

Junctional Adhesion Molecule A Expressed on Human CD34⁺ Cells Promotes Adhesion on Vascular Wall and Differentiation Into Endothelial Progenitor Cells

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Objective—To investigate the role of junctional adhesion molecule A (JAM-A) on adhesion and differentiation of human CD34⁺ cells into endothelial progenitor cells.

Methods and Results—Tissue healing and vascular regeneration is a multistep process requiring firm adhesion of circulating progenitor cells to the vascular wall and their further differentiation into endothelial cells. The role of JAM-A in platelet-mediated adhesion of progenitor cells was investigated by adhesion assays in vitro and with the help of intravital fluorescence microscopy in mice. Preincubation of human CD34⁺ progenitor cells with soluble JAM-A-Fc (sJAM-A-Fc) resulted in significantly decreased adhesion over immobilized platelets or inflammatory endothelium under high shear stress in vitro and after carotid ligation in vivo or ischemia/reperfusion injury in the microcirculation of mice. Human CD34⁺ cells express JAM-A, as defined by flow cytometry and Western blot analysis. JAM-A mediates differentiation of CD34⁺ cells to endothelial progenitor cells and facilitates CD34⁺ cell-induced reendothelialization in vitro. Pretreatment of human CD34⁺ cells with sJAM-A-Fc resulted in increased neointima formation 3 weeks after endothelial denudation in the carotid arteries of nonobese diabetic/severe combined immunodeficient mice.

Conclusion—These results indicate that the expression of JAM-A on CD34⁺ cells mediates adhesion to the vascular wall after injury and differentiation into endothelial progenitor cells, a mechanism potentially involved in vascular regeneration. Human CD34⁺ cells express JAM-A, mediating their interaction with platelets and endothelial cells. Specifically, JAM-A expressed on human CD34⁺ progenitor cells regulates their adhesion over immobilized platelets or inflammatory endothelium under high shear stress in vitro and after carotid ligation in vivo or ischemia/reperfusion injury in the microcirculation of mice. Moreover, it mediates differentiation of CD34⁺ cells to endothelial progenitor cells and facilitates reendothelialization. (*Arterioscler Thromb Vasc Biol.* 2010;30:1127-1136.)

Key Words: blood cells ■ endothelium ■ ischemia ■ monoclonal antibodies ■ platelets ■ vascular biology

As early as 1997, Asahara and colleagues¹ first reported that CD34⁺ cells isolated from human peripheral blood can differentiate into endothelial cells, contributing to neoangiogenesis. Since then, numerous experimental studies²⁻⁵ investigated and further supported the role of CD34⁺ stem cells in vascular regeneration and tissue healing. The mobilization of CD34⁺ cells expressing early cardiac, muscle, and endothelial markers into peripheral blood was reported in patients with acute myocardial infarction. After tissue ischemia, progenitor cells are mobilized from their bone marrow or peripheral niches into circulation, adhere at sites of the vascular lesion, and differentiate into a variety of mature cell types according to their origin and the local environment.^{6,7} Impairment in

this pathophysiological process, as the result of either low numbers of circulating progenitor cells or dysfunctional progenitor cells, leads to inadequate vascular repair; on coexistence with different cardiovascular risk factors, this type of impairment leads to vascular injury and atherosclerosis. The level of circulating CD34⁺ progenitor cells predicts the occurrence of cardiovascular events and death from cardiovascular causes and correlates with cardiovascular risk factors in patients with coronary artery disease.⁸ Therefore, it is not surprising that a plethora of in vivo studies and clinical trials were raised to examine the therapeutic benefits of CD34⁺ cell transplantation in ischemic disease. The transplantation of CD34⁺ cells exhibited increased potency and safety for therapeutic

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neovascularization, cardiomyogenesis, neurogenesis, and functional regenerative recovery after myocardial infarction in vivo.^{9,10} However, a major part of the molecular mechanisms underlying progenitor cell-mediated repair has not yet been elaborated.

Vascular repair is a complex process that includes mobilization, chemotaxis, adhesion, proliferation, and differentiation of progenitor cells. Although homing of CD34⁺ progenitor cells into bone marrow has been extensively studied,¹¹ domiciliation of CD34⁺ precursor cells into peripheral tissues and differentiation into endothelial cells is poorly understood. The role of platelets in domiciliation and subsequent differentiation of progenitor cells has been recently highlighted.^{12–17} Adherent platelets secrete a potent stem cell chemokine, the stromal cell–derived factor 1 (SDF-1); recruit circulating progenitor cells; and induce differentiation of the latter into endothelial cells.^{13,15} To our knowledge, the mechanisms responsible for platelet-mediated recruitment and differentiation of CD34⁺ cells have not been sufficiently elucidated.

Junctional adhesion molecule A (JAM-A; also known as JAM-1 or F11 receptor) is a cell adhesion molecule expressed on a variety of cells, including platelets playing a crucial role in platelet adhesion and vascular inflammation.¹⁸ The role of platelet-derived JAM-A in adhesion and differentiation of human CD34⁺ cells has not been described.

The present study analyzes the role of JAM-A in the adhesion and differentiation of CD34⁺ cells into endothelial progenitor cells.

Methods

A detailed “Methods” section is given in the Supplemental Material (available online at <http://atvb.ahajournals.org>).

Isolation and Culture of Platelets, Human CD34⁺ Cells, and Human Arterial Endothelial Cells

Human platelets were isolated as previously described.^{13,15} Human CD34⁺ cells were isolated from either human cord blood or bone marrow and cultured as previously described.^{14,15} Human arterial endothelial cells (haECs) were isolated and passaged according to techniques previously described.¹⁵

Adhesion Assays Under Static and Dynamic Conditions and Flow Cytometry

Evaluation of CD34⁺ cell adhesion to immobilized platelets under static and dynamic conditions (flow chamber) and to immobilized JAM-A-Fc was performed as previously described.¹⁵

To evaluate JAM-A expression on isolated CD34⁺ cells and on isolated platelets, 1-color flow cytometry was applied as previously described.¹⁵ The expression of CD146, CD31, CD34, and CD45 was determined on isolated CD34⁺ cells, haECs, and endothelial progenitor cells (EPCs).

Detection of JAM-A Protein by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblot Analysis, CFU Assay, and Reverse Transcription–Polymerase Chain Reaction

Protein detection of JAM-A on isolated platelets and human CD34⁺ cells was performed with a Western blotting detection system (Enhanced Chemiluminescence [ECL], Western Blotting Detection Kit; GE Healthcare, Buckinghamshire, UK).

To analyze the effect of JAM-A on CD34⁺ cell differentiation to endothelial progenitor cells, CD34⁺ cells were either seeded onto a

monolayer of isolated platelets over a collagen matrix, 10 $\mu\text{g}/\text{mL}$; or added onto immobilized JAM-A-Fc, as previously described.¹⁵

On differentiation of CD34⁺ progenitor cells to endothelial progenitor cell colonies, endothelial cells were further cultivated in culture flasks and analyzed for the expression of mRNA for endothelial nitric oxide synthase, CD45, platelet endothelial cellular adhesion molecule-1 (PECAM-1) (CD31), angioprotein receptor-2 (tie-2), vascular endothelial growth-1 (flk-1) (vascular endothelial growth factor-2 [VEGFR-2]), and β -actin by RT-PCR, as previously described.¹⁵

Endothelial Injury Assay and Intravital Fluorescence Microscopy

A wound-induced reendothelialization assay was performed as previously described.¹⁹

Dichlorofluorescein (DCF)-labeled CD34⁺ cells, $5 \times 10^4/250 \mu\text{L}$, were intravenously injected into C57BL/6J mice before carotid artery ligation or segmental intestinal ischemia by ligation of the supplying vessels. Before and after injury, the cell–vascular wall interactions were visualized by in vivo videomicroscopy. All images were recorded and evaluated off-line.

Evaluation of Neointima Formation

Wire-induced injury of the carotid artery was performed as previously described.²⁷ In brief, male NOD.CB17-Prkdc^{scid}/J mice were randomly assigned to receive either CD34⁺ stem cells, 5×10^5 cells/250 μL , preincubated for 30 minutes with JAM-A-Fc, 10 $\mu\text{g}/\text{mL}$ ($n=5$); or control-Fc, 10 $\mu\text{g}/\text{mL}$ ($n=6$), intravenously after denudation. After 3 weeks, the mice were euthanized and the left carotid arteries were removed, embedded in paraffin, and cut in sections. Staining with hematoxylin-eosin and elastica van Gieson reagent was performed according to standard protocols. The degree of stenosis was calculated from the neointimal area, and the original lumen area was defined as the area bounded by the internal elastic lamina.

Data Presentation and Statistical Analysis

Data are given as mean \pm SD, unless otherwise stated. For pairwise comparisons, we applied a 2-tailed unpaired *t* test. For multiple comparisons between 3 or more groups, we applied an ANOVA with a subsequent Scheffé post hoc analysis. All tests were 2-tailed, and $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed using commercially available software (SPSS version 15 for Windows; SPSS Inc, Cary, NC).

Results

Adhesion of Human CD34⁺ Progenitor Cells Over Immobilized Platelets Is Mediated Through JAM-A Under Static and Dynamic Conditions

Murine and human progenitor cells adhere to immobilized platelets in vitro^{13–15} and are recruited to arterial thrombi in vivo,¹² involving the chemokine SDF-1 binding to chemokine CXC receptor-4 (CXCR4)¹⁵ and the adhesion receptors P-selectin/P-selectin glycoprotein ligand 1^{12,13} and β_2 -integrin.¹³ Nevertheless, the counterreceptor for the β_2 -integrin on the surface of platelets has not been described until now.

Binding of the β_2 -integrin lymphocyte function–associated antigen 1 (LFA-1) to JAM-A²⁰ has been shown to enhance leukocyte interaction with platelets and endothelial cells. Because JAM-A plays a central role in cell adhesion, we investigated whether platelet-bound JAM-A plays a role in the recruitment of circulating CD34⁺ progenitor cells. At the beginning, we tested the adhesion of CD34⁺ cells on immobilized platelets under static conditions. As previously de-

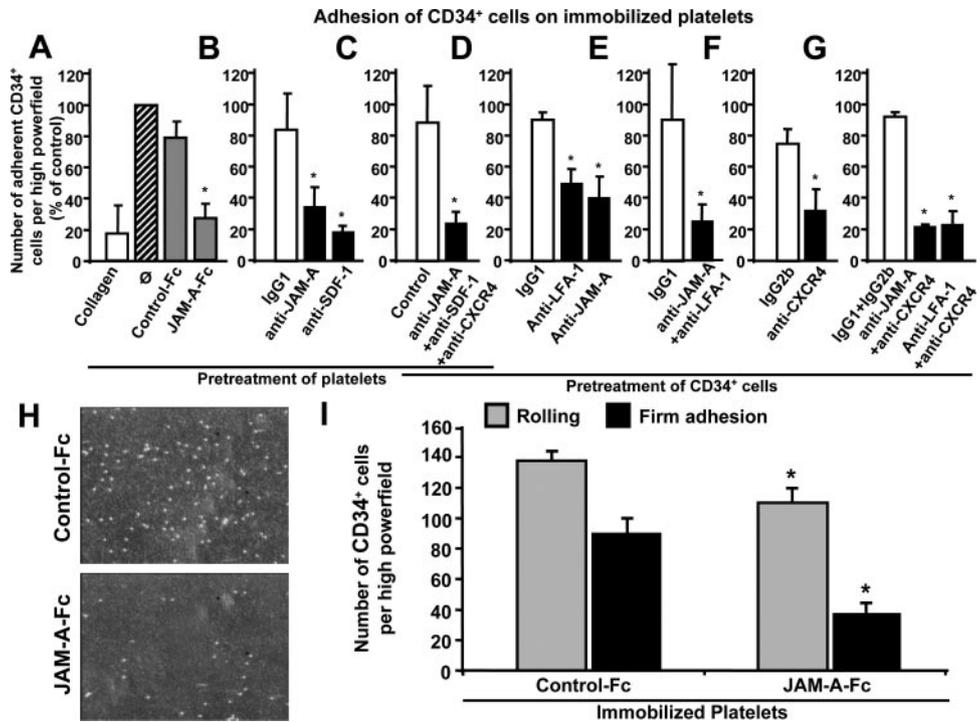


Figure 1. Adhesion of CD34⁺ cells over immobilized platelets is mediated not only through SDF-1/CXCR4, but also through JAM-A/JAM-A and JAM-A/LFA-1 interactions. A through G, Data are given as mean \pm SD of 3 to 6 independent static adhesion assay experiments. CD34⁺ cells were allowed to adhere over collagen or over immobilized platelets as described in the Supplemental Methods section. Where indicated, platelets or CD34⁺ cells were preincubated with anti-SDF-1, anti-JAM-A, sJAM-A-Fc; or anti-CXCR4, anti-LFA-1, anti-JAM-A, or a combination, respectively. * $P \leq 0.05$ vs respective control IgG, or control-Fc. H and I, Coverslips were pre-coated with collagen type I and were additionally preincubated with freshly isolated platelets to achieve adherent platelet layers, as described in the Supplemental Methods section. Immobilized platelets were subsequently preincubated with a neutralizing sJAM-A-Fc or control-Fc. Resuspended CD34⁺ cells were perfused over these coverslips. Representative phase contrast images of adherent CD34⁺ cells on immobilized platelets under high shear stress (H). Mean \pm SD of 3 independent flow chamber experiments (I). * $P \leq 0.05$ vs control-Fc.

