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3-D collagen-dependent cell surface expression of MT1-MMP and MMP-2 activation regardless of integrin β1 function and matrix stiffness

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ABSTRACT

Matrix metalloproteinases (MMPs) play roles in spatially dynamic processes, including morphogenesis, wound healing, and tumor invasion. Three-dimensional (3-D) type I collagen stimulates cellular activation of MMP-2, however, the mechanisms underlying this are controversial. The present study investigated mechanisms for 3-D collagen-induced MMP-2 activation in highly invasive human malignant mesothelioma cells. MMP-2 was effectively activated by cells cultured in 3-D collagen but not in 2-D collagen, whereas MMP-2 activation was not regulated by the flexibility of collagen. The 3-D collagen did not largely increase the gene expression of MMP-2 and MT1-MMP. However, MT1-MMP exposed to the cell surface was much increased by 3-D collagen, and loss of MT1-MMP abolished MMP-2 activation in response to 3-D collagen. MT1-MMP and integrin β1 translocated to pericellular regions interacting with collagen-coated microbeads, however their localization was different. Importantly, inhibition of integrin β1 function and expression did not affect 3-D collagen-induced cell surface localization of MT1-MMP and MMP-2 activation. Our results strongly suggest that 3-D collagen scaffolding may provide opportunity for direct and multivalent interaction with MT1-MMP, by which MMP-2 activation occur in abundant cell surface MT1-MMP-dependent manner, rather than a manner regulated by matrix stiffness and integrin β1 function.

1. Introduction

Tissues and organs are formed by specific cells and extracellular matrix (ECM) spatially organized in three-dimensional (3-D) structures. Although our knowledge about cellular behaviors is derived primarily from studies on planar tissue culture substrates, the importance of 3-D ECM has been recognized for epithelial cells and muscle cells, wherein 3-D environments promote cell polarity, differentiation, and morphogenesis [1]. Recent studies have demonstrated cellular locomotion and focal adhesion formation in 3-D ECM that differ from those in 2-D [2,3]. Mechanical properties of matrix stiffness are the proposed molecular basis of the 3-D ECM-induced morphogenesis of breast epithelial cells [4] and myotubes [5], and the differentiation of mesenchymal stem cells into specified cell types [6].

Matrix metalloproteinases (MMPs) are proteolytic enzymes with the ability to remodel the ECM as well as to regulate cellular modulators such as cell-adhesion molecules and growth factor receptors [7]. Membrane type 1 metalloproteinase (MT1-MMP) not only exhibits pericellular collagenase activity but also activates MMP-2, which is essential for skeletal development, cancer invasion, growth, and angiogenesis [8–10]. MMP-2 is secreted as a latent pro-enzyme and processed into its active form through the formation of a ternary complex composed of MMP-2, metalloproteinase inhibitor 2 (TIMP-2), and MT1-MMP [9–11]. Previous studies have demonstrated that 3-D type I collagen induced activation of MMP-2 in a wide variety of cells [12–19]. Because type I collagen is a major component of stromal tissue, and both normal and tumor cells digest collagen fibrils to grow or to invade connective tissues, 3-D collagen-dependent MMP-2 activation seems to be a fundamental mechanism in both physiological and pathological conditions. However, the mode of MMP-2 activation induced by 3-D collagen remains controversial and may use different mechanisms depending on cell types. The gene expression of MMP-2 [12,13], TIMP-2 [14], and MT1-MMP [12–15], as well as the cell surface localization [13,16,17] or processing [16,18] of MT1-MMP were increased by 3-D collagen. Collagen-induced MMP-2 activation is postulated to occur either directly or indirectly through integrin signaling [17,18]. Recent studies have demonstrated a functional interplay between MT1-MMP and integrins [19,20].

Abbreviations: 3-D, three-dimensional; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane type-1 matrix metalloproteinase; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP-2, metalloproteinase inhibitor 2.

