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Remodeling of Collagen Matrix by Human Tumor Cells Requires Activation and Cell Surface Association of Matrix Metalloproteinase-2[1]

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ABSTRACT

We assessed the functional significance of tumor cell-associated matrix metalloproteinase (MMP)-2 in extracellular matrix remodeling compared with that of the soluble enzyme by evaluating the contraction of three-dimensional collagen lattices by human glioma U251.3 and fibrosarcoma HT-1080 cell lines. In this model, the constitutive synthesis and activation of the MMP-2 proenzyme were modulated by stable transfections of tumor cells with cDNA encoding membrane type 1-MMP (MT1-MMP). The efficiency of transfected cells in contracting collagen lattices was shown to be dependent on the MT1-MMP-mediated activation of MMP-2 accompanied by cell surface association of activated MMP-2, on the cell-matrix interactions controlled by collagen-specific integrins, and on the integrity of actin and microtubule cytoskeletons. Each one of these mechanisms was essential but was not sufficient by itself in accomplishing gel contraction by MT1-MMP-transfected cells. Both MMP-2 activation and gel contraction by transfected glioma cells were inhibited by tissue inhibitor of metalloproteinase (TIMP)-2 and the recombinant COOH-terminal domain of MMP-2. However, the kinetics and mechanisms of their inhibitory effects were different, because TIMP-2 and the COOH-terminal domain of MMP-2 preferentially inhibited the MT1-MMP-dependent and autocatalytic steps of MMP-2 activation, respectively. By contrast, TIMP-1, an efficient inhibitor of soluble MMP-2 activity, failed to affect gel contraction. In addition, soluble MMP-2 activated by either organomercurials or cells was not able to induce the contraction of collagen lattices when added to transfected cells. Therefore, soluble activated MMP-2, sensitive to TIMP-1 inhibition, does not mediate collagen gel contraction by tumor cells, whereas the activity of cell surface-associated MMP-2 plays a critical role in remodeling of the extracellular matrix *in vitro*. These mechanisms of functional and spatial regulation of MMP-2 may also be applicable to different aspects of tissue reorganization *in vivo*, including cell migration and invasion, angiogenesis, and wound healing.

INTRODUCTION

Remodeling of the ECM[3] plays a critical role in the reorganization of connective tissue under both normal and pathophysiological conditions. The ECM turnover is initiated by proteolytic enzymes, mainly by the serine proteinases of the urokinase-type plasminogen activator pathway and MMPs (1). The most abundant MMP, gelatinase A or MMP-2, is secreted as a latent proenzyme by many cell types. To exert its enzymatic activity, MMP-2 requires cleavage and activation by the MT-MMPs including MT1-MMP (2). Both MMP-2 activation and MMP-2 activity are critically dependent on TIMP-2 (3). The expression of MT1-MMP and activation of MMP-2 were shown to be strongly associated with tumor invasion and metastasis (2, 4, 5), making the MT1-MMP/MMP-2 system an attractive target for the prognosis and prevention of tumor progression.

Recently, in addition to MT-MMPs (2) and TIMP-2 (3), $\alpha_v\beta_3$ integrin has been identified as a potentially critical component for docking MMP-2 at the tumor cell surfaces (6, 7). MMP-2 apparently binds near or to the ECM recognition site(s) of $\alpha_v\beta_3$ through the COOH-terminal domain of the enzyme. The binding of MMP-2 to integrins could localize ECM degradation to the tumor cell's microenvironment and thus spatially regulate the activity of the enzyme. In this regard, the proteolytic activity of MT1-MMP and MMP-2 on the cell surface has been localized to invadipodia of human RPMI7951 melanoma cells and was shown to initiate a proteolytic cascade important for cell invasion (8). Additionally, our most recent studies demonstrate that MT1-MMP and $\alpha_v/\alpha_v\beta_3$ are both required for the activation and spatial regulation of MMP-2 activity. This could represent a novel molecular mechanism for effective and selective localization of the activity of the enzyme directly at the cell surfaces.

Thus, MMP-2 activation and docking systems consist of several critical proteins, including proenzymes and activated enzymes of MT1-MMP and MMP-2, TIMPs, and integrins, all of which orchestrate a stepwise formation of heteromolecular complexes and a series of proteolytic cleavages. By these mechanisms, the latent M_r 68,000 MMP-2 proenzyme is ultimately converted into the mature active M_r 62,000 MMP-2 enzyme via the formation of a M_r 64,000 intermediate. This intermediate is generated after MT1-MMP-mediated cleavage of the NH_2-terminal part of MMP-2. The M_r 64,000 MMP-2 intermediate is characterized by some intrinsic proteolytic activity and autocatalytically generates the M_r 62,000 mature MMP-2 enzyme (3, 9). TIMP-2 plays a critical role in MMP-2 activation, because the NH_2-terminal and COOH-terminal domains of TIMP-2 complex MT1-MMP and MMP-2, respectively (10–12). The MT1-MMP/TIMP-2 heteromolecular complex is essential in activating the inhibitor-free MMP-2 proenzyme. Accordingly, the soluble preformed TIMP-2/MMP-2 proenzyme complex is largely resistant to MT1-MMP-mediated activation. Therefore, insufficiency and excess of TIMP-2 are both inhibitory for MMP-2 activation (3). In contrast to TIMP-2, TIMP-1 is incapable of a complex formation with the MMP-2 proenzyme (13). All known TIMPs inhibit activated MMP-2 species by targeting the active site of the enzyme. Specifically, the activity of MMP-2 is sensitive to inhibition by both TIMP-1 and TIMP-2 (3). MT1-MMP is also sensitive to inhibition with TIMPs; however, TIMP-1 is less effective than TIMP-2 in inhibiting MT1-MMP activity (14). The individual COOH-terminal domain of MMP-2 abolishes MMP-2 activation by competitively inhibiting the binding of the MMP-2 proenzyme to the MT1-MMP/TIMP-2 heteromolecular complex and, according to the latest findings, also by competing the binding integrin of MMP-2, $\alpha_v\beta_3$ (6, 7).

Contraction of three-dimensional gel lattices by normal and tumor cells represents a suitable *in vitro* model to study the underlying mechanisms of cell-matrix interactions. Several major events have been implicated in the contraction of cell-populated lattices. To efficiently accomplish collagen gel contraction, embedded cells should establish contacts with the substratum through the β_1 subfamily of integrins such as $\alpha_2\beta_1$ and $\alpha_1\beta_1$ (15–21). Similarly, the contraction of laminin-enriched Matrigel by human endothelial cells is dependent on $\alpha_6\beta_1$, the laminin-specific integrin (22). In addition, condensation and alignment of matrix fibrils in the direct vicinity of embedded cells as well as cell locomotion, the formation of cell clusters, and the development of contractile ring and actin cable

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[3] The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT, membrane type; mAb, monoclonal antibody; TIMP, tissue inhibitor of metalloproteinase; APMA, *p*-aminophenylmercuric acetate; TRITC, tetramethylrhodamine isothiocyanate.

are prerequisites for the efficient contraction of collagen and fibrin gels (21, 23, 24). Furthermore, the actin stress fiber network is apparently required for the contraction of collagen gels by different cell types (16, 25, 26). Additionally, microtubules are thought to function as rigid struts opposing the cellular contraction (27). Other contributors to gel contraction involve growth factors (19, 20, 28–30) and ECM proteins deposited by gel-embedded cells including collagen (31), fibronectin (32), decorin (33), tenascin (34), and osteopontin (35).

