Acquisition of Cisplatin-resistance in Malignant Mesothelioma Cells Abrogates $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ -cotransport Activity and Cisplatin-induced Early Membrane Blebbing

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Key Words

Apoptosis • Caspase-3 • Cisplatin • Malignant mesothelioma • Membrane blebbing • $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ -cotransport

Abstract

Aims: Resistance mechanisms are important limiting factors in the treatment of solid malignancies with *cis*-diamminedichloroplatinum(II) (cisplatin). To gain further understanding of the effects of acquired cisplatin-resistance, we compared a human malignant pleural mesothelioma cell line (P31) to a sub-line (P31res1.2) with acquired cisplatin-resistance. **Methods and Results:** The role of $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ -cotransport (NKCC1) activity in cisplatin-induced morphological changes and acquired cisplatin-resistance was investigated in a time-resolved manner. Acquisition of cisplatin-resistance resulted in markedly reduced NKCC1 activity, absence of cisplatin-induced early membrane blebbing, and increased basal caspase-3 activity. At equitoxic cisplatin concentrations, P31res1.2 cells had a faster activation of caspase-3 than P31 cells, but the end-stage cytotoxicity and number of cells with DNA fragmentation was similar. Bumetanide inhibition of NKCC1 activity in P31 cells repressed cisplatin-induced early-

phase membrane blebbing but did not increase P31 cell resistance to cisplatin. **Conclusions:** Together, these results suggest that active NKCC1 was necessary for cisplatin-induced early membrane blebbing of P31 cells, but not for cisplatin-resistance. Thus, acquisition of cisplatin-resistance can affect mechanisms that have profound effects on cisplatin-induced morphological changes but are not necessary for the subsequent progression to apoptosis.

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Introduction

The term apoptosis was introduced to describe a particular morphological aspect of cell death: attached cells round up and shrink, there is chromatin condensation and fragmentation of the nucleus and plasma membrane blebbing precedes the separation of the cell into membrane-bound apoptotic bodies [1]. In adherent cells, apoptotic membrane blebbing occurs in two distinct phases [2]. An early-phase membrane blebbing at the onset of the apoptotic release phase, and a late-phase membrane blebbing concurrent with phosphatidylserine (PS) exposure on the cell membrane and activation of

apoptotic caspases [2]. Although the mechanisms of apoptotic membrane blebbing are not fully understood, they appear similar for apoptotic and stress-induced membrane blebbing [3-7]. Cell membrane blebbing can commence within minutes of start of insult [8-10]. There are several reports that indicate a role for the secretory Na^+ , K^+ , 2Cl^- -cotransporter (NKCC1) in membrane blebbing via interactions with actin, the p38 mitogen activated protein kinases (p38) and extra-cellular regulated kinase 1/2 (ERK1/2) [11-14].

Potassium ions (K^+) have important regulatory roles during apoptosis. The human malignant pleural mesothelioma cell line P31 has been well characterised regarding K^+ transport mechanisms [15, 16]. At least 90 % of the K^+ transport across the cell membrane is carried out by Na^+ , K^+ , ATPase pump and NKCC1 activity [15, 16]. Pharmacological modulation of K^+ transport not only changes P31 sensitivity to apoptosis inducers, but also induces apoptosis *per se* [15, 17-19]. Efflux of K^+ is necessary for cell shrinkage, and a reduced intracellular K^+ concentration is necessary for caspase activation [20]. However, caspase activity *per se* is independent of the intracellular K^+ concentration [21]. The execution of apoptosis proceeds through signalling pathways that in most cases result in activation of the executioner caspases caspase-3 and -7 that have similar substrate specificity [22, 23]. Caspase-3 mediates the late-phase membrane blebbing [24] and the internucleosomal DNA fragmentation [22, 23], which occurs very late in apoptosis [3].

A cisplatin concentration of 10 mg/L reduces the number of P31 cells by 50 % 48 h after a 2-h exposure, and this concentration also causes extensive, dynamic membrane blebbing within minutes of start of exposure [9]. It is suggested that potassium transport has a role for tumour cell sensitivity to cisplatin [25-27], although cell lines differ in this respect [28]. Acquisition of cisplatin-resistance has been associated with a plethora of apoptosis signalling-related changes, including decreased caspase-3 activity [29, 30].

The aim of this study was to determine the role of NKCC1 in early-phase cisplatin-induced morphological changes and acquired cisplatin-resistance. A human malignant pleural mesothelioma (P31) cell line and a sub-line with acquired cisplatin-resistance (P31res1.2) were compared in a time-resolved manner regarding cisplatin-induced early-phase changes of morphology (< 30 min) and K^+ flux (< 60 min) modulation. Induction of apoptosis was determined by analysing caspase-3/7 activation over time (< 24 h), end-stage (72 h after exposure) cytotoxicity, cell morphology and DNA fragmentation.

