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Advances in Brief

Copper Deficiency Induced by Tetrathiomolybdate Suppresses Tumor Growth and Angiogenesis[1]

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Abstract

Copper plays an essential role in promoting angiogenesis. Tumors that become angiogenic acquire the ability to enter a phase of rapid growth and exhibit increased metastatic potential, the major cause of morbidity in cancer patients. We report that copper deficiency induced by tetrathiomolybdate (TM) significantly impairs tumor growth and angiogenesis in two animal models of breast cancer: an inflammatory breast cancer xenograft in nude mice and Her2/*neu* cancer-prone transgenic mice. *In vitro*, TM decreases the production of five proangiogenic mediators: (*a*) vascular endothelial growth factor; (*b*) fibroblast growth factor 2/basic fibroblast growth factor; (*c*) interleukin (IL)-1α; (*d*) IL-6; and (*e*) IL-8. In addition, TM inhibits vessel network formation and suppresses nuclear factor (NF)κB levels and transcriptional activity. Our study suggests that a major mechanism of the antiangiogenic effect of copper deficiency induced by TM is suppression of NFκB, contributing to a global inhibition of NFκB-mediated transcription of proangiogenic factors.

Introduction

Copper, an essential trace [3] element ubiquitous in the diets of humans, is an important cofactor for angiogenesis. Copper stimulates proliferation and migration of human endothelial cells (1, 2). Several reports showed a decrease in microvessel density and tumor size in penicillamine-treated, copper-deficient rabbits and rats xenografted with 9L gliosarcoma cells (3, 4). However, the molecular mechanism by which copper deficiency regulates angiogenesis remains unknown. To expedite and sustain the end point of clinical copper deficiency, we used a potent and novel copper chelator, TM,[4] developed for the treatment of Wilson's disease (5). Patients with Wilson's disease, a rare autosomal recessive disorder, have a metabolic defect of copper transport that results in life-threatening accumulation of copper in multiple organs, notably liver and the brain. TM forms a high-affinity tripartite complex with copper and albumin to chelate copper from the bloodstream. Copper status in mammals treated with TM cannot be reliably followed in the early stages of treatment by measuring total serum copper levels because the complexed copper is still detected but is not bioavailable. Serum Cp, whose synthesis is directly regulated by the bio-availability of copper to the liver, is a more accurate indicator of copper status over a wide range and thus is used as a surrogate marker of total copper status. TM safely induced copper deficiency within 2–4 weeks in humans and mice. In a recent study, we showed that copper deficiency induced by TM significantly inhibited tumor growth of head and neck squamous cell carcinoma in severe combined immunodeficient mice (6). Evidence from Phase I and preliminary results from ongoing Phase II clinical trials demonstrate that humans can withstand significant copper deficiency induced by TM with Cp reduction to 20% of baseline (7).[5] Using three-dimensional ultrasound imaging, we demonstrated a reduction in blood flow to tumor masses in patients on TM therapy (7). This evidence supports the empiric link between copper deficiency induced by TM and inhibition of tumor angiogenesis in humans.

Materials and Methods

Cell Lines. HME cells were immortalized with human papilloma virus E6/E7 and grown in 5% fetal bovine serum (Sigma Chemical Co.) supplemented with Ham's F-12 medium (JRH Biosciences) containing insulin, hydrocortisone, epidermal growth factor, and cholera toxin (Sigma Chemical Co.). The SUM149 inflammatory breast cancer cell line was developed from a primary inflammatory breast cancer tumor and grown in 5% fetal bovine serum supplemented with Ham's F-12 medium containing insulin and hydrocortisone. The HME cells were characterized as being keratin-19 positive, ensuring that they are from the same differentiation lineage as the SUM149 inflammatory breast cancer cells.

Animal Models of Breast Cancer. SUM149 cells (1×10^6 cells) were orthotopically injected into the upper left mammary fat pad of 10-week-old female athymic nude mice. Cells were trypsinized, washed, and resuspended in Hank's buffered saline solution (HBSS) at a density of 1×10^6 cells/200 μl. Mice were anesthetized using 10 mg/ml ketamine, 1 mg/ml xylazine, and 0.01 mg/ml glycopyrrolate, and an incision below the thoracic left mammary fat pad was made. Using a 27-gauge needle, the cell suspension was injected into the exposed mammary fat pad, and the wound was closed with a single wound clip. After a brief recovery period, tumor-implanted mice were randomly assigned and gavaged with water (control; $n = 7$) or 0.7 mg/day TM ($n = 7$) daily for 7 weeks. Tumor volume was measured weekly and calculated as (length $\times$ width^2)/2.