scribed,^{13,15} human CD34⁺ cells adhere onto immobilized platelets, but hardly adhere to immobilized collagen type I alone, which represents the major extracellular matrix component of the injured arterial wall ($P \leq 0.05$) (Figure 1A–G). Adhesion of CD34⁺ cells onto immobilized platelets was significantly attenuated in the presence of soluble JAM-A-Fc, but not in the presence of control-Fc protein ($P \leq 0.05$) (Figure 1A). In a similar manner, neutralizing monoclonal antibodies to JAM-A and to LFA-1 (CD11a), but not a respective isotype control IgG, inhibited the adhesion of CD34⁺ cells onto immobilized platelets ($P \leq 0.05$) (Figure 1A–G). Parallel adhesion assays with anti-SDF-1 and anti-JAM-A showed a possible synergistic role of these 2 platelet receptors in the adhesion of human CD34⁺ cells to immobilized platelets (Figure 1A–G).

To verify our findings under high shear conditions, similar to arterial flow, we conducted perfusion experiments of CD34⁺ cells over adherent platelets in a parallel plate flow chamber at a wall shear rate of 2000/s (2 Pa) (Figure 1H). A remarkable number of perfused CD34⁺ cells quickly turned into rolling and later into firm adherent cells (Figure 1H and I and Supplemental Video). Preincubation of immobilized platelets with soluble JAM-A-Fc, but not with control-Fc, attenuated rolling of CD34⁺ cells under high shear stress (Figure 1I). Moreover, significantly decreased firm adhesion of CD34⁺ cells over immobilized platelets was observed after

preincubation with soluble JAM-A-Fc (90.00 \pm 8.69 versus 37.22 \pm 6.20 for control-Fc versus JAM-A-Fc; $P \leq 0.05$; $n = 3$) (Figure 1I and Supplemental Video). The present results indicate that JAM-A regulates adhesion of CD34⁺ cells onto immobilized platelets (Figure 1). The binding characteristics of JAM-A-Fc on CD34⁺ cells are depicted in Supplemental Figure 1A.

Human CD34⁺ Progenitor Cells Express JAM-A

JAM-A has been described to be expressed on murine hematopoietic precursors only recently.²¹ However, the presence of JAM-A and its functional role on human CD34⁺ progenitor cells are not elucidated, encouraging us to investigate their possible expression on human CD34⁺ cells. Platelets constitutively express on their surface JAM-A (Figure 2A), as previously reported.^{18,22} Flow cytometric analysis of JAM-A expression on CD34⁺ cells revealed that high levels of JAM-A are expressed on their surface (Figure 2B). The presence of JAM-A in CD34⁺ cells was also verified by immunoblotting (Figure 2C). JAM-A expression levels remained unaltered under high shear stress or inflammatory conditions (Supplemental Figure 1B and C). JAM-A expression was also investigated in different CD34⁺ cell sources and subpopulations (Supplemental Figures 1D and Figure 2).

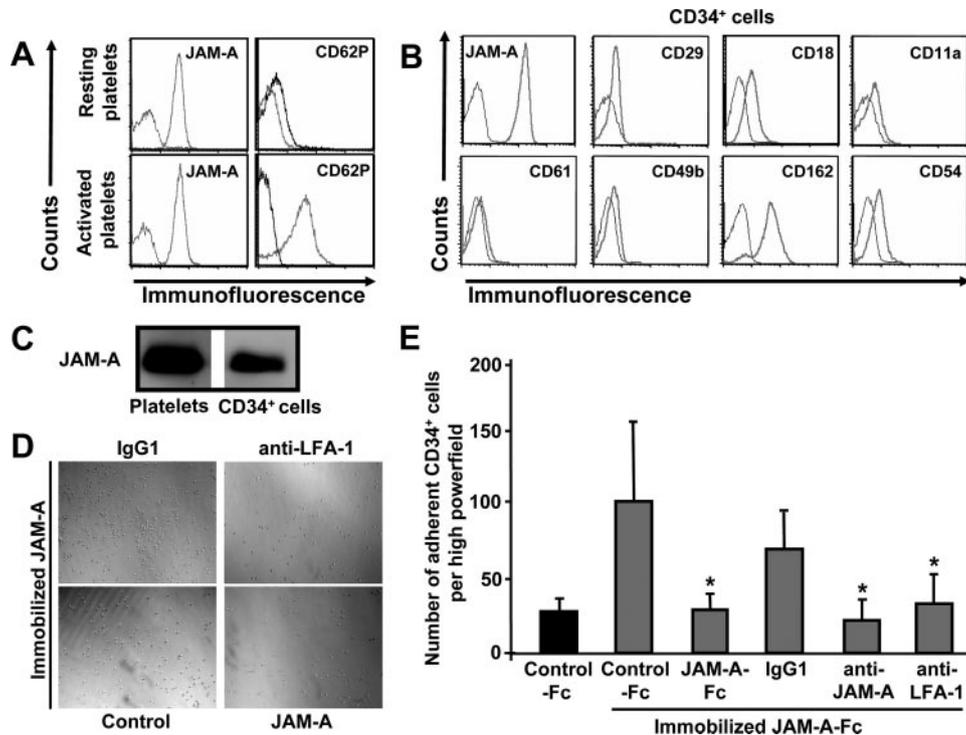


Figure 2. JAM-A is expressed not only on platelets, but also on human CD34⁺ progenitor cells. A, Representative flow cytometry histograms of expression of JAM-A (left) and P-selectin (or CD62P; right) on resting or ADP-activated washed platelets (n=6). B, Representative flow cytometry histograms of expression of JAM-A, β_1 -integrin (CD29), β_2 -integrin (CD18), LFA-1 (CD11a), β_3 -integrin (CD61), α_2 integrin (CD49b), P-selectin glycoprotein ligand-1 (PSGL-1; CD162), and intercellular adhesion molecule-1 (ICAM-1, or CD54) on human CD34⁺ cells (n=3 to 6). C, Representative immunoblotting images of JAM-A detection on platelets (positive control) and human CD34⁺ cells (n=4). D and E, CD34⁺ progenitor cell adhesion is mediated through JAM-A binding to JAM-A and LFA-1. CD34⁺ cells were allowed to adhere over immobilized JAM-A-Fc or control-Fc, as described in the Supplemental Methods section. Where indicated, CD34⁺ cells were preincubated with sJAM-A-Fc, anti-JAM-A, or anti-LFA-1. D, Representative phase contrast images of adherent CD34⁺ cells are shown. E, Data are given as the mean \pm SD of 3 independent experiments. * $P \leq 0.05$ vs respective control IgG₁ or control-Fc.

CD34⁺ Progenitor Cell Adhesion Is Mediated Through JAM-A Binding to JAM-A and LFA-1

JAM-A supports both homophilic and heterophilic interactions. JAM-A interacts with both JAM-A and the integrin LFA-1 or CD11a. In the present study, we showed that JAM-A is also present in human CD34⁺ progenitor cells. Because JAM-A binds to both JAM-A (homophilic interaction) and LFA-1 (heterophilic interaction), we asked whether a homophilic (JAM-JAM) or a heterophilic (JAM-integrin) interaction primarily regulates CD34⁺ cell adhesion. By using a static adhesion assay, we observed that CD34⁺ cells firmly adhered to immobilized JAM-A ($P \leq 0.05$; n=3) (Figure 2E). The adhesion of CD34⁺ cells to immobilized JAM-A was substantially reduced in the presence of soluble JAM-A-Fc or of a neutralizing monoclonal antibody to JAM-A (Figure 2D and E). In a similar manner, preincubation of CD34⁺ cells with a blocking anti-LFA-1 monoclonal antibody resulted in decreased CD34⁺ cell adhesion over immobilized JAM-A (Figure 2D and E). This indicates that both JAM-A and LFA-1 play a role in immobilized JAM-A-mediated CD34⁺ cell adhesion. Most of the circulating CD34⁺ cells are also positive for CD45, and both CD34⁺/CD45⁺ and CD34⁺/CD45⁻ populations were highly positive for JAM-A in healthy subjects and in patients with stable coronary artery disease

(Supplemental Figure 2A and B). Pretreatment of platelets or CD34⁺ cells with JAM-A-Fc resulted in similar inhibition levels of adherent CD34⁺ cells over immobilized platelets (Supplemental Figure 3A).

CD34⁺ Cell Differentiation Into Endothelial Progenitor Cells Is Mediated Through JAM-A

Adherent platelets cause CD34⁺ cell differentiation into endothelial cells.^{13,15} To further evaluate the molecular requirements of platelet-dependent differentiation of progenitor cells, CD34⁺ cells were allowed to adhere onto immobilized platelets or collagen (negative control) and were cultivated in endothelial cell growth medium, as previously described.¹⁵ Where indicated, sJAM-A-Fc or control-Fc was added in the cell culture system. Platelet-mediated formation of endothelial colonies of CD34⁺ cells was significantly reduced in the presence of sJAM-A-Fc, but not in the presence of control-Fc (number of colonies, 11.0 ± 1.4 versus 3.5 ± 0.7 for control-Fc versus sJAM-A-Fc; $P \leq 0.05$; n=3) (Figure 3A and B). To define the role of JAM-A on differentiation of CD34⁺ cells into endothelial progenitor cells, similar CFU assays were performed. Specifically, CD34⁺ cells were allowed to adhere onto immobilized fibronectin (positive control), control-Fc (negative control), and immobilized JAM-A-Fc; and the endothelial colonies were measured, as previously de-

