Matrix Metalloproteinase-2 Contributes to Cancer Cell Migration on Collagen

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Abstract

Matrix metalloproteinases (MMP) are central to tissue penetration by cancer cells, as tumors expand and form metastases, but the mechanism by which MMP-2 contributes to cancer cell migration is not well understood. In the present experiments, both a broad-spectrum MMP inhibitor and the isolated collagen binding domain (CBD) from MMP-2 inhibited cell migration on native type I collagen. These results verified the involvement of MMPs in general and showed that MMP-2, specifically, contributes to cell migration by a mechanism involving MMP-2 interaction with collagen. To exclude potential overlapping effects of MMP-9, additional experiments showed that MMP-2 also contributed to migration of MMP-9−/− cells. To investigate whether the homologous CBD from human fibronectin also inhibited cell migration, we first showed that fragmentation of fibronectin is a feature of breast cancer tumors and that several fragments contained the CBD. However, the recombinant fibronectin domain did not alter cell migration on collagen. This lack of effect on cell migration was explored in competitive protein-protein binding assays, which showed that the affinity of MMP-2 for collagen exceeds that of fibronectin. Furthermore, whereas the isolated MMP-2 CBD inhibited the gelatinolytic activities of MMP-2 and tumor extracts, such an inhibition was not characteristic of the corresponding fibronectin domain. Together, our results provide evidence that MMP-2 is an important determinant of cancer cell behavior but is not inhibited by the collagen binding segment of fibronectin. (Cancer Res 2005; 65(1): 130-37)

Introduction

The family of matrix metalloproteinases (MMP) consists of 23 enzymes that are characterized by their Zn^{2+}-dependence and neutral endopeptidase activities. Although these peptidases initially were associated with the cleavage of extracellular matrix molecules, particularly the collagens (1, 2), it is now recognized that the MMPs possess a broader range of functions and by proteolytic processing contribute to the control of expression and activation of chemokines, growth factors, and cellular receptors (3, 4). Based on this multiplicity of functions, the MMPs are key to normal development as well as the pathology of inflammatory diseases and cancer.

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Controlled proteolysis is required for cell migration across and through tissues in wound healing, tumor growth, and metastasis. In healing wounds, MMP-1, -3, -9, and -10 are prominently expressed in keratinocytes at the wound margin and MMP-9 and -13 in stromal fibroblasts and endothelial cells (5, 6). In addition, MMP-9 controls the rate of epithelial wound closure (7). Importantly, migration was absent from keratinocytes that did not express MMP-1 (8) and inhibited by MMP inhibitors (9).

Cancer growth and dissemination involve multiple MMPs that direct the interactions of tumor cells with the surrounding matrix environment (10, 11). Indeed, overexpression of MMP-specific tissue inhibitors of metalloproteinases reduces the malignant potential of experimental tumors (12, 13). MMP-2 has attracted attention by its association with tumor invasion and formation of metastases (14, 15). Thus, stromal cells express high levels of MMP-2 at the advancing tumor front (16), but the mechanism of the cellular interplay with the invading tumor cells is not yet understood (10).

In spite of homologies between functional domains among the MMPs, only the two gelatinases, MMP-2 and -9, contain collagen binding domains (CBD) formed by three-tandem fibronectin type II–like modules. The CBD in MMP-2 is essential to ligand interactions and substrate positioning, and the MMP-2 hydrolysis of gelatin (17) and elastin (18) is strongly reduced after deletion of the CBD. To ensure that the reduced activity of the mutated MMP-2 did not result from structural perturbations introduced by deletion of the ~20-kDa internal CBD, we recently showed that both binding and cleavage of gelatin by intact MMP-2 can be competed by soluble recombinant CBD (rCBD; ref. 19). To better understand the MMP-2 contribution to cancer cell behavior, we used the CBD as a tool to inhibit MMP-2 to test the hypothesis that MMP-2 is required for cancer cell migration across type I collagen.

The activation and catalytic activities of MMP-2 are tied to its capacity to interact with cell surface molecules. The MMP-2 can bind indirectly to membrane-type metalloproteinases in activation complexes containing TIMP-2 as the bridging molecule (20) or directly to $\alpha_v\beta_3$ integrin receptors (21). In addition, MMP-2 localizes to cell surfaces in CBD-β_1 integrin-pericellular collagen complexes (22). Saad et al. (23) recently proposed that tumor cell–associated fibronectin can release cell surface–bound pro-MMP-2 by competing its CBD-mediated binding to pericellular collagen. This mode of pro-MMP-2 release would presumably entail competition between the fibronectin and MMP-2 CBDs for binding sites on collagen.

Fibronectin contains two-tandem type II modules that have ~60% amino acid identity to the three MMP-2 type II modules and bind similar collagen types (24). Because fibronectin fragmentation is a pathophysiologic feature of several chronic inflammatory conditions including arthritis (25), poorly healing diabetic ulcers (26, 27), and periodontal disease (28, 29), the potential for competitive release of cell-bound pro-MMP-2 and disruption of MMP-2 activities by

collagen binding fibronectin fragments is of considerable biological significance and was addressed in these studies.

The present results show that MMP-2 is a key component of cancer cell migration across collagen by a mechanism involving CBD-mediated MMP-2 interactions with collagen. In addition, we show that fibronectin is degraded in tumor tissues to fragments of different masses, several of which contain the CBD. However, the isolated fibronectin CBD does not have the capacity to competitively inhibit cell migration, MMP-2 interaction with collagen, or MMP-2 hydrolysis of gelatin.