Mouse mammary tumor virus-Her2/*neu* transgenic mice were purchased from Jackson Laboratory. At ~100 days old, female mouse mammary tumor virus-Her2/*neu* mice were randomly assigned to two groups and gavaged with water (control; $n = 22$) or 0.75 mg/day TM ($n = 15$) for the entire experimental protocol. Mice were monitored weekly for overt palpable tumors, and disease-free survival curve was calculated using Log-rank analysis.

Quantification of Microvessel Density. Tumors from control or TM-treated mice were resected, immersion fixed in 10% buffered formalin, and

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[2] G. J. B. and S. D. M. are consultants and have a financial interest in Attenuor, LLC, which has licensed TM as an anticancer compound from the University of Michigan.

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[4] The abbreviations used are: TM, tetrathiomolybdate; Cp, ceruloplasmin; IL, interleukin; FGF, fibroblast growth factor; HME, human mammary epithelial; HUVEC, human umbilical vein endothelial cell; TNF, tumor necrosis factor; NFκB, nuclear factor κB; VEGF, vascular endothelial growth factor.

[5] J. A. Marrero and B. G. Redman, personal communication.

paraffin embedded. Intratumoral microvessel density was assessed with CD31 staining (DAKO) using the vascular hotspot technique. Sections were scanned at low power to determine areas of highest vascular density. Within this region, individual microvessels were counted in three separate random fields at high power (×400 magnification). The mean vessel count from the three fields was used. A single countable microvessel was defined as any endothelial cell or group of cells that was clearly separate from other vessels, stroma, or tumor cells without the necessity of a vessel lumen.

Conditioned Media from SUM149 Cells. SUM149 cells were plated at a density of 2×10^5 cells in 100 mm^2 dishes. Cells were treated with vehicle or 0.1 nM TM for 72 h. Conditioned media was collected, centrifuged for 5 min at 2500 rpm, and divided into 1-ml aliquots. Quantikine human VEGF, basic FGF/FGF2, and IL-1α ELISAs (R&D Systems, Inc., Minneapolis, MN) were used to measure protein levels of the 165 amino acid species of VEGF, basic FGF/FGF2, and IL-1α. ELISAs for IL-6 and IL-8 were performed by the University of Maryland Cytokine Core Laboratory.[6]

Rat Aortic Ring Assay. Aorta was removed from a freshly sacrificed Sprague Dawley rat and rinsed in ice-cold HBSS containing penicillin and streptomycin. Segmental rings, ~1 mm in width, were cut from the aorta and embedded in a 50-μl aliquot of 10 mg/ml Matrigel in six-well plates. Rings were incubated overnight at 37°C in serum-free media and then exchanged for conditioned media from control or TM-treated SUM149 cells. Subsequently, rings were incubated for 4 days at 37°C and analyzed by phase-contrast microscopy for microvessel outgrowth.

Transient Transfection and Reporter Gene Assay. SUM149 or HME cells (1×10^5) were transfected transiently with 1 μg of pNFκB (Clontech Laboratories, Inc.) and 0.05 μg pRL-TK (Clontech Laboratories, Inc.) with FuGene6 transfection reagent (Roche Biochemicals). pRL-TK, a *Renilla* luciferase vector, was cotransfected to normalize for transfection efficiency. After a 24-h recovery period, transfected cells were incubated in fresh medium with or without the addition of 2 nM $CuSO_4$. Cells were treated subsequently with vehicle, TM (1 nM), TNFα (2 pM), or TM and TNFα for 24, 48, or 72 h. Cells were harvested in passive lysis buffer, and the activities of the firefly luciferase and *Renilla* luciferase were quantified on a Monolight 2010 luminometer (Analytical Luminescence Laboratory) using the dual luciferase assay system (Promega Corp.).

Electrophoretic Mobility Shift Assay. Nuclear extracts from SUM149 cells were incubated with ^{32}P-labeled κB consensus sequence in a buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT for 30 min at 25°C. Protein-DNA complexes were resolved on a high ionic strength 5% polyacrylamide gel containing 0.5 × Tris-borate EDTA buffer [380 mM glycine, 45 mM Tris base (pH 8.5), 45 nM boric acid, and 2 mM EDTA]. Supershift analysis was performed as described above except nuclear extracts were preincubated with p50, RelA, p52, c-Rel, or RelB antibody (Upstate Biotechnology) for 30 min on ice.