Materials and Methods

Cell culture and experimental set-up

A human malignant mesothelioma cell line of pleural origin (P31) [31] was used to establish a cisplatin-resistant sub-line (P31res1.2) by stepwise increasing the concentration of cisplatin in the medium to a final concentration of 1.2 mg/L, in which the P31res1.2 cells were maintained. However, in all assays the control conditions were cisplatin-free medium for both P31 and P31res1.2 cells. Both cell lines were propagated at 37 °C in a humidified atmosphere containing 5 % CO_2 , grown as monolayer cultures in Eagle's minimal essential culture medium with Earl's salts supplemented with 10 % foetal calf serum, 200 $\mu\text{mol/L}$ L-glutamine, and gentamicin (MEM). To verify and briefly describe the cisplatin-resistant phenotype of the P31res1.2 cells, cisplatin-induced cytotoxicity, caspase-3/7 activation and DNA fragmentation was determined.

To investigate the role of NKCC1 in early-phase cisplatin-induced morphological changes and cisplatin-resistance, the experiments were set up in a time-resolved manner. To capture the dynamics of early-phase cell morphology changes, image analysis was performed during the initial 30 min of drug exposure. Changes in K^+ influx and efflux were determined during the initial 60 min of drug exposure. The role of NKCC1 in cisplatin-resistance was determined with a fluorescence cytotoxicity assay and determination of DNA-fragmentation.

Fluorometric microculture cytotoxicity assay

To determine cisplatin cytotoxicity, a semi-automated fluorometric microculture cytotoxicity assay (FMCA) was used [32]. Cells harvested by trypsinisation were plated in 96-well cell culture plates and maintained overnight at 37 °C in MEM. After appropriate exposures, the cells were washed with phosphate buffered saline (PBS), 10 mg/L fluorescein diacetate in PBS was added to each well and incubated for 60 min at 37 °C. Fluorescence was determined in a LS55 Luminescence spectrometer (PerkinElmer, Waltham, MA, USA) using 485 and 538 nm for excitation and emission, respectively.

Fluorometric caspase-3/7 activity assay

To determine activation of caspase-3/7 activation, a fluorometric caspase activity assay (R&D Systems) was used according to the manufacturer's instructions. Cells harvested by trypsinisation were plated on Petri dishes and maintained overnight at 37 °C in MEM to about 85 % confluence, then exposed to the drugs. Immediately after exposures the cells were lysed in lysis buffer (kit-included) for 30 min at 4 °C.

After centrifugation, the supernatant was aliquoted and stored at -80 °C until analysis. A BCA protein assay reagent kit was used according to the manufacturers' instruction (Pierce Biotechnology, Rockford, IL, USA) to determine the total protein content of lysates. 100 μg protein was mixed with assay buffer and fluorescent substrate DEVD-AFC and incubated 1.5 h at 37 °C. Fluorescence was determined in a LS55 Luminescence spectrometer (PerkinElmer) using 400 and 505 nm for excitation and emission, respectively.

TUNEL staining

DNA-fragmentation was detected using TUNEL staining (In Situ Cell Death Detection Kit, Roche) according to the manufacturer's instructions. Cells were plated as for the caspase activity assay. At appropriate times after exposures, the cells were harvested by trypsination, washed and fixed in 2 % paraformaldehyde. After permeabilisation with 0.1 % Triton X-100 in 0.1 % sodium citrate, the cells were labelled for DNA strands breaks with TMR red and analysed in a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) using the FL3 channel for detection of TMR red.

⁸⁶Rb⁺ influx and efflux determinations

The K⁺ analog ⁸⁶Rb⁺ was used to study K⁺ influx and efflux across the cell membrane. The effects of cisplatin, ouabain, a specific inhibitor of the Na⁺,K⁺, ATPase pump [33], and bumetanide, a specific inhibitor of NKCC1 activity [34], were studied. These are the two dominant K⁺ carriers in P31 cells [15, 16]. The assays were performed as described previously [15]. In short, cells were harvested by trypsination, plated in 96-well culture plates (Isoplate, PerkinElmer) and maintained 24 or 48 h at 37 °C in MEM. 48 h after plating the cells were exposed to 0.75 µCi/mL ⁸⁶Rb⁺ added simultaneously with test substances, and the cellular ⁸⁶Rb⁺ content was measured to determine ⁸⁶Rb⁺ influx. To determine ⁸⁶Rb⁺ efflux, pre-loading of the cells with 0.75 µCi/mL ⁸⁶Rb⁺ began 24 h after plating. After 24 h of pre-loading, the ⁸⁶Rb⁺-containing supernatant was removed, and then the test substances were added. To calculate ⁸⁶Rb⁺ efflux (expressed as % of total cell content), both the cell and the supernatant ⁸⁶Rb⁺ contents were measured separately. After appropriate exposures, Optiphase SuperMix Liquid Scintillation Cocktail (PerkinElmer) was used to measure cell and supernatant ⁸⁶Rb⁺ content.

