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Original Paper

Benign Pleural Mesothelial Cells Have Higher Osmotic Water Permeability than Malignant Pleural Mesothelioma Cells and Differentially Respond to Hyperosmolality

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Key Words
AQP1 • Hyperosmotic stress • Malignant pleural mesothelioma • Osmotic water permeability • Pleural mesothelial cells

Abstract
Background/Aims: Cell volume regulation is a critical mechanism for cell homeostasis and depends on the osmotic water permeability (P_f) of the cell plasma membrane. The P_f of human mesothelial cells is unknown although they contribute to serosal fluid turnover. ***Methods:*** In this study we measured the osmotic water permeability of benign human mesothelial cells (MeT-5A) and of epithelioid (M14K) and sarcomatoid (ZL34) malignant pleural mesothelioma (MPM) cells in response to acute hyperosmotic stress. We also assessed the changes in their P_f after preconditioning with 4% glucose for 24 hours. In both cases we also assessed the role of AQP1 inhibition (0.1 mM $HgCl_2$) on the P_f. Finally, we assessed corresponding changes in the AQP1 plasma membrane availability by immunofluorescence. ***Results:*** We report that MeT-5A cells have a significantly higher P_f as compared to M14K and ZL34 MPM cells [4.85E-03±2.37E-03 cm/sec (n=17) versus 2.74E-03±0.74E-03 cm/sec (n=11) and 2.86E-03±0.11E-03 cm/sec (n=11)]. AQP1 inhibition significantly decreased the P_f in all cells lines ($p<0.001$ in all cases). High glucose preconditioning for 24 hours significantly increased MeT-5A P_f ($p<0.001$), did not influence M14K P_f ($p=0.19$) and significantly reduced ZL34 P_f ($p=0.02$). Comparing cell lines after high glucose preconditioning, MeT-5A P_f was significantly higher than that of M14K and ZL34 MPM cells and the AQP1 inhibition effect was significant

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in MeT-5A and M14K cells. These results were corroborated by AQP1 immunofluorescence. ***Conclusion:*** We provide evidence for a differential regulation of P_f in benign and MPM cells that require further mechanistic investigation.

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Introduction

Pleural mesothelial cells are specialized epithelial cells of mesodermal origin lining the lungs (visceral pleura) and the thoracic wall (parietal pleura) and between which the pleural cavity is formed [1]. Under physiological conditions, a small amount of pleural fluid is present in the cavity, produced by plasma ultrafiltration from the parietal pleura capillaries [2, 3]. It is re-absorbed by the parietal pleura lymphatic stomata, by Starling forces across the visceral pleura and by mesothelial cell-mediated solute coupled liquid absorption and endocytosis [2, 3]. In several extra-pulmonary (e.g. congestive heart failure, malignancy, post-myocardial infarction, rheumatoid arthritis) and pulmonary diseases [e.g. pneumonia, lung cancer, malignant pleural mesothelioma (MPM), pulmonary embolism] pleural effusions occur (excess accumulation of pleural fluid in the pleural cavity) [4]. Depending on the underlying pathology, protein, glucose, lactate dehydrogenase and pH pleural fluid levels vary; as is the case for cytokines and growth factors that are produced locally either by pleural mesothelial cells or by leucocytes [2-8]. Thus, the pleural mesothelial cells in pathophysiological conditions can encounter various biochemical environments that may influence their functions e.g. production of cytokines, cell migration, adhesion, proliferation, mesothelial to mesenchymal transition, transport of ions [9-13].

Due to the role of pleural mesothelial cells in pleural fluid absorption, it is important to study their plasma membrane water permeability. Several studies have shown that pleural mesothelial cells express aquaporin-1 (AQP1) that plays a role in the equilibration of the osmotic gradient between the pleural cavity and the pleural capillaries in pleural effusions [3, 10, 14]. Moreover, in MPM patients the high tumor expression of AQP1 has been described as a favorable prognostic factor regarding survival [15, 16]. We previously showed a differential in the AQP1 expression with respect to MPM histological subtype (epithelioid, biphasic and sarcomatoid); more AQP1 being expressed in the least aggressive form of MPM, the epithelioid subtype [17]. Moreover, MPM AQP1 inhibition *in vitro* inhibits cell adhesion, migration and tumor sphere formation in an extracellular component type and histological type dependent manner [10]. Furthermore, AQP1 inhibition in MPM leads to decreased cell proliferation, motility and metastatic potential [18].

