Cancer Cell-associated Fibronec tin Induces Release of Matrix Metalloproteinase-2 from Normal Fibroblasts

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ABSTRACT

The bone and bone marrow are the most common sites of metastasis in breast cancer. Matrix metalloproteinases (MMPs), particularly MMP-2, produced by cancer cells or, more typically, induced in the adjacent normal stroma are necessary for the degradation of extracellular matrix essential for cancer metastasis. Here we describe a mechanism by which breast cancer cells can rapidly use MMP-2 produced by bone marrow fibroblasts (BMFs). MMP-2 is stored in an inactive conformation in association with the cell surface or extracellular matrix of BMFs. Cultures of BMFs and the human breast cancer cell line MDA-MB-231 induce release of MMP-2 into the culture supernatant without up-regulation of MMP-2 synthesis in either cell. MMP-2 is present on the surface of BMFs and is displaced by MDA-MB-231 cells or by fibroblasts or fragments of fibronec tin containing the fibronec tin type II modules. Moreover, when fibronec tin is eluted from the surface of MDA-MB-231 cells, they lose the ability to induce the release of MMP-2 from BMFs. These data are consistent with the displacement of inactive MMP-2 bound to normal fibroblasts via its collagen-binding domain by fibronec tin type II modules of cancer cell-associated fibronec tin. Cancer cells can then use the protease to facilitate tissue invasion. Because an identical mechanism can be demonstrated using fibroblasts from different sources, it is likely to be important for the rapid movement of malignant cells into a variety of normal tissues.

INTRODUCTION

Breast cancer is one of the most common forms of cancer in women, but despite being responsive to hormonal manipulation and chemotherapy, relapse after treatment is common, particularly in patients presenting with metastatic disease (1). One of the most common sites of metastasis is bone and bone marrow, which may provide a favorable microenvironment for the growth of tumor cells and act as a reservoir of disease, allowing further hematogenous spread (2, 3).

The process of tumor cell invasion and metastasis requires the degradation of connective tissue associated with vascular basement membranes and interstitial connective tissue (4, 5). Several lines of evidence strongly implicate MMPs, particularly MMP-2, in this process. These include a positive correlation between MMP-2 expression and invasive potential and the inhibition of metastasis formation in vivo by MMP inhibitors (4, 6, 7). Interestingly, MMP-2 is often associated with adjacent normal tissues rather than the tumor cells themselves, suggesting that neoplastic cells can use MMPs produced by normal cells to facilitate their egress from the tumor mass and potentially their entry into new sites (8). MDA-MB-231 and T47D cells have been shown to bind MMPs on their cell membranes by specific receptors, optimally locating these enzymes to maximize invasive potential (9).

MMPs are a family of zinc-dependent enzymes consisting of a propeptide, catalytic, hinge, and COOH-terminal (hemopexin like) domains. All MMPs are produced in latent forms requiring catalytic removal of the propeptide domain for function. MMP-2 and MMP-9 are unique because of the inclusion of three fibronec tin type II repeats within their catalytic domain (10). These repeats, referred to as the CBD, facilitate adhesion to collagen (11–13). MT-MMPs are a subfamily of MMPs which contain a transmembrane domain (14). MT1-MMP was the first physiological activator of MMP-2 discovered and is still likely to be the major activator of MMP-2 on the cell surface (15). Whereas all active MMPs can be inhibited by TIMPs, TIMP-2 has a biperic effect on MMP-2 activation, potentiating at low concentrations and inhibiting when present at high concentrations (15–17).

