Tetrathiomolybdate promotes tumor necrosis and prevents distant metastases by suppressing angiogenesis in head and neck cancer

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Abstract

Angiogenesis is well recognized as an essential process that influences not only the growth of head and neck squamous cell carcinoma (HNSCC) but also promotes its invasive and metastatic behavior. The critical role of copper in multiple facets of angiogenesis makes it an important therapeutic target. Tetrathiomolybdate is a potent copper chelator, which has shown remarkable ability to suppress angiogenesis. Although this may involve multiple mechanisms, the effects on vascular endothelial growth factor (VEGF) are pivotal. In previous work, tetrathiomolybdate suppressed production of several proangiogenic cytokines by HNSCC cell lines. Given these results, we hypothesized that tetrathiomolybdate would impair tumor growth and metastasis by HNSCC. To test this concept, we evaluated the effects of long-term tetrathiomolybdate treatment on the growth and metastatic progression of HNSCC using a xenograft animal model. The results showed that tetrathiomolybdate treatment is able to maintain effective inhibition of angiogenesis. There was a significant reduction in the tumor size and vascularity with evident gross necrosis in the tetrathiomolybdate-treated animals. These effects were highly correlated with suppression of human VEGF expressed in the developing tumors as well as the mouse VEGF levels detected in the plasma. Moreover, tetrathiomolybdate treatment drastically suppressed the development of lung metastases. Taken together, these results show that tetrathiomolybdate can act long-term as a suppressor of vascularity and inhibit the growth of metastasis in this model of HNSCC. [Mol Cancer Ther 2007;6(3):1039–45]

Introduction

Angiogenesis is a complex developmental and physiologic event that results in the formation of new blood vessels from preexisting vasculature. Under normal conditions, this process is tightly controlled by a delicate balance between the activators and inhibitors of angiogenesis (1, 2). A preponderance of current evidence supports that the growth of primary and metastatic tumors requires an ''angiogenic switch'' to be turned on and promote adequate vascularization (3–6). This vascular compartment contributes an important and unique role in sustaining the high metabolic demands and proliferative activities of cancer cells; it is also a critical player in the ability of tumor to invade tissues and metastasize to distant organs (3–6).

Despite the increasing recognition of angiogenesis as an important subversive process in tumorigenesis, developing effective targeted therapy continues to be challenging (7–10). The angiogenesis cascade is complex with multiple promoters and inflammatory cytokines involved in its activation. Moreover, there are fundamental differences between physiologic vascular development and tumor angiogenesis (11, 12). Although the molecular activation of endothelial cells to migrate, proliferate, and differentiate in the tumor environment is not fully understood, the critical role of vascular endothelial growth factor (VEGF) is well established (12, 13). VEGF is expressed in the majority of human cancers; it is particularly up-regulated in response to hypoxia, activated oncogenes, or inactivated tumor suppressor genes.

Copper is an essential trace element, which is ubiquitous in the human diet. Since the initial observation that copper depletion inhibits angiogenesis in the rabbit cornea, much has been uncovered about its role. Copper-mediated mechanisms are implicated in the expression, activation, secretion, and binding of key regulators of angiogenesis (14–16). Although several anti-copper therapies have shown tumor-suppressive effects, tetrathiomolybdate is one of the most studied (14–17). It is a powerful copper chelator that was initially introduced for the treatment of Wilson's disease, a copper storage genetic disorder. Tetrathiomolybdate has shown a promising role in suppressing tumor angiogenesis, retinal neovascularization, and pathologic inflammatory conditions (18–24) This global ability to interfere with the intertwined processes

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of angiogenesis and inflammation makes tetrathiomolybdate of great interest. Although the inhibition of VEGF and other cytokines seems to be involved, the molecular mechanisms by which copper deficiency down-regulates these processes remain unclear.

Squamous cell carcinoma (SCC) is a relatively common epithelial malignancy that can arise in several organs, including the head and neck, lung, cervix, and skin. Previous studies have shown that head and neck SCC (HNSCC) expresses elevated levels of VEGF, basic fibroblast growth factor, interleukin (IL)-1α, IL-6, and IL-8 (19, 25–27). Given that, the degree of this expression is strongly correlated with local invasion, metastatic potential, as well as resistance to radiation and chemotherapy (27–30). We proposed that tetrathiomolybdate-induced copper suppression, which interferes with such promoters of angiogenesis at multiple levels of their hierarchy, including the master switch nuclear factor-κB (17–19, 31), would affect tumor growth and progress. It is suggested that copper levels needed for cellular functions (such as a cofactor of key enzymes) are much lower than those needed for tumor angiogenesis or inflammatory response (14–16). Accordingly, tetrathiomolybdate may offer long-term tumor suppression while keeping minimal toxic effects. The objective of this study is to evaluate the effects of tetrathiomolybdate administration on the growth and metastatic progression of HNSCC using a xenograft animal model.

