Human CD34⁺/KDR⁺ Cells Are Generated From Circulating CD34⁺ Cells After Immobilization on Activated Platelets


Objective—The presence of kinase-insert domain-containing receptor (KDR) on circulating CD34⁺ cells is assumed to be indicative for the potential of these cells to support vascular maintenance and repair. However, in bone marrow and in granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood, less than 0.5% of CD34⁺ cells co-express KDR. Therefore, we studied whether CD34⁺/KDR⁺ cells are generated in the peripheral circulation.

Methods and Results—Using an ex vivo flow model, we show that activated platelets enable CD34⁺ cells to home to sites of vascular injury and that upon immobilization, KDR is translocated from an endosomal compartment to the cell-surface within 15 minutes. In patients with diabetes mellitus type 2, the percentage of circulating CD34⁺ co-expressing KDR was significantly elevated compared to age-matched controls. When treated with aspirin, the patients showed a 49% reduction in the generation of CD34⁺/KDR⁺ cells, indicating that the level of circulating CD34⁺/KDR⁺ cells also relates to in vivo platelet activation.

Conclusion—Circulating CD34⁺/KDR⁺ are not mobilized from bone marrow as a predestined endothelial progenitor cell population but are mostly generated from circulating multipotent CD34⁺ cells at sites of vascular injury. Therefore, the number of circulating CD34⁺/KDR⁺ cells may serve as a marker for vascular injury. (Arterioscler Thromb Vasc Biol. 2011;31:408-415.)

Key Words: aspirin ■ blood flow ■ diabetes mellitus ■ platelets ■ vascular biology

Numerous reports have shown an inverse correlation between the number of circulating CD34⁺/kinase-insert domain-containing receptor (KDR)⁺ cells and hemodynamic and metabolic cardiovascular risk factors, such as hypertension, hyperglycemia, hypercholesterolemia, and end-stage renal disease.1 These circulating progenitor cells are considered to be a bone marrow (BM)–derived subpopulation of the CD34⁺-positive hematopoietic stem cell fraction that coexpress vascular endothelial growth factor (VEGF) receptor type-2/KDR.2 Because KDR expression is crucial for vascular development3 and isolated CD34⁺/KDR⁺ cells may contribute to reendothelialization,4,5 these cells are considered to play a role as endothelial progenitor cells in vascular maintenance and repair.

Consequently, a reduced number of circulating CD34⁺/KDR⁺ cells was proposed to be a causal factor in the development of endothelial dysfunction and atherosclerosis.6 Recently, we showed that hemostatic factors deposited at the site of an injury, such as activated platelets, platelet products, and fibrin, support the homing of human CD34⁺ cells under flow.7 The hemostatic clot provides local signals, such as P-selectin, stromal cell-derived factor-1/CXC-chemokine ligand-12 (SDF-1/CXCL12), and VEGF, which facilitated the arrest and survival of CD34⁺ cells and induced their commitment toward an endothelial cell (EC)–like phenotype.7 Given the importance of VEGF in endothelial differentiation, we investigated the dynamics of KDR expression by CD34⁺ cells that have adhered to platelet-rich sites of EC activation under conditions of flow. Surprisingly, we observed that most, if not all, CD34⁺ cells exhibit KDR expression within 15 minutes of shear exposure. Therefore, CD34⁺/KDR⁺ cells may not be mobilized from BM as a separate progenitor lineage but are predominantly generated from CD34⁺ cells at sites of vascular injury. To address this hypothesis, we conducted a detailed ex vivo study of the early phenotypic changes that CD34⁺ cells undergo after homing. We demonstrate that CD34⁺ cells can rapidly translocate KDR from intracellular storage organelles to the cell surface.

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408
in a regulated phosphatidylinositol-3-kinase (PI3K)–dependent pathway. Then, extensive analysis of the percentage of CD34+ cells coexpressing KDR in BM, umbilical cord blood (UCB), and peripheral blood (PB) after G-CSF mobilization, in PB of control subjects and in vascular injury–prone patients with diabetes mellitus type 2 (DM2), also strongly supports the in vivo generation of CD34+/KDR+ cells in the periphery. Finally, to assess the role of platelets in the peripheral generation of CD34+/KDR+ cells in patients with DM2, we investigated the effect of aspirin (300 mg/d) in a placebo-controlled crossover study.