We have demonstrated previously the importance of MMP-2 in the invasion of BMF monolayers by the invasive breast cancer cell lines MDA-MB-231 and BT-549 (18). Using a short-term coculture system, we have also shown that contact between the breast cancer cell line MDA-MB-231 and bone marrow-derived fibroblasts results in a rapid increase in the concentration of MMP-2 in the culture supernatant (18). Here we demonstrate that the MMP-2 observed in the supernatant is primarily derived from MMP-2 bound to collagen associated with the BMF monolayer. MMP-2 is displaced from the collagen by fibronec tin bound to the breast cancer cells. Once displaced from the BMF-associated collagen, the MMP-2 can be activated by MT1-MMP/TIMP-2 complexes expressed by the breast cancer cells. This mechanism provides a rapid method by which neoplastic cells can use MMP-2 produced by normal cells to facilitate their invasion of normal tissues.

MATERIALS AND METHODS

Materials. The following antibodies were purchased: 4B4 (anti-β1) from Coulter Clones, Hialeah, FL; 5D11 (anti-MMP-2) from Oncogene Research Products Cambridge, MA; 36006.211 (anti-hMMP-2 for immunofluorescent staining) from R&D Biomedicals, Minneapolis, MN; 556000 (antihuman fibronec tin) from Cortex Biochem, San Leandro, CA; collagen antibody (MAB1334) from Chemicon International, Temecula, CA; SAMF from Sile nus, Hawthorne, Vic, Australia; and goat antimouse immunoglobulins biotin or horseradish peroxidase conjugated from DAKO Corp., Carpenteria, CA.

Marimistat was a kind gift from British Biotech Pharmaceuticals, Oxford, United Kingdom. Human recombinant MMP-9 was purchased from Chemicon International; human plasma fibronec tin and laminin was from Life Technologies, Inc., Rockville, MD; and hyaluronic acid, actinomycin-D, cyclohexamide, Triton X-100, phenylmethylsulfonyl fluoride, leupeptin, and EDTA was from Sigma Chemical Co., St. Louis, MO. Recombinant human CBDS consisting of all three fibronec tin Type II modules (Gly417-Cys631 of human MMP-2) was expressed and purified as published previously (11).

Cells. The breast cancer cell lines MDA-MB-231 and T47D were grown in RPMI containing 10% (volume for volume) FCS, 0.25 units/ml insulin, 20 mM HEPES (pH 7.0), and 4 mM L-glutamine as described previously (18). Normal

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The abbreviations used are: MMP, matrix metalloproteinase; CBDD, collagen-binding domain; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; BMF, bone marrow fibroblast; SAMF, sheep antimouse fluorescein.

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BMFs were isolated from bone marrow mononuclear cells obtained from normal allogeneic bone marrow donors with institutional ethics committee approval as described previously (19). Dermal fibroblasts, grown from human foreskins, were provided by Barry Slobedman (Westmead Millennium Institute) and gingival fibroblasts by Dr. Hans Zoellner (Westmead Hospital). All cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C. Cells were detached from flasks using 1.3 mg/ml trypsin and 0.07% (w/v) EDTA (19).

**Coculture Experiments, Zymograms, and Western Blotting.** MDA-MB-231 or T47D cells (3 x 10^6 cells in 100 μl of RPMI) were plated onto confluent BMFs in 96-well plates and incubated at 37°C for 6 h unless otherwise indicated. In some experiments, designated culture nocontact, MDA-MB-231 cells were separated from BMFs by a membrane containing 0.4-μm pores (Becton Dickinson, San Jose, CA), allowing for the diffusion of soluble factors but eliminating direct cell-cell contact. Cell lysates were prepared in 1% Triton X-100, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mg/ml leupeptin in PBS (pH 7.4) as described previously (20).

Cell surface fibronectin was removed by a 30-min exposure to 5 mM EDTA at 4°C. Where indicated, MDA-MB-231 cells were treated with 250 μg/ml fibronectin, laminin, or hyaluronic acid for 30 min at 37°C. Cells were washed twice in RPMI before plating onto BMFs. Where indicated, BMFs were preincubated with the CBD (1 mg/ml), MMP-9 (5 μg/ml, unless otherwise indicated), fibronectin (1 mg/ml, unless otherwise indicated), actinomycin-D (1 μg/ml), cycloheximide (10 μg/ml), bacterial collagenase (1 mg/ml), hyaluronidase (1 mg/ml), or BSA (1 mg/ml) for 1 h at 37°C. BMFs were washed twice with RPMI before use. The prolyl-4-hydroxylase inhibitors of collagen production, Compound I and Compound II, were generous gifts from Fibrogen (San Francisco, CA) and were used at a final concentration of 10 μM for 18 h on collagenase-treated BMFs (21).

