Expression of Junctional Adhesion Molecule-C on the Surface of Platelets Supports Adhesion, but not Differentiation, of Human CD34⁺ Cells *in vitro*

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Key Words

Platelets • CD34⁺ Progenitor Cells • JAM-C

Abstract

Background: CD34⁺ progenitor cells play an important role in haematopoiesis and vascular homeostasis. The aim of the present study was to investigate the role of platelet-derived junctional adhesion molecule-C (JAM-C) in adhesion and differentiation of human CD34⁺ cells *in vitro*, as well as its association with platelet-derived P-selectin in patients with coronary artery disease. **Methods and Results:** Using flow cytometry we observed that JAM-C expression on the surface of washed platelets is increased after activation with thrombin receptor activating peptide-6 *in vitro* and correlated with platelet-derived P-selectin expression in patients with coronary artery disease ($r=0.326$, $P=0.007$). The role of JAM-C and its counter receptor Mac-1 in adhesion of human CD34⁺ cells over immobilized platelets was investigated by using a neutralizing soluble protein (sJAM-C-Fc) and a monoclonal antibody against JAM-C or integrin Mac-1 (CD11b/CD18). Treatment with soluble JAM-C-Fc or anti-JAM-C or anti-Mac-1, but not with control-Fc or IgG1, resulted in a significantly

decreased adhesion of human CD34⁺ cells to platelets under static conditions ($P<0.05$). In order to validate our findings under high shear stress we performed flow chamber experiments. In a similar manner, inhibiting JAM-C interaction with Mac-1 resulted in a significantly decreased adhesion of CD34⁺ cells over immobilized platelets under high shear stress ($P<0.05$). Colony forming unit assays and coculture assays revealed that inhibition of JAM-C/Mac-1 axis did not influence the platelet-mediated differentiation of CD34⁺ cells to endothelial cells or to macrophages/foam cells. **Conclusions:** Taken together a platelet-derived JAM-C supports CD34⁺ cell adhesion, a mechanism potentially involved in homing as well as domiciliation of human CD34⁺ cells.

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Introduction

Platelets are crucially involved in progenitor cell recruitment on vascular wall and differentiation into endothelial cells or macrophages [1-6]. Profound impairment in neovascularization was reported in thrombocyto-

penic mice highlighting the essential role of platelets in mobilization and incorporation of hemangiocytes into ischemic limbs *in vivo* [2]. Progenitor cell adhesion over immobilized platelets is mainly based on platelet P-selectin, stromal cell-derived factor-1 (SDF-1; CXCL12) and junctional adhesion molecule-A (JAM-A) interactions with their counter receptors P-selectin glycoprotein ligand-1 (PSGL-1), CXC-receptor 4 (CXCR4) and JAM-A/ β_2 integrin, respectively [1, 3-5, 7, 8]. Platelet-mediated differentiation of human CD34⁺ cells is mainly regulated by SDF-1/CXCR4 and JAM-A/JAM-A/LFA-1 interactions [4, 5]. Platelets are required for bone marrow-derived cell recruitment into ischemia-induced vasculature *in vivo* [9]. Secretion of platelet α -granules is crucial for bone marrow-derived cell homing and subsequent angiogenesis [9]. However, the mechanisms involved in platelet interaction with human CD34⁺ cells are not fully understood.

Human junctional adhesion molecule-C (JAM-C, also known as JAM-3) is a cell adhesion molecule expressed on a variety of cells playing a crucial role in leukocyte recruitment on vascular wall, vascular permeability and vascular inflammation [10-12]. Platelets constitutively express the junctional adhesion molecule-C (JAM-C) [11, 13]. JAM-C is expressed on the majority of human, but not mouse platelets [13]. Expression of JAM-C on the surface of platelets mediates platelet interaction with leucocytes [11] and dendritic cells [14] modulating their function. JAM-C on human platelets is a counter receptor for the β_2 integrin Mac-1 [11], which is expressed on the surface of human CD34⁺ cells [15]. However, the possible role of JAM-C/Mac-1 interaction in platelet interaction with progenitor cells has not been described so far.