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In the present study, the mechanisms for 3-D collagen-dependent MMP-2 activation were investigated, using highly invasive human mesothelioma cells. We addressed whether the difference in matrix stiffness or the difference in integrin signaling between 3-D and 2-D collagen is responsible for MMP-2 activation. We obtained evidence that 3-D but not 2-D collagen induces abundant cell surface localization of MT1-MMP, by which pericellular MMP-2 activation occur in cell surface MT1-MMP-dependent manner, rather than a manner regulated by matrix stiffness/flexibility and integrin β1 function.

2. Materials and methods

2.1. Cells and materials

Human malignant mesothelioma cells (ACC-Meso-1, EHMES-1, EHMES-10, and JMN-1B) were cultured as described previously [21]. Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science. For thin layer coating, a cover glass was coated with 0.1% gelatin (Sigma), 10 μg/ml fibronectin (Sigma), collagen type I (BD Biosciences), laminin (Sigma), or collagen type IV (Sigma) in phosphate buffer saline (PBS), washed twice with PBS, and blocked with 3% bovine serum albumin (BSA) in PBS. Matrigel Matrix was obtained from BD Biosciences. For cell culture in 3-D collagen, cells were suspended at 3×10^5 cells/ml of neutralized collagen type I (2 mg/ml) (BD Biosciences) and solidified at 37 °C for 90 min. Anti-MT1-MMP (for Western blot), anti-integrin α2 (for Western blot), anti-integrin α1 (FB12 for FACS and functional blocking), anti-integrin α2 (A2-IIE10 for FACS and functional blocking; P1E6 for functional blocking), anti-integrin α3 (ASC-6 for FACS and functional blocking), and anti-integrin β1 (P5D2 for FACS, functional blocking, and immunofluorescence; B3B11 for Western blot) antibodies were obtained from Millipore. Anti-MT1-MMP antibody (hinge region, for immunofluorescence) was obtained from Abcam. Anti-TIMP-2 antibody was obtained from Anaspec, Inc.

2.2. Preparation of the polyacrylamide substrate

Polyacrylamide gels were prepared as previously established [2,5]. In brief, *N*,*N*′ methylene-bis-acrylamide (ranging from 0.03% to 1%) was added to 10% acrylamide solutions and cross-linked by the addition of 10% ammonium persulfate (1/200 vol) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (1/2000 vol). The polymerizing solution was placed on an aminosilanized glass slide and covered with coverslips coated with dichlorodimethylsilane. The polyacrylamide gel was chemically cross-linked with type I collagen by using photo-reactive sulfosuccinimidyl-6-(4-azido-2-nitrophenylamino) hexanoate (Pierce). The polyacrylamide gel was washed with PBS and blocked with 3% BSA in PBS before use.

2.3. Quantitative PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen). First-strand cDNAs were synthesized using SuperScript III Reverse Transcriptase (Invitrogen) with a random hexamer. The primer sequences were as follows: human MT1-MMP (forward primer, 5′-cactgcctacgagaggaagg-3′ and reverse primer, 5′-ttggggtactcgctatccac-3′); human MMP-2 (forward primer, 5′-atgacagctgcaccactgag-3′ and reverse primer, 5′-atttgttgcccaggaaagtg-3′); human TIMP-2 (forward primer, 5′-aaagcggtcagtgagaagga-3′ and reverse primer, 5′-cttctttcctccaacgtcca-3′); and, human GAPDH (forward primer, 5′-gagtcaacggatttggtcgt-3′ and reverse primer, 5′-gacaagcttcccgttctcag-3′). Quantitative PCR was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and a Power SYBR Green PCR Master Mix (Applied Biosystems).

2.4. Western blotting and gelatin zymography

Cells were lysed and subjected to SDS–PAGE and Western blotting as described previously [21]. Conditioned media were collected from cultures in serum-free medium, and 20 μl samples were subjected to gelatin zymography, as described previously [22].

