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A catalytically inactive gelatinase B/MMP-9 mutant impairs homing of chronic lymphocytic leukemia cells by altering migration regulatory pathways

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ABSTRACT

We previously showed that MMP-9 overexpression impairs migration of primary CLL cells and MEC-1 cells transfected with MMP-9. To determine the contribution of non-proteolytic activities to this effect we generated MEC-1 transfectants stably expressing catalytically inactive MMP-9MutE (MMP-9MutE-cells). In xenograft models in mice, MMP-9MutE-cells showed impaired homing to spleen and bone marrow, compared to cells transfected with empty vector (Mock-cells). *In vitro* transendothelial and random migration of MMP-9MutE-cells were also reduced. Biochemical analyses indicated that RhoAGTPase and *p*-Akt were not downregulated by MMP-9MutE, at difference with MMP-9. However, MMP-9MutE-cells or primary cells incubated with MMP-9MutE had significantly reduced *p*-ERK and increased PTEN, accounting for the impaired migration. Our results emphasize the role of non-proteolytic MMP-9 functions contributing to CLL progression.

1. Introduction

Chronic lymphocytic leukemia (CLL), the most common leukemia in Western countries, is characterized by the accumulation of $CD5^+$ B lymphocytes in peripheral blood and lymphoid organs [1]. Localization in these niches allows malignant cells to receive proliferative and survival signals, thus contributing to CLL progression [2]. Several molecules regulate the migration and organ localization of CLL cells, including chemokines, integrins and gelatinase-B/matrix metalloproteinase-9 (MMP-9) [3].

MMP-9 is synthesized by CLL cells and is abundant in the CLL microenvironment [4,5]. We previously showed that binding of MMP-9 to primary CLL cells or stably transfecting MMP-9 into MEC-1 cells impairs *in vivo* and *in vitro* cell migration [6]. The exact mechanism accounting for this effect is not known, but it includes regulation of relevant signalling molecules, such as RhoAGTPase, Akt, ERK and PTEN [6]. We have also shown that interaction of MMP-9 with α4β1 integrin in CLL cells induces a signalling pathway that leads to cell survival [7]. Importantly, the MMP-9 hemopexin domain or an MMP-9 mutant devoid of catalytic activity (MMP-9MutE) also induced cell survival, indicating that MMP-9 may exert functions not involving its enzymatic activity.

Non-enzymatic functions have also been reported for other MMPs [8]. For example, MMP-1 and MMP-2 induce intracellular signaling upon binding to α2β1 or αVβ3 integrins, respectively [9,10]. MMP-3 mediates epithelial cell growth by non-proteolytic mechanisms [11]. The cytoplasmic tail of MMP-14, but not the catalytic domain, was involved in macrophage invasion and myeloid cell fusion [12,13]. These evidences, together with our previous findings, highlight the multiple roles of MMPs and the need to continue studying these additional MMP properties.

To further establish the non-enzymatic functions of MMP-9 that contribute to CLL pathology, in the present report we have generated MEC-1 cells stably expressing the catalytically inactive MMP-9MutE protein and have studied their behaviour in cell migration. We demonstrate that MMP-9MutE-cells have altered migration

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regulatory pathways, resulting in impaired transendothelial migration and homing to lymphoid organs. MMP-9MutE-cells are therefore an excellent model to identify non-catalytic functions of MMP-9 contributing to CLL progression.

2. Materials and methods

2.1. Patients, cells and cell cultures

Approval was obtained from the CSIC Bioethics Review Board for these studies. Peripheral blood samples from 6 CLL patients (Table 1) were obtained after informed consent and B-lymphocytes were purified as described [5–7]. The MEC-1 cell line, established from a CLL patient [14], was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and maintained in IMDM medium (Lonza, Basel, Switzerland), 10% FBS. HUVEC were purchased from Lonza and cultured as reported [5,6]. HEK293T cells (Invitrogen, Prat del Llobregat, Barcelona, Spain) were cultured in DMEM (Lonza), 10% FBS, 1% Glutamax (Invitrogen), 10 mg/ml penicillin/streptomycin.

2.2. Antibodies and reagents

Rabbit IgG (sc-3888), rabbit polyclonal antibodies (RpAb) to MMP-9 (sc-6841R), monoclonal antibodies (mAbs) against total Akt (sc-5298), PTEN (sc-7974), RhoA (sc-418), and CD44 (sc-7297) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA Diego, CA, USA). mAbs to CD38 (16BDH), α4 (HP1/7) and β1 (Alex1/4) integrin subunits were from Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Rat mAb anti-CCR7 (3D12) was from BD Pharmigen (Franklin Lakes, NJ, USA). RpAbs against phospho-Akt (Ser473, #9271), phospho-myosin light chain (MLC, Thr18/Ser19, #3674), phospho-ERK (Thr202/Tyr204, #9101) and total ERK (#9102) were from Cell Signalling Technology, Inc. (Beverly, MA, USA). mAb to vinculin (#V9131) was from Sigma-Aldrich (St. Louis, MO, USA). HRP-labeled Abs to rabbit or mouse Ig were from Dako (Glostrup, Denmark). Alexa 488- or 647-labeled Abs, Alexa 568-Phalloidin (#A12380) and Alexa 647-Phalloidin (#A22287) were from Molecular Probes (Eugene, OR, USA). VCAM-1 (vascular cell adhesion molecule-1) and TNFα were from R&D Systems (Minneapolis, MN, USA). CCL21 was from Peprotech EC (London, UK).