There are some indications that the synthesis of MMP-2 and, more specifically, the activation of MMP-2 are critical for cell-mediated collagen gel contraction. Thus, phenanthroline, a strong chelator and a repressor of MMP activity, inhibited collagen gel contraction, whereas phorbol 12-myristate 13-acetate, an inducer of MMP-2 activation, promoted gel contraction by bovine endothelial cells (30). In two-dimensional cultures, human fibroblasts routinely do not activate MMP-2. However, embedding into three-dimensional collagen gels induces human fibroblasts to activate MMP-2 and eventually to contract collagen lattices (16). MMP-2 activation stimulated by the incorporation of fibroblasts into three-dimensional collagen correlated with up-regulation of MT1-MMP mRNA (26), whereas cytochalasin-induced MMP-2 activation in rat mesanglial cells was accompanied by down-regulation of TIMP-2 (25).

Here, we evaluate the significance of cell surface-associated MMP-2 enzyme in ECM remodeling and subsequent collagen gel contraction by human U251.3 glioma and HT-1080 fibrosarcoma cells. We demonstrate that only tumor cells capable of both sufficient synthesis and activation of MMP-2 successfully reorganize and contract three-dimensional collagen lattices. Furthermore, our results suggest that cell surface-associated MMP-2 but not the soluble enzyme specifically contributes to cell-mediated remodeling of the ECM.

MATERIALS AND METHODS

Reagents. Ascitic fluids containing function-blocking murine mAbs against human integrins α_2 (P1E6), α_3 (P1B5), and β_1 (P4C10) were from Chemicon International (Temecula, CA) and Life Technologies, Inc. (Gaithersburg, MD). Ascitic fluid induced in BALB/c mice by murine myeloma 45.6 (American Type Culture Collection, Rockville, MD) was used as a control mAb. FITC-conjugated $F(ab')_2$ fragment of sheep antimouse or goat antirabbit IgG antibodies and falloidin conjugated with TRITC, cytochalasin B, and nocodazole were from Sigma (St. Louis, MO). Polyclonal rabbit MT1-MMP-specific antibodies were a kind gift of Dr. P. Alexander (Triple Point Biologicals, Forest Grove, OR). Collagen type I (Vitrogen 100) was from Collagen Corp. (Fremont, CA). Human recombinant TIMP-1 was a generous gift from Dr. A. Doherty (Celltech, Slough, United Kingdom). Human recombinant TIMP-2 was from Calbiochem (Cambridge, MA). The TIMP-2-free MMP-2 proenzyme was isolated from the conditioned medium of p2AHT2A72 cells derived from HT-1080 fibrosarcoma cell line consequently transfected with E1A (36) and MMP-2 (10) cDNAs. When necessary, the MMP-2 proenzyme was activated for 10–30 min with 1 mM APMA in 200 mM Tris and 0.1 mM $CaCl_2$ (pH 7.5) at ambient temperature. Activation of the enzyme was verified by gelatin zymography, and the activated enzyme was extensively dialyzed against 5 mM Tris, 0.1 mM $CaCl_2$, and 0.005% Brij 35 (pH 7.5). The recombinant COOH-terminal domain of MMP-2 was isolated as the FLAG-fusion protein from the periplasmic fraction of *Escherichia coli* as described previously (3).

Cell Lines and Culture Conditions. Transfected cell lines originated from human glioma U251.3 cells (a clone derived from the parental U251MG line); fibrosarcoma HT-1080 cells (American Type Culture Collection) have been described previously (7, 10, 36, 37, 38). Glioma U-MT and fibrosarcoma HT-MT23 cell lines stably transfected with cDNA encoding MT1-MMP (2, 3) constitutively activate the MMP-2 proenzyme (7, 37). Similar to parental cells, the corresponding neo control cell lines, U-neo and HT-neo, failed to significantly activate the MMP-2 proenzyme (7, 37). Transfected cells were routinely grown in DMEM supplemented with 10% FCS (Tissue Culture Biologicals, Tulare, CA) and 0.25 mg/ml G418 (Life Technologies, Inc.) at 37°C in a humidified incubator in the presence of 7.0% CO_2.

Flow Cytometry Analysis of MT1-MMP Expression. Fluorescence-activated cell-sorting analyses were performed as described previously (39). All staining procedures were done on ice in Dulbecco's PBS supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1% BSA (DPBS/BSA; pH 7.2). Cells were incubated with 5 μg/ml polyclonal rabbit anti-MT1-MMP antibodies for 45 min followed by a FITC-conjugated $F(ab')_2$ fragment of goat antirabbit IgG antibodies (1:100) for 30 min. After the removal of unbound antibodies, cells were resuspended in DPBS/BSA supplemented with 3 μg/ml propidium iodide (Sigma), and viable cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Population gates were set by using cells incubated with normal rabbit IgG (Chemicon).

Collagen Lattice Cultures. In our studies, cells were embedded into three-dimensional collagen in the serum-free AIM-V medium (Life Technologies, Inc.). This medium allowed us to avoid the undesirable effects of FCS components such as MMPs, TIMPs, growth factors, cytokines, and ECM proteins that could affect MMP-2 activation and collagen gel contraction. The AIM-V medium does not cause apoptosis and supports cell functions including cell proliferation, migration, and invasion as well as the production and MT1-MMP-mediated activation of the MMP-2 proenzyme (7, 37).

Specifically, cells were seeded at 3×10^6 cells/100-mm Petri dish in DMEM supplemented with FCS and antibiotics. After an overnight incubation, the culture medium was exchanged with 7 ml of serum-free DMEM. After an 18-h incubation, conditioned medium was collected and stored at 4°C before analysis by gelatin zymography, and the cultures were washed with PBS and incubated in PBS for 30–45 min at 37°C. Thereafter, cells were detached by gentle pipetting, counted, centrifuged, resuspended at $1–2 \times 10^6$ cells/ml serum-free AIM-V medium supplemented with 0.25 mg/ml G418, and placed on ice. Collagen type I (3.1 mg/ml) was neutralized by mixing its eight parts with one part of 10× DMEM (ICN Biomedicals, Costa Mesa, CA) and one part of 0.1 M NaOH, resulting in a 2.5 mg/ml gel mixture. The collagen mixture was supplemented with G418. In preliminary experiments, several final concentrations of collagen and glioma U-MT cells were compared. A final concentration of 2×10^5 cells/ml in a 1.5 mg/ml collagen solution was chosen as the most effective concentration and was used thereafter in all gel contraction experiments. The test components were added to the cell-containing collagen mixture at the concentrations indicated in the text. A total volume of 0.5 ml of cell-containing mixtures was placed in each well of a tissue culture 24-well cluster (Costar, Cambridge, MA) and allowed to polymerize for 45–60 min at 37°C in a humidified air incubator, and then 0.3–0.5 ml of AIM-V medium, with or without the corresponding additives, was added into each well of the polymerized gels. After 4–5 days at 37°C in a CO_2 incubator, the collagen gels were released from the walls of the wells with a thin spatula, and plates were returned to the incubator. After an overnight incubation, the diameter of each released gel was measured, the conditioned medium was collected for further analyses, and the cultures were fixed with 4% paraformaldehyde in PBS (pH 7.2).