2.5. RNA interference

Small interfering RNA (siRNA) oligonucleotides were obtained from Nippon EGT. The siRNA sequences for human MT1-MMP and scrambled control sequences were described previously [23]. The siRNA sequences for human integrin β1 were as follows: sense, 5′-CUGUGAUAGAUCCAAUGGCtt-3′ and anti-sense, 5′-GCCAUUGGAUCUAUCACAGtt-3′. Cells were transfected with 100 nM siRNA by lipofectamine 2000 (Invitrogen) for 5 h, then medium was replaced with fresh RPMI1640 medium containing 10% FBS for 24 h before each assay.

2.6. Biotin labeling for cell surface proteins

Cells were cultured on thin layer collagen or 3-D collagen gel in serum-free medium for 40 h. The cells were harvested with trypsin/EDTA solution for thin layer collagen or 0.05% collagenase solution for 3-D collagen gel, washed twice with PBS, and surface proteins were labeled with a non-permeable sulfo-NHS-lc-lc-biotin (500 μg/ml in PBS, Pierce) at 4 °C for 1 h. After washing three times with PBS, cells were incubated with 50 mM Tris–HCl (pH 8.0)/150 mM NaCl for an additional 30 min at 4 °C. Washed cells were lysed with 500 μl of buffer composed of 20 mM Tris–HCl (pH 7.5), 1% (v/v) Triton X-100, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, pepstatin A, and leupeptin, and clarified by centrifugation. Biotin-labeled cell surface proteins were precipitated with 30 μl streptavidin–agarose (Sigma) for 4 h at 4 °C on a rotator, washed five times with lysis buffer with 500 mM NaCl and subjected to SDS–PAGE.

2.7. FACS analysis

The cells were harvested with trypsin/EDTA or 0.05% collagenase as described above, washed twice, and suspended in 10% BSA in PBS at 10^5 cells/ml. Cells were incubated with anti-integrin antibody or control mouse IgG_1 (0.5 μg per 10^5 cells in 10% BSA/PBS) and followed by secondary antibody conjugated to Alexa-488 (Invitrogen). Cells were suspended in 5 μg/ml propidium iodide/10% BSA/PBS. Cell surface expressions of integrins in viable cells were analyzed with FACS Canto (Becton Dickinson).

2.8. Collagen-coated beads and immunofluorescence

Polystyrene latex beads (Sigma, mean particle size 3.0 μm) were mixed with 1 mg/ml type I collagen in 0.02 N acetic acid or 3% BSA in PBS for 12 h at 4 °C and washed three times with PBS. Collagen- or BSA-coated beads were added to the cell culture and bound for 4 h. The cells were washed three times with PBS, fixed with 4% paraformaldehyde in PBS, and blocked with 2% BSA in PBS for 1 h at room temperature. The cells were incubated with anti-MT1-MMP and anti-integrin β1 antibodies in PBS with 3% goat serum, followed by secondary antibodies conjugated to Alexa Fluor-488 or -546. Cells were imaged using a laser-scanning confocal microscope (LMS510METASP; Carl Zeiss).

3. Results

3.1. Activation of MMP-2 by mesothelioma cells cultured on various ECMs

Human malignant mesothelioma cells expressing MMP-2 were studied for cellular processing of MMP-2. In all four lines, most MMP-2 was remained as latent form in cells cultured on a planar culture plate, but it was efficiently activated by cells cultured in 3-D type I collagen (Fig. 1A). Cellular activation of MMP-2 was not observed on the thin layer coat of type I collagen, fibronectin, laminin, or type IV collagen (Fig. 1B). Cellular activation of MMP-2 was observed when cells were cultured both within and on 3-D collagen gel (Fig. 1C), suggesting that cells did not recognize dimensionality (2-D versus 3-D). Matrigel, basement membrane fibrils largely composed of laminin and type IV collagen, did not support cellular activation of MMP-2 (Fig. 1C). These results suggested the specific regulation of MMP-2 activation by 3-D type I collagen.

