Calcium Channel Antagonists Inhibit Growth of Subcutaneous Xenograft Meningiomas in Nude Mice

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BACKGROUND
We have previously shown that calcium channel antagonists inhibit in vitro meningioma growth. This study examines the effect of calcium channel antagonists on in vivo xenograft meningioma growth.

METHODS
Meningioma cells taken from human patients were mixed with Matrigel and injected into the subcutaneous space in the flank of nude mice. These animals were treated with calcium channel antagonists in their drinking water. Tumor volumes were measured over time; comparison was made between control and treatment groups. Daily weights, average daily water consumption, and serum calcium channel antagonist levels were determined. Comparison of histology and proliferation index was made between control and treatment groups.

RESULTS
Diltiazem treatment decreased tumor growth over time compared to control groups. Increased tumor growth inhibition was seen with increasing doses (p > 0.05). Treatment with verapamil had similar effects; however, there are no statistically significant dose dependent decreases in growth with increasing verapamil doses. There were no tumor “cures” or spontaneous regression of tumor in any group including the control groups. Animal daily weight and average daily water consumption was unaffected by increasing calcium channel antagonist doses compared to control groups. Mouse serum drug levels increased with increasing doses of drug in the drinking water of treatment groups (p > 0.05). Histology and proliferative index of treatment groups were similar to control groups.

CONCLUSION
Calcium channel antagonists decrease but do not completely inhibit the growth of meningiomas in nude mice. Clinical correlations and potential applications are discussed. © 2001 by Elsevier Science Inc.

KEY WORDS
Brain tumor, calcium channel antagonist, meningioma, Matrigel, nude mouse, xenograft.

Meningiomas are thought to arise from arachnoid cap cells found in the meninges and are one of the most common primary intracranial tumors found in adults [6]. Complete surgical removal is the most accepted treatment of intracranial meningiomas [1,9,31]. The medical condition of a patient, tumor recurrence, and proximity to vascular or neurological structures can make this difficult and other adjuvant therapies would be helpful [3].

We have previously demonstrated that the calcium channel antagonists verapamil, nifedipine, and diltiazem can block primary meningioma culture growth [22,23]. An in vivo model of meningioma cell growth was developed for testing the efficacy of new therapeutic measures [21]. This study examines the effects of increasing doses of the calcium channel antagonists diltiazem and verapamil on xenograft meningioma cell growth in nude mice. These drugs were chosen based on our in vitro experiments; nifedipine was not tested in this study because of difficulties getting mice to drink nifedipine-treated water. Since these drugs can alter cardiac parameters and blood pressure, average daily water consumption and daily body weights were measured to ensure that the tumor growth inhibition was not simply due to worsening health of the treated animals. Serum drug levels were measured for correlation with therapeutic concentra-
tions in humans. Histological examination and proliferation index were examined and comparisons were performed between control and treatment groups.

**MATERIALS AND METHODS**

**TUMOR PREPARATION**

Six histologically benign tumors from six female patients aged 42–85 years of age were used for this study. Mice bearing each of these tumors were randomly mixed together in the groups studied in this article. Similar to our previous study, there was no significant difference in growth of tumor in the nude mouse between meningiomas taken from different human patients [21]. These tumor cultures were also used for in vitro growth and signal transduction pathway experiments, and characterized by electron microscopy, immunohistochemistry, and light microscopy [20–23].

The method for tumor preparation and injection has been previously described [21]. Briefly, meningiomas taken from the operating room were placed in tissue culture and used within 1–2 passages after initial removal from human patients. Approximately 1 million meningioma cells were placed in 500 μL of Matrigel. Matrigel is liquid at 4°C, but at higher temperatures quickly polymerizes into a three-dimensional gel. The flank of the athymic (nu/nu) mouse was prepped with alcohol and the cell/Matrigel mixture was injected subcutaneously using a 25-gauge needle on a tuberculin syringe.

