The Effect of Tetrathiomolybdate on Cytokine Expression, Angiogenesis, and Tumor Growth in Squamous Cell Carcinoma of the Head and Neck

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Objective: To assess the effect of tetrathiomolybdate on cytokine expression, angiogenesis, and tumor growth rate in human squamous cell carcinoma (SCC).

Design: Three human SCC cell lines were used in this study for both in vitro and in vivo investigations. Conditioned media from untreated and tetrathiomolybdate-treated cell lines were compared with regard to cytokine levels, endothelial cell chemotaxis, endothelial cell tube formation, and migration and the ability to induce angiogenesis in a rat aortic ring array. In vivo UM-SCC-38 was seeded onto tissue-engineered scaffolds and surgically implanted into the flanks of immunodeficient mice. Tumor growth rates and the level of angiogenesis were compared after 2 weeks of therapy.

Setting: A tertiary care facility.

Results: In this study, we demonstrate that tetrathiomolybdate significantly decreases the secretion of interleukin 6 and basic fibroblast growth factor by head and neck SCC (HNSCC) cell lines in vitro. Furthermore, tetrathiomolybdate treatment of HNSCC cell lines results in significantly decreased endothelial cell chemotaxis, tubule formation, and neovascularization in a rat aortic ring assay. This in vitro evidence of decreased angiogenesis by tetrathiomolybdate is confirmed in vivo by using a severe combined immunodeficiency disorder mouse model in which tetrathiomolybdate therapy is shown to prevent human blood vessel formation. Finally, human HNSCC implanted into immunodeficient mice grow to a much larger size in untreated mice compared with those treated with 0.7 mL/kg per day of oral tetrathiomolybdate.

Conclusions: These findings illustrate the ability of tetrathiomolybdate to down-regulate proinflammatory and proangiogenic cytokines in HNSCC. These observations are potentially exciting from a clinical perspective because a global decrease in these cytokines may decrease tumor aggressiveness and reverse the resistance to chemotherapy and radiation therapy seen in this tumor type.

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QUAMOUS CELL CARCINOMA (SCC) is a relatively common human malignancy that arises from the upper aerodigestive tract, lung, skin, and cervix. Several proinflammatory and proangiogenic cytokines have been detected in cell lines and tumor specimen from patients with SCC, including interleukin (IL) 1α, IL-6, IL-8, Gro-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Van Waes and colleagues have shown that human head and neck SCC (HNSCC) expresses elevated levels of proinflammatory and proangiogenic cytokines relative to controls. An increase in constitutive expression of these cytokines also results in HNSCC tumor progression, metastasis in vivo, and the development of resistance to chemotherapy and radiation therapy. Clearly, the expression of these cytokines seems to provide a survival advantage to SCC. Therefore, novel therapeutic approaches toward this cancer may involve understanding the mechanism of cytokine expression and developing an effective inhibitor.

One mechanism known to result in overexpression of proinflammatory and proangiogenic cytokines in HNSCC is the constitutive activation of the transcription factor, nuclear factor κB (NF-κB). The 5′ promoter region of genes coding for IL-1α,
IL-6, IL-8, vascular endothelial growth factor (VEGF), Gro-α, and GM-CSF in SCC contain binding domains for the NF-κB/Rel family of transcription factors.10 Inhibition of NF-κB activity using dominant-negative, mutant inhibitor-κBα, or a proteasome inhibitor (PS-341) resulted in decreased cytokine expression and decreased head and neck tumor growth in vivo.11,12 Tetrathiomolybdate, a novel therapeutic agent with efficacy against murine SCC, has similarly been shown to inhibit NF-κB activity.13,14 Using the SUM149 inflammatory breast cancer cell line and HER2/neu transgenic mice, Pan et al demonstrated that tetrathiomolybdate decreased the production of IL-1α, IL-6, IL-8, basic fibroblast growth factor (bFGF), and VEGF, resulting in decreased tumor growth and angiogenesis. The major advantages distinguishing tetrathiomolybdate from other therapeutic inhibitors of NF-κB are its low toxicity profile and oral bioavailability. In addition, tetrathiomolybdate has been shown to inhibit the extracellular release of bFGF and IL-1, thereby enhancing its antiangiogenic effects.15 We hypothesize that given the importance of elevated cytokine production in SCC, tetrathiomolybdate will be an effective anticytokine and antiangiogenic agent in this tumor type. In the present study, we investigated the effects of tetrathiomolybdate on human SCC and dermal endothelial cells by quantifying its effect on proangiogenic and proinflammatory cytokines, endothelial migration in vitro, and human SCC growth in vivo.