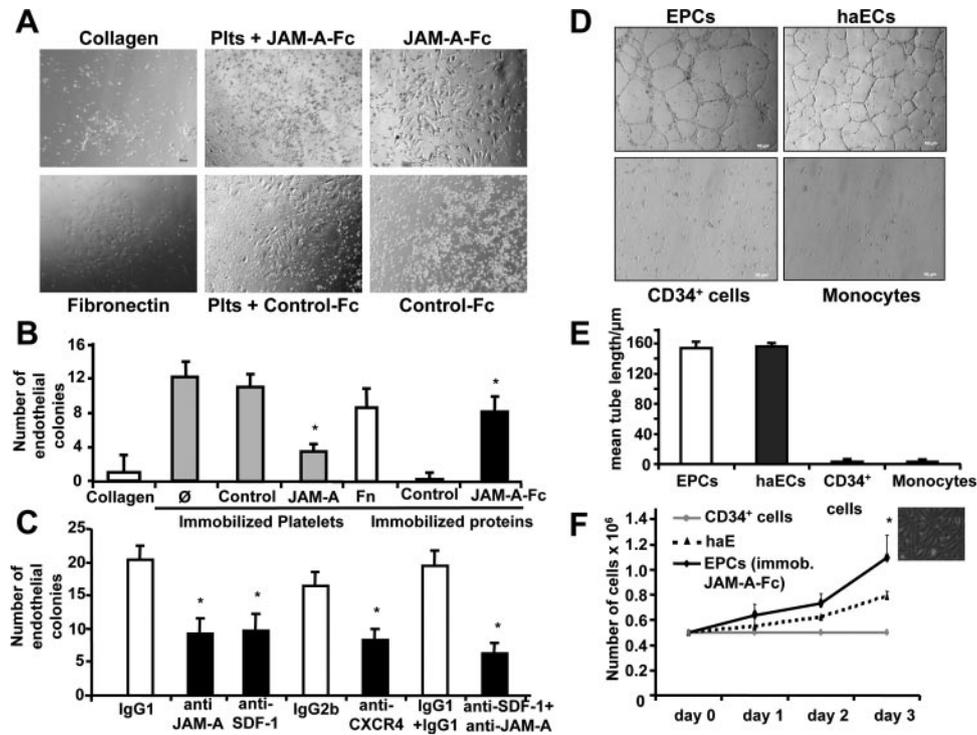


Figure 3. JAM-A induces differentiation of CD34⁺ cells into endothelial progenitor cells. A and B, CD34⁺ cells were cultivated on immobilized collagen (negative control), fibronectin (Fn, positive control), immobilized platelets in the presence of sJAM-A-Fc or control-Fc, or immobilized JAM-A-Fc or control-Fc, as described in the Supplemental Methods section. Endothelial progenitor cell CFUs were counted between days 5 and 10. A, Representative phase contrast images are shown. B, Data are given as mean ± SD of 3 independent experiments. * $P \leq 0.05$ vs control-Fc. C, CD34⁺ cells were cultivated over immobilized platelets in the presence of anti-JAM-A, anti-SDF-1, a combination of both, and anti-CXCR4. Data are given as mean ± SD of 3 independent experiments. * $P \leq 0.05$ vs control-IgG. D and E, A Matrigel angiogenesis assay was performed to investigate the ability of CFU-derived EPCs to integrate into vascular structures. Culture of EPCs and haECs within Matrigel, but not of monocytes and CD34⁺ cells, led to the formation of an extensive tubule network. D, Representative phase contrast images are shown. E, Data are given as mean ± SD of 3 independent experiments. F, Proliferation capacity of CD34⁺ cells, haECs, CFU-derived EPCs (coming from cultivation of CD34⁺ cells over immobilized JAM-A-Fc) is depicted. Cells were cultured for 3 days. Proliferation was determined by a trypan blue exclusion assay. * $P \leq 0.05$ vs haECs.

scribed.¹⁵ Immobilized JAM-A promoted the formation of endothelial colonies derived from CD34⁺ cells, when compared with control-Fc (number of endothelial colonies, 0.3 ± 0.6 versus 8.0 ± 2.0 for immobilized control-Fc versus immobilized JAM-A-Fc; $P \leq 0.05$; $n = 3$) (Figure 3B). Interestingly, immobilized JAM-A-Fc induced the differentiation of CD34⁺ cells into endothelial colonies at a similar level to immobilized fibronectin, SDF-1, and P-selectin, indicating that differentiation of CD34⁺ cells relies first on firm adhesion (Supplemental Figure 3B). Parallel CFU assays with anti-SDF-1 and anti-JAM-A showed a significant role of these 2 platelet receptors in the platelet-mediated differentiation of human CD34⁺ cells to EPCs (Figure 3C).

Next, we studied the capacity of EPCs to integrate into vascular structures through Matrigel angiogenesis assays. Incubation of EPCs and haECs, but not of monocytes and CD34⁺ cells, on Matrigel led to the formation of extensive tube formation (Figure 3D and E). Furthermore, the proliferation capacity of CD34⁺ cells, haECs, CFU-derived EPCs (derived from CD34⁺ cells incubated on immobilized JAM-A-Fc) over a 3-day period was studied. EPCs reached a significantly higher proliferation capacity after 3 days of culture when compared with mature haECs (Figure 3F).

Verification of CD34⁺ cell differentiation into endothelial progenitor cells was further performed with flow cytometry and determination of specific mRNA. The surface expression of CD146, PECAM-1 (CD31), CD34, and CD45 was tested on CD34⁺ cells, haECs, and platelet- or JAM-A-induced endothelial colonies by flow cytometry, as described in the Supplemental Online Methods section. CFU-derived EPCs exhibit similar endothelial surface markers (eg, CD146, CD144, or CD31) compared with primary endothelial cell cultures cultivated from human arteries (Figure 4A and Supplemental Figure 4). After 5 days, the morphology of initially round CD34⁺ cells on immobilized platelets and on JAM-A turned into adherent spindle-shaped cells, which were positive for von Willebrand factor, as shown by immunofluorescence microscopy (Figure 4B). Next, we analyzed whether EPCs can be activated to express activation-dependent surface markers, such as intercellular adhesion molecule-1 (CD54) and CD106. We found that stimulation of immobilized JAM-A-induced CD34⁺ cell-derived endothelial colonies with tumor necrosis factor α or interferon γ cytokines showed enhanced expression of CD54 and CD106, similar to the activation profile obtained when haECs were used or when cells were cultivated on immobilized platelets (Figure 4C). PCR analysis showed that EPCs cultivated on immobi-

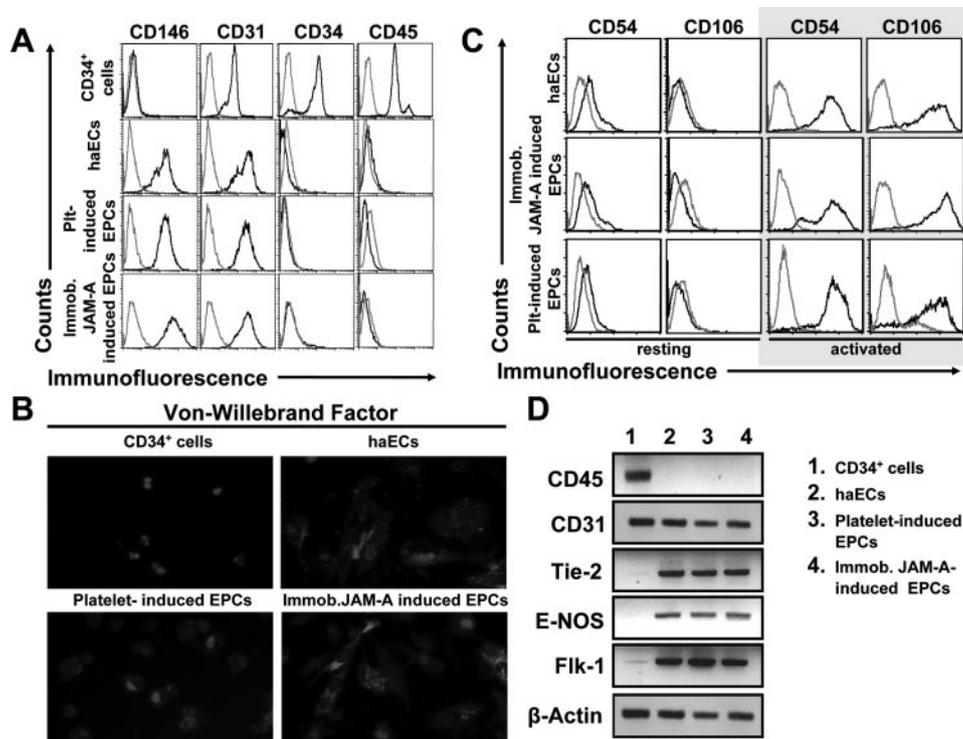


Figure 4. Verification of platelet-bound or immobilized JAM-A-induced CD34⁺ cell differentiation into endothelial progenitor cells. A, Representative flow cytometry histograms (n=3) of the surface markers CD146, CD31, CD34, and CD45, expressed on CD34⁺ cells, haECs, platelet-induced endothelial progenitor cells (EPCs), and immobilized JAM-A-induced EPCs. B, Representative immunofluorescence images (n=3) revealing the expression of von Willebrand factor (vWF) on CD34⁺ cells, haECs, platelet-induced EPCs, and immobilized JAM-A-induced EPCs. The vWF is stained red, and the nuclei are displayed blue. C, Representative flow cytometry histograms (n=3) of the 2 activation endothelial markers CD54 (intercellular adhesion molecule-1 [ICAM-1]) and CD106 on resting and tumor necrosis factor (TNF) α /interferon (INF) γ -activated platelet-induced or immobilized JAM-A-induced EPCs and haECs. D, Polymerase chain reaction analysis (n=3) of CD45, CD31, tie-2, eNOS, flk-1, and β -actin on CD34⁺ cells, haECs, and platelet-bound and JAM-A-induced EPCs.

lized JAM-A exhibit positive signals for endothelial nitric oxide synthase, endothelial angiopoietin receptor (Tie-2), and vascular endothelial growth factor receptor 2 (VEGFR-2, or flk-1), similar to those signals obtained from haECs or CD34⁺ cell-derived endothelial progenitor cells cultivated on immobilized platelets (Figure 4D).

JAM-A Is Involved in the CD34⁺ Cell-Induced Reendothelialization Process In Vitro

Endothelial progenitor cells promote neoendothelialization of the injured endothelial monolayer. Thus, we asked whether JAM-A mediates reendothelialization of an injured endothelial monolayer in a "scratch-wounded assay," as previously described.¹⁹ Primary cultures of haECs were cultivated to confluence. After scratch-induced injury of the monolayer, CD34⁺ cells, CD34⁺ cells and platelets, or platelets alone were added and the coculture was further incubated for 13.5 ± 2.6 hours (n=6) (Figure 5A). CD34⁺ cells promoted neoendothelialization of the injured zone compared with experiments in which CD34⁺ cells were absent ($P \leq 0.05$) (Figure 5A). Moreover, the addition of platelets, but not of platelets alone, in our coculture system after scratch-induced injury resulted in significantly enhanced reendothelialization compared with CD34⁺ cells alone ($P \leq 0.05$) (Figure 5A).

In the presence of soluble JAM-A-Fc, CD34⁺ cell-induced reendothelialization of the injured zone was sig-

nificantly reduced compared with Fc control experiments ($P \leq 0.05$; n=6). In a similar manner, the reendothelialization induced by a combination of CD34⁺ cells and platelets was significantly inhibited through sJAM-A-Fc ($P \leq 0.05$; n=6) (Figure 5B and C). By using rhodamine fluorescence, we could detect that JAM-A-Fc mainly inhibited the adhesion of CD34⁺ cells to endothelial cells or endothelial cells and platelets; therefore, less reendothelialization capacity was observed (Supplemental Figure 5A and B).

The interaction of circulating progenitor cells with the endothelial monolayer of the vasculature of peripheral organs is critical for homing. Previously, we showed that JAM-A-Fc reduced the reendothelialization capability in a coculture system of endothelial cells and CD34⁺ cells. Thus, we asked whether JAM-A is involved in the interaction of CD34⁺ cells with cultured haECs under dynamic shear conditions. As expected, cultured nonactivated endothelial cells do not support firm adhesion of CD34⁺ cells under arterial flow conditions (Figure 5E). However, when monolayers of haECs were activated with tumor necrosis factor α or interferon γ , the adhesion of CD34⁺ cells was significantly enhanced ($P \leq 0.05$). In the presence of soluble JAM-A-Fc, but not of control-Fc, a significant reduction of firm adhesion of CD34⁺ cells to the endothelial surface was observed ($P \leq 0.05$) (Figure 5D and E).

