Inhibitory effect of topiramate on Lewis lung carcinoma metastasis and its relation with AQP1 water channel[1]

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KEY WORDS topiramate; aquaporins; tumor metastasis; carbonic anhydrase

ABSTRACT

AIM: To study the effect of topiramate on tumor metastasis and its relation with aquaporin 1 (AQP1) water channel. **METHODS:** Lewis lung carcinoma metastatic model was used to determine the effect of topiramate on tumor growth and metastasis. Colorimetric estimation was used to investigate the action of topiramate on carbonic anhydrase (CA) activity. Western blotting and immunohistochemical analysis were used to study the influence of topiramate on AQP1 water channel expression in lungs or tumor tissues of mice bearing Lewis lung carcinoma. **RESULTS:** Treatment with topiramate (120 $mg \cdot kg^{-1} \cdot d^{-1}$, ig for 20 d) reduced the growth of primary tumor significantly ($P<0.05$). Its inhibitory rate of metastasis was 81.25 %. Topiramate inhibited CA activity in lungs of mice in a dose-dependent manner. Topiramate apparently decreased AQP1 protein expression and immunostaining in lungs or in tumor microvessel endothelial cells of mice. **CONCLUSION:** Suppression of AQP1 water channel expression may be an important pathway for the inhibitory effect of topiramate on tumor metastasis.

INTRODUCTION

Carbonic anhydrases (CA) (4.2.1.1) are a family of zinc-binding metalloproteinases that catalyze reversible hydration of carbon dioxide ($CO_2+H_2O \longleftrightarrow H^+ + HCO_3^-$). Up to now, a number of 14 different CA isozymes were described in higher vertebrates, including humans[1]. CA are abundant in a variety of tissues where they are involved in a broad range of physiological processes such as acid-base balance, carbon dioxide and ion transport, respiration, body fluid generation, bone resorption, ureagenesis, gluconeogenesis, and lipogenesis[2]. In recent years, it is reported that some of CA isozymes play important roles in tumor growth and metastasis. For example, some new CA isozymes, such as CA IX, CA XII, and CA XIV, were predominantly found to be present only in tumor cells. These and other more classical isozymes, such as CA I, CA II, and CA IV, were also shown to be present and actively involved in other types of proliferative diseases/processes, such as von Hippel-Lindau renal tumors, progressive polycystic kidney disease, acinar-ductal carcinomas of the pancreas, autoimmune or idiopathic chronic pancreatitis, and apoptosis in some human pancreatic cancer cells[3].

Microelectrode-measured pH values have indicated that the extracellular pH in solid tumors is more acidic than in adjacent normal tissue[4]. In contrast, the intracellular pH estimated by ^{31}P-magnetic resonance spectroscopy is identical or slightly more basic in tumor

[1] Project supported by the National Natural Science Foundation of China (No 39770286 and 30171090) and Rockefeller Foundation of USA (RF93063 #83).
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Received 2003-01-07 Accepted 2003-05-15

compared with normal tissue[5]. Tumor microenvironment acidity could play a predominant promoting role in tumor growth and metastasis and also could underlie resistance to radiotherapy, chemotherapy, and other nonsurgical treatments[6,7]. It has been shown that acidic pH enhances invasive behavior of tumor cell *in vitro*. Although the exact role of CA activity in carcinogenesis has not been established, Ivanov *et al* hypothesized that "tumor-associated" transmembrane isoenzymes CA IX and CA XII may be implicated in acidification of extracellular milieu surrounding the cancer cells and thus create a microenvironment conducive to tumor growth and spread[8].

CA inhibitors have been shown to inhibit tumor cell invasion *in vitro*[9], and in xenograft experiments, CA inhibitors as part of a chemotherapy regimen enhanced the effect of chemotherapy drugs and helped delay tumor growth[10]. Several 1,3,4-thiadiazole-2-sulfonamide derivatives possessing potent CA inhibitory properties act as effective *in vitro* tumor cell growth inhibitors of different leukaemia, non-small cell lung cancer, melanoma, ovarian, renal, prostate, and breast cancer cell lines[11]. Although several mechanisms of action of CA inhibitors exist, it is believed that they mainly lead to the acidification of the intra-tumoural environment ensued after CA inhibition, which may favourably influence the anticancer effect of the drug *per se* or that of another anticancer agent used concomitantly with it[11]. That seems contrary to the theory that acidic microenvironment is beneficial to tumor growth and metastasis. This fact provides a compelling argument for a more thorough evaluation of CA inhibition as a mechanism underlying or contributing to the anti-tumor activity of CA inhibitors and leads emergence of some other hypothesized mechanisms.