Western blotting

Protein expression was determined with Western blotting. Cells were plated as for the caspase activity and TUNEL assays. After 30 min of exposure, the cells were lysed with lysis buffer [50 mmol/L Tris 7.5; 1% Triton X-100; 150 mmol/L NaCl; 1 mmol/L NaF; 1 complete protease inhibitor EDTA-free cocktail tablet to 50 mL buffer, Roche] for 30 min at 4 °C. After protein concentration determination with a BCA protein assay reagent kit (Pierce Biotechnology), cell lysates and a pre-stained molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA) were separated with SDS-PAGE on 7.5 % Tris-HCl gels (Bio-Rad Laboratories) for NKCC1 (rabbit polyclonal, Abcam, Cambridge, UK) detection and on 10.5-14 % Tris-HCl gradient gels (Bio-Rad Laboratories) for p38, p-p38, ERK1/2 and p-ERK1/2 (rabbit monoclonals, Cell Signaling Technology, Boston, MA, USA) determinations. After transfer to PVDF-membranes (Bio-Rad Laboratories) and blocking, the primary antibodies were applied over night at 4 °C. HRP-conjugated secondary antibody (Cell Signaling Technology) was applied for 60 min at room temperature. Protein bands were detected with ECL Advance Western Blotting Detection Kit (GE Healthcare, UK) and visualized with the Chemidoc XRS system and Quantity One 1-D Analysis software (Bio-Rad Laboratories) and on X-ray film

(GE Healthcare). All membranes were stripped (Restore Western Blot Stripping Buffer, Pierce Biotechnology) and re-probed with actin antibody as protein loading control.

Phase-contrast microscopy and image analysis

Phase-contrast images of cell morphology were collected using a CK2 microscope (Olympus, Center Valley, PA, USA) and a Coolpix 4300 camera (Nikon, Melville, NY, USA) with an ocular adaptor UR-E4 (Nikon). Images of semi-adherent cells were collected every 5 min during exposure using a CK40 microscope (Olympus) and a DP12 camera (Olympus).

The phase-contrast image analysis technique was used as described previously [9, 16] using microscope, camera, and image analyzing system (Kontron Image System KS-400, Carl Zeiss, Jena, Germany). In short, cells suspended in 1 mL of serum-free medium were applied to an 85 µL-chamber in a microperfusion device mounted on an inverted microscope (Axiovert 100, Carl Zeiss). The cells were allowed to settle for 1 min in the chamber, then a peristaltic pump (LKB 12000 Varioperpex, LKB AB, Bromma, Sweden) was started to perfuse the cells. The entire microscope and pump system were positioned inside a thermo-regulated box set to 37 °C with 5 % CO₂. Individual cells were randomly chosen and digital image inputs of one cell at a time were taken each minute during a 5-10 min basal perfusion followed by a 30 min test perfusion. After completion of perfusion, the image sequences were re-run to perform background subtraction, contour enhancement, and object identification with interactive editing of threshold determination, and repair of any cell contour artefacts. Object identification and measuring was performed digitally. The optical halo produced around the cell due to differences in refractive indices between cell and medium was used for quantifying morphological changes. The projected cell area (PCA), and the cell shape factor (SF) was calculated for the inner and the outer border of the optical halo. A SF of 1 corresponds to a perfectly round cell, and as membrane protrusions increase, the shape factor decreases. The initial 5-10-min perfusion with MEM alone was regarded as a baseline to which the treatment period was compared for each individual cell (expressed as % of basal).

Chemicals

Culture medium (Eagle's MEM with Earle's salts) and L-glutamine were from Gibco Ltd. Foetal calf serum was purchased from Biochrom KG, cisplatin (CAS 15663-27-1) was from Bristol-Myers Squibb. Fluorescein diacetate (CAS 596-09-8), ouabain (CAS 11018-89-6), and bumetanide (CAS 28395-03-01) were from Sigma Chemical Company. ⁸⁶Rb⁺ (CAS 7791-11-9) were from GE Healthcare. All other chemicals were of analytical grade.