Glucose was shown to induce the expression of AQP1 in human peritoneal mesothelial cells *in vitro* [19]. Diabetes Mellitus (DB) is a common comorbidity in MPM [20], however no study has assessed the role of AQP1 in MPM cells osmotic water permeability (P_f) under high glucose osmotic stress. In the same context, in DB patients the development of pleural effusions is significantly more common than in non-diabetic patients [21].

Based on the above, the aim of our study was to assess the osmotic regulation of P_f of benign mesothelial cells and epithelioid and sarcomatoid MPM cells under norm-osmotic and high glucose hyper-osmotic conditions and by AQP1 inhibition to evaluate the contribution of this channel in transmembrane water transport.

Materials and Methods

Cell culture

The human cell lines MeT-5A (benign immortalized mesothelial cells), M14K (epithelioid MPM), and ZL34 (sarcomatoid MPM) were used in the study. Cell culture was performed in RPMI-1640 cell medium supplemented with 10% BCS, 1% antibiotics, and 1% L-glutamine in a 5% CO_2 humidified incubator at 37°C. The hypertonic medium for the measurement of water permeability was created by 300mOsm kg^{-1} mannitol in PBS. Control cells were maintained in normal medium while glucose preconditioned cells were

maintained in a 4% glucose (w/v)-culture medium for 24 hours prior to each experiment. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Water permeability measurements

Fluorescence measurements of cell volume were performed by the calcein quenching method as previously described [22, 23]. Cell volume changes were expressed as relative values of calcein fluorescence, an established surrogate of the cell volume fluctuations [24, 25]. Cells were grown on cover glasses and loaded with Calcein-AM (5 μM, 15min at 37°C; Invitrogen, CA, USA). The fluorescence of calcein was continuously measured with a LED light source, through a filter set #09 (BP 450 - 490 nm excitation, FT 510 nm dichroic mirror, LP 515 nm emission), a photomultiplier detector with a pinhole diaphragm in order to be able to select the cells of interest and with a 12-bit analog-to-digital converter PCL-818HG (Advantech). The data acquisition rate was set to 10 msec. A superfusion chamber was constructed as an acrylic block with T-shape current of cell medium. This design makes fast change of superfusion medium feasible and minimizes the perturbations of the specimens. The flow rate of the perfusate was set to 20 mL/min, which resulted in a complete solution exchange in the area of interest in less than 50 msec. The chamber was mounted on the stage of an inverted microscope (Axiovert 40, Zeiss, Germany; objective lens with 40x magnification; numerical aperture 0.65; thermal stabilization at 36.8 ± 0.2 °C). The water permeability was calculated from the rate of the cell volume changes under the osmotic challenge on the basis of the equation [25]: $dV/dt = -AV_wP_f\nabla\Phi$. The plasma membrane osmotic water permeability coefficient (P_f) was calculated from the time course of the cell volume change in response to an osmotic gradient. The osmotic water movement is the net flow of volume across a cell membrane in response to hydrostatic and/or osmotic pressure: $J = -L_pA\Delta\Phi$; or $dV/dt = -P_fAV_w\Delta C$. The permeability coefficient can be calculated from the slope (K_r) of the linear plot [26]: $P_f= K_r [AV_w(C_{in} – C_{out})]^{-1}$. Where J is the rate of volume flow across the membrane ($cm^3 s^{-1}$), (dV/dt) is the rate of cell volume change, L_p is hydraulic conductivity ($cm^3 s^{-1} atm^{-1}$), P_f is the osmotic water permeability coefficient ($cm s^{-1}$), A is the surface area (cm^2) which is significant for water exchange, ΔΦ is the osmotic pressure difference (atm), ΔC is the osmotic concentration difference ($Osm kg^{-1}$), and V_w is the partial molar volume of water. In the water permeability measurements, the cells were washed with PBS and were then incubated with a calcein-PBS solution for 20 minutes at 37°C. Then were incubated in a solution of PBS supplemented with 300mOsm kg^{-1} of mannitol for 30 sec (in order to balance) and then the solution was rapidly replaced with normal PBS (300mOsm kg^{-1}) and the apparent water permeability was measured. In experiments assessing the role of AQP1 in the apparent water permeability mercuric chloride ($HgCl_2$; 0.1 mM) was used to inhibit AQP1.