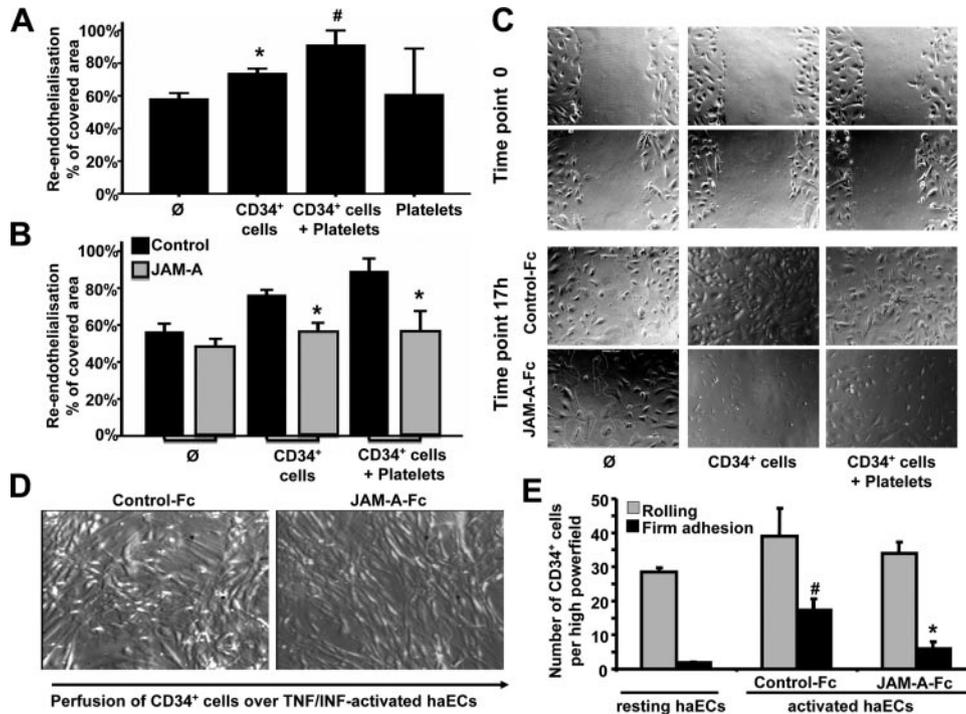


Figure 5. JAM-A is involved in the reendothelialization process through progenitor cell interaction with endothelial cells in vitro. A through C, Primary cultures of haECs were cultivated to confluence. After a scratch-induced injury of the endothelial monolayer, CD34⁺ cells, CD34⁺ cells and platelets, or platelets alone were added; and the coculture was further incubated for a mean \pm SEM of 13.5 \pm 2.6 hours (n=6). A, The mean \pm SEM of 6 independent experiments is shown. * $P \leq 0.05$ vs medium alone (\emptyset). B and C, Where indicated, the coculture system was incubated with either control-Fc or JAM-A-Fc. B, The mean \pm SEM of 6 independent experiments is shown. * $P \leq 0.05$ vs control-Fc. C, Representative phase contrast images are shown. D and E, haECs were cultured to confluence over coverslips; where indicated, they were activated with tumor necrosis factor (TNF) α /interferon (INF) γ . Resuspended CD34⁺ cells were perfused over these coverslips under high shear stress. Where indicated, CD34⁺ cells were preincubated with sJAM-A-Fc or control-Fc. D, Representative phase contrast images of adherent CD34⁺ cells on inflammatory endothelium under flow conditions. E, Data are given as the mean \pm SD of 3 independent flow chamber experiments. # $P \leq 0.05$ vs resting haECs, and * $P \leq 0.05$ vs control-Fc.

JAM-A Is Critical for CD34⁺ Cell Adhesion on Vascular Wall and Neointima Formation After Vascular Injury In Vivo

To verify our experimental results in vivo, the common carotid artery of C57BL/6J mice was injured by ligation; and DCF-stained CD34⁺ progenitor cells were injected intravenously. Preincubation of CD34⁺ cells with JAM-A-Fc resulted in decreased adhesion of progenitor cells to the injured vessel wall ($P \leq 0.05$) (Figure 6A and B). The role of JAM-A in the CD34⁺ cell-endothelium interaction was investigated in the microcirculation of the small intestine of mice after ischemia/reperfusion injury using intravital fluorescence microscopy. Enhanced platelet/endothelium adhesion occurs in the microcirculation of inflamed tissue and during reperfusion of ischemic organs.²³ The preincubation of CD34⁺ cells with JAM-A-Fc, but not of control-Fc, resulted in decreased adhesion of progenitor cells in the microvasculature ($P \leq 0.05$) (Figure 6C and D).

To evaluate the role of CD34⁺ cells and especially of JAM-A in neointima formation and in reendothelialization, we preincubated human CD34⁺ cells with either JAM-A-Fc (n=5 mice) or control-Fc (n=6 mice) and injected them into nonobese diabetic/severe combined immunodeficient mice after carotid endothelial denudation. Three weeks after denudation, the mice were euthanized; and the carotid arteries and

aortic arches were analyzed by histomorphometry and histochemistry. Hematoxylin-eosin and elastica von Gieson stainings at lesion sites show a lumen-narrowing neointima formation in all Fc-treated mice, which was significantly increased in JAM-A-Fc-treated mice ($P \leq 0.05$) (Figure 6E and F). With respect to media area, there was no significant difference between the groups.

Discussion

The major findings of the present study are as follows: (1) platelet-mediated adhesion of human CD34⁺ progenitor cells is mediated via JAM-A, (2) JAM-A is expressed on human CD34⁺ cells, (3) JAM-A interactions are mediated through progenitor cell-expressed JAM-A and β_2 -integrin LFA-1, (4) JAM-A promotes the differentiation of human CD34⁺ cells into endothelial progenitor cells, (5) JAM-A plays a role in CD34⁺ cell-mediated reendothelialization in vitro through interaction with nearby endothelial cells, (6) JAM-A is important for the adhesion of human CD34⁺ cells on the vascular wall after injury in vivo, and (7) pretreatment of CD34⁺ cells with JAM-A-Fc results in increased neointima formation after endothelial denudation in vivo.

Domiciliation of progenitor cells in peripheral tissue is a multistep cascade, including initial adhesion to the vascular wall and differentiation to vascular cells. Platelets are the first

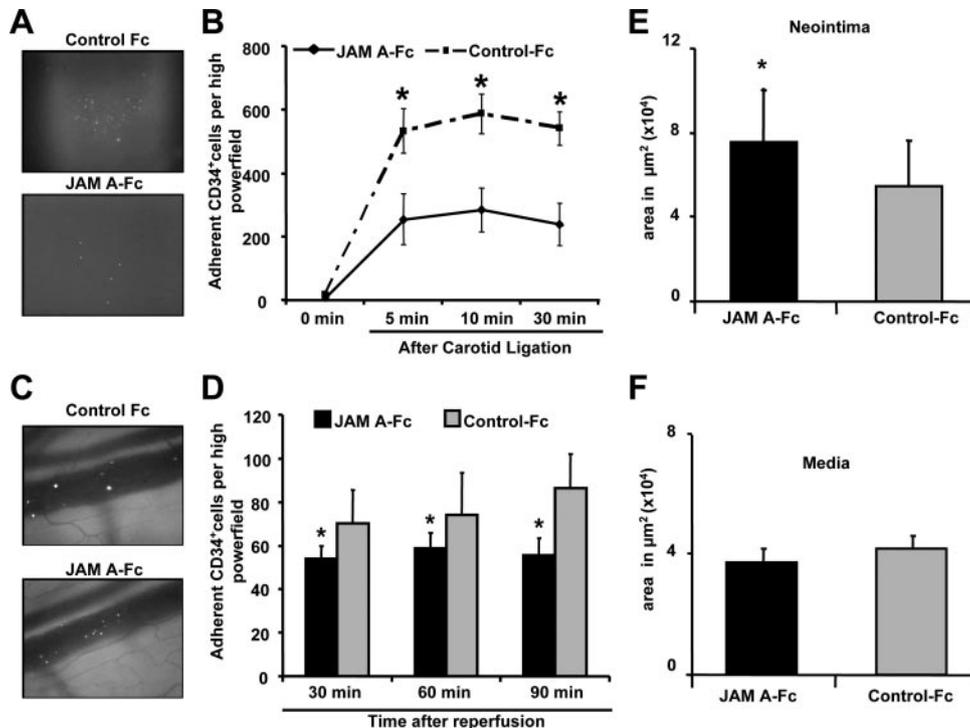


Figure 6. JAM-A is critical for CD34⁺ cell adhesion on vascular wall and neointima formation after vascular injury in vivo. A and B, In C57BL/6J mice, the common carotid artery was injured by ligation and DCF-stained CD34⁺ progenitor cells were injected intravenously. Preincubation of CD34⁺ cells with JAM-A-Fc, but not control-Fc, resulted in decreased adhesion of progenitor cells to the injured vessel wall. A, Representative intravital fluorescence microscopy images. B, Mean \pm SEM of the number of adherent CD34⁺ progenitor cells per high powerfield before and after 5, 10, and 30 after carotid ligation (n=6). C and D, Progenitor cell-microvasculature interactions were investigated in the microcirculation of the small intestine of mice after ischemia-reperfusion injury with the help of intravital fluorescence microscopy. Preincubation of CD34⁺ cells with JAM-A-Fc, but not control-Fc, resulted in decreased adhesion of progenitor cells in the microvasculature. C, Representative intravital fluorescence microscopy images of the microvasculature. D, Mean \pm SEM of adherent CD34⁺ progenitor cells 30, 60, and 90 minutes after ischemia-reperfusion (n=4 experimental animals per group) in venules. E and F, Increased neointima formation after endothelial denudation in nonobese diabetic/severe combined immunodeficient mice by pretreatment of human CD34⁺ cells with JAM-A-Fc. Histomorphometry analysis of carotid arteries 3 weeks after denudation was applied. Hematoxylin-eosin and elastica van Gieson stainings at lesion sites show a lumen-narrowing neointima formation in all Fc-treated mice (n=6); this was significantly increased in JAM-A-Fc-treated mice (n=5). Determination of the neointima (E) and media (F) shows a significant difference between the JAM-A-Fc treatment group and the control group only in the neointima. * $P \leq 0.05$.

circulating blood cells that adhere to vascular lesions and that accumulate in the microcirculation within ischemic tissue,²⁴ supporting angiogenesis²⁵ and vascular regeneration through interaction with progenitor cells.^{12,15,16} Researchers¹²⁻¹⁷ recently demonstrated that platelets influence progenitor cell recruitment and differentiation toward an endothelial phenotype. However, the underlying mechanisms are poorly elucidated.

JAM-A/JAM-1/F11R is a cell adhesion molecule expressed in epithelial, endothelial, and hematopoietic cells, such as leukocytes, platelets, and erythrocytes.²⁶ JAM-A plays a role in platelet aggregation, secretion, adhesion, and spreading.^{18,22} The role of platelet-bound JAM-A in platelet interaction with progenitor cells has not been studied until recently. To our knowledge, we show for the first time that platelet-bound JAM-A mediates the adhesion and differentiation of human CD34⁺ cells toward an endothelial phenotype. Moreover, in accordance with the recent finding that JAM-A is expressed on murine hematopoietic stem cells,²¹ we could verify the expression of JAM-A on the surface of human CD34⁺ progenitor cells. To enlighten the ligand-receptor interactions involved on JAM-A-mediated platelet

interaction with progenitor cells, we performed a series of adhesion assays showing that JAM-A binds to both CD34⁺ cell-derived JAM-A and integrin LFA-1. Moreover, immobilized JAM-A not only supported the adhesion of human CD34⁺ cells, but also promoted their differentiation to endothelial progenitor cells in a similar manner to fibronectin. Endothelial progenitor cells were positive for CD34, tie-2, endothelial nitric oxide synthase, and VEGFR-2 mRNA; however, CD34 protein was not detectable on their surface. Moreover, 2 endothelial markers (CD144 and CD146) were present on the surface of EPCs; however, intercellular adhesion molecule-1 and CD106 expression was further enhanced under inflammatory conditions, indicating that our CFU-derived EPCs present with a mature functional endothelial phenotype.

