ORIGINAL ARTICLE

Inhibition of the Growth of Squamous Cell Carcinoma by Tetrathiomolybdate-Induced Copper Suppression in a Murine Model

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Objective: To determine whether long-term therapy with tetrathiomolybdate suppresses tumor growth in an animal model.

Design: In vivo murine model.

Subjects: Thirteen 8-week-old C3H/HeJ mice, randomly assigned to a tetrathiomolybdate treatment group (n=7) or a control group (n=6).

Interventions: To render the treatment group mice copper deficient, tetrathiomolybdate (0.7 mg/d per mouse) was added to their drinking water on days 1 through 20. Control group mice received only fresh drinking water. A flank injection of 1.5×10^5 SCCVII/SF cells was administrated to all mice on day 21. The treatment group mice continued to receive daily tetrathiomolybdate throughout the remainder of the experiment (70 days). Tumor volume measurements (square of the width × length × 0.52) were taken every other day beginning on day 40.

Main Outcome Measures: Mean tumor volume differences.

Results: Mean ± SD tumor volumes on day 40 were 146 ± 263 mm^3 (n=7) and 274 ± 331 mm^3 (n=6) for the treatment and control groups, respectively. By day 54, the mean tumor volume for the treatment group was 65 ± 0 mm^3, compared with 1716 ± 960 mm^3 for the control group ($P<.001$). Treatment was withheld on day 54, resulting in a dramatic increase in tumor growth in the treatment group mice such that by day 60, there was no significant difference in mean tumor volume between groups.

Conclusion: This study demonstrates the ability of tetrathiomolybdate to maintain a significant and reversible suppression of long-term tumor growth in this murine model of squamous cell carcinoma, suggesting a potential application for the use of tetrathiomolybdate in human squamous cell carcinoma.

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ANGIOGENESIS is a tightly controlled physiological event that results in the formation of new blood vessels from preexisting host vasculature. An abundance of evidence supports the fact that angiogenesis is required for tumor growth and metastasis. Before the onset of angiogenesis, tumor size remains 1 to 2 mm, relying on simple diffusion to receive nutrients and to remove waste products.[1,2] However, once the balance is shifted in favor of angiogenesis, the angiogenic phase of tumor development begins and rapid tumor growth ensues.[1,2] A number of positive regulators of angiogenesis have been identified. These include vascular endothelial growth factor, fibroblastic growth factor, interleukin 8 (IL-8), transforming growth factors α and β, tumor necrosis factor α, angiogenin, and platelet-derived endothelial cell growth factor,[3-9] to name a few. Numerous studies have shown that increased expression of 1 or more of the factors leads to increased tumor growth and microvessel density (MVD). Factors known to up-regulate these molecules work synergistically to stimulate angiogenesis and thereby increase tumor growth.[10,11] Conversely, several classes of antiangiogenic compounds have been reported with impressive reductions in tumor growth. One such compound is tetrathiomolybdate, a potent copper chelator.[12]

Tetrathiomolybdate is an orally bioavailable compound that was initially developed for the treatment of patients with Wilson disease, a condition that leads to the abnormal accumulation of copper in several organ systems, including the liver and the brain.[12] Coincident with the development of tetrathiomolybdate, however, several studies began citing the importance of elemental copper as a critical cofactor in the process of angiogen-

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esis.[13-15] To investigate whether the copper-chelating qualities of tetrathiomolybdate translated into an antiangiogenic effect, Merajver and coworkers[16] administered tetrathiomolybdate to an orthotopic murine model of inflammatory breast cancer. They, indeed, were able to illustrate that smaller, significantly less vascularized tumors developed in tetrathiomolybdate-treated mice than in untreated control animals. As an extension of this work, our laboratory has also reported that tetrathiomolybdate therapy significantly inhibited angiogenesis and decreased tumor growth in an orthotopic murine model of head and neck squamous cell carcinoma.[17] The latter study, however, administered tetrathiomolybdate after the clinical appearance of tumor, ie, after the rapid angiogenic phase of tumor growth had begun. Our goal in the present study was to investigate whether continuous therapy with tetrathiomolybdate before tumor implantation could prevent the angiogenic phase of tumor growth and result in long-term suppression of squamous cell carcinoma in a murine model.