**Methods**
The supplemental material (available online at http://atvb.ahajournals.org) provides a full description of all methods.

**Perfusions**
Perfusions of CD34+ cells were performed as previously described.7

**Subjects and Study Design**
Circulating cells were studied in different samples: human BM, UCB, PB of G-CSF–stimulated healthy donors, PB of patients with DM2, and PB of age-matched control subjects. Subjects with DM were recruited from general practitioners affiliated with Leiden University Medical Center, Leiden, the Netherlands. Details about the randomized placebo-controlled study on the effect of aspirin in subjects with DM2 have been previously published.8

**Results**

**CD34+ Cells Homed to an EC Monolayer Under Shear Express KDR on Their Surface**
Because platelet-derived VEGF signaling through KDR is one of the most likely drivers of EC differentiation3 in vasculogenesis, we studied the dynamics of KDR expression by CD34+ cells. On freshly isolated nonsheared CD34+ (CD45dim) cells, KDR expression was not detectable above isotype (IT) levels (Figure 1A). When these cells were perfused in the ex vivo injury model, nonattaching CD34+ cells (non) still lacked KDR expression, whereas recovered CD34+ cells, which had been subjected to shear for 15 minutes (att, Figure 1A) showed a significant surface expression of KDR (P=0.042). As a control, both CD34 and P-selectin glycoprotein ligand-1 (PSGL-1) expression on nonsheared cells was confirmed (Figure 1B [P<0.001] and Figure 1C [P<0.01], respectively) but was not altered on cells subjected to shear. KDR upregulation was observed on almost all recovered/sheared cells, as evident in the histogram in Figure 1F, which shows that the entire peak has shifted to the right compared with the level of IT-incubated CD34+ cells (dashed line as a reference). For comparison, histograms are shown for KDR expression of nonperfused CD34+ cells (Figure 1D) and nonattached cells after perfusion (Figure 1E). Immunohistochemical staining of the EC-adherent shear-subjected CD34+ cells (Figure 1H) confirmed homogenous expression of KDR on the cell surface (Figure 1G).

**CD34+ Cells Harbor KDR in Intracellular Pools**
After firm adhesion of CD34+ to activated ECs, KDR was detectable after only 15 minutes of shear, suggesting the release from an intracellular storage pool rather than de novo synthesis. To demonstrate this, freshly isolated CD34+ cells were fixed, permeabilized or not, and stained for the extracellular domain of KDR and CD34. IT-matched antibodies determined background levels. Indeed, KDR expression on naïve cells was only detectable after permeabilization (Figure 1I, perm, P<0.01). In contrast, CD34 was not differentially expressed on permeabilized or nonpermeabilized cells (data not shown).

**KDR Resides With CXC-Chemokine Receptor-4 (CXCR4 or SDF-receptor) in an Early Endosomal Compartment**
Human CD34+ cells harbor intracellular CXCR4 in an early endosomal compartment, which can be functionally expressed to mediate SDF-1–dependent homing.9 To investigate whether KDR resides in similar structures, we studied colocalization of both KDR and CXCR4 with the early endosome marker-1 (EEA-1) in CD34+ cells. Confocal laser scanning microscopy of permeabilized cells showed colocalization of KDR and EEA-1 (supplemental Figure, A). CXCR4 showed similar colocalization with EEA-1 in permeabilized CD34+ cells (supplemental Figure, B). Costaining for KDR and CXCR4 confirmed colocalization in the endosomal compartment (supplemental Figure, C). IT controls were negative on both permeabilized (supplemental Figure, F) and nonpermeabilized (supplemental Figure, D, E, and G) cells.

**Homed CD34+ Cells Mobilize KDR in a PI3K-Dependent Fashion**
On homing, CD34 cells are subjected to the injury-associated microenvironment, containing activated platelets and fibrin (Figure 2A and 2B). Platelet-derived factors, in particular SDF-1, can signal through CXCR4 to initiate signal transduction pathways that prepare the cell for migration and differentiation. A common denominator of these pathways is the downstream activation of PI3K.10 To investigate whether the translocation of KDR was the result of a PI3K-specific event, we visualized PI3K-dependent activation in these cells. First, adherent CD34+ cells (Figure 2D) exhibited dense areas of polymerized filamentous actin (F-actin) in the cortical cytosol, demonstrating the activated status of the cells, which is secondary to PI3K-dependent activation involving Rac1, protein kinase C-ζ, extracellular signal–regulated kinase 1/2, and WAVE2.11 In contrast, nonsheared CD34+ cells show diffuse low-level staining of F-actin (Figure 2C).