Western Blot Analysis. Proteins were harvested from SUM149 cells using radioimmunoprecipitation assay buffer (1 × PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 0.3 mg/ml aprotinin; Sigma Chemical Co.). Aliquots (20 μg) were mixed with Laemelli buffer, heat denatured for 3 min, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Nonspecific binding was blocked by overnight incubation with 2% BSA in Tris-buffered saline with 0.05% Tween 20 (Sigma Chemical Co.). Immobilized proteins were probed using antibodies specific for p50 or RelA (Upstate Biotechnology). Protein bands were visualized by enhanced chemiluminescence (Amersham-Pharmacia Biotech).

Results and Discussion

Systemic Treatment with TM Inhibits Tumor Growth and Angiogenesis in SUM149 Xenografts. To assess the antiangiogenic action of TM *in vivo*, we used a xenograft model with a cell line derived from a patient with inflammatory breast cancer, chosen because of its highly aggressive and prominently angiogenic form of breast cancer (8). Inflammatory breast cancer is characterized by a very rapid course, typically progressing within 6 months to cause the specific clinical manifestations of erythmea, skin modules, and nipple retraction caused by tumor infiltration of lymphatic and connective tissue (9, 10). Even with multimodality treatment, the 5-year disease-free survival is <45%, making inflammatory breast cancer the most aggressive and deadly form of locally advanced breast cancer (10). SUM149 inflammatory breast cancer cells (1×10^6) were orthotopically transplanted into the mammary fat pads of 10-week-old female athymic nude mice. Mice were gavaged with water (control) or 0.7 mg/day TM starting on the day of xenograft transplantation. Cp was followed weekly and used as a surrogate marker for serum copper status. After 1 week of treatment, Cp levels of TM-treated mice were maintained at 19 ± 4% of baseline for the remainder of the experiment. The size of primary breast tumors was potently suppressed by 69 ± 3% ($P < 0.001$, $n = 7$) as a result of systemic TM therapy (Fig. 1*A*). Tumors in the control group were highly vascularized as shown by immunohistochemical staining with a CD31 antibody (Fig. 1*B*). In contrast, the smaller tumors resected from TM-treated mice were only sparsely vascularized (mean vessel count; 16 ± 2 *versus* 26 ± 3,

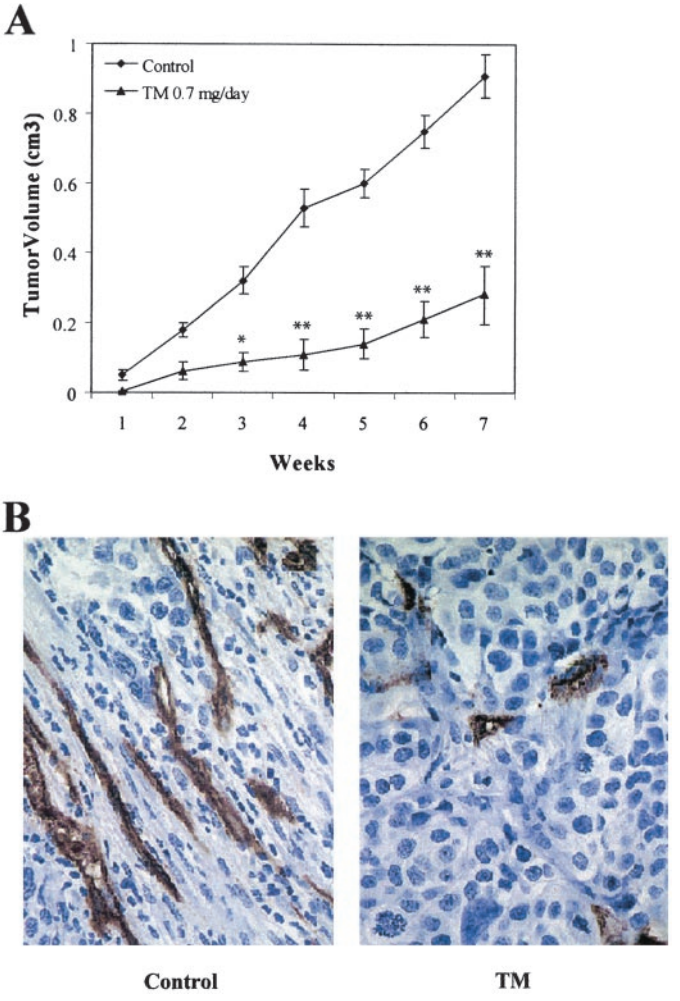

Fig. 1. Systemic treatment with TM inhibits tumor growth and angiogenesis in SUM149 xenografts. *A*, tumor growth of SUM149 xenografts. SUM149 inflammatory breast cancer cells (1×10^6) were orthotopically injected into the upper left mammary fat pad of 10-week-old athymic female mice. Mice were gavaged with water (*Control*) or 0.7 mg/day TM starting on the day of xenograft injection. Tumor volume was measured weekly and calculated as (length × width^2)/2. $*P < 0.01$, $**P < 0.001$; $n = 7$. *B*, microvessel density of SUM149 xenografts. Breast tumors from control or TM-treated mice were resected and stained with anti-CD31 antibodies. Mean vessel count was 26 ± 3 in tumors from control mice and 16 ± 2 in tumors from TM-treated mice. $P < 0.04$; $n = 3$.