Immunostaining

The rabbit polyclonal anti-AQP1 antibody (#ab15080; Abcam) was used. Cells were fixed for 30 minutes by incubation on ice with 4% paraformaldehyde on PBS and processed for immunostaining. Briefly, glasses were blocked in PBS containing 1% non fat milk for 30 minutes and incubated with the primary antibody at 4°C overnight. After 3 washes with PBS, incubation with the goat anti-rabbit IgG FITC secondary antibody followed (#554020; BD Biosciences) for 2 hours at room temperature. Pictures were taken using an Observer Z1 fluorescence microscope (Zeiss, Germany).

Statistical analyses

Data are presented as means±SD. Statistical significance was evaluated using unpaired t test with Welch correction when comparing for two variables and One-Way ANOVA when comparing three variables. A value of $p<0.05$ was considered significant.

Results

The P_f of human benign pleural mesothelial cells is significantly higher than that of MPM cells

The apparent water permeability (P_f) of benign and malignant mesothelial cells was assessed after the reaction of the cells to a rapid switch from a hyperosmotic medium (600mOsm kg^{-1}) to a normosmotic medium (300mOsm kg^{-1}). As shown in Fig. 1A, the P_f of MeT-5A cells was 4.85E-03±2.37E-03 cm/sec (n=17) that was significantly higher ($p<0.01$ in both cases) than both M14K (2.74E-03±0.74E-03 cm/sec; n=11) and ZL34 cells (2.86E-03±0.11E-03 cm/sec; n=11).

The P_f of human benign and malignant pleural mesothelial cells is mediated by the function of AQP1

$HgCl_2$ was used to assess the P_f of benign and malignant pleural mesothelial cells during concomitant inhibition of AQP1 by $HgCl_2$ treatment. As shown in Fig. 1B-D, in all the three cell lines tested, the inhibition of AQP1 resulted in a significant decrease of the P_f. More specifically, in MeT-5A cells the P_f reduced to 2.15E-03±0.92E-03 cm/sec (n=21; $p<0.001$ compared to untreated; Fig. 1B), in M14K cells the P_f was reduced to 8.63E-04± 0.32E-04 cm/sec (n=11; $p<0.001$ compared to untreated; Fig. 1C) and ZL34 MPM cells the P_f was reduced to 1.14E-03± 0.44E-03 cm/sec (n=18; $p<0.001$ compared to untreated; Fig. 1D).