A second PI3K-dependent process that follows homing is the phosphorylation of Akt/PKB (p-Akt, protein kinase B).12 Adherent cells showed a clear signal for p-Akt (Figure 2E-2G) as opposed to the nonperfused control cells (Figure 2C). The presence of membrane-associated p-Akt at the contact sites of CD34+ cells and ECs (Figure 2F and...
2G, different angles) again confirms that the CD34+/H11001 cells are rapidly activated on firm adhesion. Next, we assessed whether the KDR translocation from the intracellular compartment to the cell surface is also dependent on PI3K activation. To that end, CD34+/H11001 cells were preincubated with the PI3K inhibitor wortmannin (WM, 100 nmol/L) before and during perfusion, adherent cells were retrieved, and KDR expression was assessed by fluorescence-activated cell sorter (FACS). Inhibition of PI3K by WM showed a significantly reduced KDR signal (Figure 1J; WM versus WM; \( P < 0.03 \)), whereas the signal obtained with the IT was not affected. The specific KDR expression, calculated by subtracting the IT signals from the KDR signals (KDR-IT), revealed a decrease of 75% (\( P < 0.01 \)).

**BM, UCB, or G-CSF–Mobilized PB Contains Low Levels of CD34+/KDR**

Thus far, we demonstrated that few freshly isolated CD34+ cells express KDR. We reasoned that CD34+/KDR+ cells might be generated in vivo at platelet-rich sites of vascular injury. This hypothesis predicts that the CD34+ cell population, freshly recruited to the peripheral circulation of healthy subjects by mobilizing agents such as G-CSF, would contain low numbers of cells coexpressing KDR. In contrast, in patients with endothelial or ischemic injury, the percentage of CD34+ cells coexpressing KDR would increase because of in vivo activation of the cells on the injured platelet-rich EC surface. To validate this hypothesis, we used a detailed quantitative FACS analysis of CD34+ and CD34+/KDR+ cells. The FACS analysis was performed using a multipara-
metric gating strategy, including fluorescent microbeads, that permits the acquisition of absolute numbers of cells per volume of blood. However, because UCB is anticoagulated with a fixed amount of 8 mL of sodium citrate, the dilution of UCB samples may differ depending on the volume collected. Therefore, in this case, we expressed the number of CD34+ cells per 10^3 CD45+ leukocytes. BM samples contained 5.14 ± 0.18% CD34+ cells/10^3 CD45+ leukocytes (Figure 3, left axis), of which 0.34% ± 0.20% were CD34+/KDR+ (right axis). UCB samples contained 3.42 ± 1.35 CD34+ cells/10^3 CD45+ leukocytes, of which only 0.23% ± 0.09% coexpressed KDR. Next, we assessed that PB of control subjects (n = 15) contained 0.30 ± 0.03 CD34+ cells/10^3 leukocytes, of which 0.72% ± 0.19% were CD34+/KDR+. As expected, the number of CD34+ cells in G-CSF–mobilized PB of healthy subjects (n = 4) was largely increased, amounting to 2.27 ± 0.52 CD34+ cells/10^3 CD45+ leukocytes, which accounts for a 7.5-fold increase of CD34+ cells. Of these cells, only 0.19% ± 0.18% coexpressed KDR. Data provided as mean ± SEM.

Figure 3. The percentage of circulating CD34+/KDR+ cells per CD34+ cells progresses with diabetic conditions associated with vascular injury and platelet activation. The number of CD34+ cells (left axis, white bars) was measured (expressed as number per 10^3 CD45+ leukocytes) by FACS in BM, UCB, and PB after G-CSF treatment (cntrls+ G-CSF), in PB from patients with DM2, and in age-matched controls (cntrls). The percentage of CD34+/KDR+ cells per CD34+ cell fraction was calculated (right axis, gray bars).