3.2. Substrate flexibility in 3-D collagen-induced MMP-2 activation

To investigate whether 3-D collagen-induced MMP-2 activation is depend on substrate flexibility, Meso-1 cells were cultured on collagen-coated polyacrylamide gel with a wide range of flexibility obtained by changing the bis-acrylamide concentration from 0.03% to 1% as reported previously [2,4] (Fig. 2). Without the chemical cross-linking of collagen to polyacrylamide gel, polyacrylamide gel did not support cell adhesion (data not shown). A well-spread cell morphology, similar in appearance to cells on the 2-D culture, was seen in the case of 1% bis-acrylamide, while an insufficiently spread cell morphology similar in appearance to cells on the 3-D culture was seen in the case of 0.03% bis-acrylamide (Fig. 2A). In these conditions, activation of MMP-2 was not detected (Fig. 2B). This suggested that 3-D collagen-induced MMP-2 activation does not involve the mechanical compliance of the matrix.

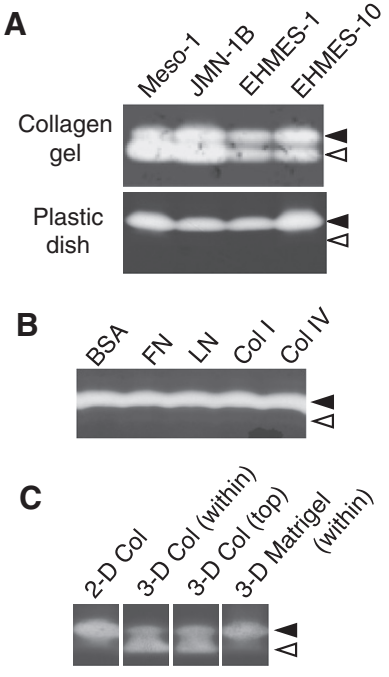

Fig. 1. Activation of MMP-2 by mesothelioma cells cultured on various ECMs. (A) MMP-2 activation in mesothelioma cells cultured in 3-D collagen gel or on a plastic dish for 2 days. (B) MMP-2 activation by Meso-1 cells cultured on a cover glass coated with various ECM. FN, fibronectin; LN, lamminin; Col I, type I collagen; and, Col IV, type IV collagen. (C) MMP-2 activation by Meso-1 cells cultured on a cover glass coated with a thin layer of collagen (2-D), within a 3-D collagen gel, on top of a 3-D collagen gel, or within 3-D Matrigel for 2 days. Conditioned media were subjected to gelatin zymography. Closed and open arrowheads indicate pro-MMP-2 and active-MMP-2, respectively.

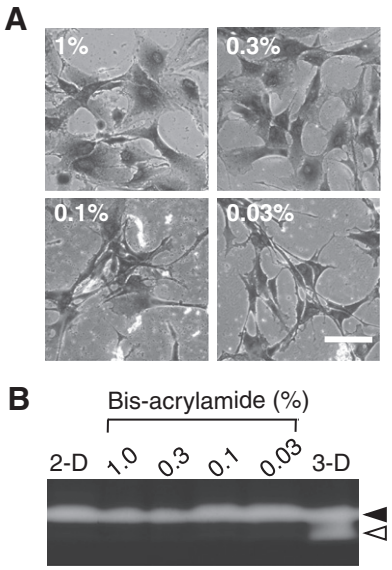

Fig. 2. Effect of matrix flexibility on MMP-2 activation. Meso-1 cells were cultured for 24 h on collagen-coated polyacrylamide gels with different elasticities prepared by varying the bis-acrylamide concentration in the polymerization reaction. Cells were fixed, stained with crystal violet, and photographed ((A), scale bar:100 μm). Conditioned media were subjected to gelatin zymography (B).