Fluorescence Staining of Collagen Gel Cultures for Actin Filaments. Glioma U-neo and U-MT cells in AIM-V medium were each combined with a gel mixture to achieve final concentrations of 5×10^4 cells/ml and 1.5 mg/ml collagen. A total of 0.2 ml of cell-containing mixture was distributed on a 22 × 22-mm coverslip (Fisher Scientific, Pittsburgh, PA) previously placed in a well of a 6-well cluster. After polymerization at 37°C, 2 ml of AIM-V medium were added to each well. After an overnight incubation, the cultures were fixed with 4% paraformaldehyde in PBS (pH 7.2) for 20 min at room temperature, washed with PBS, and permeabilized for 6 min with 0.5% Triton X-100 in PBS (pH 7.2). After washing with PBS, cell-populated gels on coverslips were preincubated with DPBS/BSA and stained with TRITC-conjugated falloidin (1:5000 in DPBS/BSA) overnight at 4°C. After extensive washing with PBS, the coverslips with gels were mounted in SlowFade Light Antifade solution (Molecular Probes, Inc., Eugene, OR) onto glass slides. Confocal images were collected with differential-interference-contrast optics and epifluorescence optics at The Scripps Institute's confocal facility. The composites of contrast-corrected images were prepared using Adobe Photoshop software (San Jose, CA).

Gelatin Zymography. To visualize the activity of secretory MMP-2, cell-conditioned medium was mixed 1:1 with 2× SDS sample buffer, and 6–16 μl of the mixture were loaded into a well of the precast 10% zymogram gel copolymerized with 0.1% gelatin (Novex, San Diego, CA). To analyze the activity of cell-associated MMP-2, cell lysates were prepared from cultures of 5×10^6 cells. After an overnight incubation, the serum-containing medium in these cultures was exchanged with 10 ml of serum-free DMEM. After an 18-h

Table 1 *Characteristics of transfected glioma U251.3 and fibrosarcoma HT-1080 cell lines*

Transfected cell lines[a]			MT1-MMP expression[b]	MMP-2 production[c] (ng/10^6 cells/h)	MMP-2 activation[d] (%)	Gel contraction[e] (%)
Origin	Name	Transfected plasmid				
Glioma	U-neo	Control	18.3	45–55	0–10	15.1 ± 2.1
U251.3	U-MT	MT1-MMP	78.1	45–55	80–90	82.4 ± 0.9
Fibrosarcoma	HT-neo	Control	26.0	50–60	5–20	22.2 ± 3.2
HT-1080	HT-MT23	MT1-MMP	93.3	50–60	75–85	67.3 ± 4.8

[a] Parental human glioma U251.3 and fibrosarcoma HT-1080 cells were transfected with the original plasmid pcDNA3neo (control), resulting in the U-neo and HT-neo cell lines, and with the pcDNA3neo/MT1-MMP plasmid (MT1-MMP), resulting in the U-MT and HT-MT23 cell lines, respectively (7, 37).

[b] MT1-MMP expression was determined by flow cytometry after staining glioma U251.3 and fibrosarcoma HT-1080 transfected cells with MT1-MMP-specific antibodies as described in "Materials and Methods." Data are expressed as the logarithmic mean of fluorescence intensity from two representative experiments in which series of glioma and fibrosarcoma cell lines were analyzed simultaneously.

[c] MMP-2 production was estimated by a comparison of gelatin zymograms of serum-free DMEM conditioned by cells with purified MMP-2 isolated from medium conditioned by p2AHT2A72 cells (10, 38). Cells were plated in serum-containing DMEM at 3×10^6 cells/10-cm Petri dish and incubated overnight. Thereafter, the medium was exchanged with 7 ml of serum-free DMEM. After an 18–20-h incubation, conditioned medium was collected and analyzed by gelatin zymography as described in "Materials and Methods." Data are presented as nanograms of MMP-2 produced by 10^6 cells per hour.

[d] MMP-2 activation was estimated by densitometric analyses of zymograms and is presented as a percentage of conversion of the M_r 68,000 MMP-2 proenzyme into both the M_r 64,000 intermediate and the M_r 62,000 mature activated MMP-2. Zymography analyses of aliquots of conditioned medium were performed 5–6 days after embedding the transfected cells into three-dimensional collagen lattices.

[e] Gel contraction was estimated as a percentage of contracted gel area: that of original gel area 18–24 hours after the release of lattices at day 4–5. Data are presented as mean ± SE from 3–16 independent experiments performed in triplicates.

incubation, cell cultures were washed three times with ice-cold PBS and scraped in 2 ml of PBS. Cell pellets were collected by centrifugation at 2000 rpm, resuspended in PBS up to a total volume of 100 μl, and mixed with an equal volume of 2× SDS sample buffer. Twelve-μl aliquots from serial dilutions of cell lysates were loaded per well of a zymography gel. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30–60 min at room temperature; incubated overnight at 37°C in 50 mM Tris buffer (pH 8.0), 5 mM $CaCl_2$, and 1 μM $ZnCl_2$; and stained with Coomassie Blue to visualize bands of gelatinolytic activity.

Data Analysis and Presentation. The values presented are mean ± SE. Statistical analysis was performed by using Student's *t* test with SigmaStat, and the data were interpolated using SigmaPlot software (Jandel Corp., Corte Madera, CA).

RESULTS

Collagen Gel Contraction by Human Glioma U251.3 Cells and Fibrosarcoma HT-1080 Cells Correlates with MMP-2 Synthesis and Activation. Contraction of three-dimensional collagen gels involves matrix reorganization by embedded cells. We used this model to evaluate the physiological significance of activation and cell surface association of MMP-2 relative to that of soluble enzyme in cell-mediated remodeling of the ECM.

For this purpose, a series of human glioma U251.3 and fibrosarcoma HT-1080 cell lines were developed in which the activation of MMP-2 was quantitatively regulated by stable transfection with MT1-MMP cDNA. These cell lines were analyzed for MT1-MMP expression and MMP-2 production and activation as well as collagen gel contraction (Table 1).

Both U251.3 and HT-1080 parental cell lines were previously found to constitutively secrete high levels of the MMP-2 proenzyme but were unable to efficiently activate MMP-2 (7, 37). Stable transfection of these cell lines with MT1-MMP generated U-MT and HT-MT23 cells that overexpressed MT1-MMP and constitutively processed about 75–90% of the secreted M_r 68,000 MMP-2 proenzyme into the M_r 64,000 activation intermediate and the M_r 62,000 mature MMP-2 (Table 1; Fig. 1, *Lanes B* and *D*). Similar to parental cell lines, control U-neo and HT-neo cells did not significantly activate MMP-2 (Table 1; Fig. 1, *Lanes A* and *C*).

To assess the efficiency of transfected cell lines in contracting collagen gels, cells were embedded into collagen type I in serum-free AIM-V medium. After embedding into collagen, U-MT and HT-MT23 cells formed interconnecting cell clusters by day 2 and a characteristic cell contractile ring by day 4. At days 4–5, all gels were released from the walls of the wells and incubated for an additional 18–24 h; thereafter, their diameters were measured, and aliquots of the supernatant were analyzed by gelatin zymography.

