Acceleration of Collateral Development by CEACAM1 Expression on CD11b+/Gr-1+ Myeloid Cells—Brief Report

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Objective—Previously, we demonstrated the relevance for endothelial carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) expression in collateral formation. However, a proarteriogenic role for CEACAM1+ myeloid cells is unknown. Here, we investigated the contribution of CEACAM1+ myeloid cells on collateral formation.

Methods and Results—Collateral growth and vascular remodeling were analyzed in CEACAM1-competent and CEACAM1 null mice after femoral artery ligation in hindlimb ischemia. Reperfusion of the adductor muscles was evaluated by Laser Doppler measurements and microcomputed tomography imaging. In CEACAM1 null mice, poor reperfusion and reduced collateral formation were observed, accompanied by reduction in arterial diameters. Using flow cytometry, we identified an increase of the muscle-resident CD11b+/Gr-1+ population in CEACAM1 null mice only, pointing toward a CEACAM1-dependent functional deviation. Direct and reciprocal bone marrow transplantations between CEACAM1-competent and CEACAM1 null mice, and antibody-mediated depletion of the CD11b+/Gr-1+ population, confirmed the requirement of CEACAM1 expression on the CD11b+/Gr-1+ population for reestablishment of perfusion after arterial occlusion.

Conclusion—CEACAM1 expression on CD11b+/Gr-1+ myeloid cells is a prerequisite for adequate collateral formation. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: arteriogenesis ■ collateral growth ■ carcinoembryonic antigen–related cell adhesion molecule 1 ■ Gr-1 ■ myeloid cells

Vascular occlusion initiates mechanisms to reestablish local blood supply. Angiogenesis, the sprouting of capillaries, is distinguished from arteriogenesis, the growth of collateral arteries from preexisting arteriolar anastomoses. During arteriogenesis, CD11b+ myeloid cells, neutrophils, monocytes, and macrophages are recruited after endothelial cell activation by hemodynamic alterations. They extravasate, accumulate in the adventitia and perivascular space, and trigger collateral growth by liberation or secretion of growth factors and matrix metalloproteinases. Recently, Kim et al demonstrated enhanced neovascularization by local injection of adductor-derived Gr-1+CD11b+ cells in murine hindlimb ischemia.

The proangiogenic role of endothelial carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) has been described. CEACAM1 is a highly glycosylated transmembrane protein of the immunoglobulin superfamily and is expressed on endothelia, epithelia, and leukocytes, including macrophages and granulocytes. Its endothelial overexpression correlated with increased tumor angiogenesis and vessel maturation in mammary carcinogenesis, whereas in CEACAM1 null hosts, tumor vessels were instable, leaky, and facilitated pulmonary metastasis. In a model for chronic cutaneous inflammation, CEACAM1+ myeloid cells support angiogenesis and wound healing. Therefore, the present study was conducted to investigate whether CEACAM1+ myeloid cells have a proarteriogenic function.

Materials and Methods

Animal Experiments

Male mice (C57BL/6J, 8–12 weeks old) were maintained according to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines for the care and use of experimental animals.

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The Materials and Methods are detailed in the online-only Data Supplement.

Results
We observed increased collateral formation in CEACAM1-competent mice 6 days after surgical occlusion of the femoral artery (Figure 1A and 1B). Immediately after ligation, perfusion rates dropped to ≈6% in both mouse lines. Three days after occlusion, reestablishment of perfusion in the adductor muscles was observed in CEACAM1-competent mice and to a significantly lesser extent, in CEACAM1 null (Ceacam1−/−) mice (22.70±2.29% versus 15.58±1.56%, respectively; Figure 1A and 1C). On day 6, acceleration of recovery and reestablishment of perfusion in CEACAM1-competent mice over Ceacam1−/− mice became even more evident (50.62±1.37% versus 33.07±2.28%; Figure 1C). Additionally, the luminal diameters of arteries were significantly increased in the adductor muscles of CEACAM1-competent mice compared with Ceacam1−/− mice (Figure 1D and 1E).