Materials and Methods

Cell Culture

UM-SCC-11B is a well-established cell line derived from a 65-year-old patient with a T2 N2a SCC of the hypopharynx (kind gift from Thomas E. Carey, Ph.D., University of Michigan, Ann Arbor, MI; ref. 32). This cell line was grown in 37°C humidified atmospheres with 5% CO_2. The cultures were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2 mmol/L L-glutamine, 50 μg/mL penicillin G, and 50 μg/mL streptomycin sulphate. Subculturing of 80% to 90% confluent cells was routinely done using trypsin-EDTA solution (0.05% trypsin and 0.53 mmol/L EDTA). At harvest, the cells were trypsinized and washed, then concentrated by centrifugation, and counted with a hemocytometer. The cells were assessed for viability by trypan blue exclusion test (>95% viable) and then resuspended for a final density of 5.0×10^6/mL using unsupplemented DMEM.

Animal Xenograft

Athymic nude mice (The Jackson Laboratory, Bar Harbor, ME), 6 weeks old, were housed in cages of five animals or less. Cages were randomized to receive either tetrathiomolybdate or autoclave sterilized water, delivered by daily p.o gavaging, resulting 16 animals in each group of the study. The treatment was initiated 2 weeks before tumor inoculation and continued until the end of the experiment. At the beginning of week 2, all animals were inoculated with 1 million cells of UM-SCC-11B cell line. Cells were delivered by s.c. flank injection in a total volume of 200 μL using 1.5 inch, 27-gauge needle. Animals were assessed weekly for tumor growth as well as body weight and general health. The tumor volume was calculated using the formula $v = w^2 \times l \times 0.52$, where w and l are the two maximum dimensions. Venous blood was collected biweekly by saphenous bleed from all mice. Tubes and needles were well heparinized to avoid activation of clotting factors. Blood samples, averaging 100 to 150 μL per mouse, were kept on ice and centrifuged at 1,000 rpm for 15 min and then at 2,000 rpm for 30 min (4°C). The pooled plasma was then aliquoted and stored at −80°C until the time of use. At the end of the experiment, week 12, the animals were euthanized using a CO_2 chamber. The tumors and lungs were first dissected and fixed with 10% buffered formalin and then processed to form paraffin-embedded tissue blocks. All animal procedures were done in compliance with the guidelines set forth by the University Committee on Use and Care of Animals at the University of Michigan.

Ceruloplasmin Assay

Plasma ceruloplasmin is a good surrogate marker for total body copper status. The baseline levels were determined for the tetrathiomolybdate group before initiation of treatment; subsequently, ceruloplasmin levels were measured biweekly over the 12-week study period. Tetrathiomolybdate treatment was started at 0.7 mg/d per mouse (previous studies has shown this to be an optimal dose) and then titrated biweekly to maintain ceruloplasmin suppressed at 20% to 30% of baseline levels (17). Ceruloplasmin was assayed based on its oxidase activity using the standard method (33). In brief, two tubes each containing 25 μL mouse plasma and 375 μL of 0.1 mol/L sodium acetate buffer (pH 5.0) were incubated for 5 min in a 30°C water bath. Prewarmed 100 μL *o*-dianisidine dihydrochloride solution (7.88 mmol/L; Sigma, St. Louis, MO) was then added to each tube and the reaction was allowed to continue for 30 min in one tube and 45 min in the other. Finally, the reaction was quenched by adding 1 mL of 9 mol/L sulphuric acid. The absorbance of both tubes was measured at 540 nm using a spectrophotometer microplate reader. Ceruloplasmin concentration in IU was calculated using the formula ceruloplasmin oxidase activity = $(A_{45} - A_{30}) \times 0.625$ units/mL, where A_{45} and A_{30} are the absorbance at 45 and 30 min, respectively.

