Influence of AQP1 on cell adhesion, migration and tumor sphere formation in malignant pleural mesothelioma is substratum and histological type dependent

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Running Title: AQP1 influence on MPM cell phenotypes

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Abstract

Malignant pleural mesothelioma (MPM) is an aggressive cancer. MPM cells express aquaporin-1 (AQP1) that in other cancers has been shown to participate in the tumor metastasis processes. However, in MPM patients AQP1 overexpression is an independent prognostic factor favoring survival. In this study we aimed at evaluating the role of AQP1 in cell adhesion, migration and tumor sphere formation in non-malignant mesothelial cells (MeT-5A) and in epithelioid (M14K) and sarcomatoid (ZL34) MPM cell lines. We used fibronectin (FN) or homologous cell-derived extracellular matrix (ECM) substratum to investigate the role of AQP1 in these experimental phenotypes, inhibiting AQP1 by $10^{-5}$M HgCl$_2$ (MC). Deposited ECM during cell culture exhibited significant concentration differences among cell types. ZL34 cell adhesion was significantly higher than MeT-5A or M14K cells on FN and ECM. MeT-5A and M14K cell adhesion on FN was sensitive to AQP1 inhibition, while AQP1 inhibition on ECM was limited to M14K cells. Wound healing in ZL34 cells was significantly higher than MeT-5A and M14K cells on FN and ECM. AQP1 inhibition significantly lowered cell migration in ZL34 cells on FN and ECM. Sphere formation was not dependent on FN or ECM in the media. AQP1 inhibition in FN media reduced sphere formation in M14K cells while in ECM, all three cell-types were sensitive to AQP1 inhibition.

Keywords: Aquaporin-1, Cell adhesion, Cell migration, Malignant Pleural Mesothelioma, Tumor sphere formation
Introduction

Malignant Pleural Mesothelioma (MPM) is a fatal malignancy of the pleural membrane, etiologically linked to asbestos exposure, with a median survival of 12-18 months following diagnosis [1,14]. The treatment is usually palliative given the advanced stage of the disease following diagnosis, however chemotherapy, radiotherapy and cyto-reductive surgery can be adopted with various results [2]. Disease severity and response to treatment is among others dependent on the histological subtype of MPM, with sarcomatoid being the most aggressive and the epithelioid being the least aggressive histological subtypes [1,14].

Improvement of patient care and extension of overall survival involves better diagnostic and prognostic biomarkers [19]. In this context it has been shown that high expression of AQP1 – determined by histology – is a significant independent prognostic factor favoring survival of MPM patients irrespective of treatment or other known prognostic factors [5]. In this study patients with larger pleural effusions had proportionately higher expression of AQP1 [7]. In MPM tissue microarrays AQP1 gene expression in MPM patients is significantly higher in epithelioid MPM as compared to biphasic and sarcomatoid in accordance with findings in immunohistochemical studies [6,7].

The role of AQP1 in pleural fluid dynamics is important in cases of pleural effusions where the osmolality difference between the parietal pleura capillaries and the pleural fluid leads to compensatory increase in the activity of pleural AQP1 [17]. Therefore, the role of pleural AQP1 in malignant pleural effusions (MPE) is potentially critical given that in a mouse model of MPE it has been
demonstrated that pleural fluid volume is directly proportional to the tumor burden in the pleural cavity [18]. This may demonstrate that increased pleural fluid volume renders new MPM tumor foci easier to develop within the pleural cavity, a process that would require MPM cell migration and adhesion to unaffected pleural surfaces.

Cell migration, adhesion and proliferation are physiological processes integral to tumor metastasis and progression that among others depend on the activity of water and ion channels and ECM components [12,13]. A recent study reported that in epithelioid MPM cells (H226), blockade of AQP1 by a pharmacological agent (AqB050) significantly decreased proliferation and migration in vitro [10]. In a melanoma and a breast cancer cell line, AQP1 over-expression significantly enhanced cell migration during wound healing [5].

The above evidence warrants the investigation of AQP1 in MPM in greater detail. Therefore, we investigated the inhibition of AQP1 in MPM cell lines during cell adhesion, wound healing and sphere formation in two different substrates, on FN and cell-derived ECM treated surfaces.