Most tumors have been shown to exhibit high vascular permeability and high interstitial fluid pressure[6,7], but the transport pathways for water movement within tumors remain unknown. Aquaporins (AQP) are a large family of membrane proteins that function as highly selective water channels. Aquaporin 1 (AQP1), the first characterized water channel protein, is the erythrocyte membrane water channel, and is also abundant in several other absorptive or secretory epithelia including the choroids plexus, the ciliary body of the eye, the gall bladder, hepatobiliary ductules, capillary endothelia, and portions of the male reproductive tract[12]. AQP1 is widely distributed in many tumors[13]. The studies of our group showed that acetazolamide (a typical CA inhibitor) inhibited the gene expression and water transport function of AQP1. We hypothesized that the suppressing action of CA inhibitors on AQP1 might be contributed to their effects on cancer invasion and metastasis.

Topiramate is a novel compound, which has been demonstrated to have a broad spectrum of antiepileptic activities both in experimental and clinical studies. This drug also has very favorable pharmacokinetic properties. It has displayed a high bioavailability, is rapidly absorbed, is excreted predominantly unchanged by the kidney and has shown good patient tolerance[14]. In recent years, many studies are focused on the effect of topiramate on central neural system, but other pharmacological actions are still poorly understood. Although topiramate is structurally novel, the sulfamate moiety is chemically related to known CA inhibitors. In fact, topiramate inhibits several carbonic anhydrase isozymes, especially CA II and CA IV. So, we hypothesized that topiramate has the similar effect on tumor growth and metastasis with CA inhibitors such as acetazolamide.

Therefore, in this study, we examined these two hypotheses by establishing Lewis lung carcinoma metastatic model and assessing the influence of topiramate on tumor metastasis and its relation with AQP1 water channel.

MATERIALS AND METHODS

Animals and Lewis lung carcinoma The female C57BL/6 mice weighing 18-20 g, were purchased from the Experimental Animal Center of Peking University (Grade II, Certificate No 11-00-0004). Lewis lung carcinoma, originally provided by Chinese Medical Science Institute was maintained in C57BL/6 mice by sc injection in the axillary region of 0.2 mL of tumor tissue homogenate [tumor tissue (g): 0.9 % sodium chloride (mL)=1:3] aseptically prepared from donors similarly inoculated for experimental tumor transplantation.

Drug treatment Topiramate was generously provided by Xi'an-Janssen Pharmaceutical Ltd and dissolved in deionized water. The mice were divided into five groups of 9 animals each, 1) control group: normal animals; 2) model group: tumor-transplanted animals with a single daily dose of vehicle; 3) TL group: tumor-transplanted animals with a single dose of topiramate (30 $mg \cdot kg^{-1} \cdot d^{-1}$, ig); 4) TM group: tumor-transplanted animals with a single dose of topiramate (60 $mg \cdot kg^{-1} \cdot d^{-1}$, ig); 5) TH group: tumor-transplanted animals with a

single dose of topiramate (120 mg·kg^{-1}·d^{-1}, ig). The treatment was initiated 1 d after tumor transplant (d 0) for a period of 20 d.

Metastases assays Three weeks after tumor transplant (d 21), the animals were killed and the weights of animal bodies, lungs, and primary tumors were measured. The lungs were removed and the number of lung metastasis was counted. Primary tumor and lungs were then surgically resected, and tissue specimens were snap-frozen in liquid nitrogen for analysis. Tumor weight index means the ratio of tumor weight to body weight (g/g)×100 %. Inhibitory effect rate of lung metastases (%)=(W_{model}-$W_{treatment}$)/(W_{model}-$W_{control}$)×100 %, here W is lungs wet weight.