**ATHYMIC MOUSE MENINGIOMA CELL GROWTH STUDIES**

Male CD-1 (nu/nu genotype) athymic nude mice of the Balb/c strain (14–16 days of age) were obtained from Charles River, housed in sterile laminar flow rooms in groups of four, and given autoclaved food and water ad libitum. All experiments were conducted in accordance with an approved institutional animal care and use committee protocol to ensure humane treatment of these animals.

Mice were inoculated as described and tumor volume measured three times a week. Average daily water consumption for groups of mice was calculated and concentration of the tested drugs in drinking water adjusted to maintain a given mg/kg/day dosage. After 1 week, mice were initially treated with 50 mg/kg/day of verapamil or diltiazem in their drinking water. This stepped increase in drug concentration prevents the 20% mortality rate observed when mice are initially treated with the higher, final concentration of drug [34].

Diltiazem was dissolved in water; verapamil was dissolved in 10% ethanol/water. Tumor-bearing control animals received equal amounts of 10% ethanol or water. Average daily mouse water consumption for each group was calculated by measuring the volume consumed by each group three times a week and dividing by the time elapsed. Drug-containing drinking water was made up fresh and changed twice a week. Drug dose concentrations were adjusted based on average daily water consumption to deliver the doses described above. Mice were weighed weekly to determine the effects of drugs on their general health.

Tumor size was measured with calipers every 3 days for up to 150 days. Tumor volume was calculated as length × width × height and expressed as cubic millimeters. The person performing these measurements was blinded to the identity of treatment groups. The experimental groups consisted of eight mice for the control group and for each treatment group. Matrigel only injections have previously been shown to create no palpable mass 15 to 30 days after injection [21]. Tumor volumes of animals in a given group on a given day after implantation were averaged, and variability expressed as standard error of the mean. Statistical significance of results was determined by one-way analysis of variance (ANOVA) and Fisher Protected Least Squares Deviation (PLSD); p values < 0.05 were considered statistically significant. After 150 days, animals were sacrificed by an overdose of sodium pentobarbital (Nembutal Sodium Solution, Abbott Laboratories, Chicago, Illinois) and histology determined. After administration of sodium pentobarbital, intracardiac aspiration of blood was performed to determine serum calcium channel antagonist concentrations.

**HISTOLOGICAL ANALYSIS**

Tumors grown in the nude mouse were fixed in 10% buffered formalin and embedded in paraffin. The sections were heated to 60°C for 20 minutes and then deparaffinized and rehydrated in a graded series of 90%, 95%, and 100% ethyl alcohols and xyylene. Hematoxylin and eosin-stained sections of mouse tumor were compared to the original pathological specimens taken from the human patients. Both whorls and psammoma bodies helped identify the mouse-grown tumors as meningiomas.
Paraffin sections were prepared from resected mouse tumors as above. All sections were deparaffinized with xylene and rehydrated in a graded series of alcohol to water. After a wash in 0.05 mol/L Tris-buffered saline (TBS) (pH 7.6), sections were immersed in 0.01 mol/L citrate buffer (pH 6.0) and microwaved two times, for 5 minutes each time, for antigen retrieval. Slides were cooled to room temperature and rinsed in TBS for 5 minutes. Endogenous peroxidase was blocked by a 10-minute incubation hydrogen peroxide at room temperature. After additional washing in TBS, nonspecific binding was blocked with 10% normal goat serum at room temperature for 10 minutes. The specimens were then incubated overnight at 4°C with mouse monoclonal MIB-1 antibody (1:50 dilution; Transduction Laboratories, Lexington, KY).

Secondary antibodies and Avidin-Biotin Complex incubations were performed with the Vectastatin ABC kit (Vector Laboratories, Burlingame, CA). After incubation with the primary antibody, the cells were washed in PBS and incubated for 30 minutes with 0.5% biotinylated antibody. The sections were then incubated for 60 minutes with Vectastain ABC Reagent (Vector Laboratories, Burlingame, CA) followed by washing in PBS for 60 minutes. The final reaction was treatment of the sections in peroxidase substrate solution 3′,3′-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories, Burlingame, CA). After counterstaining with methyl green, sections were then dehydrated in graded alcohols and xylene and coverslips were applied with dupex coverslip adhesive. Negative controls were performed as above. Light photomicrographs were obtained using a Zeiss photomicroscope and Kodak Ektachrome 160T tungsten film.

