Objective—The junctional adhesion molecule (JAM)-A on endothelium contributes to the inflammatory recruitment of mononuclear cells involving engagement of its integrin receptor lymphocyte function-associated antigen (LFA)-1. It is unknown whether these functions can be inhibited by soluble forms of JAM-A, whether JAM-A is expressed on atherosclerotic endothelium, and whether it participates in atherogenic recruitment of mononuclear cells.

Methods and Results—Adhesion assays revealed that LFA-1–mediated binding of mononuclear cells to intercellular adhesion molecule (ICAM)-1 or cytokine-costimulated endothelium was dose-dependently inhibited by soluble JAM-A.Fc (sJAM-A.Fc). Similarly, sJAM-A.Fc reduced stromal cell-derived factor (SDF)-1/H9251–triggered transendothelial chemotaxis of activated T cells and their SDF-1/H9251–triggered arrest on cytokine-costimulated endothelium under flow conditions. Immunofluorescence analysis revealed an upregulation of JAM-A on early atherosclerotic endothelium of carotid arteries from apolipoprotein E-deficient (apoE/H11002/H11002) mice fed an atherogenic diet. In ex vivo perfusion assays, pretreatment of mononuclear cells with sJAM-A.Fc inhibited their very late antigen (VLA)-4–independent accumulation on atherosclerotic endothelium of these arteries.

Conclusions—Soluble forms of JAM-A can be effectively applied to inhibit distinct steps of mononuclear cell recruitment on inflamed or atherosclerotic endothelium. In conjunction with its expression on atherosclerotic endothelium, this suggests a functional contribution of JAM-A to atherogenesis. (Arterioscler Thromb Vasc Biol. 2005;25:729-735.)

Key Words: cell adhesion molecules ▪ endothelium ▪ inflammation ▪ atherosclerosis
not regulated during lesion formation, a role of ICAM-1 is more controversial, because its expression has been associated with flow alterations independently of plasma cholesterol, whereas genetic deletion of ICAM-1 attenuated lesion formation in apoE−/− mice but not in LDLR−/− mice.

Given a recently established role of activated platelets in exacerbating atherosclerosis, it is notable that platelet adhesion on activated endothelium is mediated by homophilic interactions of JAM-A and can be inhibited with soluble forms of JAM-A, implying a role of JAM-A in inflammatory thrombosis and atherogenesis. However, the expression of JAM-A on early atherosclerotic endothelium, the contribution of JAM-A to the recruitment of mononuclear cells on inflamed or atherosclerotic endothelium, and its possible inhibition with soluble JAM-A have not been elucidated.

Methods

Cell Culture, Cell Isolation, and Reagents
Human umbilical vein endothelial cells (HUVECs), monocytic Mono Mac 6 (MM6) cells, and Jurkat T cells were cultured as described. CD4+CD45RO+ memory T cells were isolated from venous blood of healthy volunteers by negative immunomagnetic selection (Miltenyi Biotec). Tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and CD4+ were removed by flick wash. Input and adherent cells were measured by flow cytometry.

Transmigration Assay
CD4+CD45RO+ cells (0.5×10^6 cells) were pre-incubated with sJAM-A.Fc, hlgG control, JAM-A Ab, sCD30.Fc, or TS1/22 for 30 minutes, resuspended in RPMI-1640/0.5% BSA, and allowed to transmigrate across HUVEC monolayers grown on 5-μm transwell filters (Costar, Corning) toward SDF-1α (0.5 μg/mL) in the bottom chamber for 90 minutes. Input and migrated cells were counted by flow cytometry.

Adhesion Assay Under Laminar Flow Conditions
Confluent HUVECs costimulated overnight with TNF-α (100 U/mL) and IFN-γ (200 U/mL) were pretreated with SDF-1α (0.5 μg/mL) for 30 minutes. CD4+CD45RO+ cells resuspended in HMHC/0.5% BSA were pre-incubated with HP2/1, TS1/22, hlgG control, hJAM-A Ab, sJAM-A.Fc, or sCD30.Fc. T cells (1.0×10^6/mL) were perfused at 1.5 dyn/cm^2 for 5 minutes at 37°C over endothelial monolayers inserted in a parallel wall flow chamber. Cell arrest was analyzed in multiple high-power fields recorded by video microscopy. Results were expressed as cells/mm^2.