2.3. Plasmid construction and lentiviral production and infection

The preparation and characterization of MEC-1 cells stably expressing proMMP-9 (MMP-9-cells) or empty vector (Mock-cells) was previously described [6]. MEC-1 cells stably expressing the catalytically inactive proMMP-9MutE mutant were generated from the pRRLsin18.CMV.IRES.eGFP-proMMP-9 lentivirus construct by mutating the glutamate residue at position 402 in the proMMP-9 sequence to alanine, using the primers 5′-CGGCGCATGCGTTCGGCCA-3′ (forward) and 5′-TGGCCGAACG-CATGCGCCG-3′ (reverse). The mutated sequence was re-inserted into the pRRLsin18.CMV.IRES.eGFP lentivirus and confirmed by DNA sequencing (Secugen, Madrid, Spain). HEK293T cells were transiently transfected with this construct and viral supernatants were used to transfect MEC-1 cells as reported [6]. GFP-expressing cells were selected by several cell sorting steps until more than 95% of the cells were clearly positive for expression. These MEC-1 cell transfectants will be referred to as MMP-9MutE-cells.

Table 1
Clinical characteristics of CLL patients.

Patients	Sex/Age[a]	Stage[b]	Ig Status[c]	CD38	α4 subunit (%)	β1 subunit (%)
1	M/79	B/II	UM	+	30.1	95.7
2	M/86	A/I	ND	−	39.8	16.8
3	M/78	B/II	UM	+	20.0	37.5
4	M/66	B/II	Mut	+	90.9	99.4
5	M/75	ND	Mut	+	45.6	59.9
6	M/75	B/II	UM	+	75.7	73.9

[a] M, male.
[b] Stage according to references [26,27].
[c] Mut, mutated Ig; UM, unmutated Ig; ND, not determined.

2.4. Flow cytometry

1.5×10^5 primary CLL cells or MEC-1 transfectants were incubated (30 min, 4 °C) in 100 μl PBS/1%BSA with appropriate primary antibodies, washed with cold PBS and incubated (30 min, 4 °C) with Alexa 488-labeles (CLL) or Alexa 647-labeled (MEC-1) secondary Abs. Samples were analyzed on a Coulter Epics XL or FC 500 flow cytometer (Beckman Coulter, Fullerton, CA).

2.5. Immunofluorescence analyses

5×10^5 cell transfectants were added to glass coverslips previously coated with 5 μg/ml poly-lysine-1% BSA and incubated at 37 °C for 2 h. Cells were fixed with 4% paraformaldehyde, permeabilized with PBS/0.1% Triton X-100 (15 min, RT), and incubated with Alexa 568-Phalloidin for 1 h. Cells were washed with PBS/1% BSA and images acquired on a Leica TCS-SP2-AOBS-UV confocal microscope with a ×63 oil immersion objective. The LAS-AS Leica software was used for cell area determination.

2.6. DQ-gelatin degradation assay

This assay was performed as described [15,16]. Briefly, 2×10^6 MEC-1 cells were cultured in 2 ml IMDM/0.1%FBS in 6-well plates. After 24 h, the conditioned media were concentrated and added to a solution of 2.5 μg/ml DQ-gelatin (Invitrogen) in a 96 well plate. The plates were immediately placed in a microplate fluorescence reader (FL600, Biotek, Highland Park, IL, USA) and fluorescence was measured every 10 min for 2 h at 37 °C (ex 485 nm/em 530 nm).

2.7. Cell migration assays

In vivo experiments with mice were performed with the approval of the Ethics Committee of the CSIC. 5×10^6 Mock-cells, MMP-9-cells or MMP-9MutE-cells were labeled with 10 μM CFSE (Invitrogen) and injected into the tail vein of 6- to 10-week-old NOD/SCID mice. After 3 h, mice were sacrified and organs extracted and disaggregated. The number of migrated cells was determined by flow cytometry. Numbers of homed cells were normalized to the number of injected, viable CLL cells and to the total number of mouse cells in that organ. Transendothelial migration assays were performed exactly as described [5,6]. For time-lapse microscopy analyses, 5×10^4 MEC-1 cells were added to chemotaxis chambers (Ibidi, Martinsried, Germany) coated with 2 μg/ml VCAM-1. Images were acquired every 30 s for 3 h using a Leica AF6000 LX microscope (×20 objective). Mean accumulated distance, representing the movement of 40 cells, was determined using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA) and chemotaxis tool (Ibidi). Additional methods are available as Supplemental Material.

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