VEGF Expression

Immunohistochemistry staining was used to determine human VEGF expression in the xenograft tumors. Five-micrometer sections were stained using antihuman VEGF antibody at a 1:10,000 dilution (PharMingen, San Diego, CA). Appropriate negative and positive controls were done. Sections were initially scanned at low power to determine nonnecrotic areas and these were then examined at high power (×400 magnification). Four random high-power fields from each specimen were then evaluated. The degree of staining was scored as follows: grade 0 if none of the tissue was stained; grade 1 if <50% of the nonnecrotic

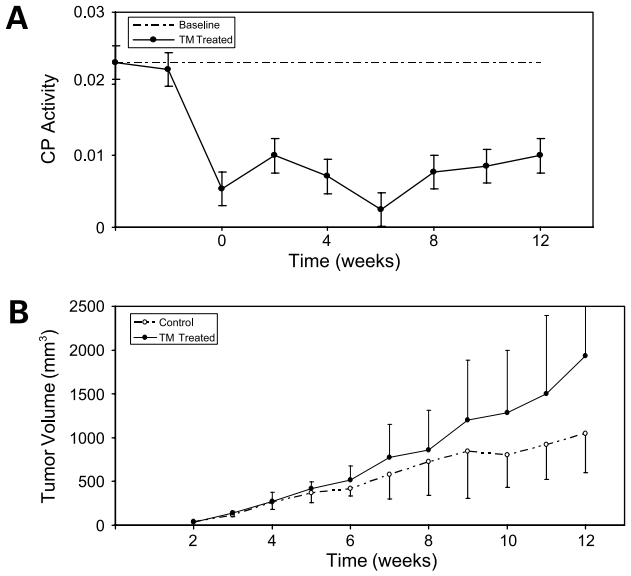

Figure 1. **A,** ceruloplasmin (*CP*) activity level in the plasma of the tetrathiomolybdate (*TM*) – treated mice. *Points,* mean (units/mL); *bars,* SD. **B,** tumor growth curves for the control and the tetrathiomolybdate-treated mice. Data are reported as median of the tumor volume with IQR (mm^3). *, $P < 0.05$.

tissue was stained; and grade 2 if at least 50% of the nonnecrotic tissue was stained (34). The results were reported for all tumors in a blinded fashion. The levels of mouse VEGF were determined in the plasma samples at baseline and subsequently at weeks 4 and 8 of the experiment. This was done in triplicates using high-sensitivity ELISA at the University of Maryland Cytokine Core Laboratory.[3]

Microvessel Density

An assessment of microvessel density in all primary tumors was determined using immunohistochemistry. Five-micrometer sections were stained using antimouse CD31 antibody at a 1:250 dilution (PharMingen). Appropriate negative and positive controls were done. The total number of stained vessels was determined in five random high-power fields (×400 magnification), and then the mean was reported in a blinded fashion for each tumor.

Lung Metastases

At the time of dissection, the lungs were examined for evidence of metastatic involvement. Five-micrometer sections were done in the middle, upper, and lower fields for all lungs. The slides were H&E stained and the entire sections were examined microscopically at low powers (×40 and ×100 magnifications) for the presence of metastases. The total number of animals with lung metastases was reported for each group in a blinded fashion.

Statistical Analysis

When appropriate, the mean was reported with the SD (±SD) and the median with the interquartile range (IQR). The proportions and rates were reported with 95% confidence intervals (95% CI). Hypothesis testing was done using *t* test for the means or the Mood's median test for the medians. Proportions or rates were tested with the test of two proportions. The statistical analysis was carried out using MINITAB 14 statistical software.

Results

Suppression of Ceruloplasmin Levels

Previous experiments using animal models have shown that p.o. tetrathiomolybdate of 0.7 mg/d is well tolerated in mice and can achieve adequate ceruloplasmin suppression. After 2 weeks of initiating tetrathiomolybdate treatment, ceruloplasmin levels were reduced to 20% to 30% of baseline levels, baseline = 0.023 ± 0.0026 and week 1 = 0.0054 ± 0.0006. This desirable target suppression was maintained throughout the experiment (Fig. 1A). Few animals in the tetrathiomolybdate group showed moderate weight loss (10–15% loss from initial body weight), but this responded promptly to dose adjustment.