Carbonic anhydrase activity assay Tissue of lungs or tumors was sonicated in sucrose solution 0.3 mol/L. The activity of CA in tissues was analyzed according to an established procedure[15]. Briefly, water was added to the sample to a total volume of 500 μL, which was continuously bubbled with CO_2. Then, 500 μL of imidazole-Tris buffer plus *p*-nitrophenol indicator (imidazole 20 mmol/L, Tris 5 mmol/L, *p*-nitrophenol 0.2 mmol/L) were added to initiate the reaction. Timing was stopped when the yellow color vanished and the solution appeared nearly colorless. A previously boiled homogenate (amount equal to each sample) was also assayed. One enzyme unit (EU) of CA activity was defined as the amount of homogenate necessary to halve the time of the control. CA activity was generally calculated from the formula:

CA (EU/mg prot)=[log(B/S)]/[(prot)log 2]

Where B and S are the times measured for paired boiled inactivated enzyme and active sample, respectively, (prot) is milligrams of protein used for the measurement.

Western blotting analysis Lung tissues were homogenized in buffer containing 0.25 mol/L sucrose and protease inhibitors. Membranes were isolated and solubilized in 1.5 % SDS[16]. Total protein concentration was measured by Lowry's method, using bovine serum albumin as the standard. Each sample containing 50 μg of protein was separated by 12 % SDS-polyacrylamide gel. Proteins were transferred electrophoretically from gels to nitrocellulose membranes. The nitrocellulose membranes were blocked in Tris-buffered saline (TBS, Tris-HCl 100 mmol/L and 0.9 % NaCl, pH 7.5) containing 5 % nonfat dry milk, followed by incubation with anti-AQP1 antibody (rabbit anti-human IgG, a generous gift from Dr Verkman, University of California, San Francisco) diluted 1:1000 in TBS containing 2.5 % nonfat dry milk at 4 ℃ overnight. The membranes were washed three times with TBS containing 0.1 % Tween-20 (TBST) and incubated for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:5000. Being washed three times with TBST, M_r 28 000 protein (AQP1) was stained with BCIP/NBT kit. Stained bands were scanned and pixel intensity was quantified using Gel Doc 2000 Image system.

Immunohistochemistry Tissues of lungs or primary tumors were cut into small blocks and transferred immediately into cold fixative (4 % paraformaldehyde in PBS 0.1 mol/L, pH 7.4). The tissue blocks were then rinsed in PBS, embedded in paraffin, and sectioned. Paraffin sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. Slides were submerged in 3 % hydrogen peroxide to quench any endogenous peroxidase activity, washed with distilled water, and heated in citrate buffer 0.01 mol/L, pH 6.0 in a microwave oven set for 15 min, then cooled and washed with PBS. Non-immune serum for blocking was applied to eliminate nonspecific staining. Sections were incubated overnight at 4 ºC with rabbit anti-human AQP1 antibody. The sections were washed with PBS and incubated with biotinylated goat anti-rabbit IgG secondary antibody for 40 min, rewashed with PBS and incubated with peroxidase-conjugated streptavidin for 40 min. The peroxidation activity was visualized by incubating the sections with a peroxidase substrate solution (DAB KIT) after sufficient washing. The sections were counterstained with hematoxylin and mounted. Non-immune rabbit serum was used as control. All controls were negative.

Statistical analysis Data were expressed as mean±SD. Comparisons of differences of metastasis number and tumor weight index between treatment group and model group were evaluated with Mann-Whitney *U*-test. The comparisons of AQP1 expression level in lungs and CA activity in tissues between treatment group and model group were done by one-way analysis of variance (ANOVA). All statistical analyses were done in SPSS version 10.0, $P<0.05$ was considered to be statistically significant.

RESULTS

Effects of topiramate on primary tumor growth and the formation of spontaneous pulmonary metastatic foci in Lewis lung carcinoma-bearing mice The Lewis lung carcinoma, when implanted sc in the axil-

lary region of mice, spontaneously metastasizes to the lungs. To determine the effects of topiramate on tumor growth and metastasis, 3 doses of topiramate were administered to Lewis lung carcinoma-bearing mice, respectively. Treatment with high dose of topira-mate (120 mg·kg^{-1}·d^{-1}, for 20 d) reduced the growth of primary tumor remarkably (P<0.05, Tab 1). The lung wet weights were decreased significantly by topiramate (120 mg·kg^{-1}·d^{-1}, for 20 d). Topiramate strongly inhibited the formation of lung metastasis in a dose-dependent manner. The inhibitory rate of lung metastasis of topiramate (120 mg·kg^{-1}·d^{-1}, for 20 d) was 81.25 %.