METHODS

CELL CULTURE

For this study, 3 human SCC cell lines (UM-SCC-9, UM-SCC-11B, and UM-SCC-38) were obtained as a gift from Thomas E. Carey, PhD (University of Michigan, Ann Arbor). UM-SCC-9 was derived from a 72-year-old patient with a local recurrence of a T2 N0 SCC of the oropharynx. UM-SCC-11B was obtained from the biopsy specimen of a 65-year-old patient with a T2 N2a SCC of the hypopharynx, and UM-SCC-38 originated from a tissue sample of a 60-year-old patient with a T2 N2a SCC of the oropharynx.16 These cell lines were cultured at 37°C (5% carbon dioxide) in Dulbecco Modified Eagle Medium (Gibco/BRL, Gaithersburg, Md), containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah) supplemented with 0.1% bovine serum albumin. A 165-µL aliquot from each of these cell suspensions was introduced into each of the upper wells of the chamber, and these were incubated for 2 hours at 37°C (5% carbon dioxide) to allow for endothelial cell adherence to the membrane. Next, the chambers were reinverted and 116 µL of control or tetrathiomolybdate-treated conditioned media were introduced into each of the upper wells of the chamber, and these were incubated for 2 hours at 37°C (5% carbon dioxide). Membranes were then scraped to remove any unincorporated endothelial cells from the lower chamber, fixed in methanol, and stained with a modified Wright-Giemsa stain. The cells that migrated through the membrane were counted in 5 high-power fields (original magnification ×200). Results were depicted as the mean number of endothelial cells that had migrated per high-power field a SEM. Experiments were performed in triplicate. All experiments were accompanied with a positive control (VEGF, 100 ng/mL) and a negative control (nonconditioned medium) to account for variable chemotaxis from one assay to the other (data not shown).

ENDOTHELIAL CELL CHEMOTAXIS ASSAY

The chemotaxis assay was performed as previously described, with minor modifications.17 Briefly, membrane filters of 0.3 µm (Osmopore, Livermore, Calif) were dipped in 2.8% glacial acetic acid for 24 hours followed by two 1-hour washings with deionized water. The filters were then dipped in 0.01% gelatin for 24 hours, dried in a laminar flow hood, and stored at room temperature until used. The assays were performed in 12-well chemotaxis chambers (Neuroprobe, Cabin John, Md). Human endothelial cells with a cell density of 1 million cells/mL were suspended in serum-free MCDB-131 medium (Sigma-Aldrich, St Louis, Mo) supplemented with 0.1% bovine serum albumin. A 165-µL aliquot from each of these cell suspensions was placed in the bottom of each well, and the membrane filters were placed over the wells. The chambers were sealed with rubber gaskets and then inverted for 2 hours at 37°C (3% carbon dioxide) to allow for endothelial cell adherence to the membrane. Next, the chambers were reinverted and 116 µL of control or tetrathiomolybdate-treated conditioned media were introduced into each of the upper wells of the chamber, and these were incubated for 2 hours at 37°C (5% carbon dioxide). Membranes were then scraped to remove any unincorporated endothelial cells from the lower chamber, fixed in methanol, and stained with a modified Wright-Giemsa stain. The cells that migrated through the membrane were counted in 5 high-power fields (original magnification ×200). Results were depicted as the mean number of endothelial cells that had migrated per high-power field a SEM. Experiments were performed in triplicate. All experiments were accompanied with a positive control (VEGF, 100 ng/mL) and a negative control (nonconditioned medium) to account for variable chemotaxis from one assay to the other (data not shown).

RAT AORTIC RING ASSAY

Aorta was obtained from a freshly killed Sprague Dawley rat and was rinsed in cold Hank’s balanced salt solution (Sigma-Aldrich), which was supplemented with penicillin and streptomycin solutions. Segmental rings of about 1 mm in width were cut from the aorta and embedded in 50-µL aliquots of BD Matrigel (Becton, Dickinson and Company, Bedford, Mass) in 6-well plates. Rings were incubated overnight in serum-free Dulbecco Modified Eagle Medium at 37°C and then exchanged with conditioned media from control or tetrathiomolybdate-treated UM-SCC cell lines. Incubation was carried out for 4 days at 37°C. Microvascular outgrowth in the form of sprouts was assessed by phase-contrast microscopy.

QUANTITATION OF NORMAL HUMAN MICROVASCULAR ENDOTHELIAL CELL TUBULE FORMATION

The effect of tetrathiomolybdate on normal human microvascular endothelial cell tubule differentiation was studied using reduced growth factor BD Matrigel on 24-well plates. Each well was plated with 25 000 endothelial cells and stimulated with...
bFGF (10 ng/mL). The wells were subdivided into 4 groups of tetrathiomolybdate treatment (0.0 nM, 0.1 nM, 1.0 nM, or 10.0 nM), and the experiments were conducted in triplicate. Cells were incubated at 37°C for 14 hours. After the completion of the incubation period, discernible tubules were counted in at least 4 random fields from each group.

