Vascular Endothelial Growth Factor-A–Induced Chemotaxis of Monocytes Is Attenuated in Patients With Diabetes Mellitus
A Potential Predictor for the Individual Capacity to Develop Collaterals

Johannes Waltenberger, MD; Juliane Lange, MD; Andrea Kranz, PhD

Background—Vascular endothelial growth factor-A (VEGF-A) acts on endothelial cells and monocytes, 2 cell types that participate in the angiogenic and arteriogenic process in vivo. Thus far, it has not been possible to identify differences in individual responses to VEGF-A stimulation because of the lack of an ex vivo assay.

Methods and Results—We report a chemotaxis assay using isolated monocytes from individual diabetic patients and from healthy, age-matched volunteers. The chemotactic response of individual monocyte preparations to VEGF-A, as mediated via Flt-1, was quantitatively assessed using a modified Boyden chamber. Although the migration of monocytes from healthy volunteers could be stimulated with VEGF-A (1 ng/mL) to a median of 148.4% of the control value (25th and 75th percentiles, 136% and 170%), monocytes from diabetic patients could not be stimulated with VEGF-A (median, 91.1% of unstimulated controls; 25th and 75th percentiles, 83% and 98%; \( P < 0.0001 \)). In contrast, the response of monocytes to the chemoattractant formylMetLeuPhe remained intact in diabetic patients. The VEGF-A–inducible kinase activity of Flt-1, as assessed by in vitro kinase assays, remained intact in monocytes from diabetic patients. Moreover, the serum level of VEGF-A, as assessed by immunoradiometric assay, was significantly elevated in diabetic patients.

Conclusions—The cellular response of monocytes to VEGF-A is attenuated in diabetic patients because of a downstream signal transduction defect. These data suggest that monocytes are important in arteriogenesis and that their ability to migrate might be critical to the arteriogenic response. Thus, we resolved a fundamental mechanism involved in the problem of impaired collateral formation in diabetic patients. (Circulation. 2000;102:185-190.)

Key Words: collateral circulation • diabetes mellitus • signal transduction • cell movement • monocytes • endothelial growth factors
past, should no longer be used to describe the stimulation of true collateral growth; the term “therapeutic arteriogenesis” seems to be more appropriate.

The cellular effects of VEGF-A are mediated via 2 distinct receptor tyrosine-kinases\(^\text{11}\) called Flt-1 (Fms-like tyrosine kinase [VEGFR1]) and KDR (kinase-insert domain-containing receptor [VEGFR2]). In previous studies on the function of KDR, we found in vitro evidence for the regulation of receptor activity under pathological conditions such as hypoxia.

Besides endothelial cells, monocytes specifically respond to VEGF-A. One of the 2 VEGF-receptors (Flt-1) is present on the surface of monocytes and mediates the chemotactic response to VEGF-A and tissue factor induction.\(^\text{18}\) What makes monocytes especially attractive is the fact that they represent the only cell type in the body that carries receptors for VEGF and, at the same time, can be obtained from individual patients for functional analysis of the VEGF receptor system. Moreover, monocytes play an important role in the angiogenic process and during collateral growth/arteriogenesis.\(^\text{19,20}\) So far, there was no experimental approach to judge the individual response to VEGF-A stimulation in a defined patient. Given the possibility of obtaining such data, this would be an extremely important piece of information in the context of stimulating collateral formation.

On the basis of these ideas, we developed and established an assay in which peripheral blood monocytes can be isolated from individuals and tested for their chemotactic response to VEGF-A in a modified Boyden chamber. We found that the specific and strong VEGF-A–induced response seen in healthy individuals is completely attenuated in patients with diabetes mellitus. We conclude that monocytes can be used to determine VEGF receptor–mediated cellular function in healthy and diseased individuals. Given the crucial role of monocytes in the development of functional collaterals, the impaired chemotactic response of monocytes to VEGF-A in diabetic patients seems to predict a reduced ability to grow collaterals. The analysis of the VEGF-A–induced migration of monocytes represents the first attempt to study the function of the VEGF system in healthy and diseased individuals.