Tab 1. Effect of topiramate on experimental tumor metastasis in mice. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 *vs* model.

Group	Number of survival	Tumor weight Index	Number of metastasis	Lungs wet weight/ mg	Inhibition rate of lung metastasis/ %
Control	9/9			174±6^{c}	
Model	6/9	29.3±1.0	22±1	370±63	
TL	8/9	25±4	14±2^{b}	282±68	44.88
TM	7/9	23±4	11±3^{c}	264±35	53.91
TH	6/9	20.7±2.4^{b}	9±2^{c}	211±25^{b}	81.25

Effect of topiramate on carbonic anhydrase activity The influence of topiramate on CA activity in lungs and primary tumors of mice bearing Lewis lung carcinoma was measured by endpoint colorimetry. Topiramate effectively decreased the activity of carbonic anhydrase in lungs, and increased doses of topiramate showed a dose-dependent inhibitory effect. The carbonic anhydrase activity in primary tumor did not change significanty after treatment with topiramate (Tab 2). This result suggested that the inhibitory effect of topiramate on carbonic anhydrase activity appeared to be tissue-specific.

Effect of topiramate on AQP1 protein expression Western blotting analysis showed that the protein level of AQP1 in lungs with metastatic tumor foci was significantly higher than that in normal lungs (P<0.01). Topiramate clearly inhibited the protein expression of AQP1 in treated group compared to the tumor transplanted model group. The dose dependence of inhibitory effect of topiramate on AQP1 protein expression can be observed in Fig 1.

Tab 2. Effect of topiramate on total CA activity in lungs and primary tumors. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 *vs* model.

Group	*n*	CA activity in lung (EU/mg protein)	CA activity in primary tumor (EU/mg protein)
Control	9	1.91±0.14	
Model	6	2.14±0.20	1.07±0.14
TL	8	1.62±0.23^{b}	0.96±0.08
TM	7	1.41±0.15^{c}	0.82±0.06
TH	6	1.22±0.21^{c}	0.80±0.06

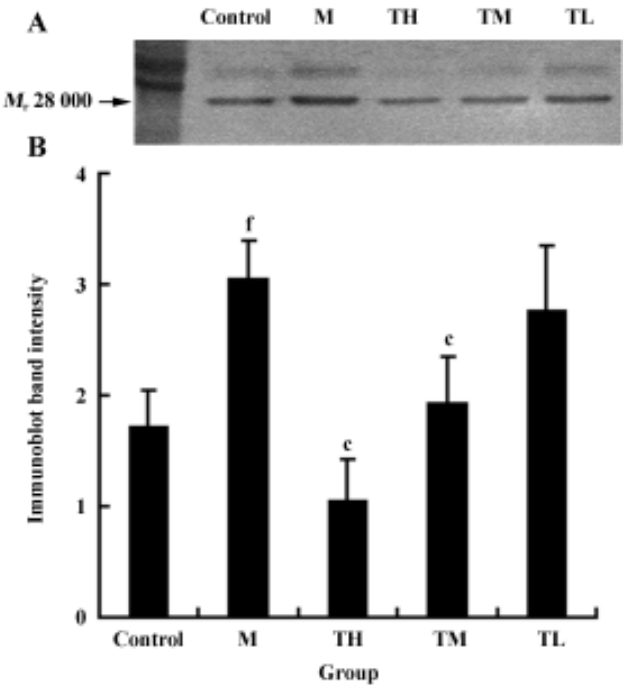

Fig 1. Effect of topiramate on protein levels of AQP1 in lungs of mice. Control: normal; M: model; TH: topiramate 120 mg·kg^{-1}·d^{-1}; TM: topiramate 60 mg·kg^{-1}·d^{-1}; TL: topiramate 30 mg·kg^{-1}·d^{-1}. A: a typical figure of Western blotting. The position of M_r 28 000 marker is indicated on the left. B: The band (M_r 28 000) of Western blotting was analyzed by densitometry. Mean±SD. n=6. ^{c}P<0.01 *vs* model. ^{f}P<0.01 *vs* control.