Materials and Methods

Collagen Purification. Acid-soluble native type I collagen was prepared from rat tail tendons as described by Piez (30) by extraction with 0.5 mol/L acetic acid and differential precipitation with 1.7 mol/L NaCl without pepsin digestion. Gelatin was prepared from the acid-soluble type I collagen by heat denaturation at 56°C for 30 minutes.

Cell Culture and Migration Assay. HT1080 fibrosarcoma, MDA-231 breast cancer, and MMP-9−/− cells were maintained in α-MEM (Sigma, St. Louis, MO) supplemented with 10% newborn calf serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The MMP-9−/− cells were established using cells released by proteolytic digestion of skin explants from MMP-9-null mice on a pure Fvb/n background (kindly provided by Dr. Zena Werb, University of California, San Francisco, CA; ref. 31). Cell migration assays used modified Boyden chambers and polycarbonate filters with 8 μm perforations (Millipore, Bedford, MA). Filters were coated overnight at 4°C with a 0.01% solution of type I collagen in acetic acid. Cells (5×10^3) were added to the upper chamber in 100 μL α-MEM containing 0.1% newborn calf serum. The lower chamber contained α-MEM with 10% newborn calf serum as chemoattractant. After 6-hour incubation (5% CO_2, 37°C), cells were fixed in methanol and stained with HemaDiff eosin and thiazine (Statlab, Lewisville, TX). The number of cells that had migrated through the membrane was quantified by counting 10 fields per membrane at a 200-fold magnification. To analyze general MMP and specific MMP-2 effects on cell migration, the MMP inhibitor BB94 (2.2 μmol/L, a gift from British Biotech Pharmaceuticals Ltd., Oxford, United Kingdom), rCBD from MMP-2 (2.4 μmol/L), or rI6I7 (2.0 μmol/L) were added individually or in combinations to the upper chamber simultaneously with the cells.

Preparation of Polyclonal Antibodies Specific for Fibronectin Fragments and rI6I7. Fibronectin was purified from human plasma by gelatin-Sepharose affinity chromatography (Amersham Pharmacia Biotech, Piscataway, NJ) by established procedures (32). In preparation for antibody production, purified fibronectin was digested with trypsin or chymotrypsin conjugated to periodate-oxidized Sepharose (33). Aliquots representing a multitude of fragments were separated from the enzymes by centrifugation after 0, 10, and 30 minutes and 1, 2, 4, 8, and 24 hours, pooled, and used as immunogen. To obtain polyclonal antibodies specific for the CBD of fibronectin, recombinant I6I7 expressed and purified detailed previously (ref. 24; see below) was used as immunogen. The titers and specificities of the antisera were monitored and verified by ELISA assays and Western blotting. For optimal antibody specificity, antisera were affinity purified against fibronectin fragments or rI6I7, respectively.

Detection of Fibronectin Fragmentation in Tumor Tissues. Extracts of experimental breast cancer tumors generated in nude mice (see below) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and probed with the affinity-purified polyclonal antibody against fibronectin fragments at a dilution of 1:500. After washes, conjugates were detected using horseradish-conjugated goat anti-rabbit antibody and enhanced chemiluminescence (Pierce, Rockford, IL) with X-Omat Blue XB-1 radiographic film (Kodak, Rochester, NY). To detect fragments containing the CBD of fibronectin (I6I7), samples were reacted with the affinity-purified antibody specific for rI6I7 at a dilution of 1:500 by the same methods. Masses of fragments were mapped relative to high molecular weight protein standards (Amersham Pharmacia Biotech) from scanned images using the Kodak 1D imaging software.

Expression and Purification of CBDs from MMP-2 and Fibronectin. The CBDs from human MMP-2 (CBD) and fibronectin (I6I7) were expressed in *Escherichia coli* Le392F′, purified, and verified according to previously reported procedures (24, 34). An additional construct encoded the fibronectin modules II1 and II2 only (rII1II2) that do not bind collagen (24).

Expression and Purification of Recombinant MMP-2 and -9. The cDNAs coding for constitutively active MMP-2 and -9 without the prodomains were amplified by PCR from MMP-2 plasmid p186.2 (ref. 35; provided by Dr. Ivan Collier, Washington University, St. Louis, MO) and MMP-9 plasmid pCEP4 (a gift from Dr. M. Seiki, University of Tokyo, Tokyo, Japan). The primers were sense: ccgctcgagTACAACTTCTTCCCTCGCAAG and antisense: cggaattcCCTGTGGGAGCCAGGGCC for MMP-2 and sense: ccgctcgagTTCCAAACCTTTGAGGGCG and antisense: cggaattcCAAAGCAGGACGGGAGCC for MMP-9. The primers contained *Xho*I and *Eco*RI sites for directional cloning into the T7-polymerase promoter-driven expression vector pRSETA (Invitrogen, San Diego, CA), which introduces a NH_2-terminal His_6 fusion tag. The recombinant enzymes were expressed in *E. coli* BL21 (DE3) pLysS and purified as detailed previously for full-length MMP-2 (19). The recombinant MMP (rMMP)-2 and -9 identities were verified by SDS-PAGE (36), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry at the University of Texas Health Science Center at San Antonio Institutional Mass Spectrometry Laboratory, and gelatinolytic activities by zymography and on fluorescent substrates (see below).

