol/L for shape factor, or (d) ondansetron 0.1 μmol/L plus EMP 40 mg/L for shape factor within the test-perifusion medium for 30 min perifusion. Mean values ± SEM. Number of cells analysed were 5 for ondansetron alone and 11 for ondansetron +EMP.

vation period (Table 1, Figure 4a). The shape factor was not influenced by serotonin (Figure 4c). When serotonin was added to the EMP-containing medium, the EMP (40 mg/L) effect was abolished with a PCA that remained close to the baseline all through the perifusion (Figure 4b). The shape factor response of EMP was not affected by serotonin (Figure 4d).

Fluorometric microculture cytotoxicity assay

Granisetron (0.1 μmol/L), ondansetron (0.1 μmol/L) and serotonin (1 μmol/L) all decreased fluorescein fluorescence to approximately 82–85% of untreated control (Figure 5). Thus the serotoninergic drugs showed a slight cytotoxic action *per se*. EMP (40 mg/L) *per se* reduced fluorescence to approximately 43% of control. When EMP was combined with granisetron (0.1 μmol/L) or ondansetron (0.1 μmol/L), the fluorescence was not changed in comparison with EMP alone. However, the addition of serotonin (1 μmol/L) to EMP, significantly increased the fluorescence to 56% of control (Figure 5). Thus, of the tested serotoninergic drugs, only serotonin (1 μmol/L) significantly protected the glioma cells against EMP (40 mg/L) cytotoxicity.

DISCUSSION

In this study, it has been shown that the size of cultured glioma cells varies instantly when perifused with EMP. The cell volume, measured as projected cell area (PCA), rapidly increased when estramustine was added to the perifusion medium. Serotonin (5-HT) and the selective 5-HT_3 receptor antagonist, ondansetron, completely eradicated estramustine-induced PCA changes whereas another selective 5-HT_3 receptor antagonist, granisetron, had no effect. The selective 5-HT_3 receptor antagonists had markedly different effects on estramustine-induced PCA without affecting estramustine cytotoxicity.

An intact cell volume is known to be of critical importance for the preservation of cell functions including growth and proliferation which includes a balanced increase in cell content of organic solutes and ions and in cell membrane surface area. Transmembrane cations fluxes of non-specific nature or via different ion channels are part of important cell functions, such as maintenance of the membrane potential and intracellular pH and volume regulation in anisotonic media [1].

During chemotherapy, the use of anti-emetics such as granisetron and ondansetron is mandatory. Their anti-emetic activity is mediated through selective antagonism of 5-HT_3 receptors on vagal afferents and at the chemoreceptor trigger zone [17, 18]. Multiple 5-HT receptor subtypes have been characterised and some have been shown to possess ion channel activity. For instance, serotonin has been found to activate a cation conducting ion channel with approximately equal permeability to Na^+ and K^+ and 5-HT-induced currents were antagonised by ondansetron [19]. The 5-HT_3 receptor subunits form a pentameric cation channel that is selectively permeable to Na^+, K^+, and Ca^{2+} ions and causing depolarisation [15, 20]. It would, therefore, not be surprising