Suppression of Tumor Growth and Metastatic Progression

Measurable tumor growth was achieved in >90% of mice by week 4 of the experiment. Survival rate over the 12-week study period was 0.88 for the tetrathiomolybdate-treated group compared with 0.75 in the control. Despite a

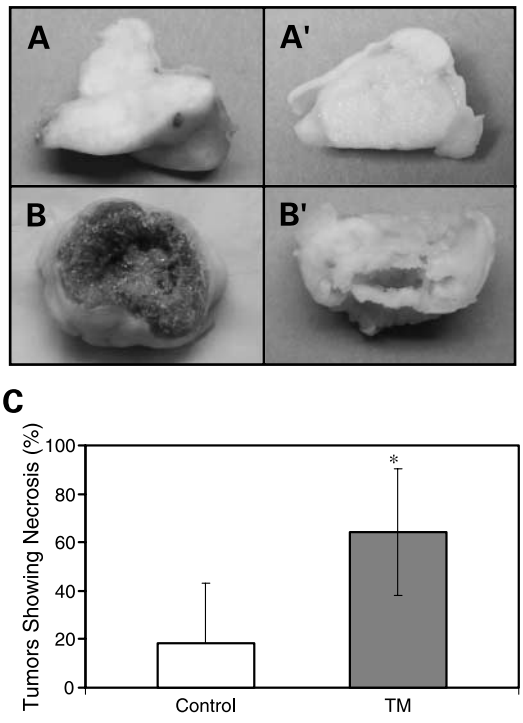

Figure 2. **A, A′** and **B, B′,** anterior and posterior views of dissected tumors from the control and the tetrathiomolybdate-treated groups, respectively. Evident tumor necrosis in the tetrathiomolybdate-treated mice. The presented tumors were matched for approximate size. **C,** percentage of tumors showing gross necrosis. *Columns,* tumors showing necrosis (%); *bars,* 95% CI. *, $P < 0.05$.

[3] http://www.cytokinelab.com

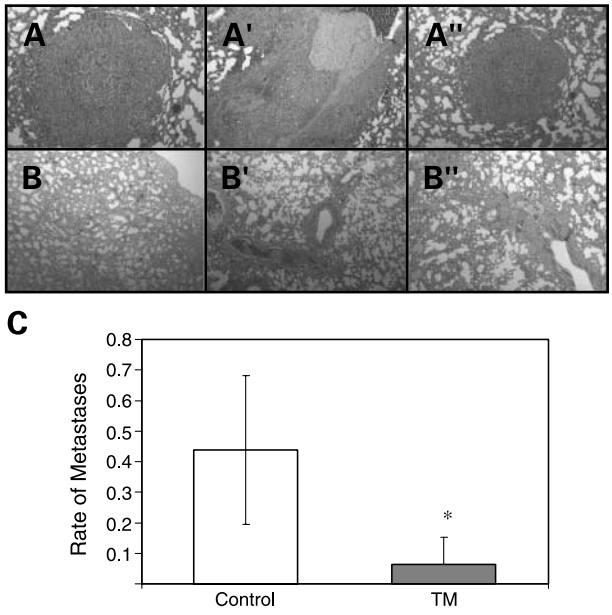

Figure 3. **A, A′, A″,** microscopic lung metastases from representative control mice. **B, B′, B″,** normal lung tissue from representative tetrathiomolybdate-treated mice. **C,** rate of developing lung metastases over the study period. *Columns,* rate of metastases; *bars,* 95% CI. *, $P < 0.05$.

lower survival rate in the controls, this was due to gavage-related complications (i.e., esophageal perforation and pneumonitis) rather than due to tumor burden. Tetrathiomolybdate treatment maintained remarkable suppression in tumor growth. Tumors in the control group continued to increase their growth rate, whereas those in the tetrathiomolybdate group reached a growth-rate limit. Figure 1B presents the median tumor volumes for both groups. At week 12, the tetrathiomolybdate group had a median tumor volume of 1,048 mm^3 (599–2,258) compared with 1,936 mm^3 (894–3,756) in the control group. This indicates a 54% reduction in tumor size ($P < 0.05$). After animal sacrifice, the dissected tumors were examined for evident necrosis. Tumors were considered to have gross necrosis if the necrotic tissue or central degeneration constituted >30% of the maximum diameter. Figure 2 indicated that the rate of observed gross necrosis was 0.64 for the tumors in the tetrathiomolybdate group compared with 0.18 in the control group ($P < 0.05$).