Statistics

For statistical analysis, the SPSS 12.0.1 for Windows program was used. The Mann-Whitney test was used for comparisons with control and for comparisons between cell lines. The level of significance for rejecting the null hypothesis of zero treatment effect was taken to be $p < 0.05$. All results are presented as means $\pm$ s.e.m. unless otherwise specified.

Fig. 1. Characterization of cisplatin cytotoxicity and induced cell death in P31 and P31res1.2 cells. (a) Cisplatin concentration-response curves. The cells were exposed to different concentrations of cisplatin for 2 h and then maintained in standard medium for 72 h prior to determining the proportion of remaining viable cells with FMCA. Means $\pm$ s.e.m. of three experiments. (b) Cell morphology at 72 h after a 2- or 6-h exposure to control or cisplatin-containing medium as indicated. Representative phase-contrast microscopy images collected with a digital camera (Nikon Coolpix 4300). N.D. = effect of exposure not determined. Bar = 50 μ m. (c) Percent TUNEL-positive cells determined after exposures as in b. Means $\pm$ s.e.m. of two to four experiments. * = $p < 0.05$; ** = $p < 0.01$ compared to control. (d) Caspase-3/7 activity determined immediately after exposures to control or cisplatin-containing medium as indicated. For each sample, 100 μ g protein was incubated for 60 min with DEVD-AFC substrate, and fluorescence was determined using 400 and 505 nm for excitation and emission, respectively. Means $\pm$ s.e.m. of three to four experiments. ** = $p < 0.01$ compared to control.

Results

Acquisition of cisplatin-resistance increases basal activity of caspase-3 but does not change DNA fragmentation

To induce acquired cisplatin-resistance, pleural malignant mesothelioma cells (P31) were maintained in increasing concentrations of cisplatin. Every month,

cisplatin cytotoxicity was monitored with FMCA until a three- to four-fold reduction in cisplatin sensitivity was reached. The resulting cell line, P31res1.2, was then maintained in 1.2 mg/L cisplatin. Quadratic curves were fitted to the FMCA data (not shown), and the 50 %-cytotoxic concentration for 2-h exposures was calculated to 15.0 mg/L for P31 cells and 45.3 mg/L for the resistant cells, when examined 72 h after end of exposure

Fig. 2. Influx and efflux of the K^+ -analog $^{86}Rb^+$ in P31 and P31res1.2 cells. Effects of the K^+ modulators bumetanide (10 μ mol/L), a NKCC1 inhibitor, and ouabain (10 μ mol/L), a Na^+,K^+ , ATPase pump inhibitor, were investigated. Time 0 = immediately after application of test substances to the cells. (a) The absolute uptake of $^{86}Rb^+$ by the two cell lines, expressed as counts per minute (cpm). Means $\pm$ s.e.m. of two experiments. *** = $p < 0.001$, compared to P31. (b) Cellular, supernatant (i.e. efflux) and total (i.e. cellular + supernatant) $^{86}Rb^+$ content of the two cell lines, expressed as counts per minute (cpm). The cells were loaded with $^{86}Rb^+$ for 24 h, time indicated is time after replacing $^{86}Rb^+$ -containing medium with $^{86}Rb^+$ -free medium. Means $\pm$ s.e.m. of two experiments. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to P31 cells. (c) Effect of K^+ modulators on the $^{86}Rb^+$ influx in the two cell lines. Means $\pm$ s.e.m. of four experiments. * = $p < 0.05$, ** $p < 0.01$, *** = $p < 0.001$, compared to control. (d) Effect of the K^+ modulators on $^{86}Rb^+$ efflux in the two cell lines. Means $\pm$ s.e.m. of four experiments. * = $p < 0.05$, ** $p < 0.01$, *** = $p < 0.001$, compared to control.

(Figure 1a). As the FMCA assay does not discriminate between cell growth inhibition and cell death, we verified that the acquired cisplatin-resistance was a consequence of reduced cell death rather than growth inhibition, by determining cell morphology and DNA fragmentation in the two cell lines 72 h after the end of the cisplatin exposure.

