Targeting the Tumor Microenvironment with Chemically Modified Tetracyclines: Inhibition of Laminin 5 γ2 Chain Promigratory Fragments and Vasculogenic Mimicry

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Abstract

The laminin 5 (Ln-5) γ2 chain and matrix metalloproteinases (MMPs) MMP-2 and membrane type 1 (MT1)-MMP act cooperatively and are required for highly aggressive melanoma cells to engage in vasculogenic mimicry when cultured on a three-dimensional matrix. Furthermore, generation of Ln-5 γ2 chain promigratory fragments by MMP-2 and MT1-MMP proteolysis is necessary for an aggressive tumor cell-preconditioned matrix to induce vasculogenic mimicry in poorly aggressive tumor cells. These observations suggest that treatment regimes that specifically target aggressive tumor cells may fail to take into account changes in the extracellular microenvironment that persist after removal or destruction of an aggressive tumor and could result in a recurrence or continuance of the tumor. As a potential therapeutic approach to address this concern, the work presented here measured the molecular consequences of adding a chemically modified tetracycline (CMT-3; COL-3) that inhibits MMP activity to aggressive metastatic melanoma cells in three-dimensional culture. COL-3 inhibited vasculogenic mimicry and the expression of vasculogenic mimicry-associated genes in aggressive cells, as well as the induction of vasculogenic mimicry in poorly aggressive cells seeded onto an aggressive cell-preconditioned matrix. Furthermore, molecular analysis revealed that COL-3 not only inhibited the generation of Ln-5 γ2 chain promigratory fragments in the aggressive cell-preconditioned matrix but also inhibited the induction of Ln-5 γ2 chain gene expression in poorly aggressive cells by the aggressive cell-preconditioned matrix. These results suggest that COL-3 (and related chemically modified tetracyclines) may be useful in targeting molecular cues in the microenvironment of aggressive tumors and could potentially be used in a combinatorial manner with other therapies that specifically target and kill aggressive tumor cells.

Introduction

The Ln-5 γ2 chain, MMP-2, and MT1-MMP act cooperatively and are required for highly aggressive melanoma tumor cells to engage in vasculogenic mimicry when cultured on a three-dimensional ECM (1). Furthermore, Ln-5 γ2 chain promigratory fragments generated in an aggressive tumor cell-preconditioned matrix by MMP-2 and MT1-MMP proteolysis induce vasculogenic mimicry in poorly aggressive melanoma cells seeded onto this matrix (1). These observations suggest that deposition of Ln-5 γ2 chain into an ECM by aggressive melanoma cells can act as a latent trigger for aggressive behavior, and less aggressive cells entering this preconditioned environment (in the presence of active MMP-2 and MT1-MMP) respond by assuming a more aggressive, vasculogenic mimicry phenotype. As a result, treatment regimens that specifically target aggressive tumor cells may fail to address changes in the ECM microenvironment that persist after removal or destruction of the aggressive cells and could result in a recurrence or continuance of the tumor. Therefore, conceptually, a therapeutic regime that targets aggressive tumor cells should also consider changes in the microenvironment of the tumor that may ultimately harbor molecular cues or triggers that can induce less aggressive cells to become more aggressive, even after the aggressive cells have been removed or killed. A key component for developing this type of therapeutic approach might involve inhibiting the generation of Ln-5 γ2 chain promigratory fragments in the ECM of aggressive tumors to produce a more benign and less inductive microenvironment.

One class of drugs that is presently in clinical trials based on their antimetastatic (2–5) and antiangiogenic (6, 7) activities is the CMTs. CMTs are potent inhibitors of MMP activity (2–7), and COL-3 (6-demethyl-6-deoxy-4-dedimethylaminotetracycline) is currently being evaluated for treatment of patients with refractory solid tumors (8–10). The work described in this study presents evidence that COL-3 treatment not only inhibits vasculogenic mimicry by aggressive/meta-

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3 The abbreviations used are: Ln-5, laminin 5; CMT, chemically modified tetracycline; ECM, extracellular matrix; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; RT-PCR, reverse transcription-PCR; VEGF, vascular endothelial growth factor; VE-cadherin, vascular endothelial-cadherin; TIE-1, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
static human melanoma cells grown in three-dimensional culture but also prevents the production of Ln-5 α2 chain promigratory fragments in the ECM and blocks the induction of vasculogenic mimicry by poorly aggressive melanoma cells placed on the aggressive cell-preconditioned matrix. These results suggest that COL-3 (and related CMTs) may provide an effective therapy for targeting molecular cues in the tumor microenvironment that could be administered in a coordinated manner with other therapies that specifically target and kill aggressive tumor cells.