**Materials and Methods**

**Cell culture.** The human cell lines, MeT-5A (benign transformed mesothelial cells), M14K (epithelioid MPM) and ZL34 (sarcomatoid MPM) were kind gifts from Dr. Ioannis Kalomenidis (University of Athens). Cell culture was performed in 10% serum-RPMI, antibiotics and L-Glutamine in a 5% CO₂, humidified incubator. Serum starvation using 0.5% serum-RPMI for 24 hours was performed
for cell synchronization. Materials were purchased from Sigma-Aldrich unless otherwise stated.

**Cell culture surface treatments.** Cell culture plastic surfaces were treated for 30 mins with FN (Calbiochem) solution (50 μg/ml FN in RPMI). Cell-derived ECM was deposited by culturing 5x10^4 cells for 48 hours in 48 well plates followed by detergent lysis, DNaseI (Roche) treatment and sterile PBS washing. ECM isolation for cell culture was done by using 0.25% TritonX-100, 1% Sodium deoxycholate in PBS and sterile filtered DNasel (schematic in Figure 1A).

**Isolation of cell-derived ECM proteins.** 10^6 cells/well were deposited in a 6 well plate and cultured for 48 hours. The deposited ECM was scraped with a sterile plastic scraper along with 0.25 ml of sterile sodium citrate buffer. ECM protein quantification was performed by the MicroBCA assay (Pierce Biotechnology).

**Drug treatments.** Sterile mercury chloride (MC; 10^{-5}M) in 10% RPMI was used for blocking AQP1.

**Reverse transcriptase PCR.** Cell monolayers were washed with PBS, lysed with QIAzol (Qiagen) and processed as per manufacturers instructions. 2μg of total RNA was reverse transcribed with random primers using RT-PCR kit (Invitrogen). Equal volumes of cDNA were then subject to PCR using Taq polymerase (invitrogen) with human AQP1 or β-Actin primers. PCR amplification algorithm was 3x, 95°C-2min, 58°C-1min, 72°C-1min followed by 27x, 95°C-1min, 58°C-30secs, 72°C-30secs. The amplified PCR products were then resolved in 2% agarose (Nippon Genetics) in TAE buffer. The primer sequences were

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\text{AQP1F} - 5'\text{CATCGGCCTCTCTGTAGCC}, \quad \text{AQP1R} - \]


5’TGCTGAAGTTGTGTGTGATCA, b-ACTF-5’CGACAGGATGCAGAAGGAGA, b-ACTR-5’TGCTTGCTGATCCACATCTG.

**Cell protein isolation and Western blotting.** Cell monolayers were rinsed with PBS and soaked in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics). The lysates were centrifuged at 15,000 rpm at 4°C for 10 min and the supernatant protein content was measured by MicroBCA assay. Equal amounts of protein (10 μg) were separated by 4–12% SDS–PAGE Bis-Tris polyacrylamide gel and then transferred to nitrocellulose membranes. Membranes were probed with rabbit anti-AQP1 (sc-20810; Santa Cruz Biotechnology) and anti-b-actin (13E5, Cell Signaling) followed by incubation with goat anti-rabbit IgG-HRP (7074S, Cell Signaling). Protein bands were revealed using chemiluminescence HRP substrate (Pierce Biotechnology) and X-ray films. The experiment was performed three times.

**Cell adhesion assays.** Synchronized cells were used for assays in 48 well plates. The plates were either treated with FN or cell-derived ECM. In each well 2.5x10⁴ were seeded in 200 μl in media alone or supplemented with MC (10⁻⁵M). Cells were allowed to attach for 90 minutes in the incubator and subsequently unattached cells were aspirated by 3x warm PBS washes. The attached cells were fixed with 4% PFA followed by staining with 0.5% crystal violet for 10 minutes. The stain was then aspirated followed by washing the plate in running tap water. The plates were dried overnight and imaged at 200x magnification. Subsequently they were de-stained with 10% acetic acid, which was subject to
OD measurements. Each experiment had a replicate of 6 or 8 and repeated 2-3 times.