Immunofluorescence
C57BL/6 apoE−/− mice (Taconic M&B, Ry, Denmark) were fed an atherogenic diet containing 21% fat, 0.15% cholesterol, and 19.5% casein (w/w; Altromin, Lage, Germany) for 6 weeks. After intraperitoneal anesthesia with ketamine/xylazine, carotid arteries of wild-type or apoE−/− mice were exposed, fixed with 4% paraformaldehyde, and embedded in paraffin; 5-μm sections were stained for mJAM-A using H202-106-7-4 (4 μg/mL), QuickKit, and Vector Red Substrate (Vector labs). Double staining for endothelial cells was performed with anti–PECAM-1 (2 μg/mL), followed by a secondary fluorescein isothiocyanate-conjugated anti-goat Ab. Isotype controls were used at equal concentrations.

Quantitative Real-Time Polymerase Chain Reaction
Total RNA of whole carotids from wild-type or apoE−/− mice was isolated and reverse transcribed using oligo-dT primers (Sensiscript, Qiagen). A 2-μL cDNA sample was incubated with 25 μL SYBR Green-containing QuantiTect Mix (Qiagen) and 0.6 μmol/L of each primer pair. Specific products were analyzed by real-time polymerase chain reaction (Tm=55°C, 45 cycles; MJ Research Opticon 2, Biozym). Used primers were 5′-AGCCAGATCACAGCTCCCTACT-3′ (mGAPDH), 5′-CCACAGCCTTGCCACGC-3′ (mJAM-A), 5′-CACCAGATTGTCAACG-3′, and 5′-CCACAGCTTTGCGCACG-3′ (mGAPDH). Target JAM-A mRNA was quantified using a standard curve and normalized to levels of GAPDH.

Ex Vivo Perfusion of Murine Carotid Arteries
After anesthesia of apoE−/− mice, the carotid bifurcation including the common carotid artery, internal carotid artery, and external carotid artery branches was exposed. Branches were ligated with sutures and a polyethylene catheter was inserted through an incision of the common carotid artery distal to a ligation. The artery was perfused with MOPS-buffered physiological salt solution with 0.5% human serum albumin using a syringe pump. Outflow was enabled by punctures in the external carotid artery and internal carotid artery. The vessel was cut distal to sutures and transferred onto a microscope stage (immersion objective 20×; Olympus BX51) superfused at 37°C with bicarbonate-buffered saline. MM6 or CD4+CD45RO+ cells (0.5×10^6 cells/mL) labeled with calcine/AM were pre-incubated with anti–PECAM-1. Cells were resuspended in MOPS buffer at 37°C and perfused at a flow rate of 3 μL/min. Adhesive interactions with the atherosclerotic vessel wall were recorded using stroboscopic epifluorescence illumination (Dreloscope 250; Drello). Cells not moving for >30 seconds were defined as adherent. Animal studies were approved by local authorities and complied with German animal protection law.
Blocking Mononuclear Cell Adhesion in Stasis

PMA-activated Jurkat T cells were allowed to adhere on immobilized ICAM-1.Fc to study inhibitory effects of soluble JAM-A in LFA-1–dependent static adhesion of mononuclear cells (Figure 1A). Pre-incubation of Jurkat T cells with sJAM-A.Fc but not with hlgG control inhibited specific LFA-1/ICAM-1 interactions, leading to a significant decrease in T cell adhesion on ICAM-1.Fc (58.8±6.4% of control, n=5; P<0.01). Treatment of Jurkat T cells with sJAM-A.Fc at concentrations ranging from 1 to 30 µg/mL further revealed that the interference with T cell adhesion in stasis was dose-dependent (Figure 1B). Similarly, the VLA-4–independent adhesion of monocytes MM6 cells on HUVECs costimulated with the proinflammatory cytokines TNF-α and IFN-γ to redistribute JAM-A to the apical surface) was significantly decreased by treatment with sJAM-A.Fc (n=4, P<0.05 versus VLA-4 mAb; Figure 1C). Pre-incubation of Jurkat or MM6 cells with sCD30.Fc, which binds to the unrelated membrane protein CD30R on mononuclear cells, did not interfere with adhesion (Figure 1A and 1C).

