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Atherogenic mononuclear cell recruitment is facilitated by oxidized lipoprotein-induced endothelial junctional adhesion molecule-A redistribution

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ABSTRACT

Background: Junctional adhesion molecule (JAM-) A is a transmembrane protein expressed in many cell types and maintains junctional integrity in endothelial cells. Upon inflammatory stimulation, JAM-A relocates to the apical surface and might thereby facilitate the recruitment of leukocytes.
Objective: Although inflammatory JAM-A redistribution is an established process, further effort is required to understand its exact role in the transmigration of mononuclear cells, particularly under atherogenic conditions.
Methods: By the use of RNA interference and genetic deletion, the role of JAM-A in the transmigration of T cells and monocytes through aortic endothelial cells was investigated. JAM-A–localization and subsequent mononuclear cell rolling, adhesion and transmigration were explored during endothelial inflammation, induced by oxidized LDL or cytokines.
Results: RNA interference or genetic deletion of JAM-A in aortic endothelial cells resulted in a decreased transmigration of mononuclear cells. Treatment of the endothelial cells with oxLDL resulted in an increase of both permeability and apical JAM-A presentation, as shown by bead adhesion and confocal microscopy experiments. Redistribution of JAM-A resulted in an increased leukocyte adhesion and transmigration, which could be inhibited with antibodies against JAM-A or by lovastatin-treatment, but not with the peroxisome proliferator activated receptor gamma-agonist pioglitazone.
Conclusions: This study demonstrates that redistribution of JAM-A in endothelial cells after stimulation with pro-atherogenic oxidized lipoproteins results in increased transmigration of mononuclear cells. This inflammatory dispersal of JAM-A could be counteracted with statins, revealing a novel aspect of their mechanism of action.

1. Introduction

Junctional adhesion molecule A (JAM-A, F11 receptor, JAM-1) is a member of the immunoglobulin superfamily (IgSF) transmembrane adhesion molecules and is expressed on a large variety of cell types, notably leukocytes, platelets and vascular endothelial cells [1]. In endothelial and epithelial cells, JAM-A maintains the integrity of the tight junctions through homophilic interactions, thereby controlling vascular and epithelial permeability [2]. Under inflammatory conditions, JAM-A has been shown to relocate to the apical surface of endothelial cells, where it becomes available for homophilic interactions with JAM-A on blood cells and for heterophilic interactions with lymphocyte functional antigen-1 (LFA-1) [3,4]. Thus, it may facilitate leukocyte adhesion and transendothelial migration [5–8]. Alternatively, JAM-A can be shed from the cell surface by metalloproteases and soluble JAM-A may modulate the actions of leukocytes distal from the site of inflammation [9]. Accordingly, elevated plasma levels of soluble JAM-A

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have been observed in individuals with coronary artery disease and strongly correlated with circulating levels of tumor necrosis factor-α (TNF-α) [10]. In addition, increased expression of JAM-A was observed in early vascular lesions of hyperlipidemic mice and in specimen of human atherosclerotic plaques [6,11] and is involved in vascular remodeling after wire injury [12]. Interestingly, the structurally and functionally related JAM-C is both upregulated and redistributed in endothelial cells after treatment with oxidized low-density lipoprotein (oxLDL), further indicating a role for JAM family members in atherosclerosis [13]. In a recent study we have demonstrated that endothelial JAM-A promotes atherogenic leukocyte recruitment under conditions of disturbed arterial flow, by redistributing from the endothelial junctions to the luminal side of the vessel wall [14].

Similar to other members of the IgSF, JAM-A consists of 2 globular extracellular domains, a transmembrane region and a relatively short N-terminus, which contains a type II PDZ domain [4,15]. The first V_H-type extracellular domain mediates homophilic interactions of neighboring JAM-A molecules in *cis*, as well as homophilic interactions of JAM-A on nearby cells in *trans* [16]. The second C_2-type domain, closest to the plasma membrane, binds to the integrin LFA-1 [7]. A previous study revealed that binding of LFA-1 to domain 2 of JAM-A caused a destabilization of the homophilic interaction through domain 1 [17]. Thus through LFA-1 binding, the tight junction barrier of JAM-A might be loosened, facilitating the advancement of leukocytes through the cellular junctions. The PDZ domain links JAM-A to a variety of intracellular proteins, notably *zonula occludens* 1 (ZO-1), which serve as anchors to the cytoskeleton and are components of the organizational tight junction scaffold [18,19]. Stimulation of endothelial or epithelial cells with TNF-α and/or interferon-γ (IFN-γ) resulted in an increase of JAM-A in the triton-X100 cell fraction, which is indicative of its dissociation from the cytoskeleton [20,21] and being liberated from cytoskeletal restraints, JAM-A might be able to guide leukocytes to junctional transmigration sites [22]. However, it is still unclear how this cellular redistribution and dispersal of JAM-A influences the subsequent recruitment of inflammatory cells. Moreover, it is of interest to investigate whether a pharmacologic attenuation of endothelial inflammation would also have effects on endothelial distribution of JAM-A and its consequences on monocyte recruitment.