**Wound healing assay.** Assays were carried out in 48 well plates pretreated with FN or with cell-derived ECM. Cells were cultured to 100% confluence in 10% RPMI and then serum starved for 24 hours followed by warm sterile PBS wash. The monolayer was then scratched in a straight line with a 20μL sterile pipette tip. The monolayers were washed with warm PBS followed by appropriate media change. Cells were examined at 100x magnification followed by image capture. The plates were then incubated for appropriate times and experiments were terminated by media aspiration, fixation with 4% PFA for 10 minutes and followed by imaging. The area of the wound was measured in ImageJ using the polygon tool. For comparisons the migration index (MI) was used where MI=(A₀−Aₜ)/A₀. A₀ represents area measured at time 0 and Aₜ represents area at the time of termination. Each experiment had a replicate n=6 or 8 and repeated 3-4 times.

**Sphere formation assay.** Synchronized cell suspensions in 10% RPMI with FN (250 ng/ml) or ECM proteins (250 ng/ml) with/without MC at a density of 2x10⁵/ml were deposited as drops in 25 μL at the bottom of sterile petri dishes. The lid received 1 ml of sterile PBS. The bottom plate was gently inverted to form hanging drops and the lid was used as the bottom. Hanging drops were incubated for 48 hours and tumor spheres were imaged at 100x magnification followed by perimeter measurements using ImageJ software. Each experiment had a replicate n=10 or 12 and experiments were repeated 2 or 3 times.
**Statistical analysis.** Analyses were performed using Prism 6 for Mac. Data comparisons were performed with one-way ANOVA and Tukey’s or Sidak’s multiple comparisons post-hoc test. All data are presented as means±SEM. Values of p<0.05 were deemed as significant.

**Results**

Isolation and quantification of cell-derived ECM. In Figure 1B-1D the progressive dissolution of a ZL34 cell monolayer is shown in a series of images (t=0,15 and 30 seconds). A corresponding video is provided in the Supplemental Materials. The deposited amount of protein on well surfaces was quantified for each cell line. MeT-5A cells produced 6.12±1.01 μg of protein/well (n=5), M14K cells produced 7.7±0.57 μg of protein/well (n=6) and ZL34 cells produced 10.19±0.19 μg of protein/well (n=6) (*p<0.05 compared to MeT-5A, ##p<0.01 compared to M14K; Figure 1E).

Evaluation of the adhesive properties of cell-derived ECM in terms of cell morphology. MeT-5A cells were circular upon adhesion to FN (Figure 1F) while on ECM coated plastic the cells attached with apparent protrusions (Figure 1G). M14K cells showed no morphological differences after adhesion to FN (Figure 1H) or ECM coated plastic (Figure 1I). The same was the case for ZL34 cells (Figure 1J and Figure 1K respectively).

MeT-5A, M14K and ZL34 cells express AQP1 both at the transcript and in the protein level. As shown in Figures 2A and 2B, all three cell lines express the AQP1 transcript and protein. Densitometry analysis of 3 replicates in each
case showed no significant differences in the expression levels among the three cell lines (data not shown).

**ZL34 cell adhesion was significantly higher than MeT-5A and M14K on both FN and ECM coated surfaces.** MeT-5A cell adhesion served as control (100±5.35%). M14K cell adhesion on FN (57±2.35%) was significantly lower compared to MeT-5A (p<0.05) and ZL34 (156±8.67%) (p<0.001) (**Figure 2C**).

ZL34 cell adhesion was significantly higher (p<0.01) compared to MeT-5A. Cell adhesion on ECM showed no significant differences between MeT-5A (100±5.51%) and M14K (86.6±4.02%). ZL34 cell adhesion (152.4±12.0%) however remained significantly higher than MeT-5A (p<0.05) and M14K (p<0.001) (**Figure 2D**).

**AQP1 inhibition lowered cell adhesion of MeT-5A and M14K.** Cell adhesion with 10% RPMI was used as control. On FN there was a significantly lower cell adhesion of MeT-5A (67.3±3.05% vs.100±5.10%; p<0.001) and M14K cells (71.2±5.89% vs.100±3.25%; p<0.005) after MC treatment. No such differences occurred in ZL34 cells (**Figure 2E**). Cell adhesion of M14K cells to ECM with MC treatment was significantly lower (88.4±4.06% vs.100±1.43%; p<0.05) while no significant differences occurred regarding MeT-5A and ZL34 (**Figure 2F**).

