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Gremlin-1 inhibits macrophage migration inhibitory factor-dependent monocyte function and survival

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ABSTRACT

Background: Monocyte migration and their differentiation into macrophages critically regulate vascular inflammation and atherogenesis and are governed by macrophage migration inhibitory factor (MIF). Gremlin-1 binds to MIF. Current experimental evidences present Gremlin-1 as a potential physiological agent that might counter-regulate the inflammatory attributes of MIF.
Methods and results: We found that Gremlin-1 inhibited MIF-dependent monocyte migration and adhesion to activated endothelial cells in flow chamber perfusion assay in vitro and to the injured carotid artery of WT and ApoE$^{-/-}$ mice in vivo as deciphered by intravital microscopy. Intravenous administration of Gremlin-1, but not of control protein, significantly reduced leukocyte recruitment towards the inflamed carotid artery of ApoE$^{-/-}$ mice. Besides, leukocytes from MIF$^{-/-}$ when administered into ApoE$^{-/-}$ mice showed lesser adhesion as compared to wild type. In the presence of Gremlin-1 however, adhesion of wild type, but not of MIF$^{-/-}$ leukocytes, to the carotid artery was significantly inhibited as compared to control. Gremlin-1 also inhibited the MIF-induced differentiation of monocytes into macrophages. Gremlin-1 substantially inhibited the anti-apoptotic impact of MIF on monocytes against BH3 mimetic ABT-737-induced apoptosis as verified by Annexin V-binding, caspase 3 activity, and mitochondrial depolarization.
Conclusions: Therefore Gremlin-1 can modulate MIF dependent monocyte adhesion, migration, differentiation and survival.

1. Introduction

Monocyte chemotaxis, adhesion and their differentiation into macrophages play a central role in deciding the onset and course of inflammation [1]. The macrophage migration inhibitory factor (MIF) is an integral component in the regulation of vascular inflammation and plaque instability [2,3]. The formation of vulnerable plaques in coronary arteries is a critical consequence of the atherosclerotic disease and enhances the risk for acute myocardial infarction [4]. Vulnerable plaques are characterized by high inflammatory activity indexed by a high content of inflammatory cells such as monocytes/macrophages, and the presence of various chemokines, cytokines and growth factors that critically contribute to plaque rupture, of which MIF appears to be a decisive one [5]. MIF is a chemokine-like protein and therefore takes over many chemokine like functions directing the chemotaxis and recruitment of monocytes and their subsequent differentiation into macrophages, thereby prompting their accumulation at the site of atherosclerotic lesions [2,3]. Association of MIF with extensive lesion development was previously described in animal models of hypercholesterolemic rabbits and mice [6,7]. Blocking MIF or its genetic deletion reduces macrophage and T-cell content of atherosclerotic plaques and attenuates progression of atherosclerosis in ApoE$^{-/-}$ mice.

Recently, plasma level of MIF has been shown to be enhanced in patients with acute myocardial infarction and is associated with the inflammatory response [8]. Also, Gremlin-1 has been described as an endogenous physiological inhibitor of MIF [9]. Gremlin-1 belongs to the DAN/Cerberus protein family, which is a member of the cysteine knot superfamily that includes, besides others, transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF). In the adult system, Gremlin-1 regulates cell proliferation and migration of cells [10–14]. Gremlin-1 binds with high affinity to MIF and reduces MIF-induced release of tumor necrosis factor-α (TNFα) from macrophages [9]. Treatment of ApoE$^{-/-}$ mice with Gremlin-1 reduces the content of monocytes/macrophages at atherosclerotic lesions and attenuates atheroprogression [9]. Given these potentials of Gremlin-1,

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we further analyzed the effect of this protein in governing monocyte functions, particularly those influenced by MIF, like migration, adhesion to endothelium and apoptosis.

2. Materials and methods

2.1. Chemicals

Recombinant Gremlin-1 was from R&D Systems, Wiesbaden, Germany (5190-GR). Recombinant human MIF was from R&D Systems, Wiesbaden, Germany (298-MF-002). INF-γ was from PeproTech, NJ, USA. TNF-alpha was purchased from tebu-bio GmbH, Germany. We used goat polyclonal anti-human MIF antibody (R&D Systems, Wiesbaden, Germany) for the detection of MIF. Rabbit polyclonal mouse anti-Gremlin-1 antibody (Abcam, Cambridge, UK) and anti-human IgG-HRP (polyclonal) (Jackson Immuno Research, PA, USA) were used to detect the $_{m}$Gremlin-1-Fc fusion protein. β-Actin antibody (Sigma-Aldrich, Steinheim, Germany) and α-actin antibody (polyclonal) (Abcam, Cambridge, UK) were used as internal loading control. Anti-mouse Ly-6G (Gr-1) monoclonal antibody (Clone RB6-8C5) was purchased from eBioscience, Inc., San Diego, CA, USA. ABT-737 was purchased from Selleckchem, FCCP from Abcam Biochemicals, staurosporine from Calbiochem, Annexin V-FITC from ImmunoTools, donkey anti-rabbit Alexa Fluor-568 antibody and TMRE from Invitrogen, rabbit monoclonal-anti-human active cleaved caspase 3 from Cell Signalling, and TO-PRO-3 iodide (Molecular probes) from Merck Group, Germany. $_{m}$Gremlin-1-Fc or control Fc were generated as described previously [9].