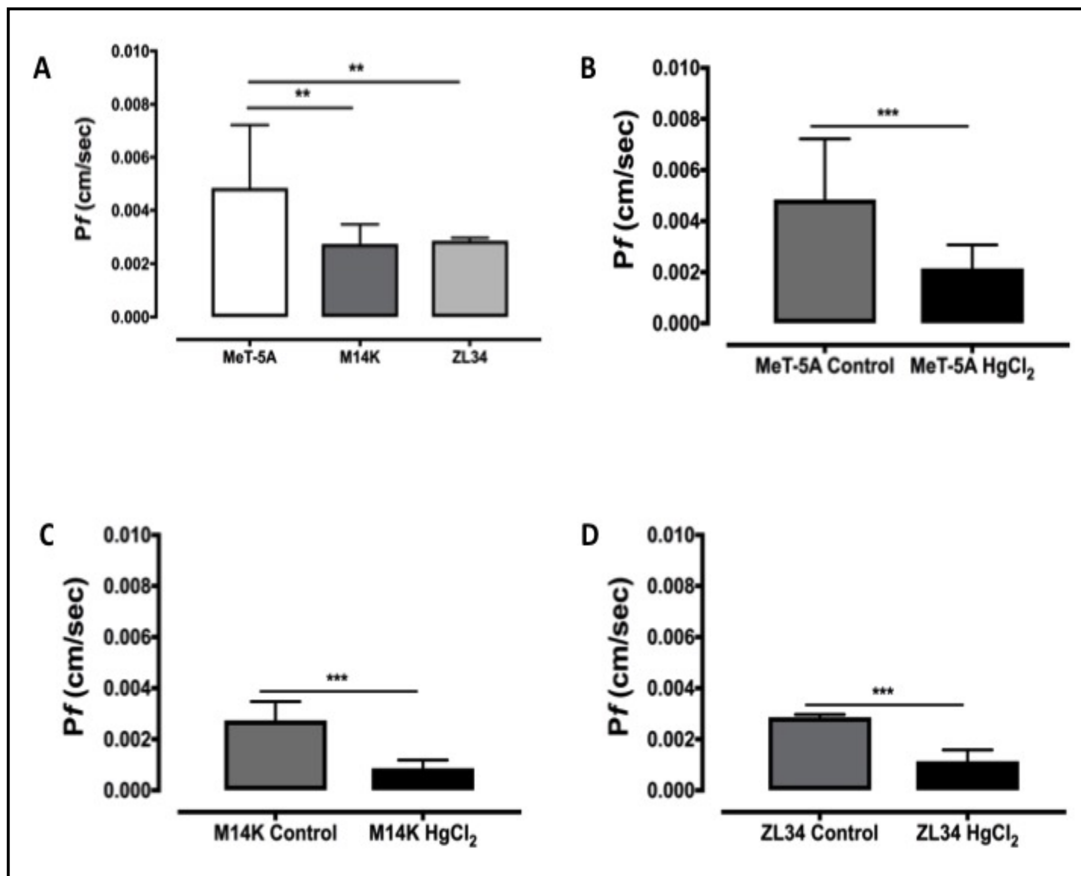

Fig. 1. (A). Osmotic water permeability (P_f) of benign (MeT-5A) and malignant (M14K and ZL34) mesothelial cells. ** $p<0.01$ as compared to MeT-5A. Comparison of P_f of MeT-5A (B), M14K (C) and ZL34 (D) cells without and with inhibition of AQP1 by $HgCl_2$. ***$p<0.001$ compared to no treatment.

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High glucose hyperosmotic stress for 24 hours significantly increased the P_f of MeT-5A, did not influence M14K cells P_f, while it significantly decreased the P_f of ZL34 cells

As shown in Fig. 2, exposure of cells to 4% glucose containing cell medium for 24 hours resulted in significantly higher P_f values in MeT-5A cells to 1.41E-02±1.11E-02 cm/sec (n=30; p<0.001 compared to Control). In M14K cells this effect was not significantly different 3.59E-03±2.33E-03 cm/sec (n=16; p=0.19 compared to Control). Finally, in ZL34 cells the hyperosmotic stress led to a significant decrease of the P_f values to 1.74E-03±1.24E-03 cm/sec (n=10; p=0.02 compared to Control).

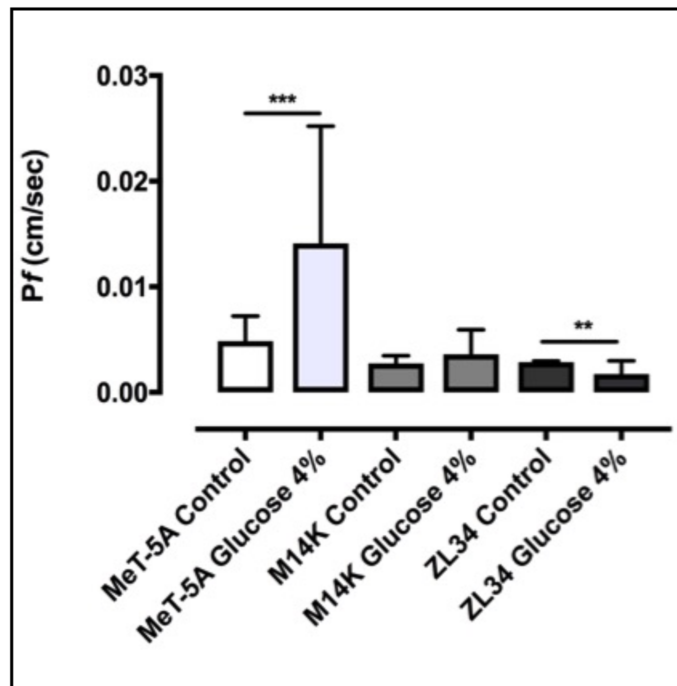

Fig. 2. Comparison of the Pf of each cells line after high glucose hyperosmotic stress for 24 hours. Differential effects of glucose are shown depending on the cell line. **p<0.01 and ***p<0.001 compared to respective control.