3.3. The central role of MT1-MMP in 3-D collagen-induced MMP-2 activation

Because transcriptional upregulation of MMP-2 [12,13], TIMP-2 [14], and MT1-MMP [12–15] have been reported in response to 3-D collagen, changes in mRNA levels for these genes were analyzed by quantitative PCR in Meso-1 cells cultured on collagen-coated plates (0 h) and in 3-D collagen for different time periods (Fig. 3A). mRNA of these genes showed less than 2-fold upregulation. Next, to analyze changes in protein levels, cell lysates or conditioned media from Meso-1 cells cultured on 2-D or 3-D for 40 h were subjected to Western blot for MT1-MMP and TIMP-2 (Fig. 3B, si-Scr). Consistent with mRNA level, MT1-MMP protein in total cell lysates was unchanged between 2-D and 3-D.

TIMP-2 was abundant in a conditioned medium of 2-D culture (Fig. 3B, si-Scr), suggesting that TIMP-2 secreted from cells does not anchor on cells in 2-D, while it effectively anchors on cells in 3-D. We tested whether the association of TIMP-2 with the cells is dependent on MT1-MMP using siRNA-mediated knockdown. In the cells treated with siRNA targeting MT1-MMP, MT1-MMP expression was strongly diminished, and this caused a remarkable decrease in cell-associated TIMP-2 levels and a lack of MMP-2 activation, even in 3-D cultures (Fig. 3B, si-MT1). These results indicated the essential role of MT1-MMP expression in the 3-D collagen-induced anchoring of TIMP-2 to cells and subsequent MMP-2 activation.

3.4. Cell surface localization of MT1-MMP in 3-D collagen

Given the essential role of MT1-MMP in 3-D collagen-induced MMP-2 activation without significant change in MT1-MMP mRNA and protein level, we test the possibility that 3-D collagen conditioning might influence cell surface localization of MT1-MMP [13,16,17]. Cell surface proteins were biotinylated in cells either cultured on collagen-coated plates (2-D) or on collagen gel (3-D)