Materials and Methods
Cell Culture. The human cutaneous (C8161) and uveal (MUM-2B and MUM-2C) melanoma cell lines have been described previously (1, 11–15) and were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bio-products, Calabasas, CA). Cell cultures were determined to be free of Mycoplasma contamination using the GenProbe rapid detection system (Fisher, Itasca, IL).

A critical aspect of this work was the careful production of three-dimensional type I collagen gels (1) as follows: 25 μl of type I collagen (average 3 mg/ml; Collaborative Biomedical, Bedford, MA) were dropped onto 18-mm glass coverslips in 12-well culture dishes and polymerized with an application of 100% ethanol for 5 min at room temperature. After washing with PBS (minus divalent cations), tumor cells were seeded onto the three-dimensional gel in complete medium. For experiments designed to analyze the ability of the cells to engage in vasculogenic mimicry using phase-contrast microscopy, cells were plated onto the three-dimensional matrix with or without 3 μg/ml COL-3 or CMT-5 (kind gifts from Calabasas, CA). Cell cultures were determined to be free of Mycoplasma contamination using the GenProbe rapid detection system (Fisher, Itasca, IL).

Western Blot and Substrate-incorporated SDS-PAGE (Zymographic) Analyses. Detection of the Ln-5 α2 chain and its cleavage fragments in three-dimensional cultures containing highly aggressive C8161 or MUM-2B cells plus and minus either COL-3 or CMT-5 was performed four times independently as follows: cells plus and minus 3 μg/ml COL-3 or CMT-5 were seeded as stated above in 12-well culture dishes with the CMT added at the time of seeding and daily thereafter. After 3 days, the cultures were washed with PBS, and then complete medium.

Results
Highly aggressive/metastatic human melanoma MUM-2B cells cultured on a three-dimensional collagen I matrix form patterned, vasculogenic-like networks, defined as vasculogenic mimicry (Fig. 1A; Refs. 1, 12–15, 17, and 18). These
cells, unlike the poorly aggressive MUM-2C cells, express Ln-5 y2 chain, which is subsequently cleaved by MMP-2 and MT1-MMP to produce promigratory fragments that become incorporated into the matrix (1). The data show that CMT-5 (control) did not disrupt vasculogenic mimicry in vitro (Fig. 1B), whereas COL-3, a potent inhibitor of MMP activity (2–7), inhibited vasculogenic mimicry by aggressive melanoma cells cultured on a three-dimensional collagen matrix (Fig. 1C). Whereas poorly aggressive MUM-2C cells do not form vasculogenic-like networks on a non-preconditioned three-dimensional matrix (Fig. 1D, inset), they did assume a vasculogenic phenotype and initiated the formation of patterned, vasculogenic-like networks when seeded onto a three-dimensional matrix preconditioned by aggressive MUM-2B cells (subsequent to removal of the aggressive cells; Fig. 1D). Also, although treatment of MUM-2B cells on

### Table 1 Vasculogenic mimicry-associated markers

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR primers</th>
<th>Amplicon size (bp)</th>
<th>Cycle no.</th>
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<tr>
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<tr>
<td></td>
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<td>MMP-2</td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>MMP-9</td>
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<tr>
<td></td>
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<td>629</td>
<td>28*</td>
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<tr>
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<tr>
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<td>721</td>
<td>30</td>
</tr>
<tr>
<td>TIE-1</td>
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<tr>
<td>GAPDH</td>
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<td>983</td>
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<tr>
<td></td>
<td>5′-CATGTTGGCCCATAGGTTAGGACAC-3′</td>
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* In the presence of 5% DMSO.
a three-dimensional matrix with CMT-5 (control) did not inhibit the preconditioned matrix from inducing vasculogenic mimicry in MUM-2C cells (Fig. 1E). COL-3 treatment inhibited the induction of vasculogenic mimicry in MUM-2C cells grown on the aggressive cell-preconditioned matrix (Fig. 1F). These results demonstrate the inductive potential of matrices preconditioned by aggressive melanoma cells and the anti-vasculogenic effects of a specific CMT.

Western blot analysis revealed that three-dimensional collagen cultures of highly aggressive/metastatic C8161 and MUM-2B cells contain Ln-5 γ2 chain plus Ln-5 γ2' and Ln-5 γ2x promigratory fragments (Fig. 2A, controls). Addition of COL-3 (but not CMT-5) resulted in a loss of the promigratory fragments (Fig. 2A). Zymography of the supernatants showed a decrease in the activity of both active and pro-MMP-2 and active and pro-MMP-9 (Fig. 2B). These data demonstrate the effectiveness of COL-3 in inhibiting MMP activity and the subsequent generation of Ln-5 γ2 and Ln-5 γ2x promigratory fragments.