The aim of the present study was to investigate the role of JAM-C in adhesion of human CD34⁺ cells over immobilized platelets under static and flow conditions and in differentiation of CD34⁺ cells into late outgrowth proangiogenic cells or macrophages/foam cells *in vitro* as well as to evaluate its association with platelet-derived P-selectin in patients with coronary artery disease.

Materials and Methods

Patients

A total of 61 consecutive patients who were referred to our hospital for symptomatic coronary artery disease and who underwent a coronary angiography were recruited into the study, as previously described [16]. All patients gave written

informed consent and the study was approved by the local institutional ethical committee. Among the study subjects, 33 patients were admitted with stable angina pectoris and 28 patients were admitted with acute coronary syndrome, as previously described [16].

Blood sample collection and whole blood flow cytometry

Arterial blood was drawn from the sheath that was introduced into the femoral artery at the beginning of coronary intervention and after administration of 2500 U of unfractionated heparin. Arterial blood was filled into 5 mL vials containing citrate phosphate dextrose adenine (CPDA) and analysed by flow cytometry according to standard methods [17-22]. Blood samples for flow cytometry (P-selectin, JAM-C) were collected before coronary intervention and analyzed immediately, as previously described [21, 22]. In brief, 10 μ L of CPDA-blood was resuspended 50:1 with phosphate-buffered saline (PBS; Invitrogen Corporation, Paisley, Scotland, UK) and was incubated for 30 min with the relevant conjugated antibodies in the dark at room temperature. After staining, the cells were fixed with 0.5% paraformaldehyde and stored at 4°C until fluorescence-activated cell sorting (FACS) was performed with a FACS-Calibur Flow Cytometer (Becton-Dickinson, Heidelberg, Germany). CD42b-FITC/PE served as a control antibody to identify the platelet population in the whole blood. Specific monoclonal antibody binding was expressed as mean fluorescence intensity (MFI) and was used as a quantitative measurement of platelet JAM-C (PE-labelled anti-human JAM-C; BD Biosciences, San Jose, USA) and P-selectin (CD62P, Immunotec, Marseille, France; clone CLB-Thromb/6; FITC) surface expression.

Isolation of platelets

Human platelets were isolated as previously described [4, 5]. 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, Germany), 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 [4, 5]. 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 15 min. 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 = 95% positive for CD34⁺ cells as determined by flow cytometry after each isolation procedure.

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 [4, 5] 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-C-Fc was performed as previously described [4, 5, 23, 24]. For static adhesion assays, isolated platelets (2x10⁸/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-C-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 control-Fc (10 µg/ml). Where indicated adherent platelets or CD34⁺ cells were pre-incubated for 30 minutes with JAM-C-Fc, anti-JAM-C (R&D Systems; Minneapolis, USA; each 10 µg/ml), anti-Mac-1 (BioLegend, San Diego, USA; 10 µg/ml). 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; clone 11711; 20 µg/ml), respectively.

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 [4, 5]. Unspecific adhesion was prevented by blocking with BSA (2%). Adherent platelets were pre-incubated for 30 min with control-Fc or JAM-C-Fc (20 µg/ml each) before perfusion was started (Fig. 1B, C). Perfusion experiments were performed at shear rates of 2 Pascal (high shear stress). To investigate CD34⁺ cell adhesion on inflammatory endothelium, haECs were immobi-

lized and cultured to confluency on coverslips. Endothelial cells were activated with 50 ng/mL TNF-α and 20 ng/mL INF-γ purchased by Peprotech Inc., New Jersey, USA). Subsequently CD34⁺ cells resuspended in IMDM medium were pre-incubated for 30 min with control-Fc, or JAM-C-Fc (20 µg/ml each) before perfusion was started (Fig. 5D, E). All experiments were recorded in real time on video-CD and evaluated off-line as previously described [4, 5].

Flow Cytometry

To evaluate JAM-C expression on isolated resting and ADP- (20 µM; Chrono-Par, Havertown, USA) or TRAP-6 (25 µM; Chrono-Par, Havertown, USA)-activated platelets one-colour flow cytometry was applied, as previously described [4, 5]. In brief, a conjugated monoclonal antibody was used to measure platelet surface expression of JAM-C (PE labelled anti-human JAM-C; 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).