Serum-free culture supernatants or cell lysates were examined by gelatin-zymography or Western blotting as described previously (18).

**Northern Blot Analysis.** MDA-MB-231 cells (1 x 10^7) and BMFs were cultured in RPMI in 75-cm² flasks separately or in coculture for 3–18 h. Cells cultured separately were combined immediately before extraction of RNA. Total RNA was isolated using a RNA isolation Kit (Advanced Biotechnologies, Surrey, United Kingdom), separated by electrophoresis on 1% agarose-formaldehyde gels, and blotted onto a Zeta-Prob membrane (Bio-Rad Laboratories, Hercules, CA). Filters were prehybridized at 50°C for 2 h. Hybridization was carried out with salmon sperm DNA (Boehringer Mannheim, Castlehill, NSW, Australia), and 0.04 mg/ml poly(A) RNA (Sigma Chemical Co.) in 2 x saline-sodium phosphate-EDTA [300 mM NaCl, 10 mM NaH2PO4, and 1 mM EDTA (pH 7.4)] before being hybridized with radiolabeled cDNA overnight under the same conditions. Labeled bands were detected by autoradiography. The MMP-2 cDNA probe was prepared from BMF RNA by reverse transcription-PCR. cDNA was prepared using oligodeoxythymidylic acid primers and Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI) and the resulting PCR product was 32P labeled using the Megaprime Kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Integrity and equal loading were assessed by subsequent hybridization with a glyceraldehyde-3-phosphate dehydrogenase probe.

**Flow Cytometry.** Indirect labeling of cells was performed as described previously (19) using antigen-specific monoclonal antibodies as the first layer and SAMF as the second layer. Cells were analyzed on a Becton Dickinson fluorescence-activated cell sorter scan flow cytometer.

**Immunoperoxidase and Immunofluorescent Staining.** Cells were fixed with 3.7% formaldehyde for 15 min at 4°C and permeabilized with 0.2% Triton in PBS for 10 min at room temperature. Cells were incubated with 0.2% FCS in PBS for 45 min, then with antibodies to MMP-2 (10 μg/ml), collagen (1 in 200), or appropriate controls for 90 min. Immunofluorescent detection was with a SAMF (1 in 200 dilution) for 1 h in the dark, and slides were mounted with Vector shield (Vector Laboratories, Inc., Burlingame, CA) before examination on an Olympus fluorescence or by confocal microscopy. Immunoperoxidase staining used biotinylated goat antiantibodies immunoglobulins (1 in 100 dilution) for 30 min followed by signal amplification using an ABC kit (Zymed, San Francisco, CA), according to the manufacturer’s instructions, and was visualized using diaminobenzidine (Sigma Chemical Co.). Cells were washed with PBS between steps and after completion of labeling.

**Statistics.** Western blots were quantified by densitometry. Data were normalized to BMFs to give a fold increase in MMP-2 levels in each condition. Student’s t tests were performed where there were only two data sets, and ANOVA for multiple comparisons at 95% significance was used where there were three or more data sets.

