Acetazolamide inhibits aquaporin-1 protein expression and angiogenesis[1]

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ABSTRACT

AIM: To study effects of acetazolamide on aquaporin-1 (AQP_1) protein expression and angiogenesis. **METHODS:** Establishing Lewis-lung-carcinoma model, the localization of AQP_1 in tumor tissues was investigated by immunohistochemical methods; The biological activity of acetazolamide was detected by endothelial cells proliferation test (MTT) assay and chorioallantoic membrane (CAM) vascular inhibition test. **RESULTS:** Immunohistochemical localization of AQP_1 in mice tumor was labeled in capillaries, post capillary venules endothelial cells. After being treated with acetazolamide, the number of capillaries and post capillary venules was significantly decreased in tumor tissue. Acetazolamide showed significant inhibitory effect on angiogenesis in CAM and endothelial cell proliferation. **CONCLUSION:** Acetazolamide might be identified and developed as one of potential lead compounds for a new therapeutic intervention in inhibiting cancer angiogenesis.

INTRODUCTION

Recently the medical remedy for malignant tumor has been concentrated to cut off the tumor nutrition supply such as inhibiting the angiogenesis and other aspects[1]. Angiogenesis, the formation of new blood vessels, is essential for tumor progression and metastasis. Vigorous neovascularization, or angiogenesis, has been associated with a poor prognosis for cancers arising at several primary sites[2]. The process of angiogenesis is required for sustaining tumor growth in the face of an accumulating cell mass and providing access to the systemic circulation so that tumor microemboli can be transported to distant sites and established metastatic foci. Angiogenesis is one of the most rapidly growing fields in basic and applied cancer research. Consequently, the modulation of tumor angiogenesis using novel agents has become a highly active area of investigation in cancer research, from the bench to the clinic. However, the great therapeutic potential of these agents has yet to be realized.

Acetazolamide is a kind of sulfanilamide served as carbonic anhydrase inhibitor and clinically used to reduce intraocular pressure in glaucoma, to correct metabolic alkalosis, and to manage cerebral edema. Parkkila *et al* have shown that acetazolamide alone could inhibit the invasive potential of cancer cells *in vitro*[3], but the mechanism of action has not be clarified.

As the first characterized water channel protein[4], AQP_1 was identified in erythrocyte membranes, renal proximal tubule, choroids plexus, eye, lung, vascular endothelium, hepatobiliary epithelium, and some tumor

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cells themselves[5]. Most tumors have been shown to exhibit high vascular permeability and high interstitial fluid pressure, but the transport pathways for water within tumors remain unknown.

In a previous study, our laboratory has demonstrated acetazolamide significantly suppressed the tumor metastases *in vivo*, and the mechanism of acetazolamide on tumor metastases could be involved in reducing AQP_1 water channel protein expression[6].

Since the preliminary evidence showing AQP_1 expression in tumor microvessels, reduced tumor growth, and angiogenesis in AQP_1-deficient mice raise the possibility that aquaporins may be involved in tumor growth and angiogenesis[7]. One of the most popular assay tissues to study angiogenic activity is the developing chick embryo. The rapidly growing extraembryonic vascular network within the CAM is used to test the effects of fluids absorbed onto a carrier or solid materials. The assay has been used to test a variety of promoters and inhibitors of angiogenesis as well as antiangiogenic strategies in cancer treatment[8].

We hypothesized that acetazolamide may have potential usefulness as an angiogenic inhibitor by regulating AQP_1 water channel function (data to be published) and protein expression. In the present study, we observed the distribution of AQP_1 in tumor tissue, spontaneous pulmonary metastasis of Lewis carcinoma in mice and detected the biological activity of acetazolamide on anti-angeogenesis.

MATERIALS AND METHODS

Lewis-lung-carcinoma *in vivo* model Female C57BL/6 mice weighing 18-20 g, were used and purchased from the Experimental Animal Center of Peking University (Grade I, Certificate No 11-00-0004). Lewis lung carcinoma provided by Chinese Medical Science Institute was maintained in C57BL/6 mice by subcutaneous injection in the axillary's region of 0.2 mL of homogenized tumor tissue [tumor tissue (g): 0.9 % sodium chloride (mL)=1:3] prepared from donors similarly inoculated for experimental tumor transplantation[6].