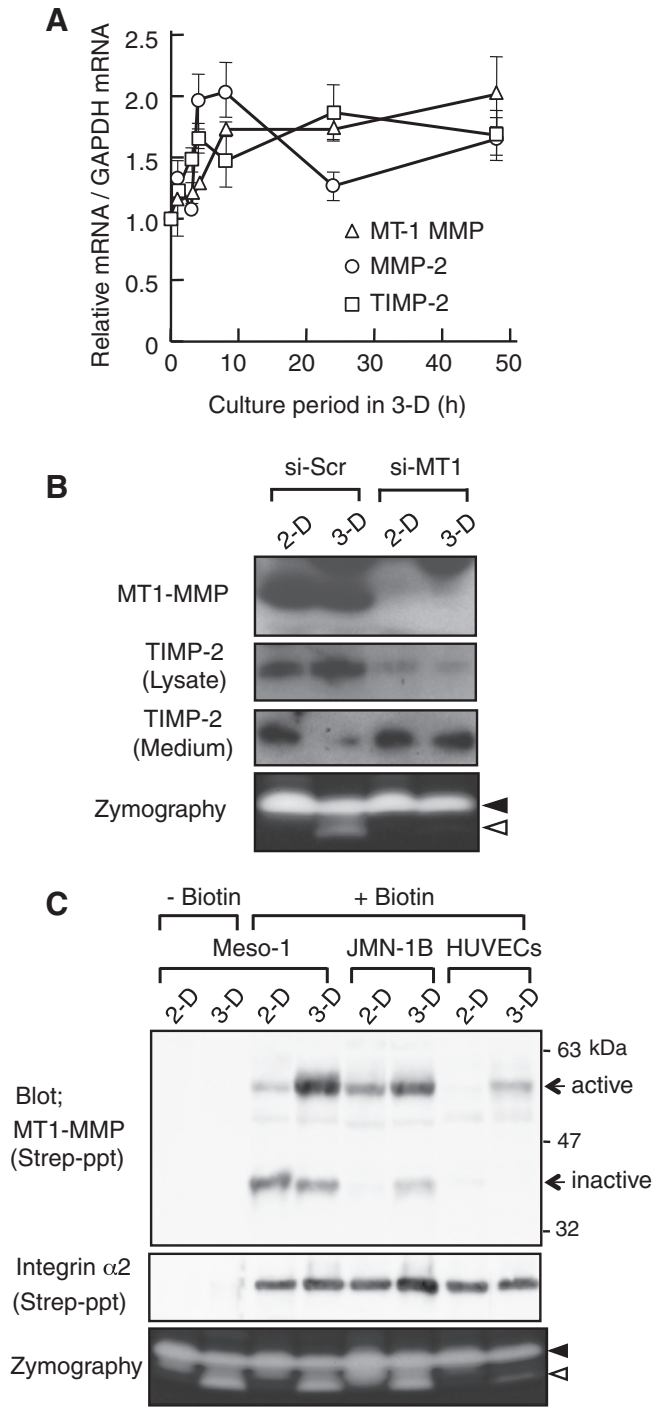

Fig. 3. Essential role of MT1-MMP and its cell surface localization in 3-D collagen-induced MMP-2 activation. (A) mRNA expression for MT1-MMP, MMP-2, and TIMP-2 in 3-D collagen. Meso-1 cells on a collagen-coated plate were harvested (0 h) and cultured in 3-D collagen for the indicated time periods. Relative mRNA levels normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels are presented as means ± SD. (B) Essential role of MT1-MMP in 3-D collagen-induced MMP-2 activation. Meso-1 cells were transfected with scrambled control siRNA (si-Scr) or siRNA targeting MT1-MMP (si-MT1), and cultured on a thin layer of collagen (2-D) or within collagen gel (3-D) for 2 days. Cell lysates were subjected to Western blotting using anti-MT1-MMP or anti-TIMP-2 antibody. Conditioned media were subjected to Western blotting using anti-TIMP-2 antibody or to gelatin zymography. (C) Increased cell surface localization of MT1-MMP in 3-D collagen. Mesothelioma cells (Meso-1 and JMN-1B) or endothelial cells (HUVECs) were cultured on 2-D or 3-D collagen for 2 days. Conditioned medium was subjected to gelatin zymography. Cell surface proteins were biotinylated, purified with streptavidin–agarose (Strep-ppt), and subjected to Western blot using anti-MT1-MMP antibody or anti-integrin α2 antibody.

for 40 h, purified, and subjected to Western blot for MT1-MMP (Fig. 3C). The active form of MT1-MMP exposed to the cell surface was much higher in the 3-D culture than in the 2-D culture of mesothelioma and endothelial cells. Integrin α2 localized on the cell surface was equivalent between 2-D and 3-D. This result suggested that the increased cell surface localization of MT1-MMP was a prerequisite for MMP-2 activation in response to 3-D collagen in cancer cell as well as normal endothelial cell.

3.5. Dispensable role of integrin β1 for the surface localization of MT1-MMP and MMP-2 activation

Cellular interaction with type I collagen is mediated largely through integrin α1β1, α2β1, and α3β1 receptors. Previous studies have indicated that crosslinking of integrin β1 could activate MMP-2 in ovarian carcinoma cells, suggesting direct involvement of integrin signaling in MMP-2 activation [17,18]. To test the involvement of integrin β1 in MMP-2 activation and cell surface localization of MT1-MMP, expression of integrin β1 was largely abolished in Meso-1 cells treated with siRNA for integrin β1 (Fig. 4A). These cells showed a round appearance both in 2-D and 3-D culture, confirming the functional knockdown of integrin β1 (Supplementary Fig. S1A). Unexpectedly, cell surface localization of MT1-MMP and MMP-2 activation were clearly induced in these cells in response to 3-D collagen (Fig. 4A). Furthermore, addition of functional blocking antibodies for integrin α1, α2, α3, or β1 could not prevent MMP-2 activation in response to 3-D collagen (Fig. 4B). Cell surface expression of integrin α1, α2, α3, and β1, as determined by FACS, showed comparative levels of these integrins between 2-D and 3-D cultures (Supplementary Fig. S1B). Taken together, these results strongly suggested that integrin β1 is dispensable for 3-D collagen-induced cell surface localization of MT1-MMP and MMP-2 activation.