METHODS

CELL CULTURE

We maintained SCCVII/SF cells in RPMI 1640 (Life Technologies, Inc, Rockville, Md) supplemented with 10% fetal calf serum, 1% dual penicillin and streptomycin, and 1% L-glutamine and cultured in 10% carbon dioxide incubators as previously described.[18] The cell-line cultures were allowed to reach a confluency of greater than 90% before animal experiments. This cell line is a highly aggressive, spontaneously arising squamous cell carcinoma syngeneic to C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Me). At harvest, the cells were trypsinized, washed in RPMI 1640 media with fetal calf serum, concentrated by centrifugation, and then counted with a hemocytometer. An assessment of cell viability was performed using trypan blue exclusion, and the cells were resuspended to a final concentration of 1.5×10^5 cells in 200 µL of RPMI 1640 solution.

TETRATHIOMOLYBDATE DOSAGE DETERMINATION

Before animal experiments, the daily water consumption of the mice was recorded for 10 days to determine the amount of water consumed per mouse per day as previously described.[17] With this information, together with the average weight of the mice, the required tetrathiomolybdate dosage of 0.7 mg/d per mouse was delivered. This optimal dosage was determined in other animal experiments by Merajver et al.[16]

IN VIVO MURINE MODEL

Thirteen mice were randomly assigned to 2 groups and identified by conventional ear punch methods. Before tumor implantation, the treatment group mice (n=7) received tetrathiomolybdate in their drinking water for 20 days. The control group mice (n=6) received fresh drinking water only during that same period. Tumors were implanted on day 21 in both groups. To establish flank tumors in the C3H/HeJ mice, the mice were fully anesthetized by means of intraperitoneal injection using a combination of ketamine hydrochloride (1 mL) and xylazine hydrochloride (150 µL) to a dose of 1 µL/g or titrated to the desired effect. The depth of anesthesia was monitored through respiratory rate, spontaneous limb movement, and tail pinch response. After an adequate plane of anesthesia was achieved to minimize animal movement, 1.5×10^5 SCCVII/SF cells were injected subcutaneously into each mouse using a 3.8-cm 27-gauge needle. The 2 groups continued to receive the previously described treatment regimen, and the flank of each mouse was examined every other day to assess tumor growth. When tumor became visible or palpable subcutaneously, 2-dimensional tumor measurements were recorded every other day on each mouse by an observer masked to the treatment being given. Tumor volumes were then calculated using the formula for a prolate spheroid (square of the width × length × 0.52). On day 54, the treatment group mice were given a 3-day drug holiday, (ie, tetrathiomolybdate delivery was withheld for 3 days). This was incorporated in to the study design to assess whether the efficacy of tetrathiomolybdate relied exclusively on inhibiting the angiogenic switch or a separate antiangiogenic mechanism. Daily tetrathiomolybdate treatments were resumed on day 57. The experiment was concluded on day 70, and the mice were killed by means of a ketamine and xylazine overdose. All animal procedures were performed in compliance with the guidelines set forth by the University Committee on Use and Care of Animals at the University of Michigan Medical School, Ann Arbor.

MVD ANALYSIS

After the mice were euthanized, the tumor masses and surrounding soft tissue were sharply excised and placed in formalin. An assessment of MVD in the primary tumors was determined by means of immunohistochemical staining for the presence of factor VIII as previously described by De Placido et al.[19] Briefly, 5-µm sections through representative invasive squamous cell cancer were cut from formalin-fixed and paraffin-embedded tissues. The slides were then pretreated with trypsin and incubated with a monoclonal antibody raised against human factor VIII–related antigen (DAKO, Copenhagen, Denmark). A standard immunoperoxidase method was used for staining (ABC Kit; Vector Laboratories, Burlingame, Calif). For each batch of slides stained, positive and negative controls were performed. Low-power microscopy (×100) was used to identify the area of the tumor as well as areas of vascularity (vascular hotspots)[19] within the tumor. The number of lumens (microvessels) per high power field (HPF) (×400) was then determined for each tumor. The average number of lumens in 7 to 10 HPF per slide was assessed and is reported as the mean MVD (mMVD)/HPF by 2 independent researchers masked to the treatment groups undergoing evaluation, as previously reported.[17]