Reduction and Alkylation of rCBD. Aliquots of rCBD were chemically treated to abrogate gelatin binding properties by reducing disulfide bonds and alkylating side groups as detailed previously (34, 37). Briefly, rCBD was equilibrated with 8 mol/L urea, 65 mmol/L DTT, 2 mmol/L EDTA, 0.5 mol/L Tris (pH 8.0) overnight, incubated for 1 hour at 50°C, and reacted with 130 mmol/L iodoacetic acid for 30 minutes at room temperature. The alkylated CBD (AlkCBD) was dialyzed against PBS [137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, 0.8 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$ (pH 7.4)].

Biotinylation of Proteins. Three milliliters of rMMP-2, rI6I7, and rCBD (200-300 μg/mL) were dialyzed against 0.1 mol/L $NaHCO_3$ and then reacted with 300 μg EZ-link Sulfo-NHS-LC-Biotin (Pierce) for 20 minutes at 22°C and for 2 hours at 4°C. Free biotin was removed and the buffer was exchanged to PBS using a PD-10 desalting column (Bio-Rad, Hercules, CA). Control assays verified that the biotinylated proteins retained their gelatin binding properties and gelatinase activities (data not shown).

Competitive Substrate Binding Assays. To characterize the relative collagen interactions of rMMP-2, rCBD, and rI6I7, 96-microwell plates were coated with native (1.0 μg per well) or denatured (0.5 μg per well) type I collagen overnight at 4°C and nonspecific binding sites were blocked with 1.5% (w/v) casein (Sigma) for 1 hour at 22°C. After thorough rinses with PBS, 1 μg per well biotinylated rMMP-2 was added in the presence of a concentration range (18- to 0-fold molar excess) of rCBD or rI6I7 in PBS with 0.5% casein (pH 7.4) and then incubated for 1 hour at 22°C. Bound rMMP-2 was reacted with alkaline phosphatase–conjugated streptavidin (Pierce) diluted 1:10,000 in PBS for 30 minutes at 22°C and quantified at 405 nm (Opsys MR, Dynex, Chantilly, VA) using 1 mg/mL *p*-nitrophenyl phosphate disodium substrate (Sigma). AlkCBD and rII1II2 were used as non-collagen binding control proteins. The binding of rMMP-2 was expressed in percentage of noncompeted rMMP-2. In assays measuring binding competition between rCBD and rI6I7, either protein was biotinylated and then incubated with a concentration range of nonlabeled competing protein as described above.

Extraction of Tumor Tissues. Enzyme activities and proteins were extracted as detailed previously (19, 38) from breast cancer tumors developed in nude mice (*neu*−/*neu*−) following s.c. injection of MDA-231 cells. Briefly, tissues were homogenized with a Polytron (PT2100, Brinkman Instruments, Westbury, NY) in ice-cold extraction buffer [50 mmol/L Tris (pH 7.5), 2 mol/L guanidine hydrochloride, 10 mmol/L $CaCl_2$] before and following addition of 0.2% Triton X-100. Nonsolubilized material was sedimented by centrifugation (100,000 × *g*) and supernatants were dialyzed against 50 mmol/L Tris (pH 7.5), 10 mmol/L $CaCl_2$, 200 mmol/L NaCl, 0.05% Brij 35, 0.02% NaN_3 at 4C. To

prevent loss of enzyme activities and unintended fibronectin cleavage, samples were processed on ice and stored at −80C until analyses.

Enzyme Activity Assays on Fluorescent Substrates. To quantify enzyme activities, rMMP-2, rMMP-9, or tumor-extracted enzyme was added to 200 μL reaction volumes containing 2 μg per well fluorescent-labeled porcine type I gelatin substrate, DQ gelatin (Molecular Probes, Eugene, OR), and assay buffer [500 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$ (pH 7.6)]. Substrate cleavage was measured at 22°C with λ_{ex} at 495 nm and $_{em}$ at 515 nm on a SpectraMAX Gemini XS plate reader (Molecular Devices, Sunnyvale, CA). Experiments analyzing the inhibitory effects of rCBD or rI6I7 on MMP-2 or MMP-9 activities monitored substrate cleavage in the presence of a concentration range of the competing domains (5-0 g). Whereas the enzyme activities generally were expressed in relative fluorescent units (RFU), the relative activities in the competition experiments were expressed in percentage of control. Rate constants were calculated within the linear range of the assays.

Results

MMP-2 Contributes to Cellular Migration on Collagen by a Mechanism Involving the CBD. Extending our earlier observation that soluble rCBD from MMP-2 disrupts MMP-2 interactions with collagen and inhibits MMP-2 activities, we tested the hypothesis that competitive inhibition by the rCBD blocks MMP-2 activities required for cancer cell migration on collagen.

Experiments done in the presence of BB94, a broad-range MMP-specific hydroxamic acid class inhibitor, confirmed that cellular MMPs were involved in cell migration on collagen. This treatment resulted in significant decreases in the number of cells migrating through native type I collagen-coated membranes to 59% of untreated control at a concentration of 500 nmol/L BB94 (data not shown) and to 40% with 2.2 μmol/L of the inhibitor (Fig. 1*A*).

To assess whether MMP-2 modified cell migration, soluble rCBD was added to block MMP-2 interactions with collagen. The rCBD reduced cell migration in a concentration-dependent manner to ~50% of control at a concentration of 2.4 μmol/L rCBD (Fig. 1*A*). To verify the specificity of the rCBD-mediated inhibition of cell migration, cells were treated with reduced and AlkCBD, which has no collagen binding properties (34). AlkCBD did not inhibit cell migration on collagen (Fig. 1*A*).