Percentage of Circulating CD34+ Cells Coexpressing KDR Is Elevated in Patients With DM2

Patients with DM2 may well display a vascular phenotype that is uniquely suited to study the correlation between vascular injury and circulating CD34+/KDR+ cells. The pathophysiological mechanisms involved in this chronic vascular disease include EC dysfunction, ongoing vascular injury, and increased platelet aggregability. To investigate whether the number of circulating CD34+/KDR+ cells in patients at risk for chronic vascular disease relates to endothelial injury and platelet activation, we performed a randomized, double-blind, placebo-controlled crossover trial in patients with DM2. All subjects received placebo and aspirin (300 mg/d; n = 20) for 6 weeks. The control subjects were originally included for a study on metabolic syndrome, for which hypertension, waist circumference, fasting blood glucose, triglyceride, and high-density lipoprotein cholesterol levels were defining variables. However, the control subjects only had 1 or 2 of these traits, whereas 3 or more traits are needed to define them as positive for metabolic syndrome. Therefore, these subjects are considered excellent controls for the patients with diabetes, who had comparable ages and anthropometric indexes.

Subject characteristics are summarized in the Table, and statistical differences between the groups are shown with an asterisk. There were no differences in age, sex, and blood pressure (either systolic or diastolic). Patients with DM2 had a higher body mass index and waist circumference and elevated levels of fasting blood glucose, homeostasis model assessment of insulin resistance, glycohemoglobin, and C-reactive protein, indicating a low-grade inflammatory state. None of the statistical differences between groups disappeared when we restricted the analysis to male subjects with DM2 only (data not shown), indicating that these differences are consistent with the diabetic profile. Circulating cells were
Plasma Levels of Soluble P-Selectin

To further support the role of platelets in the generation of CD34^+KDR^+ in vivo, we compared the levels of soluble P-selectin (sP-sel) as a marker for in vivo platelet activation^17 in the placebo and aspirin-treated patients. Aspirin treatment did not affect the average values of sP-sel between the placebo and aspirin-treated groups (51.1 ± 4.9 versus 54.3 ± 6.0 ng/mL, respectively; P = 0.437; Figure 4C). However, when looking at the subjects individually, 50% of the patients showed a reduction in sP-sel, whereas the other half showed increased sP-sel levels, confirming previous observations that the sensitivity for aspirin and, thus, the inhibition of in vivo basal platelet reactivity, in patients with DM2 is highly subject specific. To investigate the relation between effective platelet aggregation inhibition and circulating cells, we calculated the increase or decrease (Δ values) of sP-sel, CD34^+, and CD34^+KDR^+ cells for the individual patients and performed a linear regression analysis. As expected, changes in the number of CD34^+ cells did not correlate with changes in the level of sP-sel (P = 0.206, r^2 = 0.105, data not shown), indicating that CD34^+ cell numbers are not related to the degree of in vivo platelet activation. In contrast, Δ levels of CD34^+KDR^+ cells were strongly correlated with Δ levels of sP-sel after aspirin treatment (Figure 4D, P < 0.001, r^2 = 0.589). Taken together, elevated platelet activation in patients with DM2 is strongly correlated with an increased number of circulating CD34^+KDR^+ cells. Conversely, when platelet activation is inhibited, as in the “aspirin-responsive” patients with DM2, the number of circulating CD34^+KDR^+ cells is reduced accordingly.

**Discussion**

Our study supports a novel concept for the origin of circulating CD34^+KDR^+ cells (Figure 5). In contrast to concepts that CD34^+KDR^+ cells are mobilized from the BM as a unique predefined lineage with an endothelial fate, our data suggest that these cells more likely reflect the plasticity of CD34^+ cells and their capacity to respond to environmental cues. First, we determined that after G-CSF treatment, only

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**Table. Subject Characteristics at Baseline***

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n = 15)</th>
<th>Patients With DM2 (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59.3 ± 1.6</td>
<td>54.5 ± 2.1</td>
</tr>
<tr>
<td>Female sex††</td>
<td>0 (0)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.9 ± 0.4</td>
<td>31.5 ± 1.2</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>101.1 ± 1</td>
<td>106 ± 2§</td>
</tr>
<tr>
<td>Tension, mm Hg</td>
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<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>146 ± 4</td>
<td>150 ± 4</td>
</tr>
<tr>
<td>Diastolic</td>
<td>85 ± 2</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>Smoking††</td>
<td>0 (0)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>MetS†</td>
<td>0 (0)</td>
<td>18 (90)¶</td>
</tr>
<tr>
<td>Fasting blood glucose, mmol/L</td>
<td>5.0 ± 0.1</td>
<td>7.7 ± 0.2¶</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>4.9 ± 0.1</td>
<td>5.9 ± 0.2¶</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.1 ± 0.1</td>
<td>2.3 ± 0.4¶</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.7 ± 0.2</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.3 ± 0.1</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.5 ± 0.4</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>94.3 ± 3.3</td>
<td>85.1 ± 3.2</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.1 ± 0.2</td>
<td>8.9 ± 2.1¶</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; CRP, C-reactive protein; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; MetS, metabolic syndrome.