Inhibition of SDF-1α–Triggered T Cell Arrest Under Flow Conditions by sJAM-A.Fc

Given that physiological shear flow affects adhesion of mononuclear cells to activated endothelium,20 we examined arrest of CD4+CD45RO+ T cells triggered by SDF-1α on HUVEC monolayers costimulated with TNF-α and IFN-γ in a flow chamber at 1.5 dyn/cm². The experiments were performed with VLA-4–blocked lymphocytes to exclude adhesion to VCAM-1 expressed on activated HUVECs. Pretreatment of HUVECs with SDF-1α resulted in 2-fold increase in memory T cell arrest, which was LFA-1–dependent as shown with the blocking mAb TS1/22 (Figure 3A). Pre-incubation of T cells with sJAM-A.Fc significantly reduced their VLA-4–independent arrest on inflamed endothelium to levels (50.4±4.2% of control, n=4; P<0.01) seen

Transendothelial Migration of Memory T Cells Is Antagonized by sJAM-A.Fc

To further investigate the influence of sJAM-A.Fc on LFA-1–mediated steps of mononuclear cell recruitment, transendothelial chemotaxis assays were performed using HUVEC-coated transwell filters and isolated CD4+CD45RO+ memory T cells, which migrate toward the chemokine SDF-1α and express the SDF-1α receptor CXCR4.10 SDF-1α induced a 15-fold increase in transendothelial migration of memory T cells, which was largely mediated by LFA-1 as shown by inhibition with TS1/22 mAb (Figure 2). Pre-incubation of memory T cells with sJAM-A.Fc (47.6±10.6% of control,
without SDF-1α or after blocking LFA-1 (Figure 3A). This inhibition was more marked than that seen with a blocking JAM-A Ab, whereas irrelevant sCD30.Fc had no significant effects (A). Dose-dependent inhibition of CD4+CD45RO+ T-cell arrest by sJAM-A.Fc is shown for concentrations ranging from 1 to 30 μg/mL (B). T cells were pretreated with VLA-4 mAb to exclude interactions with VCAM-1 or other VLA-4 ligands. Data are given as mean ± SEM of 4 independent experiments (A) or 1 of 4 representative experiments is shown (B). Adhesion is reported as percentage of control. *P<0.05 vs control, **P<0.01 vs control.

Expression of JAM-A on Atherosclerotic Endothelium in ApoE−/− Carotid Arteries

To explore the presence of JAM-A in early stages of atherosclerosis, the expression of JAM-A was investigated by immunofluorescence staining of carotid arteries (without manifest lesions) from wild-type mice or apoE−/− mice fed an atherogenic diet for 6 weeks (Figure 4A). Compared with the endothelial marker PECAM-1, the staining for JAM-A was only slightly detected on the endothelium of carotid arteries of wild-type mice, which may reflect upregulation and apical redistribution of JAM-A after endothelial activation. Staining for JAM-A was also detectable in medial smooth muscle cells (Figure 4A). No staining was seen with isotype controls. The upregulation of JAM-A in atherosclerotic arteries of apoE−/− mice was confirmed by real-time polymerase chain reaction analysis, indicating a 2.6-fold increase of JAM-A mRNA transcripts compared with arteries of wild-type mice (Figure 4B).

Mononuclear Cell Recruitment to Atherosclerotic Endothelium Blocked by sJAM-A

To gain insights into the functional relevance of JAM-A expression in early atherogenic recruitment of mononuclear cells, carotid arteries of apoE−/− mice fed an atherogenic diet for 6 weeks were perfused ex vivo for 8 minutes with MM6 or memory T cells. Adhesion experiments using human MM6 cells on murine SV40-transformed endothelial cells under flow conditions (Figure 1A, available online at http://atvb.ahajournals.org) and ex vivo perfusion assays in carotid arteries of wild-type mice after atherogenic diet for 6 weeks revealed an upregulation of JAM-A expression colocalized with PECAM-1 (merge) on atherosclerotic endothelium. No staining was seen with IgG isotype controls. Scale bar, 20 μm (A). Quantitative real-time reverse-transcription polymerase chain reaction analysis of total RNA isolated from carotid arteries confirmed an upregulation of JAM-A mRNA expression normalized to levels of GAPDH mRNA in apoE−/− vs wild-type mice. *P<0.05 (B).
sCD30.Fc further enhanced the inhibition of their accumulation on early atherosclerotic endothelium. This inhibition was evident throughout the time course of monocyte accumulation (Figure 5B) and was also observed with memory T cells pretreated accordingly (Figure 5C). Using a combination of blocking mAbs to VLA-4 and LFA-1, the inhibition was equivalent to the combination of VLA-4 mAb and sJAM-A.Fc, thus indicating that the effect of sJAM-A.Fc was likely caused by interference with LFA-1 (Figure 5A and 5C). Thus, blockade of VLA-4 revealed a LFA-1–mediated contribution of JAM-A to mononuclear cell arrest on atherosclerotic endothelium that can be inhibited with sJAM-A.Fc.