To evaluate the endothelial phenotype of the colony forming unit-derived cells (late outgrowth proangiogenic cells), the expression of CD146 (anti-human MCAM/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 units derived by CD34⁺ cell culture over immobilised JAM-C, as previously described [4, 5].

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 late outgrowth proangiogenic cells using TNF-α (50 ng/ml) and INF-γ (20 ng/ml), as previously described [4, 5]. After CD54 and CD106 staining was performed, cells were proceeded to FACS analysis. Mouse IgG1-FITC and IgG1-PE were used as monoclonal immunoglobulin isotype control antibodies.

Colony Forming Unit Assay

To analyze the effect of JAM-C on CD34⁺ cell differentiation to endothelial progenitor cells, CD34⁺ cells were either seeded onto a monolayer of isolated platelets over a collagen matrix (10 µg/ml), or added onto immobilized JAM-C-Fc, as previously described [4, 5]. Immobilized platelets or control Fc served as positive or negative control, respectively. Where indicated adherent platelets were pre-incubated with control-Fc or JAM-C-Fc (each 20 µg/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 µg/ml hydrocortisone, 0.5 µg/ml vascular endothelial growth factor, 10 ng/ml basic fibroblast factor, 20 ng/ml R3 insulin-like growth factor-1, 1 µg/ml ascorbic acid (PromoCell, Heidelberg, Germany). Late outgrowth colony-forming units were counted between days 5 and 10 (number of endothelial colonies). 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. After washing, cells were analyzed on a FACS-Calibur flow cytometer (BD Biosciences, San Jose, USA).

Immunofluorescence microscopy

In order to test the differentiation of CD34⁺ cells to late outgrowth proangiogenic 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.

Co-culture assays and differentiation into macrophages/foam cells

CD34⁺ progenitor cells (50,000 cells) were cocultured with platelets (2x10⁸/ml) in 96-well plates precoated with 0.2% gelatin at 37°C and 5% CO₂ for 10 days, as previously described [3, 5]. Developing foam cells were counted in six windows by phase contrast microscopy, as previously described [4, 5].

Data presentation and statistical analysis

Data are presented as mean ± SD. For pairwise comparisons we applied a two-tailed unpaired t-test. For multiple comparisons between three or more groups we applied an ANOVA analysis test with a subsequent Scheffé *post hoc* analysis. All tests were two-tailed and statistical significance was considered for P values less than 0.05. All statistical analyses were performed using SPSS version 17 for mac.

Results

Human platelet expression of JAM-C is increased after TRAP-6 treatment and correlates with P-selectin expression in patients with coronary artery disease

JAM-C is constitutively expressed on the surface of platelets [11, 13]. Preincubation of washed platelets from healthy young subjects with thrombin-receptor activating peptide-6 (TRAP-6; 25μM), which specifically activates PAR-1 thrombin receptor, but not with adenosine diphosphate (ADP, 20μM), resulted in a significant increase of platelet JAM-C expression (P=0.05 for TRAP-6 vs. PBS; Fig. 1A, B). Platelet P-selectin (CD62P) was also co-evaluated in order to verify platelet activation in washed platelets treated with TRAP-6. Moreover, platelet-derived JAM-C correlated with platelet P-selectin expression in patients with coronary artery disease (n=61, r=0.326, P=0.007; Fig. 1C).

Fig. 1. JAM-C is expressed on human platelets and correlates with platelet-derived P-selectin in patients with coronary artery disease. A, Representative flow cytometry histograms of expression of JAM-C (left column) and P-selectin (CD62P; right column) on resting and TRAP-6-activated washed platelets from healthy young subjects. B, The mean and SD are being shown. C, After logarithmic transformation of the data, the Pearson correlation coefficient test was applied showing a significant correlation between platelet JAM-C and P-selectin expression in 61 patients with coronary artery disease (33 patients with stable angina pectoris and 28 patients with acute coronary syndrome).