A 2-h exposure to cisplatin increased the number of non-adherent P31 cells with most of the still-adherent cells exhibiting normal morphology (Figure 1b). A similar response was seen in the P31res1.2 cells exposed to 40 mg/L cisplatin, but P31res1.2 cells exposed to 10 mg/L cisplatin were unaffected. When the exposure time was increased to 6 h, at least half of the P31 cells exposed to 10 mg/L, as well as half of the P31res1.2 cells exposed to 40 mg/L, were released from the

substrate, and many of the still-adherent cells demonstrated an increased number of membrane protrusions. However, a 6-h exposure to 10 mg/L cisplatin did not change the morphology of P31res1.2 cells. These results indicated that cisplatin exposure induced cell death in both cell lines, possibly through induction of apoptosis. Therefore, the percent TUNEL-positive cells at 72 h after end of exposure was determined (Figure 1c). The results were in concord with the morphological changes. A 2-h exposure resulted in an increased number of TUNEL-stained cells if released cells were seen in the phase-contrast microscopy i.e. P31 cells exposed to 10 mg/L cisplatin and P31res1.2 cells exposed to 40 mg/L cisplatin. Similarly, a 6-h exposure increased the number of TUNEL-stained cells in all exposures; about 50 % of the P31 cells were TUNEL-

Fig. 3. Western blot detection of NKCC1 (~170 kDa) and phosphorylated ERK1/2 (p-ERK1/2; 44 and 42 kDa, respectively) in P31 and P31res1.2 cells. Cells were kept in serum-free medium for 60 min, and then fresh serum-free medium was applied for 30 min. The membranes were stripped and probed with an anti-actin antibody as protein loading control.

stained, as were the P31res1.2 cells exposed to 40 mg/L cisplatin. P31res1.2 cells exposed to 10 mg/L cisplatin had a lower number of TUNEL-stained cells. Based on these results, all subsequent experiments were performed using 10 mg/L cisplatin for P31 cells, and 40 mg/L cisplatin for P31res1.2 cells.

We also investigated if caspase-3 was functional in the cell lines by determining enzyme activity immediately after the end of 2, 6, or 24 h of cisplatin exposure. The P31res1.2 cells had a higher basal caspase-3/7 activity than the P31 cells (Figure 1d). After 24 h of cisplatin exposure caspase-3/7 activity was increased in both cell lines.

Acquisition of cisplatin-resistance abrogates early-phase NKCC1 activity but not NKCC1 protein expression

Compared to P31 cells, the P31res1.2 cells showed a reduced uptake of the K⁺-analog ⁸⁶Rb⁺ during the initial 60 min after addition of ⁸⁶Rb⁺ (Figure 2a). In addition, the P31res1.2 cells had accumulated less ⁸⁶Rb⁺ than the P31 cells after the 24-h pre-loading (time = 0 in Figure 2b). 15 min after removal of ⁸⁶Rb⁺, the P31res1.2 cells had similar efflux, cell content and total content as the P31 cells (Figure 2b). From 30 to 60 min after ⁸⁶Rb⁺ removal, the efflux was lower in the P31res1.2 cells, but the cellular content was only transiently decreased compared to the P31 cells. As shown previously [15, 16], both influx and efflux of ⁸⁶Rb⁺ in the P31 cells was reduced by exposure to the NKCC1 inhibitor bumetanide and the Na⁺, K⁺, ATPase pump inhibitor ouabain (Figure 2c, d). The ⁸⁶Rb⁺ influx of P31res1.2 cells was reduced by ouabain, and by the combination of ouabain and bumetanide (Figure 2c). Bumetanide alone had no effect on either ⁸⁶Rb⁺ influx or efflux of P31res1.2 cells, although bumetanide combined with ouabain transiently increased ⁸⁶Rb⁺ influx after 30 (p=0.027) and 45 (p=0.012) min, compared to the effect of ouabain alone (Figure 2c, d).

The cellular content of NKCC1 protein was determined with Western blotting. The NKCC1 antibody detected a ~170 kDa protein, corresponding to the glycosylated form of NKCC1 (Figure 3). Both cell lines had similar contents of NKCC1. Phosphorylation of

ERK1/2 was used as an indicator of NKCC1 activity. The P31res1.2 cells had about half the amount of p-ERK1/2 present in the P31 cells.

Abrogation of NKCC1 activity inhibits early-phase cisplatin-induced cell membrane blebbing

We have previously shown that ouabain exposure reduces the normal morphological changes of P31 cells during a 30-min perfusion with serum-free medium, and that cisplatin exposure induces cell shrinkage and membrane blebbing under the same conditions [9].

Because NKCC1 activity can have a role in membrane blebbing [11-14], the lack of effect of bumetanide on K⁺ fluxes in P31res1.2 cells led us to investigate the morphological response of P31res1.2 cells to cisplatin, and compare this to the response of P31 cells to cisplatin in absence and presence of bumetanide.

