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MMP2 and MMP9 participate in S1P-induced invasion of follicular ML-1 thyroid cancer cells

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ABSTRACT

The bioactive lipid sphingosine-1-phosphate (S1P) has emerged as a potent inducer of cancer cell migration and invasion. Previously, we have shown that S1P induces invasion of ML-1 follicular thyroid cancer cells via S1P receptors 1 and 3 ($S1P_{1,3}$). Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes used by cells for degradation of the extracellular matrix during invasion and migration. In the present study, we examined the role of MMP2 and MMP9 for S1P-induced invasion of ML-1 cells, and found that S1P regulates the secretion and activity of MMP2 and MMP9 via $S1P_{1,3}$. Both pharmacological inhibitors and siRNA knockdown of MMP2 and MMP9 could attenuate S1P-induced invasion. Additionally, we show that calpains and Rac1 mediate S1P-induced secretion of MMP2 and MMP9. In conclusion, MMP2 and MMP9 participate in S1P-evoked follicular ML-1 thyroid cancer cell invasion.

1. Introduction

Sphingolipids are very interesting molecules, not only because they are one of the components of the lipid membranes, but also as regulators of many central events in the cell. Sphingosine-1-phosphate (S1P) is present in the blood at high nanomolar concentrations, and has been extensively studied due to its ability to regulate cell function both during normal development and during progression of many types of diseases, including cancers. S1P exerts its effect through binding to one of its five G-protein coupled receptors ($S1P_{1-5}$), and by activating different G-proteins. However, the net result of S1P action depends on which receptor and G-protein it activates. S1P evokes migration via the pro-migratory $S1P_{1,3}$ and $G_{i/0}$ protein, while an inhibitory effect is seen when the inhibitory receptor $S1P_2$ and $G_{q/12}$ protein are activated (Selvam and Ogretmen, 2013; Pyne and Pyne, 2010).

The migration and invasion process requires proteolysis of the basement membrane and the extracellular matrix. For this purpose cells express matrix degrading and modifying enzymes. Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes of the extracellular matrix, widely used by cells during invasion and migration (Stamenkovic, 2000; Folgueras et al., 2004). The MMP family is huge and is divided into several subgroups depending on their function and localization in tissues. MMP2 and MMP9 are secreted into the extracellular space, and use type IV collagen as their substrate. Both of them have been strongly correlated with the invasiveness of many types of cancer cells (Devine et al., 2008; Yang et al., 2013; Kim et al., 2011; Rajoria et al., 2011; Zhang et al., 2012).

MMP2 and MMP9 exist as inactive zymogens with pro-peptide domains, which must be removed in order for them to become activated. Calpains, which are calcium-dependent proteolytic enzymes, have been shown to cleave and activate MMPs. Calpains regulate several essential functions in the cell, such as proliferation, angiogenesis, survival, apoptosis, as well as invasion and migration (Storr et al., 2011). Moreover, sphingolipids have been shown to regulate calpains (Kwak et al., 2012; Kang et al., 2011). In addition, Chen et al. (2013) have shown that calpains are regulating hepatocarcinoma cell (HCC) migration via increased secretion and activity of MMP2 and MMP9.

The small GTP-ase Rac1 is well characterized as a regulator of cell motility by inducing formation of lamellipodia and membrane ruffles via a subset of different signaling intermediates. Rac1 regulates several other functions, such as cell adhesion, invasion, establishment of cell polarity and cell proliferation (Murali and Rajalingam, 2014). Furthermore, Rac1 has been implicated in

Abbreviations: ERK, extracellular regulated kinase; HIF-1α, hypoxia inducible transcription factor 1α; LOX, lysyl oxidase; MMP, matrix metalloproteinase; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; S1P, sphingosine-1-phosphate; $S1P_{1,3}$, S1P receptors 1 and 3; uPA and uPAR, urokinase plasminogen activator and its receptor.

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regulating S1P-induced MMP2 and MMP9 secretion in epithelial ovarian cancer cell lines (Devine et al., 2008).

In thyroid cancer cells, we have previously shown that S1P plays a major role in regulating migration, and signaling events important for this process (Balthasar et al., 2006; Balthasar et al., 2008; Bergelin et al., 2009; Kalhori et al., 2013; Törnquist, 2012). S1P induces migration of follicular thyroid cancer cells (ML-1 cells) via $S1P_{1,3}$ and activation of downstream signaling molecules, such as ERK1/2, PKC, PI3K, Rac1 and hypoxia inducible transcription factor 1α (HIF-1α) (Balthasar et al., 2008; Bergelin et al., 2009; Kalhori et al., 2013). However, the mechanisms underlying the S1P-induced invasion of thyroid cancer cells remain poorly understood.