The rCBD and BB94 could potentially modify the cell migration by the same or different mechanisms. However, simultaneous treatment by rCBD and BB94 did not reduce cell migration over that of BB94 alone (Fig. 1*A*). This indicated that the targeted enzyme activities overlapped, with rCBD inhibiting MMP-2 and BB94 blocking MMP-2 and additional MMP activities with relatively less impact on the cell migration. In control experiments, rCBD also inhibited MMP-9 gelatinolysis, although less efficiently than MMP-2 (data not shown). To exclude the possibility that the observed effects of rCBD on cell migration were contributed by inhibition of MMP-9, which is also expressed by HT1080 and MDA-231 cells (39), our experiments showed that the migration of MMP-9−/− cells was reduced by ~50% and slightly less for wild-type cells in the presence of soluble rCBD (Fig. 1*B* and *C*).

We concluded from these experiments that cellular MMP-2 contributes importantly to cell migration on collagen.

Fragmentation of Fibronectin Is a Feature of Breast Cancer Tumors, but the Isolated CBD Does Not Impede Cell Migration. To understand whether the collagen binding by fibronectin could competitively alter cell migration in a manner similar to that of the CBD from MMP-2, we first investigated whether the tumorigenic process causes fibronectin fragmentation and specifically produces cleavage products containing the CBD of fibronectin.

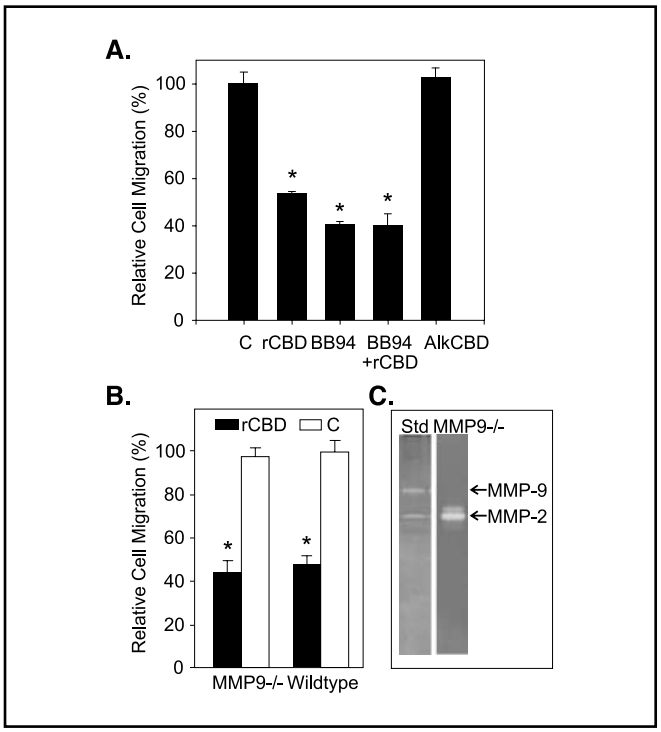

Figure 1. MMP-2 contributes to cancer cell migration on type I collagen. Cell migration was measured using Boyden chambers and native type I collagen-coated polycarbonate membranes with 0.8 μm perforations. The lower chamber chemoattractant was α-MEM containing 10% newborn calf serum. Migration was quantified by counting the number of cells migrating through the membranes after 6 hours. *A,* HT1080 fibrosarcoma cells were seeded in the upper chambers in α-MEM alone (control, *C*) or in the presence of 2.2 μmol/L BB94, 2.4 μmol/L rCBD, BB94 + rCBD, or 2.4 μmol/L alkylated, non-collagen binding rCBD. *B,* migration of MMP-9−/− cells established from MMP-9-null mice and wild-type (*Wildtype*) cells was analyzed in the presence of 2.4 μmol/L rCBD or α-MEM alone. *C,* analyses by gelatin zymography verified the absence of MMP-9 in the MMP-9−/− cells. Positions and activities of MMP-2 and -9 activity standards (*Std*). *Columns,* average cell migration (% untreated control) from three separate experiments; *bars,* SD. *, $P < 0.01$ versus control (Kruskal-Wallis and Mann-Whitney).

Western blot analyses first used the antibody raised against proteolytic fragments of fibronectin. This antibody reacted with several fibronectin fragments in extracts from three different breast cancer tumors developed in nude mice following injection with MDA-231 cells (Fig. 2*A*). The masses of the major fibronectin fragments were 159, 117, 99, and 88 kDa. Virtually no full-length fibronectin was present in the tumors, although there was positive reaction with intact fibronectin from human and murine plasma. An additional control from human gingiva showed early signs of fibronectin cleavage, presumably resulting from periodontal disease (29). Tumor samples were subsequently probed with the antibody specific for rI6I7 from the fibronectin CBD (24, 40). This antibody reacted with several distinct fibronectin fragments with masses of 184, 159, 117, 99, and 88 kDa (Fig. 2*B*) and with the fibronectin controls. For both antibodies, there were virtually no signals for fibronectin or fibronectin fragments in mouse skin (data not shown).