ANALYSIS OF DATA

In this study, mean tumor volume was assessed as a measure of tumor growth during the course of treatment. Two-dimensional external measurements were performed every other day. Microvessel density counts from all mice in each group were also analyzed. A logarithmic transformation was applied to the response (tumor volume) to achieve normality for analysis. Data were analyzed using repeated measures analysis. A mixed model with random intercept and slope was fitted to the data while adjusting for the log scale tumor volume at day 40. The model assumes that treatment and control groups have different quadratic tumor growth coefficients. Furthermore, tumor growth rates in the treatment group changed after treatment was withheld on day 54. Comparisons regarding the tumor MVD counts of the 2 groups were performed using the *t* test.

We performed statistical analysis using PROC MIXED in SAS version 8.2 (SAS Institute Inc, Cary, NC). A 2-tailed *P* value of .05 or less was considered to be statistically significant. All data are presented as mean ± SD.

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RESULTS

IN VIVO MURINE MODEL

Measurable tumor growth was achieved in 6 (100%) of the 6 control group mice by day 35, whereas only 5 (71%) of 7 tetrathiomolybdate-treated mice had measurable tumor growth by day 35. All of the mice had measurable tumor growth by day 40. The tumor volume was significantly greater in the control group compared with the treatment group from day 40 through day 60. **Figure 1** shows tumor volume over time for each group. The mean tumor volumes were 146±263 mm^3 (n=7) for the treatment group and 274±331 mm^3 (n=6) for the control group on day 40. On day 54, the mean tumor volumes were 65±0 and 1716±960 mm^3 for the treatment and control groups, respectively. After the treatment was withheld, tumor growth rates for the tetrathiomolybdate-treated group dramatically increased. The results suggest that the treatment group had significantly slower tumor growth rates before day 54 compared with those of the control group ($P<.001$). However, after the termination of tetrathiomolybdate treatment on day 54, the tumors grew more rapidly in the treatment group, presumably due to the removal of the inhibitory effects of tetrathiomolybdate. By day 60, the tumor volumes between groups were no longer statistically significant. **Figure 2** shows the fitted population average tumor volume for each group with 95% confidence intervals. At day 60, the confidence intervals of the 2 groups overlap.

MVD COUNTS

Tumor vascularity was assessed by factor VIII analysis at the completion of the experiment. The MVD in the control group was 74.60±7.74 mMVD/HPF. The tetrathiomolybdate-treated group had a mean vessel count at the end of the experiment of 83.96±9.83 mMVD/HPF. A positive control of normal, nonneoplastic tissue resulted in a count of 6.6±3.4 vessels/HPF, which was also included for comparison. There was no significant difference between the 2 groups of mice with regard to tumor MVD at the completion of the experiment. We know from previous experience[17] that tetrathiomolybdate decreases tumor vascularity; therefore, this finding illustrates that discontinuation of tetrathiomolybdate treatment results in a rapid neovascularization of the tumor implant.

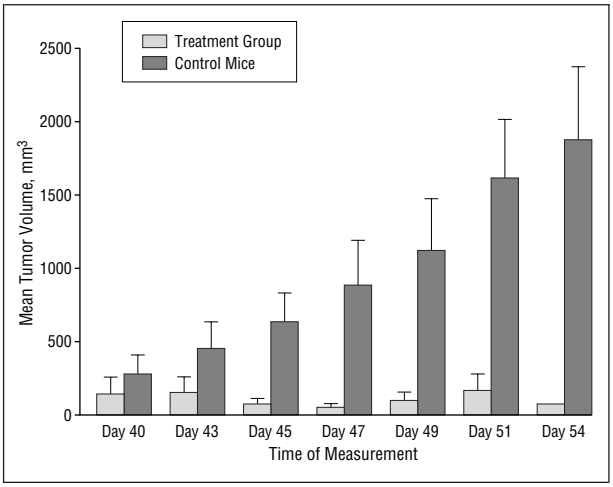

Figure 1. Graphic comparison of tumor volumes in the control group vs the tetrathiomolybdate-treated group. There was a dramatic inhibition of tumor growth in the tetrathiomolybdate-treated animals until day 54, when the tetrathiomolybdate therapy was discontinued for a period of 3 days. Bars represent mean±SD.