Drug preparation and treatment Acetazolamide was purchased from Sigma and given at a volume of 0.1 mL per mice (40 $mg \cdot kg^{-1} \cdot d^{-1}$, ig). Control mice received the same volume of vehicle by ig.

Immunohistochemistry On d 21 of treatment, the mice were killed and primary tumors were then surgically resected. The samples were fixed in 4 % formaldehyde, embedded in paraffin and sectioned at 5 μm. Routine histology was carried out after haematoxylin and eosin staining of the sections. Coronal sections were mounted onto APES-coated slides, deparaffinnized, rehydrated, and incubated in 3 % hydrogen peroxide to quench any endogenous peroxidase activity, washed with distilled water and TBS and digested with 0.1 % pancreatin for 30 min. Normal rabbit serum was applied to eliminate nonspecific staining. Sections were incubated overnight at 4 ºC with optimally diluted rabbit anti-human AQP_1 antibody (a gift from Professor Verkman AS, University of California, San Francisco, USA). The sections were washed with TBS and incubated with biotinylated goat anti-rabbit IgG (Bio-RAD, USA) for 30 min, rewashed, and incubated with peroxidase-conjugated streptavidin for 30 min. The peroxidation activity was visualized by incubating with a peroxidase substrate solution (DAB kit). The sections were counterstained with hematoxylin and mounted. Appropriate diluted solutions of rabbit IgG was used as controls in the immunohistochemical localization of AQP_1[9].

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay Human endothelial cells (2×10^4), ECV304 in 2 mL RPMI-1640-10 % fetal calf serum (FCS) was added into wells of a 24-well plate (6 well×4 group), which was a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal human umbilical cord. The plate was incubated at 37 ºC, 5 % CO_2, 95 % humidity for 6 h. Stock solutions of acetazolamide was prepared in dimethylsulfoxide (Me_2SO) and aliquots of stock were then added to culture medium to yield final concentrations of 1×10^{-7}, 1×10^{-6}, or 1×10^{-5} mol/L, Me_2SO alone was added to control medium and was present at a concentration of 0.1 %, and cultured for 72 h, which was administered twice during the 72 h period: the first dose at the beginning of the assay and the second after 24 h. After growing for 72 h, 50 μL MTT solution (1 g/L)(Sigma) was added to each well. The plate was then incubated at 37 ºC for 4 h in dark. Addition of 150 μL Me_2SO was mixed well with MTT and kept at room temperature for 15-30 min. The plate was shaken until the precipitate was dissolved. Absorbance was measured at 570 nm. The experimental procedures were repeated three times.

Chick chorioallantoic membrane (CAM) preparation Stock solutions of acetazolamide were

prepared in Me_2SO and yielded final concentrations of 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol/L(ie, 0.44 ng/CAM, 4.4 ng/CAM, 44 ng/CAM), Me_2SO alone was control medium and was present at a concentration of 0.1 %. Fertilized eggs obtained from Chinese Agriculture University with 30 eggs in each group and livability ≥50 % were used. The eggs were incubated at 37 ºC, 60 %-70 % humidity for 5 d. On d 6, a small window was made in the region of air sac of the eggs and then continues to incubate for 24 h. On d 7, the shell membrane was peeled off to expose the chorioallantoic membrane of chick embryo. Sterile 6 mm diameter circular filter paper discs soaked with total 20 μL different concentration medium were applied onto the surfaces of the growing CAMs. The windows were sealed and the eggs were incubated for anther 72 h. On d 10, from the windows added solution methanol: acetone=1:1 (v/v) to fix for 15 min. Then the CAMs were separated and kept in filter papers to screen and preserve[10].

The numbers of blood vessels around the filter papers within 1 mm were counted under microscopy. The blood vessels were divided into three sizes according to diameter as 1 mm, 1-0.1 mm and <0.1 mm.

Statistical analysis Data were expressed as mean±SD. All statistical analysis were done in SPSS version 10.0. $P<0.05$ was considered to be statistically significant.