[6] Internet address: http://www.cytokinelab.com.

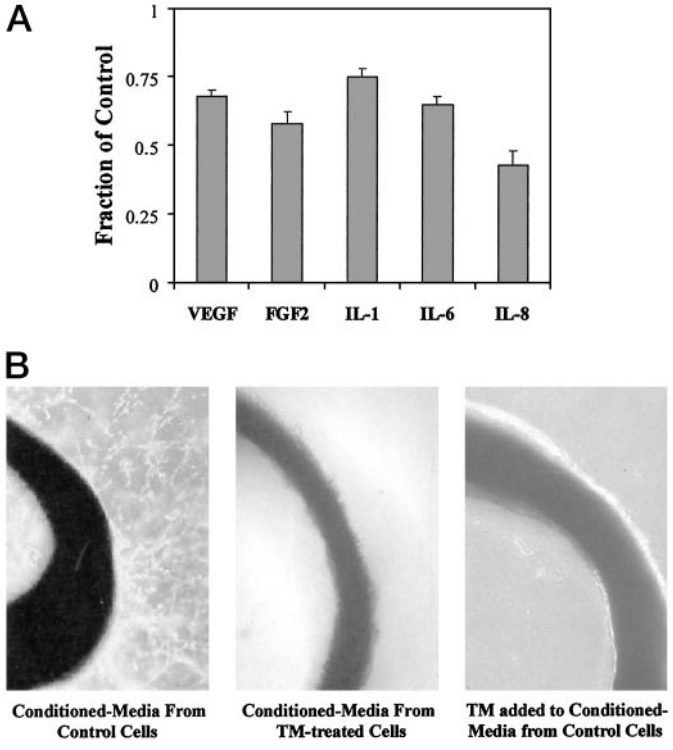

Fig. 2. TM decreases proangiogenic mediators and inhibits *in vitro* angiogenesis. *A*, levels of proangiogenic mediators in conditioned medium from SUM149 cells. Conditioned media from control and TM-treated (0.1 nM) SUM149 cells were analyzed for VEGF, FGF2, IL-1α, IL-6, and IL-8 by ELISA. Data are presented as a fraction of control SUM149 cells. *B*, rat aortic ring assay. Segments of rat aorta were embedded in Matrigel and incubated in the corresponding conditioned media for 4 days at 37°C. Microvessel outgrowth was analyzed by phase-contrast microscopy. Data are representative of three independent experiments.

$P < 0.04$, $n = 3$), providing further evidence that copper deficiency induced by TM is antiangiogenic.

TM Decreases Proangiogenic Mediators and Inhibits *in Vitro* Angiogenesis. A possible explanation for the antiangiogenic action of TM is that copper deficiency induced by TM may result in a shift of overall balance toward inhibition of angiogenesis as a consequence of a decrease in proangiogenic factors, increase in antiangiogenic factors, or a combination of both. Consistent with this hypothesis, TM-treated SUM149 cells released significantly lower amounts of five potent proangiogenic mediators: (*a*) VEGF; (*b*) FGF2; (*c*) IL-1α; (*d*) IL-6; and (*e*) IL-8 ($P < 0.05$; Fig. 2*A*). Moreover, IL-6 and IL-8 mRNA expression was decreased by TM (data not shown). To test whether the decrease in proangiogenic mediators was sufficient to impair the angiogenic potential of the conditioned media, we used the rat aortic ring assay, an *in vitro* model of angiogenesis that resembles the outgrowth of primordial vessels from a host vessel under an appropriate stimulus (Fig. 2*B*). Rat aortic rings incubated with conditioned media from TM-treated SUM149 cells showed a marked reduction in vessel outgrowth when compared with rings incubated with conditioned medium from untreated SUM149 cells. In addition, primordial blood vessels failed to grow when conditioned media from untreated SUM149 cells were supplemented with TM before incubation with aortic rings. Because copper may be involved in the regulation of the biological activity of the prototype members of the FGF gene family (11), we tested if copper deficiency could suppress the stimulatory effect of exogenous FGF2 on HUVECs. TM blocked FGF2-stimulated HUVEC tubule formation in a dose-dependent manner, without affecting HUVEC proliferation (data not shown). Although previous studies have suggested that copper may be required for endothelial cell proliferation and migration (1, 2), our study indicates, for the first time, that absence of copper is able to block exogenous FGF2-mediated organization of endothelial cells into a primordial vessel network. Our data demonstrate that copper deficiency inhibits tumor angiogenesis by two different mechanisms: (*a*) reduction in the level of extracellular proangiogenic mediators released by cancer cells; and (*b*) inhibition of endothelial cell differentiation.