AQP1 distribution in lung and primary tumor of mice in model group or treated group was assessed by immunohistochemical analysis. AQP1 was abundant in peribronchiolar capillaries, postcapillary venules, bronchiolar basal membrane and alveolar epithelial cells (Fig 2). Treatment with topiramate did not alter the localization of AQP1, but decreased the immunostaining of AQP1 in lung, which was consistent with the result of

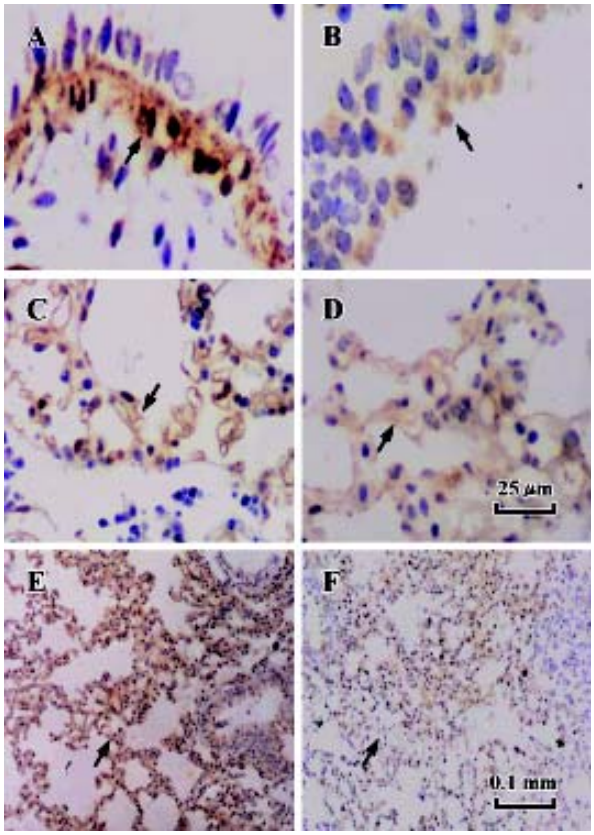

Fig 2. Immunohistochemical localization of AQP1 in lungs of mice. AQP1 positive staining was found in bronchiolar basal membrane (A and B), alveolar epithelial cells (C and D), and capillaries (E and F). Abundant AQP1 was seen in model group (A, C, and E), and decrease in labeling (B, D, and F) was seen in topiramate (120 mg·kg^{-1}·d^{-1})-treated group. (A, B, C, and D) ×400; (E and F) ×100.

Western blotting. In primary tumor, AQP1 was present in capillaries and postcapillary venule endothelial cell, but absent in tumor cell. Inhibition of AQP1 protein expression could be also obtained by treatment with topiramate in tumor. At the same time, we observed that the vasculature in tumor was decreased after treatment with topiramate (120 mg·kg^{-1}·d^{-1}, for 20 d, Fig 3).

DISCUSSION

The main purpose of this study was to test the hypothesis that topiramate, an anticonvulsant compound with multiple mechanisms of action, would prevent tumor metastasis, and study the role of AQP1 in it. The present results indicated that treatment with topiramate significantly reduced the spontaneous lung metastases and lung nodule formation. The inhibitory rate of lung metastasis by the high dose of topiramate was 81.25 %.