Adhesion of human CD34⁺ progenitor cells over immobilized platelets is mediated through JAM-C/Mac-1 interactions under static and dynamic conditions

Binding of β_2 integrin Mac-1 ($\alpha_M\beta_2$) to JAM-C has been shown to enhance leukocyte interaction with plate-

Fig. 2. Platelet-mediated adhesion of CD34⁺ cells is facilitated by JAM-C/Mac-1 interactions under static conditions. A, Representative phase contrast images of adherent CD34⁺ cells on immobilized platelets under static conditions. B, Mean and SD of 3 independent static adhesion assay experiments are presented. CD34⁺ cells were allowed to adhere over immobilized platelets as described in Methods. Where indicated cells were pre-incubated with sJAM-C-Fc, anti-Mac-1, anti-JAM-C or respective isotype controls. Blockage of platelet-bound JAM-C or of CD34⁺ cell-bound β_2 integrin Mac-1 (CD11b) resulted in a significant decrease of adherent CD34⁺ cells over immobilized platelets. Scheffé post hoc analysis: * $P \leq 0.05$ vs. respective control IgG1 or control-Fc. C, D and E, Adhesion of human CD34⁺ cells over immobilized JAM-C-Fc vs. control-Fc (C) and in the presence of neutralizing soluble JAM-C-Fc or control-Fc (D,E) was evaluated under static conditions. * $P \leq 0.05$ vs. respective control.

lets and endothelial cells [10-12]. Since JAM-C play a central role in cell adhesion and transmigration, we investigated whether platelet-bound JAM-C play a role in recruitment of circulating CD34⁺ progenitor cells *in vitro*. At the beginning we tested the adhesion of CD34⁺ cells on immobilized platelets under static conditions. Adhesion of CD34⁺ cells onto immobilized platelets was significantly attenuated in the presence of soluble JAM-C-Fc, but not in the presence of control-Fc ($P=0.05$; Fig. 2A, B). In a similar manner, neutralizing monoclonal antibodies (mAb) to JAM-C and to β_2 integrin Mac-1 (CD11b/CD18), but not a respective isotype control IgG1, attenuated the adhesion of CD34⁺ cells onto immobilized platelets ($P=0.05$, Fig. 2A, B). Using a static adhesion assay we observed that CD34⁺ cells firmly adhered to immobilized JAM-C ($P=0.05$; Fig. 2C). Adhesion of CD34⁺ cells to immobilized JAM-C was substantially re-

duced in the presence of soluble JAM-C-Fc, but not of control-Fc ($P \leq 0.05$; Fig. 2D).

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 2,000 s⁻¹ (Fig. 3A, B). A remarkable number of perfused CD34⁺ cells quickly turned into rolling and later into firm adherent cells. Pre-incubation of immobilized platelets with soluble JAM-C-Fc, but not with control-Fc, attenuated the firm adhesion of CD34⁺ cells over platelets under high shear stress (control-Fc vs. JAM-C-Fc: mean $\pm$ SD: 90 $\pm$ 8.69 vs. 60.17 $\pm$ 11.55; $P=0.05$; $n=3$; Fig. 3A, B). No effect was observed regarding CD34⁺ cell rolling (data not shown). In order to evaluate the possible role of endothelial JAM-C in CD34⁺ cell adhesion over activated endothelial cell with TNF- α (50 ng/ml) and INF- γ (20

Fig. 3. Platelet-derived, but not endothelial-derived, JAM-C mediates adhesion of CD34⁺ cells under high shear stress. A and B, Cover slips were precoated with collagen type I and were additionally preincubated with freshly isolated platelets to achieve adherent platelet layers as described in Methods. Immobilized platelets were subsequently preincubated with sJAM-C-Fc or control-Fc. Resuspended CD34⁺ cells were perfused over these cover slips. A, Representative phase contrast images of adherent CD34⁺ cells on immobilized platelets under high shear stress. B, Mean and SD of 3 independent flow chamber experiments are shown. students' t-test: *P=0.05 vs. control-Fc. C and D, Cover slips were precoated with human arterial endothelial cells (haECs) and were additionally preincubated with TNF- α (50 ng/ml) and INF- γ (20 ng/ml) for 6h before perfusion experiments of human CD34⁺ cells were started. C, Representative phase contrast images of adherent CD34⁺ cells on activated haECs under high shear stress. D, Mean and SD of 3 independent flow chamber experiments are shown. Student's t-test: P>0.05 vs. control-Fc.

ng/ml), perfusion experiments were conducted showing that endothelial JAM-C is not involved in adhesion of human CD34⁺ cells over activated endothelial cells (P>0.05; Fig. 3C, D).