*Data are given as mean ± SEM unless otherwise indicated.
†P < 0.01. §P < 0.05. ¶P < 0.001.

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**Figure 4. Assessment of absolute and Δ values of circulating cells and sP-sel. A through C, Absolute numbers are shown of CD34^+ cells (number/mL) (A), CD34^+KDR^+ cells (number/mL) (B), or sP-sel (ng/mL) (C) in the placebo or aspirin-treated period. Mean ± SEM levels are presented with bars, and results per subject are shown, connecting placebo- with aspirin-obtained values. The probability values are calculated comparing placebo with aspirin. D, Linear regression is shown between Δ values of sP-sel and CD34^+KDR^+ cells.**
CD34+/KDR+ cell fraction does not increase as a result of mobilization from BM but may rather be generated in the periphery from CD34+ cells immobilized at platelet-rich sites of vascular injury.

We demonstrated that the absolute number of CD34+ cells/per 10^3 CD45+ leukocytes was significantly reduced in patients with DM2 compared with controls (Figure 3). This is in line with many studies that have consistently demonstrated a reduction of these cells in patients with DM2 and in other patient groups with an increased risk for chronic vascular disease.27

In contrast, we show that the percentage of CD34+ cells coexpressing KDR is increased in patients with DM2; and this paralleled absolute numbers of these cells (data not shown). This seems contradictory to published results; however, in many reports, patients received aspirin27 or platelet aggregation inhibitors,28 and this may explain why these articles document lower amounts of CD34+/KDR+ cells in their patient cohorts.

The link between CD34+/KDR+ cell numbers and platelet activation in the patients with DM2 was only revealed when we distinguished patients who responded to aspirin from nonresponders. This may be explained by the fact that, in patients with coronary artery disease, diabetic patients have a high prevalence of laboratory-defined aspirin resistance.18

Our ex vivo studies revealed that surface expression of KDR is already detectable within 15 minutes after adhesion to platelet-rich EC surfaces. This short time span indicates that KDR mobilization is not mediated by increased transcription, as was proposed in another model in which CD34+ cells were subjected to shear for 24 hours.29 Indeed, we detected an intracellular pool of KDR, associated with an EEA compartment, in which CXCR4 has also been localized.30 Like other growth factor receptors, KDR30 and CXCR43 are subject to a low constitutive rate of endocytosis. This internalization involves the formation of endocytic vesicles from clathrin-coated pits that subsequently fuse with early (EEA-1–positive) endosomes, from which the receptors may serve 2 important purposes. First, it desensitizes the cell to avoid premature activation by growth factors. In particular, for CD34+ stem cells, this may be essential to maintain plasticity during their trafficking from and toward BM niches. Second, when cells are recruited to sites of inflammation or tissue repair, targeted recycling is essential to generate polarity of the cells. Active recycling of KDR and CXCR4 to the leading edge may sensitize the cell to VEGF- and SDF-1–induced chemotactic signals.32 VEGF and SDF-1 play important roles as chemoattractants in progenitor cell mobilization and homing, and both factors may also serve as EC differentiation factors.33,34 Because VEGF and SDF-1 are actively released from activated platelets, the rapid upregulation of KDR and CXCR4 by CD34+ cells that have adhered to platelet-rich sites of EC injury may well further stimulate these cells to enter an EC fate.7 The fact that WM inhibited KDR expression indicates that recycling of this receptor is the result of PI3K-dependent signaling pathway(s) that follow on