Embedding cells into collagen and the release of gels did not affect the level of MMP-2 production and activation of any of the cell transfectants tested. Effective gel contraction was induced only by U-MT and HT-MT23 cells that were capable of both the synthesis and activation of MMP-2 (Fig. 2, *B* and *D*; Table 1). A slight activation of the MMP-2 proenzyme in three-dimensional cultures of U-neo and HT-neo control cells correlated with <25% contraction of collagen lattices (Fig. 2, *A* and *C*; Table 1). Thus, the contraction of collagen gels by U-MT and HT-MT23 cells correlated with their ability to produce and activate the MMP-2 proenzyme.

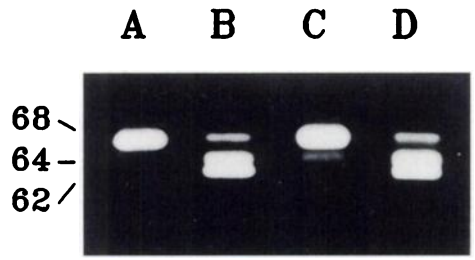

Fig. 1. Activation of MMP-2 by transfected human glioma U251.3 cells and fibrosarcoma HT-1080 cells. Transfected cells [U-neo cells (*Lane A*), U-MT cells (*Lane B*), HT-neo cells (*Lane C*), and HT-MT23 cells (*Lane D*)] were embedded into three-dimensional collagen and incubated in serum-free AIM-V medium. At day 5, 5-μl aliquots of conditioned media were analyzed by gelatin zymography as described in "Materials and Methods." The apparent molecular weight of the MMP-2 species (in thousands) is shown on the *left*.

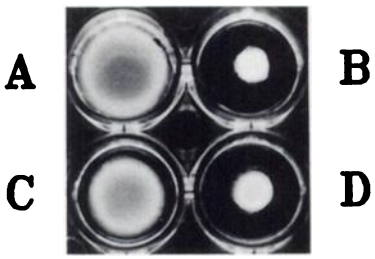

Fig. 2. MT1-MMP-transfected glioma U-MT and HT-MT23 cells efficiently contract collagen lattices. Transfected cells [U-neo cells (*A*), U-MT cells (*B*), HT-neo cells (*C*), and HT-MT23 cells (*D*)] were embedded into collagen gels and incubated in the wells of 24-well clusters as described in "Materials and Methods." At day 5, gels were released from the walls of the wells and returned to the incubator. Photographs were taken from the gels fixed in 4% paraformaldehyde 20 h after the release of the gels.

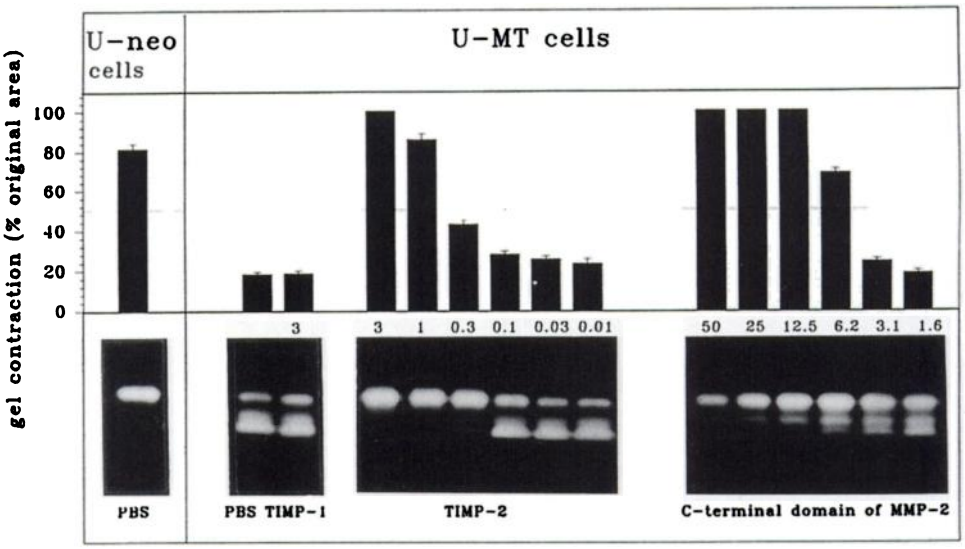

Fig. 3. Activation of MMP-2 and collagen gel contraction by glioma U-MT cells are inhibited by TIMP-2 and the COOH-terminal domain of MMP-2 but are not affected by TIMP-1. Control U-neo cells and MT1-MMP-transfected U-MT cells were embedded into three-dimensional collagen with and without TIMP-1, TIMP-2, and the COOH-terminal domain of MMP-2 at the final concentrations (micrograms/milliliter) indicated at the *bottom* of the bars. At day 5, the gels were released and incubated for an additional 18 h, and the diameter of the gels was measured. Gel contraction was determined as a percentage of gel area:that of the original gel area. Data are expressed as mean ± SE from a representative experiment performed in triplicate. Gelatin zymograms of conditioned media (5 μl/lane) are shown *below* the corresponding bars.

Inhibition of MMP-2 Activation Blocks Gel Contraction by Glioma U-MT Cells. To additionally verify that MMP-2 activation is essential for matrix remodeling and subsequent collagen gel contraction by tumor cells, we further assessed the effects of TIMP-2 and the recombinant COOH-terminal domain of MMP-2.

The inhibitory effects of TIMP-2 on MMP-2 activation have been attributed to the inhibitor's complex formation with the MMP-2 proenzyme and the inhibition of both MMP-2 and MT1-MMP activities (3, 13, 14). As expected, MMP-2 activation and gel contraction by U-MT cells were inhibited by TIMP-2 in a concentration-dependent manner (Fig. 3). At concentrations of 1–3 μg/ml, *i.e.*, a 7–20× molar excess over secretory MMP-2, TIMP-2 inhibited MMP activation and gel contraction by U-MT cells to U-neo control levels. Characteristically, U-MT cells in the presence of TIMP-2 failed to form cell clusters or develop a contractile ring, thus morphologically resembling U-neo cells. Importantly, increasing concentrations of TIMP-2 preferentially inhibited the MT1-MMP-dependent conversion of the MMP-2 proenzyme into the M_r 64,000 activation intermediate (Fig. 3), apparently due to the inhibitor's complex formation with the MMP-2 proenzyme and the inhibition of MT1-MMP activity.