TM Suppresses NFκB Protein Levels and Transcription. Evidence linking uncontrolled NFκB activity to oncogenesis has emerged in recent years. The NFκB transcription factor is known to regulate genes important for invasion, angiogenesis, and metastasis. These include proangiogenic factors, such as VEGF, IL-6, and IL-8, matrix metalloproteinases, urokinase plasminogen activator, and cell adhesion molecules, such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (12–16). Recently, blocking NFκB activity in human ovarian cancer cells was reported to inhibit VEGF and IL-8 expression, resulting in a decrease in tumor angiogenesis (17). To investigate the NFκB dependence of proangiogenic factors in SUM149 cells, we transfected these cells with the super-repressor IκBα (S32AS36A). Conditioned media from SUM149 super-repressor IκBα clones had significantly lower amounts of VEGF, IL-6, and IL-8 in comparison with the empty vector-transfected SUM149 cells; 45 $\pm$ 5% inhibition for VEGF, 37 $\pm$ 4% inhibition for IL-6, and 58 $\pm$ 5% inhibition for IL-8 ($P < 0.05$, $n = 3$). Because TM was able to decrease these proangiogenic factors to a similar extent, we sought to determine whether copper deficiency induced by TM is modulating NFκB-mediated signaling. SUM149 and nontumorigenic-immortalized HME cells were transiently transfected with pNFκB, a vector that contains four tandem copies of the κB consensus sequence upstream of the luciferase reporter gene (Fig. 3). Endogenous NFκB activity was shown to be 2.5-fold higher in SUM149 cells in comparison with HME cells. This is consistent with our observation that p50 protein levels were significantly higher in SUM149 cells (data not shown). Several studies using human breast cancer cells also reported that overexpression of p50 results in constitutive NFκB activity (18, 19).

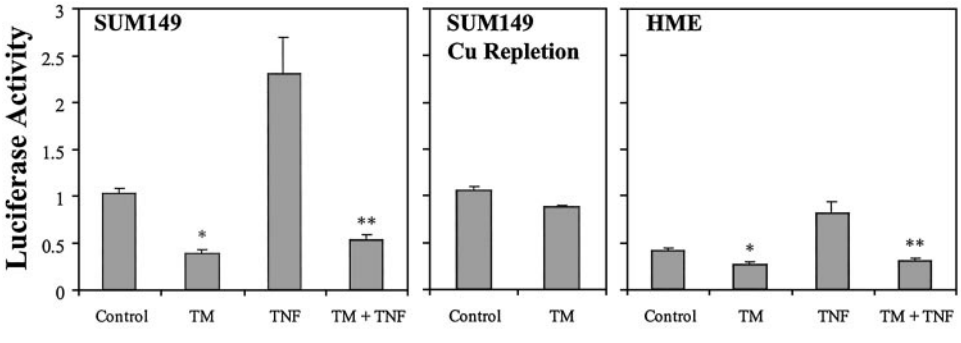

Fig. 3. TM suppresses NFκB-dependent transcription. SUM149 or HME cells were transfected with pNFκB (Clontech Laboratories, Inc.), a vector that contains four tandem copies of the κB consensus sequence fused to a TATA-like promoter region from the herpes simplex virus thymidine kinase promoter. pRL-TK (Promega Corp.), a *Renilla* luciferase vector, was cotransfected into the cells to normalize for transfection efficiency. After a 24-h recovery period, transfected cells were incubated in fresh medium with or without the addition of 2 nM $CuSO_4$. Cells were treated subsequently with vehicle, TM (1 nM), TNFα (2 pM), or TM and TNFα for 24 h. *$P < 0.001$; $n = 6$, vehicle *versus* TM. **$P < 0.001$; $n = 6$, vehicle or TNFα *versus* TM + TNFα.

