Importance of Junctional Adhesion Molecule-C for Neointimal Hyperplasia and Monocyte Recruitment in Atherosclerosis-Prone Mice—Brief Report

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Objective—Although junctional adhesion molecule (JAM)-C has been implicated in the control of inflammatory leukocyte recruitment, its role in neointima formation after arterial injury has not been elucidated.

Methods and Results—In apolipoprotein E–deficient (Apoe<sup>−/−</sup>) mice fed an atherogenic diet, antibody blockade of JAM-C significantly reduced neointimal hyperplasia after wire injury of carotid arteries without altering medial area and decreased neointimal macrophage but not smooth muscle cell (SMC) content. An increased expression of JAM-C was detected in colocalization with luminal SMCs 1 day after injury and neointimal SMCs after 3 weeks. Blocking JAM-C inhibited monocyctic cell arrest and leukocyte adhesion to carotid arteries perfused ex vivo and in vivo. Furthermore, monocyte adhesion to activated coronary artery SMCs under flow conditions in vitro was diminished by blocking JAM-C.

Conclusions—Our data provide the first evidence for a crucial role of JAM-C in accelerated lesion formation and leukocyte recruitment in atherosclerosis-prone mice. (Arterioscler Thromb Vasc Biol. 2009;29:1161-1163.)

Key Words: leukocyte recruitment ■ adhesion molecules ■ remodeling ■ inflammation ■ restenosis ■ atherosclerosis

The recruitment of inflammatory cells to the vessel wall determines neointimal hyperplasia after injury. A key role herein can be ascribed to junctional adhesion molecules (JAMs) of the immunoglobulin superfamily, which mediate firm arrest but also subsequent transmigration of leukocytes across endothelial cells (ECs). JAM-C has been identified to be localized in tight junctions of ECs, in smooth muscle cells (SMCs), but also human platelets and blocking antibodies to JAM-C were shown to inhibit leukocyte accumulation in different mouse models of inflammation. In Apoe<sup>−/−</sup> mice, JAM-C was described to be highly expressed in ECs and intimal SMCs. Recently, genetic deficiency in JAM-C was demonstrated to result in respiratory, digestive, and immune disorders, unveiling its importance in diverse biological processes.

We here addressed the role of JAM-C in neointima formation after wire-induced arterial injury in Apoe<sup>−/−</sup> mice using a blocking antibody.

Materials and Methods
For detailed Materials and Methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Mouse Model of Carotid Artery Injury
Apoe<sup>−/−</sup> mice on high-fat diet received monoclonal H33 or isotype control 3/wk and underwent wire-induced injury of carotid arteries. Movats’ pentachrome and immunofluorescence staining was performed on paraffin-embedded artery sections.

Adhesion Assay, Ex Vivo Perfusion, and Intravital Microscopy
MonoMac6 cell adhesion to human coronary SMCs was analyzed in a parallel-wall flow-chamber. Leukocyte arrest to carotid arteries was analyzed ex vivo or in vivo.

Results and Discussion
Carotid arteries of Apoe<sup>−/−</sup> mice fed an atherogenic diet and treated with a specific neutralizing anti–JAM-C (H33) or isotype control antibody were analyzed 3 weeks after wire-induced denudation-injury. Compared with control-treated mice, a significant reduction in neointimal plaque formation but not medial areas was observed in H33-treated mice (Figure 1A and 1B). Quantitative immunostaining revealed a significant decrease in the relative content of neointimal macrophages (Figure 1C and 1D), whereas SMC content was...
not altered (Figure 1E). The inhibition of plaque formation may thus be attributable to an attenuation of monocyte infiltration.

In contrast to healthy vessels, atherosclerotic arteries display increased expression of JAM-C in EC layers and intimal SMCs, and oxLDL induces the redistribution of JAM-C from inter endothelial contacts supporting leukocyte recruitment. Compared to marginal staining in uninjured arteries, an enhanced expression of JAM-C could be observed in injured segments of Apoe−/− carotid arteries (not shown). One day

Figure 1. Carotid arteries of Apoe−/− mice treated with isotype control or H33 (n=6 each) 3 weeks after wire-induced injury. Quantification of pentachrome stained neointimal and medial areas; representative images (A and B). Neointimal Mac-3+ macrophage staining (red) relative to neointimal area; representative images (C and D). Alpha-smooth-muscle actin (SMA)+ SMC content relative to neointimal area (E). *P<0.01.