Using direct and reverse bone marrow transplantation between CEACAM1-competent donors and Ceacam1−/− recipients, we demonstrate that transfer of CEACAM1+ BM reconstituted adequate collateral formation, irrespective of the Ceacam1 genotype of the recipient. On the contrary, introduction of CEACAM1 null grafts into wild-type (WT) mice compromised arteriogenesis, and the number of collaterals was decreased opposed to WT or Ceacam1−/− mice that received WT BM (WT+WT BM, 9.8±0.5; Ceacam1−/−+WT BM, 9.8±0.7; WT+Ceacam1−/− BM, 6.4±1.0; Figure 2A−2C). Thus, we characterized myeloid and hematopoietic cell populations in peripheral blood, BM, and adductors of the different mouse lines.

In the adductors of Ceacam1−/− mice, 7 days after ligation of the femoral artery, only the CD11b+Gr-1+ population exhibited a relative local increase (Figure 2D; Figure I in the online-only Data Supplement). Antibody-mediated depletion of this population conveyed proof that perfusion recovery is sensitive toward elimination of CEACAM1-expressing CD11b+Gr-1+ cells but not CEACAM1-negative CD11b+Gr-1+ cells (Figure 2E).

Discussion
We describe for the first time that CEACAM1+ myeloid cells are essential for adequate arteriogenesis in a model of murine hindlimb ischemia and that their presence increases the number of collateral arteries and collateral vessel calibers. Preexisting collateral diameters in naïve mice were not altered in CEACAM1 null mice, immediately after vessel occlusion, which is different from platelet endothelial cell adhesion molecule−knockout mice compared with wild-type littermates. Therefore, the immunoglobulin family members platelet endothelial cell adhesion molecule and CEACAM1 mediate their proarteriogenic effects via different mechanisms or differentially modulate the arteriogenic activity of myeloid cells. Because only transplantation of WT BM into Ceacam1−/− mice reintroduced complete recovery in the arteriogenic response, we confirmed the requirement for CEACAM1+ BM−derived cells for resolution of inflammation during wound healing and vascular remodeling. Accumulation of the CD11b+Gr-1+ population in the collateral region of Ceacam1−/− mice suggests, more probably, a negative regulatory role of CEACAM1−/CD11b+Gr-1+ cells on collateral growth or an ineffective compensatory response to their functional deficiency. Congruently, depletion

Figure 1. Carcinoembryonic antigen−related cell adhesion molecule 1 (CEACAM1) deficiency impairs collateral formation. A, Microcomputed tomography analyses, (B) numbers of collaterals, (C) perfusion recovery, (D) arterial diameters, and (E) endothelial (CD31, green) and pericyte staining (NG2, red) of arteries in the adductors of CEACAM1-competent and Ceacam1−/− mice at indicated time points (C) or 7 days after femoral artery ligation. The blue bars in A indicate the location of femoral artery ligation, whereas the arrows point to collateral vessels. Data shown include the means±SEM from at least 6 mice (A, B, and C) or 3 mice (D and E) per group. Ceacam1−/− indicates CEACAM1 null mice.
of CEACAM1+/CD11b+/Gr-1+ corrupted reestablishment of perfusion, whereas depletion of CEACAM1−/CD11b+/Gr-1− did not convey any effects. Because the monocytic CD11b+/Ly-6C+ and granulocytic CD11b+/Ly-6G+ populations did not show any deviations in their distribution or relative quantities in the different reservoirs, the CD11b+/Gr-1+ cells most likely belong to a myeloid-derived suppressor cell population. This is in agreement with a previous report stating that vessel maturation is poor in the presence of CEACAM1−CD11b+Gr-1+ cells.11 Functional characterization of CEACAM1+/CD11b+/Gr-1+ cells in vascular remodeling requires further investigation.

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Disclosures

None.

References

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Supplemental Material

Supplemental Methods.

Generation of Ceacam1⁻/⁻ mice

Ceacam1⁻/⁻ mice were generated by homologous recombination-assisted deletion of exons 1-4 and insertion of a Neo cassette using Sv129 ES cells (Genoway, Lyon, France). Backcrossing was performed for 12 generations onto C57BL/6J background. Mice were genotyped by polymerase chain reaction using a four-primer PCR using primers Ex5zuHorN1 (5'-GGT CAC AGA GTC TAG TTC TT-3') and HorN1 (5'-CTA AAG CGC ATG CTC CAG ACT GCC-3') to identify the knockout allele (430 bp), and PN5 (5'-TAC ATG AAA TYG CAC CAG TCG C-3') and PN8 (5'-CTG CCC CTG GCG CTT GGA-3') to identify the wild type allele. The construct for the targeted deletion is shown in Supplementary Figure II.