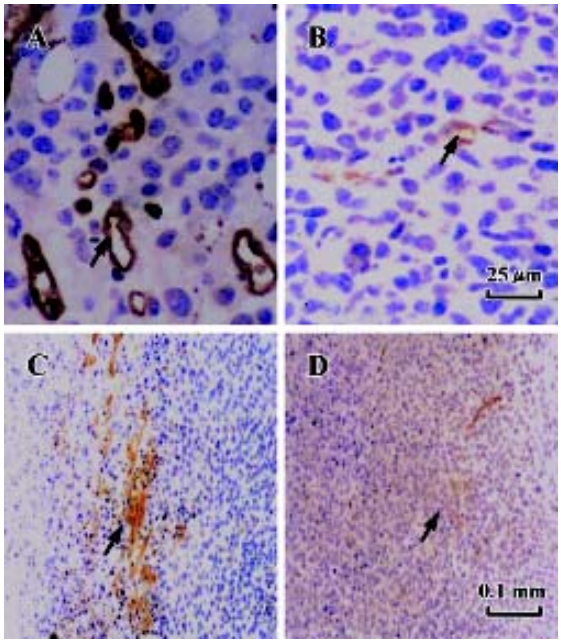

Fig 3. Immunohistochemical localization of AQP1 in capillaries and postcapillary venule endothelial cells in primary tumors. Abundant AQP1 was seen in model group (A and C), and decrease in labeling (B and D) was seen in topiramate (120 mg·kg^{-1}·d^{-1})-treated group. (A and B)×400; (C and D)×100.

The effect is surprising. The inhibitory effect of topiramate on metastasis of Lewis lung carcinoma raised an interesting question: what is the target for topiramate?

As we know, the development of metastases is a highly selective process dependent on complex interactions between tumor cells and their unique microenvironment that is characterized by low acidic excellular pH and altered hydrostatic pressure. Tight control of pH homeostasis in tumors is achieved by proton extrusion mechanisms including plasma membrane proton pumps, proton channel/proton wires, sodium/proton exchangers, and monocarboxylic acid transporters[17]. The extremely efficiency of cell surface CA may play an important role in controlling the levels of protons and bicarbonate in the immediate vicinity of the tumor cells by sensing pH and tipping the proton balance across the cell membrane[2]. In the present study we found that topiramate decreased the activity of CA in lungs of mice bearing Lewis lung carcinoma in a dose-dependent manner, but had no influence on CA in tumor tissues that might be related with the different distribution of CA isozymes in lungs and tumors, and the selective action of topiramate on different isozymes. So, the suppressing effect of topiramate on some CA isozymes activity may be a mechanism of its inhibitory effect on

tumor metastasis.

Ivanov *et al*[17] reported that the high vascular permeability and high interstitial fluid pressure of most tumors might result from activation, as a consequence of the acidic tumor microenvironment, of trans-membrane aquaporins that are widely distributed in tumors. The results of Western blotting analysis in this study demonstrated that AQP1 protein expression was higher in the lungs of mice bearing Lewis lung carcinoma than that of normal mice. This is consistent with our previous studies[18] and indicates the possibility that tumors growing may have a higher water permeability since AQP1 is generally responsible for water movement across cellular membrane. Furthermore, the up-regulated AQP1 expression in tumors may reflect a role of this water channel in pathological processes of tumors including the development of effusions or edema. The exact role of AQP1 in tumor growth and metastasis is being studied through gene transfection *in vitro* by our lab. Treatment with topiramate dramatically suppressed the expression of AQP1 in lungs and in vascular endothelial cells of tumor tissues. This finding indicated that topiramate inhibited tumor metastasis, at least in part, by directly suppressing the protein expression of AQP1. The previous studies demonstrated that acetazolamide could inhibit the expression of AQP1 in mRNA and protein levels. Acetazolamide and topiramate all belong to CA inhibitors, so we postulate that topiramate has the same mechanism with acetazolamide.

Anti-angiogenic therapy is one of the most promising strategies to inhibit tumor growth and metastatic dissemination. Most solid tumors cannot expand without generation of new blood vessels ensuing an adequate supply of oxygen and nutrients[19]. Furthermore, the formation of metastasis by solid tumors also appears to be dependent on neovascularization of the primary tumor[20]. We found that the vasculature was decreased in tumor tissues after treatment with topiramate. AQP1 protein is strongly expressed in most microvascular endothelia where it plays an important role in sustaining the normal function of endothelia[21]. The relationship between AQP1 expression and water transport within tumors and the tumor vasculature remains to be explored in detail. Topiramate might reduce the number of microvessles in tumor by inhibiting the expression of AQP1.

To our knowledge, this is the first demonstration of the effect of topiramate on tumor metastasis. From the results of this study, we conclude that suppression of AQP1 water channel expression may be an important pathway for its inhibitory effect on tumor metastasis.

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