2. Materials and methods

2.1. Reagents and cell culture

Dharmacon® small interfering RNA (siRNA) pools targeting JAM-A or laminin and FITC-conjugated phalloidin were purchased at Thermo Scientific (Pittsburg, PA). Antibodies against mouse and human JAM-A were purchased at Hycult Biotech (Uden, Netherlands) and mAb TS1/22 against CD11a was isolated from hybridoma-conditioned medium. Fab fragments were generated by papain digestion of JAM-A antibodies (243 and 246) and of mAb TS1/22[22]. Cytokines and chemokines were from Peprotech (Rocky Hill, NJ). Low-density lipoprotein (LDL) was prepared from freshly prepared human plasma by ultracentrifugation in a KBr density gradient and oxidized with $CuCl_2$ as described [23]. Fusion protein of human IgG Fc and JAM-A (JAM-A.Fc) was isolated from stably transfected CHO supernatants [6]. All other reagents were purchased at Sigma–Aldrich (St. Louis, MO) and were of the highest purity available.

Peripheral blood mononuclear cells (PBMC) were isolated from fresh buffy coats by density gradient centrifugation. T cells were isolated using magnetic beads (Life Technologies, Carlsbad, CA) and were cultured up to 48 h in RPMI medium containing 10% fetal bovine serum and 25 ng/ml interleukin 2. Mouse aortic endothelial cells (MAoECs) were isolated from JAM-A$^{-/-}$ and JAM-A$^{+/+}$ apolipoprotein E–deficient (Apoe$^{-/-}$) mice according to Kreisel et al. [24], and maintained in RPMI 1640 containing 20% FCS, 0.1% 2-mercaptoethanol and 50 μg/ml endothelial cell growth supplement (BD Biosciences, Franklin Lakes, NJ). Human aortic endothelial cells (HAoEC) were purchased from Promocell (Heidelberg, Germany) and cultured in proprietary MV2 media. Blood and tissue donations by humans and animals, respectively, were approved by the local ethics boards of the RWTH, LMU and Maastricht universities and the local governments.

2.2. Stimulation and transfection of endothelial cells

HAoEC were untreated, treated with TNF-α (10 ng/ml), without or with CCL2 (100 ng/ml) or CXCL12 (500 ng/ml), or transfected with siRNA using a nucleofector™ with a reagent kit for endothelial cells (Lonza, Basel, Switzerland). The cells were subsequently cultured in 5 μM pore size Transwell® filters (Corning, Tewksbury, MA) or collagenized glass slides until confluency. MAoECs (JAM-A$^{+/+}$ Apoe$^{-/-}$ or JAM-A$^{-/-}$Apoe$^{-/-}$) were grown on collagenized glass slides and were activated for at least 4 h with TNF-α (25 ng/ml). For some experiments, JAM-A$^{-/-}$Apoe$^{-/-}$ MAoECs were transfected with siRNA or a pcDNA3-based expression vector containing a cDNA for human JAM-A using a nucleofector. Cells were analyzed by flow cytometry with proper gating and appropriate isotype controls on a FACSCalibur (BD Biosciences, Franklin Lakes, NJ).

2.3. Rolling/adhesion/transendothelial migration experiments

Real time measurement: rolling and adhesion – Human monocytes or T cells (each 0.25×10^6/ml) were untreated or treated with oxLDL (10 μg/ml, 16 h) and were applied on HAoECs untreated or treated with oxLDL (10 μg/ml), TNF-α and IFN-γ (10 ng/ml and 20 ng/ml, 4 h, respectively) or oxLDL (10 μg/ml) + JAM-A.Fc (15 μg/ml). Assays were performed and recorded under shear stress (1.5 dyn/cm^2) using a syringe pump (WPI, Berlin, Germany) in a laminar flow chamber for 3 min. Leukocytes loosely adhering to ECs for maximum 3 s were counted as rolling leukocytes, whereas stable adhesion of leukocytes throughout the whole measurement counted as adherent leukocytes.

Real time measurement: *transendothelial migration* – Human monocytes (0.7×10^6/ml) or T cells (0.5×10^6/ml) were untreated or treated with oxLDL (10 μg/ml, 16 h) and were applied on HAoECs or MAoECs untreated or treated with JAM-A.Fc, JAM-A D1.Fc, JAM-A D2.Fc (all 15 μg/ml; specifically blocking JAM-A homophilic or heterophilic interactions, respectively) without or with LDL, oxLDL (both 10 μg/ml, 16 h), TNF-α and IFN-γ (10 ng/ml and 20 ng/ml, 4 h, respectively) or oxLDL plus JAM-A.Fc (10 μg/ml, 15 min). After application of leukocytes to the confluent EC monolayer, time-lapse images were recorded (Cell^M system, Olympus, Tokyo, Japan). Leukocytes showing transmigration were counted and related to adherent cells.

Transwell® assay – PBMC (1×10^5) were added in the filter inserts containing confluent HAoEC and were allowed to transmigrate towards CCL2 (100 ng/ml) or CXCL12 (500 ng/ml) to attract monocytes or T-cells, respectively, for 90 min at 37 °C. The transmigrated leukocyte subtypes were gated and counted using flow cytometry.

2.4. Fluorescent bead assay

HAoEC were treated without or with LDL, oxLDL (both 10 μg/ml, 16 h) or TNF-α and IFN-γ (10 ng/ml and 20 ng/ml respectively, 4 h) in the absence or presence of cytochalasin D, jasplakinolide (both 50 nM, 24 h), lovastatin or pioglitazone (both 1 μM, 24 h pre-incubation) and were subsequently incubated with fluorescent