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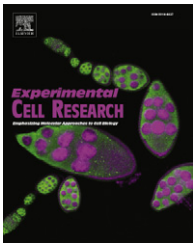

Research Article

Protein disulfide isomerase-mediated disulfide bonds regulate the gelatinolytic activity and secretion of matrix metalloproteinase-9

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ABSTRACT

Matrix metalloproteinase-9 (MMP-9) is one of the major MMPs that can degrade extracellular matrix. Besides normal physiological functions, MMP-9 is involved in metastasis and tumor angiogenesis. Although several inhibitors of MMP-9 have been identified, *in vivo* regulators of MMP-9 activation are unknown. In the present study we intended to investigate novel therapeutic target protein(s) that regulate MMP-9 activation and/or secretion. We have identified protein disulfide isomerase as a novel upstream regulator of MMP-9. Mass spectrometric analysis of post-translational modification in MMP-9 confirmed six disulfide bonds in the catalytic domain and one disulfide bond in the hemopexin domain of MMP-9. Establishment of cells that overexpressed wild-type and mutant forms of MMP-9 revealed that 'cysteine-switch' and disulfide bonds within the catalytic domain are necessary for the secretion and intracellular trafficking of MMP-9. However, the disulfide bond of the hemopexin domain and other cysteines have no significant role in secretion. These insights into the secretion of MMP-9 constitute the basis for the development of potential drugs against metastasis.

Introduction

Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix (ECM). MMPs play critical roles in pathological process like inflammation, arthritis and metastasis, besides normal physiological functions such as embryonic development, reproduction and tissue remodeling [1]. MMP-9 is under tight control by cytokines and inflammatory cells and by dysregulation of oncogenes and tumor suppressor genes [2]. MMP-9 promotes tumor progression and tumor-induced

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Abbreviations: MMP, matrix metalloproteinase; PDI, protein disulfide isomerase; ECM, extracellular matrix; DTT, dithiothreitol; FN-type II, Fibronectin type-II.

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angiogenesis by degrading most of the ECM substrates such as collagenase-treated collagen, aggrecan and fibronectin [3–6]. MMP-9 is not constitutively expressed in normal cells; rather it is expressed in tumor cells. MMP-9 is overexpressed in skin carcinogenesis [7], lung carcinoma [8], breast cancer [9], hepatocellular carcinoma [10], gliomas, and giant cell tumor of bones. Therefore, elucidating the regulatory mechanism of MMP-9 activation and secretion is a prerequisite for potential drug therapy.

Human MMP-9 is composed of 707 amino acids, which includes 19 cysteine residues [11]. Two of these cysteine residues are located in the signal peptide and this region is cleaved during MMP-9 translation. MMP-9 is synthesized and secreted as an inactive zymogen, pro-MMP-9 [12]. Inactive state of pro-MMP-9 is maintained by the interaction between a cysteine-sulfhydryl group of Cys99 in the propeptide domain and the zinc ion bound to the catalytic domain, known as the 'cysteine-switch' mechanism [13,14]. Catalytic activity of MMP-9 is regulated at the level of transcription, protein secretion, zymogen activation and finally by inactivation of the activated enzyme [5]. Following synthesis and secretion, MMPs can be activated by disruption of the cysteine-switch. Cysteine-switch modification can be done by physiological (*e.g.*, oxidant, disulfides, electrophiles) or non-physiological (*e.g.*, alkylating agents, and heavy metals) means; that leads to inter or intramolecular autolytic cleavage of the pro-domain [5,13]. Alternatively, the pro-domain can be directly cleaved by other proteases like furin, plasmin, and other MMPs [15,16]. In the case of MMP-9, activation can be achieved by several MMPs such as, MMP-2, MMP-3, and MMP-13 [17–19]. However, activated MMP-9 can in turn be inhibited by the universal protease inhibitor α-2 macroglobulin and/or specific tissue inhibitors of MMPs (TIMP), particularly TIMP-1 [20]. MMP-9 has unique structural features which confer distinct functions. Besides the pro-peptide and hemopexin domain it contains three fibronectin type-II (FN-type II) repeats in the catalytic domain like that of MMP-2; moreover, it has a unique collagen V-like domain. The hemopexin and catalytic domains are connected by a highly flexible O-glycosylated domain [21]. MMP-9 degrades denatured collagen or gelatin through binding of FN-type II to the substrates [5]. Although the FN-type II of human fibronectin has two disulfide bonds, it has not been experimentally validated whether there are disulfide bonds in the FN-type II repeats of MMP-9. However, it has been found that cysteines of MMP-9 contribute to the formation of homomultimers and heteromultimers with neutrophil gelatinase B-associated lipocalin [22]. Recently, mutagenesis was used to study these disulfide-bridged multimers [23]. In the present study we focused on the role of disulfide bonds in the monomeric MMP-9.