The direct role of the COOH-terminal domain of MMP-2 is intrinsically distinct from that of TIMP-2. The individual COOH-terminal domain of MMP-2 is a specific inhibitor of MMP-2 binding to TIMP-2 and integrin $\alpha_v\beta_3$. This domain does not inhibit or directly bind MT1-MMP (3, 6, 7, 10). Accordingly, when the COOH-terminal domain competitively inhibits MMP-2 binding to the MT1-MMP/TIMP-2 complex, it prevents the conversion of the M_r 68,000 MMP-2 proenzyme to the M_r 64,000 MMP-2 activation intermediate (3). Our most recent observations also demonstrate that the COOH-terminal domain of MMP-2 competes the binding of activated MMP-2 to the cell surfaces, thus blocking the further autocatalytic conversion of the M_r 64,000 intermediate into the M_r 62,000 mature MMP-2.[4]

The recombinant MMP-2 COOH-terminal domain blocked both MMP-2 activation and gel contraction in a dose-dependent manner (Fig. 3). Gel contraction correlated directly with the extent of MMP-2 activation. The COOH-terminal domain preferentially inhibited the MT1-MMP-independent M_r 64,000→M_r 62,000 step of MMP-2 activation. These findings are in agreement with our earlier data that show that this particular step of MMP-2 activation by HT-1080 plasma membranes was most sensitive to inhibition with the COOH-terminal domain of MMP-2 (3). As shown by zymography, the conversion of the M_r 68,000 proenzyme to the M_r 64,000 intermediate is not inhibited at those concentrations of the COOH-terminal domain that are substantially inhibitory for both gel contraction and the M_r 64,000→M_r 62,000 conversion of MMP-2 (Fig. 3). At higher concentrations of the COOH-terminal domain, the TIMP-2/MT1-MMP-dependent conversion of the M_r 68,000 proenzyme to the M_r 64,000 intermediate of MMP-2 was blocked, apparently due to the saturation of available TIMP-2 (3).

The effects of another inhibitor of MMP-2 activity, TIMP-1, were also examined. TIMP-1 is an inhibitor of several MMPs that binds only the activated forms of MMP-2 (1). At 3 μg/ml (*i.e.*, about 22× molar excess to MMP-2), TIMP-1 altered neither the extent of MMP-2 activation nor the efficiency of lattice contraction by glioma U-MT cells (Fig. 3). To verify the functional activities of TIMP-1 and TIMP-2 used in our gel contraction experiments, we analyzed their inhibitory effects on APMA-induced activation of MMP-2. At a relatively low concentration, *i.e.*, 5× molar excess over MMP-2, both inhibitors efficiently blocked APMA-induced activation of MMP-2 (data not shown). Because TIMP-1 did not inhibit gel contraction at 22× molar excess to MMP-2, these results indicate that soluble MMP-2 does not significantly mediate gel remodeling and gel contraction. This conclusion is supported by two separate lines of evidence, as shown below.

Activity of Soluble MMP-2 Does Not Induce Collagen Gel Contraction by Glioma U-neo Cells. To confirm the above-mentioned findings, we further assessed whether collagen gel contraction by tumor cells could be induced by soluble, externally activated MMP-2. To this end, U-neo and U-MT cells were embedded into collagen with and without APMA-activated MMP-2. Gelatin zymography confirmed the expected levels of APMA-activated MMP-2 added to the cultures and the additional amounts of MMP-2 secreted by cells during a 5-day incubation (Fig. 4, *inset*). At a 0.01–3.0 μg/ml range of concentrations that broadly overlapped with the levels of MMP-2 secreted by transfected glioma cells, the APMA-activated enzyme did not affect collagen lattice contraction, *i.e.*, it neither induced nor inhibited gel contraction by U-neo and U-MT cells, respectively (Fig. 4; shown are the effects of 3.0 μg/ml APMA-activated MMP-2). To confirm the results of these experiments with cell-activated MMP-2, AIM-V medium conditioned by U-MT cells was added to gels populated with U-neo and U-MT cells. Again, no effects on gel contraction were observed in these experiments (data not shown). These results demonstrate that externally added soluble MMP-2

[4] E. I. Deryugina, M. A. Bourdon, T. I. Postnova, J. W. Smith, and A. Y. Strongin. MT1-MMP and integrin $\alpha_v\beta_3$ cooperate in activation of MMP-2 and directional migration of tumor cells, submitted for publication.

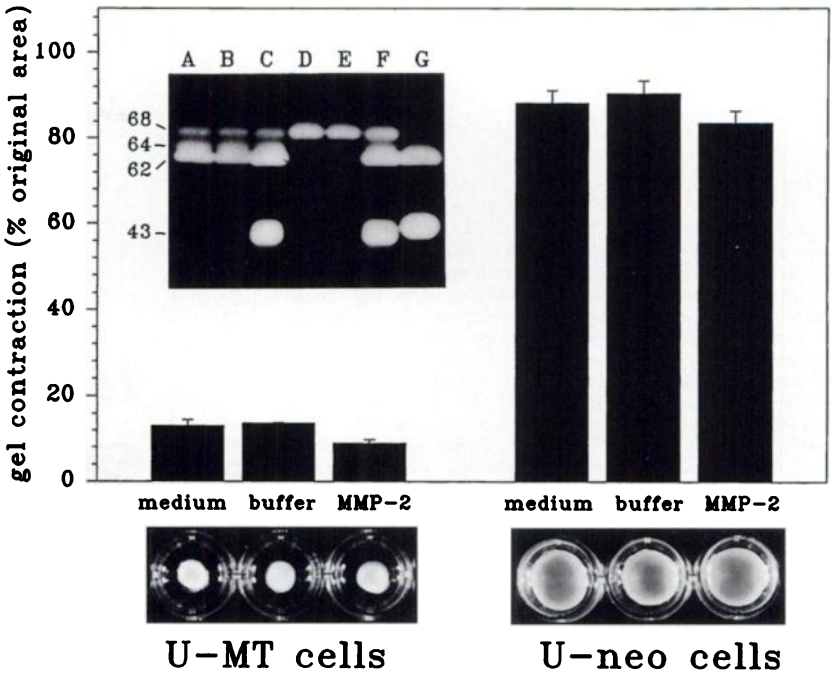

Fig. 4. Soluble APMA-activated MMP-2 does not induce gel contraction by glioma U-neo cells. Control U-neo cells and MT1-MMP-transfected U-MT cells were embedded into collagen and incubated in AIM-V medium alone (*medium*), in the presence of 3 μg/ml APMA-activated MMP-2 (*MMP-2*), or in corresponding amounts of the buffer used for the dialysis of activated MMP-2 (*buffer*). At day 5, the gels were released from the walls of the wells. After an overnight incubation, the diameter of each gel was measured, and conditioned medium was collected for zymography analysis. Gel contraction was determined as a percentage of gel area:that of the original gel area. Data are expressed as mean ± SE from a representative experiment performed in triplicate. Photographs of the representative gels fixed with 4% paraformaldehyde are shown at the *bottom* of the corresponding bars. *Inset*, zymogram of conditioned media (5 μl/lane) from U-MT (*Lanes A–C*) and U-neo (*Lanes D–F*) cultures incubated in AIM-V medium (*Lanes A* and *D*), in the presence of buffer used for the dialysis of activated MMP-2 (*Lanes B* and *E*), or with 3 μg/ml APMA-activated MMP-2 (*Lanes C* and *F*). *Lane G*, APMA-activated MMP-2 at a final concentration of 3 μg/ml, 5 μl/lane. The apparent molecular weight of MMP-2 species (in thousands) is indicated on the *left*.

enzyme is incapable of stimulating the specific matrix remodeling that is required for subsequent collagen gel contraction by glioma cells.