Induction of arteriogenesis and perfusion measurement

Hind limb ischemia was induced in mice by femoral artery ligation as previously described. Briefly, mice were anesthetized with 8 mg/kg body weight xylazine and 100 mg/kg ketamine intraperitoneally and the right femoral artery was ligated twice proximal to the superficial epigastric artery using 7-0 silk sutures (Ethicon, Livingston, Scotland). Before and after as well on day three and six blood flow was measured using the Laser Doppler O2C (Oxygen to See; LEA Medizintechnik, Gießen, Germany). Therefore mice were anesthetized with isoflurane inhalation and kept on a warming plate with 37 °C. The shallow well LF-2 was fixed with tape on the footpad of the mice. Blood flow was measured three times alternately on the right and left footpad as arbitrary units [AU]. Perfusion was expressed as ratio of the flow of the occluded and non-occluded hind limb.

Evaluation of collateral growth with µCT analysis

Seven days after femoral artery occlusion, mice were anesthetized, perfused first with pre-warmed saline containing heparin via the left ventricle and subsequently with 45 % barium sulfate in 9 % galantine. After hardening of the galantine on ice the legs were dissected, fixed overnight in 4 % paraformaldehyde and transferred in 70 % ethanol. Using Micro Computer Tomography (µCT; 1CT40, Scanco, Bassersdorf, Switzerland), lower limbs were scanned (55 kV/145 μA, voxel size 15 μm) and a three-dimensional reconstruction of the blood vessel microstructure was performed. Finally corkscrew collaterals were calculated blinded, without
knowledge of the type of mice investigated.

**Immunohistology of arteries in the adductor muscle**

Seven days after femoral artery ligation of WT and *Ceacam1* mice animals were euthanized and perfused with saline. The adductor muscles were dissected, placed in O.C.T. compound (Sakura, Zoeterwoude, Netherlands) and snapfrozen in liquid nitrogen. 7-µm cryosections were fixed with ice-cold acetone and stained with antibodies binding PECAM1 (rat anti-CD31, unlabeled, Acris, Herford, Germany) and NG2 (rabbit anti-NG2, unlabeled, Millipore, Schwalbach, Germany). As secondary antibodies, goat anti-rat Alexa488 (Invitrogen, Life technologies, Carlsbad, CA) and swine anti-rabbit TRITC (DAKO, Glostrup, Denmark) were used. Counterstaining of nuclei was performed with DAPI (Invitrogen). Slides were mounted with Aqua PolyMount (Polysciences, Warrington, PA) and visualised with the Leica microscope DM5000 B connected with the camera DFC 360 FX (Leica, Wetzlar, Germany). Pictures were taken with Leica software AF 6000 and statistic calculations of capillary densities or vessel diameters larger than 15µm were performed with ADOBE photoshop software. For assessment of vessel wall thickness, the CD31-positive endothelial as well NG2-positive pericyte cell layers were included. To determine vessel lumen dimension and sizes, the vessel lumens were highlighted in ADOBE photoshop software, and the diameters of the marked vessels were approximated by calculation of a size-matched circle. Only vessels with a diameter larger than 15 µm were considered, since smaller vessels are pre-capillaries and not arterioles.