Figure 2. Immunofluorescence staining for JAM-C, α-SMA+ SMCs, CD31+ ECs, and DAPI cell nuclei in Apoe−/− carotid arteries after injury (A). Ex vivo adhesion of MonoMac 6 cells (arrowheads, B), and intravital microscopy of leukocyte adhesion (C) in Apoe−/− carotid arteries 1 day after injury treated with H33 or isotype control, **P<0.0001. Adhesion of MonoMac 6 cells pretreated with/without anti-Mac-1 antibody or isotype control (hatched bar) to oxLDL-stimulated SMCs pretreated with/without H33 or isotype control (black bar) in vitro, **P<0.001 vs untreated (D). n=3 to 4, *P<0.05.
after injury, double-immunofluorescence staining revealed the expression of JAM-C in colocalization with luminal SMCs (Figure 2A), whereas remaining ECs could only rarely be detected (not shown). This is in line with previous findings that SMCs can replace ECs in denuded vessels and display a proadhesive phenotype with a constitutive upregulation of different chemokines\(^1\) and implies that luminal SMCs can express and present JAM-C to circulating leukocytes. Three weeks after injury the expression of JAM-C was detected in colocalization with luminal ECs but also SMCs and neointimal plaque SMCs (Figure 2A).

After injury, the exposed subendothelial matrix can trigger the adhesion of platelets, which support leukocyte recruitment.\(^1,12\) On human platelets, JAM-C serves as a ligand for the adhesion of platelets, which support leukocyte recruitment. JAM-C on SMCs supports monocyte recruitment in vivo,\(^1\) and in vitro, SMCs bearing JAM-C on their luminal surface enable monocytes to adhere to the SMCs.\(^3\) The expression and interaction of endothelial JAM-C with Mac-1 on leukocytes supports monocyte recruitment in vivo,\(^1,12,5\) and in vitro, Mac-1 on SMCs supports monocyte recruitment,\(^12\) whereas remaining ECs could only rarely be detected (not shown). This is in line with previous findings that SMCs can replace ECs in denuded vessels and display a proadhesive phenotype with a constitutive upregulation of different chemokines\(^1\) and implies that luminal SMCs can express and present JAM-C to circulating leukocytes. Three weeks after injury the expression of JAM-C was detected in colocalization with luminal ECs but also SMCs and neointimal plaque SMCs (Figure 2A).

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Disclosures
None.

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Material and Methods

H33 antibody and specificity

The anti-JAM-C antibody (clone H33, full name CRAM-13H33) was generated by Aurrand-Lions et al. The specificity of H33 binding selectively to JAM-C was tested in the murine squamous cell carcinoma cell line KLN205 mock-transfected or stably transfected with a JAM-C\textsuperscript{EGFP} expressing fusion vector. The molecular weight of the fusion protein containing JAM-C (30 kDa) and EGFP (40 kDa) is approximately 70 kDa. In conjunction with EGFP, flow cytometry confirmed the detection of a specific signal for JAM-C by H33 in comparison to isotype control on JAM-C\textsuperscript{EGFP}-expressing cells, while an antibody to integrin α\textsubscript{v} (RMV 7) produced a staining in both mock and JAM-C\textsuperscript{EGFP}-transfected cells serving as a positive control; antibody binding was detected using an Phycoerythrin-conjugated anti-rat secondary antibody (Jackson ImmunoResearch, Ref #112-116-143) (Online Data Supplement Figure 1A). A specific band at the expected size of the fusion protein containing JAM-C (70 kDa) was in addition revealed by H33 in lysates of JAM-C\textsuperscript{EGFP} but not mock-transfected KLN205 cells by Western Blotting after immunoprecipitation of JAM-C, performed as described, while JAM-C could not be detected using anti-integrin or isotype control antibodies (Online Data Supplement Figure 1B). Furthermore, FACS analyses revealed a specific staining for JAM-C using H33 on primary lung microvascular endothelial cells (LMECs) isolated from wild type (Jam-C\textsuperscript{+/+}) but not JAM-C-deficient (Jam-C\textsuperscript{-/-}) mice\textsuperscript{3,5} compared to control antibodies directed against L-selectin (Mel14, rat IgG2a). Staining for CD31 (GC51, rat IgG2b) on both Jam-C\textsuperscript{+/+}
and Jam-C+/- LMECs mice in comparison to isotype matched control antibodies directed against Mac-1 (M1/70, rat IgG2b) served as a positive control; primary antibody binding was detected using an Phycoerythrin-conjugated secondary anti-rat antibody (Online Data Supplement Figure 1C). Together, these data confirm binding of H33 specifically to JAM-C. In addition, H33 was shown to block JAM-B/JAM-C interactions5, angiogenesis and leukocyte homing to tumors4.