**RESULTS**

**BMP Cell-associated MMP-2 Is the Source of the Increased MMP-2 Detected in Coculture Supernatants.** Coculture of BMFs and the breast cancer cell lines T47D and MDA-MB-231 result in an increase in the level of both pro and active forms of MMP-2 in the culture supernatant (Fig. 1A). This effect was not generalized because it was not observed in the pre-B cell line NALM6 (data not shown). The substitution of fibroblasts from other sources, including dermal- and gingival-derived fibroblasts, as well as the human BMF cell line,
levels were not the results of increased synthesis (Fig. 2A). Analysis of cell lysates obtained from these cultures showed a consistent increase in the level of MMP-2 in culture supernatants of MDA-MB-231 cells, BMFs, and MDA-MB-231 cells that had been cultured separately or in direct contact (Fig. 2A). Preincubation with the transcription inhibitor, actinomycin D, or the translation inhibitor, cyclohexamide (CHX) as described in “Materials and Methods.” In B, BMFs were treated with Actinomycin D (Act D) or Cyclohexamide (CHX) as described in “Materials and Methods” before the addition of MDA-MB-231 cells. Culture supernatants were collected after 6-h coculture and analyzed by Western blot.

FS-1, resulted in a similar increase in MMP-2 in the culture supernatant (Fig. 1B and data not shown). In contrast, substitution of murine fibroblasts for the human BMFs eliminated the effect (data not shown). Direct contact coculture of MDA-MB-231 cells with BMFs resulted in a 5.7 ± SE 1 (P < 0.0001, n = 22)-fold increase in MMP-2 in the culture supernatant. Although both pro and active forms of MMP-2 were up-regulated, quantitation was performed on the proenzyme only because of the need for bands on autoradiographs to be in the linear range for densitometric analysis. In the absence of direct contact between the two cell types, MMP-2 up-regulation in cocultures was significantly reduced, although a slight increase in MMP-2 levels was always detected, suggesting a minor role for a soluble mediator (Fig. 1C).

No change in the level of mRNA for MMP-2 was detected as a result of MDA-MB-231 cell coculture with BMFs at any time point tested from 3 to 18 h, despite significant increases in MMP-2 protein levels in culture supernatants (Fig. 2A). Preincubation with the transcription inhibitor, actinomycin D, or the translation inhibitor, cyclohexamide, failed to inhibit coculture-induced elevation of MMP-2 protein in culture supernatants, confirming that increased MMP-2 levels were not the results of increased synthesis (Fig. 2B). These data suggest that the increased level of MMP-2 in supernatants of cocultures was derived from cellular stores.

Zymograms of culture supernatants of MDA-MB-231 cells, BMFs, and cocultures of the two cell types showed a clear increase in the levels of MMP-2 present in the culture supernatants of cocultures. Analysis of cell lysates obtained from these cultures showed a concomitant decrease in the level of MMP-2 associated with the cellular fraction (Fig. 3, A and B). Before coculture, MMP-2 was present in the cell-associated fraction of BMFs but not MDA-MB-231 cells, suggesting that the MMP-2 was fibroblast derived. Confocal microscopy of BMFs showed that on BMFs, MMP-2 was primarily associated with the plasma membrane (Fig. 3C).

MMP-2 Is Bound to BMFs through Its CBD. It has been reported previously that MMP-2 can be held on the cell surface by binding to cell-associated collagen through the CBD (12). The failure to detect MMP-2 at the cell surface of MDA-MB-231 cell supernatants is in agreement with the absence of MMP-2 in the cell lysates of these cells (Fig. 3A). Pretreatment of BMFs with CBD before coculture completely inhibited the cell-cell, contact-induced appearance of MMP-2 in the culture supernatant (Fig. 4B). This was not attributable to the process of washing cells or the presence of any protein because pretreatment of BMFs with BSA had no effect of MMP-2 production after coculture (Fig. 4C). This suggests that the increased MMP-2 detected in supernatants from cocultures of MDA-MB-231 cells and BMFs originated from BMF cell surface-associated MMP-2 and that MMP-2 binds to the cell via its CBD.