All lungs were examined grossly as well as microscopically to evaluate metastatic progression (Fig. 3). The rate of developing lung metastases over the study period was 0.06 in the tetrathiomolybdate group compared with 0.44 in the control, showing an 86% relative risk reduction in metastatic progression ($P < 0.05$).

Suppression of Tumor Angiogenesis

The results of microvessel count were summarized in Fig. 4. The mean of microvessel count per high-power field was 5 ± 1 for the tetrathiomolybdate group compared with 17 ± 2 for the control. This indicated a 70% reduction in microvessel density ($P < 0.0001$). Furthermore, VEGF activity was assessed in the tumors and the mouse plasma. Tetrathiomolybdate treatment maintained remarkable reduction in human VEGF expression, and 18% showed grade 2 staining in tetrathiomolybdate-treated mice compared with 79% in the control ($P < 0.001$). The results were summarized in Fig. 5 with representative fields from size-matched tumors. Tumor progression was accompanied by an increase in circulating mouse VEGF levels in the control mice, baseline = 19 ± 3 pg/mL, week 4 = 110 ± 15 pg/mL, and week 8 = 120 ± 14 pg/mL. Nonetheless, this trend was attenuated in the tetrathiomolybdate-treated mice, baseline = 27 ± 1 pg/mL, week 4 = 95 ± 10 pg/mL, and week 8 = 112 ± 15 pg/mL. Adjusted for baseline levels, the results showed a 20% reduction in circulating VEGF at week 4 ($P < 0.01$) and 13% at week 8 ($P < 0.05$) compared with the control (Fig. 5).

Discussion

Angiogenesis is a complex event that requires coordination from a variety of growth factors and cell adhesion molecules to stimulate endothelial cells to form vascular structures. It is now well recognized that angiogenesis is an essential player in the growth and invasive behavior of many tumors. Tetrathiomolybdate is a potent copper chelator, which has shown the ability to suppress angiogenesis. In a recent study, tetrathiomolybdate showed inhibitory effects on several proangiogenic cytokines produced by HNSCC cell lines (19). UM-SCC-11B cell line was of particular interest because it expressed high levels of

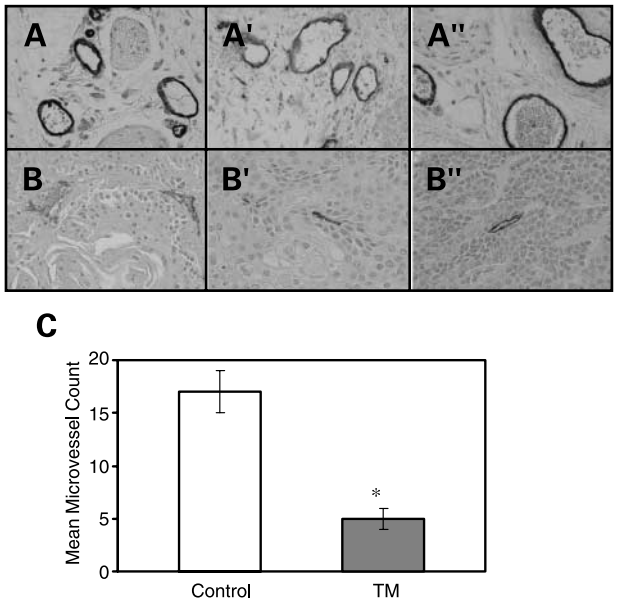

Figure 4. **A, A′, A″** and **B, B′, B″,** representative fields illustrating microvessel density assessment after staining with CD31. Representative high-power fields from tumors developed in the control (**A, A′, A″**) and tetrathiomolybdate-treated (**B, B′, B″**) mice. Microvessels were sparse, smaller, and poorly developed in the tetrathiomolybdate-treated mice. **C,** *Columns,* mean of microvessel count per high-power field reported; *bars,* SD. *, $P < 0.0001$.