Mouse model of carotid artery injury

Female, 8 week old Apoe+/- mice (C57Bl/6 background) were fed an atherogenic diet containing 21% fat starting 1 week before transluminal carotid artery injury induced by a 0.014 inch angioplasty guide-wire in anesthetized mice (100 mg/kg ketamine/ 10 mg/ kg xylacine, i.p.)6. Mice received i.p. injections of monoclonal antibody against JAM-C (H33, 50 µg/mouse, 3/week) or isotype control antibody (rat IgG2a) starting with 2 injections peri-operatively. After 1 day or 3 weeks, arteries were harvested by in situ perfusion fixation with 4% paraformaldehyde and embedded in paraffin. Animal experiments were approved by local authorities and complied with the German animal protection law.

Morphometry, immunohistochemistry and immunofluorescence stainings

Serial sections (5µm, 250-500µm from the bifurcation) stained with Movats pentachrome were analyzed by planimetry. The relative macrophage and SMC content was determined by antibody staining for Mac-3 (#550292, BD Pharmingen) detected by alkaline-phosphatase enzyme (Vector Laboratories) and counter-staining by Mayers haemalaun and α-smooth muscle actin (1A4; Dako) detected by FITC-conjugated secondary antibody (Jackson ImmunoResearch). For double immunofluorescence stainings, primary affinity purified rabbit
anti-JAM-C antibody (Nr. 526, provided by Prof. Imhof), anti-SM α-actin (α-SMA), anti-P-selectin (clone C-20, Santa-Cruz Biotechnology), anti-CD31 (M-20, Santa-Cruz Biotechnology), or appropriate isotype control antibodies were detected by secondary anti-rabbit Cy-3 and anti-mouse- or anti-goat FITC-antibodies (Jackson ImmunoResearch). Cell nuclei were counterstained with DAPI (Vectashield, Vector Labs). Images were visualized using a Leica DMLB microscope (Leica Microsystems, Wetzlar) and 10x0.30 numeric aperture or 40x0.75 Leica objectives. Images were captured using a JVC digital camera KY-F70B 3-CCD (Vitor, Tokyo) and processed using Diskus software.

Cell culture and flow adhesion assay

Human coronary artery SMCs (Promocell), MonoMac6 cells and human umbilical vein endothelial cells (HUVECs) were maintained as described. MonoMac6 cell adhesion to SMC was analyzed in parallel wall flow-chambers. SMCs expressed smooth muscle specific α-actin but not vWF and similarly to vWF+ HUVECs displayed a strong expression of JAM-C, as analyzed by RT-PCR (see Online Data Supplement Figure 2). Confluent SMCs were activated by oxLDL (10 µg/ml, overnight) and pre-treated with/without H33 or isotype control (10 µg/ml, 30 min). MonoMac6 cells (0.5x10^6/mL) were pre-treated with/without anti-Mac-1 antibody (CBRM1/29, 20 µg/ml) or isotype control (mouse IgG1) and perfused over SMC monolayers (1.5 dyn/cm^2) in the continuing presence of the antibodies. The number of cells firmly adherent to SMCs was quantified by analysis of images recorded with a 3CCD video camera and recorder (JVC, Wayne, NJ).
Reverse-transcriptase and real time PCR

For RNA analysis, total RNA was isolated from HUVECs and SMCs, reverse-transcribed into cDNA using Mo-MLV RT (Invitrogen). RT-PCR was performed using specific primer pairs (hJAM-C forward: GAA GCC AGT GAC CCC TGT CTG TAG AG, reverse: CAT CTG GTT TCC CTG GGT TCT TG; hcalponin forward: GCA GAT GGG CAC CAA CAA AGG AGC, reverse: CCT TGG GGG AAC AGG GAA GG; hSM α-actin forward: GAT GGC TTT GGG CAG CTT GGC AG, reverse: GAG GCA GGC TAA GCG GG A TGG; hvWF forward: GTT GTG GGA GAT GTT TGC CTA CG, reverse: GAG AAC CTC ATG GTA CAC AAC AGA GC). Products were separated by agarose gel electrophoresis. The expression levels of the target genes were in addition quantified by real time PCR analysis using the QuantiTect SYBR-Green PCR kit (Opticon MJ Research) and specific primer pairs (Jam-C, Hs00230289_m1; 18sRNA, Hs99999901_s1, Applied biosystems).