Like MMP-2, MMP-9 contains three fibronectin type II modules, whereas fibronectin contains two such modules. These are highly homologous to those observed in MMP-2. To extend the recombinant CBD findings, we pretreated BMFs with MMP-9. This dramatically diminished contact-induced increases in MMP-2 in culture supernatants (Fig. 5, A and B). As little as 80 ng/ml MMP-9 was able to attenuate the coculture-induced increase in MMP-2. Thus, MMP-9 competes with MMP-2 for binding to the cell surface by the CBD. Although a small amount of active MMP-9 could be detected in the MMP-9 added to the BMFs, the addition of the MMP inhibitor Marimistat (1 μg/ml) did not reduce the inhibitory effect of MMP-9 treatment on MMP-2 production. This confirms that the effect of MMP-9 was not attributable to its protease activity. MDA-MB-231 cells produce MMP-9 that is detected in the culture supernatant but not in the cell-associated fraction (Fig. 3A). It is possible that MMP-9 reported previously (12). The failure to detect MMP-2 in CBD-treated MDA-MB-231 cell supernatants is in agreement with the absence of MMP-2 in the cell lysates of these cells (Fig. 3A). Pretreatment of BMFs with CBD before coculture completely inhibited the cell-cell, contact-induced appearance of MMP-2 in the culture supernatant (Fig. 4B). This was not attributable to the process of washing cells or the presence of any protein because pretreatment of BMFs with BSA had no effect of MMP-2 production after coculture (Fig. 4C). This suggests that the increased MMP-2 detected in supernatants from cocultures of MDA-MB-231 cells and BMFs originated from BMF cell surface-associated MMP-2 and that MMP-2 binds to the cell via its CBD.

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Coculture and coculture of MDA-MB-231 cells and BMFs pretreated with BSA (1 mg/ml; MDA-MB-231 cells (Coculture)), BMFs, coculture of MDA-MB-231 cells and BMFs (CBD/Coculture), and coculture of MDA-MB-231 cells and BMFs pretreated with recombinant CBD (MDA-MB-231 cells, BMFs, and coculture of BMFs and MDA-MB-231 cells (Coculture)).

There was no evidence for the presence of cell-associated MMP-9 from zymograms of MDA-MB-231 cell lysates (Fig. 3A). This makes it unlikely that cell-associated MMP-9 is responsible for the increases in MMP-2 that result from cell-cell contact.

Fibronectin was found to be associated with MDA-MB-231 cells by flow cytometry (Fig. 7A). Surface-associated fibronectin was eluted from MDA-MB-231 cells by incubating the cells at 4°C in the presence of EDTA. Removal of surface fibronectin was confirmed by flow cytometry (Fig. 7A). MDA-MB-231 cells that had been denuded of surface fibronectin were completely unable to induce increased levels of MMP-2 in culture supernatants from cocultures with BMFs (Fig. 7, B and C). Replacement of cell surface-bound fibronectin on MDA-MB-231 cells restored the ability of these cells to induce increased MMP-2 production in cocultures with BMFs. Readdition of laminin and hyaluronic acid had minimal effect on the MMP-2 content of coculture supernatants (Fig. 7B). This suggests that MDA-MB-231 surface-associated fibronectin is sufficient to induce the increased levels of MMP-2 observed in cocultures of MDA-MB-231 cells and BMFs. Although this does not prove that fibronectin is the only factor involved in eluting MMP-2 from the cell surface, it indicates that the collagen involved is not held in the monolayers by other matrix components, including fibronectin.

Presence of Fibronectin on the Surface of MDA-MB-231 Cells. The requirement for direct contact between the MDA-MB-231 cells and BMFs for maximal up-regulation of MMP-2 suggests that the factor involved in eluting MMP-2 from the cell surface is bound to the MDA-MB-231 cell surface. There was no evidence for the presence of soluble fibronectin only reduced the contact-dependent increase in supernatant MMP-2 levels at high concentrations (0.5–1 mg/ml; Fig. 5, C and D).