0.19%±0.18% of the mobilized peripheral CD34+ cells coexpress KDR. This level of expression is similar to that of the CD34+ population in BM, which amounts to 0.34%±0.19% and confirms that during mobilization, little “activation” of these cells occurs. Second, we demonstrated that most, if not all, CD34+ cells can rapidly upregulate KDR expression once recruited to platelet- and fibrin-rich EC surfaces. Third, in patients with DM2 who are known to display ongoing endothelial injury with associated platelet aggregation, we observed a 5-fold increase in the percentage of circulating CD34+ cells coexpressing KDR compared with control subjects. Moreover, treatment with aspirin (300 mg/d) reduced the number of circulating CD34+/KDR+ cells by 47% in these patients, concomitant with markers of systemic platelet activation. Together, these data support our hypothesis that, also in vivo, CD34+ cells are generated from BM niches. Second, when cells are recruited to sites of vascular injury, we propose that most of the CD34+/KDR+ cells will remain associated with the vascular wall to participate in vascular repair (Figure 5; arrow a), whereas a fraction of these cells may reenter the circulation from the injured vascular wall secondary to shear or mechanical stimuli (Figure 5, arrow b), thereby reflecting the rate of conversion of CD34+ cells at sites of vascular injury. We propose that most of the CD34+/KDR+ cells will remain associated with the vascular wall to participate in vascular repair (Figure 5; arrow a), whereas a fraction of these cells may reenter the circulation from the injured vascular wall secondary to shear or mechanical stimuli (Figure 5, arrow b), thereby reflecting the rate of conversion of CD34+ cells at sites of vascular injury. Thus, the number of CD34+/KDR+ cells may provide an index of systemic vascular injury.

Interestingly, in conditions of acute vascular ischemia and EC injury, an increase in the number of circulating CD34+/KDR+ cells has been observed (eg, thermal injury,19 radiation-induced endothelial injury,20 sepsis,21 acute ischemic stroke,22 acute myocardial infarction,23 and even brief exposure to second-hand smoke24). Some of these studies reported that the acute elevation of circulating CD34+/KDR+ cells was associated with a concomitant increase in levels of mobilizing factors, such as VEGF and SDF-1, suggesting a causal relationship.21,25 Furthermore, markers of endothelial damage/dysfunction (ie, von Willebrand factor and soluble E-selectin) and the platelet marker sP-selectin26 were reported to be elevated, suggesting that these acute conditions are associated with in vivo vascular injury and platelet activation. These data are in agreement with our concept that the
signaling” could be one mechanism responsible for the exposure of the adherent CD34

If, indeed, CD34+/KDR+ cells represent the provasculogenic component in these therapies, the potential of the CD34+ cells may have been underestimated because the CD34+/KDR+ cell fraction and the CD34+/KDR- cells, which compose >99% of the circulating stem cells in healthy individuals, may participate in a provasculogenic response.

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Disclosures
None.

References


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Methods

Isolation of CD34+ cells from cord blood

Umbilical cord blood (UCB) was collected immediately after delivery with written approval in bags containing citrate, phosphate and dextrose (Maco Pharma, Tourcoing, France). The mononuclear cell fraction was isolated by gradient separation (Ficoll, Amersham, The Netherlands). CD34+ cell isolation was performed using the CD34 progenitor cell isolation kit (Miltenyi, Biotech, The Netherlands). Purity of the cells accounted for ≥ 95% as assessed by fluorescence activated cell sorter (FACS) analysis (BD Biosciences), using FITC-labeled antibody against CD34. CD34+ cells were kept at 4°C at a stock concentration of 10^7/mL in RPMI containing 1% human serum albumin (HSA, Sigma Aldrich, Germany) until they were used for perfusion experiments.

Perfusions

Perfusions of CD34+ cells were performed as previously described.1 In short, a confluent monolayer of EC was made procoagulant after 6 h stimulation with recombinant tumor necrosis factor-α (10 ng/mL, Boehringer Ingelheim, Germany) and the tissue factor-expressing EC were pre-perfused with platelet rich plasma prepared from low molecular weight heparin anti-coagulated whole blood, leading to the deposition of fibrin-rich platelet thrombi. This ex vivo perfusion model, mimicking a vascular injury, was subsequently perfused with CD34+ cells, isolated from fresh UCB at a shear rate of 1 dyne/cm^2. Adhered CD34+ cells were then subjected to an
increased shear rate of 2 dyne/cm² for 15 minutes. The shear-subjected, adherent CD34⁺ cells were either fixed with 0.5% glutaraldehyde for 10 minutes or the adherent cells were detached and recovered using flow-buffer containing 5 mmol/L EDTA.