Semi-quantitative RT-PCR was then used to examine changes in the expression of vasculogenic mimicry-associated markers in the highly aggressive/metastatic C8161 and MUM-2B cells in response to COL-3 or CMT-5 when cultured on collagen three-dimensional matrices (Fig. 3A) and in poorly aggressive MUM-2C cells cultured on a MUM-2B cell-preconditioned matrix in the presence or absence of COL-3 or CMT-5 (Fig. 3B). Highly aggressive cells treated with CMT-5 (control) in three-dimensional culture showed little change in the expression of Ln-5 γ2 chain, MMP-2, MMP-9, MMP-14, VE-cadherin, VEGF-C, or TIE-1 in either cell line. In contrast, COL-3 treatment resulted in decreased expression of MMP-2, MMP-9, MMP-14, VE-cadherin, and VEGF-C in C8161 cells and MMP-2, MMP-9, VE-cadherin, VEGF-C, and TIE-1 in MUM-2B cells (Fig. 3A). The relative change in expression compared with the untreated control cells (normalized to 100% and corrected for loading against GAPDH expression) are summarized in Table 2. MUM-2C cells, which do not express Ln-5 γ2 chain when cultured on a collagen three-dimensional matrix (Fig. 3B), express Ln-5 γ2 chain when cultured on a matrix preconditioned by MUM-2B cells. Whereas addition of CMT-5 to the MUM-2B cells cultured on a collagen three-dimensional matrix did not inhibit the subsequent ability of the preconditioned matrix to induce Ln-5 γ2 chain expression in MUM-2C cells, COL-3 treatment inhibited the induction of Ln-5 γ2 chain expression in MUM-2C cells by the preconditioned matrix (Fig. 3B). These results show the inductive influence of matrices preconditioned by aggressive melanoma cells and the inhibitory effects of COL-3 on matrix-induced gene expression.

**Discussion**

Previous work demonstrated that Ln-5 γ2 chain is significantly overexpressed (up to 50-fold) in highly aggressive melanoma cells.
compared with poorly aggressive human melanoma cells (1). When deposited into the ECM by the aggressive cells, laminin was shown to colocalize with the de novo generated tubular networks in vitro characteristic of melanoma vasculogenic mimicry (1) and in a manner similar to laminin-associated patterned networks in histological sections of aggressive melanoma tumors. Furthermore, inhibition of MMP-2 and MT1-MMP activity prevented generation of Ln-5 γ 2 chain promigratory fragments and resulted in a loss of the vasculogenic phenotype by aggressive cells. A significant observation from this previous work was that less aggressive melanoma cells, which do not engage in vasculogenic mimicry on three-dimensional matrices in vitro, assumed the more aggressive vasculogenic phenotype when cultured on three-dimensional matrices preconditioned by aggressive melanoma cells. These results suggest that a therapeutic regime designed to specifically target aggressive tumor cells without addressing changes in their microenvironment could result in conditions where less aggressive cells, which may not be killed by the initial treatment regime, become more aggressive as they encounter the modified microenvironment. Because a key component of these events is the presence and level of activity of MMP-2 and MT1-MMP, the current study sought to examine the potential therapeutic consequence of inhibiting these specific MMPs with respect to maintaining the vasculogenic phenotype of aggressive melanoma cells and inducing this phenotype in poorly aggressive melanoma cells.