MMP-2 Is Bound to BMFs through Collagen. BMFs have collagen within their extracellular matrix as determined by immunocytochemistry (Fig. 6A). It is likely that the CBD of MMP-2 binds to the cell surface of BMF through cell surface-associated collagen. Treatment of the BMFs with collagenase, but not hyaluronidase, significantly attenuated the increase in MMP-2 content of supernatants induced by coculture with MDA-MB-231 (Fig. 6, B–D). Removal of collagen from the BMF monolayer was confirmed by immunocytochemistry (Fig. 6A). BMFs were treated with bacterial collagenase and cultured overnight with or without the inhibitors of collagen synthesis Compound I and Compound II to determine whether inhibiting collagen synthesis would prolong impaired MMP-2 release, supporting an essential role for collagen in the release of MMP-2 into culture supernatants. MMP-2 release on coculture returned after overnight culture only if inhibitors of collagen synthesis were not present, indicating that collagen is involved in the process of MMP-2 release (Fig. 6E). The specificity of collagenase was confirmed by the lack of effect of collagenase on BMF-associated fibronectin as assessed by flow cytometry (data not shown).

Treatment of BMFs with antibodies to the β1-integrin chain before the initiation of cocultures had only a marginal effect on MMP-2 up-regulation (data not shown). It is likely that the collagen involved is not held in the monolayers by β1-integrins alone but that it is integrated into the BMF extracellular matrix, adhering to a number of matrix components, including fibronectin.

Presence of Fibronectin on the Surface of MDA-MB-231 Cells. The requirement for direct contact between the MDA-MB-231 cells and BMFs for maximal up-regulation of MMP-2 suggests that the factor involved in eluting MMP-2 from the cell surface is bound to the MDA-MB-231 cell surface. There was no evidence for the presence of cell-associated MMP-9 from zymograms of MDA-MB-231 cell lysates (Fig. 3A). This makes it unlikely that cell-associated MMP-9 is responsible for the increases in MMP-2 that result from cell-cell contact.

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BMFs. Cancer cell line MDA-MB-231 and bone marrow-derived fibroblasts (BMFs) interact to facilitate their invasion of normal tissues. This ability of breast cancer cells to induce the release of MMP-2 was not restricted to BMFs but was observed in all normal fibroblasts and the human fibroblast cell line tested. We conclude that this mechanism is involved in the metastasis of malignant cells to many tissues and is not restricted to bone and bone marrow metastasis.

The ability of malignant cells to influence MMP-2 production by adjacent normal cells is well established. However, this process has only been demonstrated after >48 h of coculture and has been dependent on increased synthesis of MMP-2 (8, 22, 23). The mechanism by which MMP-2 is regulated in normal tissue by malignant cells is likely to be important for the escape of neoplastic cells from tumor margins, allowing invasion of adjacent normal tissues and possibly entry into the blood or lymphatic systems. However, it is difficult to imagine these mechanisms being involved in the infiltration of malignant cells at distant sites where a much more rapid regulation of MMPs is likely to be required. In this study, we report a mechanism by which malignant cells can rapidly use MMPs pro-

Fig. 6. BMF MMP-2 is bound to collagen. A, immunoperoxidase staining of BMFs with anticollagen I antibody (a), anticollagen I antibody (b), or collagenase-treated BMFs stained with anticollagen I antibody (c). Arrow, collagen fibrils present in the BMF layer. B, Western blot of MMP-2 in 6-h culture supernatants of MDA-MB-231 (MDA), BMFs, coculture of MDA-MB-231 and control (Coculture), or collagenase-treated (Collagenase/Coculture) BMFs. C, Western blot of MMP-2 in 6-h culture supernatants from MDA-MB-231 (MDA), BMFs, coculture of MDA-MB-231 cells and control (Coculture), or hyaluronidase-treated BMFs (Hase/Coculture). D, quantitation of Western blots of MMP-2 in culture supernatants. Mean and SE of normalized data from five independent experiments are shown. *P < 0.04 compared with coculture. E, Western blot of MMP-2 in 6-h culture supernatants. Culture supernatants were from MDA-MB-231 cells (MDA) or BMFs cultured alone or cocultures of MDA-MB-231 and BMFs (Coculture). Where indicated, confluent BMFs were treated with collagenase and then cultured overnight in medium containing vehicle only (Cont), Compound I (Comp I, 10 μM), or Compound II (Comp II, 10 μM), as indicated. One of two experiments is shown.