Collagen Gel Contraction by Glioma U-MT Cells Correlates with Cell Association of Activated MMP-2. To determine whether cell-mediated gel contraction correlates with the association of activated MMP-2 at cell surfaces, we analyzed cell lysates of U-neo and U-MT glioma cells by gelatin zymography (Fig. 5). Only the M_r 68,000 MMP-2 proenzyme was revealed in the cell lysates of control U-neo cells, indicating that the intracellular pool and any cell surface-associated MMP-2 consisted of the proenzyme only. In contrast, both the M_r 64,000 and M_r 62,000 activated MMP-2 species and MMP-2 proenzyme were found in U-MT cell lysates. Thus, specific association of MMP-2 with MT1-MMP-transfected cells correlates with cell-mediated matrix remodeling and collagen gel contraction. The amounts of MMP-2 identified in glioma cell lysates corresponded to ~60–80 ng/5 × 10^6 cells (50,000–100,000 molecules/cell) and constituted 0.5–1% of total secretory MMP-2 found in the conditioned medium (~7–15 μg/5 × 10^6 cells; Fig. 5).

Collagen Gel Contraction by Glioma U-MT Cells Requires Integrin-mediated Cell-Collagen Interactions and the Integrity of Actin and Microtubule Cytoskeletons. To confirm that our experimental cell system properly responded to some critical stimuli reported for other cell types (16, 17, 26, 27), we assessed whether gel contraction by MT1-MMP cell transfectants was dependent on integrin-mediated cell-matrix interactions and the integrity of actin and microtubule cytoskeletons.

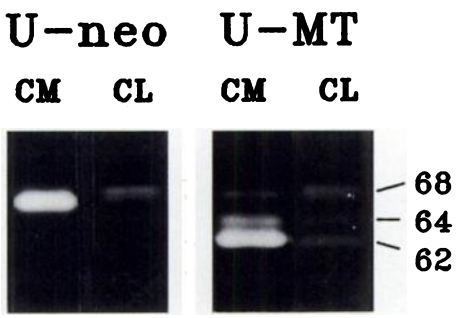

Fig. 5. Activated MMP-2 is specifically associated with MT1-MMP-transfected glioma U-MT cells. Conditioned medium (*CM*) and cell lysates (*CL*) were prepared and analyzed by gelatin zymography as described in "Materials and Methods." Visualized are bands of gelatinase activity associated with 3 μl of medium conditioned by ~1.5 × 10^3 cells and 12 μl of lysate from ~3.3 × 10^4 cells.

Collagen lattices populated with glioma U-MT and U-neo cells were incubated with control or function-blocking mAbs specific for human integrins α_2, α_3, and β_1. In the presence of integrin-specific antibodies, both U-neo and U-MT cells remained round, without characteristic spreading within the collagen matrix. In contrast to control mAb 45.6, the integrin-specific mAbs completely inhibited gel contraction by U-MT cells (Fig. 6). None of these antibodies significantly affected the extent of MMP-2 activation by U-neo and U-MT cells (Fig. 6). Thus, even in the presence of activated MMP-2, U-MT cells failed to contract the gels if cell-collagen interactions mediated by collagen-specific integrins were disrupted.

To identify polymerized actin, glioma cells embedded into three-dimensional collagen were stained with TRITC-labeled phalloidin. Confocal microscopy showed a well-developed actin filament network in both U-neo and U-MT cells (Fig. 7, *A* and *C*, respectively). Actin filaments were readily seen in the cytoplasm as stress fibers that ended up as large bundles in cell protrusions. The actin filament network in both U-neo and U-MT cells was sensitive to inhibition with cytochalasin B (Fig. 7, *B* and *D*, respectively). At a concentration of 1 μg/ml, cytochalasin B affected neither the production nor extent of MMP-activation by both U-neo and U-MT cells but completely inhibited gel contraction by U-MT cells (Fig. 8). The inhibition of gel contraction by U-MT cells was accompanied by a rapid depolymerization of actin filaments. One h after treatment with 1 μg/ml cytochalasin B, U-MT cells did not reveal any intact actin stress fibers (Fig. 7, *D*). Accordingly, U-MT cells failed to contract collagen lattices if treated with 1 μg/ml cytochalasin B 4 h before the release of gels.

Nocodazole, an inhibitor of microtubule polymerization, severely suppressed MMP-2 production by glioma cells when used at micromolar concentrations. In contrast, at 200 nM, this inhibitor did not significantly affect MMP-2 production and activation but caused a complete inhibition of gel contraction by U-MT cells. Similar to cytochalasin B, nocodazole did not induce any significant MMP-2 activation in U-neo cells (Fig. 8).

Taken together, our data indicate that in addition to efficient MMP-2

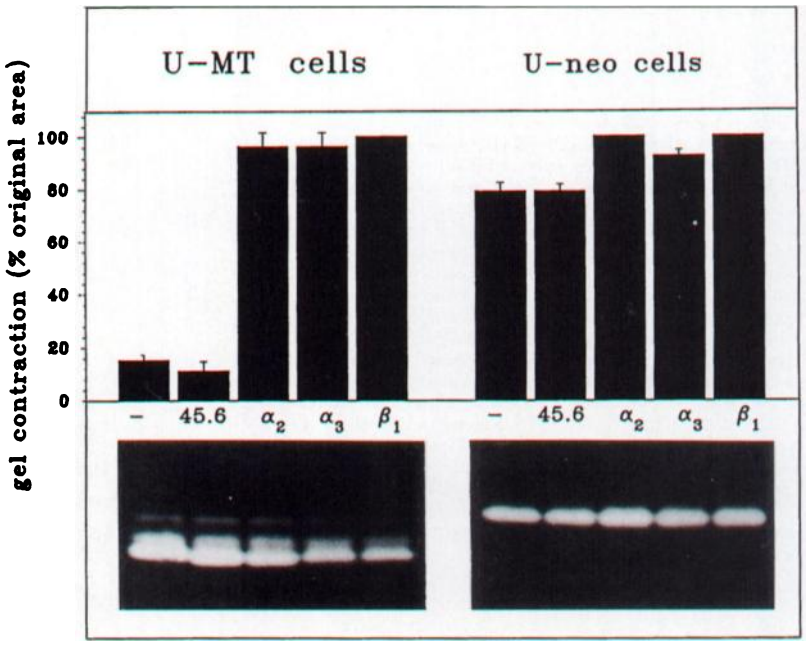

Fig. 6. Gel contraction and activation of MMP-2 by transfected glioma cells in the presence of integrin-specific function-blocking mAbs. Control U-neo cells and MT1-MMP-transfected U-MT cells were embedded into three-dimensional collagen and cultured with or without (−) mAbs added as ascitic fluids to a final concentration of 1:100. At day 5, the gels were released from the walls of the wells. After an overnight incubation, the diameter of each gel was measured, and conditioned medium was collected for zymography analysis. Gel contraction was determined as a percentage of gel area:that of the original gel area. Data are expressed as mean ± SE from a representative experiment performed in triplicate. The specificity of mAbs added to cultures is indicated at the *bottom* of the corresponding bars (*45.6*, control mAb; α_2, mAb P1E6; α_3, mAb P1B5; β_1, mAb P4C10). Gelatin zymograms of conditioned media (5 μl/lane) are shown *below* the corresponding bars.

synthesis and activation and cell surface association of activated MMP-2, integrin-mediated cell-collagen interactions and the integrity of actin and microtubular cytoskeletons are also prerequisites for effective collagen gel contraction by tumor cells.