Previously, we showed that platelet-derived SDF-1 regulates the adhesion and differentiation of CD34⁺ cells to EPCs.²⁷ The Rap1 pathway is involved in both SDF-1-stimulated integrin activation and JAM-A downregulation, indicating a possible link between these 2 important receptors.^{28,29} In accordance with these findings, the present study results indicated parallel adhesion and CFU assays with

SDF-1; JAM-A showed a possible synergistic role of these 2 platelet receptors in the platelet-mediated adhesion and differentiation of human CD34⁺ cells to EPCs.

Hematopoietic and endothelial progenitor cells are recruited to ischemic regions that modulate vasculogenesis, accelerating reendothelialization and limiting atherosclerotic lesion formation.^{6,30–32} Clinical trials^{33,34} indicate a beneficial effect of intracoronary infusion of progenitor cells on myocardial function in patients with ischemic heart disease. However, the potential determinants and receptors involved in reendothelialization are still unknown.³⁵ In the present study, we showed that soluble JAM-A-Fc could inhibit the CD34⁺ cell-induced reendothelialization in vitro and enhance neointima formation in vivo, indicating that JAM-A may play a critical role in reendothelialization.

The present findings imply that JAM-A mediates recruitment of circulating CD34⁺ cells toward the injured vessel wall (vascular lesion and inflamed endothelium) and that JAM-A induces differentiation of CD34⁺ cells into endothelial progenitor cells and supports reendothelialization.

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Disclosures

None.

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Junctional Adhesion Molecule A Expressed on Human CD34⁺ Cells Promotes Adhesion on Vascular Wall and Differentiation Into Endothelial Progenitor Cells

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Supplement Material

Junctional adhesion molecule-A expressed on human CD34⁺ cells promotes adhesion on vascular wall and differentiation into endothelial progenitor cells

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Supplemental Methods

Generation of soluble Fc-fusion proteins

Cloning of soluble human JAM-A-Fc

Fc fusion proteins were generally created as described before ¹. To generate a soluble form of JAM-A, we fused the extracellular domain of these proteins to the human Fc domain. In detail, we initially amplified the Fc from a human heart cDNA library (Clontech, Palo Alto, CA). The PCR fragment was cloned into the plasmid pcDNA5-FRT (Invitrogen, Karlsruhe,

Germany) using NotI/XhoI. For cloning of the extracellular domains, total RNA from cultured megakaryocytes was isolated (RNeasy Mini Kit, Qiagen, Hilden, Germany) according to the manufacturers recommendations. Reverse transcription was performed (Omniscript RT Kit; Qiagen) using 2 µg RNA at 37°C over night. 100 ng of the cDNA was used as a template in PCR amplification of the human extracellular domains cDNA using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). For JAM-A the PCR fragments were cloned BglIII/NotI into the plasmid pcDNA5-FRT-Fc (BamHI/NotI). For cloning of a soluble Fc control fragment the leader peptide of CD40 was amplified from a human heart cDNA library (Clontech, Palo Alto, CA). The PCR fragment was cloned into the plasmid pcDNA5-FRT-Fc with KpnI/NotI. The accuracy of all plasmids was confirmed by sequencing (MediGenomix, Martinsried, Germany).

Generation of stable expressing CHO cells

Flp-InTM-CHO cells (Invitrogen, Karlsruhe, Germany) were cotransfected with a 9:1 ratio of the plasmids pOG44: each recombinant pcDNA5-FRT-Fc (Invitrogen, Karlsruhe, Germany) at a confluency of 70 %. 24 hours after transfection the cells were washed and fresh medium was added. 48 hours after transfection, the cells were split 1 to 20 into fresh medium containing 500 µg/ml hygromycin. Hygromycin-resistant foci were isolated, expanded and tested for the expression of soluble recombinant Fc fusion protein by western blot.

Purification of control Fc and JAM-A-Fc

The supernatant of cultured stable transfected CHO cells was collected, centrifuged (3800 g, 30 min, 4°C), and filtrated (0.2 µm). Soluble control Fc or soluble human recombinant protein (JAM-A-Fc) was precipitated by addition of 1.2 vol ammonium sulfate solution (761g/L) and stirring over night at 4°C. The proteins were pelleted by centrifugation (3000 g, 30 min, 4°C), dissolved in 0.1 vol PBS and dialyzed in PBS over night at 4°C. The protein solution was

clarified by centrifugation (14000 g, 30 min, 4°C), filtrated (0.2 µm), and loaded on Protein A-columns (HiTrap™ Protein A HP, Amersham Pharmacia Biotech AB, Uppsala, Sweden), equilibrated with binding buffer (20 mM sodium phosphate buffer pH 7.0, 0.02% NaN₃). The column was washed with binding buffer until OD280 < 0.01 and eluted with elution buffer (100 mM glycine pH 2.7). The eluted fractions (900 µl) were neutralized with 100 µl of neutralization buffer (1 M Tris/HCl pH 9.0, 0.02% NaN₃), pooled, dialyzed in PBS over night at 4°C, aliquoted and frozen at -20°C. The column was neutralized by binding buffer and washed with 20% (v/v) ethanol. After SDS-PAGE, the proteins were detected using Coomassie staining or with horseradish peroxidase conjugated goat anti-human IgG antibody (Fcγ fragment specific; 109-035-098; Dianova, Hamburg, Germany).

Moreover, as further source of our fusion proteins and verification of our results, we purchased JAM-A-Fc (R&D Systems; Minneapolis, USA; 10 µg/ml). Experiments with the purchased fusion proteins resulted in identical results like the ones taken place with our generated JAM-A-Fc.

Isolation of platelets

Human platelets were isolated as previously described^{2, 3}. Briefly, venous blood was drawn from the antecubital vein of healthy volunteers and collected in acid citrate dextrose (ACD)-buffer. After centrifugation at 430 g for 20 min, platelet-rich plasma (PRP) was removed, was added to Tyrodes-HEPES buffer (HEPES, 2.5 mM/L (Carl Roth GmbH, Karlsruhe, Gemrnay), NaCl, 150 mM/L, KCl, 1 mM/L, NaHCO₃, 2.5 mM /L, NaH₂PO₄, 0.36 mM/L, glucose 5.5 mM/L (Sigma, Steinheim, Germany), BSA 1 mg/ml, pH 6.5), and was centrifuged at 900 g for 10 min. After removal of the supernatant, the remaining platelet pellet

was resuspended in Tyrodes-HEPES buffer (pH 7.4 supplemented with 1 mM/L CaCl₂ and 1 mM/L MgCl₂).

Isolation and culture of human CD34⁺ cells

Human CD34⁺ cells were isolated either from human cord blood or bone marrow and cultured as previously described^{2, 4}. Human mononuclear cells were obtained from human umbilical cord blood, peripheral blood or bone marrow by density gradient centrifugation on Biocoll separation solution (Biochrom, Berlin, Germany) at 600 g for 15min. CD34⁺ cells were enriched by immunoaffinity selection (CD34 Progenitor Cell Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. For cell culture, IMDM with Glutamax was used, supplemented with 5% heat-inactivated fetal calf serum, 100 U/ml penicillin-streptomycin, 1% MEM-vitamines and 1% non-essential amino acids (all purchased from Invitrogen, Karlsruhe, Germany). The isolated cells were $\geq 95\%$ positive for CD34⁺ cells as determined by flow cytometry after each isolation procedure. Populations of CD34⁺ cells originated from cord blood or peripheral blood or bone marrow presented with similar results in our *in vitro* experiments.

Isolation and culture of human arterial endothelial cells

HaECs were isolated from adult human iliac arteries of bypass recipients by mechanically removing the endothelial layer as previously described^{5, 6} and were cultured in endothelial growth medium (Vasculife, Lifeline Cell Technology, Walkersville, USA) containing 2% fetal calf serum on culture flasks (Greiner, Frickenhausen, Germany) coated with 2% gelatin. The cells were propagated in growth medium renewed each 2 to 3 days. When the cells had become confluent, they were detached by treatment with trypsin/EDTA and were passaged with a split ratio of 1:5. Cells were kept at 37°C under 5% CO₂/95% air. HaECs were identified by immunocytochemical staining against the von Willebrand factor (Boehringer,

Mannheim, Germany) and their characteristic “cobblestone” growth pattern with contact inhibition between cells. Routine stainings with the DNA dye DAPI (4', 6-diamino-2-phenylindole-dihydrochloride; Boehringer, Mannheim, Germany) were used to exclude mycoplasma contaminations.

Adhesion assays under static and dynamic conditions

Evaluation of CD34⁺ cell adhesion to immobilized platelets under static and dynamic conditions (flow chamber) and to immobilized JAM-A-Fc was performed as previously described². For static adhesion assays, isolated platelets (2×10^8 /ml) were allowed to adhere to 96-well plates pre-coated with collagen type I (20 µg/ml) for 2 hours to achieve a monolayer of immobilized platelets. JAM-A-Fc (10 µg/ml) was analogously immobilized after 2-hour incubation in a 96-well plate. Unspecific adhesion was prevented by blocking with BSA (2%) for 30 minutes afterwards. Subsequently, CD34⁺ cells resuspended in IMDM medium were added and incubated for 30 minutes. After two gentle washing steps with PBS, residual adherent CD34⁺ cells were counted by direct phase contrast microscopy. As negative control, similar experiments were performed with CD34⁺ cells adherent to collagen (20 µg/ml; Figure 1A) or control-Fc (10 µg/ml; Figure 2E). Where indicated adherent platelets or CD34⁺ cells were pre-incubated for 30 minutes with JAM-A-Fc, anti-JAM-A, anti-SDF-1, anti-CXCR4 (R&D Systems; Minneapolis, USA; each 10µg/ml), anti-CD11a (BioLegend, San Diego, USA; 10 µg/ml), or a combination of the above mAbs. As a control, adherent platelets, or CD34⁺ cells were pre-treated with control-Fc (10 µg/ml), isotype control IgG1 (R&D Systems; Minneapolis, USA; clone11711; 20 µg/ml), or isotype control IgG_{2B} (R&D Systems; Minneapolis, USA; clone11711; 20 µg/ml), respectively (Figure 1A; 2A; Supplemental Figure 2A).

To evaluate CD34⁺ cell adhesion to immobilized platelets under flow conditions, platelets were allowed to adhere to collagen-coated glass coverslips and then used in a flow

chamber (Oligene, Berlin, Germany), as previously described ². Unspecific adhesion was prevented by blocking with BSA (2%). Adherent platelets were pre-incubated for 30 min with control-Fc or JAM-A-Fc (20 µg/ml each) before perfusion was started (Figure 1 B,C). Perfusion experiments were performed at shear rates of 2 Pascal (high shear stress). To investigate CD34⁺ cell adhesion on inflammatory endothelium, haECs were immobilized and cultured to confluency on coverslips. Where indicated, cells were activated with 50 ng/mL TNF α and 20 ng/mL INF γ (the referred cytokines were purchased from Peprotech Inc., New Jersey, USA). Subsequently CD34⁺ cells resuspended in IMDM medium were pre-incubated for 30 min with control-Fc, or JAM-A-Fc (20 µg/ml each) before perfusion was started (Figure 5 D, E). All experiments were recorded in real time on video-CD and evaluated off-line as previously described ².