RESULTS

Localization of AQP_1 in tumor tissues Immunohistochemical localization of AQP_1 in mice tumor was labeled in capillaries, postcapillary venules (Fig1). Immunostaining showed strongly positive for AQP_1 in mice bearing Lewis lung carcinoma, but negative in mice treated by acetazolamide. And the results also indicated the number of capillaries and postcapillary venules were significantly decreased in tumor tissue.

Effect of acetazolamide on endothelial cells proliferation Significant difference was observed between the control and the treated cells by acetazolamide. This result indicated that acetazolamide could inhibit human endothelial cells proliferation, and among the concentration of 1×10^{-7}-1×10^{-5} mol/L, appeared a concentration-dependent depression activity (Fig 2).

CAM vascular inhibition test When acetazolamide among the dose of 0.44-44 ng/CAM was tested on d 7 CAM embryos, it resulted in dramatical angiogenesis inhibition as well as vessel regression after 72 h, and mainly reduced the number of microvasculature (Fig 3). This result also indicated that the anti-angio-

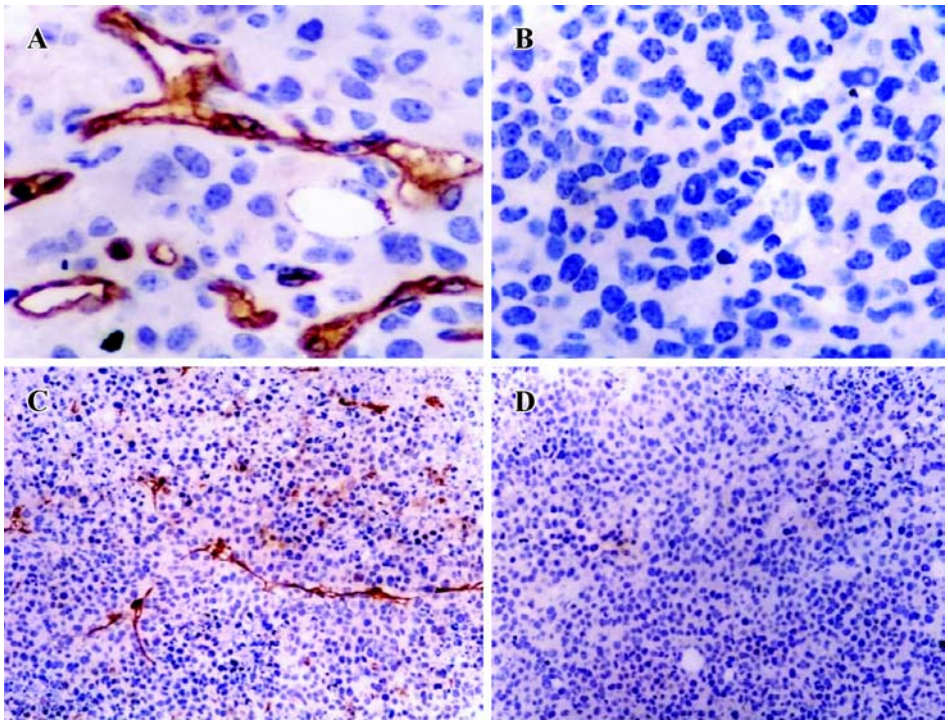

Fig 1. Expression of AQP1 in capillaries (A, B) and postcapillary venules endothelial cell (C, D) of primary tumor. A and C: Untreated; B and D: Treated with acetazolamide.

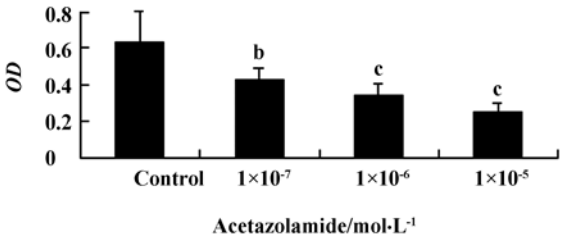

Fig 2. Effect of acetazolamide on cultured ECV304 growth detected by MTT method. *n*=6. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* Control.