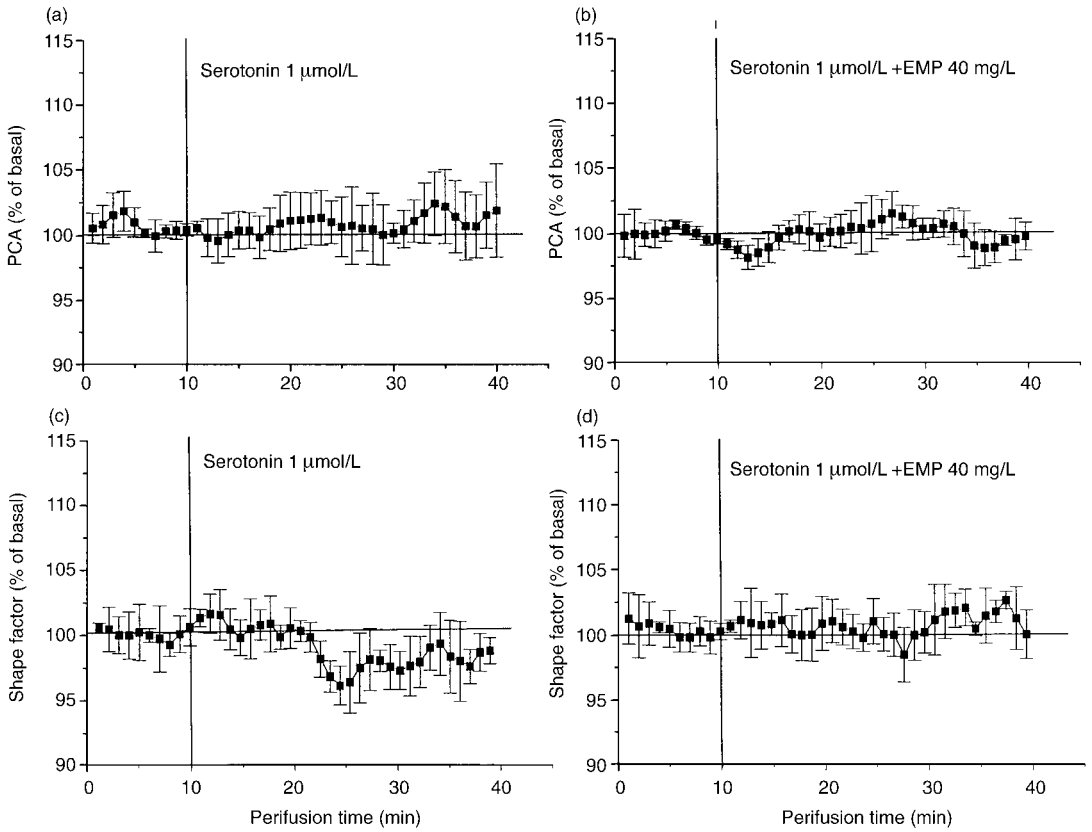

Figure 4. Effects of serotonin on PCA and shape factor and EMP-induced cell geometry change. Glioma cells were harvested and introduced into a perifusion chamber and the geometry of an individual cell was monitored by computerised image processing. After 10 min of basal medium perifusion, the medium was abruptly changed to either (a) serotonin 1 μmol/L for PCA, (b) serotonin 1 μmol/L plus EMP 40 mg/L for PCA, (c) serotonin 1 μmol/L for shape factor, or (d) serotonin 1 μmol/L plus EMP 40 mg/L for shape factor within the test-perifusion medium for 30 min perifusion. Mean values ± SEM. Number of cells analysed were 5 for serotonin alone or in combination with EMP.