SEVERE COMBINED IMMUNODEFICIENCY DISORDER MOUSE MODEL OF HUMAN ANGIOGENESIS

All animal procedures were performed in compliance with the guidelines set forth by the University Committee on the Use and Care of Animals at the University of Michigan Medical School. One million endothelial cells were seeded onto poly-lactic acid, highly porous scaffolds (6 × 6 × 1 mm) and surgically implanted on the dorsum of immunodeficient mice.18 Beginning 10 days prior to implantation and continuing thereafter, the mice were gavaged with either tetrathiomolybdate (0.7 mg/kg per day; n=5) or water (control; n=5). After 2 weeks, the animals were humanely killed, and the implants were excised, fixed in 10% buffered formalin, and embedded in paraffin. Five-micrometer cryotome sections were cut and immunohistochemical staining was performed using antihuman CD31 antibodies (Pharmigen, San Diego, Calif). Microvessel density was assessed for each experimental condition using the vascular hot spot technique in 10 high-power fields (original magnification ×200), as previously described.14

To evaluate the effect of tetrathiomolybdate on the endothelial cell viability and proliferation during the process of neangiogenesis in this model, duplicate slides were cut and stained with Apoptag (Chemicon International, Temecula, Calif) and Ki-67, respectively. A fixed number of endothelial cells from 10 random high-power fields (original magnification ×200) were then counted, and the proportion of apoptotic and proliferating cells was determined.

IN VIVO SEVERE COMBINED IMMUNODEFICIENCY DISORDER MOUSE MODEL OF HUMAN SCC

Ten severe combined immunodeficiency disorder (SCID) mice were tagged by conventional ear-punch methods and randomly assigned to receive placebo (n=5) or 0.7 mg/kg per day of tetrathiomolybdate (n=5) by gavage, as previously described.15,19 On day 0 of the experiment, 150 000 UM-SCC-38 cells in 200 µL of RPMI (Life Technologies Inc, Gaithersberg) were injected into the flank of each mouse using a 1.5-in (3.81-cm), 27-gauge needle. The mice were subsequently given treatments, monitored daily, and weighed weekly, and 2-dimensional tumor measurements were recorded. Tumor volumes were calculated from these measurements using the following formula (square of the width in centimeters) × (length in centimeters) × 0.52. At day 45 of the experiment, the mice were humanely killed by approved methods prior to tumor ulceration. Each individual tumor was fixed in 10% formalin and paraffin
embedded. Five-micrometer sections of each tumor specimen were cut and stained with antibody to CD31 as per manufacturer's specifications (Pharmigen). The density of vessels for each tumor was determined by counting the number of vessel lumens per high-power field (original magnification \( \times 200 \)) in 10 fields per specimen.

**RESULTS**

**TETRATHIOMOLYBDATE DECREASES CYTOKINE LEVELS IN UM-SCC CELL LINES**

To determine whether tetrathiomolybdate inhibits cytokine secretion, the cells were treated with 4 different concentrations of tetrathiomolybdate (0.0nM, 0.1nM, 1.0nM, and 10.0nM) for 72 hours, and the conditioned media were analyzed for cytokine content. As shown in Figure 1, secretion of bFGF and IL-6 significantly decreased in all 3 cell lines in a dose-dependent manner. With regard to the effects of tetrathiomolybdate on IL-8 and VEGF concentrations, there was a trend toward decreased secretion of these proteins, but the changes did not reach statistical significance (\( P \geq .05 \)) (Figure 2).

Similarly, the effects on Gro-\( \alpha \) secretion were modest, as illustrated in Figure 3.

**EFFECT OF TETRATHIOMOLYBDATE ON ENDOTHELIAL CELL MIGRATION**

To investigate whether the effect of tetrathiomolybdate on proangiogenic cytokines secreted a biologically relevant effect on the endothelial cell compartment, we studied human microvascular endothelial cell migration through gelatin membranes. This, in effect, is a functional, in vitro assay measuring the tumor’s proangiogenic capacity. As shown in Figure 4, tetrathiomolybdate caused a dose-dependent decrease in endothelial cell migration in all 3 cell lines. The maximum effect of tetrathiomolybdate was observed at the 10.0nM dose. UM-SCC-38 showed a more modest but still significant (\( P < .05 \)) effect on endothelial cells.