2.2. Cell isolation and cell lines

Human monocytes were isolated from peripheral venous blood by adherence following Ficoll-Paque purification of peripheral blood mononuclear cells as described previously [15]. Human umbilical vein endothelial cells (HUVEC) were cultivated until confluence in complete endothelial cell basal medium (PAA) containing supplementary growth factors and 10% FCS. Human aortic endothelial cells (HAECs) were cultured in complete endothelial cell basal medium (PAA) containing growth factors, 10% human serum and 10% FCS and grown to confluence.

2.3. Monocyte chemotaxis and monocyte adhesion to endothelial monolayer under flow

Human monocytes were isolated from buffy coats and from peripheral venous blood samples by adherence after Ficoll-Paque purification of peripheral blood mononuclear cells as described [15]. Cell migration was performed using 48-well-modified Boyden chamber (Neuro Probe Inc., Gaithersburg, USA) with two compartments separated by a 5 μm polycarbonate membrane. Monocytes (20,000 cells per well) were added to the upper compartment, and medium containing either Gremlin-1 (2 μg/mL, R&D Systems, Wiesbaden, Germany, 5190-GR), MIF (50 ng/mL, R&D Systems, Wiesbaden, Germany, 298-MF-002) or Gremlin-1 (2 μg/mL) + MIF (50 ng/mL) (10 min preincubated) or medium alone was added to the lower chamber. After 6 h of incubation at 37 °C, the chemotaxis chamber with filter was removed. The migrated monocytes were fixed with methanol and stained on the filter with May-Grünwald and Giemsa reagent. The filter was photographed and migrated cells were counted.

For the flow chamber assay ECV 304 cells were cultivated until confluence on cover slips as described [16]. ECV-304 cells were either kept under resting condition or activated with TNF-α (50 ng/mL) and INF-γ (25 ng/mL) for 6 h. Human monocytic cells (Mono-Mac 6) were perfused over the endothelial monolayer at a specified shear rate, in the presence or absence of MIF (50 ng/mL), Gremlin-1 (2 μg/mL) or Gremlin-1 (2 μg/mL) + MIF (50 ng/mL). Adhesion of monocytes was analyzed after 10 min of perfusion as described [17].

2.4. Cloning, expression and purification of fusion protein $_{m}$Gremlin-1-Fc and Fc

A murine Gremlin-1-Fc fusion protein consisting of the leader sequence of IgG kappa (IgK), the fragment crystallizable region of human IgG2 (Fc) with the hinge region, and the extracellular domain of murine Gremlin-1 ($_{m}$Gremlin-1-Fc) and a corresponding IgG2 Fc control protein without the $_{m}$Gremlin-1 were designed and cloned into the pcDNA5/FRT vector (Invitrogen) for expression under control of the CMV promoter. For amplification, competent *Escherichia coli DH5α* (Stratagene) were transformed with these expression vectors, and the amplified plasmids were isolated using Midi Prep Kit (Qiagen). The identity of the constructs was verified by DNA sequencing (Eurofin MWG Operon, Germany).

Stable cell lines for the expression of $_{m}$Gremlin-1-Fc fusion protein and Fc control protein, respectively, were generated with the Flp-In™ system and Flp-In™-CHO cells, respectively, following the recommendations of the manufacturer (Invitrogen GmbH, Germany). $_{m}$Gremlin-1-Fc stable expressing cell lines were cultivated in HAM's-F12 medium (Biochrom AG, Germany) containing 10% FCS, 1% P/S and 250 μg/mL Hygromycin B at 37 °C and 5% CO_2. For lab scale expression of $_{m}$Gremlin-1-Fc and Fc, 3×10^6 cells were seeded on T-160 cell culture flasks in serum free CHO (A) medium (Gibco, Scotland). After 8 days of incubation, cell culture supernatants of the stable Flp-In™ CHO expression cells producing $_{m}$Gremlin-1-Fc or Fc, respectively, were collected. The supernatants were purified using Protein G agarose beads (Pierce, USA) following the recommendations of the manufacturer. Purified protein was pooled, dialyzed in PBS overnight at 4 °C, and frozen at −80 °C until use. Protein concentrations were determined with the anti human IgG ELISA (Helvetica Healthcare, Switzerland) following the instructions of the manufacturer. Purity of $_{m}$Gremlin-1-Fc was verified by SDS-PAGE.

2.5. ELISAs

The following proteins were quantified by ELISA: human MIF (Ray Biotech Inc., GA, USA) and human IgG (Helvetica Healthcare, Geneva, Switzerland).

2.6. In vitro model of macrophage and foam cell generation

The effects of Gremlin-1 on the formation of macrophages/foam cells from human monocytes were studied in vitro as described [18]. The formation of macrophages and foam cells were analyzed in the absence or presence of Gremlin-1 (1 μg/mL) by morphological assessment and anti CD68-staining. For the detection of MIF, supernatants were analyzed using anti human MIF ELISA.