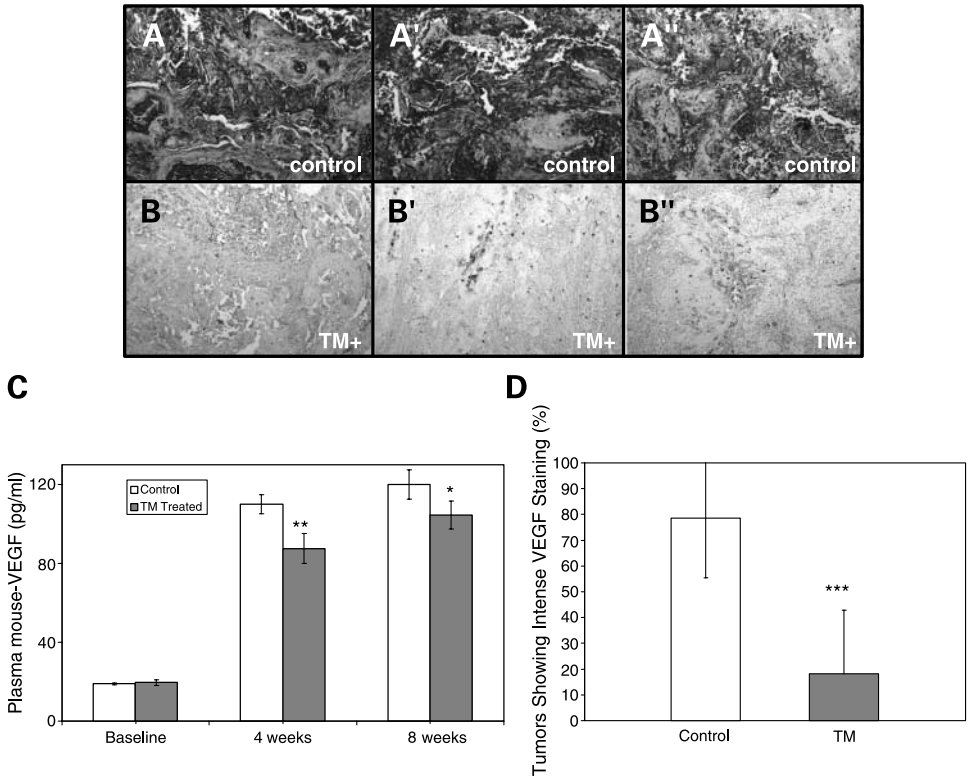

Figure 5. **A** and **B,** representative high-power fields for human VEGF staining in the control (**A**, **A′**, **A″**) and tetrathiomolybdate-treated (**B**, **B′**, **B″**) mice. **C,** plasma levels of mouse VEGF detected using high-sensitivity ELISA. Tetrathiomolybdate treatment showed modest decrease in the tumor-associated induction of plasma VEGF. *Columns,* mean (pg/mL); *bars,* SD. **, $P < 0.01$; *, $P < 0.05$. **D,** percentage of tumors showing intense human VEGF staining (grade 2). There was a significant reduction in VEGF staining for the tetrathiomolybdate-treated mice. *Columns,* tumors showing intense VEGF staining (%); *bars,* 95% CI. ***, $P < 0.001$.

proangiogenic factors, which were notably suppressed with tetrathiomolybdate treatment. The current study expanded this work by investigating the long-term effects of tetrathiomolybdate in a xenograft animal model of UM-SCC-11B. It is believed that copper levels needed for physiologic functions are lower than those favored by tumor angiogenesis (14–16). Therefore, preferential targeting of tumor neovascularization can be achieved with minimal systemic toxicity. In this study, the long-term p.o. intake of tetrathiomolybdate was well tolerated with no significant effects on the behavior or general health of the animals. Ceruloplasmin levels were effectively suppressed over the study period. The tumor growth rate was restricted in the tetrathiomolybdate-treated animals; this achieved a reduction in tumor volume, which was more evident as the control tumors progressively advanced in size. These results suggest that copper is a rate-limiting factor in angiogenesis. It seems that higher copper levels are needed during rapid growth compared with early stages of tumor development.