**Cell characterization and enumeration by FACS**

Freshly isolated CD34⁺ cells, not subjected to flow, or CD34⁺ cells either non-attached during perfusion or attached and recovered after perfusion, were incubated with blocking-buffer (phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 2% fetal calf serum (FCS); 30 min at 4°C) and then incubated with an isotype-matched phycoerythrin (PE)-, peridinin chlorophyll protein-cyanine dye (PerCP-Cy5)- or fluorescein isothiocyanate (FITC)-labeled mouse immunoglobulin (mlgG) or specific antibodies: PE-labeled mlgG directed against the extracellular domain of KDR (R&D Systems, Abingdon, UK), Pacific Blue-labeled mlgG against CD45 (Dakocytomation, Glostrup, Denmark), PerCP-Cy5-labeled mlgG against CD34 (BD Biosciences, Breda, The Netherlands) or FITC-labeled mlgG against PSGL-1 (KPL-1, St Cruz, CA, USA). FACS-analysis was performed using a multi-parametric gating strategy based on the International Society of Hematotherapy and Graft Engineering.² During FACS-analysis, progenitor cells were characterized as events located in the lymphocyte gate (based on forward- and side scatter patterns) and expressing CD34⁺ and CD45⁻. Of these gated cells KDR-, CD34- and PSGL-1-expression was determined and expressed as MFI. Per experiment, triplicate perfusions were performed and experiments were performed in duplicate. In a separate experiment, freshly isolated CD34⁺ cells were fixed in 3.7% paraformaldehyde (PFA) for 10 minutes at 4°C, washed in FACS-buffer (PBS
containing 1% BSA and 0.05% Na-azide) and half of these cells were permeabilized using 0.5% Triton X-100 dissolved in FACS-buffer. These cells were incubated with blocking-buffer and subsequently incubated with pure mIgG directed against the extracellular domain of KDR (Reliatech), FITC-labeled mIgG against CD34 (BD Biosciences) or isotype-matched controls. Binding of pure mIgG was detected with Alexa 647-labeled anti-mIgG antibody (Molecular Probes, Leiden, The Netherlands). During FACS-analysis, cells were selected in the lymphocyte gate and analyzed for expression of CD34 and KDR (expressed as MFI). For the inhibition of PI3K, CD34+ cells were incubated with wortmannin (100 nM for 30 min at 37°C) prior to perfusion. Attached cells were harvested and stained for FACS-analysis parallel to non-treated CD34+ cells. A detailed description of the FACS-based enumeration of circulating CD34+ and CD34+/KDR+ cells has been published by van der Klaauw et al.3 For the calculation of the number of CD34+ cells per 10^3 CD45pos-leukocytes, the events of CD34+ cells was devided by the events of CD45pos-leukocytes, multiplied by 1000.

Confocal Laser Scanning Microscopy

For the detection of surface-expressed KDR on shear-subjected CD34+ cells, glass coverslips containing EC-adherent CD34+ cells were fixed with 0.5% glutaraldehyde, washed with PBS, incubated with blocking-buffer and subsequently incubated with mIgG against the extracellular domain of KDR (Reliatech). Binding of this primary antibody was detected with Alexa 647-labeled anti-mIgG and fluorescent micrographs were made, combined with Nomarski projection using CSLM (LSM 510, Zeiss, Germany). For the detection of F-actin and p-Akt, glutaraldehyde-fixed coverslips, containing EC-adherent CD34+ cells, were incubated with Triton to permeabilize the cells (as described in the section “Cell characterization and
enumeration by FACS”), and subsequently incubated with blocking-buffer, phalloidin-
methylrhodamine (Sigma-Aldrich, Germany) and rabbit-IgG (rIgG) directed against p-
Akt (Cell Signalling, Bioké, The Netherlands). Binding of the primary antibody was
detected with Alexa-647 labeled anti-rIgG (Molecular Probes, Leiden, The
Netherlands). For staining of early-endosome-associated proteins, freshly isolated
CD34+ cells were fixed in 3.7% PFA and either or not permeabilized with Triton. Cells
were blocked and incubated with mlgG against KDR (Reliatech) or mlgG against the
CXC chemokine receptor-4 (CXCR4; R&D Systems), both in combination with rIgG
against early endosome antigen-1 (EEA-1, kindly provided by Dr. S. Urbé,
Physiological Laboratory, University of Liverpool, UK). Binding of primary antibodies
was detected with secondary antibodies against mlgG labeled with Alexa-647 and
anti-rIgG labeled with Alexa-568 (both from Molecular Probes). Fluorescent images
were combined with Nomarski projection using CLSM.

