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Matrix metalloproteinase-2: A key regulator in coagulation proteases mediated human breast cancer progression through autocrine signaling

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ABSTRACT

Aims: Cell invasion is attributed to the synthesis and secretion of proteolytically active matrix-metalloproteinases (MMPs) by tumor cells to degrade extracellular matrix (ECM) and promote metastasis. The role of protease-activated receptor 2 (PAR2) in human breast cancer migration/invasion *via* MMP-2 up-regulation remains ill-defined; hence we investigated whether TF-FVIIa/trypsin-mediated PAR2 activation induces MMP-2 expression in human breast cancer.

Main methods: MMP-2 expression and the signaling mechanisms were analyzed by western blotting and RT-PCR. MMP-2 activity was measured by gelatin zymography. Cell invasion was analyzed by transwell invasion assay whereas; wound healing assay was performed to understand the cell migratory potential.

Key findings: Here, we highlight that TF-FVIIa/trypsin-mediated PAR2 activation leads to enhanced MMP-2 expression in human breast cancer cells contributing to tumor progression. Knock-down of PAR2 abrogated TF-FVIIa/trypsin-induced up-regulation of MMP-2. Again, genetic manipulation of AKT or inhibition of NF-κB suggested that PAR2-mediated enhanced MMP-2 expression is dependent on the PI3K-AKT-NF-κB pathway. We also reveal that TF, PAR2, and MMP-2 are over-expressed in invasive breast carcinoma tissues as compared to normal. Knock-down of MMP-2 significantly impeded TF-FVIIa/trypsin-induced cell invasion. Further, we report that MMP-2 activates p38 MAPK-MK2-HSP27 signaling axis that leads to actin polymerization and induces cell migration. Pharmacological inhibition of p38 MAPK or MK2 attenuates MMP-2-induced cell migration.

Significance: The study delineates a novel signaling pathway by which PAR2-induced MMP-2 expression regulates human breast cancer cell migration/invasion. Understanding these mechanistic details will certainly help to identify crucial targets for therapeutic interventions in breast cancer metastasis.

1. Introduction

Development and maintenance of multicellular eukaryotes are attributed to cell migration. The orchestrated cellular movement from one location to another is critical for embryonic development, wound healing and immune responses [1–3], defects in which results in severe consequences like intellectual disability, vascular disease, tumor formation and metastasis [4–6]. Metastasis, a phenotype of cancer aggressiveness, is the spread of cancer from the originating site to a site away from the primary tumor. This requires alteration of cellular actomyosin dynamics to break initial attachment, invade into circulation followed by migration and adherence to a different body part, forming a secondary tumor [7]. Invasion is facilitated by the secretion of active matrix-metalloproteinases (MMPs) which degrade extracellular matrix (ECM) and promote metastasis [8]. MMPs are calcium-dependent zinc-containing endopeptidases belonging to metzincin superfamily produced by the cells in zymogen form (Pro-MMPs) [9]. Based on substrate specificity, MMPs are categorized into different types as collagenase (MMP1, MMP8, MMP13), gelatinase (MMP-2, MMP9), stromelysin (MMP3, MMP10, MMP11), matrilysin (MMP7, MMP-26), enamelysin (MMP-20), metalloelastase (MMP12), membrane-type MMPs (MMP14, MMP15, MMP16, MMP17, MMP-24, MMP-25) etc. Previous studies have demonstrated that MMP-2 participates in breast cancer invasion under various physiological/pathological conditions [10]. Membrane type1 MMP (MT1-MMP, MMP14) is responsible for activating latent MMP-2 (Pro-MMP-2). Tissue inhibitors of metalloproteinase-2 (TIMP-2)

Abbreviations: PAR2, protease-activated receptor2; TF, tissue factor; FVIIa, activated factor VII; MMP-2, matrix metalloproteinase-2; rMMP-2, recombinant MMP-2; HSP27, heat shock protein 27; RT-PCR, reverse transcriptase-polymerase chain reaction; NF-κBI, NF-κB inhibitor

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promote the activation by acting as a bridging molecule tethering Pro-MMP-2 and MT1-MMP. Then pro-MMP-2 is cleaved by an adjacent TIMP-2-free MT1-MMP thereby forming active-MMP-2 [11].

Studies reveal that a positive correlation exists between blood coagulation and cancer progression [12]. Tissue Factor (TF), a transmembrane glycoprotein localized in the extravascular region, is the key initiator of blood coagulation. Upon vascular injury, TF directly contacts with the clotting protease factor VIIa (FVIIa) forming TF-FVIIa complex which converts soluble fibrinogen to insoluble fibrin to cease bleeding [13]. Apart from coagulation, the complex has also been reported to induce signaling events in cancerous development and metastasis, *via* activation of a G-protein coupled receptor (GPCR) family protein, protease-activated receptor2 (PAR2) [14]. PAR2 is activated by trypsin [15] leading to cell proliferation, metastasis, angiogenesis etc.

Previous studies have demonstrated that PAR2 activation promotes cancer progression by increasing cancer cell metastasis [16]. MMP-2 has been shown to contribute to cancer cell invasion by ECM degradation through matrix proteolysis [17]. Here, we elucidate that TF-FVIIa/trypsin-mediated PAR2 activation promotes MMP-2 expression in human breast cancer cells *via* PI3K/AKT pathway, followed by IκBα degradation and translocation of NF-κB into the nucleus. The secreted MMP-2 cleaves ECM to promote tumor invasion. The role of MMP-2 in inducing cellular signaling in the context of cell migration is poorly understood. Here, we show that MMP-2 promotes p38 MAPK phosphorylation, which activates downstream molecules MK2 and HSP27. HSP27 activation leads to actin polymerization [18] which is critical for lamellipodia formation during cell migration [19]. Our study first time reveals that coagulation factors-mediated PAR2 activation leads to cancer progression *via* MMP-2, by not only enhancing its expression to degrade ECM to facilitate invasion but also inducing autocrine signaling to promote cell migration *via* actin rearrangements.