Results

GROWTH STUDIES

Figure 1 shows the effects of diltiazem on tumor growth in nude mice injected with 1 million meningioma tumor cells in a Matrigel matrix. Results are presented as tumor volume over time. Mice were treated with either water only (n = 8) or water containing a dose of diltiazem 50 mg/kg (n = 8). Mice treated with diltiazem had statistically decreased growth over time (Day 0, \( p = 0.1083 \); Day 30, \( p = 0.0156 \); Day 60, \( p < 0.0001 \); Day 90, \( p < 0.0003 \); Day 120, \( p < 0.0025 \)) as indicated by one-way analysis of variance (ANOVA) and Fisher PLSD compared to groups receiving water only at the majority of time points studied after the first week after implantation. Brackets represent standard error of the mean (SEM).
this time. This is an inherent difficulty with this tumor model and is also demonstrated in Figure 2.

Figure 2 displays the effects of verapamil on tumor growth in nude mice presented as tumor volume over time. Water only (n = 12) and 10% EtOH groups (data not shown here). Mice treated with verapamil 50 and 100 mg/kg had statistically decreased growth over time (Day 0, p = 0.3981; Day 30, p = 0.0557; Day 60 p < 0.0001; Day 90, p < 0.0021; Day 120, p = 0.0015) as indicated by one-way analysis of variance (ANOVA) and Fisher PLSD, compared to control groups receiving 10% ethanol at the majority of time points studied after the first week after implantation. There are no statistically significant dose-dependent decreases in growth with increasing verapamil doses. Growth in groups receiving verapamil 200 mg/kg (n = 8) is not significantly different from the 50 and 100 mg/kg groups; however, there is a 50% mortality rate by 40 days after beginning treatment in the 200 mg/kg group, and the data from this group is not shown here. Brackets represent standard error of the mean (SEM).

WEEKLY WEIGHTS

There was no consistent statistical difference between average weekly total body weights or mice treated with either water only (n = 8), water containing 10% ethanol (n = 8), water containing a dose of diltiazem 50, 100, or 200 mg/kg (n = 8 for each group) or water containing a dose of verapamil 50, 100, or 200 (n = 8 for each group) (data not shown).

DAILY WATER CONSUMPTION

The average daily water consumption of differing doses of diltiazem and verapamil was compared to water and 10% ethanol controls and calculated as average daily water consumption. Groups receiving 10% ethanol or verapamil dissolved in 10% ethanol had statistically decreased daily water consumption (p > 0.05). Groups receiving verapamil 200 mg/kg had further decreased daily water consumption compared to 10% ethanol control groups (p > 0.05). Groups receiving diltiazem 100 and 200 mg/kg had decreased daily water consumption compared to water control groups (p > 0.05) (data not shown).

SERUM DRUG LEVELS

Average serum concentrations of diltiazem or verapamil were determined after 130–140 days of continuous treatment with these drugs. Results are pre-
presented in Figure 3 as average serum concentration in ng/ml units. Serum drug levels of groups receiving doses of 100 mg/kg of both verapamil and diltiazem were statistically increased \((p < 0.05)\) compared to groups receiving 50 mg/kg. Serum drug levels in groups receiving 200 mg/kg were not statistically increased compared to the 100 mg/kg groups.

**HISTOLOGY AND Ki-67 LABELING INDEX**

Histologically, treatment groups appear to have demonstrated less tumor growth but no evidence of cell death or necrosis. It appears that the cells formed small pockets of growth within the Matrigel matrix, but this growth was arrested in the treatment groups. In contrast, the control groups demonstrated more consolidated growth of tumor cells replacing the Matrigel background (Figure 4A).