**Methods**

**Characterization of Patients and Healthy Volunteers**

Patients with diabetes mellitus (n = 16; 6 men and 10 women) and a mean age of 68.3 ± 10.4 years were studied; this group included both patients with insulin-dependent (n = 10) and non–insulin-dependent diabetes mellitus (n = 6). Patients with underlying inflammatory or malignant disease and those who smoked were not included in this study. The glycosylated fraction of the major component of adult hemoglobin (HbA1c) was elevated in all patients; this elevation ranged from 7.0% to 11.2%, with a median of 8.5% (25th and 75th percentiles, 7.9% and 9.3%, respectively). Healthy volunteers (n = 14; 5 men and 9 women) were included in our study as a control group. Their mean age was 56.4 ± 4.0 years. Informed consent was obtained from patients and healthy volunteers according to the requirements of the local ethical committee.

**Isolation of Monocytes from Peripheral Venous Blood**

Monocytes were isolated from 60 mL of heparinized venous blood samples using a slightly modified version of the method of Denholm and Wolber.\(^\text{21}\) In brief, density centrifugation was performed using the Ficoll separation solution with a density of 1.077 g/mL (Biochrom) to isolate mononuclear cells. In a second round of centrifugation, monocytes were enriched using Percoll separation solution with a density of 1.139 g/mL (Sigma) before washing and resuspending the cells in DMEM (Biochrom). The purity of the extracted monocytes was up to 93%, as determined by analysis with a fluorescence-activated cell sorter using an antibody recognizing CD14 (M14-FITC, Coulter Electronics). The vitality of the isolated monocytes was assessed by trypan blue exclusion; it was always >90%.

**Monocyte Migration**

Monocyte chemotaxis was quantitated using a modified 48-well Boyden chamber (Nuclepore) and polycarbonate membranes with a pore diameter of 5 μm (Nuclepore). Monocytes were seeded in a concentration of 5 × 10⁵ cells/mL in DMEM and allowed to migrate for a total of 3 hours in the humidified incubator (37°C; 5% CO₂). Adherent cells on the filter membrane were fixed in 99% ethanol for 10 minutes and stained using Giemsa dye before scraping off cells at the upper side of the filter membrane. For a quantitative assessment of migrated cells, a total of 15 high power fields from 3 different wells (5 each) were counted. Cell migration was stimulated with either VEGF-A165 (0.1 to 10 ng/mL; this was kindly provided by Denis Gospodarowicz, Chiron, Emeryville, Calif) or formylMetLeuPhe (fMLP, 10⁻⁸ mol/L; Sigma).

**Immunoprecipitation and In Vitro Kinase Assay**

Isolated monocytes were preincubated for 5 minutes with 100 μmol/L Na₃VO₃ to inhibit phosphatase activity. Cells were stimulated for 3 minutes at 37°C with 50 ng/mL VEGF. After washing with ice-cold PBS containing 100 μmol/L Na₃VO₃, cells were solubilized in a lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl [pH 7.4], 1% CHAPS [Sigma], 10 mmol/L EDTA, 10% glycerol, 100 μmol/L Na₃VO₃, 1% Trasylol [Bayer], and 1 mmol/L PMSF). The cell lysates were centrifuged at 10 000g for 15 minutes, and phosphotyrosine-specific immunoprecipitation was performed using the 4G10 monoclonal antibody (UBI) and a rabbit anti-mouse antiserum (Sigma). Immunoprecipitates immobilized on Protein A-Sepharose CL 4B (Pharmacia) were used for the immune complex kinase assay, which was performed for 7 minutes at room temperature in 25 μL of 50 mmol/L HEPES buffer (pH 7.4) containing 10 mmol/L MnCl₂, 1 mmol/L dithiothreitol, and 5 μCi of (γ⁻³²P]ATP (Amersham). The samples were separated by SDS-PAGE (5 to 15% gradient) before the gels were incubated for 30 minutes in 2.5% glutaraldehyde, washed 2 times for 15 minutes in 10% acetic acid and 40% methanol, treated for 1 hour at 55°C in 1 mol/L KOH to remove serine-bound phosphate,\(^\text{22}\) washed 3 times for 20 minutes in 10% acetic acid/40% methanol, dried, and exposed to Hyperfilm MP (Amersham). Radioactive bands were quantitated on a Fuji Phosphorimager.