Finally, to investigate further the relationship between MT1-MMP, integrin β1, and collagen, the distribution of MT1-MMP and integrin β1 on the cell membrane was analyzed after being attached to collagen-coated beads by immunofluorescence. Collagen-coated beads would effectively bind to Meso-1 cells, but none of the BSA-coated beads would (Fig. 4C). Cell surface MT1-MMP was increased at the peripheral layer of collagen-coated beads (Fig. 4D). Cell surface integrin β1 was localized at the base of the collagen-coated beads (Fig. 4D, arrowhead). The differential localization of MT1-MMP or integrin β1 with pericellular collagen suggests the direct interaction of MT1-MMP with collagen.

4. Discussion

3-D collagen induced transcriptional upregulation of MMP-2 [12,13], TIMP-2 [14], and MT1-MMP [12–15]. In the present study, a slight increase in MMP-2, MT1-MMP, and TIMP-2 mRNA and protein expression was observed, however, this could not explain the activation of MMP-2 that occurs almost exclusively in 3-D collagen. The ratio between TIMP-2 and MT1-MMP determines the activity of MT1-MMP [10,24]. Although we observed increased association of TIMP-2 to cells in 3-D, this was a consequence of MMP-2/TIMP-2/MT1-MMP ternary complex formation, because the association between TIMP-2 and cells, as well as MMP-2 activation, was abolished in cells devoid of MT1-MMP expression. Surface biotinylation analysis clearly demonstrated that 3-D collagen significantly increased the amount of MT1-MMP on mesothelioma and endothelial cell surfaces. Taken together, these results strongly suggest that cell surface localization of MT1-MMP leading to ternary complex formation with TIMP-2 and MMP-2 is a critical regulatory step in 3-D collagen-dependent MMP-2 activation.