Several previous studies have shown that an increased expression and activity of both MMP2 and MMP9 correlate with an enhanced invasion of thyroid cancer cells (Rajoria et al., 2011; Yang et al., 2013; Zhang et al., 2012). However, the significance of MMP2 and MMP9 for S1P-induced invasion and migration of thyroid cancer cells has not been elucidated. The aim of this present study was to investigate the ability of S1P in regulation of the expression, secretion and activity of MMP2 and MMP9. We were also interested in the importance of these degrading enzymes for S1P-induced invasion of ML-1 cells. Our results show that S1P increases the secretion and activity of MMP2 and MMP9 via $S1P_{1,3}$, and that S1P-evoked invasion and migration of ML-1 cells is partially mediated by MMP2 and MM9. The S1P-induced MMP2 and MMP9 secretion was, at least in part, mediated by calpains and Rac1, and inhibition of calpains blocked S1P-induced invasion.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fatty acid-free BSA and SB-3CT were purchased from Sigma Aldrich (Schnelldorf, Germany). Penicillin/streptomycin, trypsin and L-glutamine were from Invitrogen (Carlsbad, California, USA). Type IV collagen was purchased from Becton Dickinson Biosciences (Bedford, MA, USA). Transwell inserts for invasion assays were from Corning (Corning, NY, USA). S1P was from Enzo Life Sciences (Plymouth, PA, USA). VPC23019 was from Avanti Polar lipids (Alabaster, AL, USA). GM6001 was from Merck (Darmstadt, Germany). The rabbit β-actin antibody and rabbit MMP2 antibody were from Cell Signaling Technology (Danvers, MA, USA). The HRP-conjugated goat anti-rabbit antibody was from Bio-Rad Laboratories (Hercules, California, USA). FTY720 was purchased from Selleckchem (Houston, USA). CAY10444 was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The rabbit MMP9 antibody and the calpain activity assay kit were from Abcam (Cambridge, MA, USA). The BCA Protein Assay kit (bicinchoninic acid) was from Pierce Biotechnology (Rockford, IL, USA). siRNA against MMP2 (ON-TARGET SMART pool siRNA: ACAAGAACCAGAUCACAUA), MMP9 (ON-TARGET SMART pool siRNA: GCAUAAGGACGACGUGAAU) and a negative non-targeting siRNA control were from Dharmacon (Dallas, TX, USA). The CellTiter 96 AQueous One Solution proliferation kit was from Promega (Madison, WI, USA). Nitrocellulose transfer membrane was from Whatman (Maidstone, UK). The Western Lightning Plus-ECL kit was from Perkin Elmer (Waltham, MA, USA). The Amaxa electroporation device and Amaxa Cell Line Optimization Nucleofector Kit were from Lonza (Basel, Switzerland). ALLN and Rac1 inhibitor (W56) were from Calbiochem (Darmstadt, Germany).

2.2. Cell culture

The human follicular thyroid cancer cells (ML-1 cells) were a kind gift from Dr. Johann Schönberger (University of Regensburg, Germany; Schonberger et al., 2000). The authenticity of the cell line was verified at the CU Cancer Center, DNA Sequencing and Analyses Core (University of Colorado Denver, USA). The ML-1 cells were cultured in DMEM medium supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. The cells were allowed to grow in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37 °C. FTC-133 thyroid follicular cancer cells were obtained from Banca Biologica e Cell Factory, National Institute for Cancer Research (Genova, Italy). The cells were grown in DMEM-Ham's F12 medium (1:1) supplemented with 10% FBS and 2 mM L-glutamine.

2.3. Cell invasion assay

For the invasion experiments, 6.5-mm-diameter Transwell inserts with 8 μm pore size were used (Corning Costar, Bodenheim, Germany). The day before an experiment, the medium was changed to serum-free medium (SFM) and the membranes were coated with 5 mg/cm^2 collagen IV. Prior to an experiment, the cells were treated with the indicated inhibitor for the times indicated. The cells were harvested in SFM medium and 200,000 cells were added to the upper chamber. DMEM medium containing 10% lipid stripped FBS (LS-FBS) with or without S1P (100 nM) was used as chemoattractant. The indicated inhibitors were present in both chambers throughout the entire experiments. The inserts were incubated in a water-saturated incubator at 37 °C in 5% CO_2 and 95% air for 6 h. The cells that had migrated to the lower surface of the filter were fixed with 2% paraformaldehyde in PBS for 10 min, and stained with 0.1% crystal violet in 20% methanol for 5 min. After removing the nonmigratory cells with a cotton swab, the chambers were allowed to dry over night. The migrated cells were counted using 40× magnification in 8 fields in a straight line bisecting the membrane.

2.4. Western blot analysis

ML-1 cells and FTC cells were grown as described earlier in medium containing 5% LS-FBS for 1 day. At the day of the experiment, cells were treated with the indicated concentrations of S1P. In some experiments cells were preincubated with the $S1P_{1,3}$ inhibitor VPC-23019 (1 μM), the $S1P_{1,3}$ functional antagonist FTY720 (10 μM), the $S1P_3$ antagonist CAY10444 (10 μM), Rac1 inhibitor (20 μM) or ALLN (50 μM) for 1 h, and then incubated with S1P (100 nM) for 4 h. In the experiments in Fig. 3 cells were pretreated with the S1P receptor inhibitors as described earlier, and then stimulated with 100 nM S1P for 48 h. The inhibitors were present throughout the entire experiments. Thereafter medium was collected, and cells were then washed with ice-cold PBS, and lysed in lysis buffer (10 mM Tris pH 7.7, 150 mM NaCl, 7 mM EDTA, 0.5% NP-40, 0.2 mM PMSF, 0.5 μg/ml leupeptin). Cell lysates were centrifuged at 13,000 rpm for 15 min at 4 °C, whereafter the pellets were removed and supernatants were collected. Protein concentrations were determined with the BCA Protein Assay kit, thereafter Laemmli sample buffer (LSB) was added and the lysates boiled. Western blot analysis was performed as described previously (Balthasar et al., 2008). The primary antibodies used were anti-MMP2, anti-MMP9 and β-actin (1:1000 in 5% BSA in TBST (Tris-buffered saline (NaCl, 150 mM; Tris-base, 20 mM (pH 7.5)) containing 0.1% Tween 20. The secondary antibodies used were peroxide-conjugated goat anti-rabbit antibodies (1:3000 in 5% milk in 0.1% TBST). The amounts of secreted MMPs were normalized against total protein concentrations in the plates. In experiments where the expression of MMPs was investigated, the results were normalized against β-actin.

2.5. Transfection of ML-1 cells

The cells were transfected with 100 nM siRNA against MMP2 and MMP9. For control purpose, a non-targeting siRNA negative control