**Immunoradiometric Assay**

The VEGF-A concentration was analyzed in serum samples of all diabetic and nondiabetic subjects. Samples were stored at −20°C until analysis. An immunoradiometric assay was performed with 2 monoclonal antibodies specific for VEGF-A, which were generously supplied by Genentech Inc (South San Francisco, Calif). We used the monoclonal antibody B2.6.2 to recognize VEGF-A₁₆₅ and VEGF-A₁₈₉ and the monoclonal antibody A4.6.1 to recognize VEGF-A₁₂₀, VEGF-A₁₆₅, and VEGF-A₁₈₉.\(^\text{23}\) The 96-well plates (Maxisorp, Nunc) were coated with B2.6.2 (5 μg/mL) in 50 mmol/L carbonate buffer (pH 9.6) for 16 hours at 4°C. After washing with 0.03% Tween 80 in PBS (pH 7.4), the plates were blocked for 1 hour at 25°C using PBS (pH 7.4) with 0.5% bovine serum albumin and 0.03% Tween 80. Plates were washed before the addition of serum samples or the VEGF-A₁₂₀ control (range, 5 pg/mL to 11 ng/mL) and incubated for 2 hours at 25°C; all experiments were performed in triplicate. After a washing step, the monoclonal anti-VEGF antibody A4.6.1, [²²³I]-labeled using the Chloramin-T method,\(^\text{24}\) was added to each well.
by guest on August 31, 2017 http://circ.ahajournals.org/ Downloaded from

counted using an automated master, LKB-Pharmacia). The sensitivity of the assay was 20 pg/mL. No cross-reactivity was found with the closely related platelet-derived growth factor-BB.

Statistical Analysis

Results of the migration assays and the VEGF-A serum levels were analyzed using a 2-sided exact Wilcoxon test for unpaired samples. Data for each group of patients were described as medians and quartiles (25th and 75th percentiles). In the case of the migration testing was primarily performed for a VEGF-A concentration of 1 ng/mL.

Results

VEGF-A–induced monocyte migration was assessed using a modified Boyden chamber assay. The migration of monocytes from healthy volunteers (n=5) were then discarded, the plates were washed, and the wells were counted using an automated γ-counter (LKB Wallac 1277 GammaMaster, LKB-Pharmacia). The sensitivity of the assay was 20 pg/mL. No cross-reactivity was found with the closely related platelet-derived growth factor-BB.

Different concentrations of VEGF-A were supplied to the upper and/or lower compartments of the modified Boyden chamber. The results are presented as the mean±SEM of migrated cells in 15 high power fields counted for each sample. Data from a representative experiment are shown. Relative changes in cell number were virtually identical in 3 independent experiments, but the absolute number of cells per high power field varied slightly between different individuals.

Figure 1. Monocyte chemotaxis analysis. Monocytes were isolated from peripheral blood samples and stimulated with either VEGF-A (1 ng/mL; A) or fMLP (10⁻⁸ mol/L; B) using the modified Boyden chamber. A total of 15 high power fields were counted for each sample, and the quartiles and 5%/95% values are given in a box plot. The Wilcoxon test for unpaired samples was used to estimate statistical significance.

(5×10⁴ cpm/well) and incubated for 2 hours at 25°C. Supernatants were then discarded, the plates were washed, and the wells were counted using an automated γ-counter (LKB Wallac 1277 GammaMaster, LKB-Pharmacia). The sensitivity of the assay was 20 pg/mL. No cross-reactivity was found with the closely related platelet-derived growth factor-BB.

Table, the maximal induction of migration occurred in the presence of a positive concentration gradient between the 2 compartments. In the presence of equal concentrations of VEGF-A, no enhanced migratory response could be observed. These results indicate that VEGF-A can activate a true chemotactic response in monocytes with no appreciable chemokinetic activity.