**Flow cytometric analyses**

Single cell suspensions from peripheral blood, BM and the adductor muscles at the site of femoral artery ligation were analysed by flow cytometry seven days after ligation. Femurs were flushed and passed through a 70-µm cell strainer (BD, Heidelberg, Germany) to obtain single cell suspensions. Adductor muscles were resected, weighed and digested using a cocktail of 10 U/ml dispase (BD Pharmingen), 0.2 mg/ml CollIV (Worthington, Lakewood, NJ) and 0.1 mg/ml DNAse I (Roche Diagnostics, Mannheim, Germany) in DMEM for 1 hour at 37 °C with constant shaking. The suspension was passed through a 70-µm cell strainer. Unspecific binding of antibodies was blocked using Cohn-II (Sigma-Aldrich, Steinheim, Germany). The following monoclonal antibodies were used: FITC-labeled anti-CD11b (BD), PE-labeled anti-Gr-1 (BD), Alexa647-labeled anti-F4/80 (BioLegend, San Diego, CA), Alexa647-labeled anti-CEACAM1 (CC1, a kind gift from K. Holmes), PE-labeled anti-Ly-6C
(BD), PE-labeled anti-Ly-6G (BD), PE-labeled anti-CCR2 (R&D, Minneapolis, MN), Alexa647-labeled anti-CD206 (eBioscience, San Diego, CA), PE-labeled anti-sca-1 (BD), FITC-labeled anti-CD31 (BD), APC-labeled anti-VEGFR1 (R&D) and PE-labeled anti-CXCR4 (eBioscience). As controls the appropriate isotype antibodies were used. Dead cells were excluded by 7-AAD (BD) staining. Analyses were performed with a FACScalibur (BD) flow cytometer using CellQuestPro™ (BD) software. To quantitate CD11b+/Gr-1+ and CD11b+/Ly-6C+ populations, all live cells were included in the gate; in double labelings for CD11b+/Gr-1+ or CD11b+/Ly-6C+, these double positive cells were quantified in dot plot analyses as calculated by the CellQuestPro™ software.

**Bone marrow transfer**

BM chimeras were generated as previously described⁵. Briefly, WT and Ceacam1−/− mice were irradiated with 8 Gy. One day after, mice received 1x10⁷ BM cells from either WT or Ceacam1−/− mice. Transfer efficiency was checked 60 days thereafter by flow cytometric analyses. To distinguish between donor and recipient cells the two allelic forms CD45.1 and CD45.2 were used.

**Ablation of Gr-1+ cells**

To systemically deplete Gr-1+ cells in vivo, 150 µg of the monoclonal antibody RB6-8C5 (Gr-1, eBioscience) were injected intraperitoneally one day before and two and five days after femoral artery ligation. As a control, the appropriate isotype antibody was used. To monitor the depletion of Gr-1 positive cells, polymorphonuclear cells from blood smears one day after each bolus were counted.

**Statistical analyses**

Statistical analyses were carried out following F-test analyses with the Mann-Whitney U test. \( P \) values less than 0.05 were considered to be statistically significant.
**Supplemental Figure Legends.**

**Supplemental Figure I: Analyses of myeloid cells and hemangiogenic progenitor populations.** Flow cytometric analyses of BM, peripheral blood and muscles seven days after femoral artery ligation of WT and Ceacam1⁻/⁻ mice. Myeloid cells and endothelial progenitors did not reveal differential amounts or distribution of CD11b⁺/Ly-6C⁺ and CD11b⁺/Ly-6G⁺ cells (A, C, E). No quantitative differences in the presence of type 1 macrophages (identified by CD11b and CCR2) or type 2 macrophages (identified by CD11b and CD206) were found (E). CD31⁺ cells were only increased in BM (p=0.0219) (B) from Ceacam1⁻/⁻ mice but not in peripheral blood (D) or adductor muscles (F). Likewise, no spatio-quantitative differences were found for sca-1⁺ and VEGFR1⁺/CXCR4⁺ populations. Data shown include the mean ± SEM from at least 4 mice per group.

**Supplemental Figure II: Transgenic construct for the generation of CEACAM1-knockout mice.** Two SpeI restriction sites are located upstream and downstream of exons 1-5 from the murine Ceacam1 gene (upper panel). They comprise 11.9 kb. The recombinant allele with insertion of the Neo cassette and deletions of exons 1-4 can be identified by a 6.8 kb SpeI restriction fragment. Recombinant alleles were identified by primers HOR-N1 and Ex5zuHOR-N1, encompassing the overlapping fragment between the Neo cassette and exon 5 of the Ceacam1 gene. The location of the primers is indicated by green arrows (lower panel).
Supplemental Figure I
Supplemental Figure II