Because several fibronectin fragments in the tumors contained the CBD of fibronectin, we analyzed the effects of recombinant I6I7 on cell migration. However, in spite of collagen binding properties similar to the rCBD (24), the rI6I7 consistently did not alter the migration of either HT1080 fibrosarcoma or MDA-231 breast cancer cells (Fig. 2*C*). To understand the basis for this difference in the cellular response to rI6I7 and rCBD and to analyze differential effects of rI6I7 and rCBD on MMP-2 catalytic activities, we

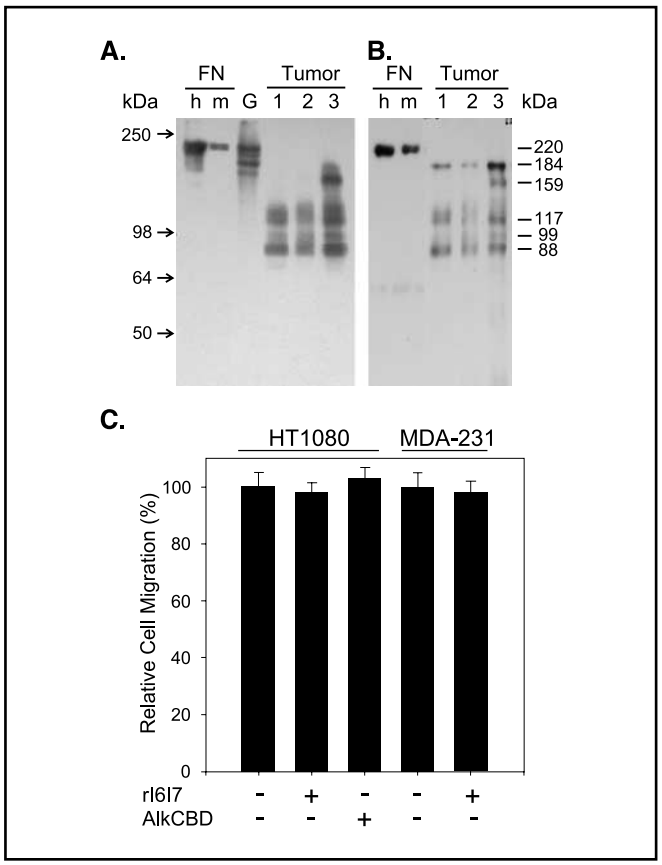

Figure 2. Fibronectin fragmentation in tumor tissues and effects of the fibronectin CBD on cell migration. Extracts were generated from experimental tumors in three different nude mice (*Tumors*, *1*, *2*, and *3*) resulting from s.c. injection of MDA-231 breast cancer. Samples were transferred to polyvinylidene difluoride membranes and probed for fibronectin fragmentation by Western blotting. *A*, protein bands detected with an affinity-purified polyclonal antibody raised against tryptic and chymotryptic fibronectin fragments. *B*, proteins reacting with an affinity-purified polyclonal antibody specific for the CBD of fibronectin (rI6I7). Controls were purified fibronectin (*FN*) from human (*h*) and murine (*m*) plasma. *C*, effects of soluble rI6I7 (2 μmol/L) and negative control AlkCBD (2.4 μmol/L) on migration of HT1080 and MDA-231 cancer cells on native type I collagen-coated polycarbonate membranes in Boyden chambers in 6-hour migration assays relative to untreated cells. *Columns*, average cell migration from two independent experiments in triplicate; *bars*, SD.

proceeded to characterize the collagen binding properties of these two functional protein domains relative to full-length MMP-2.

The Collagen Binding Affinities of the MMP-2 Type II Modules Exceed Those of the Corresponding Modules in Fibronectin. The interactions of rCBD and rI6I7 with native and heat-denatured forms of type I collagen were investigated in competitive protein binding assays (Table 1).

The rCBD competed virtually all rI6I7 binding to native type I collagen (~86%) at equimolar concentrations (Table 1). By comparison, the non-collagen binding AlkCBD control protein did not alter the rI6I7 binding to native type I collagen, thereby verifying the specificity of the reaction. Conversely, rI6I7 did not reduce the binding of rCBD to native type I collagen with up to an 11.6-fold molar excess of competing rI6I7.

Because a major substrate of MMP-2 is denatured type I collagen, gelatin, we analyzed the relative interactions of rI6I7 and rCBD with this form of collagen (Table 1). The rCBD also efficiently competed the rI6I7 binding to gelatin. At an rI6I7/rCBD molar ratio of 1:0.7, the rI6I7 binding to gelatin was reduced by ~34%, increasing to ~80% at 1:2.8. With an 11.6-fold molar excess of rCBD, only ~10% rI6I7 bound gelatin and AlkCBD did not block the rI6I7 binding at this concentration. By comparison, even an 11.6-fold molar excess of rI6I7 had no effect on the rCBD binding to gelatin. In further support of these observations, the non-collagen binding rII1II2 (24, 40) likewise did not reduce gelatin binding by the rCBD.

The results from assays with isolated CBDs translated to full-length MMP-2 interactions with type I collagen. At equimolar concentrations, the rCBD competed the binding of MMP-2 to native type I collagen by ~60% (Fig. 3*A*). A higher concentration of rCBD was required to compete MMP-2 binding to denatured type I collagen. An 18-fold rCBD molar excess reduced the MMP-2 binding by ~51% (Fig. 3*B*). This is consistent with the exposure of a larger number of cryptic binding sites for MMP-2 and fibronectin on collagen on unwinding of the triple helix (34, 41). In contrast to rCBD, the rI6I7 did not reduce MMP-2 binding to either form of type I collagen when included in the assays at the same molar ratios (Fig. 3*A* and *B*).

Thus, the rI6I7 from fibronectin was incapable of competing rCBD or rMMP-2 binding to native or denatured type I molecules.

The Fibronectin CBD, rI6I7, Does Not Inhibit the Gelatinolytic Activities of Recombinant MMP-2 and Tumor Extracts. Because soluble rCBD disrupts the substrate binding and gelatinolytic activities of MMP-2 (19), we analyzed whether rI6I7 likewise could inhibit MMP-2 hydrolysis of gelatin.