Protein disulfide isomerase (PDI) is an ER localized protein with oxidation, reduction and isomerization activities. PDI maintains proper protein folding by forming disulfide bonds in the nascent proteins through oxidoreductase activity [24]. Overexpression of PDI is observed in invasive tumors [25,26]. Thus we hypothesized a positive correlation between PDI and MMP-9 in tumor cells. Disulfide bonds are important for structural integrity and functional regulation of proteins. It is also crucial for the stabilization, secretion and induction of conformational changes to bind with the target protein [27].

In the present study, we discovered PDI as a novel regulator for MMP-9 activation and secretion. Through mass spectrometric analysis, we also identified six disulfide bonds in the catalytic domain of monomeric MMP-9. It was found that the cysteine-switch and disulfide bonds in the catalytic domain are necessary for the secretion of MMP-9. On the contrary, the disulfide bond in the hemopexin domain and other cysteines exerted either negligible or no effect on MMP-9 secretion. Thus our results suggest that inhibitors against PDI or disulfide bond formation may offer effective therapeutic potential for cancer metastasis.

Materials and methods

Construction of plasmids and establishment of stable cell lines

To examine the effect of PDI on MMP-9 activation and secretion, we constructed FLAG-tagged PDI vector into pCI-Neo (Promega) vector. The FLAG epitope was introduced between Gln^{501} and Lys^{502} within human PDI. A stable cell line expressing PDI-FLAG was established by transfecting pCI-PDI-FLAG into HT1080 cells using Effectence Transfection Reagent (QIAGEN) and was designated as HT1080-PDI-FLAG cells. Cells transfected with the pCI-Neo vector were designated as HT1080-Mock cells. The human MMP-9 gene was cloned into the pcDNA3.1/Myc-His (+) vector (Invitrogen), as described before [28]. A series of MMP-9 mutants was constructed where all cysteine residues, except the two in signal peptide, were substituted with serine by PCR site-directed mutagenesis using the overlap extension technique [29]. Permanent cell lines expressing MMP-9 were established by transfecting pcDNA3.1/Myc-His (+)-MMP-9 into HT1080 cells. The cells transfected with pcDNA3.1/Myc-His (+) were designated as HT1080-Neo and those expressing high levels of MMP-9 were designated as HT1080-MMP-9-MH cells. We also established the stable cell lines that expressed mutant forms of MMP-9 and were designated as HT1080-MMP-9-MH/C99S, HT1080-MMP-9-MH/C230S, HT1080-MMP-9-MH/C244S, HT1080-MMP-9-MH/C256S, HT1080-MMP-9-MH/C271S, HT1080-MMP-9-MH/C288S, HT1080-MMP-9-MH/C302S, HT1080-MMP-9-MH/C314S, HT1080-MMP-9-MH/C329S, HT1080-MMP-9-MH/C347S, HT1080-MMP-9-MH/C361S, HT1080-MMP-9-MH/C373S, HT1080-MMP-9-MH/C388S, HT1080-MMP-9-MH/C468S, HT1080-MMP-9-MH/C516S, HT1080-MMP-9-MH/C674S, and HT1080-MMP-9-MH/C704S.

Gelatin zymography

Exponentially growing cells were seeded at 1.5×10^5 cells per well in 6-well plates (Sumilon) for 12 h. After cell attachment, the conditioned media were exchanged for serum-free medium for 24 h. The conditioned media were collected and centrifuged at 15,000 rpm for 15 min and loading buffer without 2-mercaptoethanol was added to the supernatants that were subsequently electrophoresed on an SDS-polyacrylamide gel containing 0.1% gelatin at 4 °C. After electrophoresis, the gel was washed twice with a 2.5% Triton X-100 for 1 h and incubated overnight in incubation buffer [50 mmol/L Tris–HCl (pH 7.5), 200 mmol/L NaCl, and 10 mmol/L $CaCl_2$] at 37 °C. Then, the gel was stained with Coomassie Brilliant Blue G-250 [28]. For cell lysate preparation, cells were washed with PBS and 2-mercaptoethanol-free loading buffer was added after sonication at 4 °C.

RNA interference

HT1080-Mock and HT1080-PDI-FLAG cells (1.5×10^4) were seeded in a 6-well plate and incubated for 24 h. Then the cells were transfected with 200 pmol siRNA using Lipofectamine2000 (Invitrogen)