As proof of principle, CMTs were used to inhibit melanoma cell-associated MMP activity. Previously, CMTs were shown to inhibit human tumor cell invasion (2–4), metastasis (2–5), and angiogenesis (6, 7) and, for some derivatives, the expression of MMP-2 and MMP-9 (2, 19, 20). Recent studies have also shown that CMT-8 and estrogen can promote wound healing in ovariectomized rats by normalizing wound bed total collagen content and structure and recovering the expression and processing of Ln-5 γ 2 (21), indicating differences in the specific activities of CMTs. Because the ability of tetracyclines to inhibit MMP activity is distinct from their antimicrobial activity, modified forms of tetracyclines have been generated that are no longer effective as antibiotics but retain their ability to inhibit MMP activity. Some of these modified derivatives can attain a higher concentration in serum with a longer half-life and much greater potency than the unmodified forms (22). As such, CMTs provide a family of potent inhibitors of MMP activity (and expression) that can be used in long-term treatment regimes without concern for the side effect of generating antibiotic-resistant microorganisms that could result from long-term antibiotic therapy. Based on the history of therapeutic use of tetracycline and its derivatives in patients, a nonantibiotic, chemically modified derivative (CMT-3 or COL-3) that inhibits MMP-2 (2–4, 19, 20) and MT1-MMP (3) activity associated with tumor cell invasion (2–4), metastasis (2–5), tumor angiogenesis (6, 7), and vasculogenic mimicry (1) is presently in clinical trials in patients with refractory solid metastatic tumors (8–10). The work presented here extended these observations by examining the potential therapeutic use of COL-3 compared with CMT-5 (a modified tetracycline that does not inhibit MMP activity or expression) on melanoma cell vasculogenic mimicry and the ability of an aggressive cell-preconditioned matrix to induce this vasculogenic-like phenotype in poorly aggressive cells. Thus, the purpose of the current study was to examine the molecular underpinnings of COL-3 inhibition of
vasculogenic mimicry with respect to MMP activity and select vasculogenic-associated genes. The results demonstrate that COL-3 inhibited both vasculogenic mimicry in highly aggressive melanoma cells cultured on a three-dimensional collagen matrix and the induction of vasculogenic mimicry in poorly aggressive melanoma cells seeded on an aggressive melanoma cell-preconditioned matrix (Fig. 1). Coincident with these results was a decrease in MMP-2 and MT1-MMP activity and a decrease in Ln-5 \( \gamma^2 \) and Ln-5 \( \gamma^2 \times \gamma^2 \) promigratory fragments in the aggressive cell-preconditioned matrix (Fig. 2). Furthermore, semiquantitative RT-PCR showed that COL-3 treatment resulted in a decrease in expression of MMP-2, MMP-9, and MT1-MMP, as well as a decrease in expression of other vasculogenic markers previously identified in aggressive cells (Fig. 3). These results suggest that COL-3 inhibited vasculogenic mimicry in aggressive cells by inhibiting MMP expression and activity and the production of Ln-5 \( \gamma^2 \) and Ln-5 \( \gamma^2 \times \gamma^2 \) promigratory fragments generated from Ln-5 \( \gamma^2 \) chain expressed and deposited into the matrix by aggressive cells. Because COL-3 reduced the levels of Ln-5 \( \gamma^2 \) and Ln-5 \( \gamma^2 \times \gamma^2 \) promigratory fragments in the aggressive cell-preconditioned matrix, it could no longer induce vasculogenic mimicry in the less the aggressive cells. RT-PCR also revealed that COL-3 treatment down-regulated the expression of previously identified vasculogenic mimicry markers in the aggressive cells, including VE-cadherin and TIE-1, and the lymphangiogenic marker VEGF-C (Table 2; Refs. 12, 14, and 15). In the case of VE-cadherin, COL-3 completely inhibited its expression, suggesting its potential importance as a suppressor of the vasculogenic phenotype.

Whereas it was previously shown that less aggressive melanoma cells cultured on a three-dimensional matrix pre-conditioned by aggressive melanoma cells assumed the more aggressive (i.e., vasculogenic mimicry) phenotype (1), a key observation from the present work was that poorly aggressive MUM-2C cells cultured on a three-dimensional matrix pre-conditioned by the aggressive MUM-2B cells were induced to express higher levels of the Ln-5 \( \gamma^2 \) chain. This observation demonstrated that poorly aggressive melanoma cells are not only responding physically to their environment but also respond to cues and signals that can result in the induction of genes and proteins associated with an aggressive, vasculogenic mimicry phenotype and may suggest a feedback loop that continues to promote the aggressive phenotype while also inducing poorly aggressive cells to become more aggressive. An important extension of this finding, in terms of a therapeutic treatment that targets the microenvironment of an aggressive tumor, is that COL-3 (but not CMT-5) treatment of the aggressive cells on the three-dimensional matrix before addition of the poorly aggressive cells inhibited the induction of Ln-5 \( \gamma^2 \) chain in the poorly aggressive cells.

In conclusion, COL-3, a CMT presently in clinical trials for treatment of patients with refractory solid tumors, inhibited MMP activity and vasculogenic mimicry associated with aggressive melanoma tumor cells cultured on a three-dimensional matrix. Furthermore, COL-3 treatment prevented an aggressive cell-preconditioned matrix from inducing vasculogenic mimicry and the expression of Ln-5 \( \gamma^2 \) chain in poorly aggressive melanoma cells. Although serious side effects have been reported in some clinical trials using COL-3 (8–10), our work suggests that the optimum dosage and/or delivery schedule of CMT necessary to inhibit the inductive nature of the modified ECMs could be well below the amount of CMT presently used as a primary antimetastatic/antiangiogenic compound. Because tetracyclines have been used successfully to treat a variety of diseases using prolonged applications without the severity of side effects observed in some of the above-mentioned clinical trials (for example, see Refs. 23 and 24), these results suggest that COL-3 (and related CMTs) may be therapeutically useful as adjuvant therapy combined with other treatment modalities to specifically target the tumor cell microenvironment to inhibit the promotion or recurrence of certain aggressive cancer tumors.

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References


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