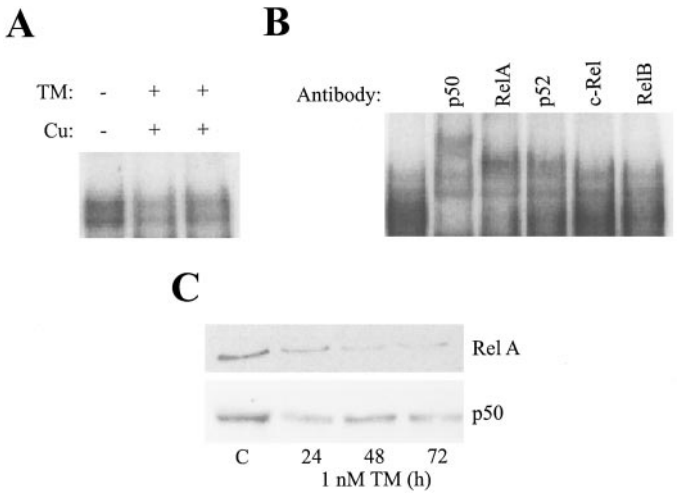

Fig. 4. TM decreases NFκB binding and protein levels. *A*, gel shift analysis. SUM149 cells were treated with vehicle or TM (1 nM) with or without the addition of 2 nM $CuSO_4$ for 72 h. Nuclear proteins were extracted, incubated with ^{32}P-labeled κB consensus sequence, and resolved by EMSA. *B*, identification of NFκB binding complex. Supershift analysis was performed by preincubating nuclear extracts with p50, RelA, p52, c-Rel, or RelB antibody (Upstate Biotechnology) for 20 min on ice. *C*, p50 and RelA protein levels. SUM149 cells were treated with vehicle or TM (1 nM) for 72 h. p50 and RelA levels were determined by Western blot analysis. Data are representative of three independent experiments.

After treatment for 24 h, TM inhibited luciferase activity by 62 ± 2% ($P < 0.001$, $n = 6$) in SUM149 cells and 34 ± 2% ($P < 0.001$, $n = 3$) in HME cells. Moreover, TM completely blocked TNFα-stimulated NFκB activity in both cell lines. Similar results were observed at 48 and 72 h demonstrating that TM is also able to inhibit NFκB activity on a sustained basis without affecting cell survival under these conditions. In Fig. 4*A*, we analyzed the binding of nuclear proteins from SUM149 cells to a labeled oligonucleotide spanning the κB consensus sequence. Extracts from TM-treated cells showed a decrease in nuclear protein binding to the κB consensus sequence. In addition, supershift analysis revealed that the predominant NFκB components in SUM149 cells are p50, p52, and RelA. When cells were cultured with added copper (2 nM $CuSO_4$ addition), TM partially lost its ability to regulate κB binding and NFκB transcriptional activity. In addition, copper repletion partially reversed TM inhibition of IL-6 and IL-8 mRNA expression (data not shown), consistent with restoring NFκB's ability to enhance transcription of these genes. Interestingly, p50 and RelA protein levels were reduced after treatment with TM in SUM149 cells, suggesting that TM may be suppressing NFκB activity by decreasing levels of NFκB component proteins.

Our data clearly demonstrate that NFκB signaling is suppressed after TM treatment *in vitro*. Because VEGF, IL-6, and IL-8 are NFκB-regulated genes, we anticipate that the decreased secretion of these proteins may be a direct consequence of TM's ability to inhibit NFκB activity. This leads to the suggestion that TM is limiting tumor neovascularization by repressing the ability of cancer cells to release NFκB-dependent inflammatory cytokines and proangiogenic mediators into the extracellular compartment and thus limiting the autocrine and/or paracrine effects of these proteins to stimulate angiogenesis in the tumor microenvironment. TM also retards the release of IL-1α in SUM149 cells. Consistent with our observation, the release of IL-1α is dependent on the oxidative function of intracellular copper and blocked with TM treatment.[7] Interestingly, IL-1α was reported to enhance NFκB activity resulting in an increase in IL-6 (20). IL-1 is able to induce the expression of VEGF and FGF2 in human endothelial and melanoma cells (21, 22). From these observations, we further hypothesize that TM may be limiting tumor angiogenesis by directly blocking the release of IL-1α from tumor cells to prevent additional NFκB stimulation in tumor and endothelial cells in close proximity.