2. Materials and methods

2.1. Cell culture

The human breast cancer cell lines, MDAMB231 and MCF7 were purchased from American Type Culture Collection (ATCC) and maintained in standard DMEM (Gibco, Invitrogen) whereas; SKBR3 cells (ATCC) were cultured in McCoy's 5 A medium. All the growth media were supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin solution (Invitrogen).

2.2. Western blot analyses

- *Analysis of MMP-2 expression*

In order to determine MMP-2 expression, cells were seeded onto 24-well plate and allowed to reach 80% confluency. Cells were serum starved for 2 h followed by the treatment of human recombinant FVIIa (100 nM) or trypsin (5 nM). After 16 h of incubation, cells were lysed with laemmli buffer and proteins were subjected to western blot analysis for analyzing MMP-2 content. Alternatively, cells were pre-treated with various inhibitors for an hour unless specified otherwise followed by the addition of stimulus. For knock-down studies, cells were transfected with MMP-2 siRNA or AKT siRNA or their scrambled form for 48 h followed by the treatment with the ligands as described above.

- *Determination of intracellular activation of signaling molecules*

To determine the role of AKT/NF-κB axis in MMP-2 expression, cells were seeded onto 24 well-plates and allowed to reach 80% confluency. Serum starvation was given with serum-deprived DMEM media for 2 h followed by treatment of FVIIa or trypsin. AKT and IκBα phosphorylation was analyzed after 10 min and 30 min respectively of ligands treatment, whereas nuclear translocation of NF-κB was monitored an hour post-ligands addition. NF-κBI (blocks nuclear translocation of NF-κB thereby preventing its transcriptional activity) was introduced an hour before ligands treatment.

- *Determination of intracellular signaling molecules activation by MMP-2*

Cells were grown in 24 well-plates and serum starvation was given for 2 h followed by the treatment of human recombinant MMP-2 (rMMP-2; 30 ng/ml). Inhibitors like SB203580 or PF-3644022 hydrate were introduced an hour before rMMP-2 addition. Cells were lysed after the indicated time period and the activation of p38 MAPK (15 min), MK2 (30 min) and HSP27 (1 h) was analyzed by phospho-specific antibodies against these proteins using western blot approach. The following phospho-antibodies were applied: phospho-AKT (Ser 473), phospho-IκBα (Ser 32), phospho-p38 MAPK (Thr180/Tyr182), phospho-MK2 (Thr334), and phospho-HSP27 (Ser82). Normalization was carried out with the non-phospho form of the target proteins. MMP-2, Histone H3, GAPDH, TF, PAR2 and β-actin antibodies were used as per the requirements. Antibodies were purchased from Cell Signaling Technology.

In another approach, rMMP-2 was pre-incubated with MMP-2 catalytic inhibitor ARP 100 for 10 h. MDAMB231 cells were serum starved for 2 h followed by the addition of rMMP-2 ± ARP 100. After 15 min of incubation, cells were lysed and P38 MAPK phosphorylation was analyzed by western blotting.

- *The standard protocol of western blotting*

Western blot was performed by the standard western blotting protocol. Briefly, cells were lysed and the lysates were boiled at 95 °C for 5 min followed by the separation of proteins by SDS-PAGE. Proteins were transferred onto PVDF membrane and the membrane was blocked with 5% BSA in TBS. The membrane was incubated with primary antibody (1:1000) overnight at 4 °C followed by washing with TBST [TBS-Tween 20 (0.1%)] and secondary antibody (1:5000; Sigma) treatment for an hour. After TBST washing, the membrane was subjected to development by ECL method. Densitometric scanning of blots was performed by Image J and graph was prepared by using GraphPad Prism5.

2.3. Gelatin zymography

Cells were seeded onto 60 mm dishes and serum starvation was given followed by FVIIa or trypsin treatment. After 16 h, cells were lysed with lysis buffer [Tris (50 mM), NaCl (150 mM), 1% NP-40, $MgCl_2$ (10 mM) and DTT (5 mM) containing protease inhibitor (Roche)] with periodic agitation and centrifuged at 16,000 rpm for 15 min to separate cell debris. Proteins in the supernatant were mixed with laemmli buffer (2×) and loaded onto SDS gel containing gelatin. The gel was washed with 2.5% Triton X-100 and incubated overnight with incubation buffer (NaCl, $CaCl_2$, Tris, and NaN_3). Staining was done with Coomassie brilliant blue (G-250) and after destaining images of the gels were taken from which band intensity was measured and accordingly graph was prepared. Zymography was also performed with proteins in the cell-supernatant to detect the presence of active MMP-2 in the extracellular medium.

2.4. Semi-quantitative (reverse transcriptase) PCR and quantitative (real time) PCR analysis

Cells were cultured in 60 mm dishes and challenged with FVIIa or trypsin along with untreated control for 12 h and total RNA was extracted by conventional TRIZOL method (Invitrogen). Inhibitors were introduced an hour before ligands addition. Reverse transcription was performed to synthesize first strand cDNA using oligo-dT as the primer (GCC Biotech). PCR amplification of MMP-2 gene was performed with human specific MMP-2 primers: forward 5′- GTGCTGAAGGACACACT