Drug effect on cell morphology was first determined by phase-contrast microscopy without image analysis to decide exposure combinations to investigate with the phase-contrast image analysis. Both bumetanide 10 and 100 μmol/L induced small membrane blebs and membrane protrusions in P31 cells (Figure 4). When bumetanide was combined with cisplatin, membrane blebbing was absent. Not any of the exposures induced membrane blebbing or protrusion in the P31res1.2 cells. These concentrations also have similar inhibitory effects on NKCC1 activity in the P31 cells [16, 19, 35] demonstrating that there was little risk of non-specific effects of bumetanide regardless if 10 or 100 μmol/L bumetanide were used in the experiments.

In P31res1.2 cells, there was a very small increase in PCA during control conditions but the SF fluctuated more than in P31 cells due to changes in roundness of the P31res1.2 cells (Figure 5a, b). Cisplatin exposure did not change the P31res1.2 PCA and did not cause any

Fig. 4. Phase-contrast images of cells collected every 5 min during exposures as indicated. Cisplatin exposures were 10 mg/L for P31 cells and 40 mg/L for P31res1.2 cells. Prior to exposure the cells were kept in serum-free medium for < 60 min, and exposures were done in serum-free medium. Arrowheads indicate large membrane blebs; arrows indicate small membrane blebs and membrane protrusions. Bar = 10 μ m.

cell membrane blebbing or other membrane protrusions (Figure 5b), consistent with the results in Figure 4. Bumetanide exposure of P31 cells caused a PCA decrease, and consistent with the results in Figure 4 there was membrane blebbing after 15 min of exposure (Figure 5b). When bumetanide and cisplatin were combined, the PCA continued to increase, although less than under control conditions, and the changes in SF were eliminated due to a marked reduction in membrane blebbing.

Despite the conflicting observation that bumetanide exposure alone caused membrane blebbing in P31 cells, the similarity of the morphology for P31res1.2 cells exposed to cisplatin and P31 cells exposed to cisplatin in the presence of bumetanide (Figure 4 and 5) indicated that NKCC1 activity has a role in cisplatin-induced acute membrane blebbing. We therefore investigated if this was

reflected in the effect of cisplatin on the uptake and efflux of $^{86}\text{Rb}^+$. In the P31 cells, the effect of bumetanide on the $^{86}\text{Rb}^+$ influx and efflux was similar to that shown in Figure 2. In the P31res1.2 cells, bumetanide surprisingly caused a small reduction in $^{86}\text{Rb}^+$ efflux immediately at the start of the experiment (Figure 6). In P31 cells, exposure to cisplatin caused a transient decrease in $^{86}\text{Rb}^+$ uptake and an initial increase in $^{86}\text{Rb}^+$ efflux (Figure 6). When cisplatin and bumetanide were combined, $^{86}\text{Rb}^+$ influx was inhibited already at start (time 0). At 15 and 30 min, the effect of the combination was similar to that of bumetanide alone. In the P31res1.2 cells, cisplatin reduced $^{86}\text{Rb}^+$ influx after 30 min of exposure. The $^{86}\text{Rb}^+$ efflux was not affected by cisplatin exposure. However, the combination of cisplatin and bumetanide reduced $^{86}\text{Rb}^+$ efflux for the entire 30 min studied.

Fig. 5. Morphological responses of P31 and P31res1.2 cells during exposure to control, 10 mg/L cisplatin (P31), 40 mg/L cisplatin (P31res1.2), 100 μ mol/L bumetanide (P31), or 100 μ mol/L bumetanide + 10 mg/L cisplatin (P31) in serum-free medium. Image analysis of bumetanide and bumetanide+cisplatin exposure were not performed on the P31res1.2 cells as phase-contrast imaging showed no changes in cell membrane morphology. Images were collected every 60 s during the basal period (-5 to 0 min, when the cells were flowed with serum-free medium) and the following exposure period. (a) Representative cells of four to ten cells analysed per exposure. Bar = 5 μ m. N.D. = effect of exposure not determined. (b) Quantification of the morphological changes found in a. PCA = projected cell area, and SF = shape factor, a parameter describing the roundness of the cells and the presence of membrane protrusions. A reduction in SF = increased presence of protrusions. The basal level = -5 to 0 min i.e. the initial flowing of serum-free medium without any substances added. Data is presented as means of four to ten cells analysed, the s.e.m.-bars are excluded to make the data more legible.

Early-phase cisplatin-induced membrane blebbing was not associated with caspase-3/7 activity or changes in p38 or ERK1/2 activities

Caspase-3 activity mediates the late-phase apoptotic membrane blebbing [19]. There was no activation of caspase-3/7 in the cells after 30 min of cisplatin exposure (not shown). Furthermore, the removal of serum during 60 min prior to the 30-min exposure did not change caspase-3/7 activity (not shown).