An interesting finding of this study is the remarkable gross necrosis observed with long-term suppression of angiogenesis. It is clear that the gross necrosis in tetrathiomolybdate-treated tumors correlated with the decreased tumor vascularity. Tumors from tetrathiomolybdate-treated mice showed a 70% reduction in microvessel count; the vessels were also visibly smaller and poorly developed compared with the control. Although there are several promoters of angiogenesis, the role of VEGF is pivotal. Initially discovered as a tumor-secreted vascular permeability factor, VEGF now is recognized as a copper-dependent multifunctional cytokine. It is required for the proliferation, migration, and adhesion of endothelial cells, as well as the remodeling of the extracellular matrix (3). Therefore, it was fundamental to evaluate VEGF activity in this study. Previously, *in vitro* monolayer cultures of UM-SCC-11B showed some suppression of VEGF in response to tetrathiomolybdate treatment (19). In the current xenograft model, tetrathiomolybdate treatment suppressed VEGF expression in the developing tumors despite evident gross necrosis. This was an important observation because the hypoxia generated in tissues undergoing necrosis is a strong inducer of VEGF. The main regulators of such cellular responses are transcription factors known as hypoxia-inducible factors. Recent studies have shown the ability of copper to regulate VEGF expression by using the hypoxia pathways (35). This was further supported by showing the ability of copper to suppress the proteasomal degradation of hypoxia-inducible factor-1 (36). These findings are particularly important because they suggest that tetrathiomolybdate-induced copper deficiency may function by inhibiting hypoxia-inducible factors and therefore blocking the hypoxic responses and associated VEGF up-regulation. Nonetheless, this hypothesis requires future evaluation.

Given the complexity of copper functions, it is anticipated that tetrathiomolybdate effects will involve multiple and intertwined targets. One such target recently uncovered is the nuclear factor-κB ''master-switch'' transcription factor (17, 31). Nuclear factor-κB oversees the expression of many

factors and cytokines involved in angiogenesis and cell proliferation and inflammation. These include VEGF, IL-1, IL-6, IL-8, and, more recently, hypoxia-inducible factor-1 (37). This upstream global suppression may not only target the tumor cells but can also interfere with the recruitment and activation of the host stromal and inflammatory cells promoting angiogenesis. This hypothesis resonates well with the dramatic and progressive induction in the mouse VEGF levels observed with tumor development; tetrathiomolybdate treatment seems to interfere with this induction, causing a modest suppression in mouse VEGF levels compared with the control.

The important link between angiogenesis and the invasive behavior of tumor cells has received increasing attention in the literature (3, 31, 38, 39). In this study, tetrathiomolybdate suppression of angiogenesis was associated with a reduction in metastatic spread to the lungs. Although this can be explained by the evident decrease in the size and vascularity of the primary tumors, other mechanisms may also be involved. Several reports in the literature suggest that copper as well as VEGF are important activators of matrix metalloproteinases, which are needed for extracellular matrix remodeling during angiogenesis (39, 40). Matrix metalloproteinases are known mediators in the invasive behavior of tumor cells, with a recent member identified as a specific and critical player for developing lung metastases (39). Thus, tetrathiomolybdate may act by inhibiting matrix metalloproteinases and therefore suppressing the ability of tumor cells to invade the normal tissues and blood vessels.

One of the challenges in antiangiogenic therapy is the development of resistance (7, 8). The tumor microenvironment is complex and in constant evolution. Targeting a specific component of this process may eventually lead to an adapted tumor microenvironment, either through the selection for favorable mutations or by shifting the angiogenesis pathway through alternative players. In this study, tetrathiomolybdate treatment sustained suppression of angiogenesis over a 12-week period, resulting to evident necrosis and reduction in tumor size. VEGF was also effectively suppressed despite these hypoxic conditions. Tetrathiomolybdate seems to suppress angiogenesis at multiple levels of its hierarchal regulation. This is particularly important if long-term antiangiogenic therapy is to maintain suppression in the face of a highly adaptive tumor environment.

A large body of evidence supports the fact that tumor cells orchestrate an induction in angiogenesis, which is at least in part played out by the host, to sustain their growth and progression. It is anticipated that therapies targeting angiogenesis are more likely to have tumorostatic rather than tumorotoxic effects. In this study, tetrathiomolybdate treatment was able to maintain potent inhibition of angiogenesis, potentially by targeting multiple facets of this process. Tetrathiomolybdate treatment showed the ability to suppress the human VEGF expressed in the tumor as well as the mouse VEGF detected in the plasma. This global targeting of angiogenesis was not only growth limiting for developing tumors but was also capable of drastically suppressing metastatic progression, the main cause of death in humans with HNSCC. It is likely that this is due to combined effects on the tumor size and vascularity as well as the invasive behavior of tumor cells. VEGF is a critical promoter of angiogenesis and seems to be an important target for copper suppression. The results of this study support a potential application of copper deficiency for long-term treatment to inhibit metastasis.

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