**Discussion**

Our data reveal an involvement of JAM-A in LFA-1–mediated steps of mononuclear cell recruitment on inflamed endothelium, ie, adhesion and transmigration, which can be dose-dependently inhibited with a soluble form of JAM-A. Evidence for an upregulated expression of JAM-A on early atherosclerotic endothelium and the inhibition of mononuclear cell accumulation on early atherosclerotic endothelium of ex vivo perfused carotid arteries in apoE−/− mice by pretreatment with sJAM-A.Fc further imply a role of JAM-A in initial atherogenesis.

An infiltration with T cells accompanied by the production of cytokines, such as INF-γ, is thought to be instrumental in controlling the development and progression of atherosclerotic lesions. Besides a dominant role of VCAM-1 in atherosclerotic plaque formation, ICAM-1 upregulated at atherosclerosis-prone sites may contribute to the inflammatory recruitment of T lymphocytes. Endothelial ICAM-1 exerts its adhesive function by engaging LFA-1 on leukocytes. The pre-incubation with sJAM-A.Fc inhibited adhesion of T cells to immobilized ICAM-1 in a dose-dependent manner, as well as adhesion of monocyte MM6 cells to proinflammatory stimulated HUVEC monolayers. In principle, cell–cell adhesion can be supported by homophilic JAM-A interactions in trans, as shown for JAM-A–mediated arrest of platelets to cytokine-activated endothelium. With the presence of immobilized ICAM-1 as an exclusive substrate for T cell adhesion, an interference of homophilic JAM-1 interactions by sJAM-A.Fc can be excluded in our assay. This indicates that sJAM-A.Fc exerts its inhibitory function by antagonizing the LFA-1/ICAM-1 interaction via binding to LFA-1 and corroborates recent findings that JAM-A is a specific ligand for LFA-1 and interacts with its I domain, which has been recently demonstrated in solid-phase binding assays with LFA-1 I domain locked in open, active conformation. However, it cannot be excluded that soluble JAM-A may act by engaging JAM-A on the surface of mononuclear cells, thereby triggering JAM-A–mediated signaling events. This may occur through a cis interaction with the cytoplasmic part of the αL chain and may result in altered LFA-1 conformation or signal transduction affecting LFA-1 affinity for ICAM-1, as has been described for increased LFA-1 binding capacity after engagement of PECAM-1. JAM-A has been implicated in inflammatory leukocyte diapedesis. Accordingly, we found that sJAM-A.Fc is effective in blocking migration of memory T cells across HUVEC monolayers in response to SDF-1α. Using a specific mAb confirmed a major role of LFA-1 in transendothelial migration, which may be mediated by the dynamic regulation of its avidity. Although homophilic JAM-A interactions between leukocytes and endothelium may also contribute to transmigration, the inhibition of LFA-1 avidity by sJAM-A.Fc in adhesion assays infers that this function is targeted to impair transmigration. As observed by live imaging, LFA-1 is enriched near the tail region of transmigrating neutrophils, where it might interact with a ring structure transiently formed by endothelial JAM-A at sites of diapedesis and closed soon thereafter. Blocking leukocytic LFA-1 or JAM-A with sJAM-A.Fc might impede interactions in tunnel structures decorated by endothelial JAM-A. A slight increase in endothelial permeability in the presence of sJAM-A.Fc (data not shown) is unlikely to account for inhibitory effect on transmigration, because only T cells were preincubated with sJAM-A.Fc. The notion that homophilic interactions of JAM-A or cis interactions with LFA-1 may also contribute to transmigration is supported by preliminary findings that soluble forms of JAM-A with single domain deletions (deficient in interacting with either JAM-A or LFA-1) both exerted inhibitory effects (L.F., G.O., C.W.,...
unpublished data). Moreover, this would also enable a selective interference with LFA-1 interactions but not homophilic JAM-A interactions to avoid deleterious effects on endothelial integrity in potential therapeutic applications of sJAM-A.