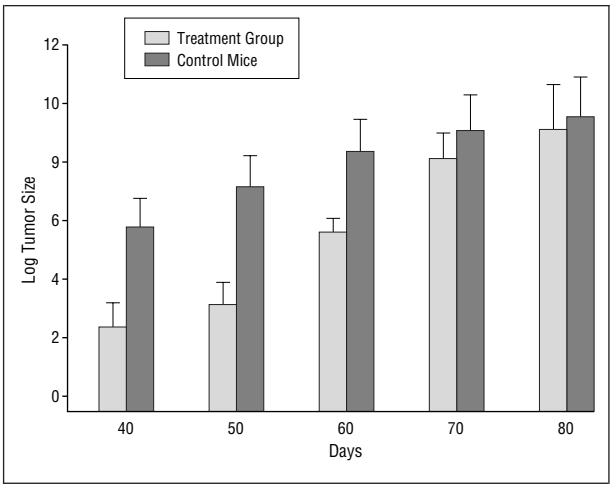

Figure 2. Growth curves of the control and tetrathiomolybdate-treated animals through the course of the entire experiment. Dramatic tumor growth inhibition caused by tetrathiomolybdate is seen until day 54, when the animals were given a 3-day drug holiday. The tetrathiomolybdate-treated tumors then grew dramatically so that by day 60, there was no significant difference between groups. Bars represent means; limit lines, 95% confidence intervals.

COMMENT

The biological behavior of tumor progression depends heavily on angiogenesis; however, angiogenesis is not required for the initial events of tumorigenesis. Folkman[1] labeled this as the prevascular phase of tumor growth. Tumors in this phase can only grow to a size (1-2 mm) at which they can receive nutrients and dispose of waste by diffusion alone. Beyond this prevascular phase, tumors become vascularized and assume a more aggressive phenotype. Nearly 20 biological modifiers have been identified to date, which initiate angiogenesis by direct action on local endothelial cells or by stimulating local inflammatory cells to induce angiogenesis.[20] Because of the inherent complexity of the angiogenic process, the ideal antiangiogenic therapy would target multiple activators of angiogenesis rather than a single, albeit potent biological modifier, such as vascular endothelial growth factor or thrombospondin.

Because copper is a required cofactor for the function of many biological mediators of angiogenesis, a copper suppression approach to antiangiogenic therapy could prove to be highly effective under this hypothesis. The initial evidence pointing to the importance of copper in angiogenesis was illustrated in a study conducted by Gullino.[21] In that elegant analysis, it was clearly shown that copper was the only mineral present in the rabbit cornea during neovascularization of an implanted tumor.[21] Expanding on this intriguing finding, Brem et al[22] showed

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that anticopper therapy using penicillamine inhibited the growth and vascularization of gliosarcoma in a well-proved animal model.[22] The use of the potent copper chelator tetrathiomolybdate as an antiangiogenic therapeutic agent was first introduced by two of us (S.D.M. and G.J.B.).[16] Merajver and colleagues[16] illustrated the dramatic efficacy of tetrathiomolybdate as an antiangiogenic compound for the treatment of orthotopic inflammatory breast cancer in an HER2/Neu transgenic mice model. Our laboratory further illustrated that tetrathiomolybdate was a potent inhibitor of orthotopic squamous cell carcinoma in a murine model.[17] Furthermore, in vitro studies showed that copper was required for endothelial cell proliferation, migration, and organization into primordial vessel networks.[22-24] This promising observational data resulted in a phase 1 trial of tetrathiomolybdate for metastatic solid tumors. The results of that trial illustrated that humans can withstand significant copper deficiency (ie, a reduction of serum ceruloplasmin, a surrogate marker of copper status, down to 20% of baseline, which is a level producing antiangiogenic effects) without major adverse effects.[25] Results of 3-dimensional ultrasonography also showed a significant decrease in tumor blood flow in patients receiving tetrathiomolybdate therapy.