**ZL34 wound healing was significantly higher than MeT-5A and M14K.** Wound closure of ZL34 on FN at 4 hours (MI=0.69±0.03) was significantly higher than MeT-5A (0.18±0.01, p<0.001) and M14K (0.13±0.01, p<0.001) (**Figure 3A**). Regarding ZL34 cells complete wound closure was observed at 4.5-5 hours on FN while on ECM it was observed after 8 hours. There were significant
differences in comparisons between ZL34 (MI=0.95±0.01) and MeT-5A (0.24±0.01, p<0.001) and M14K (0.32±0.01, p<0.001) (Figure 3B). Also M14K MI was significantly higher than MeT-5A (p<0.05).

Migration index was lowered on ECM substratum. The MI on FN was higher compared to ECM (Figure 3C). In MeT-5A at 8 hours on FN MI=0.35±0.01 was significantly higher than on ECM (MI=0.24±0.02, p<0.001). In M14K at 8 hours on FN, MI=0.35±0.02 was significantly higher than on ECM (MI=0.32±0.01, p<0.01). In ZL34 the comparison was performed at 4 hours and on FN MI=0.69±0.04 was significantly higher than on ECM MI=0.51± 0.01 (p<0.001).

AQP1 blockade lowered the MI in ZL34 cells. After treatment with MC there were no significant differences in MeT-5A and M14K cells. In ZL34 cells the MI was significantly lowered on FN (control MI=0.63±0.05 versus MC MI=0.43±0.03, p<0.001) (Figure 3D). Similarly, on ECM, MeT-5A and M14K showed no response to MC treatment while in ZL34 MI was significantly lowered from 0.95±0.03 (10%) to 0.86±0.02 (MC) (p<0.05; Figure 3E).

Sphere formation was substratum independent. There were no significant differences in sphere perimeters regarding all cell lines both in FN and ECM groups (Figure 4A-4B).

AQP1 blockade during sphere formation was substratum and cell type dependent. Sphere formation in FN supplemented media during AQP1 inhibition reduced the size only of M14K spheres as the perimeter (in pixels) was significantly decreased from 1060±15.91 (10%) to 744±17.76 (MC), (p<0.001; Figure 4C). No differences occurred in MeT-5a and ZL34. In contrast sphere
formation in ECM supplemented media during AQP1 inhibition resulted in significant reduction of the sphere sizes in all cells line (Figure 4D). In MeT-5A the reduction was from $1275 \pm 33.99$ (10%) to $557 \pm 29.45$ (MC) pixels ($p<0.001$), in M14K from $1486 \pm 96.47$ (10%) to $512 \pm 31.37$ (MC) pixels ($p<0.001$) and in ZL34 from $2167 \pm 98.24$ (10%) to $1409 \pm 61.7$ pixels (MC) ($p<0.001$).

Discussion

We evaluated the role of AQP1 in the context of cell adhesion, wound healing and sphere formation in MeT-5A, M14K and ZL34 cell lines. AQP1 blockade reduced the cell adhesion of MeT-5A and M14K cells during adhesion on FN substratum while this effect was limited only to M14K cells during adhesion on ECM substratum. During wound healing AQP1 function was critical only in ZL34 cells irrespective of the substratum. In the context of sphere formation with FN, M14K spheres were smaller due to AQP1 inhibition, and this effect extended to MeT-5A and ZL34 as well in the presence of ECM.