Table 1. Competition between rI6I7 and rCBD for binding to native and denatured type I collagen

Test protein	Competing protein	Molar ratio*	Protein binding†
Native type I collagen			
rI6I7	rCBD	1:1.0	12.6 ± 1.4‡
	AlkCBD	1:1.0	101.0 ± 4.6
rCBD	rI6I7	1:1.3	101.5 ± 1.0
		1:11.6	105.8 ± 2.6
	AlkCBD	1:11.6	108.6 ± 5.5
Denatured type I collagen			
rI6I7	rCBD	1:0.7	66.5 ± 3.5‡
		1:2.8	19.9 ± 3.8‡
		1:11.6	10.4 ± 0.8‡
	AlkCBD	1:11.6	102.0 ± 2.2
rCBD	rI6I7	1:11.6	105.0 ± 2.2
	rII1II2	1:11.6	105.0 ± 1.1
	AlkCBD	1:11.6	100.0 ± 4.0

NOTE: Biotinylated recombinant I6I7 or CBD were added in the presence of a concentration range (11.6-0 mol/L fold) nonlabeled competing CBD or control protein to 96-microwell plates coated with native (1 μg per well) or denatured (0.5 μg per well) type I collagen. After incubation for 1 hour at 22°C and thorough washes, bound protein was quantified at 405 nm following reaction with alkaline phosphatase–conjugated streptavidin and *p*-nitrophenyl phosphate disodium substrate.

*Molar ratios for test: competing proteins that had statistically significant effects on protein binding or up to a maximum ratio of 11.6.

† Average ± SD binding of test protein in competitive binding assays expressed in percentage of control without competitor. Calculated from two to three independent experiments ($n = 3$).

‡ $P < 0.01$ versus noncompeted control (Kruskal-Wallis and Mann-Whitney).

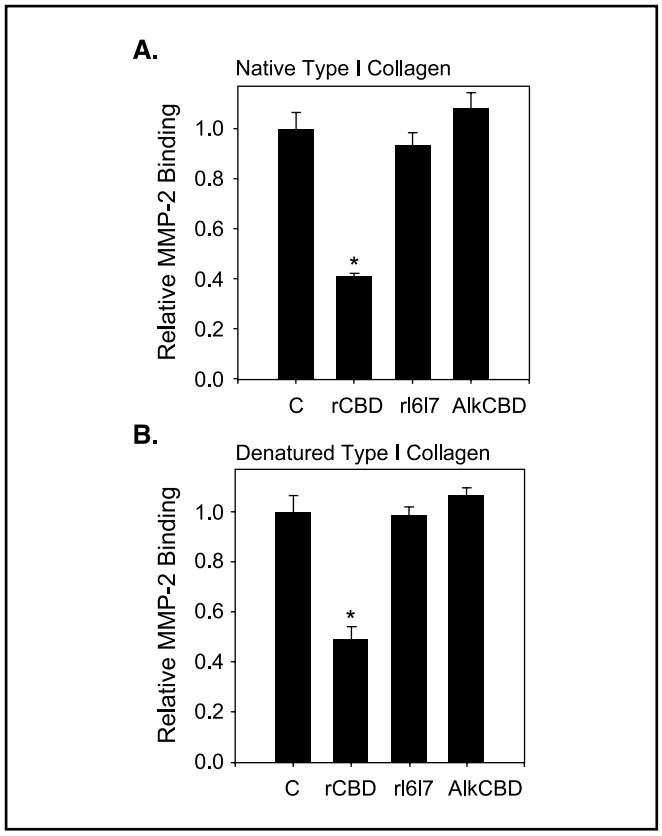

Figure 3. MMP-2 interactions with collagen in the presence of CBDs from fibronectin and MMP-2. rMMP-2 interactions with native (*A*) and denatured (*B*) forms of type I collagen were competed with soluble CBDs from MMP-2 (rCBD) and fibronectin (rI6I7). Biotinylated rMMP-2 was added to 96-microwell plates previously coated with 0.5 μg per well of native or denatured type I collagen alone or in the presence of the competing CBDs rCBD and rI6I7. Non-collagen binding AlkCBD served as negative control. Based on preceding competitive binding assays, the competing domains were added at equimolar amounts for native type I collagen assays and at an 18-fold molar excess in assays with denatured type I collagen. After final rinses, bound rMMP-2 was quantified with alkaline phosphatase–conjugated avidin and *p*-nitrophenyl phosphate disodium substrate at 405 nm. *Columns,* average of triplicate wells from three individual experiments relative to noncompeted rMMP-2; *bars,* SD. *, $P < 0.01$ versus control (Kruskal-Wallis and Mann-Whitney).

In the presence of competing rI6I7 at molar ratios up to 9-fold that of rMMP-2 (Fig. 4*A*), the rI6I7 did not inhibit degradation of gelatin by MMP-2 (Fig. 4*A*). This result is consistent with the inability of rI6I7 to disrupt MMP-2 binding to collagen. The rCBD, serving here as a positive control (19), reduced the hydrolysis of gelatin by MMP-2 by >60% as measured after 4 hours (Fig. 4*A*). The rate constants were 56.7×10^{-3} and 32.8×10^{-3} RFU/s for reactions containing rI6I7 and rCBD, respectively, and 60.3×10^{-3} RFU/s for control rMMP-2 (Table 2).