DISCUSSION

In this study, we addressed the functional significance of cell surface-associated MMP-2 relative to its soluble form in tumor cell-

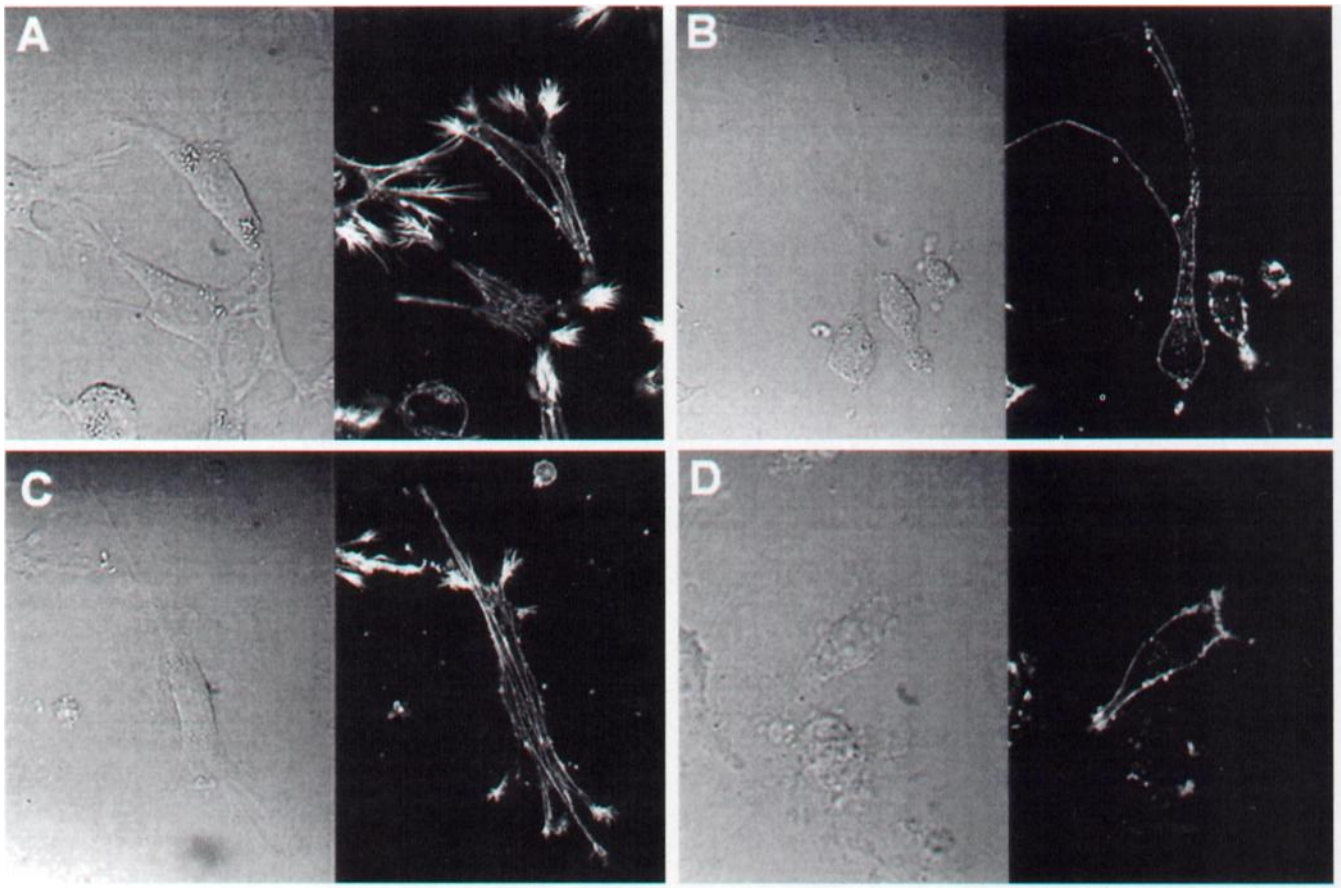

Fig. 7. The actin filament network in glioma cells within three-dimensional collagen is sensitive to cytochalasin B. MT1-MMP-transfected U-MT (*A* and *B*) and control U-neo (*C* and *D*) glioma cells were embedded into collagen gel and incubated on coverslips overnight in serum-free AIM-V medium. To reveal actin, the cells were stained with TRITC-falloidin as described in "Materials and Methods." One h before staining, cytochalasin B was added to cell cultures at a final concentration of 1 μg/ml (*B* and *D*) from a stock solution in DMSO (1 mg/ml). DMSO was added at a final concentration of 0.1% (v/v) to control cultures (*A* and *C*). Confocal images of cells were collected with differential-interference-contrast (*A–D, left panels*) or epifluorescence optics (*A–D, right panels*).

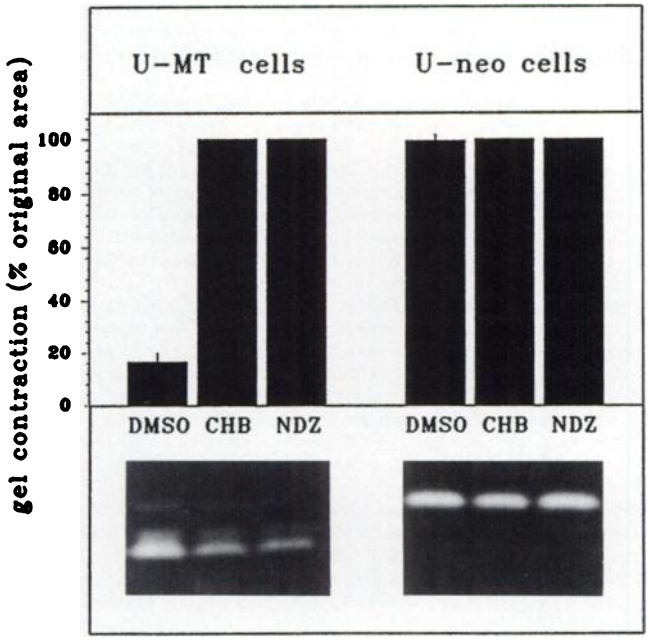

Fig. 8. Gel contraction by U-MT glioma cells depends on the integrity of the actin and microtubule cytoskeletons. Control U-neo cells and MT1-MMP-transfected U-MT cells were embedded into collagen gels and incubated in serum-free AIM-V medium with cytochalasin B (*CHB*; 1 μg/ml) or nocodazole (*NDZ*; 200 ng/ml) added from the inhibitors' stock solutions in DMSO. DMSO was added at a final concentration of 0.1% (v/v) into control cultures (*DMSO*). After a 4-day incubation, all gels were released from the walls of the wells and allowed to contract for additional 18–24 h; then, the diameter of each gel was measured, and the conditioned medium was analyzed by gelatin zymography. Gel contraction was determined as a percentage of gel area:that of the original gel area. Data are presented as mean ± SE from a representative experiment performed in triplicate. Gelatin zymograms of conditioned media from U-neo and U-MT cell cultures are shown at the *bottom* of the corresponding bars.

mediated ECM remodeling by using contraction of collagen lattices populated with tumor cells as a suitable *in vitro* model (40).