Platelet isolation and flow cytometric analysis

Platelet-rich-plasma was prepared from 8 week old Apoe−/− mice and platelets were isolated by centrifugation at 1250 g. Platelets were left untreated or incubated with thrombin (1 U/mL, 30 min, 37°C), and reacted with saturating concentrations of affinity purified anti-JAM-C antibody (rabbit anti-mouse, Nr. 526) and detected with FITC-conjugated secondary Ab and stained with anti-CD41-PE (BD Biosciences). Probes were analyzed using a BD FACSaria System (BD Biosciences) and experiments were analyzed via FlowJo Software (Tree Star).

Ex vivo perfusion and intravital microscopy of carotid arteries

Carotid arteries of Apoe−/− mice were isolated for ex vivo perfusion or intravital microscopy one day after wire-injury. After transfer onto a microscope stage, arteries were perfused with
MOPS-buffered physiological salt solution (4 µL/min), and after pre-incubation with H33 or isotype control antibodies (10 µg/ml, 30 min), perfused with calcein-AM labeled MonoMac6 cells (10⁶/mL, Molecular Probes) for 8 min. For intravital microscopy, rhodamin-6G (Molecular Probes) was administered i.v. to Apoe⁻/⁻ mice injected with H33 or isotype control (10 µg/ml, 30 min before and after wire-injury) to label circulating leukocytes, and injured common carotid arteries were exposed. Leukocyte arrest to arteries was analyzed by epifluorescence microscopy (Zeiss Axiotech, x20 water immersion).

**Statistical Analysis**

Data represent mean±SEM and were analyzed by 2-tailed Student’s t-test using GraphPad Prism (InStat software; GraphPad).

**References**

**Online Data Supplement Figure 1**

The murine squamous cell carcinoma cell line KLN205 was mock transfected or stably transfected with a JAM-C<sup>EGFP</sup> expressing vector (A,B). Flow cytometric analysis was performed to detect EGFP in conjunction with JAM-C by H33 or integrin α<sub>V</sub> by RMV 7 in comparison to isotype matched control antibodies. Representative dot plots are shown (A). JAM-C was immunoprecipitated from cell lysates and Western Blotting was performed, revealing a specific signal at the expected size of JAM-C (70 kDa) using H33 in JAM-C<sup>EGFP</sup> but not mock-transfected KLN205, while no signal for JAM-C could be detected using integrin α<sub>V</sub> or isotype control antibodies. The band at about 75 kDa represents unspecific binding (B). Flow cytometry of primary lung microvascular endothelial cells (LMECs) revealed the expression of JAM-C by H33 (black line) on Jam-C<sup>+/+</sup> LMECs but not Jam-C<sup>-/-</sup> mice, while CD31 (black line) could be detected on Jam-C<sup>+/+</sup> and Jam-C<sup>-/-</sup> LMECs mice in comparison to the respective isotype matched control antibodies (filled grey profile). Representative histograms are shown (C).
Online Data Supplement Figure 2

HUVECs and SMCs display a robust expression of JAM-C mRNA (459 base pair product), as evaluated by RT-PCR analysis. In addition, hCASMCs show a strong mRNA expression of the smooth muscle cell markers SM α-actin (358 base pairs) and calponin (471 base pairs) but not of the endothelial cell marker von Willebrand factor (vWF, 479 base pair). In contrast, HUVECs show no transcription of SM α-actin or calponin but a strong expression of vWF.
Online Data Supplement Figure 3

One day after injury, double-immunofluorescence staining revealed the accumulation and aggregation of P-selectin$^+$ platelets in some injured segments of Apoe$^{-/}$ carotid arteries (green), while no co-staining with JAM-C (red) could be detected; cell nuclei were stained by DAPI (blue, A). Flow cytometry of mouse platelets did not reveal any surface expression of JAM-C on unstimulated (control) or thrombin-activated mouse platelets (B).