These observations obtained with recombinant MMP-2 translated to experiments using gelatinolytic activities extracted from the same breast cancer tumors that showed fibronectin fragmentation (Figs. 2*A* and *B* and 4*B*). Phenylmethylsulfonyl fluoride inhibited ~50% of the gelatinolytic (non-MMP) activities in the tumor extracts. Most (>80%) of the remaining gelatin hydrolysis was blocked by the specific MMP inhibitor 1,10-*o*-phenanthroline and therefore could be ascribed to MMPs in the extracts. The presented results were subsequently obtained with phenylmethylsulfonyl fluoride in the reactions (Fig. 4*A*). Under these conditions, rI6I7 did not inhibit gelatin degradation, whereas rCBD reduced the gelatin degradation by ~65% at the 4-hour time point (Fig. 4*B*). The corresponding rate constants were 40.8×10^{-3} and 28.3×10^{-3} RFU/s for activities in tumor extracts containing rI6I7 and rCBD, respectively, compared with 58.3×10^{-3} RFU/s for control reactions (Table 2).

Discussion

Intricate interactions among extracellular matrix molecules, cellular receptors, and proteolytic activities guide cell behavior in such processes as tissue expansion and penetration in cancer, tissue remodeling, and wound healing (1). The proteinases enable cells to migrate across matrices and through tissues by modifying extracellular matrix components. Pilcher et al. (8) found that keratinocytes require MMP-1 activity for migration across native type I collagen. MMP-1 cleaves the native collagen molecules only into $^3/_4$ and $^1/_4$ fragments that are unstable at body temperature and unwind and denature into the constituent collagen α-chains. The denatured α-chains are susceptible to hydrolysis by MMPs, particularly the gelatinases, MMP-2 and -9. In the context of cancer, MMP-2 is a key enzyme in metastasis and is abundantly expressed at the advancing front of tumors (15, 42). Localized proteolysis at the cell matrix interface is important to controlling cellular behavior

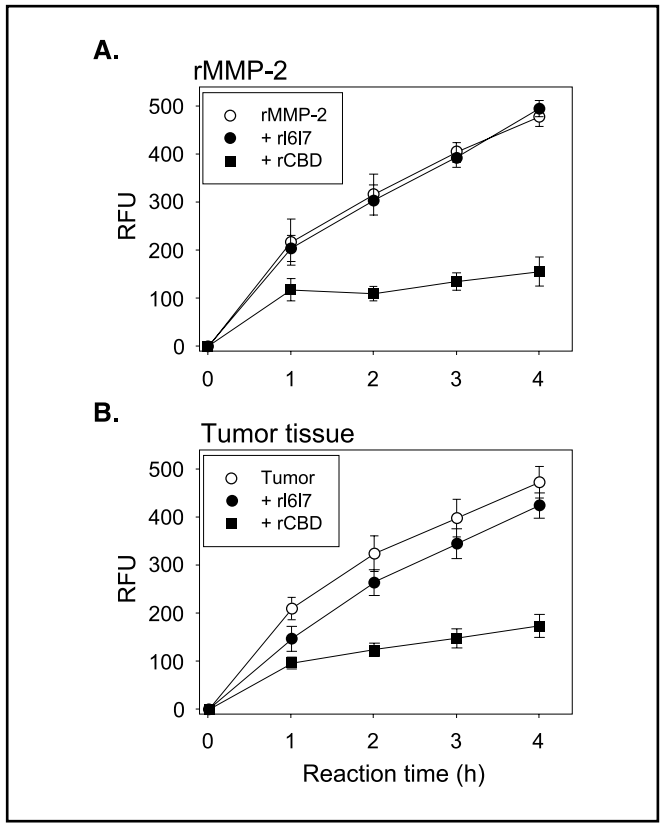

Figure 4. Gelatinolytic activities of rMMP-2 or tumor extracts in the presence of rCBD from rMMP-2 or rI6I7 from fibronectin. *A,* recombinant MMP-2 (140 nmol/L) was reacted with 2 μg per well fluorescent-labeled gelatin alone (*rMMP-2*) or competed with rCBD (*+rCBD*) or rI6I7 (*+rI6I7*) added to 9-fold molar concentrations over rMMP-2. Substrate hydrolysis was monitored with λ_{ex} at 495 nm and λ_{em} at 515 nm for 4 hours at 22°C. *B,* in similar type assays with enzyme activities from tumor extracts, 2 mmol/L phenylmethylsulfonyl fluoride was added to block >80% of non-MMP activities. Competing recombinant rCBD or rI6I7 were added at the same concentrations (1.2 μmol/L) after titration of reactions to yield activities comparable with rMMP-2 (*A*). *Points,* averages from duplicate assays; *bars,* SD.

Table 2. Competitive inhibition by fibronectin- and MMP-2-derived CBDs of MMP-2 and gelatinolytic activities extracted from breast cancer tumors

Test protein	Control	Competing domain	
		rI6I7	rCBD
Recombinant MMP-2, rate of cleavage ($\times 10^3$ RFU/s)	60.3	56.7	32.8
Tumor extract, rate of cleavage ($\times 10^3$ RFU/s)	58.3	40.8	28.3

NOTE: The cleavage of a quenched fluorescent-labeled gelatin incubated with either recombinant MMP-2 or tumor extracts alone (control) and in the presence of a 9-fold molar excess of rI6I7 or rCBD (1.2 μmol/L) was monitored for 1 hour at 22°C. Assays with tumor extracts contained 2 mmol/L phenylmethylsulfonyl fluoride to block non-MMP activities and were titrated to yield activities corresponding to rMMP-2. Averages from duplicate assays.

(2, 43). Based on the established cell surface positioning of MMP-2 (20–22), we hypothesized that MMP-2 contributes to cell migration across collagen matrices. Importantly, because we found that isolated, soluble rCBD can compete MMP-2 interactions with native type I collagen and gelatin and thereby inhibit its catalytic activities (19), competition with the CBD domain presented a unique tool for analyzing MMP-2 contributions to cell migration.