The growth pattern of MPM includes multiple nodules within the pleural cavity that progressively grow contiguously to form a solid tumor [11]. Hence, the process of cell adhesion is important both in the initial spreading of the MPM tumor cells within the cavity and in the tumor progression. Contrary to our findings, AQP1 over-expression in 4T1 mammary gland and B16F10 melanoma cells did not affect cell adhesion to FN, laminin-1 or collagen-1 [5]. In MPM cell adhesion the role of AQP1 is largely unknown. Our results showed that in M14K epithelioid MPM cells, adhesion was AQP1-dependent, in ZL34 sarcomatoid
MPM cells adhesion was AQP1-independent while in benign MeT-5A cells AQP1 inhibition was only evident on FN substratum. These data suggest a cell and substratum dependent role for AQP1 in cell adhesion of pleural benign and malignant cells. Previously, ZL34 and M14K cell adhesion on FN substrate showed that there was no difference between the two in serum-deprived media [12]. However, our experimental design involved FBS supplemented media and demonstrated that there is an MPM histological type dependence of cell adhesion, where the more aggressive sarcomatoid type adheres significantly more than the less aggressive epithelioid type and than benign mesothelial cells. Potentially this may explain why MPEs due to sarcomatoid MPM typically present with copious but acellular or low cellular pleural effusions. To our knowledge this is the first study demonstrating differences regarding this phenotype depending on MPM histological type and in a more clinically relevant cell adhesion assay design using homologous cell-derived ECM substrate.

AQP1 over-expression in 4T1 and B16F10 cell lines enhanced wound-healing. AQP1 was localized at the cell lamellapodia at the wound edge implicating a functional aspect of AQP1 during migration [5]. Conversely, mouse AQP1 null proximal tubule cell wound healing was significantly lower compared to wild type cells while adenovirus mediated AQP1 expression restored the wound closure speed [4]. Our results are partially in agreement, with significant lowering of MI in ZL34 cells during AQP1 inhibition irrespective of the substratum. AQP1 inhibition in MeT-5A and M14K cells had no effect on MI irrespective of the substratum. However, this is in contrast to the inhibition of cell migration in H226
MPM cells (epithelioid) after AQP1 inhibition with AqB050 [10]. This discrepancy may be due to the different inhibitory agents and different substrata used in our study. Previously it has been shown that cell migration and invasion increased according to the aggressiveness of the cell type in the following pattern: benign mesothelial<epithelioid<biphasic [3]. This finding was attributed to the epithelial-mesenchymal transition process during MPM disease progression [3]. Similarly we have shown that ZL34, a sarcomatoid MPM cell line, demonstrates significantly higher MI in comparison to MeT-5A and M14K cell line.

MPM spheres have been shown to have systematic differences in expression profiles of pathways governing cell signaling, proliferation, motility and morphology as compared to cell monolayers [8]. Tumor initiating cells (TICs) from sphere cultures demonstrated that TICs are more efficient in xenograft tumorigenesis compared to adherent cells [15]. This highlights the importance of tumor spheres in MPM modeling. AQP1 inhibition with AqB050 reduces sphere colony formation of H226 cells, however colonies were generated on agarose substratum [10]. We have shown that sphere formation is sensitive to AQP1 in M14K (in FN) while AQP1 inhibition leads to reduction of sphere perimeter in MeT-5A, M14K and ZL34 in the presence of ECM.

Finally, in the current study we demonstrated that the use of homologous cell-derived ECM in the examined phenotypes might bare insights to more clinically relevant results. The differences in cell adhesion, migration and tumor sphere formation by the influence of FN and cell-derived ECM strengthens the notion that our proposed model should be the experimental design of choice.
given that it adds an *in-vivo* experimental layer to *in-vitro* studies regarding MPM. In line with this FN has been reported to be expressed in mesothelial cells and to enhance cell migration and motility [9,11,12]. We have also determined differences in the amount of ECM produced by benign, epithelioid and sarcomatoid cell lines. Previous studies have shown differences in the production of laminin, FN and collagen IV proteins among epithelioid, biphasic and sarcomatoid MPM cell lines [16].

In conclusion our study provides insight into the role of AQP1 in MPM pathobiology and an influence of its role depending on the cell-substrate interaction. The molecular mechanisms governing these effects need further characterization. Furthermore proteomic analyses identifying the ECM composition per each cell line will help elucidate the exact role of the AQP1-ECM interactions.

**Acknowledgements**

This work was supported by a Hellenic Thoracic Society 2012 Research Award (to E. Apostolidou and S.G. Zarogiannis).
References


Figure legends

Figure 1: (A) Schematic of ECM isolation and uses of prepared surface. (B-D) Time-lapse images of a ZL34 monolayer subjected to detergent lysis. (E) Quantitative comparison of deposited ECM protein ($\mu$g/mL)$\pm$SEM, n=5-6 and *p<0.05 versus MeT-5A, ##p<0.01 versus M14K. (F) MeT-5A adhesion on FN and (G) ECM coated plastic. (H) M14K adhesion on FN and (I) ECM coated plastic. ZL34 adhesion on FN (J) and ECM (K) coated plastic.