**Scanning electron microscopy**

After perfusion, samples were dehydrated, critical-point dried (Bal-Tec CPD 030,
Liechtenstein) and coated with gold/palladium using a sputtercoater (EMI-TEC
K500X, Berlin, Germany). Samples were evaluated at 5 kV using a JEOL-JSM-6700F
scanning electron microscope (Jeol Ltd, Japan).

**Subjects and study design**

Circulating cells were studied in different samples: human BM, UCB, PB of G-CSF-
stimulated healthy donors, PB of diabetes mellitus type 2 (DM) patients and PB of
age-matched control subjects. BM and UCB was obtained from subjects with
informed consent. G-CSF mobilization was performed in healthy subjects, prepared
to donate stem cells for allogeneic peripheral blood stem cell transplantation. Stem cell donors entered the study according to a protocol approved by the institutional medical ethics committee. Stem cell donors received a single dose of 10 µg/kg/day recombinant human G-CSF (Filgrastim; Amgen, Thousand Oaks, CA) one and five days before large-volume leukapheresis. PB samples were assayed for numbers of CD34⁺- and CD34⁺/KDR⁺-cells one day before leukapheresis.

Subjects with DM were recruited from general practitioners affiliated to the Leiden University Medical Center (The Netherlands). Details about the randomized placebo-controlled study on the effect of aspirin in subjects with type 2 diabetes have been published previously.⁴ All subjects gave written informed consent and the study was approved by the institutional review committee and performed in accordance with the Declaration of Helsinki. The aspirin-study had a prospective, randomized, placebo-controlled, double-blind, crossover design. All subjects (n=20) received one period placebo and the other period aspirin (300 mg/day). The first treatment period with aspirin or placebo for 6 weeks was followed by a washout period of 4 weeks. Thereafter, those assigned to placebo in the first period received aspirin for 6 weeks and those assigned to aspirin received placebo for additional 6 weeks. At each visit, EDTA-anticoagulated PB samples were drawn from antecubital veins.

The age-matched controls comprised male subjects above 50 years of age with mild visceral obesity, but without metabolic syndrome (MetS),⁵ type 2 diabetes mellitus, manifest cardiovascular disease, use of statins or non-steroidal anti-inflammatory drugs, current smoking, familial history of premature cardiovascular disease and severe obesity (BMI>40 kg/m²). All control subjects gave informed consent and the study complied with the Declaration of Helsinki.
**Measurement of sP-sel and VEGF.**

sP-Sel and VEGF was measured in plasma using commercially available ELISA kits (both R&D Systems), according to the instructions of the manufacturers. Measurements were performed in duplicate and average values were used.

**Statistical Analysis**

Data are expressed as mean ± standard error of the mean (SEM). Statistical one-way analysis was performed by analysis of variance (ANOVA), unpaired t-test analysis when the distribution was parametric or Mann-Whitney analysis when the distribution was non-parametric, using the computer program GraphPad Prism 4.0 for Windows. A value of P<0.05 indicated statistical significance.

Effects of aspirin versus placebo on circulating cells were estimated using paired samples t-tests, since the differences in circulating progenitor cells between treatment with aspirin and placebo were normally distributed (data not shown). Correlations with delta sP-sel and delta VEGF were calculated using linear regression analysis.
Reference List


**Supplemental Figure I.** KDR expressed by CD34⁺ cells is associated with an early endosomal compartment. To study co-localization of KDR with an early endosome compartment, CD34⁺ cells were fixed and either permeabilized (no asterix) or non-permeabilized (asterix). Cells were stained for (A and D) KDR (green) and EEA-1 (red), for (B and E) CXCR4 (green) and EEA-1 (red) or for (C) CXCR4 (green) and KDR (red). Background levels were visualized with (F and G) mIgG (green) and rIgG (red). Fluorescent imaging was combined with Nomarski projections, leading to co-localizing signals in yellow. Scale bars represent 5 µm.