Stress-induced membrane blebbing is shown to occur when p-ERK1/2 is low, and p-p38 is increased [5]. Therefore, the effect of bumetanide and cisplatin on p-ERK1/2 and p-p38 expression was determined with

Western blotting. There were no significant changes in p-ERK1/2 or p-p38 after 30 min of cisplatin exposure (not shown).

Abrogation of NKCC1 activity does not affect P31 cell resistance to cisplatin

To investigate if NKCC1 activity has a role in cisplatin-resistance, the effects of bumetanide and cisplatin exposure on cell number and DNA fragmentation in P31 and P31res1.2 cells were determined. A long-time exposure study was performed, exposing the cells continuously for 72 h before determining cell viability with FMCA. Due to the long exposure time,

Fig. 6. Effects of the NKCC1 inhibitor bumetanide (10 $\mu\text{mol/L}$) on cisplatin-induced changes in influx and efflux of the K^+ -analog $^{86}\text{Rb}^+$ in P31 and P31res1.2 cells. P31 cells were exposed to 10 mg/L cisplatin, P31res1.2 cells to 40 mg/L cisplatin. Time 0 = immediately after application of test substances to the cells. Means $\pm$ s.e.m. of four experiments. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, compared to control, or as indicated. 48 h after cell plating to 96-well plates, the cellular content of $^{86}\text{Rb}^+$ was determined as a measure of influx after simultaneous addition of $^{86}\text{Rb}^+ \pm \text{K}^+$ modulators (upper panels). 24 h after cell plating to 96-well plates, the cells were loaded with $^{86}\text{Rb}^+$ for 24 h. The $^{86}\text{Rb}^+$ efflux was then determined after exposure to $^{86}\text{Rb}^+$ -free medium $\pm \text{K}^+$ modulators (lower panels).

the cisplatin concentrations in these experiments were reduced to 0.2 mg/L for P31 cells, and to 3 mg/L for P31res1.2 cells. Bumetanide alone decreased the percent viable cells in both cell lines (Figure 7a). In P31 cells, the combination of bumetanide and cisplatin increased cytotoxicity of each drug alone, but the effect was less than additive. When P31 cells were exposed to bumetanide for 3 h, and analyzed for DNA fragmentation 48 h after that, bumetanide *per se* decreased the percent of TUNEL-stained cells, but did not change the effect of cisplatin (Figure 7b). In P31res1.2 cells, bumetanide had no effect on the percent of TUNEL-stained cells.

Discussion

The acquisition of cisplatin-resistance in cultured malignant mesothelioma cells resulted in abrogation of $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ -cotransport (NKCC1) activity, inhibition of early-phase cisplatin-induced membrane blebbing, and an increased basal caspase-3 activity. At equitoxic cisplatin concentrations, P31res1.2 cells had a faster activation of caspase-3 than P31 cells, but the effect on end-stage cytotoxicity and number of cells with DNA fragmentation was similar. The early-phase cisplatin-induced membrane blebbing of the P31 cells was probably a stress-induced response. This response was

Fig. 7. Effects of the NKCC1 inhibitor bumetanide on cisplatin cytotoxicity and DNA fragmentation to P31 and P31res1.2 cells. (a) The percent of viable cells after 72 h of exposure was determined with FMCA. Means $\pm$ s.e.m. of six to seven experiments. ** = $p < 0.01$, *** = $p < 0.001$ compared to control, unless otherwise indicated. (b) The percent of cells with fragmented DNA was determined 48 h after a 3-h exposure. Means $\pm$ s.e.m. of five to seven experiments. * = $p < 0.05$, ** = $p < 0.01$, compared to control, unless otherwise indicated.

dependent on NKCC1 activity, but not necessary for the subsequent progression to apoptosis.

NKCC1 activity was necessary for cisplatin-induced early-phase membrane blebbing in P31 cells. The P31res1.2 cells expressed similar amounts of NKCC1 as the P31 cells, but there was very little bumetanide-responsive NKCC1 activity during the initial 60 min after medium change. The P31res1.2 cells did not respond to cisplatin exposure by cell membrane blebbing. However, four findings show that the NKCC1 in P31res1.2 cells still can be activated. First, the addition of bumetanide during ouabain exposure inhibited the K^+ influx more than ouabain alone after 30 and 45 min of exposure. Second, bumetanide exposure could transiently inhibit K^+ efflux during the first minutes of exposure. Third, the addition of bumetanide to ouabain or cisplatin inhibited K^+ efflux, although neither of the substances had any effect alone. Fourth, the proportion of viable P31res1.2 cells decreased after a 72-h exposure to bumetanide. These effects of bumetanide exposure are most likely due to inhibition of activated NKCC1. Bumetanide has a higher affinity for activated NKCC1 than for basal NKCC1 [36]. In the early G1 phase of the cell cycle, NKCC1 is activated, and inhibition impairs cell proliferation [37]. Conversely, over-expression of the NKCC1 gene causes cell proliferation [38].