Figure 2: (A) Representative image of AQP1 and $\beta$-Actin PCR product from cDNA of each cell line. (B) Western blot image of AQP1 and $\beta$-Actin protein from each cell line. (C) Mean cell adhesion$\pm$SEM, n=6-8 as % of control MeT-5A adhesion on FN substratum and (D) ECM substratum. *p<0.05, **p<0.01 versus MeT-5A and ####p<0.001 versus M14K. (E) Mean cell adhesion$\pm$SEM (n=6-8) after AQP1 inhibition on FN and (F) on ECM substratum. *p<0.05, ***p<0.05 and ****p<0.001.

Figure 3: (A) Mean MI$\pm$SEM, n=6-8, comparisons on FN substratum at 4 hours, ****p<0.001 versus MeT-5A, ####p<0.001 versus M14K on FN. (B) Mean MI$\pm$SEM, n=6-8, comparisons on ECM substratum at 8 hours, *p<0.05, ****p<0.001 versus MeT-5A and ####p<0.001 versus M14K. (C) Mean MI$\pm$SEM, n=6-8, of each cell line as a comparison between FN and ECM substratum **p<0.01, ****p<0.001. (D) Mean MI$\pm$SEM, n=6-8, comparisons on FN after 4 hours
AQP1 inhibition, ****p<0.001. (E) Mean M±SEM, n=6-8, comparisons on ECM after AQP1 inhibition, *p<0.05.

Figure 4: (A) Mean perimeter measurements (pixels)±SEM, n=8-9 of each cell line in FN or ECM. Sphere formation of MeT-5A in FN (B1) and ECM (B2), M14K in FN (B3) and ECM (B4), ZL34 in FN (B5) and ECM (B6). (C) Mean perimeter measurements±SEM, n=8 during AQP1 inhibition, versus control in media with FN **** p<0.001 and (D) in media with ECM versus controls (n=5-6) ****p<0.001.
Figure 1: MeT-5A, M14K, and ZL34 cell culture conditions and ECM protein concentration levels.

Panel A: Diagram illustrating the process from cell culture to retained ECM and cell adhesion.

Panel B-D: Images showing different stages of the process.

Panel E: Bar graph comparing ECM protein levels in MeT-5A, M14K, and ZL34 cells, with significant differences indicated by * and **.
Figure 2

(A) Western blot analysis of AQP1 and β-Actin in MeT-5A, M14K, and ZL34 cells.

(B) Western blot analysis of AQP1 and β-Actin in MeT-5A, M14K, and ZL34 cells.

(C) Bar graph showing the percentage of cell adhesion with FN in MeT-5A, M14K, and ZL34 cells.

(D) Bar graph showing the percentage of cell adhesion with ECM in MeT-5A, M14K, and ZL34 cells.

(E) Bar graph showing the percentage of cell adhesion with FN in 10% MC, 10 M MC, 10% MC, 10 M MC, and 10% MC, 10 M MC conditions for MeT-5A, M14K, and ZL34 cells.

(F) Bar graph showing the percentage of cell adhesion with ECM in 10% MC, 10 M MC, 10% MC, 10 M MC, and 10% MC, 10 M MC conditions for MeT-5A, M14K, and ZL34 cells.
Figure 3
Figure 4

(A) Bar graph showing sphere perimeter (pixels) for different cell lines (MeT-5A, M14K, ZL34) under FN and ECM conditions. The graph compares cell lines at various concentrations (10%, 10^5 M, 10^6 M).

(B) Microscope images of cell aggregates labeled B1 to B6, with scale bars indicating 100 μm.

(C) Bar graph showing sphere perimeter (pixels) for different cell lines under varying concentrations (10% MC, 10^5 M, 10^6 M). The graph illustrates statistical significance with asterisks.

(D) Expanded view of the bar graph from (B), highlighting the statistical significance (****) for MeT-5A, M14K, and ZL34 at different concentrations.