Flow Cytometry

To evaluate JAM-A expression on isolated CD34⁺ cells and on resting and activated isolated platelets with ADP (20 µM; Chrono-Par, Havertown, USA) or TRAP (25 µM; Sigma, Steinheim, Germany) one-colour flow cytometry was applied as previously described ². In brief, a conjugated monoclonal antibody was used to measure CD34⁺ cell and platelet surface expression of JAM-A (PE labelled anti-human F11 Receptor; BD Biosciences, San Jose, USA). Platelet activation status was determined through co-estimation of P-selectin expression (anti-CD62P-FITC; Immunotec, Marseille, France; clone CLB-Thromb/6). JAM-A expression level of CD34⁺ cells in comparison to other cells adhesive molecules constitutively expressed by progenitor cells was further investigated by FACS analysis. CD34⁺ (isolated from umbilical cord blood) were labelled with JAM-A PE, beta-1 integrin (CD29), beta-2 integrin (CD18 FITC; BD Pharmingen, San Jose, USA), LFA-1 (CD11a PE clone HI111, Biolegend, San Diego, USA), beta-3 integrin (CD61 FITC; BD Pharmingen, San Jose, USA), alpha-2 integrin (CD49b, clone HAS3; R&D Systems; Minneapolis, USA),

P-selectin glycoprotein ligand-1 (PSGL-1; CD162 PE BD Pharmingen ,San Jose, USA) and ICAM-1 (CD54 FITC; Beckman Coulter, USA). Mouse IgG1-PE and IgG1-FITC used as isotype control antibodies and binding properties were evaluated by flow cytometry.

Expression of CD146 (anti-humanMCAM/CD146-FITC; R&D Systems, Minneapolis, USA), CD31 (anti-CD31-FITC; Immunotec, Marseille, France), CD34 (anti-CD34-FITC; clone 8G12; BD Biosciences, San Jose, USA) and CD45 (anti-CD45-PE; Immunotec, Marseille, France) was determined on isolated CD34⁺ cells, human arterial endothelial cells and colony forming unit assays-derived endothelial progenitor cells (platelet-induced differentiation to EPCs and immobilized JAM-A-induced differentiation to EPCs). CD34⁺ cells and human arterial endothelial cells were used as negative and positive control, respectively. EPCs derived from colony forming unit assays were further tested for expression of VE-Cadherin through flow cytometry. EPCs and haECs were labelled with monoclonal anti-human CD144 PE (clone TEA 1, Beckman Coulter, USA) and binding was measured by flow cytometry. IgG1-PE was used as monoclonal immunoglobulin isotype control antibody.

To measure the ability of the newly differentiated endothelial progenitor cells to get activated, expression of CD54 (anti-CD54-FITC; Immunotec, Marseille, France) and CD106 (anti-CD106-PE; BD Biosciences, San Jose, USA) was determined on resting (non-activated) and activated CD34⁺ cell-derived endothelial progenitor cells and human arterial endothelial cells using TNF- α (50 ng/ml) and INF- γ (20 ng/ml). After CD54 and CD106 staining was performed, cells were proceeded to FACS analysis, as previously described ². Mouse IgG1-FITC and IgG1-PE were used as monoclonal immunoglobulin isotype control antibodies (Figure 4 C).

To analyze whether JAM-A expression changes under inflammatory or shear stress conditions CD34⁺ cells were preincubated with one of the following cytokines (all purchased from R&D Systems; Minneapolis, USA) over a period of 2 or 6 hours: TNF- α (100ng/mL), INF- γ (100ng/mL), IL-1 β (10ng/mL), IL-6 (10ng/mL), LPS (100ng/mL) or SDF-1

(100ng/mL; Sigma-Aldrich, Munich, Germany). Subsequently, cells were stained with mouse anti-human JAM-A-PE and expression levels were measured by FACS analysis. To investigate JAM-A expression of CD34⁺ cells under high shear conditions, CD34⁺ cells were exposed to shear stress in an aggregometer (Chronolog, Havertown, USA) under stirring conditions (1200 rpm) for 5 or 30 min, and subsequently labelled with JAM-A PE.

For evaluation of JAM-A-Fc binding on human CD34⁺ cells, fluorescein-isocyanate (Sigma-Aldrich, Munich, Germany) was incubated with 100µg purified JAM-A-Fc (R&D Systems; Minneapolis, USA) or control-Fc (R&D Systems; Minneapolis, USA) protein and different fractions were collected by a Sephadex G-25M column (Pharmacia). Protein concentration was determined by Bradford. Next, human CD34⁺ cells were incubated with sJAM-A-Fc-FITC and binding properties were evaluated by flow cytometry. To further examine the interaction of progenitor cell-derived JAM-A with sJAM-A-Fc, progenitor cells were preincubated with sJAM-A-Fc or control-Fc before staining with JAM-A-PE antibody and binding properties were evaluated by flow cytometry. Mouse IgG1-PE was applied as monoclonal immunoglobulin isotype control antibody.

Comparison data of JAM-A expression on CD34 positive cells derived from bone marrow, peripheral blood and umbilical cord blood were obtained by FACS analysis. CD34⁺ cells were labelled with anti-JAM-A-PE or isotype-IgG PE and binding properties were evaluated by flow cytometry.

Detection of JAM-A protein by SDS-PAGE electrophoresis and Immunoblot analysis

Cells were lysed for 30 minutes on ice with a lyses buffer containing 10mM Tris-HCL pH 7.2; 100 mM NaCl, 0.1% NP-40, protease inhibitor cocktail (Roche, Germany) and 0.1 mM PMSF. Cell debris was spun down at 1400 rpm for 5 minutes at 4°C. Protein content was determined by the Bradford method (BioRad, Munich, Germany). Protein expression was assayed by Western-blot and Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

(SDS-PAGE). Equal amounts of protein (25 μg) from human cord blood CD34⁺ progenitor cells and platelets were separated on a 10% gel, transferred to a Nitrocellulose membrane (Hybond Amersham Bioscience, UK) and blocked in 5% milk for one hour at 37°C. Then, membranes were incubated overnight at 4°C with a mice anti-human JAM-A (1:50 Hycult biotechnology) primary antibody. As a secondary antibody an anti-mouse IgG-HRP was used for 1h at RT (1:5000 GE Healthcare, UK). Protein detection was made with an ECL-Plus western blotting detection system (Amersham Bioscience, UK).

Colony Forming Unit Assay

To analyze the effect of JAM-A on CD34⁺ cell differentiation to endothelial progenitor cells, CD34⁺ cells were either seeded onto a monolayer of isolated platelets over a collagen matrix (20 $\mu\text{g}/\text{ml}$), or added onto immobilized JAM-A-Fc or control-Fc, as previously described ². Immobilized collagen type I (BD Biosciences, San Jose, USA) and immobilized control Fc served as negative control, while immobilized fibronectin served as positive control. Where indicated adherent platelets were pre-incubated with control-Fc or JAM-A-Fc (each 20 $\mu\text{g}/\text{ml}$). Subsequently, the cells were cultivated for several days in endothelial cell growth medium MV2 containing 5% heat-inactivated fetal calf serum, 5.0 ng/ml epidermal growth factor, 0.2 $\mu\text{g}/\text{ml}$ hydrocortisone, 0.5 $\mu\text{g}/\text{ml}$ vascular endothelial growth factor, 10 ng/ml basic fibroblast factor, 20 ng/ml R3 insulin-like growth factor-1, 1 $\mu\text{g}/\text{ml}$ ascorbic acid (PromoCell, Heidelberg, Germany). Endothelial progenitor cell colony-forming units were counted between days 5 and 10 (number of endothelial colonies, Figure 3 A, B). To determine the expression of endothelial cell markers, cells were washed, resuspended in PBS and incubated with the respective labelled antibody for 30 min at room temperature (as previously described under “Flow Cytometry”). After washing, cells were analyzed on a FACS-Calibur flow cytometer (BD Biosciences, San Jose, USA). To further investigate the role of SDF-1, JAM-A and CXCR4 on CD34⁺ cell differentiation to endothelial progenitor cells, CD34⁺ cells were

preincubated with antibodies against JAM-A, SDF-1, CXCR4 (each 20 $\mu\text{g}/\text{mL}$) and seeded onto a monolayer of isolated platelets over a collagen matrix (20 $\mu\text{g}/\text{ml}$). As a control, CD34^+ cells were pre-treated with isotype controls IgG1 (R&D Systems; Minneapolis, USA; clone11711; 20 $\mu\text{g}/\text{ml}$), or isotype control IgG_{2B} (R&D Systems; Minneapolis, USA; clone11711; 20 $\mu\text{g}/\text{ml}$), respectively. Endothelial progenitor cell colony-forming units were counted between days 5 and 10 (number of endothelial colonies). In another set of experiments, JAM-A Fc, SDF-1 and P-selectin (each 20 $\mu\text{g}/\text{mL}$) were immobilized on 24 well plates at 4°C over night. Immobilized fibronectin served as positive control, while immobilized collagen served as a negative control. Subsequently, CD34^+ cells were cultivated for several days in endothelial cell growth medium. Endothelial progenitor cell colony-forming units were counted between days 5 and 10.

Immunofluorescence microscopy

In order to test the differentiation of CD34^+ cells to endothelial progenitor cells, a rabbit anti-human vWF monoclonal antibody (Dako Cytomation) and a secondary sheep anti-rabbit mAb (Sigma-Aldrich, Munich, Germany) were used. CD34^+ cells were co-incubated with medium or platelets for 10 days on chamber slides and processed for immunofluorescence microscopy. Between each incubation step, cells were gently washed with PBS.

Reverse Transcription – Polymerase Chain Reaction

Upon differentiation of CD34^+ progenitor cells to endothelial progenitor cell colonies, endothelial cells were further cultivated in culture flasks and analyzed for the expression of mRNA for eNOS (endothelial nitric oxide synthase), CD45, PECAM-1 (CD31), tie-2 (endothelium specific receptor tyrosine kinase 2), flk-1(VEGFR-2) and β -actin by RT-PCR as previously described². Briefly, mRNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed using the Im PromII Reverse Transcription

System (Promega, Mannheim, Germany) and aTaq Polymerase (Promega, Mannheim, Germany). Annealing temperature was 68.4°C for 30 seconds, extension at 72°C for 45 seconds, with a final 5 minutes extension at 72° C. The primer sequences were as follows: eNOS forward 5`-GGA AAA GGC CAG GGC TCT GCT GGA GC-3`, reverse 5`-GAA CAC CAG CTC GCT CTC CCT AAG CTG-3`, CD45 forward 5`-GAG GAG GAA ATT GTT CCT CGT CTG ATA AGA-3`, reverse 5`-CTG GCA GGT AAG GCA GCA GAG CTG TGG-3`, CD31 (PECAM-1) forward 5`-GTT GTA TGA GGT CCA GAT TAT CCT GTC-3`, reverse 5`-GCT GAG GAC ACT TGA ACT TCC GTG TAC TGC`; tie-2 forward 5`-GAC GTA GTA GGA CGA TGC TAA TGG AAA GTC-3`, reverse 5`-GTG TAC TTC TAG AAT ATC AGG TAC TTC ATG C-3`, flk-1 (vascular endothelial growth factor-1) forward 5`-CTT CTC TAG ACA GGC GCT GGG AGA AAG AAC-3`, reverse 5`-CAC GTT GAG ATT TGA AAT GGA CCC GAG ACA TG-3`, β -actin (internal control), forward 5`-ACC TTC AAC ACC CCA GCC ATG-3`, reverse 5`-GCT CGG TCA GGA TCT TCA TGA GG-3`.

Angiogenesis Assay *in vitro*

A Matrigel angiogenesis assay (BD, Biosciences) to investigate the ability of CFU derived EPCs to integrate into vascular structures, was performed according to the instructions of the manufacturer. In brief, CFU derived EPCs were obtained from colony forming unit assays. Confluent cells were detached by treatment with trypsin/EDTA and counted in a hemacytometer. Human arterial endothelial cells (haECs 1×10^5 cells/ml) monocytes (1×10^5 cells/ml) and umbilical cord blood isolated CD34⁺ (1×10^5 cells/ml) cells were seeded in endothelial basal medium supplemented with growth factors and 10% FCS (Vasculife, Lifeline Cell Technology, Walkersville, USA) on Matrigel basement membrane matrix (BD Biosciences). The length of tube-like structures was measured by light microscopy after 24 hours in a blinded fashion.