TM Prevents the Development of *de Novo* Clinically Overt Tumors in Her2/*neu* Transgenic Mice. Our results suggest that the inhibition of proangiogenic factors within the tumor microenvironment accompanies copper deficiency. This leads to the corollary that malignant clones that arise in a copper-deficient milieu may not be able to stimulate sufficient neovascularization for growth beyond a few millimeters. We designed an experimental protocol to determine the effectiveness of TM in retarding or preventing the growth of mammary tumors in female Her2/*neu* transgenic mice. Extensive clinical studies have shown that overexpression of Her2/*neu* in patients with breast cancer correlates overall with poorer prognoses (23, 24). Female Her/*neu* mice develop focal mammary adenocarcinomas, which eventually metastasize to the lungs (25). These mice develop single or multifocal mammary tumors approximately at 205 days of age (25). By initiating TM treatment 90–120 days before tumors became clinically evident, we surmised that the TM-treated mice would be copper deficient throughout the key period of tumor development when angiogenesis is required for continued tumor growth. Female Her2/*neu* transgenic mice (~100 days old) were gavaged with water (control) or 0.75 mg/day TM ($n = 22$ for control group and $n = 15$ for TM group). Cp levels were maintained at 10–30% of baseline for the entire protocol. Depending on the individual mouse, 2–4 weeks were required to achieve the anticipated end point of copper depletion. We chose this end point to investigate whether the level of copper deficiency that would be tolerable in humans could inhibit the angiogenic switch in Her2/*neu* mice. TM-treated and control mice did not differ in weight or general health as evidenced by social behavior and level of activity. Fig. 5*A* depicts the Kaplan-Meier plot for disease-free survival of TM-treated and control mice. By 218 days, 50% of the control mice and none of the TM-treated mice had overt tumors. Log-rank analysis demonstrated that the TM-treated mice had a statistically significant prolongation of disease-free survival compared with the control group ($P < 0.0147$). At the conclusion of the experiment, with a median follow-up time of 221 days, palpable tumors were not observed in the TM-treated mice that remained copper deficient with Cp levels ≤30% of baseline. However, when TM-treated mice were released from therapy, measurable tumors were observed by 13 ± 5 ($n = 5$) days postrelease. The restoration of copper in these mice, previously copper deficient for >7 months, appears to be sufficient to enable tumor growth to proceed at the normal rate within 2 weeks. Therefore, it is clear that these TM-treated Her2/*neu* mice retained the capacity to develop macroscopic mammary tumors, and copper deficiency appears to act as a barrier for their appearance. It is important to note that Her2/*neu*-positive breast cancer cells have enhanced NFκB activity (26, 27). In light of our observations, this implies that in a copper-deficient environment, nascent breast cancer tumors with Her2/*neu* overexpression have impaired ability to activate the angiogenic switch attributable, in part, to decreased production of proangiogenic factors, perhaps as a consequence of NFκB inhibition by TM.

To investigate the effects of chronic TM administration on the mammary gland, we analyzed mammary gland whole mounts and the microscopic characteristics of tumors resected from the Her2/*neu* mice. Mammary glands of TM-treated Her2/*neu* mice showed regions containing multiple thickened end buds (Fig. 5*B2*). These large buds were widespread throughout the mammary glands of TM-treated Her2/*neu* mice. Histological analyses of these structures clearly

[7] A. Mandinova, S. Bellum, C. Bagala, R. Soldi, I. Micucci, M. Landriscina, F. Tarantini, I. Prudovsky, and T. Maciag. The stress-induced release of the pro-inflammatory cytokine IL1α is Cu^{2+}-dependent. Proc. Natl. Acad. Sci. USA, submitted for publication, 2002.