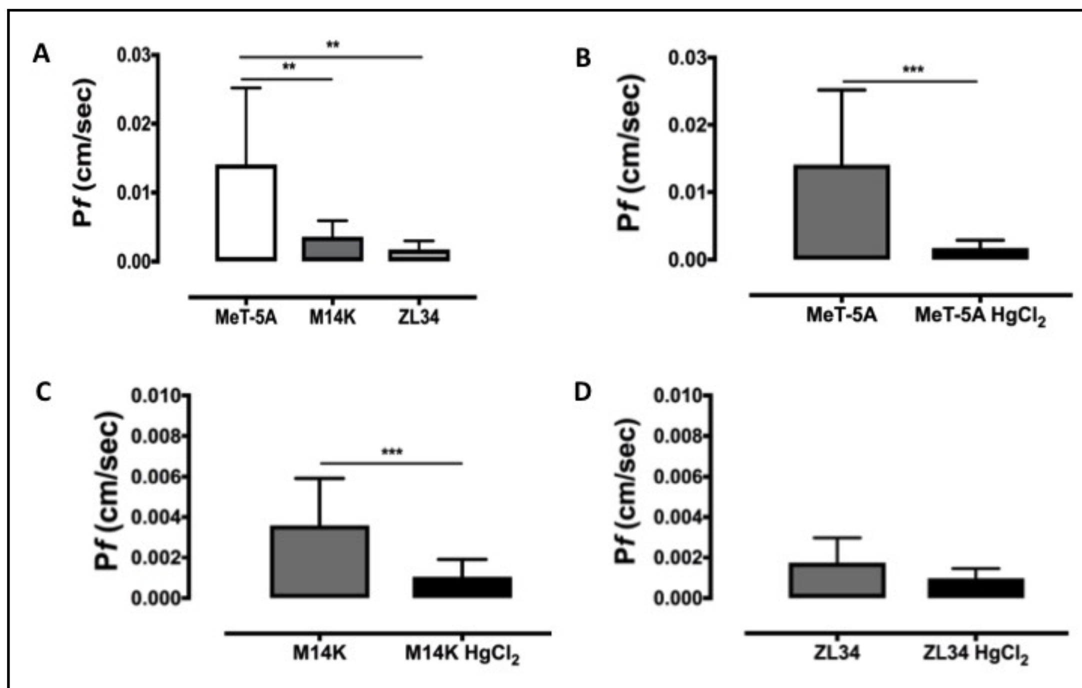

Fig. 3. (A). Osmotic water permeability (P_f) of benign (MeT-5A) and malignant (M14K, ZL34) mesothelial cells that were exposed to high glucose hyper-osmotic stress for 24 hours. ** p<0.01 as compared to MeT-5A. Comparison of P_f of MeT-5A (B), M14K (C) and ZL34 (D) cells without and with inhibition of AQP1 by $HgCl_2$. ***p<0.001 compared to no treatment.

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The P_f of human benign pleural mesothelial cells is significantly higher than that of MPM cells after high glucose hyperosmotic stress for 24 hours and is mediated by AQP1 in benign and epithelioid MPM cells

The P_f of benign and malignant pleural mesothelial cells was also assessed after a 24-hour high glucose hyperosmotic stress with and without AQP1 by $HgCl_2$. As shown in Fig. 3A, the P_f of MeT-5A cells was 1.41E-02± 1.11E-02 cm/sec (n=30) that was significantly higher ($p<0.01$ in both cases) than both M14K (3.59E-03± 2.33E-03 cm/sec; n=16) and ZL34 cells (1.74E-03± 1.24E-03 cm/sec; n=10). As far as the inhibition of AQP1 is concerned it resulted in a significant decrease of the P_f in benign cells and epitheliod MPM cells. More specifically, in MeT-5A cells the P_f reduced to 1.71E-03±1.16E-03 cm/sec (n=12; $p<0.001$ compared to