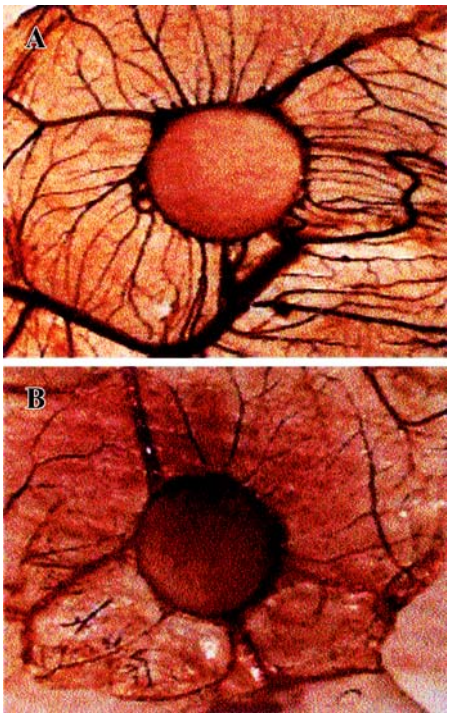

Fig 3. Effect of acetazolamide on angiogenesis in CAM assay. A: Control; B: Treated with acetazolamide.

genic/vessel regression activities of acetazolamide were dose-dependent (Tab 1).

Tab 1. Effect of acetazolamide on angiogenesis in CAM assay. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* C. AL: 0.000044 μg; AM: 0.0044 μg; AH: 0.044 μg.

Group	*n*	Number of blood vessels		
		>1 mm	1-0.1 mm	<0.1 mm
Control	16	2.9±1.4	12.2±4.5	45.1±9.5
AL	15	2.9±2.3	11.7±4.6	37.4±12.8^{b}
AM	19	2.5±2.1	7.6±2.6^{c}	36.3±12.1^{b}
AH	21	1.7±1.1^{b}	5.9±2.3^{c}	27.0±7.9^{c}

DISCUSSION

The results consistent with our hypothesis that acetazolamide showed a significant inhibitory effect on angiogenesis in C57BL/6 mice bearing Lewis lung carcinoma on chorioallantoic membrane and on proliferation of endothelial cells. This suggested that acetazolamide might be an effective inhibitor of endothelial cells and has potential therapeutic usage on the angiogenesis of cancer. Our findings extend the pharmacological mechanism of acetazolamide on tumor metastasis. The molecular mechanisms of acetazolamide-induced anti-angiogenesis are not well understood.

At present, acetazolamide as a carbonic anhydrase (CA) inhibitor is mainly used for edematous diseases such as glaucoma, mountain sickness, congestive heart failure-induced or drug-induced edema. The investigations of our laboratory have indicated that acetazolamide inhibited gene expression of AQP_1 in rat kidney[11, 12].

AQP_1 is abundant in the microvasculature epithelial cells. The results in the present study raise the possibility that the treatment of acetazolamide for tumor metastasis may be partly due to the suppression in the AQP_1 gene expression. There are evidences showing that AQP_1 plays an important role in several water balance disorder diseases[13]. This action suggests new therapeutic targets for human diseases in the future. Most tumors have been shown to exhibit high vascular permeability and high interstitial fluid pressure[14]. In reviewing the published studies on AQP_1 and carbonic anhydrase especially carbonic anhydrase II and carbonic anhydrase IV, we found that they shared many common biological characteristics. For instance, they are all widely distributed in fluid transporting tissues including the kidney, lung, brain, eye, erythrocytes, colon, pancreas, and some glands and having the function of facilitating reabsorption and secretion of water[15]. In addition, they increase in response to estrin[16] and developmentally increase after birth[17]. That AQP_1 is capable of transporting CO_2[18], and that CO_2 is a substrate of carbonic anhydrase in catalyzing the formation of HCO_3^-, suggest a close relationship on the biological characteristics between carbonic anhydrase and aquaporins.

Tumor angiogenesis is a complex, multi-step process that characterizes the development of tumor's circulatory system and the supply of nutrients that required for growth. Angiogenesis inhibition can lead to tumor regression and, in some cases, to complete elimination

of the tumor[19]. Investigators around the world have focused their interest the mechanisms of tumor angiogenesis and suggested that anti-angiogenesis could prove effective in the treatment of cancer[20]. Acetazolamide repress the ability of the endothelial cell to participate in the angiogenic process, which may generate tumor treatment effects that do not lead to the generation of drug resistance. That is exciting because acetazolamide might be identified as one of potential lead compounds for development of new drugs for therapeutic intervention of cancer in future.

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