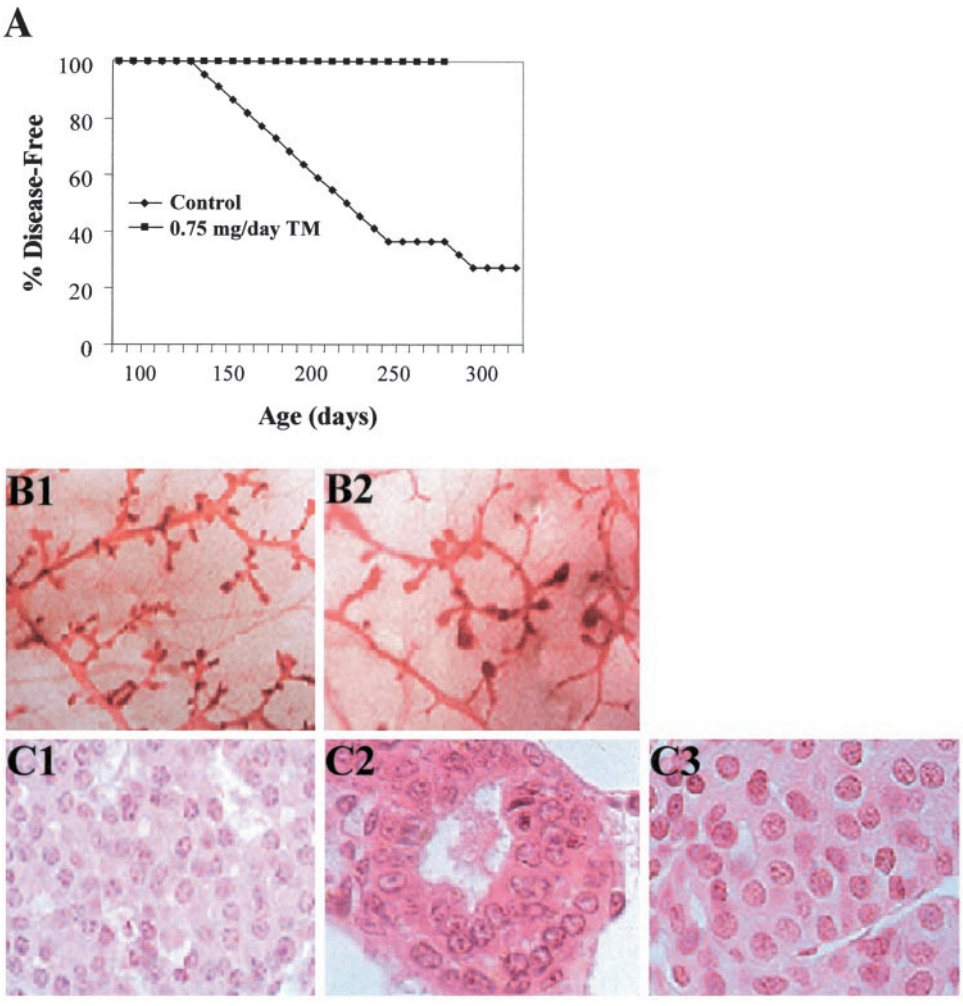

Fig. 5. TM prevents the development of *de novo* clinically overt tumors in Her2/*neu* transgenic mice. *A*, time to first tumor in Her2/*neu* transgenic mice. Female Her2/*neu* transgenic mice (~100 days old) were gavaged with water (*Control*) or 0.75 mg/day TM ($n = 22$ for vehicle group and $n = 15$ for TM treatment group). Cp levels were maintained at 10–30% of baseline for the entire experiment. Mice were monitored for the appearance of overt tumors, and disease-free survival was analyzed using Kaplan-Meier plot. *B*, whole mammary gland mounts. Mammary glands from control (*B1*) or TM-treated (*B2*) mice were fixed in a mixture of one part glacial acetic acid and three parts 100% ethanol for 60 min. The glands were washed for 15 min in 70% ethanol and rinsed with distilled water for 5 min. Whole mammary gland mounts were stained with alum carmine overnight and washed with 70, 90, and 100% ethanol for 15 min each. Subsequently, the mounts were washed with toluene for 15 min and stored in methyl salicylate. *C*, microscopic analysis of H&E-stained whole mammary gland mount. *C1*, control Her2/*neu* mouse (×100 magnification). *C2*, TM-treated Her2/*neu* mouse (×100 magnification). *C3*, TM-treated/release Her2/*neu* mouse (×100 magnification).

showed several discrete microscopic foci of hyperplastic or neoplastic epithelium interspersed among scant normal ducts lined by a single layer of normal cells. These foci contained pleomorphic cells, with increased cytoplasmic clearing and some prominent reddish nucleoli, often filling the duct entirely. These neoplastic foci also were observed in the mammary glands of vehicle-treated and TM-treated/release Her2/*neu* mice. The rapid development of overt tumors in mice released from TM treatment strongly suggests that the cells of the microtumors in TM-treated mice are neoplastic. Interestingly, the morphology of the cells from the microtumors of TM-treated mice is strikingly similar to the cells from the bulky tumors that developed in vehicle- or TM-treated mice that were released from therapy (Fig. 5*C1-3*). This observation further supports our supposition that these microtumors are comprised of completely transformed cells, but failed to acquire neovascularization, and thus have remained noninvasive and relatively contained in a dormant state with minimal or no angiogenesis.

Taken together, these results support our initial clinical observations indicating that copper deficiency induced by TM is a potent approach to inhibit tumor angiogenesis with minimal adverse effects. TM exerts its antiangiogenic action at least in part through restriction of the extracellular appearance of proangiogenic factors and suppression of NFκB activity. The observation that TM suppresses NFκB activity is potentially exciting from a clinical perspective because constitutive NFκB activity is linked to the development of resistance to chemotherapy or radiotherapy (28, 29). Inhibition of NFκB by TM, in turn, may restore the sensitivity of resistant cancer cells and recapitulate the efficacy of chemotherapy or radiotherapy-induced apoptosis. Moreover, the strong suppressive effect on tumor growth when the angiogenic switch is inhibited before or at the time of neoplastic transformation in mammary epithelium suggests a promising role for TM as a chemopreventative agent for use in carriers of cancer susceptibility genes.

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