The migration assays included HT1080 fibrosarcoma cells, which express several MMPs at high levels, including the MMP-2 and -9 (39). Both soluble rCBD and the broad, hydroxamic acid–type MMP inhibitor BB94 (44) inhibited cell migration on native type I collagen-coated surfaces and thereby verified the contribution of MMPs in general and MMP-2 specifically to cell migration. Simultaneous treatment with rCBD and BB94 had little additive effect over BB94 alone, indicating that the two compounds acted on similar enzyme targets to inhibit the proteolytic modification of the native type I collagen matrix to a molecular form that supports cell migration. Although MMP-2 can cleave collagen triple helices, the rate is slower than that of MMP-1, likely resulting from differences in the K_m for MMP-2 (K_m = 8.5 μmol/L) and MMP-1 (K_m = 1.0 μmol/L; ref. 45). It is therefore plausible that the rCBD may have interfered with the matrix modification by inhibiting primarily MMP-2 gelatinolysis following initial collagen cleavage by MMP-1 or other collagenases and, to a lesser extent, MMP-2 cleavage of native collagen molecules. Finally, addressing the substrate overlap between MMP-2 and -9, our observation that the migration of MMP-9−/− cells also was decreased in the presence of the rCBD confirmed the involvement of the MMP-2 in cell migration and eliminated the possibility that the cellular effects resulted from rCBD inhibition of MMP-9.

In the context from studies by others focusing on the roles of MMP-1 (8, 46) and MMP-9 (7) in cell migration, the present experiments on MMP-2 indicate that cell migration involves several MMPs in a manner that may be both substrate and cell type dependent.

Our detection of fibronectin fragmentation in tumors may be biologically significant because peptide segments from fibronectin differentially affect both cell behavior and MMP expression. For example, the IIICS and CS-1 segments of the fibronectin heparin binding region modify MMP expression (47) and splicing differences of the V region influence tumor cell invasion and apoptosis (48, 49). Due to the high level of homology between the type II modules in fibronectin and MMP-2 and the similarity between the collagen binding properties (24), our working hypothesis was that collagen binding fibronectin fragments could alter cell migration by competing for MMP-2 binding sites on substrate molecules. In support of this biological rationale, our analyses detected several 88- to 184-kDa mass range fibronectin cleavage fragments in experimental breast cancer tumors that contained the ~24.6-kDa rI6I7, which corresponds to the shortest collagen binding segment of fibronectin (24, 40).

Unexpectedly, the rI6I7 did not inhibit cell migration on native type I collagen matrices at molar concentrations that yielded strong effects with the rCBD from MMP-2. To understand the basis for the differences between the two CBDs, we did a series of competitive protein-protein binding assays. Whereas the rCBD effectively competed rI6I7 binding to native and denatured type I collagen, the rI6I7 was virtually incapable of reducing rCBD binding to these forms of type I collagen. These results are consistent with the apparent K_ds of interaction for the rI6I7 and rCBD that are 3.0×10^{-7} and 0.3×10^{-7} mol/L for interactions with native type I collagen and 3.7×10^{-7} and 0.15×10^{-7} mol/L for denatured type I collagen, respectively (24). These observations of isolated fibronectin and MMP-2 CBD interactions corresponded well to results from experiments using full-length MMP-2. Neither rI6I7 nor AlkCBD had the capacity to compete binding of full-length MMP-2 to native or denatured type I collagen. Moreover, the rI6I7 did not inhibit cleavage of a fluorescent-labeled gelatin substrate even at molar ratios of rI6I7 up to 9-fold that of recombinant MMP-2, whereas the rCBD substantially reduced the activity of its parental MMP-2 (19). Likewise, rI6I7 did not reduce gelatinolytic activities extracted from tumor tissues.

From these experiments, we concluded that rI6I7, by virtue of its lower affinity, was incapable of competing the CBD-mediated MMP-2 interactions with collagen and consequently the hydrolysis of this substrate. The results also may explain the differences in cellular responses to the two CBDs. Thus, contrary to soluble rCBD, the affinity of rI6I7 for native type I collagen or gelatin was insufficient to inhibit MMP-2-mediated substrate modification in the cell migration system.

Our results complement the experiments on MMP-2 release from pericellular collagen of stromal cells by cancer cell–derived fibronectin reported by Saad et al. (23). Interestingly, those investigators found that plasma fibronectin did not release cell surface–bound MMP-2 from stromal cells co-cultured with MDA-231 cells, whereas MMP-2 was released by co-culture with untreated MDA-231 cells or MDA-231 cells from which cellular fibronectin was first depleted and then replenished (23). Our data imply that the lack of effect of plasma fibronectin originated from its lower affinity for collagen relative to the MMP-2 and was not structural in nature, because the collagen binding region of fibronectin generally is not thought to differ between plasma and cellular fibronectin (50, 51). It is perceivable, however, that cell-associated fibronectin could act by other mechanisms, such as interaction with cell membrane proteins or receptors to induce a cascade of events that ultimately results in the release of cell surface–bound MMP-2.

In summary, our results provide evidence that MMP-2 contributes to cell migration across native type I collagen by a

mechanism that involves collagen interactions via the CBD of the enzyme. Further, we have shown that fibronectin fragmentation is a feature of tumorigenesis and produces peptides that contain the CBD of the molecule. However, due to its lower affinity for collagen relative to MMP-2, the isolated CBD from fibronectin does not inhibit cell migration on collagen or MMP-2 gelatinolysis.

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