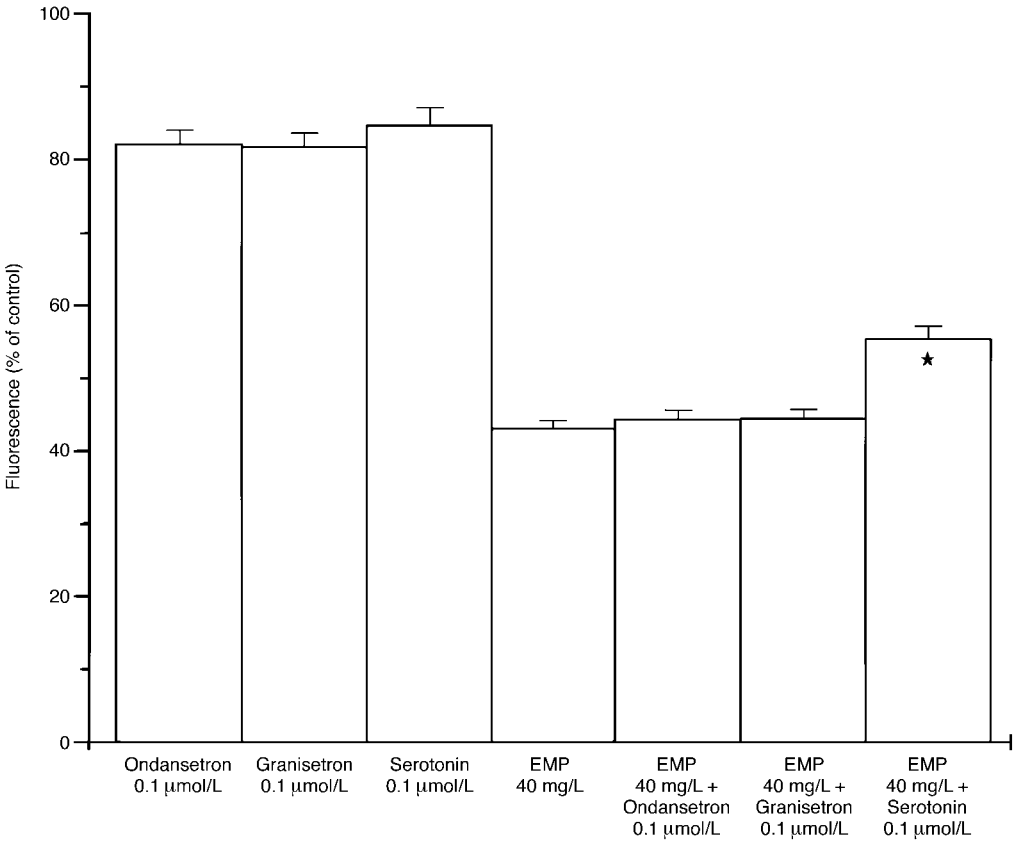

Figure 5. FDA fluorescence from glioma cells after 24 h incubation in microculture with ondansetron 0.1 μmol/L, granisetron 0.1 μmol/L, or serotonin 1.0 μmol/L, with or without EMP 40 mg/L. After incubation culture plates were centrifuged, wells rinsed with PBS and FDA added. After 60 min further incubation, the fluorescence of each well was determined. Mean values ± SEM. *n*=48, 4 separate experiments with 12 replicates each. **P*<0.001 compared with estramustine alone.

if serotonin or the selective 5-HT_3 receptor antagonists interferes with cell volume regulation and cellular effects of drugs such as estramustine, that also affects transmembrane cation fluxes. We have shown that ondansetron was inert and non-interactive with irradiation and several anticancer agents on human lung cancer cells and Chinese hamster fibroblasts, whereas granisetron potentiated the cytotoxic effect of epirubicin on fibroblasts and had a seemingly additive effect to estramustine cytotoxicity on lung cancer cells [15], the first time that potential clinical significant differences between the highly selective 5-HT_3 receptor antagonists had been suggested. At that time, pre-clinical studies had shown that ondansetron had a weak antagonistic activity on 5-HT1b, 5-HT1c, adrenergic α1 and μ opoid receptors whilst granisetron was supposed to be more selective than ondansetron with respect to 5-HT_3 receptors. However, both drugs have a selectivity ratio of approximately 1000:1 for the 5-HT_3 receptors with respect to any other type of receptor and, therefore, it was regarded unlikely that these minor differences would have clinical relevance [21, 22]. To further strengthen the possibilities of clinical differences in the interaction of 5-HT_3 receptor antagonists and toxic agents, Toral and co-workers, using other toxic agents, showed that ondansetron had a unique ability to block voltage-gated potassium channels induced by 5-HT receptor selective neurotoxins of TE671 human neuroblastoma cells. This property was not shared with the other 5-HT_3 receptor ligands which led them to suggest that the potassium-channel-blocking activity was not mediated through 5-HT_3 receptors [20].