Proliferation index, measured by MIB-1 antibody labeling of the Ki-67 antigen in both treatment and control groups, was 1–2%. This is similar to that found in primary benign human meningiomas [17]. Because the baseline proliferative index of the control groups was so low, no statistical difference was noted between treatment and control groups (Figure 4D). In contrast, as a positive control, glioblastoma cells grown in nude mice have a very high MIB-1 labeling index.

**DISCUSSION**

**CALCIUM CHANNEL ANTAGONIST IN VITRO GROWTH INHIBITION**

We have previously demonstrated that the growth stimulation of meningiomas in culture by serum and growth factors can be inhibited by calcium channel antagonists [22,23]. Similar to our growth factor studies, gastrin-induced proliferation of a pancreatic cell line can be inhibited by calcium channel antagonists [5]. Prostate and human lung carcinoma cell culture cell proliferation can be inhibited by verapamil [4,46]. Primary cultures of human glial tumors show reversible growth inhibition when treated with verapamil [33]. In a similar study, verapamil and nifedipine inhibited DNA synthesis and growth of human glioma cells [29]. Verapamil inhibits murine B16 melanoma and colon adenocarcinoma C26 tumor metastasis by altering platelet aggregation [36]. Verapamil reversibly decreased human melanoma cell invasion and metastasis in a dose-dependent manner [37].

Carboxyamido-triazole (CAI), an agent that is an antagonist of calcium-sensitive signal transduction pathways, has both in vitro and in vivo anti-proliferative and anti-metastatic properties [19,24,26–28,36]. This agent is a synthetic inhibitor of nonexcitable calcium channels that reversibly inhibits angiogenesis, tumor cell proliferation, and
Expression of matrix metalloproteinase, which is important in metastasis, invasion, and tumor growth, is inhibited by CAI treatment; this was felt to be secondary to inhibition of calcium influx [26].

CALCIUM CHANNEL ANTAGONIST CELL EFFECT ON IN VIVO GROWTH STUDIES

There is very limited information available concerning meningioma tumor xenograft nude mouse studies, especially those involving calcium channel antagonists. Diltiazem or verapamil (doses of 3.5 mg/day dissolved in drinking water) inhibits the growth of HT-39 breast tumor cells implanted in athymic mice by roughly 50% [34]. Athymic xenograft human glioma cell growth is inhibited by intraperitoneal injection of verapamil 25–50 mg/kg every other day; verapamil also enhances the anti-proliferative effects of the nitrosurea 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU) [7]. Nude mice bearing intraperitoneal OVCAR-3 human ovarian carcinoma had longer survival times if treated with the carboxyamide-amino-imidazole L651582. As mentioned previously, this agent targets calcium-sensitive signal transduction pathways and has both anti-proliferative and anti-metastatic properties [28].

SERUM DRUG LEVELS

Serum drug levels of roughly 150 ng/mL after administration of at least 100 ng/mL verapamil, and
approximately 75 ng/mL for diltiazem after similar dose administration were measured in mice in our study. Therapeutic serum levels of the calcium channel antagonists used for the treatment of hypertension or cardiac irregularities are roughly 50 to 200 ng/mL [15]. Clinically achievable and tolerable plasma levels of verapamil in human patients are approximately 100 to 400 ng/mL for verapamil and 50 to 200 ng/mL for diltiazem [1,16,18]. CSF concentrations are roughly one tenth of those found in serum [10]. However, meningiomas may be highly vascular lesions and are found outside the blood brain barrier, and it is conceivable that a peritumoral concentration roughly equivalent to that in serum might be achieved. In fact, co-administration of diltiazem has been suggested as a strategy for increasing intra-tumoral blood flow with subsequent increases in intra-tumor anticancer drug concentrations [38]. This makes the use of these agents attractive for multimodality therapy, increasing blood flow while also exerting an antiproliferative effect on the tumor.