Flow chamber experiments with T cells and inflamed endothelium confirmed the inhibitory effect of sJAM-A.Fc on leukocyte recruitment under more physiological conditions. This was performed under blockade of VLA-4, because an apical redistribution of JAM-A essential for its function in leukocyte recruitment requires costimulation with TNF-α and IFN-γ,7 which also upregulates VCAM-1 expression.26 Similar to static assays, the SDF-1α–triggered memory T-cell arrest on inflamed endothelium in flow was inhibited by sJAM-A.Fc, a JAM-A Ab, or an LFA-1 mAb. The inhibition of arrest by sJAM-A.Fc was dose-dependent, with a maximum of 75%. The high degree of inhibition achieved is likely because of the fact that blocking LFA-1 by sJAM-A.Fc, as seen in static assays on ICAM-1.Fc, may impair its engagement not only with JAM-A but also with ICAM-1 or ICAM-2 expressed on inflamed endothelium and contributing to efficient arrest.

This is the first report to our knowledge demonstrating the expression of JAM-A on atherosclerotic endothelium in carotid arteries of apoE−/− mice and providing evidence that it is substantially upregulated compared with wild-type mice. Expression was also less pronounced in apoE−/− mice not fed a Western diet, indicating that similar to VCAM-1, JAM-A upregulation depends on hyperlipidemia.13 The faint JAM-A staining in arteriole endothelium of wild-type mice may be related to the physiological context of JAM-A expression, which is predominantly found in vasculature with prominent tight junctions, eg, in endothelium forming the blood–brain barrier.27 In turn, the upregulation of JAM-A expression in hyperlipidemia may correspond to a different functional relevance under inflammatory conditions that require further characterization.

Ex vivo perfusion assays investigating mononuclear cell accumulation to carotid arteries of apoE−/− mice14,28 revealed an involvement of JAM-A in the atherogenic recruitment of monocytes and T cells, and thus in the pathogenesis of atherosclerosis. In accordance with a prominent function of the VLA-4/VCAM-1 axis,14 arrest of mononuclear cells on early atherosclerotic endothelium was inhibited by a VLA-4 mAb, unaffected by sJAM-A.Fc alone, but further reduced by a combination of sJAM-A.Fc and VLA-4 blockade on monocytes. Thus, an adjuvant role of JAM-A throughout the time course of accumulation was disclosed by blocking VLA-4. The combinatorial effect of LFA-1 and VLA-4 mAbs to MM6 and T-cell arrest on atherosclerotic endothelium revealed that sJAM-A.Fc may exert its function by blocking leukocyte LFA-1 receptors. The fact that sJAM-A.Fc reduced static adhesion of MM6 cells on proinflammatory costimulated HUVECs similar as observed in ex vivo perfusion experiments with atherosclerotic arteries under flow conditions suggests that the involvement of JAM-A in mononuclear cell recruitment is less dependent on shear conditions than previously observed for Mac-1/JAM-C interactions.29 The expression pattern of JAM-A in carotid arteries of apoE−/− mice suggests that atherosclerotic activation leads to an upregulation of endothelial JAM-A, but also to an apical redistribution as seen after inflammatory costimulation with TNF-α and IFN-γ.7 Consequently, luminal presentation constitutes a prerequisite for the role of JAM-A supporting VCAM-1 in atherogenic arrest of mononuclear cells and possibly for haptotactic guidance of arrested cells to sites of diapedesis. In light of its involvement in postadhesive steps of recruitment, eg, diapedesis, which are not ideally represented in the ex vivo perfusion model, the participation of JAM-A may be more prominent in vivo.

In addition to a direct function of JAM-A in recruitment of mononuclear cells, its involvement in platelet adhesion to endothelium14 could further suggest a role in contact-dependent deposition of platelet chemokines and other platelet-mediated mechanisms enhancing mononuclear cell arrest.17,28,30 To confirm the relevance of these mechanisms for atherosclerotic lesion formation in vivo, studies using mice with genetic deletion of JAM-A, blocking antibodies, or sJAM-A.Fc in apoE−/− mice are clearly warranted.

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Involvement of JAM-A in Mononuclear Cell Recruitment on Inflamed or Atherosclerotic Endothelium: Inhibition by Soluble JAM-A
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