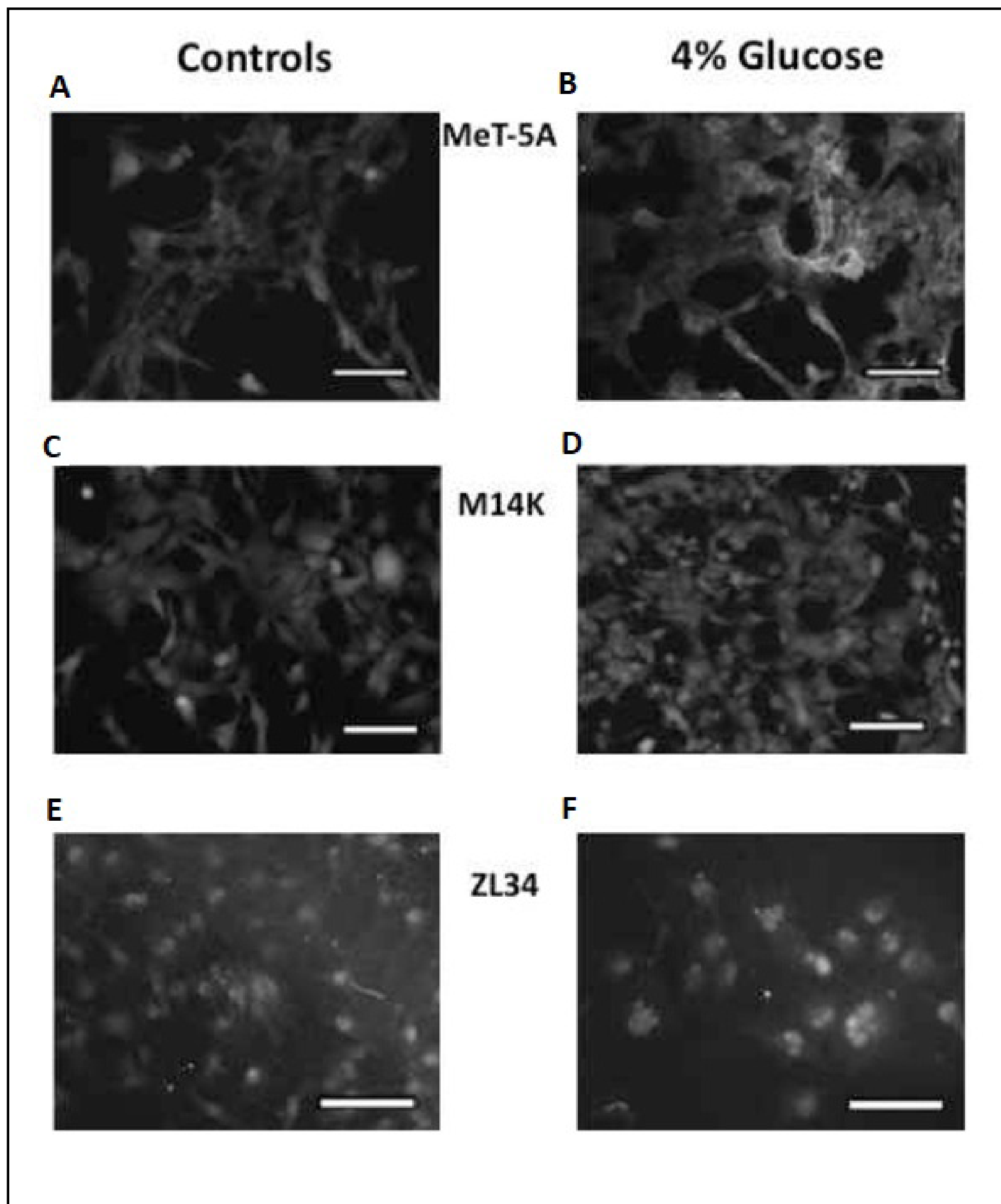

Fig. 4. Immuno-staining for AQP1 in MeT-5A, M14K, ZL34 cells in control (A, C, E) and high glucose hyperosmotic conditions (B, D, F) respectively. Scale bar is 50 µm.

Control; Fig. 3B), in M14K cells the P_f was reduced to 1.04E-03± 0.86E-03 cm/sec (n=16; p<0.001 compared to Control; Fig. 3C). In the case of ZL34 MPM cells the P_f was not reduced significantly (9.71E-04±0.48E-04 cm/sec; n=10; p=0.09 compared to Control; Fig. 3D).

P_f effects of high glucose hyperosmotic stress for 24 hours in MeT-5A, M14K and ZL34 correspond to similar changes in the AQP1 protein expression as evidenced by immunofluorescence

In Fig. 4A-4F the intensity of AQP1 immunostaining of MeT-5A, M14K and ZL34 cells in control conditions (Fig. 4A, 4C and 4E respectively) and after high glucose induced hyperosmotic stress for 24 hours incubation with 4% glucose solution (Fig. 4B, 4D and 4F respectively). The intensity of staining is increased in MeT-5A cells, it is similar in M14K cells and it is reduced in ZL34 cells following the trend seen in the corresponding P_f measurements.