In contrast to the VEGF-A–induced effects, the VEGF-independent chemotactic response of monocytes to the tripeptide fMLP (10⁻⁸ mol/L) remained intact in diabetic patients at 235% (25th and 75th percentiles, 158% and 357%); the control group had a response of 304% (25th and 75th percentiles, 262% and 420%) (Figure 1B). No statistically significant difference existed between the 2 groups.

The VEGF-A–inducible kinase activity of Flt-1 remained fully intact in monocytes from diabetic patients, as assessed by the in vitro kinase assay (Figure 2). VEGF-A could induce similar levels of tyrosine phosphorylation in all monocyte preparations analyzed. No differences existed in monocytes isolated from healthy control subjects.

The VEGF-A serum level was measured in blood samples from all individuals tested. The median VEGF-A serum level in healthy individuals was 98 pg/mL (25th and 75th percentiles, 75 and 137 pg/mL). In contrast, the VEGF-A serum

<table>
<thead>
<tr>
<th>VEGF-A</th>
<th>None</th>
<th>0.03</th>
<th>0.3</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower compartment: VEGF-A, ng/mL</td>
<td>None</td>
<td>65±4</td>
<td>66±4</td>
<td>61±4</td>
</tr>
<tr>
<td>0.03</td>
<td>72±4</td>
<td>69±4</td>
<td>66±3</td>
<td>67±3</td>
</tr>
<tr>
<td>0.3</td>
<td>80±5</td>
<td>81±6</td>
<td>64±5</td>
<td>62±3</td>
</tr>
<tr>
<td>3</td>
<td>106±5</td>
<td>97±5</td>
<td>70±5</td>
<td>64±3</td>
</tr>
</tbody>
</table>

Different concentrations of VEGF-A were supplied to the upper and/or lower compartments of the modified Boyden chamber. The results are presented as the mean±SEM of migrated cells in 15 high power fields counted for each sample. Data from a representative experiment are shown. Relative changes in cell number were virtually identical in 3 independent experiments, but the absolute number of cells per high power field varied slightly between different individuals.

Compared with the unstimulated 100% control, VEGF-A–stimulated (1.0 ng/mL) monocyte migration measured 91.1% (25th and 75th percentiles, 83% and 98%). When monocytes were stimulated with VEGF-A at 0.1 ng/mL, this value measured 91.1% (25th and 75th percentiles, 87% and 99%); it measured 91.8% (25th and 75th percentiles, 82% and 100%) at 0.3 ng/mL VEGF-A and 87% (25th and 75th percentiles, 76% and 97%) at 10 ng/mL VEGF-A. All these values were significantly below the results obtained for healthy individuals (P<0.0001). No differences existed between monocytes from patients with insulin-dependent and non–insulin-dependent diabetes mellitus.

To clarify whether the migration of monocytes across the porous filter membranes depended on the presence of a VEGF-A gradient between the lower and the upper compartment, we performed a checkerboard analysis. As shown in the Table, the maximal induction of migration occurred in the presence of a positive concentration gradient between the 2 compartments. In the presence of equal concentrations of VEGF-A, no enhanced migratory response could be observed. These results indicate that VEGF-A can activate a true chemotactic response in monocytes with no appreciable chemokinetic activity.

In contrast to the VEGF-A–induced effects, the VEGF-independent chemotactic response of monocytes to the tripeptide fMLP (10⁻⁸ mol/L) remained intact in diabetic patients at 235% (25th and 75th percentiles, 158% and 357%); the control group had a response of 304% (25th and 75th percentiles, 262% and 420%) (Figure 1B). No statistically significant difference existed between the 2 groups.

The VEGF-A–inducible kinase activity of Flt-1 remained fully intact in monocytes from diabetic patients, as assessed by the in vitro kinase assay (Figure 2). VEGF-A could induce similar levels of tyrosine phosphorylation in all monocyte preparations analyzed. No differences existed in monocytes isolated from healthy control subjects.