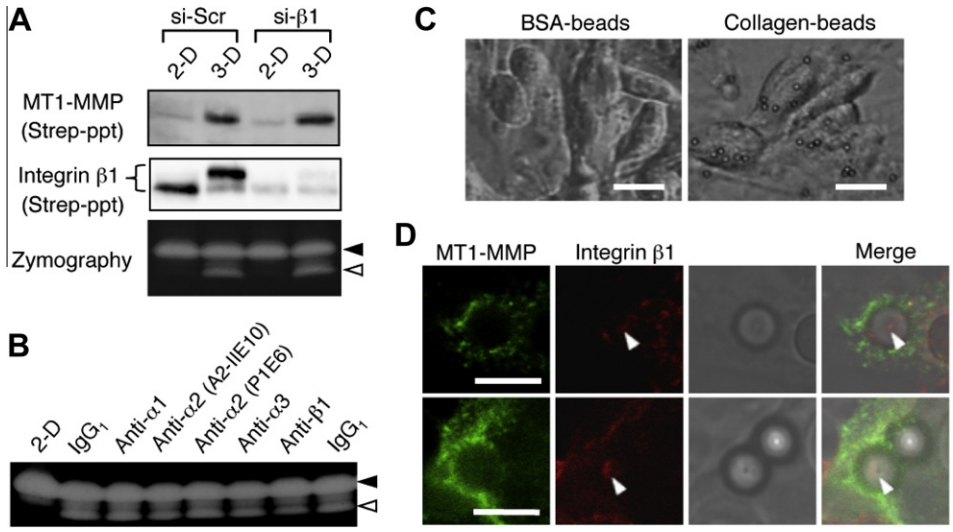

Fig. 4. Dispensable role of integrin β1 on cell surface localization of MT1-MMP and MMP-2 activation. (A) Meso-1 cells transfected with control siRNA (si-Scr) or integrin β1-targeting siRNA (si-β1) were cultured on 2-D or 3-D collagen for 2 days. Cell surface proteins were biotinylated, purified with streptavidin–agarose (Strep-ppt), and subjected to Western blot using anti-MT1-MMP antibody or anti-integrin β1 antibody, while the conditioned media were subjected to gelatin zymography. Note that cell preparation from the 2-D culture with trypsin resulted in cleavage of integrin β1. (B) Meso-1 cells were cultured on 2-D or 3-D collagen with 20 μg/ml of functional blocking anti-integrin antibody or control mouse IgG_1 for 2 days. Conditioned media were subjected to gelatin zymography (C, D) Cell surface distribution of MT1-MMP and integrin β1 upon attachment of collagen coated-beads. Collagen- or BSA-coated beads were added to a culture of Meso-1 cells for 4 h. The cells were photographed (C) and subjected to immunocytochemistry (D) with anti-MT1-MMP (green) or anti integrin β1 (red) antibody. Scale bars: 50 μm (C) or 5 μm (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Clustering of integrin β1 increased cell surface MT1-MMP, colocalization of MT1-MMP with integrin, and facilitated activation of MMP-2 [17,18]. MT1-MMP and integrin αVβ3 play a cooperative role in MMP-2 activation [19]. Association of MT1-MMP with β1 or αVβ3 integrins controls the internalization of MT1-MMP in endothelial cells [20]. Thus, it has been postulated that collagen-induced activation of MMP-2 occurs either directly or indirectly through integrin signaling. Here, cell surface localization of MT1-MMP and activation of MMP-2 were clearly induced in mesothelioma cells even when expression of integrin β1 was substantially abolished. Cell surface expressions of integrin α1, α2, α3, and β1 were equivalent between 2-D and 3-D. Although these results do not exclude the possibility that integrin signals can stimulate MMP-2 activation, the results of the present study showed that integrin β1 is dispensable for 3-D collagen-induced MT1-MMP surface localization and MMP-2 activation, at least in mesothelioma cells.

The differential localization of MT1-MMP or integrin β1 with pericellular collagen suggests the direct interaction of MT1-MMP with collagen, rather than integrin β1-mediated association. Previous studies have indicated that collagen can associate directly with the hemopexin domain of MT1-MMP, and addition of recombinant hemopexin domain inhibits collagen-induced MMP-2 activation [25]. The addition of type I collagen to cell cultures blocked internalization of MT1-MMP through the hemopexin domain, thereby leading to increases in surface MT1-MMP and MMP-2 activation [16]. Considering these reports and the results of the present study, it seems apparent that collagen might directly interact with MT1-MMP, leading to increased surface expression of MT1-MMP and MMP-2 activation.

What is the difference between 2-D and 3-D collagen that affects differential regulation of cell surface localization of MT1-MMP and MMP-2 activation? The results using collagen-coated gels with different degrees of stiffness have suggested that mechanical properties of extracellular scaffolding were not involved in MMP-2 activation. One possible explanation is that the amount of immobilized collagen on the culture plates is insufficient for MMP-2 activation, because tissue culture plates have a limited protein binding capacity, on the order of ∼μg, which is much less than the amount, on the order of ∼mg, of 3-D collagen gel.

In conclusion, the present study has demonstrated that 3-D collagen-dependent MMP-2 activation depends on neither integrin β1 nor collagen flexibility in malignant mesothelioma cells. Cell surface localization of MT1-MMP available for ternary complex formation with TIMP-2 and MMP-2 is a critical regulatory step in 3-D collagen-dependent MMP-2 activation. Collagen in a 3-D scaffold may provides opportunity as dense and multivalent scaffold capable of directly interacting with MT1-MMP, by which MMP-2 activation occur in abundant surface MT1-MMP-dependent manner.

Disclosure statement

The authors have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.050.

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