The increased response of P31 cells to bumetanide exposure complies with the increased proliferation rate of these cells compared to the P31res1.2 cells (unpublished data).

The mechanisms of cisplatin-induced early-phase membrane blebbing appear very similar to stress-induced membrane blebbing. Increased p38 activation concurrent with decreased ERK1/2 activity results in increased activation of HSP27 and increased actin-polymerisation activity [5]. Both p38 [11] and actin polymerization [12] can activate NKCC1 [12], and NKCC1 can activate ERK1/2 [13, 38]. These interactions emphasize the role of NKCC1 in cell membrane blebbing. However, a 30-min cisplatin exposure was not sufficient to affect either p38 or ERK1/2 activity in these cells. This suggested that NKCC1 can have a role in cell membrane blebbing independent of p38 and ERK1/2 activity, although that remains to be shown.

In addition, we found that P31 cell acquisition of cisplatin-resistance resulted in elevated basal caspase-3 activity, in contrast to the reduced caspase-3 activity reported in other cisplatin-resistant cells [29, 30]. It is tempting to speculate that the deregulated K^+ transport of P31res1.2 cells caused the increased basal caspase-3 activity. A reduced intracellular K^+ concentration is necessary for activation of caspase-3, although not

necessary for maintaining enzyme activity [21]. Although the P31res1.2 cells had a reduced accumulated content of $^{86}\text{Rb}^+$, the cell content of $^{86}\text{Rb}^+$ was only transiently lower than in the P31 cells, probably due to a reduced efflux. No conclusions can therefore be drawn regarding the intracellular K^+ concentration of P31res1.2 cells compared to the P31 cells. It is possible that the continuous exposure to cisplatin results in an increased caspase-3 activation, but that the activity remains below the threshold level for execution of apoptosis. This could be due to over-expression of caspase-inhibiting proteins, e.g. the X-linked inhibitor of apoptosis protein (XIAP) [39]. Another interesting mechanism of caspase-3 regulation is the WNK3 inhibition of caspase-3 activation [40]. In HeLa cells, down-regulation of WNK3 results in increased caspase-3 activation [40]. Kinase-active WNK3 phosphorylates and activates NKCC1, and kinase-dead WNK3 reduces NKCC1 phosphorylation [41]. Although not investigated here, reduced WNK3 activity could explain the low NKCC1 activity and increased caspase-3 activity in the P31res1.2 cells.

The onset of caspase-3 activation after cisplatin exposure was more rapid in the P31res1.2 cells, with significant activity already after 6 h of exposure. In P31 cells, the caspase-3 activity after 6 h of cisplatin exposure was still lower than the basal activity in P31res1.2 cells. Despite these differences in caspase-3 activity, there was still DNA fragmentation in about 50 % of the cells of both cell lines 72 h after end of a 6-h exposure. Although caspase-3 undoubtedly has a role in late apoptotic membrane blebbing [22], early-phase membrane blebbing is independent of caspase activation [5, 6]. Our results support this as we found no changes in caspase-3

activity in the P31 cells during membrane blebbing. Additionally, no membrane blebbing occurred in the P31res1.2 cells, which have an increased basal caspase-3 activity. These results suggested that the main consequences of acquired cisplatin-resistance in P31res1.2 cells are to be found in the earlier stages of apoptosis signalling, prior to caspase-3 activation, rather than after.

Conclusion

The acquisition of cisplatin-resistance in human malignant pleural mesothelioma P31 cells reduced the NKCC1 activity, disrupted the K^+ -regulation, and increased the basal caspase-3 activity. Despite these changes, the execution of apoptosis in response to equitoxic concentrations of cisplatin was similar in both cell lines. The cisplatin-induced membrane blebbing of the P31 cells was probably a stress-induced response, dependent on active NKCC1, but not necessary for the subsequent progression to apoptosis. Thus, acquisition of cisplatin-resistance can affect mechanisms that have profound effects on cell morphology but are not necessary for the subsequent progression to apoptosis.

Acknowledgements

The authors thank Le Thu Trinh for skilful technical assistance. The study was supported by the Lions foundation, the County Council, and the Faculty of Medicine, Umeå University, Umeå, Sweden.

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