The VEGF-A serum level was measured in blood samples from all individuals tested. The median VEGF-A serum level in healthy individuals was 98 pg/mL (25th and 75th percentiles, 75 and 137 pg/mL). In contrast, the VEGF-A serum
levels of diabetic patients were significantly elevated (median, 153 pg/mL; 25th and 75th percentiles, 106 and 230 pg/mL; \( P = 0.0088 \)) (Figure 3). VEGF-A serum levels of female diabetics were higher than those of male diabetics (230 versus 149 pg/mL; \( P = \text{NS} \)), just as VEGF-A serum levels in healthy female individuals were higher than those in healthy male individuals (116.5 versus 88 pg/mL; \( P = \text{NS} \)).

**Discussion**

In the present study, we showed for the first time that the cellular response to the angiogenic factor VEGF-A strongly depends on the integrity of the studied cells. Although the Flt-1–mediated chemotactic response to VEGF-A was strong and consistent in all monocytes isolated from healthy volunteers, monocytes from patients with diabetes mellitus did not properly respond, and they lacked the ability to migrate toward VEGF-A in a chemotaxis assay. Moreover, our data on the VEGF-A–induced and Flt-1–mediated signal transduction defect in diabetic patients provide novel insight into the mechanism of how monocyte function is impaired in diabetic patients.

The recruitment of monocytes is thought to be an important step during collateral formation secondary to regional myocardial ischemia. Increased shear stress in preformed epicardial collaterals somewhat distant to the actual area of hypoxia and ischemia paves the way for monocyte accumulation, their maturation to macrophages, and the release of growth factors, which creates an inflammatory environment. In contrast to true angiogenesis, the process of arteriogenesis, which describes the growth of collateral vessels, seems to be critically dependent on monocytes and on proper monocyte function. Therefore, our ex vivo assay using the VEGF-A stimulation of monocytes may have a predictive value for a patient’s ability to develop collateral circulation to an ischemic area in the heart or in the peripheral circulation. In fact, during the initial review process of this article, a study was published in *Circulation* demonstrating that the ability of diabetic patients with coronary artery disease to develop coronary collaterals is significantly impaired. These data are compatible with our hypothesis of growth factor–induced monocyte migration as a predictor of an individual’s ability to develop collateral circulation. Therefore, our novel ex vivo assay is a good candidate for a surrogate assay of the process of arteriogenesis. Likewise, our data may serve as a molecular explanation for the reduced collateralization seen in diabetic patients.

Because VEGF-dependent monocyte function is severely reduced in monocytes from diabetic patients, our data suggest that VEGF-A and its receptor Flt-1 might indeed be critically involved in stimulating the process of arteriogenesis. VEGF-A could stimulate arteriogenesis in the following 2 different and independent ways. (1) Direct VEGF-A action stimulates the endothelium and promotes vascular remodeling. (2) VEGF-A promotes an indirect mode of activation by stimulating monocyte recruitment to the vessel wall. These monocytes and developing macrophages are vehicles for a number of vascular growth factors that are produced by these cells and released at the site of activation, such as vascular growth factors (including VEGF-A), basic fibroblast growth factor, transforming growth factor-β, and epidermal growth factor.

Our model, however, does not exclude the functional involvement of other growth factors and cytokines in the process of arteriogenesis. For example, monocytes could be recruited to the vessel wall by monocyte chemoattractant protein-1. On the basis of recent data, this protein could also act as a molecular mediator (it can be induced by VEGF-A). Because of the limited number of monocytes obtained from each preparation and the need for triplicate analysis and the inclusion of proper controls, we have not been able to test the

![Figure 2](image-url) Phosphotyrosine blot analysis of monocytes. Effect of VEGF-A (50 ng/mL for 10 minutes) stimulation on monocytes from diabetic and nondiabetic individuals. Tyrosine-phosphorylated proteins were immunoprecipitated using a phosphotyrosine-specific antibody (4G10, UBI), and an in vitro kinase reaction was performed. Proteins were then separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and detected using autoradiography. The relative activation of 3 individual proteins (p210, p120, and p69) was quantitated using phosphostorage technology.