Proliferation Assay

The proliferation capacity of EPCs, haECs, CFU derived EPCs (derived from coincubation with platelets or grown on immobilized JAM-A Fc) and CD34⁺ cells was examined by trypan blue exclusion as previously described ⁷. Cell counting was performed in a hemacytometer over a period of 3 days.

Endothelial injury assay

A wound-induced re-endothelialisation assay (scratch assay) was performed as previously described ⁸. Briefly, human arterial endothelial cells (5×10^5 cells/well) were seeded into 24-well plates and cultured overnight at 37°C with 5% CO₂. When the cells were 80% confluent, a straight scratch was gently made through the central axis of the plate using a micropipette tipp. The plates were rinsed with PBS, and serum-low endothelial growth media (Vasculife, Lifeline Cell technology) was added. After injury of the endothelial monolayer, CD34⁺ cells or CD34⁺ cells and platelets, platelets or medium without cells were added. Subsequently, the coculture system was further incubated for 13.5 ± 2.6 hours with either control-Fc or JAM-A-Fc (20 µg/mL). To identify migrating CD34⁺ cells in the microscope cells were prestained with rhodamine. The acellular area created by scratching was calculated by Axiovision software (Zeiss, Germany). Photographs were taken at the time of the wound (0 hours) and after 13.5 ± 2.6 hours.

Intravital fluorescence microscopy

C57Bl/6J mice were anesthetized by intraperitoneal injection of midazolame (5 mg/kg body weight; Ratiopharm), medetomidine (0.5 mg/kg body weight; Pfizer) and fentanyl (0.05 mg/kg body weight; CuraMed Pharma GmbH) and placed on a heating pad for maintenance of body temperature between 36°C and 37°C. Polyethylene catheters (Portex) were implanted

into the left jugular vein for injection of the DCF-labeled (5-carboxyfluorescein diacetate succinimidyl ester) stem cells ($5 \times 10^4/250 \mu\text{l}$).

The common carotid artery was dissected free and ligated vigorously for 5 min to induce vascular injury. For intravital microscopy of the intestine a transverse laparotomy was performed and a segment of the jejunum was gently exteriorized. Segmental intestinal ischemia was induced by ligation of the supplying vessels and the segment itself for 1 hour. Before and after injury, the cell- endothelium interaction was visualized by in vivo video microscopy using a Zeiss Axiotech microscope (water immersion objective: 20X, W 20X/0.5; Zeiss) with a 100 W HBO mercury lamp for epi-illumination. All images were recorded and evaluated off-line.

Evaluation of Neointima Formation

Wire-induced injury of the carotid artery was performed as described before ⁹. In brief, male NOD.CB17-Prkdc^{scid}/J mice were anesthetized by intraperitoneal injection of a solution of midazolame (5mg/kg body weight; Ratiopharm, Ulm, Germany), medetomidine (0.5 mg/kg body weight, Pfizer, Karlsruhe, Germany), and fentanyl (0.05 mg/kg body weight; CuraMed Pharma, Karlsruhe, Germany). The left carotid artery was exposed and a transverse arteriotomy was made in the internal carotid artery. The denudation was performed with a 0.014-in flexible angioplasty guidewire by triple withdrawal of the wire. The mice were randomly assigned to receive either CD34⁺ stem cells (5×10^5 cells/250 μl) preincubated for 30 min with JAM-A-Fc (10 $\mu\text{g/ml}$, n = 5) or control-Fc (10 $\mu\text{g/ml}$, n = 6) intravenously after denudation. After 3 weeks the mice were sacrificed and the left carotid arteries were removed and embedded in paraffin blocks and cut into 5- μm sections and stained with hematoxylin-eosin and elastica van Gieson reagent according to standard protocols. Six of the elastica van Gieson stained sections upstream of the carotid bifurcation were used for quantification of neointima formation. The area of the lumen and the areas bounded by internal elastic lamina

(IEL) and external elastic lamina (EEL) were measured planimetric with NIS-Elements Imaging Software (Nikon GmbH, Düsseldorf, Germany). Neointimal area was calculated by subtracting lumen area from the IEL, and medial area by subtracting the EEL from the IEL area. The degree of stenosis was calculated from neointimal area and the original lumen area defined as area bounded by the internal elastic lamina.

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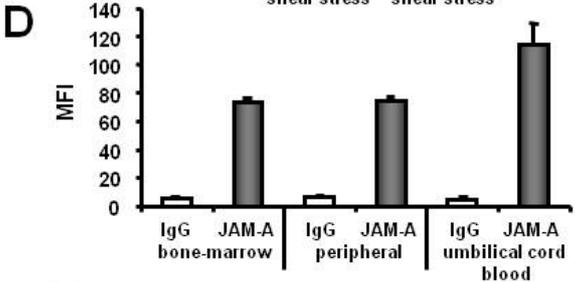
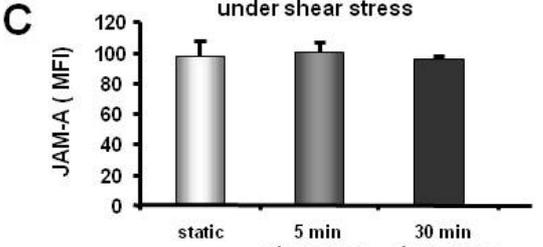
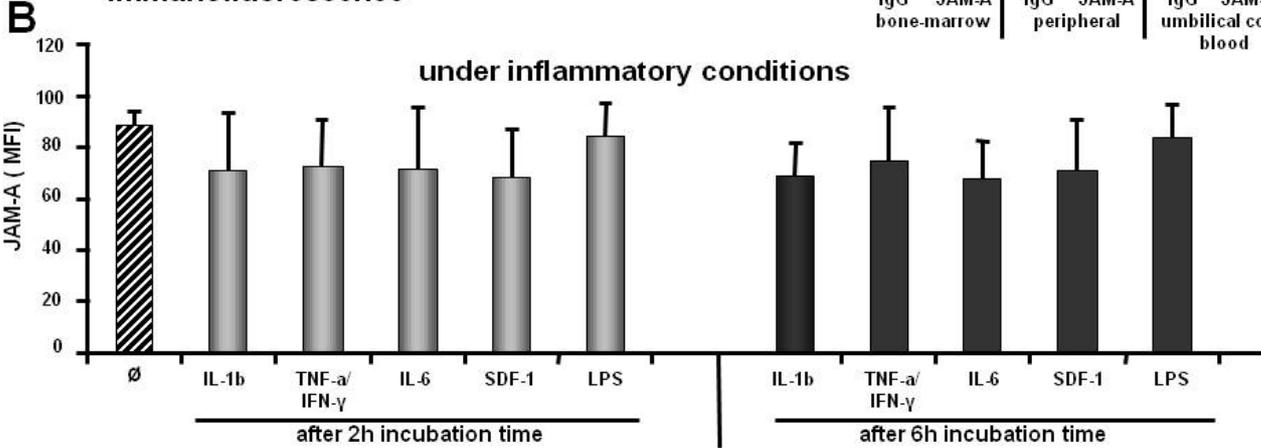
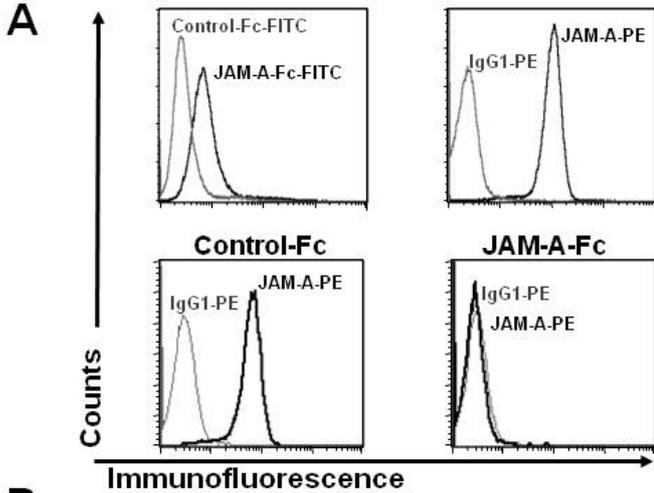
Supplemental Video and Figures

Supplemental Video. Adhesion of human CD34⁺ progenitor cells over immobilized platelets is mediated by JAM-A under high shear stress.

Supplemental Figure I. Binding properties of JAM-A-Fc on human CD34⁺ progenitor cells and expression of JAM-A under high shear stress and inflammatory conditions. A, JAM-A-Fc protein or control-Fc were conjugated with fluorescein-isocyanate (FITC) and human CD34⁺ cells were incubated with JAM-A-Fc-FITC or control-Fc-FITC and binding properties were evaluated by flow cytometry. Soluble JAM-A-Fc-FITC binding to CD34⁺ cells revealed significantly higher mean fluorescence intensity (MFI) compared to control-Fc-FITC (JAM-A-Fc-FICT vs. control-Fc-FITC: mean±SD: 10.8±2.9 vs. 4.23±0.32; P≤0.05; panel upper left). To further examine the interaction of progenitor cell-derived JAM-A with sJAM-A-Fc, progenitor cells were preincubated with sJAM-A-Fc before staining with mouse anti-human JAM-A-PE monoclonal antibody. Expression levels of JAM-A on CD34⁺ cells was analysed by FACS (panel upper right). Preincubation of human CD34⁺ cells with sJAM-A-Fc (panel lower right), but not with control-Fc (panel lower left), resulted in a significantly decreased binding of anti-JAM-A-PE antibody to human CD34⁺ cells. Representative flow cytometry histograms are shown of n=3 independent experiments. B, Expression levels of JAM-A on human CD34⁺ cells under inflammatory conditions. To analyze whether JAM-A expression changes under inflammatory conditions, CD34⁺ cells were preincubated with one of the following cytokines over a period of 2 or 6 hours: TNF-alpha (100ng/ml), INF-gamma (100ng/ml), IL-1beta (10ng/ml), IL-6 (10ng/ml), LPS (100ng/ml) or SDF-1 (100ng/ml). Subsequently cells were stained with mouse anti-human JAM-A-PE and expression levels were measured by FACS analysis. No significant changes in JAM-A surface protein expression could be observed after stimulation with any of the above cytokines. C, To analyze

whether JAM-A expression changes under shear stress, CD34⁺ cells were exposed to stirring conditions (1200rpm) for 5 or 30 min and then JAM-A expression was determined by FACS analysis. No significant changes in JAM-A protein expression was observed after 5 or 30 min of high shear stress. D, JAM-A surface expression on human CD34⁺ cells coming from bone marrow, umbilical vein cord blood and peripheral blood are depicted. Mean and SD of 3 independent experiments are shown.