DISCUSSION

Here we identify a novel mechanism by which breast cancer cells use MMP-2 produced by normal fibroblasts to facilitate their invasion of this tissue. We have shown previously that coculture of the breast cancer cell line MDA-MB-231 and bone marrow-derived fibroblasts results in an increase in MMP-2 in culture supernatants in a manner that is largely, although not entirely, dependent on direct contact between the two cell types (18). Here we show that the increase in MMP-2 in coculture supernatants is not attributable to increased levels of transcription but to the release of cell-associated MMP-2 from the BMFs. This release and subsequent activation of MMP-2 occurs rapidly, making this a mechanism by which metastasizing malignant cells could use MMP-2 produced by adjacent normal cells to facilitate their invasion of normal tissues. This ability of breast cancer cells to induce the release of MMP-2 was not restricted to BMFs but was observed in all normal fibroblasts and the human fibroblast cell line tested. We conclude that this mechanism is involved in the metastasis of malignant cells to many tissues and is not restricted to bone and bone marrow metastasis.

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Fig. 7. MDA-MB-231 cell surface-bound fibronectin is sufficient to increase culture supernatant MMP-2 levels in cocultures of MDA-MB-231 cells and BMFs. A, flow cytometric analysis of cell surface-bound fibronectin on MDA-MB-231 cells. Staining with fibronectin antibody is shown by the thick line and negative control antibody by the thin line. a, MDA-MB-231 cells; b, MDA-MB-231 cells after treatment with EDTA at 4°C to remove fibronectin. B, Western blot of 6-h culture supernatants from MDA-MB-231 cells (MDA), BMFs, coculture of MDA-MB-231 cells and BMFs (Coculture), coculture of cold/EDTA-treated MDA-MB-231 cells and BMFs (EDTA Treatment), and coculture of cold/EDTA-treated MDA-MB-231 cells to which fibronectin (1 mg/ml; FN Readdition), laminin (1 mg/ml; LM Readdition), or hyaluronic acid (1 mg/ml; HA Readdition) was added. C, quantitation of Western blots of MMP-2 in 6-h supernatants from MDA-MB-231 cells, BMFs, coculture of MDA-MB-231 cells and BMFs (Coculture), coculture of BMFs and cold/EDTA-treated MDA-MB-231 (EDTA Treated), and coculture of BMFs and cold/EDTA-treated MDA-MB-231 cells to which 1 mg/ml fibronectin was added (FN Readdition). Mean and SE of normalized data from six independent experiments are shown. *P < 0.04 compared with coculture. n.s., nonsignificant.
duced by adjacent normal fibroblasts. Increased levels of MMP-2 have been detected within 1 h of the initiation of contact and do not require new protein synthesis. The fibroblast origin of the MMP-2 is suggested by the decrease in fibroblast-associated MMP-2 and the absence of detectable MMP-2 in the MDA-MB-231 cells, whereas confocal microscopy suggests that this MMP-2 is surface membrane associated.