Discussion

In this study we evaluated the differences in the P_f of benign pleural mesothelial and epithelioid and sarcomatoid MPM cells. We found that in normal conditions, the P_f of benign cells is significantly higher from both epithelioid and sarcomatoid type of MPM cells. However, the effect on P_f was different in each cell line after their exposure to a hypertonic environment created by high glucose or mannitol for 24 hours prior to the experiment. Namely, the P_f of benign cells increased significantly after the hyperosmotic stress, the P_f of epithelioid MPM cells was unaffected while that of sarcomatoid MPM cells decreased significantly. Finally, these differences were reflected also after AQP1 immunostaining.

The P_f values of mesothelial cells were significantly lower than that of other epithelial cells such as the kidney OMCD principal epithelial cells that we have previously assessed [23-25, 27]. This difference is expected because pleural mesothelial cells are normally facing a steady mucosal environment due to the stable composition of the pleural fluid in contrast to kidney OMCD principal epithelial cells that are challenged constantly with pro-urine of great variability in terms of osmolality (from 100-1400mOsm kg^{-1}) [25]. Therefore, the kidney cells would be expected to have higher P_f in order to adjust rapidly to drastic changes of the apical extracellular osmolality.

This is the first study describing differences in the P_f between normal mesothelial and MPM cells. No other similar study exists in order to compare our findings, however a relevant study in human glioma showed that several cell lines maintain their AQP1 expression when grown in culture while others do not [28]. Also very few studies have investigated the AQP1 mediated P_f differences in cancer cells by means of either silencing or inducing overexpression of AQP1 [28-31]. In all these studies as expected over-expression of AQP1 and silencing, increased and decreased respectively the P_f of the studied cells. In such studies the loss of AQP1 results in the reduction of cell phenotypes that promote cancer processes such as angiogenesis, cell proliferation, migration and invasion. Indeed, one of the key functions that depend on the regulation of cell volume in cancer cells is that of cell migration and invasion [32]. In our experiments we speculate that the changes in the water permeability are mediated by changes in the expression levels of AQP1 and this notion is supported by our results regarding AQP1 immunostaining in MeT-5A cells.

However, in a previous study of our group we demonstrated that inhibition of AQP1 in the same cell lines used in the current experiments led to the inhibition of cell migration only in the ZL34 cell line [10]. One difference compared to the current study is the lower concentration of the AQP-1 inhibitor $HgCl_2$ used (0.01mM as compared to 0.1mM in the current study), that is much less cytotoxic but leads to nearly 40% inhibition of AQP1 water conductance as compared to 100% inhibition of AQP1 in the case of 0.1mM that is close to 100% [33].

As noted previously pleural mesothelial AQP1 is important since it mediates the faster resolution of aniso-osmotic pleural effusions with regards to the plasma of the pleural capillaries [14]. Regarding MPM, increased expression of AQP1 in biopsies of MPM patients has been shown to correlate with increased survival [16]. Additionally a specific pattern of expression was shown analogous to the histological subtype severity: higher AQP1 in epithelioid MPM with better prognosis than in sarcomatoid MPM with worse prognosis. How does AQP1 increased expression confer a survival advantage to MPM patients is not known, however it may have to do with the fact that it may lead to faster resolution of pleural effusion in MPM that reduce the capacity of the tumor to spread in the thoracic cavity [10, 17]. Diabetes is a common comorbidity in MPM therefore, the differential response of M14K and ZL34 cells in terms Pf and AQP1 expression due to high glucose hyperosmotic stress indicates impairment in the mechanism that regulates AQP1 expression and water permeability in M14K and ZL34 cells and potentially may be of clinical importance [20]. Based on our experimental approach we cannot speculate on the clinical relevance of such findings, however they indicate that there is a cellular mechanism that regulates water permeability as response to high tonicity and/or high glucose extracellular environment. This hypothesis requires further study especially under the rationale that several malignancy promoting cell phenotypes depend on the effective cell volume regulation.

Conclusion

In conclusion we have demonstrated that there is a difference in the Pf of benign mesothelial, epithelioid and sarcomatoid MPM cells. Furthermore, hyperosmotic stress yields different responses in each cell type that was accompanied by corresponding changes in the expression of AQP1. Further studies are needed for the dissection of the molecular events that mediate our findings.

Acknowledgements

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Disclosure Statement

The authors declare no conflicts of interest.

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