Supplemental Figure I



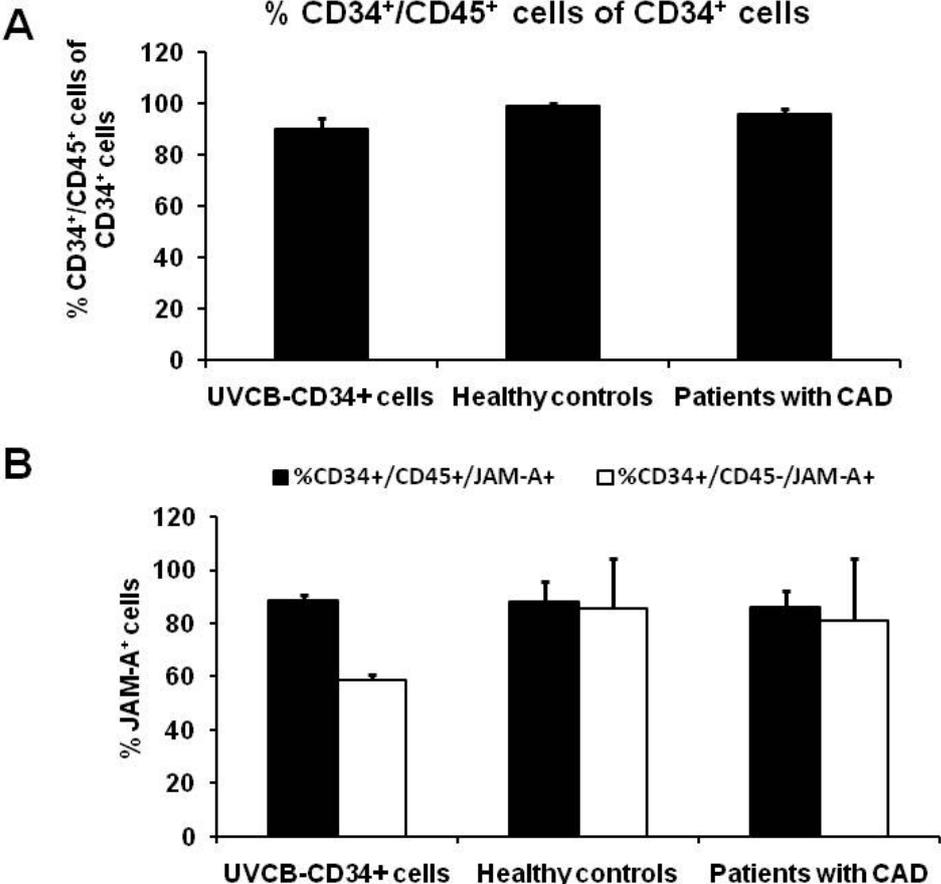
Supplemental Figure II. JAM-A expression on CD34⁺/CD45⁺ and CD34⁺/CD45⁻ cells.

The proportion of CD34⁺/CD45⁺ and CD34⁺/CD45⁻ cells, as well as the subpopulations CD34⁺/CD45⁺/JAM-A⁺ and the CD34⁺/CD45⁻/JAM-A⁺ were calculated *in vitro* and *ex vivo* in healthy subjects and patients with stable coronary artery disease by flow cytometry.

In vitro: Three independent flow cytometry experiments were conducted to evaluate the proportion of cells positive for CD45 and JAM-A using isolated human umbilical vein cord blood (UVCB) CD34⁺ cells with the help of a MACS isolation kit. Mean±SD: 89.8±4.5% of isolated CD34⁺ cells were positive for CD45. 88.3±2% of CD34⁺/CD45⁺ cells were positive for JAM-A. From the CD34⁺/CD45⁻ population, 58.6±2.02% cells were also positive for JAM-A.

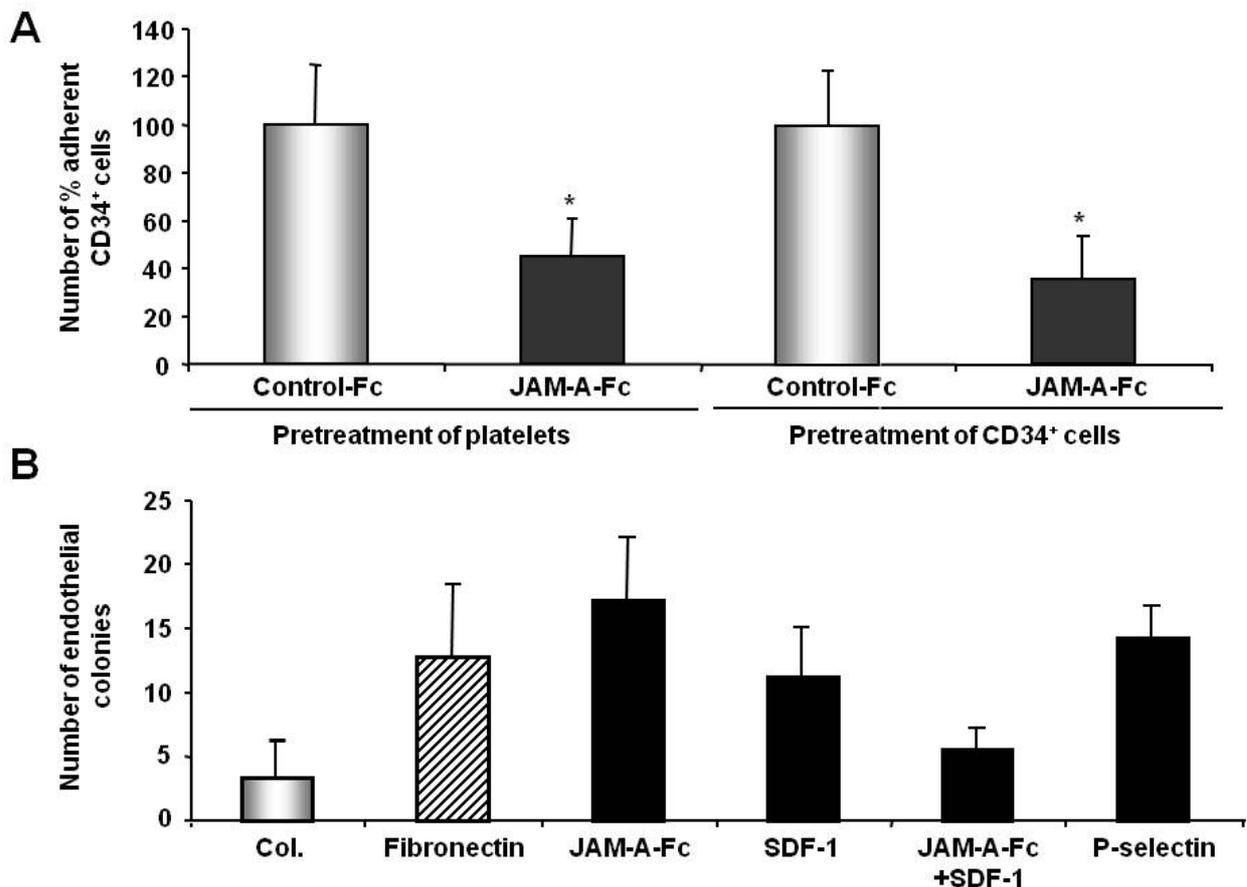
Ex vivo in healthy subjects and patients with coronary artery disease: Proportion of CD34⁺/CD45^{+/-} subpopulations was calculated by flow cytometry. Blood was taken from healthy donors (n=6) and patients with stable coronary artery disease (CAD; n=4). Peripheral blood mononuclear cells (PBMCs) were prepared by isolation over sterile Ficoll/Hypaque gradients, as previously described (Stellos K, Eur Heart J, 2009). For FACS analysis mononuclear cells were coincubated with CD34-APC, CD45-FITC, and JAM-A-PE. Healthy subjects: Mean±SD: 98.8±1.4% of CD34⁺ cells were positive for CD45. 88.4±7.2% of CD34⁺/CD45⁺ cells were positive for JAM-A. From the CD34⁺/CD45⁻ population, 85.6±18.7% cells were also positive for JAM-A. Patients with stable coronary artery disease: Mean±SD: 95.95±2.1% of CD34⁺ cells were positive for CD45. 82.2±6% of CD34⁺/CD45⁺ cells were positive for JAM-A. From the CD34⁺/CD45⁻ population, 81.04±23.3% cells were also positive for JAM-A.

Supplemental Figure II



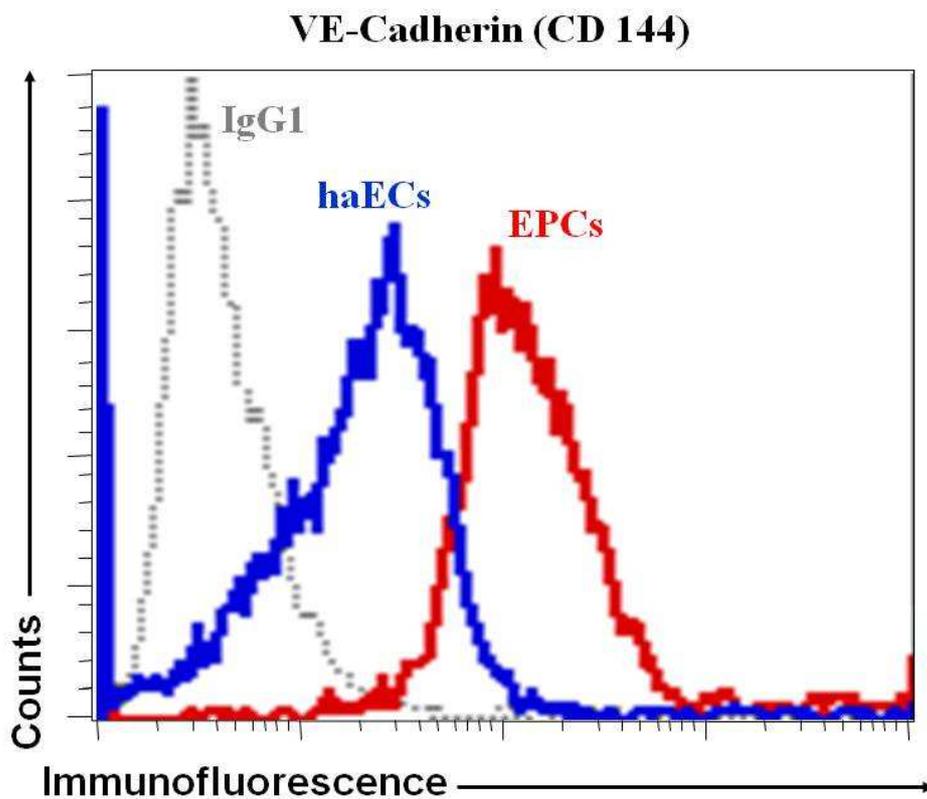
Supplemental Figure III. JAM-A-Fc in adhesion and differentiation of human CD34⁺ cells. A, Mean and SD of 8 independent static adhesion assay experiments are presented. CD34⁺ cells were allowed to adhere over immobilized platelets as described in Methods. Where indicated platelets or CD34⁺ cells were pre-incubated with sJAM-A-Fc, or control-Fc. *P \leq 0.05 vs. respective control-Fc. B, CD34⁺ cells were cultivated over immobilized fibronectin, immobilized JAM-A-Fc, immobilized SDF-1, or immobilized JAM-A-Fc with immobilized SDF-1, or immobilized P-Selectin. Endothelial progenitor cell colony forming units were counted between days 5 and 10. Mean and SD of 4 independent experiments are shown.

Supplemental Figure III



Supplemental Figure IV. VE-Cadherin expression in endothelial progenitor cells. EPCs derived from colony forming unit assays were further tested for expression of VE-Cadherin through flow cytometry. Both EPCs and mature human arterial endothelial cells were positive for VE-Cadherin. A representative FACS histogram is shown (of n=3).

Supplemental Figure IV



Supplemental Figure V. JAM-A is involved in the CD34⁺ cell-induced re-endothelialisation process *in vitro*. A and B, Primary cultures of human arterial endothelial cells (haECs) were cultivated to confluency. After a scratch-induced injury of the endothelial monolayer, CD34⁺ cells or CD34⁺ cells and platelets, were added and the co-culture was further incubated for 13.5±2.6 hours (n=5). CD34⁺ cells were stained before with rhodamine (red). Where indicated the coculture system was incubated with either control-Fc or JAM-A-Fc. Representative fluorescence images are shown. Cells in red indicate CD34⁺ cells. B, Spindle-like cell most probably coming from a CD34⁺ cell. Using rhodamine fluorescence we could detect that JAM-A-Fc mainly inhibited the adhesion of CD34⁺ cells to endothelial cells or endothelial cells and platelets and therefore less reendothelialisation capacity was observed. However, most of the cells covering the scratch lesion were coming from nearby proliferating human arterial endothelial cells indicating that CD34⁺ cells in this coculture system *in vitro* play a role in reendothelialisation through mainly paracrine way than self-differentiation to endothelial cells. At the present assay we could show that adhesion of progenitor cells on endothelial cells is perhaps the first step of a cascade resulting in proliferation and reendothelialisation of the vascular injury area *in vitro*. A possible explanation of JAM-A-Fc effect could be that it prevented the adhesion, i.e. interaction of progenitor cells with endothelial cells.

Supplemental Figure V