![Figure 3](image-url) VEGF-A serum levels in diabetic and nondiabetic individuals as determined by immunoradiometric assay (described in Methods). Quartiles and 5%/95% values are given in a box plot. The Wilcoxon test for unpaired samples was used to estimate statistical significance.
response of monocytes from diabetic individuals toward other factors. However, this will be the subject of a future study.

In diabetic patients, the decreased chemotactic response of monocytes to VEGF-A is a consequence of impaired VEGF-A–induced and Flt-1–mediated signal transduction. Although the activation of tyrosine phosphorylation seems to remain fully intact, the signal does not reach the (intact) cytoskeletal components responsible for migration. Strong evidence indicates that the impaired VEGF-A–induced and Flt-1–mediated effect is selective and that the investigated monocytes are basically intact; for example, the potent and unspecific tripeptide fMLP can stimulate a proper chemotactic response in these cells. Therefore, our findings suggest a signal transduction defect is responsible for the impaired monocyte migration.

It is presently unclear whether the impaired VEGF-A–induced response of monocytes reflects or predicts an impaired endothelial response to VEGF-A. It may well be that the Flt-1–mediated response of VEGF-A in the endothelial cells of diabetic individuals is impaired as well. However, because most of the VEGF-A–induced responses in the endothelium are mediated by KDR and because KDR is not expressed in monocytes, our finding of impaired monocyte migration in diabetic individuals does not necessarily predict an impaired endothelial response to VEGF-A. There are many possible explanations for why arteriogenesis might be impaired while angiogenesis, in particular diabetic retinopathy, is stimulated in these patients. Although Flt-1–mediated responses are impaired in patients with diabetes mellitus, which leads to reduced monocyte migration and impaired arteriogenesis (as shown in this article), the angiogenic response of endothelial cells may be enhanced secondary to elevated VEGF-A levels. Another possible explanation might be a different degree of involvement of monocytes in arteriogenesis and angiogenesis. It is tempting to speculate that monocyte migration is the rate-limiting step in arteriogenesis, whereas the involvement of monocytes at sites of angiogenesis reflects the inflammatory nature of this process.

In the present study, we showed that the serum level of VEGF-A is significantly elevated in diabetic patients; this is similar to discoveries in the ocular fluid of patients with proliferative diabetic retinopathy. VEGF-A levels are raised under diabetic circumstances as a direct consequence of elevated glucose concentrations.

In addition, the fact that angiogenesis is promoted in the form of diabetic retinopathy raises questions about differences in the pathogenesis of diabetic retinopathy and other forms of angiogenesis. Proliferative diabetic retinopathy is preceded by a long period of microvascular damage. After decades of chronic changes, microvascular occlusion eventually results in ischemia, which leads to the secretion of VEGF-A from the retina and to the development of abnormal angiogenesis within the isolated compartment of the eye. It is conceivable that elevated VEGF-A levels in diabetic individuals acting on KDR may compensate for the impaired activity of any Flt-1–mediated cellular response.

Taken together, these data indicate that the cellular response of monocytes to VEGF-A is attenuated in diabetic patients due to a downstream signal transduction defect. Therefore, we postulate that the VEGF-A–induced and monocyte-dependent process of collateral formation is severely impaired in diabetic patients and that VEGF-A–based therapeutic strategies to enhance tissue perfusion should give better results in patients not suffering from diabetes mellitus.

Acknowledgment

This study was supported in part by grants Wa734/2-1 and Wa734/2-4 from the Deutsche Forschungsgemeinschaft and by the Sonderforschungsbereich SFB451 project B1 (all to J.W.). We would like to acknowledge the kind gift of recombinant VEGF-A from Denis Gospodarowicz, Chiron, Emeryville, Calif. We thank Hedwig Frank for performing the checkerboard analysis.

References

Vascular Endothelial Growth Factor-A–Induced Chemotaxis of Monocytes Is Attenuated in Patients With Diabetes Mellitus: A Potential Predictor for the Individual Capacity to Develop Collaterals

Johannes Waltenberger, Juliane Lange and Andrea Kranz

_Circulation_. 2000;102:185-190
doi: 10.1161/01.CIR.102.2.185

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/102/2/185

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/