JAM-C does not influence platelet-mediated CD34⁺ cell differentiation into endothelial cells or macrophages/foam cells in vitro

To further investigate the role of JAM-C in platelet-dependent differentiation of progenitor cells into endothelial cells, CD34⁺ cells were allowed to adhere onto immobilized platelets and were cultivated in endothelial cell growth medium, as described in Methods. Where indicated sJAM-C-Fc or control-Fc were added in the cell culture system. Platelet-mediated formation of endothelial colonies of CD34⁺ cells was not altered in the presence of sJAM-C-Fc compared to control-Fc (control-Fc vs. sJAM-C-Fc: mean number of colonies $\pm$ SD: 11 $\pm$ 1.4

vs. 10.5 $\pm$ 2.1; P>0.05; n=3; Fig. 4 A, B). In order to further define the role of JAM-C on differentiation of CD34⁺ cells into endothelial cells, similar colony forming unit assays over immobilized JAM-C-Fc or control-Fc were performed. Immobilized JAM-C slightly, but significantly, promoted formation of endothelial colonies derived from CD34⁺ cells, when compared to control-Fc (immobilized control-Fc vs. immobilized JAM-C-Fc: mean number of endothelial colonies $\pm$ SD: 0.3 $\pm$ 0.6 vs. 2.3 $\pm$ 0.6; P=0.05; n=3; Fig. 4C, D).

Colony forming units were further cultivated till confluency and light microscopy photos were taken indicating the differentiation into spindle-like endothelial cells (Fig. 4E). Verification of CD34⁺ cell differentiation into endothelial cells was further performed with flow cytometry. In specific, the surface expression of CD146, PECAM-1 (CD31), CD34, and CD45 was tested on CD34⁺ cells, human arterial endothelial cells and plate-

Fig. 4. Platelet-derived JAM-C does not influence differentiation of CD34⁺ cells into endothelial cells or into macrophages/foam cells. A and B, CD34⁺ cells were cultivated over immobilized platelets (A, B) in the presence of sJAM-C-Fc or control-Fc, as described in Methods. Preincubation of immobilized platelets with sJAM-C-Fc did not alter the number of CD34⁺ cell-derived endothelial colonies compared to control-Fc. A, Representative phase contrast images are shown. B, Colony forming units were counted between days 5 and 10. Mean and SD of 3 independent experiments are shown. C and D, CD34⁺ cells were cultivated on immobilized control-Fc or immobilized JAM-C-Fc, as described in Methods. Cultivation of human CD34⁺ cells over immobilized JAM-C resulted in slightly increased endothelial colony formation. C, Representative phase contrast images are shown. D, Mean and SD of 3 independent experiments are shown. *P=0.05 vs. control-Fc. E, Endothelial colonies were cultivated till confluency. Representative phase contrast images are shown. F, Representative flow cytometry histograms (of n=3) of the surface markers CD146, CD54, CD106, CD31, CD34 and CD45 (black line) vs. isotype control IgG (grey line) expressed on immobilized JAM-C-induced late outgrowth colony-derived resting and activated endothelial cells. Two activation endothelial markers CD54 (ICAM-1) and CD106 on resting and TNF- α /INF- γ activated late outgrowth colony-derived endothelial cells were also evaluated. G and H, Co-culture experiments of platelet thrombi with human CD34⁺ cells were applied to evaluate the differentiation of the latter into macrophages/foam cells, as described in Methods. Co-incubation of the co-culture experiments with sJAM-C-Fc did not alter the number of CD34⁺ cell-derived macrophages/foam cells compared to control-Fc.

let-induced or JAM-C-induced endothelial colonies by flow cytometry, as described in Materials and Methods. When CD34⁺ cells were cultivated to form colony forming units on immobilized JAM-C, they exhibit typical en-

dothelial cell surface markers such as CD146 or CD31, but not the leukocyte marker CD45 or the stem cell marker CD34 (Fig. 4F). After 10 days the morphology of initially round shaped CD34⁺ cells on immobilized platelets or

immobilized JAM-C turned into adherent spindle-shaped cells, which were positive for vWF, as shown by immunofluorescence microscopy (data not shown). Next we analyzed whether CD34⁺ cell-derived endothelial progenitor cells can be activated to express activation-dependent surface markers such as ICAM-1 (CD54) and CD106. We observed that stimulation of CFU-derived late outgrowth proangiogenic cells with TNF- α /INF- γ cytokines resulted in an enhanced expression of both CD54 and CD106 (Fig. 4F).