**TUMOR MODEL**

Studies of meningioma cells in culture have the disadvantage that they do not allow study of in vivo characteristics of these tumors such as morphology, neovascularization, or growth characteristics [30]. Matrigel-augmented meningioma has a high rate of growth in culture, tumor enlargement is progressive over time, and the histology of the xenograft tumor closely resembles that of the original human tumor [21]. However, there are concerns that Matrigel enhances tumorigenicity or might modify the biological characteristics of the original tumor. In fact, Matrigel may increase the drug resistance of tumors in vivo to chemotherapeutic agents [14,35], or transform pre-malignant cells into aggressive tumors [12,13]. In our original description of this model, Matrigel did not seem to alter the original histology of the human meningiomas implanted in nude mice [21]. In support of this, the Ki-67 labeling index of the tumors in this model was similar to published MIB-1 labeling indices; 0.75 ± 0.21 for benign, 3.2 ± 0.57 for atypical, and 6.04 ± 1.48 for malignant meningiomas (p < 0.0066) [17]. The slow growth of these tumors and the low proliferation index makes interpretation of treatment efficacy difficult. The proliferation index of the control groups was low at baseline, therefore measurement of changes in the treatment groups is difficult. The slow rate of tumor growth complicates direct tumor size measurement. This is both a criticism and support of this model; the model seems to approximate the natural history of benign meningiomas, but makes in vivo treatment efficacy difficult to evaluate.

**CLINICAL POTENTIAL**

The accumulation of molecular events during cancer progression changes the signaling homeostasis of cancer cells through mutation, altered gene expression, and the development of autocrine loops upon which these cells depend. Signal transduction therapy, such that used in this study, is an attempt to suppress hyperactive or aberrantly active signaling pathways in malignant cells [24]. Redundancy in signaling pathways in nonmalignant cells can theoretically maintain checks and balances that protect against normal cell toxicity during continuous exposure to signal modulatory drugs [8,32]. Signal transduction therapy with agents targeting calcium influx and calcium influx-driven downstream signaling events may offer alternative approaches for cancer treatment [2,8,24,32]. Phase I clinical trial with carboxyamido-triazole (CAI), an agent that targets calcium-sensitive signal transduction pathways for patients with refractory solid tumors, resulted in “disease stabilization and improvement in performance status” in 49% of patients studied [24]. Ubiquitous normal tissue toxicity, anticipated to occur with signal transduction inhibitors, was not seen with CAI therapy [24]. This is supports other observations that normal cells have redundant signaling pathways that allow them to maintain biochemical homeostasis [27,32].

**Conclusions**

Calcium channel antagonists diltiazem and verapamil inhibit the growth of meningioma cells in culture. It seems that this effect can be demonstrated in a xenograft mouse meningioma tumor model. The animals tolerated this treatment at drug levels similar to those achievable in human patients. This suggests a possible role for these drugs as an adjuvant therapy for recurrent or unresectable meningiomas.

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**COMMENTARY**

Meningiomas have been considered as primarily a surgical issue for many years. Recurrences and atypical behavior are, however, much more common than had initially been appreciated. Radiotherapy has been a modestly useful adjunct, but chemotherapy has played only a minimal role in these more complex cases.

In this work, the authors describe a potential role for calcium channel antagonists in decreasing the growth of meningiomas. The studies carried out in a nude mouse model system demonstrate a decrease in the growth of implanted human meningiomas. Although clearly an early-stage study, this work suggests another potential approach for dealing with complex meningiomas.

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The authors have extended their initial in vitro experience with monitoring the effects of calcium channel antagonists on meningioma cultures with this in vivo study in nude mice. The data indicate that these agents appear to slow the growth of meningiomas with minimal toxicity.

Because calcium channel blockers are in widespread use for the treatment of cardiovascular disease, it should be relatively easy to establish a randomized trial and to determine whether these agents are clinically useful.

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Do not protect yourself by a fence, but rather by your friends.

—CZECH PROVERB

Do not stand in a place of danger trusting in miracles.

—ARAB PROVERB