In a second approach human monocytes were seeded out (5×10^4 cells/well in a 96 well plate) in foam cell medium (RPMI 1640, +1% Sodium Pyruvate, +1% L-Glut., 1% P/S, 1% NEAA, 20% human serum) to generate foam cells. At day 1, 5 and 10 the cells were stimulated with different cytokines as the following: Gremlin-1 (1 μg/mL, R&D Systems, Wiesbaden, Germany, 5190-GR), MIF (10 ng/mL, R&D Systems, Wiesbaden, Germany, 298-MF-002), Gremlin-1 (0.5 μg/mL) + MIF (10 ng/mL) (10 min preincubated), blocking MIF antibody (25 μg/mL, provided by Prof. Jürgen Bernhagen, RWTH Aachen University, Germany, clone IIID9), isotype control: normal mouse IgG (25 μg/mL) (Santa Cruz Biotechnologies, sc-2025) or medium alone. At day 4 in every setting the cells were fed with acLDL (80 μg/mL, Kalen Biomedical). The cells were photographed and counted at day 10 to get the number of macrophages/foam cells per visual field.

2.7. Secretion of MIF from activated monocytes

Human monocytes (3×10^5/well) were stimulated for 24 h with LPS (1 μg/mL, Sigma-Aldrich) and medium control. After incubation time as indicated cell supernatants were collected and cell lysates were generated. For the generation of cell lysates, the cells were once washed with PBS. The cells were lysed afterwards for 45 min on ice using RIPA buffer (150 mM NaCl, 50 mM tris, 0.1% tris, 0.5% sodium-desoxycholat, 1% Triton X-100, protease inhibitors (1:25); pH 8.0). The lysate was centrifuged (15 min, 4 °C, 10,500 ×*g*) and the supernatant was collected and frozen at −20 °C. Cell lysates and culture supernatants were analyzed by immunoblotting using antibodies to MIF (polyclonal, R&D Systems, Wiesbaden, Germany), a loading control beta-Actin (Abcam, polyclonal, Ab1801) and by ELISA (MIF, Ray Biotech Inc., GA, USA).

2.8. SDS-PAGE and immunoblotting

For immunoblot analysis lysates of human monocytes were separated on SDS-PAGE according to Lämmli [19]. Protein concentration was determined using Biorad Protein Assay with protein standard BSA (Sigma) and measurement of absorption at 405 nm. The samples were diluted with Lämmli buffer (1×, +5% mercaptoethanol) and heated for 10 min up to 95 °C. Thirty micrograms of total protein were separated on a 15% SDS-polyacrylamide gel electrophoresis (PAGE) (Invitrogen). Blotting of the protein onto a polyvinylidene difluoride membrane (PVDF, Immobilon, Millipore) was performed using Semi Dry Tranfer Cell System (Peqlab). We used goat polyclonal anti-human MIF antibody (R&D Systems, Wiesbaden, Germany) for the detection of MIF. Rabbit polyclonal mouse anti-Gremlin-1 antibody (Abcam, Cambridge, UK) and anti-human IgG-HRP (polyclonal) (Jackson Immuno Research, PA, USA) were used to detect the $_{m}$Gremlin-1-Fc fusion protein. β-Actin antibody (Sigma-Aldrich, Steinheim, Germany) and α-actin antibody (polyclonal) (Abcam, Cambridge, UK) were used as internal loading control. For the detection of antibody binding, corresponding secondary fluorescence labeled antibodies and the Odyssey infrared imaging system (LI-COR, Bad Homburg, Germany) were used. Bands were quantified using ImageJ software (National Institutes of Health, USA).

2.9. Animals

Male apolipoprotein E deficient mice B6.129P2-Apoe^{tmUnc} (ApoE$^{-/-}$) were purchased from Jackson laboratories. Regarding the long time drug treatment studies with $_{m}$Gremlin-1-Fc, intraperitoneal administration of $_{m}$Gremlin-Fc (1 μg/g body weight; n = 8) or Fc (equimolar, n = 8) was initiated at the age of 10 weeks. The proteins were given three times per week for 4 weeks in total. After the last administration the mice were sacrificed in general anesthesia. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the German law for the welfare of animals. Animal studies were approved by the local authorities (regional board Tübingen).

2.10. Determination of leukocyte adhesion and intravital microscopy

Leukocyte adhesion to the carotid artery of C57Bl/6J (Charles River) and ApoE$^{-/-}$ mice was analyzed in vivo by use of intravital video fluorescence microscopy [20–22]. All mice were fed normal chow. In brief, mice were anesthetized by intraperitoneal injection of a solution of midazolam (5 mg/kg body weight; Ratiopharm), medetomidine (0.5 mg/kg body weight; Albrecht), and fentanyl (0.05 mg/kg body weight; CuraMed Pharma GmbH). One day prior to intravital microscopy, the mice received 2 μg/g b.w. $_{m}$Gremlin-1-Fc or