**TETRATHIOMOLYBDATE INHIBITS ANGIOGENESIS IN IN VITRO AND IN VIVO MODELS**

As shown in Figure 5, rat aortic ring incubated with tetrathiomolybdate-treated conditioned media from the HNSCC cells lines showed a remarkable decrease in vessel outgrowth compared with the ring incubated with untreated conditioned media from UM-SCC cell lines. Further evidence that tetrathiomolybdate inhibits angiogenesis in vitro is illustrated by the significant inhibition of endothelial cell differentiation into tubule networks at doses of 0.1nM and 1.0nM (Figure 6 and Figure 7). After showing an effect in vitro, it was critical to show that tetrathiomolybdate had similar antiangiogenic effects in vivo. Using a well-developed model of in vivo angiogenesis, we studied the effect of tetra-
thiomolybdate on the endothelial cell compartment alone without tumor stimulus. As shown in Figure 8, the implant in the control group of mice was highly vascularized compared with the copper-deficient, thiomolybdate-treated mice. Statistical analysis showed a 72% reduction in vessel formation with thiomolybdate treatment (P=.005) (Figure 9). Furthermore, only 2% of the cells were Apoptag positive, and an equivalent number (2%) were actively proliferating, as illustrated by Ki-67 stains. These data illustrate that thiomolybdate does not result in endothelial cell apoptosis but rather prevents endothelial cell proliferation and organization into tubular networks. This recapitulates the in vitro findings outlined previously.

TETRATHIOMOLYDBDATE INHIBITS THE GROWTH OF HUMAN SCC IN VIVO

After illustrating potent anticytokine and antiangiogenic effects in a variety of in vivo and in vitro assays, it was critical to assess the ability of thiomolybdate to inhibit human tumor growth in vivo. In vivo studies using UM-SCC-38 in SCID mice resulted in significant inhibition of tumor growth (2475 vs 414 mm³; P=.005).
COMMENT

Tetrathiomolybdate is a potent copper chelator initially developed for the treatment of Wilson disease.\textsuperscript{20} However, owing to the critical importance of copper in the proliferation and migration of human endothelial cells, tetrathiomolybdate was investigated as an antiangiogenic agent.\textsuperscript{21,22} It was found that oral administration of tetrathiomolybdate safely and rapidly induced copper deficiency within 2 to 4 weeks in mice and humans. This degree of copper deficiency resulted in a global inhibition of angiogenesis and inhibition of a very aggressive,
molybdate had a documented reduction in blood flow to important ly, however, patients treated with tetrathiomolybdate had mi nimal and rapidly reversible adverse effects. More can tolerate copper reduction to 20% of baseline with involving a variety of solid tumors reveals that humans have illustrated that tetrathiomolybdate is able to sig nificantly decrease IL-1 and FGF1 secretion but also reduces the expression of synaptotagmin 1, S100A13, and phosphatidylserine and abrogates the recruitment of macrophages to the site of injury in a model of vascular stenosis. A second major mechanism of tetrathiomolybdate effect is inhibition of NF-κB activity. Using SUM 149 inflammatory breast cancer cell lines, Pan et al illustrated that tetrathiomolybdate was able to dramatically decrease the levels of proangiogenic mediators via inhibition of NF-κB transcription. Specifically, p50 and RelA protein levels were reduced after treatment with tetrathiomolybdate, and there was a decrease in nuclear binding to the κB consensus sequence, resulting in the observed decrease in cytokine levels. Furthermore, tetrathiomolybdate treatment was able to prevent the growth of mammary tumors in HER2/neu transgenic mice, which are known to have enhanced NF-κB activity.

In the present study, we have shown that tetrathiomolybdate is able to decrease the expression of proangiogenic and proinflammatory cytokines of head and neck cancer cells in vitro. Furthermore, in reliable functional assays of angiogenesis capacity, tetrathiomolybdate treatment resulted in a dramatic decrease of endothelial cell migration and abolished new vessel sprouts from rat aorti c rings in response to conditioned media from head and neck cancer cell lines. We also illustrated that tetrathiomolybdate prevents endothelial cell differentiation into tubule networks in vitro and in vivo. Finally, and most importantly however, these in vitro results translated into decreased growth and angiogenesis of human SCC in an in vivo SCID mouse model. These findings are consistent with those seen in human inflammatory breast cancer and add further evidence to the potential therapeutic utility of this compound. Also, as can be inferred from the decrease in proangiogenic cytokine and FGF1 levels, tetrathiomolybdate appears to function through similar mechanisms in HNSCC as in other tumor types. These observations are potentially exciting from a clinical perspective because the level of angiogenesis and constitutive NF-κB activity in HNSCC has been linked to increased tumor aggressiveness, decreased survival, and resistance to chemotherapy and radiation therapy. Future and ongoing investigations will more specifically delineate the mechanism of effect of tetrathiomolybdate in HNSCC and also determine whether copper deficiency can restore chemosensitivity and radiosensitivity to resistant cancer cells.

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