In view of this encouraging observational data, it is important to establish the molecular mechanism of the antiangiogenic effects of tetrathiomolybdate. Several recent studies have begun to elucidate the cellular effects of tetrathiomolybdate. Pan et al[24] have shown that SUM149 inflammatory breast cancer cells produced significantly lower amounts of vascular endothelial growth factor, fibroblastic growth factor 2, IL-6, and IL-8 in vitro, compared with untreated controls. This finding suggests that copper may be a necessary factor for proper expression and/or secretion of these proangiogenic compounds. Furthermore, Landriscina et al[26] independently reported that copper was essential to the extracellular release of fibroblastic growth factor 1 and IL-1a, providing additional support for the role of copper deficiency in inhibiting key events associated with tumor angiogenicity. In addition, tetrathiomolybdate was found to inhibit the activity of the transcription factor NFκB.[24] This family of transcription factors is known to regulate genes important for invasion, angiogenesis, and metastasis, including vascular endothelial growth factor, IL-6, IL-8, matrix metalloproteinases, urokinase plasminogen activator, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1.[24] The aggregate of these findings suggest that tetrathiomolybdate inhibits multiple proangiogenic factors within the tumor microenvironment. This leads to the corollary that malignant tumors arising in a copper-deficient milieu may not be able to stimulate sufficient neovascularization to grow beyond a few millimeters.

Our present study was designed to investigate this possibility, ie, to render mice copper deficient before tumor implantation and evaluate whether this could indefinitely suppress tumor growth by inhibiting the switch to the angiogenic phase of tumor growth. It is an extension of our earlier work in which we were able to demonstrate the efficacy of tetrathiomolybdate as a therapeutic agent in already established tumors in an orthotopic murine model of head and neck squamous cell carcinoma. Our most significant finding is that tetrathiomolybdate dramatically suppresses tumor growth in mice, which were rendered copper deficient before tumor implantation. Moreover, the tumors remained suppressed for as long as the mice received tetrathiomolybdate therapy. This ability to maintain long-term tumor suppression is an attractive characteristic of tetrathiomolybdate, given its minor adverse effect profile. However, tetrathiomolybdate did not appear to inhibit the angiogenic switch. Discernible tumor nodules developed in the tetrathiomolybdate-treated mice (which one would not expect with switch inhibition), but the nodules remained small. The likely explanation for this phenomenon is the inherent complexity of the angiogenic process. Although tetrathiomolybdate was able to inhibit the expression of multiple proangiogenic compounds, others likely remained unaffected, thus allowing the tumors to become vascularized. The potent and global inhibition of angiogenesis by tetrathiomolybdate, however, kept these tumors from growing in their expected rapid manner. Once the tetrathiomolybdate therapy was discontinued and systemic copper was repleted during the drug holiday, the tumors grew at a rate greater than that of the control tumors. This rapidity in growth rate was reflected in the tumor MVD at the conclusion of the experiment, which demonstrated no significant difference in vessel density between the control and treatment groups.

Based on the findings of this study, a number of future investigations are being undertaken to identify the optimal use of this promising compound. First and foremost, in patients who are at a high risk for locoregional recurrence or distant metastases, long-term tetrathiomolybdate therapy may indefinitely prevent microscopic residual tumor or microscopic metastases from leaving the prevascular phase. This effect could potentially maintain tumor foci at a clinically irrelevant size, have a favorable impact on recurrence rates, decrease the development of distant metastases, and ultimately improve long-term survival. Second, it has been clearly documented that tumor sensitivity to cytotoxic therapy, whether it be chemotherapy or radiation therapy, is negatively correlated to a tumor's angiogenic potential.[27-29] Therefore, by inhibiting a tumor's ability to express proangiogenic molecules in response to cytotoxic therapy, tetrathiomolybdate may act to sensitize tumors to these proven treatment modalities and improve their efficacy. Other antiangiogenic agents have been used in this manner and proved highly effective in this regard.[29,30] Clearly, much remains to be learned regarding tetrathiomolybdate's effect on human squamous cell carcinoma of the head and neck, but preliminary studies indicate that it may have a future role in the treatment of this relentless malignancy.

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