MMP-2 can associate with the cell surface through a number of mechanisms. MMP-2 can bind through its COOH-terminal, hemopectin-like domain to the integrin αvβ3 and MT1-MMP-associated TIMP-2 (16, 24). TIMP-2, which is directly associated with the cell surface (25), may also bind MMP-2. MMP-2 can also associate with the cell surface of gingival fibroblasts through the binding of the CBD of MMP-2 to cell-associated collagen I, V, and X (12). BMFMs express αvβ3 and MT1-MMP and contain collagen (Fig. 6A), making it likely that MMP-2 can bind to BMFs by a number of mechanisms. In this study, pretreatment of BMFs with recombinant CBD completely inhibited contact-induced elevation of MMP-2 in coculture supernatants, suggesting that MMP-2 liberated as a result of breast cancer cell/fibroblast contact binding to BMFs via its CBD. Other molecules containing fibronectin type II domains homologous with the CBD of MMP-2, including MMP-9 (containing three fibronectin type II modules) and fibronectin (containing only two modules), similarly prevented contact-mediated elevation of MMP-2. Breast cancer cells produce MMP-9, providing a possible explanation for the small increase in MMP-2 detected in culture supernatants from cocultures where direct contact was prevented, but transfer of soluble factors was possible (Figs. 1 and 2A; Ref. 18). MMP-9 binds collagen type I with much greater affinity than MMP-2, consistent with our observation that very small amounts of MMP-9 could elute MMP-2 from the BMF cell surface (26). Removal of fibronectin-associated collagen by enzyme treatment and/or blockade of synthesis also eliminated the contact-mediated increase in soluble MMP-2. Therefore, we conclude that MMP-2 eluted from BMFs by breast cancer cell contact is bound to fibroblast-associated collagen through the CBD.

Fibroblasts were detected on the surface of breast cancer cells, presumably bound via the fibronectin receptors expressed by these cells, including the integrins α5β1 and αvβ3 (18). The elimination of increased soluble MMP-2 when fibroblast had been removed from the surface of breast cancer cells before coculture, and the restoration of the effect after readdition of fibroblast, suggests that fibroblast is the primary protein involved in the displacement of MMP-2 from fibroblast-associated collagen. However, we cannot exclude a role for other breast cancer cell surface molecules in this process. It is worth noting that soluble fibroblast was much less effective at eluting fibroblast-associated MMP-2 than cell-bound fibroblast. It is possible that this is attributable to changes in the conformation of fibroblast after cell association (27). Indeed, the conformation of fibroblast is important for the binding of collagen, and cell surface association of fibronectin results in changes in its structure (28).

MMP-2 bound to cell-associated collagen remains in the inactive form even after stimulation with concavalin A (12). After coculture with breast cancer cells, inactive MMP-2 is released from BMFs, allowing activation after association with the MT1-MMP/TIMP-2 complexes expressed by MDA-MB-231 cells. As a result, both inactive and active MMP-2 levels are increased in the coculture supernatant (Fig. 1). Although BMFMs express MT1-MMP and TIMP-2, these molecules do not appear to be involved in activation of MMP-2 in cocultures, because coculture with T47D cells results in increased inactive MMP-2 only. In addition, when fibronectin was added back to MDA-MB-231 cells after cold/EDTA treatment, activation of MMP-2 did not occur despite the restoration of coculture-induced elevation of MMP-2 levels in the culture supernatant. This is consistent with the removal of TIMP-2 from MT1-MMP on the MDA-MB-231 cells as a result of the removal of Zn2+ (29). Removal of TIMP-2 would be expected to reduce the efficiency of activation of MMP-2 either by preventing the transient surface association of MMP-2 to MT1-MMP through TIMP-2 or by the inactivation of MT1-MMP by autolysis in the absence of TIMP-2 (17).

We have demonstrated previously that MMP-2 significantly contributes to the invasion of BMFs by MDA-MB-231 cells (18). As a result of this study, we conclude that cell contact between breast cancer cells and BMFs results in the rapid release of inactive membrane-associated MMP-2. Once released, MMP-2 may then associate with breast cancer cell-associated MT1-MMP/TIMP-2 complexes facilitating its activation and subsequent invasion of normal tissue by the malignant cells. Because most malignant cells will have surface-associated fibronectin, this is likely to be an important mechanism for the development of metastases in many cancers.

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Cancer Cell-associated Fibronectin Induces Release of Matrix Metalloproteinase-2 from Normal Fibroblasts

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