In order to evaluate the differentiation of CD34⁺ cells into macrophages/foam cells *in vitro*, platelet thrombi were cultivated with CD34⁺ cells for 10 days, as previously described [25, 26]. In accordance with the CFU results, JAM-C did not influence platelet phagocytosis and foam cell formation (Fig. 4G, H).

Discussion

The major findings of the present study are: 1) JAM-C expression on platelets is increased after PAR-1 activation and correlates with P-selectin expression in patients with coronary artery disease; 2) platelet-mediated adhesion of human CD34⁺ progenitor cells is facilitated by platelet-JAM-C binding to Mac-1; 3) JAM-C does not influence the platelet-mediated differentiation of human CD34⁺ cells either into endothelial progenitor cells or to macrophages/foam cells *in vitro*.

Human CD34⁺ cells are pluripotent progenitor cells giving birth to cells of haematopoietic cell lineage [27]. Increasing evidence the last years has proven that bone marrow-derived CD34⁺ cells are mobilised after acute ischemia supporting among others angiogenesis, neurogenesis after ischemic stroke, myocardial healing after myocardial infarction and vascular regeneration after vascular injury [28-30]. Transplantation of human CD34⁺ cells is an established routine therapy after bone marrow irradiation or myeloablative anticancer chemotherapy in patients with different forms of cancer including multiple myeloma, lymphoma, acute leukemia and breast cancer [6, 31]. The last 6 years accumulating evidence supported the usage of human CD34⁺ cells in patients with ischemic or dilative cardiomyopathy and peripheral vascular disease, although the first clinical results did not reach the high expectations of the scientific community. The regenerative potential of stem and progenitor cells is a multistep process including mobilization of progenitor cells from bone marrow or peripheral niches to peripheral circulation, chemotaxis to target tissue, adhesion on vascular

wall, survival and engraftment into local tissue, differentiation into mature functional cell types and proliferation. Engraftment and homing after transplantation of CD34⁺ cells in bone marrow in patients with cancer as well as domiciliation of the mobilised CD34⁺ cells in peripheral vasculature after acute ischemia are pathophysiological phenomena sharing a common step: adhesion of CD34⁺ cells on vascular wall.

The third member of the JAMs family, JAM-C, also called JAM-3, was initially described as a novel counter-receptor on platelets for the leukocyte β_2 integrin Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) [7, 10]. Recent reports highlight the role of JAM-C in maintaining the integrity and function of myelinated peripheral nerves, monocyte and neutrophil transendothelial migration in inflammation and vascular endothelial permeability [10, 12, 33].

Platelets are crucially involved in all phases of progenitor cell recruitment on vascular wall [25, 27, 34, 35]. We and others have previously shown that murine and human progenitor cells adhere to immobilized platelets *in vitro* and are recruited to vascular wall *in vivo* involving the chemokine stromal cell-derived factor-1 binding to CXCR4 and the adhesion receptors P-selectin/P-selectin glycoprotein ligand-1, as well as binding of JAM-A to JAM-A or to LFA-1 [1-6]. At the present study we also investigated the effects of platelet-bound JAM-C on progenitor cell recruitment and differentiation. We could show for the first time that platelet-bound JAM-C partially mediates platelet-induced adhesion of human CD34⁺ cells, but no effect was observed regarding the platelet-promoted differentiation into endothelial cells or macrophages/foam cells.

Taken together, the present findings imply that platelet-derived JAM-C mediates recruitment of circulating CD34⁺ cells towards immobilized platelets, which may play a critical role in engraftment/domiciliation of circulating CD34⁺ progenitor cells on vascular wall. Further studies are needed to elucidate the *in vivo* relevance of our findings.

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