To evaluate the significance of MMP-2 activation in cell-induced collagen gel contraction, we analyzed a series of transfected tumor cell lines derived from human glioma U251.3 cells and fibrosarcoma HT-1080 cells. In these cell lines, MMP-2 activation was regulated by stable transfection with MT1-MMP (7, 37). Our data demonstrated that only MT1-MMP transfected cell lines capable of MMP-2 activation, efficiently mediated matrix remodeling, and contracted collagen lattices. Thus, constitutive production of the MMP-2 proenzyme accompanied by its MT1-MMP-mediated activation ensured effective collagen gel contraction by MT1-MMP-transfected glioma U-MT cells and fibrosarcoma HT-MT23 cells.

Recently, we demonstrated that an increase in collagen degradation by MT1-MMP-transfected glioma cells was proportional to MMP-2 activation and sensitive to TIMP-2 inhibition (7). In the current studies, both MMP-2 activation and gel contraction by U-MT glioma cells were inhibited by TIMP-2 in a dose-dependent manner. The kinetics of TIMP-2 inhibition indicates that the MT1-MMP-mediated step of MMP-2 activation (*i.e.*, the conversion of the M_r 68,000 MMP-2 proenzyme to the M_r 64,000 intermediate) is most sensitive to this inhibitor. Another specific inhibitor, the COOH-terminal domain of MMP-2, was distinct from TIMP-2 in terms of MMP-2 inhibition. At high concentrations, the COOH-terminal domain of MMP-2 also completely blocked MMP-2 activation and collagen gel contraction by U-MT cells. However, lower concentrations of the COOH-terminal domain preferentially blocked the autocatalytic conversion of the M_r 64,000 MMP-2 intermediate to the M_r 62,000 mature MMP-2, whereas the MT1-MMP-dependent M_r 64,000→M_r 62,000 step was less sensitive to its inhibition. These data directly correlate with our most recent findings that the autocatalytic M_r 64,000→M_r 62,000 step of MMP-2 activation is mediated by $\alpha_v/\alpha_v\beta_3$ integrins and does not occur independently of cell surfaces.[4] According to our model (7), the excess of the individual COOH-terminal domain should competitively inhibit MMP-2 binding to $\alpha_v/\alpha_v\beta_3$ and reduce the levels of the cell-associated M_r 64,000 and M_r 62,000 active MMP-2 species. The distinct mechanisms and kinetics of inhibition of MMP-2 activation by TIMP-2 and the COOH-terminal domain of MMP-2 explain why similar levels of gel contraction by U-MT cells in the presence of inhibitors corresponded to different amounts of activated MMP-2 found in conditioned media.

In contrast to the effects exerted by TIMP-2 and the individual COOH-terminal domain of MMP-2, TIMP-1 did not inhibit gel contraction by U-MT cells. However, both TIMP-2 and TIMP-1, at a 5× molar excess to MMP-2, inhibited APMA-induced MMP-2 activation, which is in agreement with the data reported by other authors (1, 13, 41). Therefore, the resistance of cell-mediated collagen gel contraction to a 22× molar excess of TIMP-1 relative to secretory MMP-2 strongly indicates that soluble activated MMP-2 does not contribute significantly to matrix remodeling *in vitro*. This finding also suggests a protection of cell surface-associated active MMP-2 species from their inhibition by TIMP-1. These conclusions were further strengthened by findings indicating that soluble MMP-2 enzyme, either APMA- or cell-activated, failed to induce gel contraction by tumor cells. Indeed, if the cell surface-associated MMP-2 comprises the main activity involved in cell-mediated matrix remodeling, it follows that once released by cells, activated MMP-2 species have relatively low affinity for rebinding to cell surfaces. Accordingly, we speculate that the MT1-MMP-dependent cleavage of the MMP-2 proenzyme is followed by an immediate docking of the M_r 64,000 MMP-2 activation intermediate to $\alpha_v/\alpha_v\beta_3$ integrins at tumor cell surfaces.[4] Such mechanisms could at least partially protect activated MMP-2 from inhibition by TIMPs.

In agreement with the above-mentioned considerations, MT1-MMP-dependent activation of MMP-2 correlates with cell surface association of the activated MMP-2 enzyme. Correspondingly, only the MMP-2 proenzyme was found in cell lysates of control glioma cells, whereas the M_r 68,000 MMP-2 proenzyme and the M_r 64,000 and M_r 62,000 activated MMP-2 species were revealed in cell lysates of MT1-MMP-transfected glioma cells. Because soluble MMP-2 did not induce gel contraction by control cells, it indicates that cell-associated activated MMP-2 is essential in mediating remodeling of the ECM and contraction of collagen lattices. Because cell-associated MMP-2 represents only about 1% of total MMP-2 produced by glioma cells, it would seem that regulation of MMP-2 activity is mediated by controlled cell surface activation. Studies are in progress to assess whether disruption of the association of activated MMP-2 with cell surfaces would affect the migratory, invasive, and ECM remodeling abilities of tumor cells.

It should be stressed that in our experimental system, transfected cells properly responded to major functional stimuli that are essential for the efficient contraction of cell-populated collagen lattices. Thus, the disruption of cell-matrix interactions with function-blocking mAbs specific for the collagen receptor integrins α_2, α_3, and β_1 completely prevented both the spreading of glioma-transfected cells within three-dimensional collagen gels and subsequent gel contraction. In agreement with the earlier data (16, 25, 26), the integrity of the actin cytoskeleton was also a prerequisite for collagen gel contraction by transfected cells in our cell model. Accordingly, depolymerization of actin filaments with cytochalasin B completely abrogated gel contraction. Unlike fibroblasts (27, 42), glioma cell-mediated gel contraction was sensitive to the disruption of the microtubule network by nocodazole at nanomolar concentrations. It has been shown that microtubule assembly could be affected by nanomolar concentrations of nocodazole (43). Because microtubule or microtubule-associated proteins may influence the dynamic properties of the actin cytoskeleton (27), this, in turn, could have affected gel contraction by cells.

Overall, our data indicate that collagen gel contraction by tumor cells is regulated by a coordinated interplay of integrin-mediated cell-matrix interactions, the integrity of the actin and microtubule cytoskeletons, cell rearrangements within collagen, and constitutive synthesis and cell surface activation of MMP-2. All of these parameters are essential but are not sufficient by themselves for collagen gel contraction by cells. In fact, the disruption of at least one of these fundamental mechanisms could result in a total inhibition of cell-mediated collagen gel contraction.

In conclusion, our results indicate that MMP-2 activity at cell surfaces, but not the soluble enzyme, is critical for remodeling of the ECM by tumor cells. The results could also explain a lack of direct correlation between the stage of tumor progression and the levels of soluble TIMPs and MMPs (1, 4). It remains to be determined whether mechanisms governing the spatial and functional regulation of MMP-2 activity are also critical for the ECM remodeling involved in the migration and invasion of tumor cells *in vivo*.

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Remodeling of Collagen Matrix by Human Tumor Cells Requires Activation and Cell Surface Association of Matrix Metalloproteinase-2

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