In a recent study, we found that bumetanide and furosemide, inhibitors of Na^+, K^+, Cl^- cotransport and ouabain, an inhibitor of Na^+, K^+, ATPase, completely blocked the estramustine-induced cell volume increase [3]. We showed that acute changes in cell size correlated strictly with the concentration of estramustine in the perifusion systems. The Na^+, K^+, Cl^- cotransport and Na^+, K^+, ATPase blockers were shown to have dramatic effects on cancer cell morphology and seemed to interact with the estramustine response. It, therefore, seemed possible that blockage of Na^+, K^+, ATPase and/or Na^+, K^+, Cl^- cotransport activation by estramustine could affect morphological changes and cytotoxicity of the drug.

In this study, serotonin and the specific 5-HT_3 receptor antagonist, ondansetron, completely eradicated estramustine-induced PCA changes whereas another specific 5-HT_3 receptor antagonist, granisetron, had no effect on estramustine-induced increase of glioma cell volume. In contrast to both serotonin and granisetron, ondansetron also significantly reduced the shape factor of cells incubated with estramustine, i.e. decreased the roundness. We believe that these morphological effects are to be sought in diverging effects of the serotoninergic drugs on cellular cation transmembrane transport systems. Our finding of ondansetron's ability to completely block EMP-induced volume increases could have an parallel in Toral and co-workers finding of ondansetron's unique ability to block voltage-gated potassium channels induced by 5-HT receptor selective neurotoxins of TE671 human neuroblastoma cells. This property was

not shared by the other 5-HT_3 receptor ligands, suggesting that the potassium-channel-blocking activity was not mediated through 5-HT_3 receptors [20]. Moreover, 5-HT induced currents of a murine neuroblastoma cell line was antagonised by ondansetron [19] and cation channels opened by 5-HT were blocked by ondansetron [23]. Data obtained in this work points to a strong possibility that ondansetron possesses a distinct potassium channel blocking activity and that this property differs from that of granisetron. This suggestion is supported by the fact that ondansetron completely abolishes the reduction of ^{86}Rb influx by estramustine alone whereas granisetron did not (data not shown). In contrast to results from previous studies [8, 15], the suggested relationship between estramustine-induced cell volume changes and cytotoxicity was not confirmed with this glioma cell line, as the selective 5-HT_3 receptor antagonists had markedly different effects on estramustine-induced PCA changes without affecting estramustine cytotoxicity. The previously observed correlation between estramustine-induced cell volume increase and cytotoxicity could possibly be related to the specific cell type studied. Further studies on cell volume regulation of other cell lines and its correlation to serotoninergic modulation of cancer-drug cytotoxicity seem necessary before conclusions on the relationship could be drawn.

Estramustine is a complex between oestradiol-17β and the alkylating agent nor-nitrogen mustard and is widely used in the treatment of advanced prostatic cancer [24, 25]. Estramustine phosphate is specifically metabolised by glioma cells *in vitro* [26] and *in vivo* in rats [27] and humans [28]. In addition to an interaction with microtubules and related processes, it has also been suggested that estramustine cytotoxicity may involve a direct interaction with cell membrane components [12, 29–32]. It is, therefore, of interest to emphasise that the cytoskeleton has been found to be a modulator of ion transport and cell volume regulation, as ion exchangers and ion pumps are directly associated with the cytoskeleton [33, 34]. As we found diverging effects of the tested 5-HT_3 receptor antagonist drugs on both PCA and shape factor, the eventual effects on the cytoskeleton of the agents combined with estramustine could also be a future investigative line to follow.

The present study suggests that there exists an interaction between serotoninergic drugs and the mechanisms regulating cell volume induced by estramustine as both serotonin and ondansetron prevented glioma cell volume increases induced by the anticancer drug. Further studies on serotoninergic agents, interference with the antitumour efficacy of cancer chemotherapy are warranted, as are investigations of the interrelationship between serotoninergic agonists/antagonists, ion flows, cytotoxicity and cell volume regulatory mechanisms under the influence of anticancer drugs.

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